Proceedings of

THE FIRST INTERNATIONAL CONFERENCE ON TOXIC DINOFLAGELLATE BLOOMS

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Proceedings of

THE FIRST INTERNATIONAL CONFERENCE ON TOXIC DINOFLAGELLATE BLOOMS

Managing Editor

VINCENT R. LoCICERO

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INTRODUCTION

The objectives of the First International Conference on Toxic Dinoflagellate Blooms were (1) to convene the worlds' foremost experts and researchers in toxic dinoflagellate blooms, (2) to share experiences and advances in the related disciplines, and (3) where possible, to identify areas of promising or needed research. These proceedings are evidence of the realization of our objectives.

The high quality of the accomplishments recorded here is a tribute to those who persisted in this work in spite of limited financial support and little scientific recognition. Now that these toxic "red tides" can be shown to be an increasingly severe public health menace around the world, it is obvious that increased support must be forthcoming to mount an intensified and expanded research effort to combat this problem. This was the clear plea arising from the participants in the conference and from the scientific evidence they brought.

Many deserving papers were not presented orally beacuse of time limitations. However, they are printed here in the hope that this document will thereby become the most comprehensive and useful possible reference on the present status of work in the field.

Particular appreciation must be accorded to the members of the conference committee for their cooperation and dedication to the success of this conference, and to Dr. E. R. Pariser, Marine Advisory Services Office, MIT Sea Grant Program; W. H. Weston, Professor Emeritus Harvard University, and Dr. L. Loeblich, Biological Laboratories, Harvard University, for their assistance in the scientific editing of manuscripts.

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DINOFLAGELLATE BLOOMS - AN OVERVIEW [1]

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CANADA

[1]Conference Chairman's opening address to the International Conference on toxic Dinoflagellate blooms.

NOV. 4, 1974. Boston, Mass.

Good morning. Ladies and Gentlemen. It is indeed a great pleasure for me to welcome all of you to this first International Conference on Toxic Dinoflagellate Blooms. My first task as the Conference Chairman is entirely pleasurable; it is to express on your behalf the deep appreciation and gratitude to our hosts, the Massachusetts Science and Technology Foundation and the Massachusetts Institute of Technology Sea Grant Program for sponsoring and arranging to hold this important conference in this great city of Boston. We are also grateful to the various governmental and non-governmental organizations who provided funds to cover the cost of this conference.

Although a few local meetings or symposia dealing primarily with red tide outbreaks in the Gulf states have been held earlier (Galveston, Texas, 1958, St. Petersburg, Florida, 1964; Sarasota, Florida, 1974,) this Conference to the best of my knowledge, represents the first comprehensive interdisciplinary meeting ever to be held on the subject of 'Toxic dinoflagellate blooms' and associated phenomena, and brings together currently leading international experts in this field. The major objectives of this Conference are to evaluate the state of the art in this field, to identify gaps in our knowledge, to set forth requirements for needed research, and to design effective control and management schemes which will protect the public health, the environment, the fishery resources, and the coastal economics from negative effects of such blooms. It is my sincere hope that during the next couple of days and even after this conference, both formal and informal discussions within such a large, highly qualified and diversified a group will at least stimulate and clarify thinking about this problem.

The association of toxic marine dinoflagellate blooms, particularly of the genus *Gymnodinium* and the genus *Gonyaulax* with 'red tide' and mass mortality of marine animals is well documented and the overall impact of such 'red tide' outbreaks particularly in Florida and California in terms of economic losses is common knowledge. Certain toxic dinoflagellates have also been implicated with the accumulation of paralytic toxin in molluscan shellfish in various parts of the world and the world-wide incidence of known paralytic shellfish poisonings (PSP) to humans have been estimated to be in the vicinity of 1600 cases, including 300 fatalities. In addition, several cases of mortality of domestic animals, fish, birds and other marine animals have been directly linked with PSP.

Although this Conference is intended to focus attention on the various problems caused by blooms of toxic marine dinoflagellates, the agenda before us is more oriented towards discussion of paralytic shellfish poison problems. Several of the contributed papers deal with biology, chemistry, toxicology, pharmacology and management of PSP and their inclusion in the program is intentional for several very good reasons. First, there is evidence that paralytic shellfish poisoning outbreaks are increasing in intensity and spreading to new areas. Second, these outbreaks are world-wide in occurrence and an organized international effort is needed to comprehend the complexities of this phenomenon and to map out strategy for the protection of public health and the fishery industry. Thirdly, the inter-disciplinary aspects of PSP problem

have never been discussed in a common forum. And finally, a severe PSP outbreak in the autumn of 1972 along the New England coast which had had no previous history of such toxic blooms has revived interest in toxic dinoflagellate blooms in general and their hazards to public health and fisheries in particular. The 1972 'red tide' outbreak along the New England coast resulted in complete closure of the entire 3200 Km Massachusetts coastline for harvesting shellfish and declaration of a public health emergency by the State Governor. Besides 26 reported cases of poisoning, the shellfish industry suffered severe economic losses and the public confidence in the safety of fishery products was momentarily shaken.

I was associated for a number of years with the PSP research program in eastern Canada where this phenomenon is of regular occurrence during the summer months, however, it is not my intention this morning to talk about our PSP problem since most of this information is already available in the published literature and adequately summarized in the Fisheries Research Board of Canada Bulletin 177 an offprint of which is already in your hands. Instead, I wish to confine my remarks to an overview of dinoflagellate blooms as a biological phenomenon, without trying to steal the thunder from other speakers who will be dealing with this aspect in a more specific way.

The occurrences of toxic dinoflagellate blooms in the sea, often leading to the well known 'red water or red tide' conditions are not new. They have been noticed, sometimes recorded, and generally ignored since biblical times. In several regions of the world 'red tide' outbreaks have been responsible for harmful effects on public health, large-scale fish mortalities and destruction of other marine life, and have spelled disaster for coastal fishing, tourist and recreational industries. These negative effects are not necessarily confined to blooms of toxic dinoflagellates. Non-toxic dinoflagellate blooms are also important in view of the fact that secondary effects of decomposition and decay of such blooms may jeopardize the survival of many coastal marine organisms. Wherever this happens, it results in a drastic change in the ecological balance of the inshore coastal areas and it may take several years before the affected marine communities are able to establish themselves in those areas.

The spectacular growth of marine dinoflagellates resulting in 'red tide' conditions is generally confined to coastal waters or to those regions of the sea where active upwelling takes place. Sporadic occurrences and generally short duration of such red water blooms have usually caught the scientific community off guard and have discouraged study of this phenomenon on a systematic and continuing basis. Consequently, there are very few published records where such blooms have been explained on the basis of environmental factors prevailing prior to or during the bloom period. The events preceding a red tide have generally been subjects of considerable speculations and vague generalizations and despite several studies on growth requirements of red tide dinoflagellates under laboratory and natural conditions the question why a dinoflagellate species grows 'wild' creating red tide conditions in the sea has remained unanswered. Extrapolations of laboratory results to explain natural

red tide occurrences have not been altogether satisfying.

There is little doubt that the abundance and seasonal distribution of marine dinoflagellates are intimately related to temperature, salinity, light, nutrients and current regimes in the sea. High temperature, high light intensity and a relatively stable water column are some of the factors that stimulate their growth in nature. however, the exact mechanism which permits the development of an extensive monospecific dinoflagellate bloom with virtual exclusion of other phytoplankton species is still very much an enigma and requires further studies. I feel that for too long, study of the 'red tide' phenomenon has been carried out in much too narrow, much too conservative and parochial a manner. We have been looking at a limiting factor rather than concentrating on interaction of multiple factors which create conditions conducive to a monospecific dinoflagellate bloom. We have tended to approach this problem as if the cause of headache was a lack of aspirin.

Some of you may not entirely agree with me, but I subscribe to the idea that the development of a 'red tide' bloom should be looked upon as a process comprising two main components, (1) initiation of a bloom, and (2) subsequent development and continuation of this bloom to the extent that it becomes visible. There is sufficient evidence to support the fact that conditions for initiating a bloom may not necessarily be the same required for continuation of a bloom. Initiation of a bloom appears to be largely a matter of 'biological conditioning' of sea water which favours a more or less exponential growth of a dinoflagellate species and involves largely biological and chemical factors. The subsequent continuation of the bloom appears to be largely influenced by physical factors like winds, tides, convergence, divergence, temperature or salinity-induced stratification, Langmuir convection cells, stability of the water column etc. Positive phototactic behaviour of many dinoflagellate species may also contribute towards this concentrating mechanism.

Since red water blooms are essentially a coastal phenomenon, it has been suggested that the growth of dinoflagellates to bloom proportions may be dependent upon, or at least influenced by production processes characteristic of coastal waters. Coastal waters exhibit a higher concentration and wider variety of nutrients, trace metals, particulate and dissolved organic matter, and other biologically active substances than those found in the open sea. This enrichment is primarily due to drainage from land and/or due to local upwelling and there is reasonable evidence to suggest that dinoflagellate blooms are associated with 'biological conditioning' of inshore coastal waters brought about by introduction of organic compounds of terrigenous origin.

I wish to expand on the role of land drainage in the development of dinoflagellate blooms because the introduction through land drainage of significant amounts of terrigenous organic matter which includes a number of biologically active substances has in my opinion the most profound impact on production processes in coastal waters. Although species specific diversity in growth requirements is to be expected, a number of laboratory studies have shown that marine dinoflagellates generally thrive best under conditions of

low salinity and high organic enrichment. Both these conditions prevail in coastal waters, particularly in areas of river discharge and/or after a heavy rain-fall. Indeed sufficient indirect evidence is available to suggest that heavy rain-fall or land drainage is a prerequisite to most, if not all dinoflagellate blooms. A great bulk of terrigenous organic matter in coastal zone is represented by humic substances which are efficient natural trace metal chelators. My own work with such substances as well as recent published and unpublished experimental data from several laboratories suggest that increased production of planktonic algae in general, and of marine dinoflagellates in particular is linked with aquatic humus present in coastal waters.

Another aspect of the influence of land drainage on red tide development is related to the entry of pollutants in coastal waters. In some areas pollution has been blamed for red tides and concern has been expressed that increasing pollution of inshore coastal waters may increase the frequency of occurrence of red tide outbreaks. There is little doubt that waste discharges introduced from land modify quantitatively as well as qualitatively the planktonic population in coastal waters. Since dinoflagellate blooms correspond to high organic loading in sea water, sewage outfall discharges introducing substantial amounts of organic material could create conditions favourable to growth of marine dinoflagellates and thus set the stage for the development of severe red tide blooms of toxic dinoflagellates. Recent observations in Tokyo Bay, Japan and Oslofjord in Norway which are subject to considerable urban discharge appear to suggest that the frequency of red tide outbreaks is on the increase.

Development of a dinoflagellate bloom can be looked upon both as a product as well as a process of the general eutrophication of the coastal ecosystem. The interaction of the various conservative and radical environmental factors which brings about eutrophication should also be able to explain how and why a 'red tide' bloom is generated. Recently modest attempts have been made to understand the interplay of the various factors involved by constructing ecological models. Working with models is an excellent way of developing an appreciation of the whole system. The general idea behind any ecological model is elucidation of complexities and interactions of a system which would permit development of predictive capability that could be applied to real life situations. Unfortunately, unlike physical systems, the complexities in a biological system are magnified several-fold due to the fact that there is an enormous variety of interactions between chemical, physical and biological components of that system. Some of these are either not identified properly, or are measured imprecisely, thus making a biological model an elegant but entirely useless tool. Because the degree of sophistication required to develop predictive models has not been developed by many of us biologists who have very little idea of what level of detail or resolution is required in a predictive model, modelling in biological systems appears to be more an intuitive art than a sober science. Those model makers who work in glorious isolation and are content with the 'black box' philosophy are, in my opinion, wasting not only their time but also that of others. I believe that a close integration between experimentalist, field

observer and the theoretician is an essential prerequisite for meaningful predictive models applicable to 'red tide' phenomenon.

Finally, I would like to say that in present day context, research and management are interdependent; research information is vital for development of regulations, guidelines, and general management policy. Research on dinoflagellate blooms and associated phenomena is demanding of a variety of disciplines and this is quite evident from the full and varied agenda ahead of us. During the next three days you will be exposed to a number of papers on biological, ecological, chemical, toxicological and management aspects of dinoflagellate blooms. Most of this work is relatively recent and has been carried out against generally serious odds of insufficient or interrupted funding and general lack of appreciation of this problem by administrators and the public. I have over the years looked with concern, bewilderment and if I may say so, with certain amount of dismay at the attitude of research administrators with respect to research on red tide blooms. In this country as well as elsewhere the interest and involvement of various agencies in red tide research has tended to follow the same sporadic and unpredictable frequency as that of a 'red tide'. Both the federal and state agencies responsible for funding have treated this problem as a 'political football' and have regarded research as a convenient faucet to be turned off or on at will to suit their purpose. This has resulted in irreparable damage to serious studies of this phenomenon on a systematic and continued basis especially when one realizes that it takes 2-3 years to assemble a good team of researchers and another 4-5 years or more of diligent investigations to bring a study to a close. Despite these odds considerable progress in our understanding of this pehnomenon and its public health and economic implications has been made. There are obvious gaps in our knowledge of certain aspects which must be filled in order that we may develop a better scientific basis for our management programs. I am confident that our discussions and deliberations during the course of this conference will lead us to a fuller realization of the magnitude of the problems associated with dinoflagellate blooms, as well as deficiencies in our approach to these problems and possible remedies. It is my earnest hope that upon termination of this Conference, we will be able to come out with a clear road map which should help not only the individual scientist in assessing and reorienting his research program, but also those who are involved in formulating policies to safeguard public health and to prevent economic losses. If this Conference succeeds in generating a better public understanding of the facts associated with red tide phenomenon and is able to arouse interest and awareness among various Federal and State officials, leaders of the Fishery industry, Scientists, Engineers and the like, it should be regarded as a major first step. I once again extend a sincere welcome to you all and wish you every success in your endeavors. Thank you!

SESSION SUMMARY

OCEANOGRAPHIC CONDITIONS ASSOCIATED WITH RED TIDE BLOOMS

Session Chairman

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Since the beginnings of biological oceanography, studies of factors controlling the blooming of phytoplankton have dominated the research scene.

Early in the research it became apparent that changes in population abundance were a general phenomena of lakes and oceans, but the interpretation of causes, with prediction as the goal, was complicated by the interrelationship between physical and biochemical factors. Today the appearance and disappearance of different species is still one of the principle problems in phytoplankton ecology. It becomes more perplexing but no less interesting when a single species of dinoflagellate blooms at the expense of all obvious external growth factors.

Some ecologists believe that large concentrations of dinoflagellates are "accumulations" of algae brought about by an interaction between behavior and hydrography. Other ecologists feel that blooms occur because of complex biochemical relationships with water chemistry. To the reader this might signify a wide degree of polarization, in reality most ecologists feel that "blooms"are explained by both theories. Models of blooms include inputs such as hydrography, biochemistry, behavior, as well as the physical chemistry of the water masses.

This section of the conference was composed of researchers actively engaged in developing predictive models of plankton blooms and/or assessing the influence of hydrographic and meteorological phenomena.

The specific subjects that were addressed are:

1) Common external contributory factors to blooms.

2) The roles of temperature, salinity and nutrients.

3) The implication of oceanographic factors such as vertical mixing, advection and stability of the water column with the occurrence of blooms.

4. The influence of meteorological, and physical conditions on blooms.

5) Industrial/ human factors related to blooms.

6) Sequential relationships of meteorological, hydrographic or biological-chemical conditions upon the cessation or limitation of bloom size.

Practically every paper in this session emphasizes some factor of physical oceanography which can concentrate red tide organisms. Such features as convergences, vertical mixing, convective cells and internal waves have been implicated. However, most speakers have been emphatic that concentrating mechanisms alone cannot explain the bloom phenomena: All concentrating mechanisms coupled directly or indirectly with enrichment is a necessity.

On the Atlantic coast those present when the 1972 outbreak occurred have asked the question as to why was 1972 different from preceeding years. Two things stand out, (1) persistant wind conditions favorable for near shore upwelling, (2) Hurricane Carrie and the winds and rain associated with same. Both of these events provided the mechanism for enrichment of the water column at a time when growth conditions for *Gonyaulax tamarensis* were favorable. On the Pacific coast of the United States the appearance of blooms

of *Gonyaulax polyedra* appears to be correlated with the intensity and duration of upwelling along the coast, while on the west coast of Florida vertical mixing and/or seasonal cooling correlate with the rate of change of water temperature. An extensive record of past occurrences has allowed Florida workers to develop a predictive model.

In the geographical areas experiencing red tide problems external activities such as dredging and the discharge of human and other wastes have aggravated the problem.

The question that continues to nag oceanographers is why any of the above mentioned events lead to a unialgal patch of red tide organisms. This is where behavior of the organism (vertical migration) becomes important. Researchers on the Pacific Coast believe that daily migration provides a selective advantage for the dinoflagellates, in that they can seek out nutrient rich water at depth during the hours not favorable for photosynthesis. There is also increasing evidence that the solo nature of species in patches is maintained by the excretion of growth inhibitors of other phytoplankton and, perhaps, selective grazing.

The papers of the session emphasize the complexity of the problems. A major part of this is the difficulty in studying the problem in the field. As one participant put it, "Progress in this area of research is slow, but the fact is as doctors we only get to see our red tide patients once or twice in several years."

PHYSIOLOGICAL ECOLOGY OF *GONYAULAX POLYEDRA* A RED WATER DINOFLAGELLATE OF SOUTHERN CALIFORNIA

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ABSTRACT

Gonyaulax polyedra is frequently a dominant species in red tides off Southern California. Scientific observations of the species composition of blooms off La Jolla were begun in the 1920's and physiological measurements have been carried out since the 1950's. The latter include studies of bioluminescence and the periodicity of cell division, vertical migration, proximate chemical composition, and nitrogen assimilation.

Causative mechanisms of local red tides appear to differ from those off Florida. Here nutrient enrichment via upwelling is more important than freshwater runoff. The physical concentration of organisms into dense, visible patches has been shown by Kamykowski (14) to depend upon a daily vertical migration through the thermocline.

As a first approximation the coastal surface waters off Southern California may be regarded as oligotrophic with respect to ambient concentrations of nutrients. At invervals these waters are enriched by the admixture of nutrient-rich water from depth. This enrichment promotes phytoplankton growth: either diatom or dinoflagellate blooms may result. The dinoflagellate blooms are associated with steep, shallow thermoclines, a nutrient-depleted, shallow mixed layer (< 10 m) but with nutrient-rich water below the shallow thermocline and within the depth range of the diurnal vertical migration of the dinoflagellates. We hypothesize that the vertical migration along with certain idiosyncracies in their metabolism of nitrogen (notably a lack of circadian periodicity and of ammonium-repression of the enzyme nitrate reductase) provide an advantage for dinoflagellates over coastal diatoms when the above-mentioned physical conditions prevail.

Introduction

Gonyaulax polyedra Stein is one of the most abundant phytoplankters in the nearshore coastal waters of Southern California. Reid, *et al.* (24) found it to be among the ten dominant species (when dominance was based upon biomass as carbon) in about one-half of the samples studied for the period April through September, 1967 from a station 1.4 km. from the beach off La Jolla. Its abundance decreased progressively at stations 4.6 and 12.1 km offshore. Between the inshore and offshore stations the water depth increased from 20 to greater than 300 meters indicating the narrow continental shelf in this region.

The surface waters off Southern California alternate between oligotrophic (ambient nitrate and chlorophyll concentrations less than 1 μg atom liter $^{-1}$ and 1 μg liter $^{-1}$, respectively) and more nearly eutrophic states. Surface nitrate may reach 10-20 μg atom liter^{-1} and phytoplankton chlorophyll up to 20 μg liter^{-1} in diatom blooms or as much as several hundred μg liter^{-1} in dinoflagellate red water blooms. There is little freshwater runoff from rivers in this semiarid region. Rainfall is typically of the order 25 cm year^{-1} with a winter maximum. Approximately 2 x 10^9 gallons day^{-1} of sewage flows into

the coastal waters, largely from the metropolitan areas of Los Angeles, Orange County, and San Diego. But even nutrient additions from sewage are expected to be small compared with nutrients advected through Southern California coastal waters by the California Current. Upwelling is characteristic of this and other eastern boundary currents (30). Increases in the standing stocks of phytoplankton seem to be closely related to inputs of nutrients into the surface waters via upwelling. Indeed Kofoid (16) early attributed a causal relationship of *G. polyedra* blooms to upwelling and more recent observations by Clendenning (6) and Holmes *et al.*, (13) confirm his suggestion.

Gonyaulax polyedra has been the most characteristic organism in local red water dinoflagellate blooms, as recorded by Allen (1) in more than twenty years of observations of phytoplankton collected off the Scripps Institution pier. But other dinoflagellates may also be dominant in such blooms. Among these are *Prorocentrum micans* (1), *Cochlodinium sp.* and *Gymnodinium sp.* (13), *Gymnodinium flavum* (17, 29) and *Ceratium spp.* (*C. furca* and *C. dens*) (7). These organisms and *Gymnodinium splendens* are also frequent components of *G. polyedra* blooms (13).

The decline of local blooms has been related to water exchange, to grazing by the holozoic dinoflagellates *Polykrikos sp.* (13), *Noctiluca sp.* and *Fragilidium heterolobum* (5), and to rotifers and holozoic ciliates, including tintinnida. The latter are often abundant in such blooms. Their ingestion of *G. polyedra* has been observed in red water samples examined in the laboratory.

Observations on local red water blooms predate the Scripps Institution of Oceanography (established in 1912). In addition to the early published work of Kofoid (16) and Allen (1) there is considerable unpublished local lore and natural history. This information is valuable but difficult to attribute to its source. Therefore the general oceanographic characteristics commonly associated with *G. polyedra* blooms and blooms of other red water dinoflagellates will be listed without complete attribution. Most of these characteristics were included in Ryther's 1955 review (25) and in Holmes *et al.* 1967 (13). Dense, visible red water blooms are associated with calm, warm weather in the "upwelling season" of April-November when there is a steep, shallow thermocline. The dominant dinoflagellates are vertically migrating species. Red patches are often not apparent early in the day but appear mid-morning, suggesting that either their upward migration or their aggregation in the horizontal sense takes place in the morning. Patches are restricted to inshore areas, usually within 5 km of the beach. Patches of red water are most frequently observed in areas near submarine canyons (as off the Scripps Institution) or south of headlands. The areal extent of the blooms may be very great in the longshore direction. Clendenning (6) reported a more-or-less continuous bloom for over 200 km along Baja California (Mexico) and Southern California (USA). Physical concentration mechanisms must be involved to achieve such high cell densities as are often observed since the cellular content of nitrogen in blooms may exceed that initially available in the water column (13).

Table I

Red water blooms noted by staff members of the Food Chain Research Group in the period December 1966 to September 1974 off Southern California.

Date	Place	Comment
3 Sept. 67	Seal Beach	*Ceratium spp.* Vertical migration studied
15 Febr. 68	Sorrento Slough	Red patches
27 Mar. 68	La Jolla	*G. polyedra, Prorocentrum micans, Ceratium spp.*
17-20 May 68	La Jolla	*Ceratium* (2 spp.), *P. micans*
26 Febr.-11 Mar. 69	La Jolla	*G. polyedra, C. furca, C. fusus, P. micans,* Surface silicate concentrations 9-13 µg atom liter^{-1} in patches. Cell conc. up to 10^{11} cells liter^{-1}.
27 May 69	La Jolla-San Diego	*P. micans*
3 Sept. 70	Newport Beach	Dense, bright-red patches
22-28 Oct. 70	La Jolla	*G. polyedra*
9 Dec. 70	La Jolla	*G. polyedra, Peridinium spp.*
22-27 Aug. 71	San Diego Bay	*Mesodinium rubrum, C. furca*
24 Apr. 72	La Jolla	Mixture of dinoflagellates
8-28 Aug. 74	La Jolla	*G. polyedra, Fragilidium heterolobum,* red patches seen off SIO 9-18 Aug. 72
16 Sept. 74	El Segundo (Los Angeles area)	Dense red water in three bands 1-2 km apart on 1-5 km offshore. Bands parallel to the coastline.

Red water blooms have not been spectacular off La Jolla since 1965-66, as might be expected since the Food Chain Research Group set up for a major study in 1967. Casual observations since then have indicated occasional minor blooms here (Table 1) although more extensive blooms have taken place in the period in the Los Angeles area. However, considerable information on the composition and physiology of *G. polyedra* and other local bloom organisms, particularly *Gymnodinium splendens*, has developed from studies of laboratory cultures and the Scripps Institution's 70 m^3 volume "Deep Tank". Additional insight has accrued from a theoretical study of the role of semidiurnal internal tides in physically concentrating phytoplankton see Kamykowski (14) and from the MESCAL cruises off Baja California of the University of Washington upwelling program (see Walsh *et al.* (28)).

Role of Vertical Migration in Nutrient Assimilation

The vertical migration of *G. polyedra* and *Cachonina niei*, a dinoflagellate isolated by A. R. Loeblich, III from the Salton Sea, was studied in the Scripps Institution "Deep Tank" by Eppley, et al, (7). A pump and hose plumbed to a fluorometer were used to provide a continuous depth profile of chlorophyll fluorescence at intervals over the day-night light cycle. The chlorophyll fluorescense recordings served to indicate the depth of the migrating dinoflagellate populations in the 10 meter depth of the tank. *Gonyaulax polyedra* migrated downward at night and upward in the daytime, reaching maximum surface concentrations in the afternoon. Nitrogen-starved cells largely lost their ability to undergo the vertical migration. *Cachonina niei* behaved similarly but continued its migrations even in N-depleted water. Rates of migration were about 1 meter $hour^{-1}$. The downward rate of migration of a natural assemblage of *Ceratium spp.* was about 2 m hr^{-1}. Similar values were reported in the laboratory study of Hand *et al.* in 1965 (10) and earlier by Hasle in 1950 (12) for natural assemblages. It seems quite likely that these organisms may traverse a depth range of the order 10-15 meters over a 24 hour period.

The vertical migration of *G. polyedra* and other red water dinoflagellates has clear survival value in reaching water depths where nutrients are available. Holmes et al (13) found nitrate concentrations in a red water patch as follows: surface 0.07, 7 meters 0.13, and 12 meters 7.68 µg atom $liter^{-1}$. Armstrong and LaFond (3) found similar high nutrient levels associated with the thermocline in Southern California coastal waters. But this advantage would accrue only if the dinoflagellates can assimilate nutrients at night. Since circadian periodicity in nitrate, ammonium assimilation and in nitrate reductase activity was demonstrated in natural assemblages of marine diatoms with maximum activity about noon and minimal activity about midnight, this question is not trivial. Experiments were carried out in August, 1974 with a natural red water bloom dominated by *G. polyedra* (Table 2). Circadian periodicity in assimilation of nitrate is evident, as with the diatom crops, but the amplitude of the variation from day to night was less (1.5 to 3 fold, rather than 5-10 fold).

Table 2.

Circadian periodicty in nitrate uptake rate of *Gonyaulax polyedra* in samples of a red water bloom. The sample was maintained at 18°C on a light-dark cycle (lights on 0600, off 2030; light intensity approx 0.03 cal cm^{-2} min^{-1}). 13-14 August, 1974.

Time	Cells $liter^{-1}$	Nitrate uptake rate μmoles $liter^{-1}$ hr^{-1}	Nitrate reductase activity μmoles NO_2 formed $liter^{-1}$ hr^{-1}
Midnight	2.52×10^6,*	1.08	0.40
Noon	2.77×10^6†	2.96	0.62
Midnight	2.49×10^6	0.92	1.3
Noon	2.77×10^6	1.44	0.31

* Cell carbon and nitrogen contents were 3040 and 285 picog. $cell^{-1}$, respectively. Chlorophyll *a* content was 13.8 picog. $cell^{-1}$.

† Cell carbon and nitrogen contents were 2840 and 273 picog. $cell^{-1}$, respectively. Chlorophyll *a* content was 12.4 picog. $cell^{-1}$.

This, along with the enhanced assimilation rates of N-starved cells on exposure to nutrients, suggests that the migrating dinoflagellates may indeed be able to assimilate ample nutrients for sustained growth at night if they can reach depths where nutrients are available. If they cannot, it is expected that the ability of *G. polyedra* to migrate will be lost and the bloom will dissipate or that the dinoflagellates will persist at greater depths where light and nutrients are available.

It is interesting to note that the amplitude of the circadian periodicity in chlorophyll fluorescence was also much less in dinoflagellates than was found with diatom crops in Chesapeake Bay by Loftus and Seliger (19).

Another physiological idiosyncracy of the dinoflagellates we have studied, *Peridinium triquetrum* and *G. polyedra*, is the apparent lack of repression of nitrate reductase by ammonium (11). In marine diatoms the enzyme disappears rapidly from cells growing with ammonium ion present at concentrations greater than about 1 μg atom $liter^{-1}$, even in the presence of nitrate. In these dinoflagellates, however, it appears that the enzyme is synthesized whenever nitrate is present and persists even in its absence and when growth is at the expense of ammonium. Further, natural red water samples of *G. polyedra* have not shown the marked circadian periodicity in enzyme activity observed with diatoms (Table 2). This may provide additional survival advantage for

vertically migrating dinoflagellates that encounter nitrate only in the descent to nutrient-rich water at night in that their enzymatic machinery for nitrate assimilation is already present and need not be synthesized afresh each night.

Loftus *et al.* (19), working with Chesapeake Bay blooms, have found species differences among dinoflagellates in the kinetic parameters for the photosynthetic utilization of CO_2. The high pH values (8.5 or so) observed in daylight in dense red water patches here suggest that similar physiological adaptations may be expected in our local species.

The ability of red water blooms to sustain themselves in waters with depleted nutrients at the surface (except for silicate that is not apparently assimilated by dinoflagellates and was found at elevated levels at the surface in red water blooms by Armstrong, *et al.* (4), see Table 1, thus finds an explanation. Nonmigrating phytoplankton in bloom proportions would lack this advantage.

Diatom blooms off Southern California are also related to upwelling and other nutrient inputs and might be expected if vertical water mixing is of sufficient intensity to bring nutrients to the surface. Dinoflagellate blooms might be more likely to develop if the upwelling intensity were such that nutrient enrichment did not reach the surface but rather 5-10 m depth, with an overlying steep, shallow thermocline. The dinoflagellates could then take advantage of their vertical migration capacity.

Physical Concentration Mechanisms and Vertical Migration

Ryther (25), Pomeroy et al (23), Seliger et al (26), Loftus et al (21), and other authors have pointed out that some mechanism of physically concentrating dinoflagellates at the surface must operate in red water blooms to account for the high cell densities observed. Nutrient concentrations available in the water are insufficient, even in the entire water column from surface to bottom, to account for the nutrient content of the cells. Kamykowski (14) has shown by mathematical analysis that semidiurnal internal tides will strongly concentrate organisms that carry out vertical migrations through the thermocline or other density gradient if the waters above and below the discontinuity move with different velocities (Figure 1).

Kamykowski's model provides a particularly attractive hypothesis for explaining the surface aggregations of dinoflagellates observed in local red water blooms for several reasons. 1) It provides the several-fold concentration factor needed. 2) It predicts that the red water patches will occur in bands oriented parallel to the shore (in September, 1974 we observed three such bands about 1-2 kilometers apart in Santa Monica Bay) 3) The model requires both vertically migrating organisms and a strong, shallow discontinuity layer, consistent with earlier observations, in order for organisms to aggregate. 4) The model also explains why blooms would be restricted to shallow, inshore waters and why we often observe deep maxima (15-40 m) in chlorophyll fluorescence profiles due to dinoflagellates, without surface blooms, in the

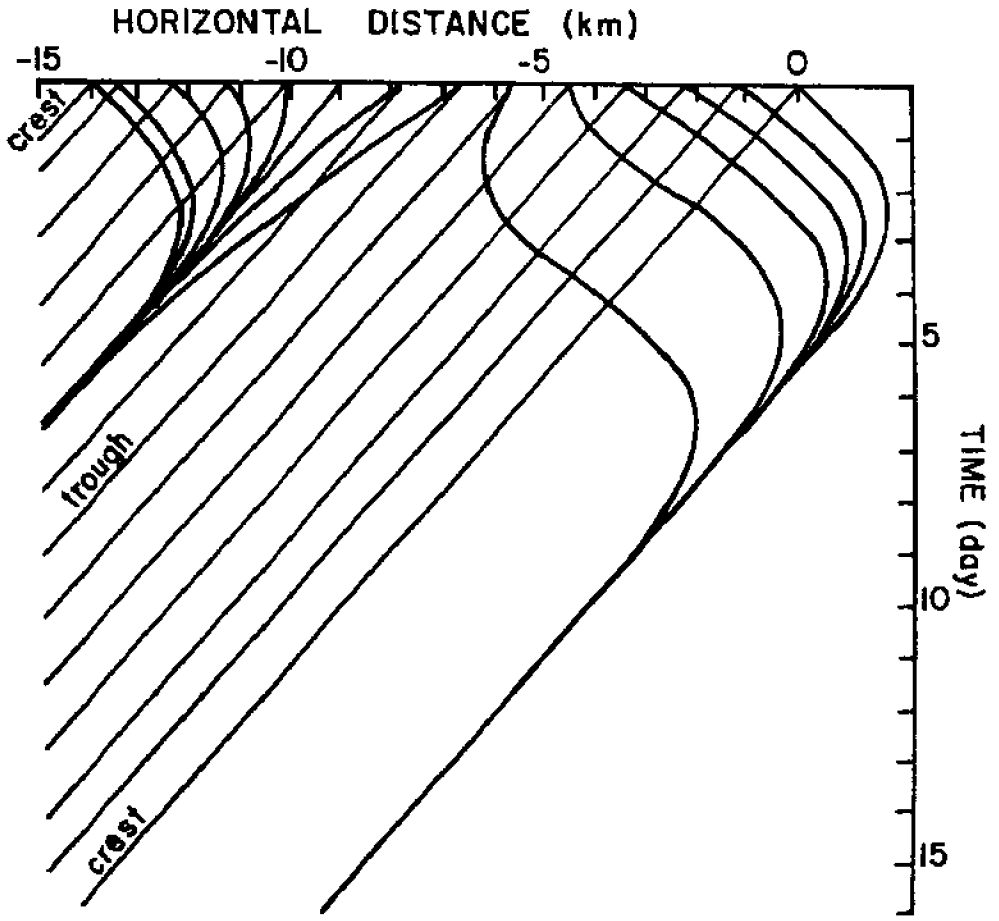

Figure 1. Surface patterns of thermocline-crossing phytoplankton undergoing diurnal vertical migrations. The surface patterns perpendicular to the shore (X-axis) are sampled at 24 hr. intervals for 16 days. The oblique lines connect the spatial positions of the various initial sampling points along the wave length of the semidiurnal internal tide with the time of each simulated sample. A very strong convergence occurs with vertically migrating phytoplankton effectively forming one band per wave length near the trough by the 9th day. Simulated conditions: internal wave amplitude 4.5 m centered at a mean thermocline depth of 10.5 m; phytoplankton swimming velocity 1.0 m hr^{-1} above the thermocline, 0.5 m hr^{-1} below the thermocline, on a 12-hr day : 12 hr night cycle. From Kamykowski (14).

absence of steep, shallow thermoclines. In passing it may be noted that local blooms may be of such density as to increase the surface temperature a degree or more via light absorption and this serves to positively reinforce a steep, shallow thermocline.

Needs for Further Understanding

It is clear that the above arguments and hypotheses are useful toward understanding red water blooms off Southern California. But from this distance it is not clear to what extent, if any, they may apply to blooms of

Gymnodinium breve off Florida (27) or to blooms of other *Gonyaulax* species in temperate waters such as Puget Sound or off New England, or for that matter to blooms in Japanese inland waters or in Southern California harbors and shallow embayments. They may be most pertinent to coastal regions with eastern boundary currents, as the Benguela (9).

Furthermore, many questions remain for the region in question. For example, *Gymnodinium splendens* has not been recorded as a dominant species in local blooms, although it is often present and may reach moderate cell densities and form nearly unialgal chlorophyll maxima at 15-30 m depth according to Lasker (18) and Kiefer and Lasker (15). Forward (8) has investigated the phototactic responses of this species and reported that it is positively phototactic (only) and that the light sensitivity of the response shows circadian periodicity with a maximum sensitivity in the first 4 hours of the light period. A vertically migrating bloom of this species was studied in the Gulf of California by Kiefer and Lasker (15).

Also lacking is an explanation for the apparently downward directed swimming at night observed by Kiefer and Lasker for *G. splendens*, for *G. polyedra* and *C. niei* in the deep tank and for *Ceratium spp.* in a natural bloom off Newport Beach California (7,15). Is there a geotactic or a very light sensitive negative phototaxis at night or is the apparently directed swimming downward the result of accelerated sinking? Clearly this downward swimming is equally as important to the hypothesis as the positive phototaxis. The inoculum of dinoflagellates at the commencement of a bloom remains for speculation but may play an important role in determining species dominance. We need more information of which species form resting cysts that settle to the bottom and the conditions under which they release motile vegetative cells.

Importance of Dinoflagellates to the Food Web

First feeding anchovy larvae (*Engraulis mordax*) have been supported in the laboratory with *Gymnodinium splendens* as the food organism. On shipboard the larvae successfully fed only in water samples taken from the chlorophyll maximum, observed at a few meters depth when the chlorophyll maximum contained 20-400 food organisms per ml of minimum cell diameter 40 µm (18). In March and April, 1974 the chlorophyll maxima observed in the nearshore waters off Southern California were composed almost exclusively of *G. splendens* and permitted successful larval feeding (18). Juvenile copepods also aggregate in such chlorophyll maximum zones at depth according to Mullin and Brooks (22) and Anderson *et al.* (2) and require relatively high concentrations of food particles for maximum rates of feeding.

Corresponding concentrations of the chain diatoms *Chaetoceros* sp. and *Thalassiosira* sp., of the appropriate physical size, were not utilized by the anchovy larvae (18), although such diatoms provide adequate food for the copepod *Calanus helgolandicus* (22). It appears as if dinoflagellates 40 µm or greater in diameter and present in concentrations of at least 2×10^4 liter^{-1} may

play a special role in the early growth of the northern anchovy off Southern California. The dinoflagellate species characteristic of red water blooms meet these requirements and their role in the food web of larval anchovy is under active investigation.

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OCEANOGRAPHIC FACTORS ASSOCIATED WITH NEW ENGLAND RED TIDE BLOOMS

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ABSTRACT

The coastal region from Rye, New Hampshire to Cape Ann, Massachusetts, the site of *Gonyaulax tamarensis* blooms in 1972 and 1974, is described in terms of its hydrographic, biological and meteorological characteristics. The influence of the Merrimack River is considered. Oceanographic features associated with these and other red tides are discussed. Actual salinity and temperature distributions are considered less important than: (1) a sequence of coastal upwellings which enrich the surface waters; (2) a period of warm dry weather which increases thermal stratification and results in increased concentrations of sewage-derived nutrients in river water; and, (3) rainfall which assists in the movement of dinoflagellates into the estuaries. Diurnal vertical migration is proposed as a mechanism for the buildup of a near unialgal population of *G. tamarensis* within the estuaries. The potential impact of a proposed nuclear power plant on future red tides in the region is discussed.

INTRODUCTION

Red tides develop in specific locations in response to the presence of oceanographic conditions conducive to their growth. Due to the uniqueness of each red tide, in terms of major species, location and time of year, no single set of factors is expected to be responsible in each case.

Environmental conditions, apparently optimal for the proliferation of *Gonyaulax tamarensis*, were present in northern New England waters during 1972 and 1974. *Gonyaulax tamarensis* can cause paralytic shellfish poisoning and, although present in small numbers in previous years, never developed into a serious health hazard prior to the red tide outbreak in September 1972. This paper describes the physical-chemical conditions and phytoplankton populations characteristic of the Merrimack River coastal region, the apparent point of origin of the recent red tide blooms. The specific events which preceded and accompanied red tide blooms in New England in 1972 and 1974 are discussed and compared with those described from previous red tides. The influence of proposed environmental modifications of the Merrimack River coastal region on future red tides is also considered.

Description of Northern Massachusetts-New Hampshire Coastal Zone

The coastal region from Rye, N. H. to Cape Ann, Mass. (Fig. 1) which my students and I have been studying since August, 1971*, appears to be the site of the initial development of the recent New England Red Tides. Since the oceanographic characteristics of this region are evidently suitable for the

* These studies have been supported by funds from the UNH Sea Grant-Raytheon Program, NOAA Environmental Research Laboratory and the Massachusetts Department of Natural Resources.

development of red tides, they are considered in detail in this section.

The coastline of the region consists mostly of low, sandy barrier beaches which protect extensive *Spartina* marshes and estuarine areas. The coastal region contains the Merrimack River, the major river between Cape Cod and Cape Elizabeth (Saco River). More than 1.3 million persons live within the 5010 square mile (13,000 km^2) Merrimack River drainage basin (2) which comprises eight percent of the entire drainage basin of the Gulf of Maine (6). The Merrimack River dominates the physical, chemical and biological characteristics of the surrounding coastal region.

The magnitude of several adjacent rivers can be compared with the Merrimack River on the basis of mean flow data from 1942, (14) which showed

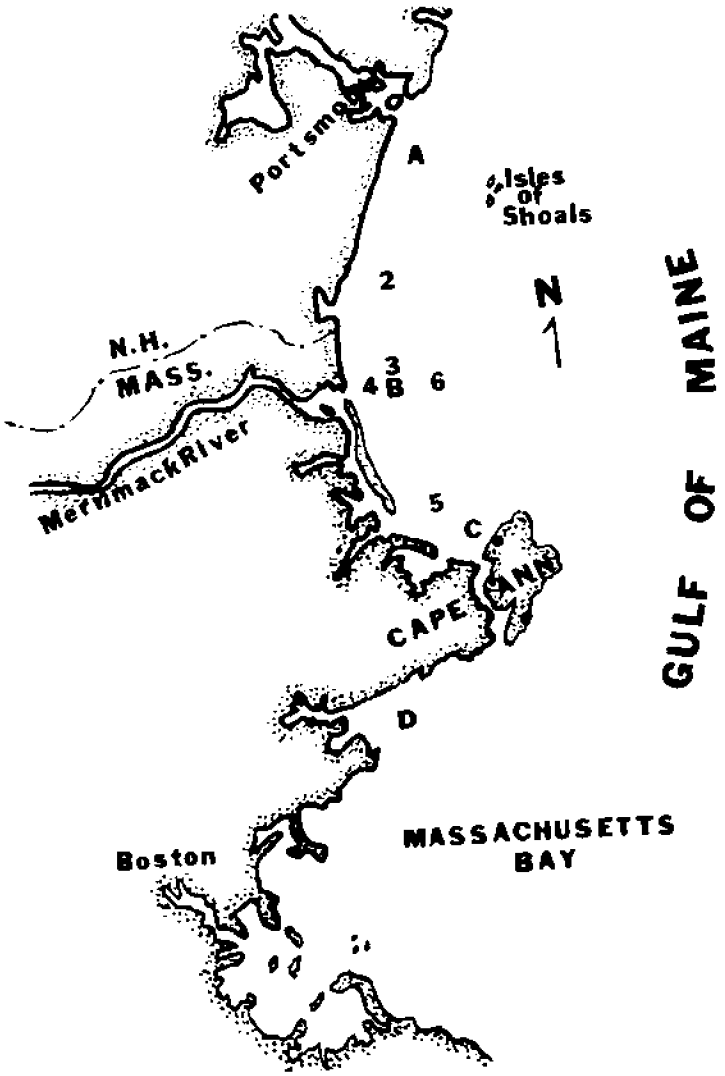

Figure 1: Map of Northern Massachusetts and New Hampshire Coastal Region. A. Rye, N. H.; B. Newburyport, Mass.; C. Annisquam, Mass.; D. Salem, Mass. Stations 2-6 occupied 1973-74.

the Piscataqua River with 491 sec-ft (8.3×10^5 1/m), the Ipswich River with 163 sec-ft (2.8×10^5 1/m), and the Merrimack River at Lowell, 5910 sec-ft (100×10^5 1/m). Average annual rainfall in the region is 43 inches (1.09 m) (3) which is distributed rather uniformly over the entire year. River runoff exhibits a definite seasonal cycle coinciding with the period of major snow melt during the spring (Table 1).

Table 1

Water Discharge Data of Merrimack River Below Concord River at Lowell, Mass. in CFS (cubic ft. per second)

Date	1945/46[1]	1970/71[2]
October	4266	3093
November	6047	4257
December	10041	4969
January	8856	4169
February	7148	5235
March	19200	9843
April	9739	21700
May	11869	16510
June	8536	3511
July	2695	1567
August	4181	2109
September		2074

Average discharge for 48 year period ending 1971 = 7055 CFS

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Winds follow the same general patterns as elsewhere in the Gulf of Maine (14) (15) mostly from the west, with considerable seasonal variation: in Fall and Winter, mainly from north to west; in Spring, winds are variable and, during the Summer, winds blow from the southwest and southeast quadrants.

Non-tidal currents tend to move the surface coastal waters in the region counter-clockwise, to the southwest along the coast; bottom waters move either to the southwest or toward the shore according to Bigelow (6), Day (10), and Graham (15). Graham also noted that bottom water which

originated in the northern Gulf of Maine moved during the summer through Jeffrey and Scantum Basin and toward the shore near the mouth of the Merrimack River.

Winds can generate surface currents up to 2% of wind velocity, and consequently, non-tidal currents are stronger in the fall and winter because of higher wind velocities from the northern quadrant. Currents are increased during the spring due to increased snow-melt runoff piling up along the shoreline. Winds from the southern quadrant, common in the late spring and summer, reduce and even reverse the slow-moving, counter-clockwise, surface current along the coast. The most prominent feature of the water circulation along the western coast of the Gulf of Maine is upwelling (15).

Mulligan and deLara (23) have recently reviewed the phytoplankton and hydrographic conditions in the region (Table 2).

Table 2

Characteristics of Coastal Stations Located 1-3 Miles (1.6 - 4.8 kms) from Shore between Cape Ann, Mass. and Rye, N. H. (Mulligan and de Lara, 1974)

Salinity (°/oo)	9.3 - 33.3
Temperature (°C)	1 - 18.1
Depth of euphotic zone (m)	5 - 20
Chlorophyll *a* (mg. m^3)	0.3 - 15.5
N-NO_3 mg at. l	0.00 - 9.64[1]
P-PO_4 mg at. l	0.050 - 1.175[1]

[1]data from Clay (unpublished)

Salinities measured from August 1971 to July 1972 ranged from 9.3 to 33.3 °/oo. Highest salinities were recorded in January and February. Manohar-Mararaj and Beardsley (21) estimated that the Merrimack River contributed over 90% of the fresh water in Massachusetts Bay. Ice-flows and low salinity surface water from the Merrimack River were observed six miles offshore and could be seen moving southeastward around Cape Ann into Massachusetts Bay (Mulligan, unpublished). Consequently, the surface waters off and to the south of the Merrimack River displayed the lowest salinities. During the springtime, the entire water column off Annisquam had reduced salinites when compared with conditions present at coastal stations to the north.

Water temperatures ranged from 1-18.1 °C over this period and were slightly lower in the northern portion of the region throughout the year. The coastal waters to the north of the Merrimack River (to a depth of 20 meters) were generally well mixed. Thermal stratification developed during the warmer months and became well established, especially at the mouth of the Merrimack River and in the waters to the south.

The depth of the euphotic zone varied from 5 - 20 meters with the lowest transparencies occurring during the spring and fall. Silt contributed by the Merrimack River was largely responsible for the minimal spring transparencies.

Phosphate and nitrate profiles were obtained at five coastal stations from October, 1973 - April, 1974 (Clay, unpublished). The concentrations ranged from 0.050 - 1.175 µg at $P\text{-}PO_4$/l and 0.00 to 9.64 µg at $N\text{-}NO_3$/l. Although the concentrations of both inorganic compounds were reduced in the spring, nitrate values reached undetectable levels during plankton blooms. Vacarro (38) has shown that $N\text{-}NH_4$ is abundant in the North Atlantic and may be an important source of nitrogen during certain times of the year with maximum concentrations occurring in the summer.

Chlorophyll *a* concentrations in the region reached a high of 15.5 mg/m^3 in mid-April, 1972 in the surface waters south of the Merrimack River. Although chlorophyll *a* concentrations were especially high during the spring and fall, some high values were also noted during the summer. Increased concentrations of chlorophyll *a* in deep waters occurred simultaneously with a decline in surface pigment concentrations. Highest chlorophyll *a* concentrations in May (10.0 mg/m^3) were present in bottom waters off the Merrimack River. Fluorimetric measurements of chlorophyll *a* concentrations obtained by Wells (unpublished) in the spring of 1974 ranged from 0.4 - 9.6 mg/m^3, and showed that the algal population reached maximum levels in the inshore waters in March and at a later date at the offshore stations. From March to May, 1974 the highest concentrations of chlorophyll *a* were often found in the bottom waters.

This coastal region from Rye, N.H. to Cape Ann, Mass. is especially important as it has been identified by Bigelow (5) as one of two major points of origin for the spring diatom bloom in the Gulf of Maine. Although some discrepancies were noted, the phytoplankton population of the region showed general agreement with the earlier descriptions of Bigelow. Diatoms are the major group of phytoplankton throughout the entire year.

The spring bloom occurred in April, 1972 with *Chaetoceros debilis*, *C. compressus* and *Thalassiosira nordenskioldii* the dominant diatoms at 726, 265 and 59 cells/ml, respectively. Highest concentrations occurred in the southern portion of the region. *Skeletonema costatum* was the major summer form reaching a maximum concentration of more than 2000 cells/ml in the surface waters at Annisquam in late June, 1972. This organism comprised 92% of the phytoplankton population at that time. *Leptocylindrus danicus*, *L. minimum* and *Guinardia flaccida* were the dominant fall organisms. *Thalassionema nitzschioides* occurred throughout the year reaching a maximum population of 240 cells per ml in October, 1971. Dinoflagellates generally were a minor part of the phytoplankton in the coastal region. Even at the height of the September, 1972 red tide bloom, the maximum coastal population of *Gonyaulax tamarensis* observed in the water column was 132.5 cells/ml. Freshwater forms were frequently collected off the Merrimack River and at

Annisquam. The phytoplankton populations were greatly reduced during the winter months.

Phytoplankton surveys were carried out in the region in 1973 - 1974 by Beauregard, Parker and Kouloheras (unpublished). Parker and Kouloheras reported an unusual spring population which was similar to that recorded in Massachusetts Bay during the same period by Parker and Mulligan. (26) and Mulligan and Wessel (24). *Chaetoceros socialis* (218 cells/ml) was the major diatom in the first portion of the bimodal spring bloom which occurred from Feb. 28 to March 28, 1974. *Phaeocystis pouchetti* was dominant at all stations on April 26, 1974, reaching a population maximum of 6900 cells ml. Parker describes the 1974 spring bloom in the Merrimac River coastal region as developing sequentially from the southwest to the northeast, supporting the theory that this region is an initial site of the spring bloom in the Gulf of Maine. On the last date that the entire phytoplankton population was analyzed, April 25, 1974, the concentration of *Skeletonema costatum* had reached 92 cells/ml.

Review of Gonyaulax tamarensis Red Tides in Northern New England

There have been three major red tides in this region within recent years: clam flats in northern New England were closed due to the presence of toxic levels of paralytic shellfish poison producing organisms in September, 1972 and in May and August of 1974. Although data on population distribution of the causative organisms are incomplete, the events which preceded each red tide appear to follow a specific pattern.

Gonyaulax tamarensis was noted in small numbers in samples collected in Boston Harbor and north of Cape Ann during the summer of 1972 (22). The organism increased in population north of Cape Ann but continued at low levels to the south. Since both regions were subject to the same meteorological conditions* , evidently these meteorological conditions, although unique (August, 1972 was one of the dryest on record since 1885) were not sufficient to account for the 1972 red tide. Mulligan (22) suggested that an upwelling which was noted on August 9 at Annisquam was perhaps the necessary additional ingredient for the development of the red tide to the north of Cape Ann.

The 13 day period from August 20 - September 2, 1972 was relatively dry and had an average air temperature 3.0 °C above normal. A heavy rainfall (3.8 inches; 9.7 cm) occurred on September 3. The clam flats in northern Massachusetts were closed on September 14, 1972 while those in New Hampshire and Maine were closed on subsequent days due to a high level of *Gonyaulax tamarensis* toxin in the clams.

* Meteorological records from U. S. Weather Station Blue Hills Observatory, Milton, Massachusetts.

Chlorophyll *a* transects through the region on August 12 and September 17, 1972 showed a population maximum first in southern waters and later in waters to the north, suggesting a northward movement of the population (Fig. 2). Lowest salinity surface water was observed along the coast to the north of the Merrimack River station on September 17, 1972.

When the *G. tamarensis* population was mostly in bottom water at Annisquam on September 17, 1972, a possible indication of nitrogen limitation (11), the population at Rye, N.H. appeared in a healthy condition, concentrated at the surface (Table 3). On this date, Rye surface water had a temperature of 15.0°C and a salinity of 29.4 °/oo, while Annisquam surface water registered 15.5°C and 29.7 °/oo. The highest proportion of *G. tamarensis* cysts was noted on this date in the surface waters at the mouth of the Merrimack River where the temperature was 15.8°C and the salinity 27.1 o/oo. Although Prakash et al. (31) suggest low temperature as a causative factor for cyst formation, some other factor(s) must have been operating in this instance. The temperature and salinity values registered north of Cape Ann were similar to those recorded to the South of Cape Ann where no proliferation of *G. tamarensis* developed. *Skeletonema costatum* was the dominant organism in the surface waters along the coast throughout the period of the red tide bloom

A phytoplankton and hydrographic study of the region north and south of the Merrimack River was also conducted in 1973-74. Apparent upwellings were noted in the spring throughout the region; the first portion of the spring diatom bloom on March 28, 1974 occurred simultaneously with increased

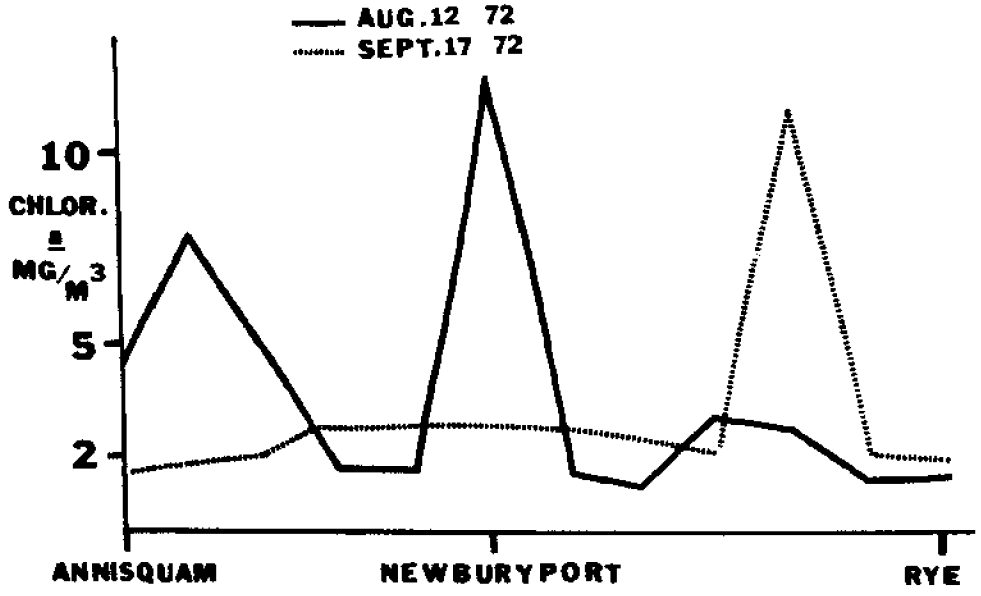

Figure 2: Chlorophyll *a* concentration (mg/m^3) in surface waters 1 mile from shore during 1972 Red Tide.

Table 3

Characteristics of Northern New England Coastal Waters During the Red Tides of 1972, 1974

Date	Location	Salinity o/oo	Water Temperature °C	Air Temperature °C** + 3.0	Chlorophyll *a* mg. m3	*Skeletonema costatum* cells/ml	*Gonyaulax tamarensis* cells/ml	Condition, *G. tamarensis* cells
Sept. 14 - 15, 1972	*							
Sept. 17 1972	Rye, surface	29.4	15.0		1.8	313.9	119.8	Healthy
	Newburyport, surface	27.1	15.8		2.6	210.5	70	nearly half encysted
	Annisquam, surface	29.7	15.5		1.5	670.0	26.8	unhealthy
	Annisquam, bottom	30.3	11.0		1.3	19.1	132.5	few cysts, not migrating, perhaps nutrient limited
	Salem Harbor, surface	29.4	16.2		12.5	2760.0	5.1	apparently healthy
May 15, 1974	Essex, surface	26.5	9.5		4.5		6.0	apparently healthy
May 31, 1974	*			+ 2.6				
June 4,	Newburyport, surface	21.9	12.4		-		-	
	Essex, surface	24.3	12.4		-		-	
	Annisquam, surface	24.7	11.9		-		-	
Aug. 26,	*			+ 2.3				

* Dates of closing Northern New England (*Mya arenaria*) clam flats due to high levels of *Gonyaulax tamarensis* toxin.

** Average air temperature during 10 day period preceding Red Tides recorded at Blue Hill Observatory, Milton, Mass.

concentrations of $N\text{-}NO_3$ and $P\text{-}PO_4$ (Clay, unpubl.). *Gonyaulax tamarensis* was observed (6 cells/ml) in the surface water off Ipswich Bay on May 16, 1974. Phytoplankton were not counted after this date.

Surface temperature and salinity off Ipswich, Mass. on May 16, 1974 were 9.5°C and 25.6 °/oo respectively. May 22, 1974 ended a 10-day period when rainfall was practically zero and the air temperature averaged 2.6 °C above normal. It is likely that both the temperature and salinity of the coastal waters increased during this 10-day period. The northern New England clam flats were closed on May 31 due to toxic levels of *Gonyaulax tamarensis*. On June 4, after 13 days of cold rainy weather, the surface temperature and salinity off Ipswich Bay were recorded as 12.4°C and 24.3°/oo. It appears therefore, that the coastal *Gonyaulax tamarensis* population of May, 1974 was developing at or in excess of temperatures of 9.5 °C and salinities of 25.6 °/oo. Nitrate nitrogen and reactive phosphorous readings in the water column at most stations located 1-3 miles (1.6-5.4 km) from the coast in the spring of 1974, showed $N\text{-}NO_3$ dropping to undetectable levels and $P\text{-}PO_4$ declining to 0.15-0.25 µg-at/1 on April 25 (Clay, unpublished).

The northern New England clam flats were again closed to digging on August 26, 1974. August was a relatively dry month and until August 17, the air temperature averaged 0.7 °C above normal. A heavy rainfall on August 17, was followed by a warm 10-day period when the average air temperature was 2.3 °C above normal. A very light rain occurred on August 23, 1973. No hydrographic or phytoplankton samples were collected during this period but on the basis of previous records, surface temperatures were expected to range from 16-18°C and the salinity values to approximate 30 °/oo.

DISCUSSIONS AND CONCLUSIONS

Although the three New England red tides present somewhat of a mystery, nonetheless, they possess certain features in common.

1. They occurred during the period when *G. tamarensis* has been reported to be present in the Gulf of Maine (16).
2. They occurred in a region in which upwellings are a dominant feature.
3. They closely followed major phytoplankton blooms (Sept. 1972; May 1974).
4. They followed periods of reduced rainfall and warm weather which stratified and stabilized the water column and delivered drainage water more concentrated in organic and inorganic substances.
5. They occurred during periods when water currents are minimal and when surface waters are most likely to move in a northerly direction.
6. They developed in coastal waters and moved into estuaries where further growth and concentration occurred.

The September 1972 and August 1974 red tides of *Gonyaulax tamarensis* occurred during the warmest period of the year as is the typical case (25, 31).

The May 1974 red tide is not without precedence since similar blooms occur in European waters during the spring (41).

In September 1972 and May 1974 red tide blooms occurred when the water temperatures were 15 °C and 9.5 °C and the salinities were 29.4 and 25.6 °/oo. During the August, 1974 red tide, salinity and temperature conditions were probably similar to those in September 1972. These values approximate those reported to accompany Bay of Fundy red tide blooms; *Gonyaulax tamarensis* dominates when the surface waters have a temperature of 11.3 °C and a salinity of 31.9 °/oo (Prakash, 1967). Growth at all these temperatures and salinities can occur under laboratory conditions but optimal laboratory growth occurred at 15-19 °C and 15-23 °/oo (29). However, clones of this organism are likely present in nature which exhibit best growth over a wide range of temperatures and salinities. Clonal differences of *G. tamarensis* are being investigated in the laboratory of T. Smayda at the Narragansett Marine Laboratory (pers. comm.).

That salinity and temperature are not the factors which control the initiation of the red tide blooms is clear from the fact that *G. tamarensis* does not reach bloom proportions each year, and even when it does bloom, the blooms are geographically restricted in scale and do not occupy all regions where apparently appropriate salinities and temperatures are present. Since the region to the south of Cape Ann contained a seed population of *G. tamarensis*, was subject to the same warm, dry period, and supported blooms of *Skeletonema costatum*, the presence of these conditions is evidently not sufficient to initiate red tide blooms.

The coastal region of the Gulf of Maine is characterized by frequent upwellings (15). Upwellings were shown by Mulligan (22) to occur prior to the September 1972 red tide. Upwellings bring organic and inorganic substances into the euphotic zone. The blooms of phytoplankton which accompanied or preceded the red tide blooms also contributed a great deal of dissolved and particulate organic matter. Ignatiades (19) showed that *Skeletonema costatum*, (the major organism in the summer plankton in the region), growing under field conditions in July, contributes 15.9-18.5% of its organic production to the surrounding waters. Carlucci and Bowes (7) demonstrated that *Skeletonema costatum* released utilizable quantities of the vitamins thiamin and biotin. These substances stimulate the growth of *G. tamarensis* according to Prakash (29).

Gonyaulax polyedra, which causes red tides on the west coast of the United States, has been shown by Walsh *et al.* (39) to increase in concentration in newly upwelled water, and to be replaced by a typical diatom flora as the upwelled water ages. The red tides in the vicinity of the Peru current are stimulated by a reduction in upwelling intensity and replace the typical diatom flora (18).

Actual nutrient concentrations are seldom measured during red tide blooms, and even when available, represent the differences between the amounts supplied and the amounts removed and provide little information on

phytoplankton productivity Holmes *et al.* (17) state that the nitrogen concentration within the water column is not sufficient to support U. S. west coast red tides and suggested some vertical and horizontal concentration of the population occurred. The nutrient data of Clay (unpublished) from the Merrimack River region, spring of 1974, show more rapid depletion of $N\text{-}NO_3$ than $P\text{-}PO_4$. Although Clay showed $N\text{-}NO_3$ concentrations were generally at undetectable levels in late spring, Vacarro (38) indicated that $N\text{-}NH_4$ concentrations in the surface waters of the North Atlantic are highest in the summer months, and may be an important source of nitrogen during that period.

Although upwelled water may provide an initial supply of nutrient-rich water, subsequent and continuous enrichments are contributed by river water. For example, many of the 1.3 million persons within the Merrimack River drainage basin are serviced by sewage systems which deliver waste water to the Merrimack River. Periods of reduced rainfall, which preceded all three red tides, result in river runoff water with higher concentrations of waste water constituents. In addition, the reduced volumes of stream water reach higher temperatures during these warmer than average periods. Therefore, lenses of nutrient-rich, warm river runoff are continuously contributed to the coastal waters from the estuaries.

The coastal population of *G. tamarensis* increased under conditions of increased nutrients and increased stability of the heated, upwelled water. Wyatt and Horwood (42) have postulated that increased vertical stability is an important prerequisite for red tide development. Plesset and Winet (27) noted that vertical stability is a requirement for bioconvection, a process that aids bloom formation in flagellated algae.

Rainfall aids the movement of red tide organisms into the estuaries where further development occurs. Rainfall also contains inorganic nitrogen which is often the critical limiting factor to algal growth in coastal marine waters according to Ryther and Dunstan (35). As the rainfall moves over and through the soil, organic and inorganic substances are dissolved and delivered to the population of *G. tamarensis* now situated within the estuaries. Both the reduced salinities and increased temperature of the estuaries and the increased concentrations of organic substances should stimulate growth of *G. tamarensis* as observed in laboratory by Prakash (29) and Prakash and Rashid (30) and in field studies with other *Gonyaulax* spp. by Conover, (8).

It should be noted that when the red tide populations were developing along the coast, they were not, numerically, the major constituent of the phytoplankton. It was only when *G. tamarensis* formed surface streaks or moved into the estuary that a near unialgal population developed. This organism probably is capable of physiological responses similar to those of *G. polyedra*, which Eppley *et al.* (11) have shown to execute large (10 m), diurnal vertical migrations under proper nutrient conditions. The migrations likely result in more rapid cell increases within the estuaries than would occur entirely from cell division. Downward vertical migrations place the organisms

into the landward flowing salt wedges which ultimately deliver them to the heads of the estuaries. These migrations help to explain the tremendous rise in *G. tamarensis* numbers relative to those of *Skeletonema costatum* within the estuaries. Red tide organisms have also been suggested to favor interfaces of different water masses by Ryther (33), Pomeroy *et al.* (28), Lefevre and Grall (20), and this mechanism may also assist in the further augmentation of the estuarine populations.

The offshore red streaks or patches which were observed in mid-September, 1972 in the coastal waters from Cape Ann, Mass. to the Isles of Shoals, N.H. by Mulligan (22) were populations of *G. tamarensis* concentrated either (1) in situ, along the complicated upwelling boundary region (37, 34, 13) or (2) within water masses of river origin (36). Ford (14) has described the formation and movement of pools of Merrimack River water to 30 miles (48 km) from shore. He also showed that a strong onshore wind of 2 days duration in August had vertically mixed offshore waters and crowded new estuarine water along the shore (a red tide concentration mechanism suggested by Ryther (33).

During typical mid-summer days at the seashore (in the absence of strong winds associated with weather fronts) gentle breezes blow over the cooler waters toward the land. Since the dinoflagellate population, if present, would be in surface waters at this time, they would be moved shoreward. At night, when the winds blow offshore, the dinoflagellates have migrated downward in the water column and are not influenced by the offshore water movement at the surface.

It appears that, of all of the potential red tide causative factors examined, only coastal upwellings which occur with regularity at Annisquam and to the north of Cape Ann during the period optimal for growth of *Gonyaulax tamarensis*, were essential to the development of northern New England red tides. Future red tide studies should examine the movement of water masses during spring and summer. Also, changes in phytoplankton composition and distribution within the water column must be carefully monitored to determine estuarine and coastal concentration mechanisms.

Figure 3 attempts to summarize the conditions which led to the development of the New England red tides. Since large concentrations of *G. tamarensis* were not recorded prior to September 1972, but have appeared twice since that time, the initial bloom could have sensitized the region for future red tides. Larger quantities of cysts of *Gonyaulax tamarensis* are now present in the region as a consequence of the 1972 red tide. The reservoir of dormant cells from the 1972 red tide should permit the delivery of a more concentrated initial inoculum of *G. tamarensis* to the surface waters, thereby facilitating the 1974 and subsequent red tide blooms.

A large nuclear power plant is proposed for the Merrimack River coastal region which could have a profound effect on the frequency of future red tides. Plans call for the outflow to be situated about 6 miles (9.6 kms) to the north of the Merrimack River at Seabrook, New Hampshire, 4000 feet (1.2 km)

offshore of the largest, most productive, soft shelled-clam (*Mya arenaria*) flats in New Hampshire. This proposed power plant will remove and reintroduce into the environment 780,000 gallons (2.9×10^6 liters) of sea water per minute which will have been heated 22°C above the ambient sea water temperature. The volume of water from this plant equals 1/4 of the average flow of the Merrimack River, 3.5 times the average output of the Piscataqua River, and more than 10 times the average output of the Ipswich River. This power plant discharge will be continuous; will always be much hotter than that of the Merrimack River; and during warm periods of the year will even exceed the discharge volume of the Merrimack River.

The continually added hot bottom water will eventually surface and intensify thermal stratification and since living organisms present in the intake water will be cooked and killed, the discharge water will contain higher concentrations of organic substances than are present in surrounding waters. The discharge from the power plant will produce a continuous upwelling of heated, nutrient rich water. Because of the sensitivity of the region to red tide

Figure 3

Sequence of Events Leading To The Development of New England Red Tides

Coastal upwelling ⟶ bring cysts of *G. tamarensis* into euphotic zone; provide nutrients for growth. Coastal water mass moves north from Cape Ann in the late spring and summer.

↓

Warm, dry period ⟶ provides stratified water column; causes increased temperatures and increased concentrations of nutrients in river water.

↓

Rainfall ⟶ increases movement of coastal populations of *G. tamarensis* into estuaries where growth and mechanical concentration of populations through vertical migration occurs; delivers dissolved organic and inorganic substances which promote growth of *G. tamarensis*.

↓

G. tamarensis removed from water and concentrated by filter feeding organisms.

↓

New England red tide (paralytic shellfish poisoning)

Figure 3: Sequence of events leading to development of New England Red Tides.

blooms, a nuclear power plant designed to release massive quantities of heat and nutrients to the waters which surround it, should probably be either located elsewhere or redesigned.

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RED TIDES IN THE LOS ANGELES-LONG BEACH HARBOR

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ABSTRACT

The major organism producing red tides in the San Pedro Bay area is *Gonyaulax polyedra*. Extracts from laboratory cultures are toxic to fish, but assays on similar extracts from cells harvested from red tides show a very wide range of toxicity.

Out studies on red tides are made for predictive purposes and in order to establish the sequence of events leading to red tides in a highly developed industrial-commercial area. The blooms can follow certain weather conditions which cause stirring of the shallow harbor and resuspension of organic detritus from the sediments. There is also an apparent relationship between the patterns of waste discharge and the appearance of red tide. Increased discharge at the opening of the anchovy season is followed in some conditions by a bloom in the outer harbor. Regular monitoring is carried out of coliform bacteria and total plate count of marine microbes. Of particular interest are the recent measurements of bacteriophages present in greater numbers than anticipated and which are being investigated for their role in bacterial breakdown and resultant mineralization processes. Controlled laboratory cultures of dinoflagellates are used to test the influence of compounds from the natural environment and of breakdown products from cultures of the marine bacteria on patterns of growth and toxicity.

Dinoflagellates are normal components of the phytoplankton in southern California inshore coastal waters throughout the year. Sporadic "blooms" occur, however, which may produce localized patches or cover large areas with muddy, reddish colored waters.

Conditions which may account for the sporadic blooms are beginning to be delineated, so that prediction of blooms may be possible.

With minor exceptions, red tides are restricted by the longshore current to a narrow band of inshore waters along the coast, or to headlands or embayments. Thus, two of the three initial conditions postulated by Ryther (4) are present: one, the normal dinoflagellate population, and two, a reduced or restricted circulation which prevents a reproducing population from dispersing.

The third condition is a supply of nutrients, although in local waters enriched by sewer outfalls nutrients are probably not a limiting factor. In certain coastal areas, bottom nutrients may be redistributed in surface waters by upwelling, and extensive blooms may occur. Other possible sources of nutrient input might include storm water runoff, but this does not seem to coincide with blooms in local southern California waters.

Although sporadic blooms may occur throughout the year, the most predictable and extensive episode is likely to be in late summer and early fall. The Los Angeles-Long Beach Harbor is a relatively shallow dredged basin with low tidal flushing and low current velocities (less than 0.5 knots near the Los Angeles entry) (3). The principal driving mechanism is wind, which is usually southwesterly and light. The bottom sediments are mostly fine black silt,

contaminated with a high incidence of trace and heavy metals, chlorinated hydrocarbons, microbial populations, and organic detritus (2).

Stirring of the harbor appears to be a fourth condition for blooms. In the late summer and early fall thermal inversion such as can be found in fresh water lakes may occur. Chilling of surface waters causes warmer bottom waters to rise, resuspending bottom sediments and nutrients.

At various times throughout the year, the wind direction changes to the northeast and strong, warm, gusty "Santa Ana" winds blow from the desert areas. These cause stirring of the harbor and also may spread clouds of dust over the waters. Red tide blooms may follow such winds within a week or two.

Another potential triggering mechanism may be warm water effluents from cooling systems, such as are found at power plants and oil islands, or from sewer and industry outfalls. Warm ponds or patches of water may provide a microcirculation, and may also stimulate reproductive cycles in the phytoplankton.

The presence of the organisms, existing low circulation, adequate nutrients, stirring mechanisms, and possibly warm temperature areas all appear to be conditions conducive to local red tide blooms. But these conditions do not provide an adequate explanation of the mechanisms involved in the bloom.

Various organisms have been associated with phytoplankton blooms in the harbor. On several occasions a localized bright "green tide" patch was observed which consisted of a euglenoid bloom. Other greenish and brownish blooms occur occasionally, but the most pronounced and widespread red blooms are caused by *Gonyaulax polyedra*. Densities of these organisms may range from less than one to 15,000 cells per ml.

Within the Los Angeles-Long Beach Harbor, several sites have been identified as potential foci of red tide occurrence. These include the area around Fish Harbor, Channel 2 in Long Beach, and near the mouth of the Los Angeles River in the bay where the oil islands are located.

The Fish Harbor area has been studied extensively by our group, Harbors Environmental Projects. It is sponsored by the USC Sea Grant program and by the Allan Hancock Foundation, with funds from the Los Angeles Harbor Department, the Army Corps of Engineers, the Tuna Research Foundation and Pacific Lighting International. A pilot project was begun almost five years ago to conduct biological baseline and monitoring studies in the area. This has since been expanded to include microbiology, benthic organisms, phytoplankton productivity, zooplankton, fish populations, invertebrate settling potential, physical water quality, nutrient chemistry, hydrology, sediment chemistry, biomass and bioassay.

Fish Harbor consists of two basins; outer Fish Harbor, which contains a yacht anchorage, and Inner Fish Harbor around which the canneries are located.

Wastes from the canneries and fishing boats were formerly dumped into Fish Harbor, but some years ago the cannery discharges were relocated to the east, in the vicinity of the Terminal Island primary treatment sewage outfall. The

area is very shallow, since dumping of dredge spoil and land fill was permitted in the area. Thus conditions of high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are present (2).

Drogue and current meter studies (5) show that the waste input is on the north edge of a clockwise gyre, which under normal southwest winds, carries the waste waters east toward the navy mole. The falling tide pulls the water mass toward Angels Gate, and the incoming tide directs it back into Fish Harbor or up the main channel. Thus poor mixing and dispersal result, but normally the receiving waters have sufficient capacity to maintain required water quality criteria of 5 ppm[1] at 1,000 feet from point of discharge. Under Santa Ana (NE) wind conditions, wastes tend to remain in the outfall area rather than be dispersed and diluted in the gyre.

Oxygen depletion in these waters may lead to sulfide production by bacteria and a "white tide" composed of a mixture of bacteria and colloidal sulfur may result.

The normal seasonal wind and water changes, temperature changes and nutrient input may cause localized blooms at any time of the year. However, in the late summer and early fall, natural turnover may coincide with the opening of the anchovy (wet fish) season in mid-September. This results in a cannery waste discharge increase from about 2 MGD[2] to about 10 MGD, and has in the past reached as high as 25 MGD. In 1974, however, the fishermen were on strike and no anchovies were processed, but a severe red tide occurred. It appeared first in the Long Beach area and ultimately covered all the adjacent inshore waters of both south and west coasts.

A sequence of events has been noted in the oxygen curves for the Fish Harbor area. Shortly after the appearance of white tide, red tide begins to appear in waters peripheral to the white tide. Dissolved oxygen (DO) drops rapidly in the white tide area from 7-8 ppm to zero or near zero. White tide then begins to disappear and red tide proliferates. Daylight oxygen readings climb rapidly and readings may reach 14-16 ppm. At night, the phytoplankton apparently utilize the oxygen, because oxygen is greatly reduced just before dawn.

Red tides in the Los Angeles-Long Beach Harbor have been cited as being involved in fish kills on several occasions. However, circumstances suggest that these may be due to oxygen depletion rather than to toxicity of the phytoplankton. Fish have been observed swimming through the red tide in the early stages of a bloom with no apparent problem. As the bloom becomes senescent and oxygen levels fall, fish in the bloom area appear at the surface gasping, as though for oxygen. Chamberlain (1) suggests that at the air-water interface minute amounts of dissolved oxygen may be present. Chamberlain

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[1] ppm = parts per million

[2] MGD = million gallons per day

transferred some apparently moribund fish to well oxygenated aquaria, and they recovered, with normal swimming behavior.

Cells from red tides in the harbor have been recovered during the past four years and extracted for toxins. Crude chloroform-methanol extracts were added to tanks containing mosquito fish *Gambusia affinis*, at a concentration of 0.5%. Extracts from blooms in the late summer of 1971 and 1972 showed ample evidence of toxicity, but blooms from 1973 and 1974 showed greatly reduced toxicity. This suggests that the toxicity is induced by some external factor which may be incorporated into the phytoplankton.

Regardless of whether the biological kills are due to oxygen depletion or toxicity, they set the scene for another bacterial bloom. Mussels (*Mytilus*) on pilings show gills clogged mechanically by the *Gonyaulax* cells; they smother and fall to the bottom, where they decay. Given (pers. comm.) reported a white, presumably bacterial film over much of the bottom, along with remains of decaying organisms when he conducted a diver survey in Long Beach following the red tide.

At times the bacterial-red tide cycle will not produce a bloom when it might be expected. Seasonal wind or rain storms from the northwest or south and peak high tides may cause sufficient mixing and dispersal to break the cycle. Such storms may at the same time cause stirring, but if the water temperature decreases as well, bacterial activity may decrease sufficiently to prevent bloom conditions.

In normal bacterial cycles, mass, sudden die-off rarely occurs. Rather, mixed populations will move in the direction of dominance by one or several species until nutrients are exhausted. Research is presently underway to identify factors which might serve to cause a restricted cycle in the marine bacteria. We have recently detected higher levels of bacteriophage activity in the harbor than would normally be anticipated in marine waters, especially in the Fish Harbor area. Plaque formation of up to 40 plaques per ml. have been recorded in culture tests, whereas less than 1 per ml would be expected in coastal marine waters (C. Frey, pers. Comm.).

In attempting to correlate these observations with studies in the laboratory we have been hindered by the absence of axenic cultures of the causative organism *Gonyaulax polyedra*. Paralled experiments have been carried out on another dinoflagellate, *Gymnodinium breve*, which is responsible for the ichthyotoxic red tides of the Gulf of Florida. We have these in pure culture, bacteria free and in defined growth medium.

Earlier studies showed that when cultures of *G. breve* were made bacteria free by the action of antibiotics and grown in a minimal experimental medium (MEM) there was a delay time of up to 8 days before the cells entered a logarithmic phase of growth. This delay was immediately eliminated if very low (hormonal) levels of gibberellic acid were added to the medium (now labelled MEG).

During the past months we have extended these studies to investigate the effect of adding naturally occurring marine bacteria to the axenic cultures

grown in Minimal (MEM) medium. When contamination by the bacteria is permitted,the delay time of the growth phase after the procedure of subculture is reduced and the addition of gibberellic acid has little or no effect.

Thus we believe that the presence of natural bacteria provides an environment probably by the release of some chemical compound at low level which stimulates the division of the cells in culture. It is possible that some such event occurs in nature as suggested by the bacterial bloom in Fish Harbor running ahead of a red tide there. Thus the link between bacterial blooms and red tide blooms appears to be consistent and probably significant.

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HYDROGRAPHIC FACTORS AFFECTING THE DISTRIBUTION AND MOVEMENT OF TOXIC DINOFLAGELLATES IN THE WESTERN GULF OF MAINE

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ABSTRACT

Although "red tide" blooms of the marine dinoflagellate, *Gonyaulax tamarensis* (recently reidentified as *G. excavata*) are common occurrences in the waters of the Bay of Fundy and eastern Maine, until recently they were rare in the western Gulf of Maine. During September, 1972 the first reported bloom of this toxic dinoflagellate occurred in the coastal waters from southern Maine to Cape Ann, Massachusetts resulting in a widespread outbreak of paralytic shellfish poisoning (PSP) which eventually contaminated shellfish as far south as Cape Cod, Massachusetts. Most severely affected were the blue mussel *Mytilus edulis* and the soft-shelled clam *Mya arenaria* which attained observed PSP levels of 2,900 μg/100g meat and 4,600 μg/100g meat respectively in the Hampton Harbor estuary, New Hampshire. The PSP levels remained above 80 until May, 1973 when the flats were reopened to harvesting. In late May - early June, 1974 another smaller bloom occurred with PSP levels of 348 for *M. arenaria* and 499 for *M. edulis;* the flats were closed for several weeks then reopened. In late August, 1974 a third bloom similar to the 1972 one occurred; by early September PSP levels of 550 for *M. arenaria* and 8400 for *M. edulis* were recorded but fortunately by late October toxicity levels again dropped below 80.

Extensive oceanographic data from studies conducted by Normandeau Associates, Inc. in New Hampshire coastal waters document the hydrographic framework of each of these three blooms. Data on rainfall, runoff, water temperature, salinity, nutrients, and currents suggest that coastal upwelling played a key role in the development of these *G. tamarensis* blooms. Beginning with stratified coastal waters, local winds drive the warm, upper layer offshore and as a compensatory flow the lower layer of cool, salty water is carried shoreward along the bottom. This mechanism may concentrate the dinoflagellates in coastal waters where they have sufficient nutrients and light as well as proper conditions of temperature and salinity to bloom. In attempting to explain how the first bloom of this type occurred in these waters in 1972, it is possible that a *G. tamarensis* bloom could have been pushed across the Gulf of Maine from eastern Canada by northeast winds preceding Hurricane Carrie. Offshore winds and subsequent upwelling then brought the dinoflagellates into the coastal waters where the bloom ensued. The later blooms in 1974 could have come from dormant cysts which were triggered by upwelling.

INTRODUCTION

Until recently major toxic dinoflagellate blooms in the Gulf of Maine were confined to the waters of eastern Canada. In the Bay of Fundy to the north of the Gulf, where the accumulation of paralytic toxin in molluscan shellfish during the summer months is an annual phenomenon, high toxicities in shellfish occur simultaneously with the appearance of large numbers of

dinoflagellates in the coastal plankton. Occasionally, certain marine dinoflagellates produce blooms that impart a reddish or yellowish-color to the ocean but no such discoloration has ever been reported with the blooms in the Bay of Fundy. Not until 1961 was it shown that the marine dinoflagellate *Gonyaulax tamarensis* was the causative organism (1). Its distribution especially in offshore waters is still poorly understood.

The first reported bloom of this toxic dinoflagellate in the western Gulf of Maine in the coastal waters from southern Maine to Cape Ann, Massachusetts occurred during September, 1972. This bloom resulted in a widespread outbreak of paralytic shellfish poisoning (PSP) which eventually contaminated the waters as far south as Cape Cod, Massachusetts. Most severely affected were the blue mussel *Mytilus edulis* and the soft-shelled clam *Mya arenaria*, both remaining toxic until late April, 1973 (Figure 1). No further blooms occurred until late May - early June, 1974 when a small outbreak was observed in the waters from New Hampshire to Cape Ann. Fortunately it only lasted a few weeks. In late August, 1974 a third bloom occurred which resulted in PSP levels as high or higher than those of 1972. Although this bloom was widespread throughout the western Gulf of Maine, it only persisted for about 2 months and by late October most areas were again open for harvesting of *M. arenaria* (Figure 1). What triggered these blooms has been a subject of considerable interest. Recent papers by Mulligan (2) and Sasner, Ikawa, and Barrett (3) have explored various factors which may have been responsible for the 1972 bloom.

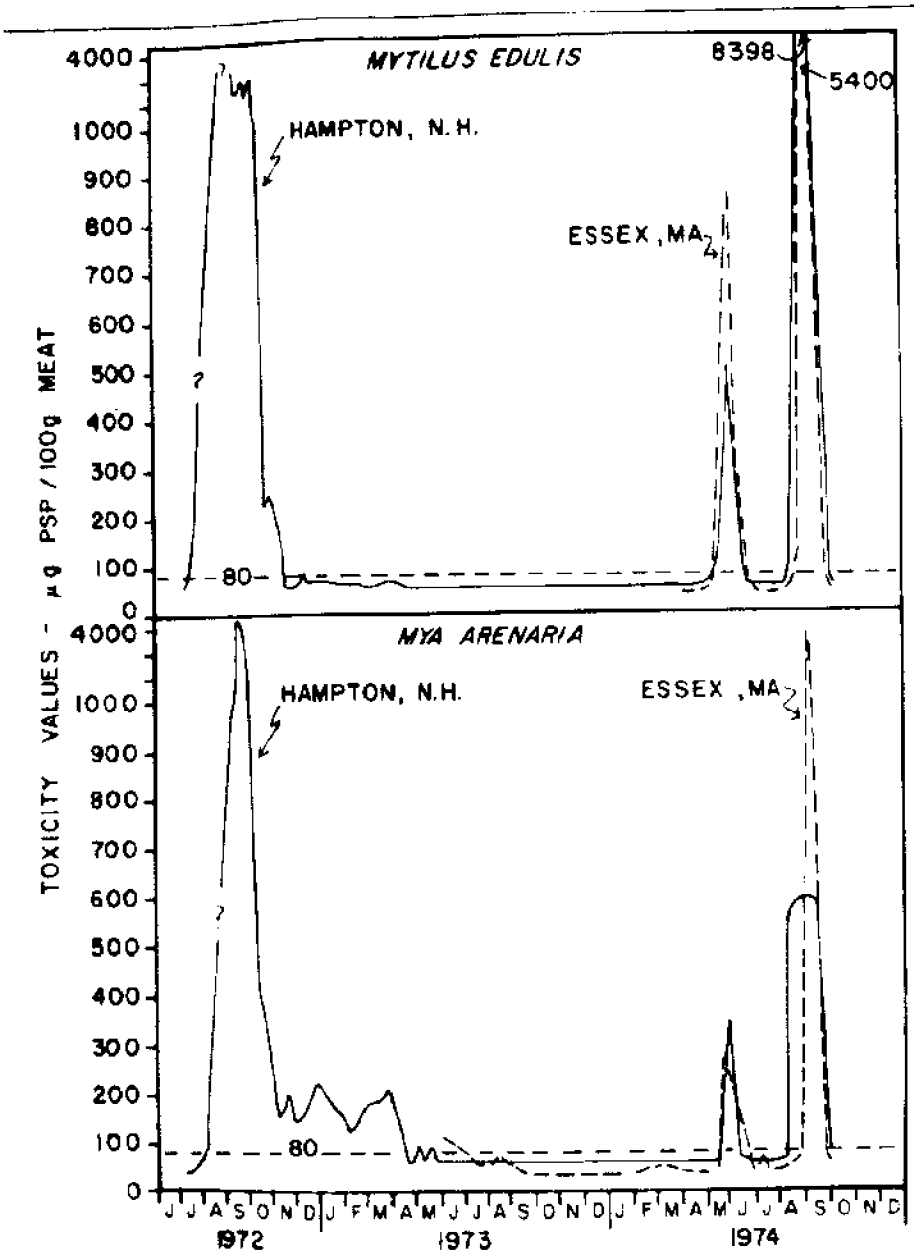
Throughout the period of these three toxic dinoflagellate blooms Normandeau Associates, Inc. (NAI) has been conducting extensive hydrographic and biological studies in the western Gulf of Maine and New Hampshire coastal waters out to about 42 km (26 miles) offshore. This study program which is being funded by Public Service Company of New Hampshire was designed to help evaluate the possible environmental effects of their proposed nuclear power plant at Seabrook, N.H. by documenting the existing year-round hydrographic characteristics of these waters with base-line oceanographic data.

The purpose of this paper is: (1) to examine in detail the hydrographic framework of the two toxic dinoflagellate blooms which occurred in 1974, (2) to re-examine the circumstances surrounding the 1972 bloom, and (3) to determine the reasons why no bloom occurred during 1973. Although many different factors interact to help trigger such blooms, the extensive hydrographic data collected in these waters by NAI since September, 1972 suggest that upwelling played a key role during each bloom and was absent during August and September, 1973 when no bloom occurred.

STUDY AREA

The Gulf of Maine which forms the eastern coast of New England is a roughly rectangular embayment stretching about 320 km west to east from Cape Cod to Cape Sable, Nova Scotia and about 190 km north to south from

istal Maine to Georges Bank. The flow of water in the Gulf is very strongly luenced by tides and at most locations the tidal currents comprise the ater part of the total water movement. However, this tidal movement is erimposed on a more general counterclockwise scheme of net non-tidal culation which undergoes an annual cycle (4). The exchange of waters ween the Gulf and the North Atlantic is restricted by Georges Bank.



gure 1: History of PSP toxicity levels in *Mytilus edulis* and *Mya arenaria* from Hampton Harbor estuary, N. H. and Essex estuary, Mass. from 1972-1974; data from Sasner and Ikawa of University of N.H., N.H. Division of Public Health, and Mass. Dept. of Public Health.

The drift-bottle studies of Day (5) and Bumpus and Lauzier (6) show that beginning in winter there is an indraft of water off of Cape Sable into the eastern Gulf where the flow bifurcates northward into the Bay of Fundy and westward along the coast of Maine. In the western Gulf the flow becomes southward along the coast past Cape Ann and seaward out beyond Cape Cod. By February several irregular eddies develop in the central portion of the Gulf and water spills out into the Atlantic across Georges Bank. In the early spring freshwater runoff is discharged from coastal estuaries, helping to set in motion an eddy which rapidly develops into a huge counterclockwise gyre encompassing the entire Gulf of Maine by the end of May. The less-saline waters of the Bay of Fundy flow southward along the western coast of Nova Scotia and join the westward flow of this gyre. Drift-bottles released by NAI along the New Hampshire coast in late spring, 1973, at the time of year when the flow reaches its peak, were carried southeastward across the Gulf to Nova Scotia in only 71 days. In the summer the pattern of the spring gyre persists but its drift rate gradually decreases. By fall the southern side of the Gulf's counter-clockwise flow breaks into a southerly drift across Georges Bank.

Circulation studies in the western Gulf of Maine by Graham (7) and drift-bottle releases by NAI out to 42 km offshore of New Hampshire show that net non-tidal drift of near-surface water varies from about 1.8 - 5.5 km/day, and may reach 13 km/day during favorable wind conditions. Drift-bottles released by NAI generally moved either southerly and southeasterly off Cape Ann out of the study area or southwesterly toward shore. During winter months drifters are sometimes carried northward apparently by southwest winds and a small current eddy which develops off Cape Ann. In contrast, the year-round pattern of bottom circulation documented by NAI's releases of sea-bed drifters shows generally either a strong southwestward component toward shore or a southward movement off Cape Ann. These near-bottom flows are much weaker than surface flows (about 0.2 km/day). These data show a general shoreward component of saline near-bottom water which may be a flow compensating for the seaward drift of near-surface freshened water from coastal estuaries (8).

In general the hydrography of the New Hampshire coastal waters (Figure 2) is quite variable, with the fields of temperature, salinity, density, and dissolved oxygen showing pronounced seasonal variations. Data from April, 1973 through April, 1974 taken at low water from a hydrographic station 2 km offshore show the pronounced thermal stratification which develops in late May and persists through late September (Figure 3). During the fall the water column is nearly isothermal but as the winter progresses the surface waters actually become colder than those at depth. Lowest salinities occur during the spring when fresh-water runoff is greatest. Density values are somewhat variable but show the same general cycle of stratification during the year. Highest concentrations of dissolved oxygen occur during the spring when photosynthesis is most pronounced, resulting in supersaturated conditions up

to about 144%. Lowest concentrations, which result in about 80% saturation, occur during the summer and early fall.

Currents are very dynamic throughout the water column with nearsurface shearing and reverse flow at depth frequently present. About half of the time on a year-round basis, weak reversing tides cause the water to flood to the north and ebb to the south roughly parallel to the shoreline. Northeast and

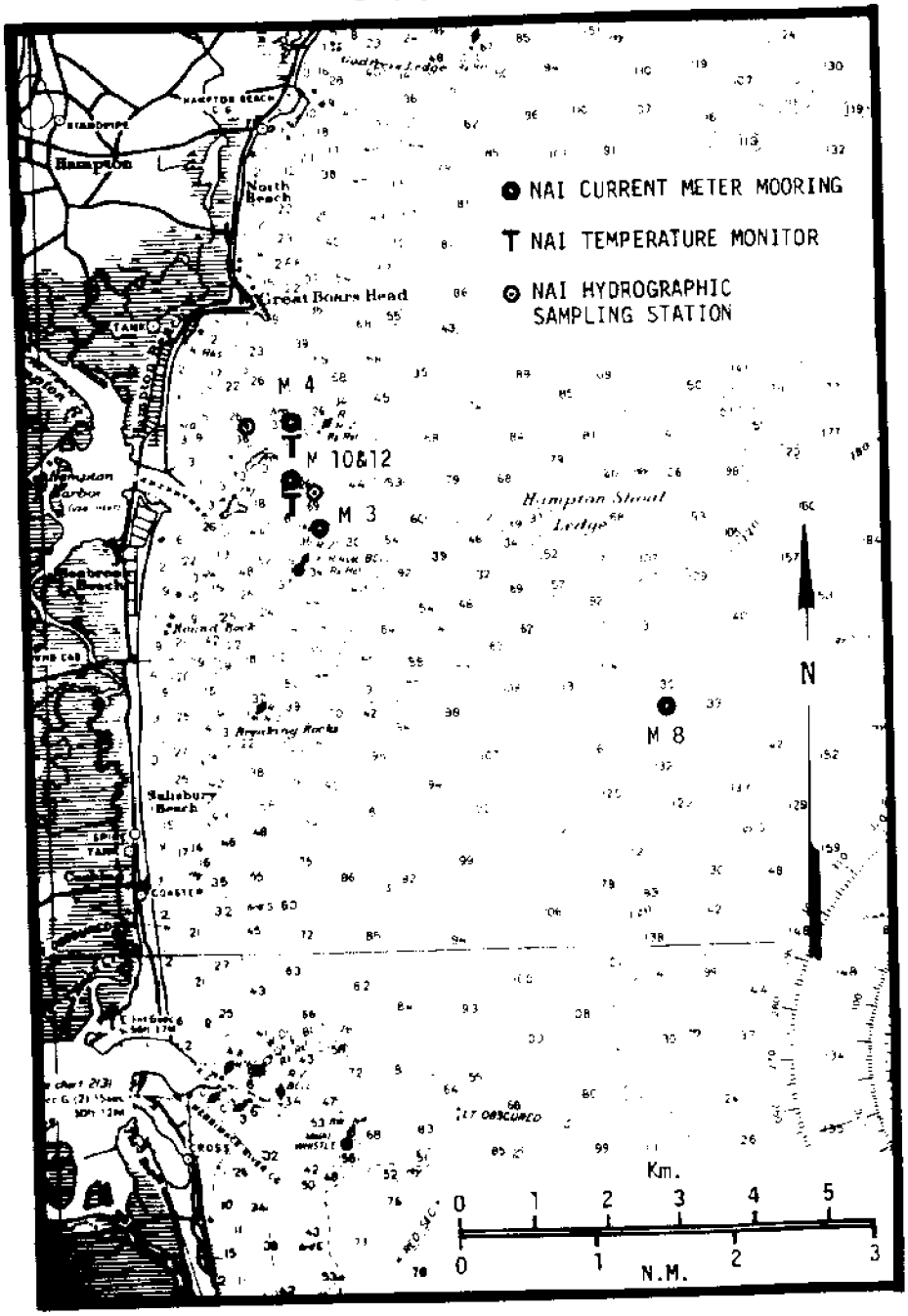


Figure 2: Location map for selected NAI current meter moorings, temperature monitors, and hydrographic sampling stations off Hampton Beach, N.H.

southwest winds which occur during storms scattered throughout the remainder of the year generate their own water movement to the south and north respectively, masking out these tidal flows. This variability is typical of coastal waters where wind, tides, waves (surface and internal), bottom topography, river runoff, evaporation, precipitation, isolation, proximity to land, and advection all affect the hydrography to varying degrees in time and space. In general, the various hydrographic features of these waters are characteristic of temperate coastal areas (9).

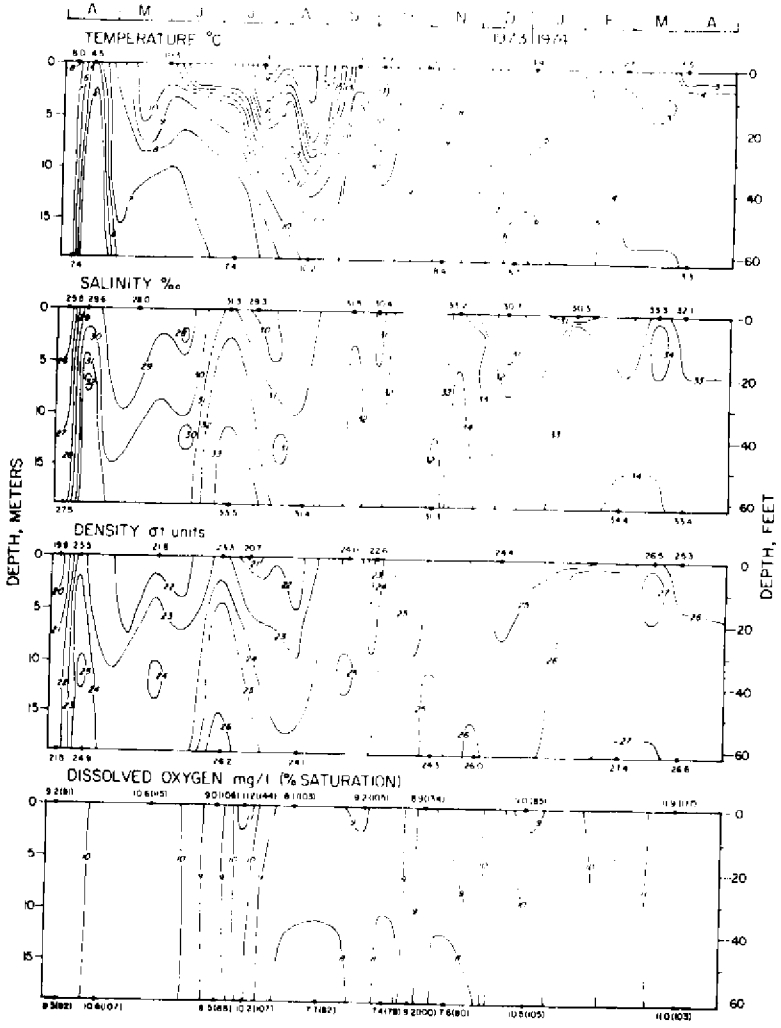


Figure 3: Annual variation in hydrographic parameters of waters 2 km off Hampton Beach, N.H.; measurements were made biweekly at low water throughout the year.

METHODS OF STUDY

NAI's oceanographic study program of the New Hampshire coastal waters has included: (1) continuous year-round monitoring of currents and water temperature from a number of moored offshore buoy systems out to 7.5 km offshore, (2) monitoring of local winds and tides, (3) monthly plankton cruises out to 42 km offshore, (4) biweekly or monthly slack-water surveys, (5) periodic 13-hour anchor stations at offshore locations, (6) release of more than 2,500 drift bottles and 2,250 sea-bed drifters, and (7) a mid-summer thermal infrared survey.

For each of the three toxic dinoflagellate blooms the following data have been compiled:

1. Toxicity level - PSP level in μg/100g meat for *M. edulis* and *M. arenaria* from Hampton Harbor estuary, N.H.

2. Rainfall - approximate rainfall for eastern N.H. from NOAA daily weather maps;

3. Runoff - U. S. Geological Survey discharge data from the Merrimack River at Lowell, Mass. (Station 1-1000);

4. Temperature - continuous data from NAI temperature monitors at mooring 4 (-5.8 m below MLW) or mooring 10 (-4.8 m) or selected hydrographic station data from surface, midwater, and nearbottom depths;

5. Salinity - from selected hydrographic station data for surface, midwater, and near bottom depths; and

6. Nutrients - total nitrogen (comprising total nitrate and nitrite) and total phosphorus (comprising total orthophosphate and summed organic phosphate) from selected hydrographic station data.

For the time periods when current meter data were available, the daily 24-hour net-drift in nautical miles per day was calculated for both a nearshore site about 2 km east of Hampton Beach, N.H. and for a deeperwater site about 7.5 km offshore (Figure 2). For the former site, data from near-surface waters were compiled from moorings 4 and 12; from mid-depth waters, moorings 3 and 10 were utilized. For the latter site, data from two depths at mooring 8 were used. Net-drift vectors were plotted sequentially as a function of time.

RESULTS

Bloom of May-June, 1974

Beginning in May, 1973 when the Hampton Harbor estuary, New Hampshire and Essex Estuary, Massachusetts were reopened to Shellfish harvesting after having been closed by prolonged toxic effects of the 1972

bloom, both the blue mussel, *M. edulis* and the soft-shelled clam, *M. arenaria* had PSP levels below 58 (Figure 1). In Figure 4 where toxicity levels in shellfish found in Hampton Harbor, rainfall, runoff, water temperature from off Hampton Beach, salinity and nutrients are plotted as a function of time, several relationships are apparent. Rainfall was generally very small, but there was substantial runoff from the Merrimack River, the single largest freshwater

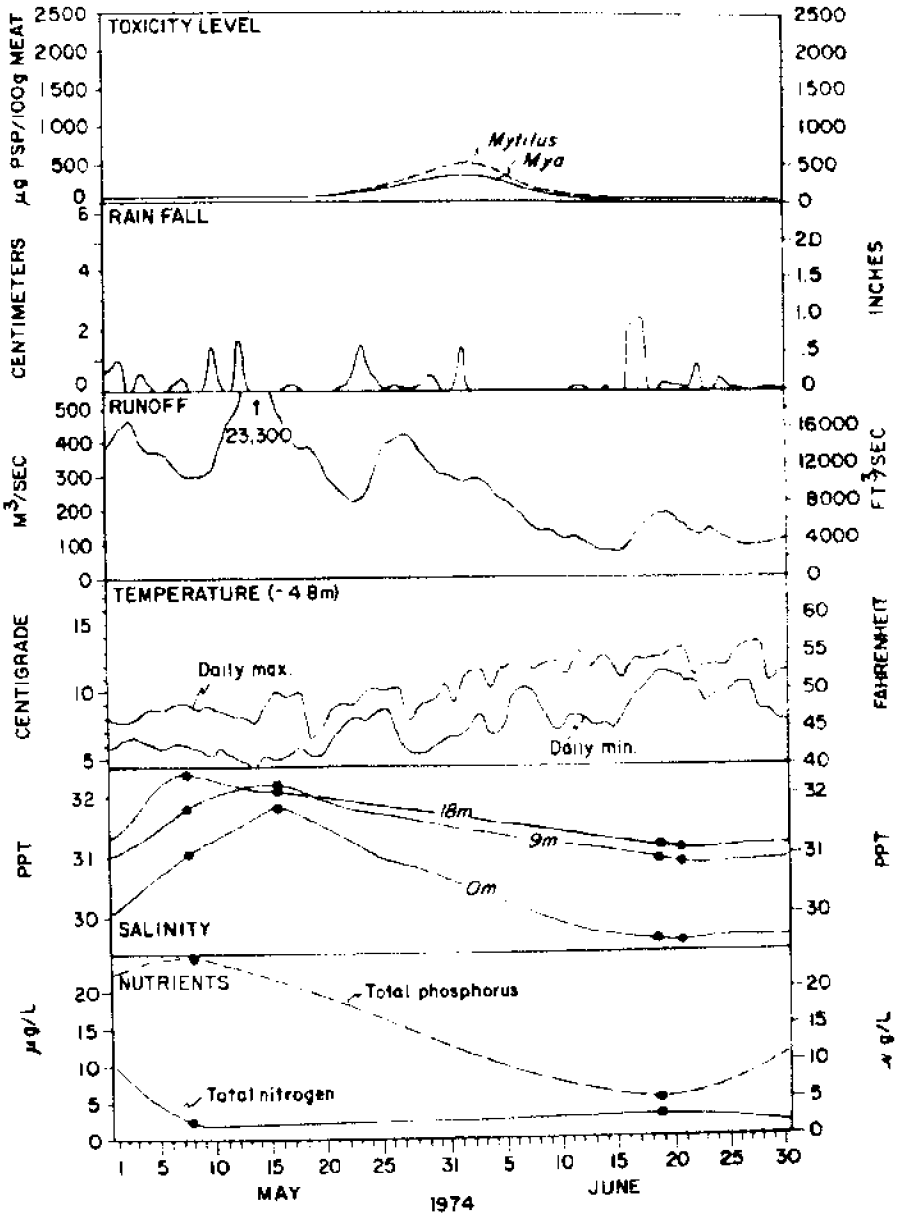


Figure 4: Toxicity levels of shellfish in Hampton Harbor, local rainfall, runoff from the Merrimack River, temperature at mooring 10, high water salinities 2 km offshore, and nutrients 1 km offshore for May and June, 1974.

input to the western Gulf of Maine (10). During mid-May discharge reached 660 m^3/sec (23,300 ft^3/sec), but by early June was back down to more typical levels for this time of year. Although the data are limited, this freshwater input to the coastal waters was manifested in the subsequent drop in surface salinity (0m) from 31.8 to 29.6 °/oo (PPT) and somewhat smaller drops at mid-depth (-9 m), and near-bottom (-18 m). Likewise the nutrient data are limited but in general total nitrogen remained quite low throughout the period; total phosphorous started high (24 µg/L), but by June 19 was down to 7 µg/L.

Between May 20 and 29 the bloom was first observed at Essex, Massachusetts and shortly thereafter at Hampton Harbor. Because of their fast rate of filtering, *M. edulis* picked up the largest amounts of the potent endotoxin synthesized by *G. tamarensis* in the shortest time and by June 2 had PSP levels of 499 µg toxin per 100g of shellfish meat (values greater than 80 are indicative of shellfish that are unsafe for human consumption by U.S. Public Health Service Standards). In contrast *M. arenaria* picked up the toxin more slowly, only reaching a PSP level of 348 before the bloom dissipated. By June 17 PSP values for both organisms were again below 58.

Examination of the continuous temperature data from -4.8 m depth at mooring 10 (1.8 km offshore; Figure 2) and the 24-hour net-drift data from selected offshore current meters (Figure 5) shows that coastal upwelling occurred immediately prior to the bloom. That is, for about 7 days preceding May 20, the near-surface waters at mooring 12 showed a persistent net northeastward drift offshore. At mooring 10 slightly deeper in the water, a similar pattern occurred, whereas offshore about 7 km at mooring 8, flows were generally southwestward. During the same period the temperature monitor showed a pronounced change in its daily variations; with a drop in the daily maxima and a rise in the daily minima, the daily range became much smaller (Figure 4). These data appear to indicate upwelling. The warm surface layer above the sharp seasonal thermocline was carried offshore by the wind-driven northeastward flowing surface currents. A southwestward compensatory counter-flow at depth carried colder, saltier water toward shore. This upwelling mechanism could have transported the offshore waters, which presumably were carrying encysted cells of *G. tamarensis* down from the Bay of Fundy, into the coastal zone where increased light, freshened salinities, nutrients, and water temperatures appear to have been favorable for a bloom of toxic dinoflagellates. Additional upwelling in early June probably reinforced the bloom, but fortunately the toxicity levels did not go any higher. By late June Hampton Harbor flats were again reopened to shellfish harvesting. Although upwelling seemed to play a key role in triggering the bloom, it was probably augmented by the high runoff and other peripheral effects in mid May.

Bloom of August-September, 1974

Toxicity levels remained below 58 until about mid August when another

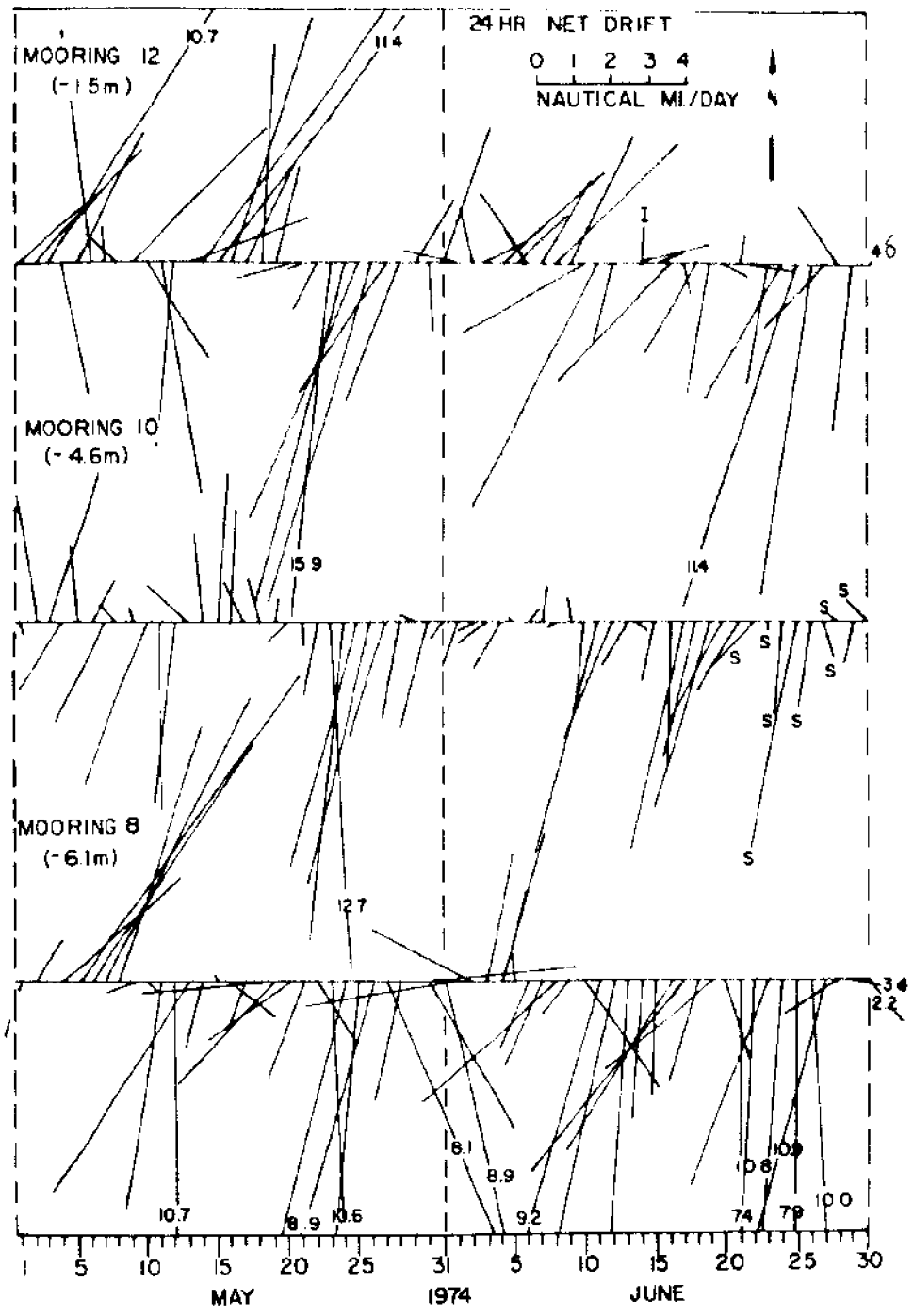


Figure 5: Sequential 24-hour net-drift vectors for May and June, 1974 from moorings 12 and 10 located 2 km offshore and mooring 8, 7.5 km offshore; I = data incomplete and S = speed data incomplete, assume 0.1 kts.

bloom began. Figure 6 shows how rapidly *M. edulis* picked up the toxin in the Hampton Harbor estuary attaining a level of 8,398 μg/100g meat on September 3, 1974. At about the same time levels almost as high (7,600) were found in blue mussel samples from Rye Harbor, New Hampshire. At both Hampton Harbor and Essex, Massachusetts *M. arenaria* took up the toxin more slowly but did not reach levels this high or as high as those of 1972

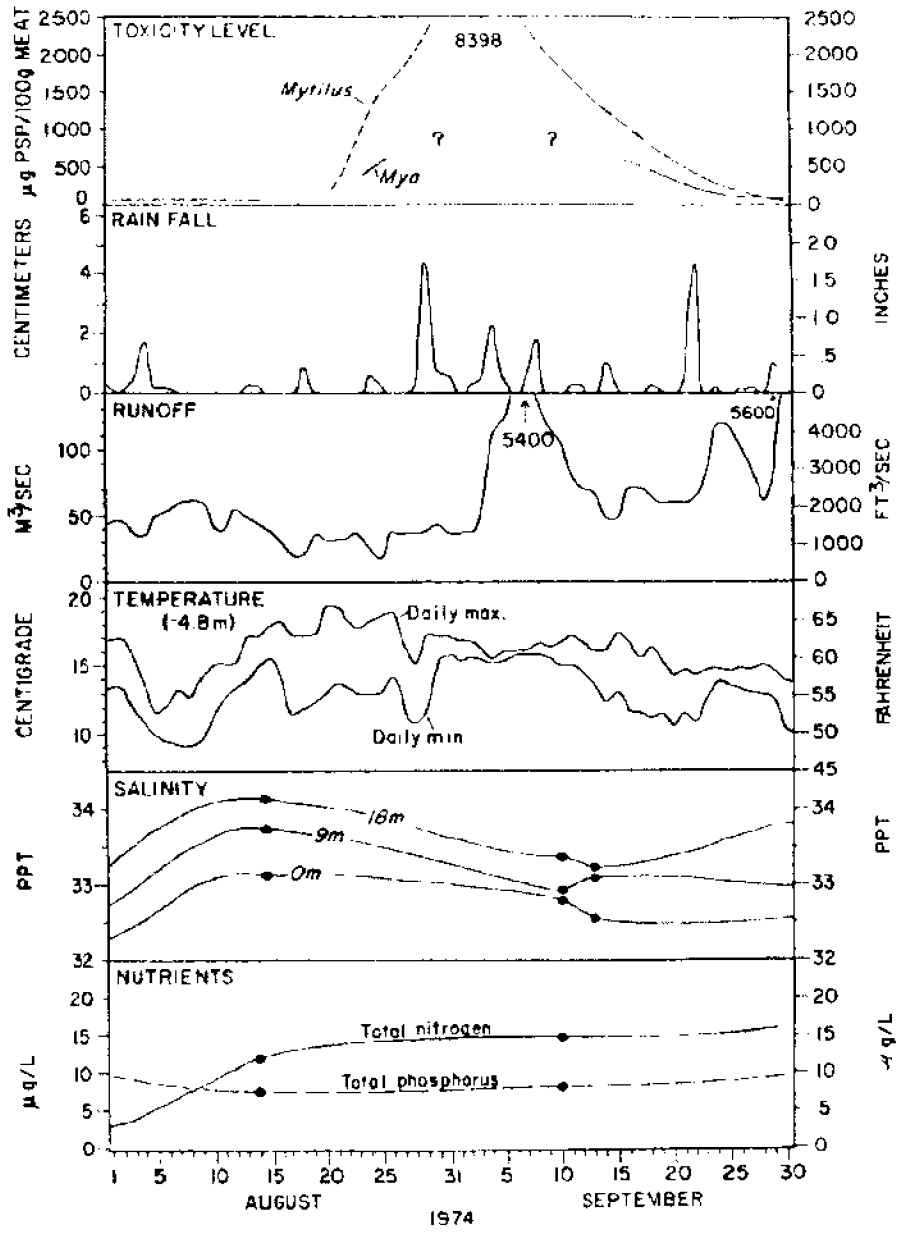


Figure 6: Toxicity levels of shellfish in Hampton Harbor, local rainfall, runoff from the Merrimack River, temperature at mooring 10, high water salinities 2 km offshore, and nutrients 1 km offshore for August and September, 1974.

(Figure 1). Rainfall was very low during this period and runoff was close to record drought levels (about 25 m^3/sec or 1,000 ft^3/sec). Water temperature and salinities were quite high until late in the month when another period of coastal upwelling took place (Figure 7). Starting August 17 the near-surface net-drift at mooring 12 was again offshore toward the northeast, whereas

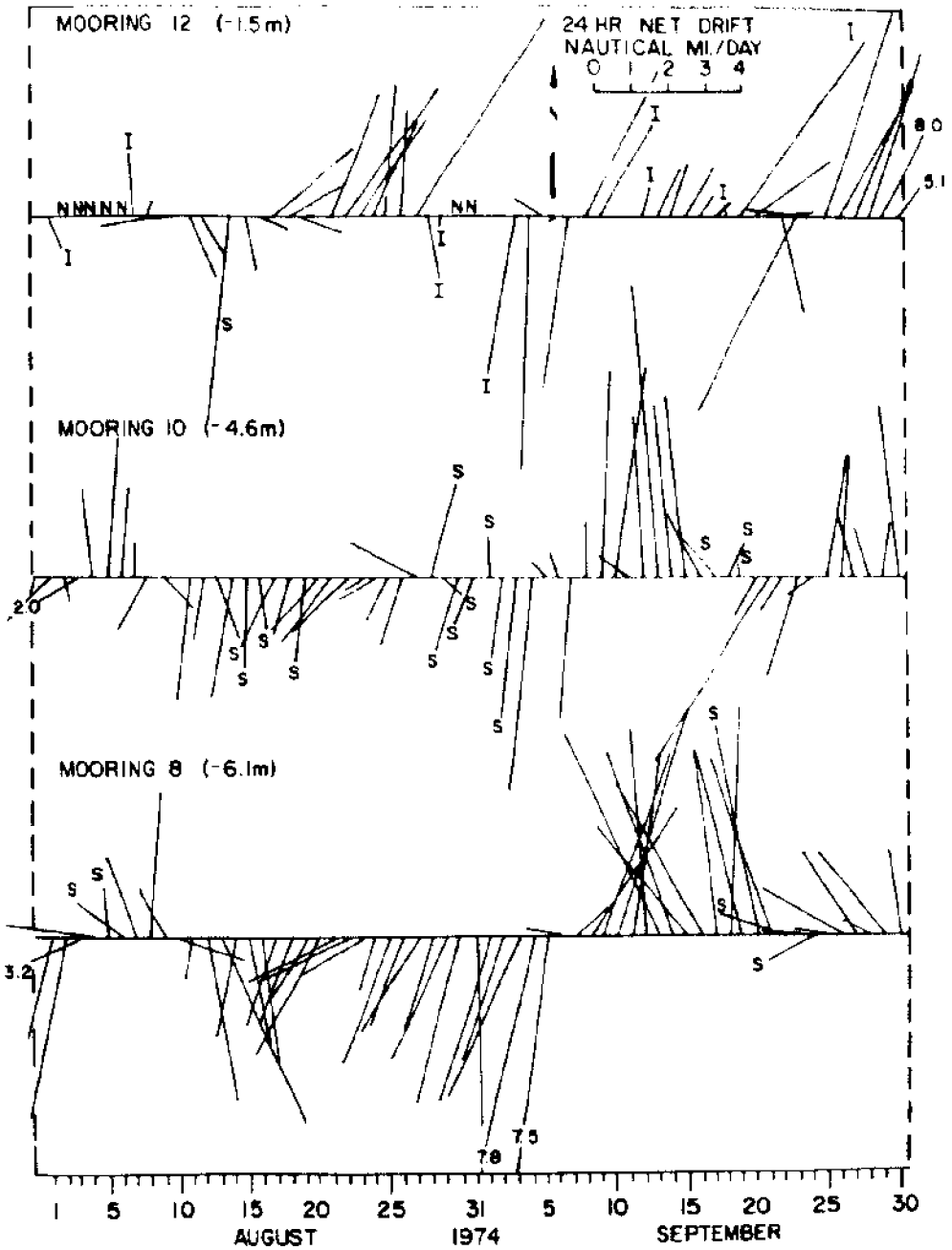


Figure 7: Sequential 24-hour net-drift vectors for August and September, 1974 from moorings 12 and 10 located 2 km offshore and mooring 8, 7.5 km offshore; N = no data, I = data incomplete and S = speed data incomplete, assume 0.1 kts.

flows at depth were southwestward toward shore. This pattern persisted at least until August 28 when the current meter at mooring 12 began to malfunction; it is thought that this pattern may have continued even longer. As the upwelling progressed into early September, the temperature pattern shifted abruptly to a lower daily maxima and a higher daily minima, indicating a well-mixed thermal distribution (Figure 6). It should be noted that at this time all 6 of the NAI temperature monitors, which are located at various distances and depths offshore, showed this same thermal pattern with a range of temperatures around 15-16°C. This phenomenon occurred immediately prior, and concurrent to, the toxic dinoflagellate bloom. Some rainfall and heavy runoff (153 m^3/sec or 5400 ft^3/sec) in early September helped lower the offshore salinities but this occurred well after the bloom was in progress. Another period of upwelling in September again upset the typical temperature pattern of a high daily maxima and low daily minima. As the warm stratified surface water was carried offshore, deeper water upwelled with vertical mixing taking place along the coast. From the limited nutrient data no apparent changes in total nitrogen and total phosphorus were detected as the bloom progressed. Fortunately the bloom did not persist and by late October PSP toxicities of shellfish were again below 80. With this latter bloom it should be noted that neither rainfall or runoff seemed to play a role in triggering the event.

Bloom of August-September, 1972

Although available hydrographic data from this bloom period are not as comprehensive as from later ones, similar patterns are evident. The bloom, which was the first of its kind ever observed in these waters, apparently began early in September. By September 10, bioassays of *M. arenaria* from Hampton Harbor showed PSP levels of 970. The digging of clams and harvesting of shellfish was immediately banned in all or portions of the three afflicted states - New Hampshire, Massachusetts and Maine. As much as 10 days prior to the nearshore bloom, local fishermen reported several patches of colored water offshore. On September 14 and 15 during the peak of this bloom, streaks of visible reddish water several km long were observed from aircraft and boats within 3-4 km of the New Hampshire and northern Massachusetts coastline (3). Spectacular phosphorescence similar to that observed in eastern Canada (1) was also observed offshore during an NAI plankton cruise on September 13, 1972. As the bloom progressed the highest recorded PSP levels from Hampton Harbor were 2,926 for *M. edulis* and 4,620 for *M. arenaria*. Because these data are limited it is possible that much higher levels actually occurred.

Figure 8 shows that rainfall was very low until September 3 when Hurricane Carrie dropped nearly 10.2 cm (4 in.) of rain. August, 1972 was actually the driest August recorded at Boston since 1883. Runoff was moderate in early August then quite low in the middle of the month; in late August another

peak discharge occurred reaching 152 m^3/sec (5350 ft^3/sec) before tapering off to about average summer conditions (11). Offshore salinities were slightly below average for the summertime but rose sharply in late September along with a small temperature drop which appeared to follow an upwelling event. Although no current meter data are available, wind records from the NOAA daily weather maps (12) show several days of northeast winds during Hurricane Carrie early in September. In attempting to explain why no previous blooms of this type had occurred in these waters, we postulate that at this time there may

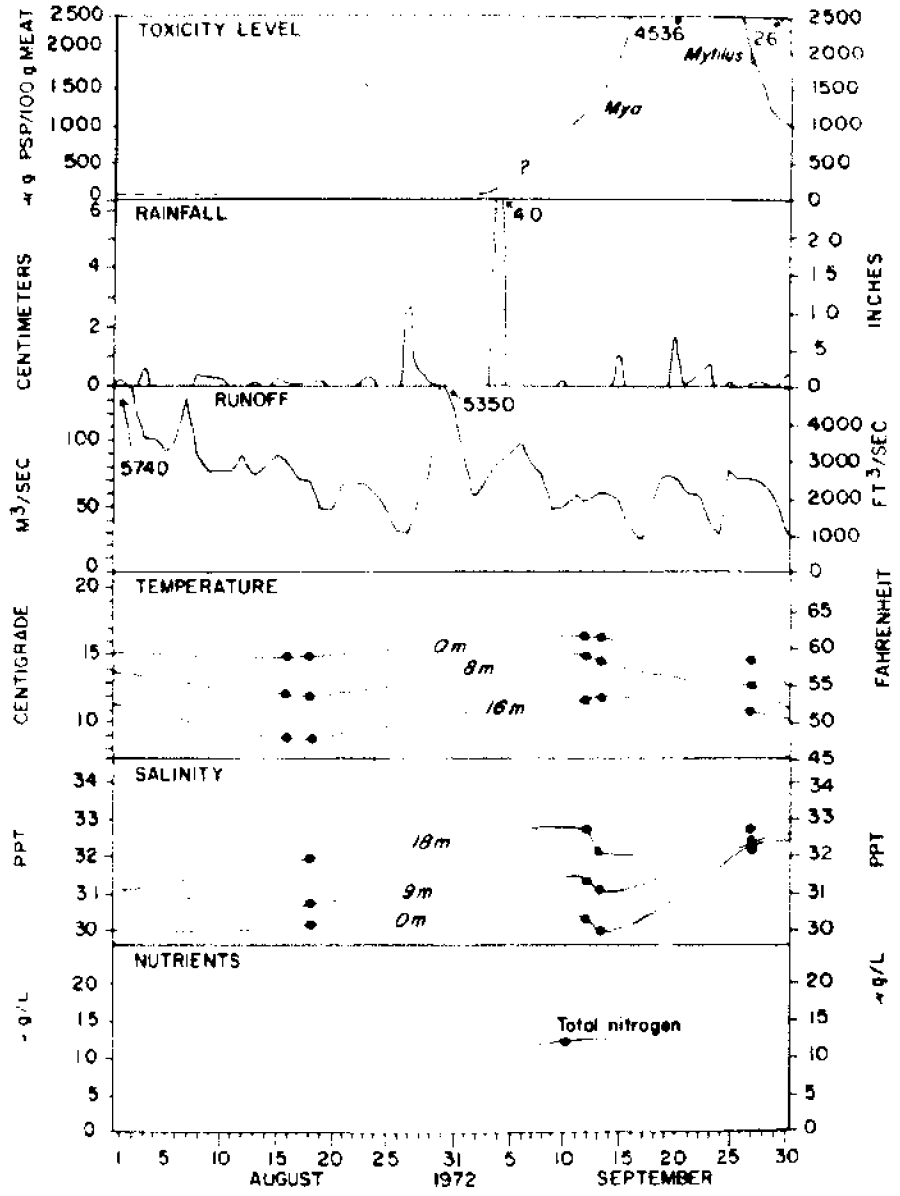


Figure 8: Toxicity levels of shellfish in Hampton Harbor, local rainfall, runoff from the Merrimack River; temperature, salinity and nutrients from selected stations 2 km offshore for August and September, 1972.

have been a *G. tamarensis* bloom occurring in the waters off eastern Maine. These storm winds may have carried the water mass with the toxic organisms southwestward across the Gulf. Then with almost 10 days of persistent west to southwest winds, upwelling of deep waters carried these organisms into the Cape Ann area and concentrated them in the coastal waters. Unfortunately the other data, such as pertain to nutrients, are not comprehensive enough to further document conditions favoring this phenomenon.

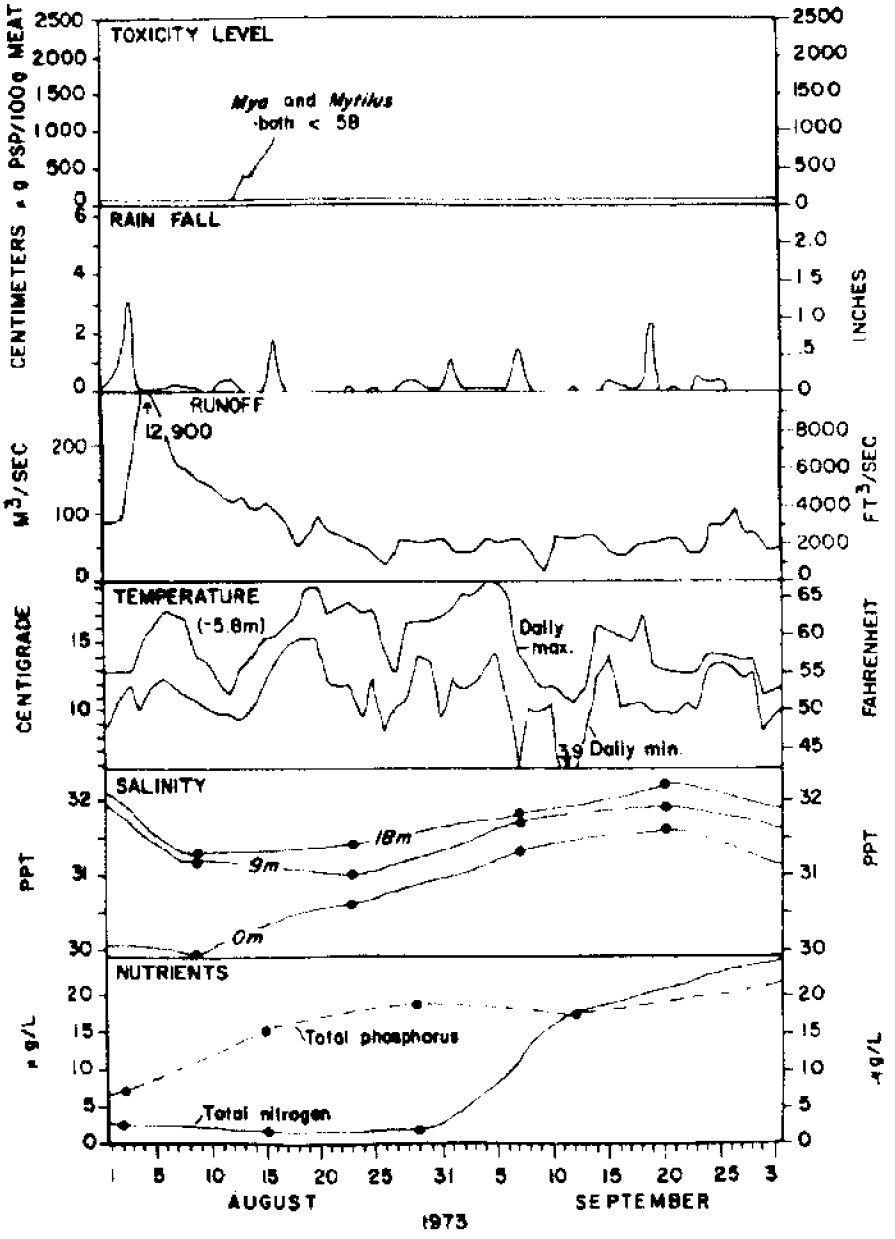


Figure 9: Toxicity levels of shellfish in Hampton Harbor, local rainfall, runoff from the Merrimack River, temperature at mooring 4, high water salinities 2 km offshore, and nutrients 1 km offshore for August and September, 1973.

Mulligan (2) also suggested upwelling off Cape Ann as one of the factors responsible for the bloom. In addition, he felt that the drier-than-normal August weather may have promoted encystment and thus indirect expansion of the resident dinoflagellate population. When *G. tamarensis* found itself in the coastal water, the bloom occurred. The winds and the heavy rainfall from Hurricane Carrie may have increased the normal flushing rates of local estuaries, thereby drawing greater volumes of coastal water, rich with *G. tamarensis* into areas populated with *M. arenaria* and *M. edulis*. The bloom may also have been augmented by dredging operations which were taking place near Annisquam, Massachusetts (3). With this unusual combination of conditions plus the mixing of ocean water with runoff water which is normally rich in inorganic and organic nutrients, the *Gonyaulax* population developed to bloom proportions in the brackish, nutrient-rich water of the estuaries and coastal areas. *M. edulis* toxicities from Hampton Harbor remained above 80 until November 21, 1972 whereas *M. arenaria* was above this level until April 19, 1973 (Figure 1). The *M. arenaria* in the Essex River to the south did not drop below 80 until June 25, 1973.

Non-Bloom of August-September, 1973

For two years out of the past three, toxic dinoflagellate blooms have occurred in the late summer. In hopes of gaining further insight into the triggering mechanisms of such blooms, we examined the August-September, 1973 hydrographic data when no bloom was observed. Figure 9 shows that toxicity levels for both *M. edulis* and *M. arenaria* in Hampton Harbor were below 58 for the entire period. Rainfall was sparse (about the same as during August-September, 1972 with the exception of Hurricane Carrie), whereas runoff was very high in early August (about 365 m^3/sec or 12,900 ft^3/sec) but tapered off to typical late summer levels. Temperature patterns showed considerable daily variation with the suggestion of a small upwelling period late in September. The high runoff depressed surface salinities down to 30 °/oo early in August but they gradually rose again to 31.5 °/oo by the end of the summer. Total nitrogen was quite low but rose sharply during early September; in contrast total phosphorus levels remained quite high from early August through the rest of the period. Examination of 24-hour net-drift plots in Figure 10 for current meters located comparably to the 1974 ones (Figures 2, 5, and 7) indicates no apparent period of offshore nearsurface flows and landward bottom flows that could have triggered an upwelling period and upset the pattern of coastal thermal stratification.

DISCUSSION

From these data it appears that the upwelling phenomenon, a natural feature of the waters of the Bay of Fundy (13) where paralytic shellfish poisoning is a common occurrence, may be a key factor in the development of the three *G.*

tamarensis blooms in the western Gulf of Maine. Only recently has upwelling in these waters been documented (7, 14, 15). Upwelling is essentially an ascending motion in coastal waters of some minimum duration and extent whereby water from subsurface layers is brought into the surface layer along

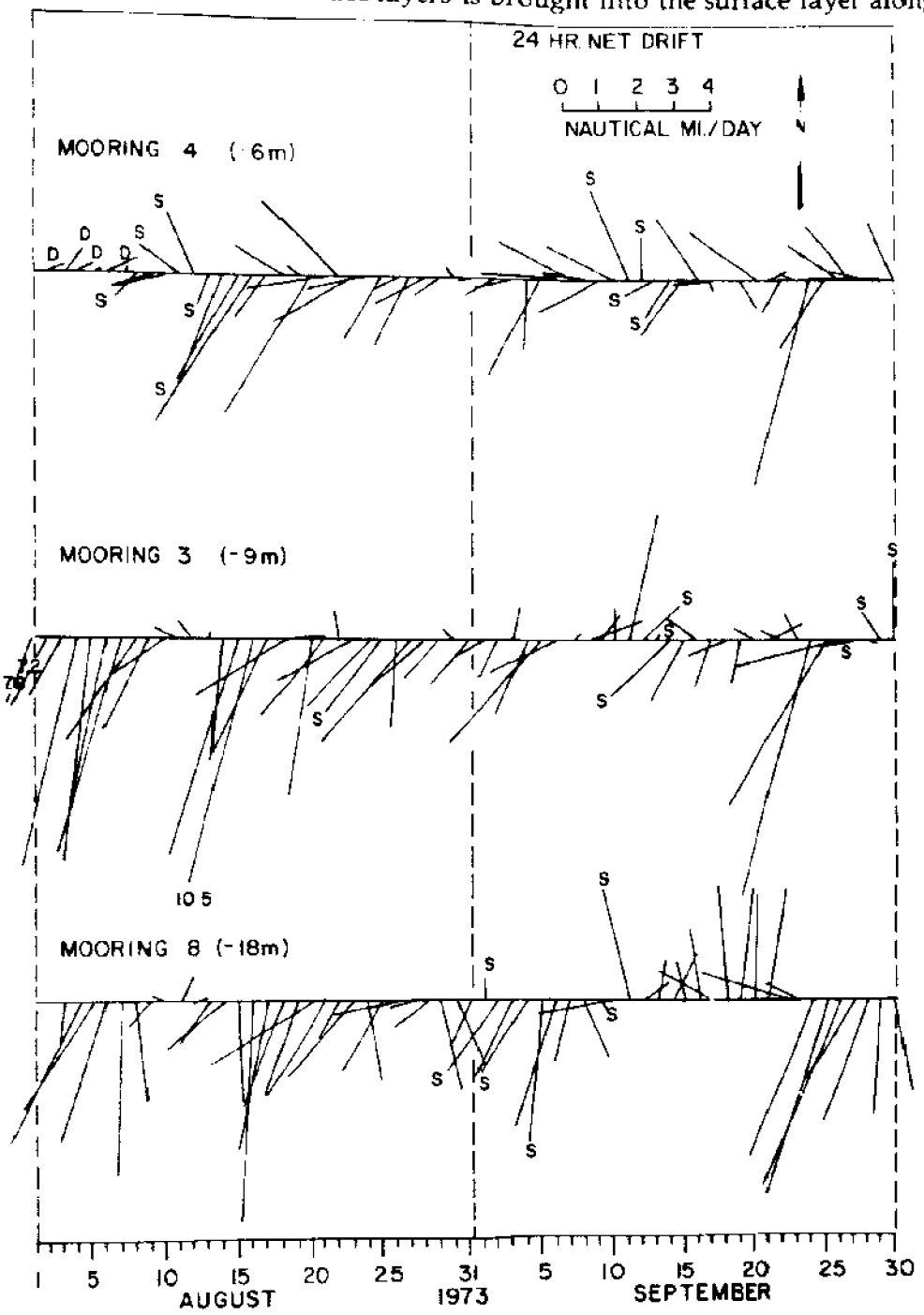


Figure 10: Sequential 24-hour net-drift vectors for August and September, 1973 from moorings 4 and 3 located 2 km offshore and mooring 8, 7.5 km offshore; D = direction data incomplete and S = speed data incomplete, assume 0.1 kts.

the coast and is subsequently removed from the area of upwelling by horizontal flow (16). Classically the phenomenon of upwelling has been ascribed to divergence in the surface water layer. The resulting offshore Ekman transport is generally associated with eastern boundary currents and with equatorward winds. But upwelling is known to occur under other circumstances and in other oceanographic regimes. (15).

Bigelow (4) noted shallow upwelling along the northwestern shores of Massachusetts Bay during the summer in association with westerly and southwesterly winds. This upwelling lowered surface temperatures by 4°C down to about 12°C in July and 10°C in August and caused an increase in surface salinity. These observations were almost identical to those described above from 1972-1974. Graham (7) suggested that upwelling is the most prominent feature of the coastal circulation of the western Gulf of Maine and found it associated with shoreward drift along the bottom and surface drift parallel to the shore offshore. Longard and Banks (17) found that when westerly winds were re-established at Sambro light vessel (Nova Scotia) after a period of easterlies, upwelling occurred near the coast with a subsequent decrease in surface water temperature at the light vessel. From oceanographic data collected at the Boston light vessel from 1955 through 1972, Kangas and Hufford (15) felt that localized upwelling occurs during most summers at the light vessel station in Massachusetts Bay in association with predominately westerly to southeasterly winds during the summer months.

Apparently these coastal waters first have to be well stratified before upwelling can occur. The surface winds drive the upper, warm freshened layer offshore and as a compensatory flow the deeper, cool salty water moves shoreward near the bottom. Once the stratification is upset nearshore, winds and waves help to mix the water column vertically. Under such conditions the *G. tamarensis* cells can be transported landward from mid depth and concentrated in the coastal region where they may encounter sufficient nutrients and light to bloom. In the Bay of Fundy high temperature, high light intensity and a relatively stable water column stimulate their growth (1). They can flourish in low concentrations of nitrogen and phosphorus and shown by the relatively large populations of dinoflagellates in temperate coastal waters of eastern Canada during summer, when these nutrients are at their lowest level. The largest populations seemed to occur with temperatures of about 10-12°C much like those off the New England coast.

SUMMARY

During August and September, 1972 the first major toxic dinoflagellate bloom of *G. tamarensis* ever observed in the western Gulf of Maine occurred. It may have been first initiated by a period of northeast winds which could have carried a "seed" population from eastern Maine and the Bay of Fundy across the Gulf of Maine. Offshore winds then triggered a period of coastal upwelling, drawing in and concentrating the dinoflagellates in the nearshore

waters off Cape Ann. A heavy rainfall and moderate runoff after a late-summer peak discharge may have added additional nutrients to the system. Temperature, salinity, and nutrient data are not very comprehensive but suggest favorable conditions for the bloom. In August and September, 1973 when no bloom occurred, no apparent coastal upwelling was observed. Rainfall was sparse but runoff was very high in early August and then tapered off to average late-summer levels. Temperatures showed typical summer thermal stratification; salinities were low then increased as the runoff effects dissipated. Nutrient data showed moderate total phosphorus but low total nitrogen until early September. During May and June, 1974 a small bloom occurred in conjunction with a coastal upwelling period, low rainfall, high to moderate runoff, fairly high salinities and total phosphorus, and low total nitrogen levels. This bloom may have been augmented by the presence of residual cysts from the earlier bloom (2). The most recent bloom which occurred in August and September, 1974 was coincidental to a period of coastal upwelling with moderate rainfall, generally low runoff, high salinities, and moderate nutrient levels. Recent studies by MIT in Massachusetts Bay during the spring of 1974 showed that nitrogen levels dropped as the bloom progressed, suggesting that the system may be nitrogen limited. Variations in total phosphorus seemed to have little effect (B. Pearce, personal communication).

In conclusion it appears from these data that coastal upwelling may have played a key role in the development of three toxic dinoflagellate blooms of *G. tamarensis* in the waters of the western Gulf of Maine during 1972-1974. Additional studies are needed to further document these findings and evaluate the interrelationship of other hydrographic parameters such as rainfall, runoff, temperature, salinity, and nutrients. Assuming that cysts of *G. tamarensis* have now been introduced into the coastal waters of Massachusetts, New Hampshire, and Maine because of the 1972 bloom, it is possible that toxic dinoflagellate blooms may continue to occur with some regularity during the warmer months whenever offshore winds create local coastal upwelling.

ACKNOWLEDGMENTS

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TEMPERATURE PATTERNS IN THE LONG-RANGE PREDICTION OF RED TIDE IN FLORIDA WATERS*

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ABSTRACT

Reliable techniques for predicting red tide would have significant practical value. Toxic blooms of *Gymnodinium breve* Davis along the central west coast of Florida not only place severe economic stress upon tourist-oriented communities but also hold the potential for serious public health problems. A strong empirical relationship has been discovered between patterns of surface water temperature and later major eruptions of red tide in the Gulf of Mexico near Tampa Bay. Predictive value of this technique lies in the ability to complete by early April all computations suggestive of major red tide during the next 12 months. Easily recognized indicator patterns of water temperatures during the period 19 January - 2 April have preceded by varying time intervals each and every one of the five occurrences of major red tide in the past 17 years at Egmont Key, Florida. Preliminary considerations strongly suggest recurrence of a serious bloom of *G. breve* there in 1975.

The developed predictive relationship is totally empirical and considered highly local in its application. Cause-effect dependency is neither claimed nor denied. This technique in no way conflicts with earlier causal models considering environmental conditions that immediately precede toxic dinoflagellate blooms.

INTRODUCTION

The term "red tide" is loosely applied to regions of biologically discolored sea water often associated with signs of extreme toxicity. When conditions in the sea are optimum for growth and/or aggregation of certain poisonous microorganisms, dinoflagellates in particular, populations rapidly increase by several orders of magnitude. Resulting concentrations of associated toxins then reach levels that are hazardous for marine life and for man.

Valuable planning use could be made of a dependable predictive technique by municipalities concerned with sanitation and pollution control and especially by public and private health agencies. I present here a method for forecasting major red tides in Florida gulf waters, predicated for the first time upon simple, standard oceanographic measurements.

The unarmored dinoflagellate, *Gymnodinium breve* Davis, is the primary causative agent of noxious red tides in waters of the Gulf of Mexico along the central west coast of Florida (3, 1, 8). Fish kills commence as organism concentrations rise above about 2.5×10^5 per liter and become massive with higher counts that at times reach many millions per liter (11, 13). The unsightly and unsanitary, malodorous conditions produced by tons of dead fish at the water's edge during such "blooms" place severe economic stress upon adjacent tourist-oriented communities (4). More importantly, the potential for serious human health problems has now become acutely apparent (5).

Many unsuccessful attempts have been made to relate onset of red tide to a host of environmental parameters. No significant correlations were found (9)

between organism abundance and the more likely required chemical nutrients for the 7-year period (1954-1961). These workers did find thru multiple curvilinear correlation that 61% of monthly variability of *G. breve* was associated with changes in salinity, temperature, onshore winds over 7 knots, and abundance of organisms the previous month. Unfortunately, the complex nature of such multi-dimensional relationships renders them of very limited predictive value.

Measured availability of recognized growth stimulants such as chelated iron has been proposed as an indicator of predisposition to red tides in certain regions off the Florida west coast (6). For example, a significant bloom would be expected near Charlotte Harbor following any three-month delivery to that area of at least 235,000 pounds of iron, measured well up the Peace River at Arcadia. Concomitant requirements were optimum water temperatures of 16-27°C and the absence of disturbing high winds within 150 miles of the harbor. These authors recognized the possible link between their "iron index" and other environmental parameters such as rainfall and the associated flushing into the sea of such vital chemicals as chelating agents.

WORKING HYPOTHESIS

Earlier workers generally have attempted to correlate onset of red tide with measured quantities or concentrations of suspected chemical and physical determinants. Blooms have been considered largely the result of random causative perturbations, principally nutritive, in the environment. In contrast, I envision red tide as essentially of kinetic origin with its appearance resulting from periodic shifts in rates of a complex interplay of highly competitive chemical and physical processes. It then reasonably follows that dinoflagellate blooms might be highly relatable to first, and possibly second, differentials with respect to time of chemical and physical parameters where correlations have been found to be insignificant in terms of absolute values. For example, rate of change of temperature would more likely be a governing influence on red tide than actual measured degrees.

Additionally, patterns of change in environmental parameters are considered to be equally important as rates of change. Thus, seasonal variations in measured values of chemical and physical factors establish after a time a pattern of change taken as average or normal for that particular region of water. Progressions thru the ranges in values of the various parameters are made at rates resulting from a complex combination of influences. Marine life prospering over long periods of time in such an area would then be those forms capable of accepting normal endemic variations in vital influences, both as to patterns of change and rates of change.

Simply stated, my hypothesis is as follows: In regions where red tides periodically occur, the probability of blooms reaching major proportions would be higher when certain conditioning factors more closely follow long-term average patterns and/or rates of change. Once requirements have

been met for disposition towards red tide, the actual timing of the occurrence would then depend upon short-range triggering influences such as nutrient availability, competitor pressure, and/or optimum physical conditions.

Temperature was deliberately taken as the parameter of choice for testing my hypothesis because of its fundamental role in the kinetics of chemical, physical, and biological processes in general and its recognized influence upon phytoplankton production in the sea (2). Blooms of *G. breve* have been observed in Florida waters over a temperature range of 15-33° C, but these organisms do not thrive at the temperature extremes nor when subjected to abnormally high rates of cooling (10, 12, 13, 11). No previous relationships have been developed between onset of red tide and temperatures within this range, which covers essentially the full seasonal variation along the Tampa Bay region of Florida.

METHODS AND RESULTS

For many years, the U. S. Weather Bureau (Tampa, Florida) has monitored surface sea temperatures off the U. S. Coast Guard Light Station at Egmont Key in the Gulf of Mexico near the mouth of Tampa Bay. The principal data base for this study was taken from their daily climatological records (Weather Bureau Form 5371), beginning with the year 1948. These data represent average surface water temperatures taken by thermometer read to the nearest degree Fahrenheit.

Daily water (surface) temperatures for the arbitrarily selected period of 1948 thru 1970 were used to develop the average annual cycle graphed as the smooth lower curve in Fig. 1. The curve is not symmetrical, for the sea at Egmont Key cools far more rapidly in the fall then it heats in the spring. Highest temperatures are normally realized in early August, with the low point reached in mid-January.

Periods of maximum and minimum rates of temperature change during the mean annual cycle are more clearly seen in the upper curve of Fig. 1. This rate curve is a plot of coefficients of regression of 1948-1970 average daily temperatures on time for 10-day periods culminating with each day of the year. The average rate of temperature change is zero in mid-January and early August, corresponding to minimum and maximum temperatures, respectively. Maximum heating rate occurs about the first of May, and cooling occurs most rapidly in early November. Periods of maximum rate appear as spring and fall inflection points in the lower temperature curve. Note in particular that the sea at Egmont Key heats many more days than it cools, i.e., 210 versus 156 days.

Rate of Temperature Change

Intense blooms of *G. breve* appear strongly related to rate of temperature change although rectilinearly unrelated to actual degress of temperature. All 12 months of the calendar year have been associated with Florida red tides

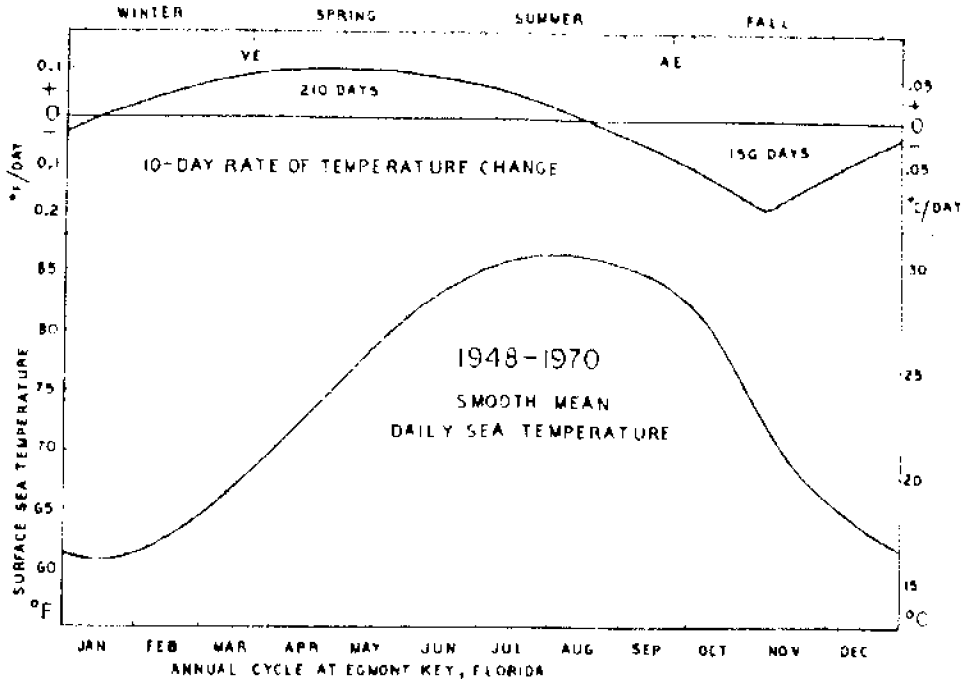

Figure 1. Smooth Mean Daily Sea Temperatures and Rates of Temperature Change at Egmont Key, Florida Calculated from U. S. Weather Bureau Measurements over the 23-Year Period 1948 thru 1970.

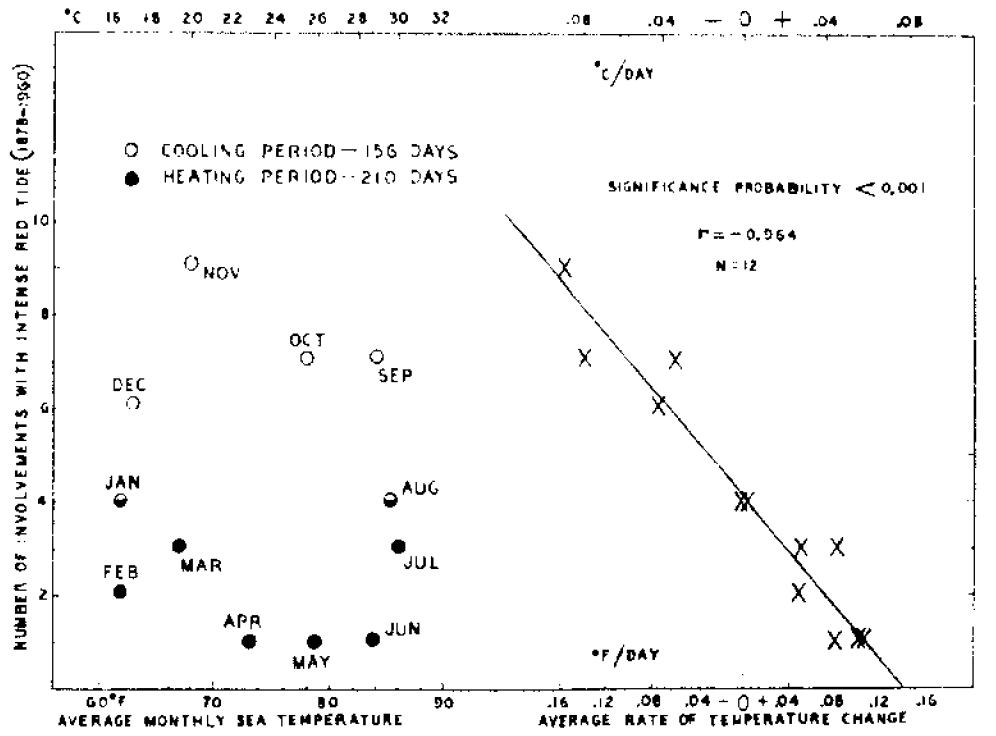

Figure 2. Monthly Occurrences of Intense Red Tide in Florida Waters as Related to Sea Temperatures and Rates of Temperature Change.

with varying frequencies. The cyclic relationship on the left in Fig. 2 was obtained by plotting (a), the number of involvements of each month of the year with intense red tide in Florida waters during the period 1878-1960 (7), against (b), the corresponding average sea temperature estimated for each month from the temperature curve of Fig. 1. Approximate symmetry of the cyclic plot about a mid-range value of approximately 75° F (24°C) indicates minimal dependence of incidence of major red tide upon temperature within the range of occurrence in Florida waters, i.e., about 59-91° F (15-33° C). On the other hand, the shading of the points indicates that the cooling part of the thermal cycle (August thru January) has seen far more intense toxic episodes than the general period of warming (February thru July). This is in spite of the fact that the heating period is almost 40 percent longer than the cooling period.

Additionally, regression (linear plot on the right in Fig. 2) of monthly red tide incidence upon estimated rate of temperature change (from the upper curve in Fig. 1) shows a strikingly intense negative correlation connoting a significance probability of less than 0.001.

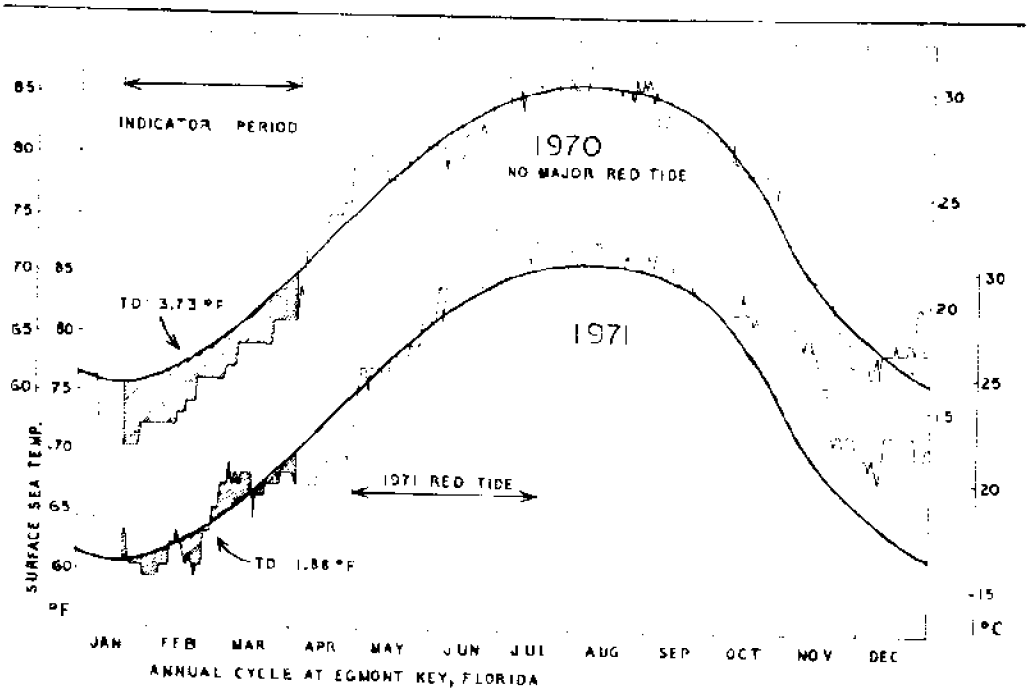

Figure 3. Typical Patterns of Daily Mean Surface Sea Temperatures (Egmont Key, Florida) in Comparison to the Average Annual Cycle. The data were treated in °F, as actually recorded by the U. S. Weather Bureau. Darkened areas signify deviations of temperature from the average cycle during the indicator period, 19 January - 2 April. Magnitudes of the deviations were calculated in terms of "temperature departure (TD)", as defined in the text. Note the relatively minor variations in 1971 compared to the previous year. Also, as seen on other occasions, the major red tide of 1971 followed a lengthy period of minimal temperature change.

Daily temperatures at Egmont Key were superimposed upon the average annual cycle (lower curve of Fig. 1) to produce plots typified in Fig. 3 by patterns for 1970 and for 1971, a year during which major red tide occurred at Egmont Key. In general, daily surface sea temperatures were found to deviate much more widely from the average between the dates of maximum cooling and maximum heating, i.e., November thru April. Furthermore, the magnitudes of such deviations seen in plots covering a number of years were found to be rhythmical and highly associated on a time scale with occurrence of major red tide in the immediate area of Egmont Key. The rhythm was found to be particularly pronounced during an "indicator period" that is the time of normal warming prior to reaching a sea temperature of 70° F (21.1°C), i.e., 19 January thru 2 April.

I devised a modification of standard deviation, the "temperature departure (TD)", to quantify variations in patterns of daily water temperatures from the smooth lower curve of Fig. 1. Temperature departure measures magnitude of deviation from a normal temperature curve and does not differentiate between directions of deviation, i.e., warmer or cooler. The TD is given by the formula,

$$\left[\Sigma(T-T_a)^2 / (N-1)\right]^{1/2},$$

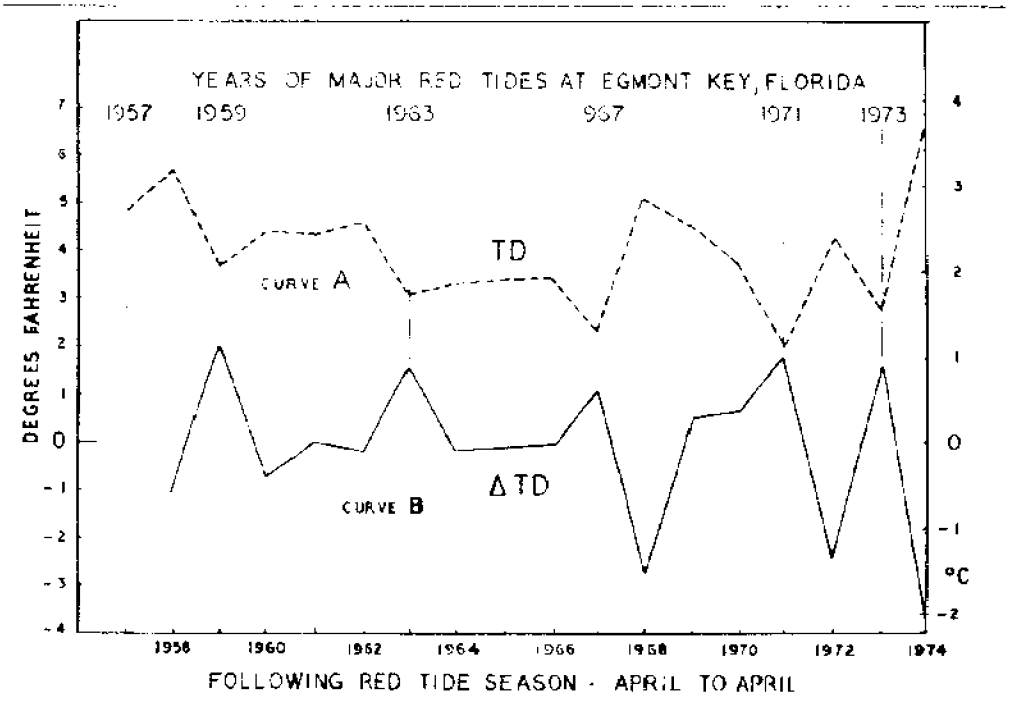

Figure 4. Temperature Departures (TD) and Values of ΔTD (TD of Previous Year Minus that of Subject Year) during Indicator Periods of 19 January - 2 April and the Strong Empirical Relationship to Subsequent Occurrence of Major Red Tide in the Area of Egmont Key, Florida.

where T is the measured daily mean water temperature, T_a is the long-term average temperature for that day (taken from the smooth mean curve in Fig. 1), and N is the number of days in the indicator period (74, except for one additional in leap years). Values for TD generally were in the range 1.9-6.5° F (1.1-3.6° C), with zero indicating no deviation at all in temperature pattern from that of the long-term average during the indicator period 19 January - 2 April.

Temperature data at Egmont Key were only complete enough to permit TD calculations for indicator periods since 1957, seen as Curve A in Fig. 4. Curve A is then a quantitative expression of the rhythm easily observed subjectively in plots (such as those of Fig. 3) covering a wide span of years. There is a suggestion in Curve A of a two-year cycle that often degrades to a four-year pattern. Minima seen in the plot indicate relative close approaches to the normal early warming pattern of sea temperatures. In each such instance, a major red tide erupted later that year (April to April) involving Egmont Key. Furthermore, no serious outbreaks occurred during years not showing minima in the plot of temperature departures.

A plot of the form of Curve A does not permit correlation with major red tide initiation during two consecutive years. However, each minimum in Curve A is associated with a marked improvement in TD over the previous year. The basic predictive plot then became that of Curve B in Fig. 4, which considers a delta temperature departure (ΔTD), i.e., TD for the previous year minus the subject year. For example, Fig. 3 indicates a ΔTD for 1971 of 3.73-1.88 = 1.85°F. A minimum value of ΔTD that excluded only the peak years coinciding with occurrence of major red tide was set at about 0.75° F (0.42° C).

CONCLUSIONS AND DISCUSSION

Although admittedly heavily burdened with assumptions, the plots of Fig. 2 strongly suggest a link between rates of change of water temperature and support (not to be confused with initiation) of intense blooms of *G. breve*, the causative agent of Florida red tide.

Close approach to a normal (average) pattern of water temperature change during the late winter months has been found highly indicative of impending *G. breve* blooms of major proportions along the central west coast of Florida. Evidencing the strength of this relationship was the occurrence of a major red tide in the region of Egmont Key following each and every instance (total of 5) in the past 17 years that temperature departures during the indicator periods (19 January - 2 April) improved by at least 0.75° F in consecutive years.

Predictive value of this relationship is enhanced by an ability to complete by 2 April all calculations suggestive of major red tide during the next 12 months. Even though major blooms started in one year have occasionally persisted into the next, actual initiation of a major red tide in Florida waters has not been reported during the January-thru-March indicator period. Also, major red tides at Egmont Key are not known to have been initiated in successive years.

The demonstrated strong relationship between patterns of sea temperature and subsequent major red tide is totally empirical, and cause-effect is neither claimed nor denied. It may well be that this is only a temperature-handle on some other causatively related parameter, such as movements of water masses or a complexity of meterological factors.

Calculations strongly indicate (Fig. 4) that it is highly unlikely for initiation of a major bloom of *G. breve* to occur again prior to April 1975 in the region of Egmont Key, Florida. On the other hand, the outlook might not be so optimistic for later in 1975. The high temperature departure of 6.46°F (3.59°C) noted for 1974 would require a minimum of 5.71° F (3.17° C) for 1975 so as not to exceed for ΔTD the value of 0.75° F (0.42° C) that has been shown to be highly indicative of impending major red tide. Unfortunately, deviations of this magnitude from the normal pattern of water temperature have been noted only twice in the past 18 years.

Since red tides are often highly localized, patterns of water temperature can be taken as anticipatory signs of major blooms only within the geographical limits for which the data are representative. Correlation has been demonstrated only for the immediate region of Egmont Key, Florida, and only for major blooms of *G. breve*. It remains to be tested whether similar signs can be recognized in temperature patterns of other localities which will portend major shifts in populations of these and/or other marine microorganisms.

FUTURE PLANS

Surface sea temperatures at Egmont Key will continue to be monitored to test the predictive quality of the demonstrated association between patterns of rising temperatures in the spring and subsequent occurrence of major red tide.

Applications of this general approach to prediction capability will be made in other geographical areas affected by periodic blooms of toxic marine microorganisms, especially those localities where historical temperature data are already available. Temperature monitoring will be initiated in some areas, especially along the gulf coast of central Florida, where early red tide prediction would be of particular practical and/or scientific significance.

Temperature data will continue to be examined in terms of rates of change immediately preceding, during the course of, and in association with the demise of major *G. breve* blooms. For example, note in Fig. 3 the lengthy period of minimal temperature change (followed by a rapid rise) immediately preceding major red tide at Egmont Key in 1971. Thus, properly interpreted temperature data may be of value in detecting imminence of a major bloom as well as its long range prediction.

Utilization of remote sensing of sea temperatures from orbiting or stationary satellites and/or high altitude aircraft would permit greater geographical coverage. Such techniques should also provide insight into the direction and rate of movement of surface isotherms and their effects upon temperature patterns observed at a fixed monitoring station.

Water temperature was only the first parameter chosen to be examined in terms of association between rates and/or patterns of change and occurrence of major red tide. The Mote Marine Laboratory has already commenced application of this approach to the mass of chemical data that has been generated over the past three years by a weekly water sampling program conducted in collaboration with the University of South Florida.

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THE LIMITATIONS OF PHYSICAL MODELS FOR RED TIDES

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INTRODUCTION

Two types of oceanographic processes have been used to account for the occurrence of red tides on a purely physical basis. These are the circulation patterns observed in shallow water, and in deep water above the pycnocline, and the processes which lead to an increase in vertical stability in the water column. These are not always distinct, and the two tend to occur simultaneously in many oceanographic situations. But it is useful to make the distinction for didactic purposes, and there are situations where one or other is relatively weakly developed. Routine synoptic surveys rarely provide information on small-scale physical features of the marine environment since sampling programs are generally too coarse in both space and time to reveal them, even when the observations necessary for their identification and description are made. Red tides and related phenomena are for the most part small-scale features of the sea when viewed against the physical background, however dramatic they may sometimes appear to those directly involved. Any attempt to explain red tides, and more broadly the distributions of plankton populations in limited sea areas, must take account of the advective and diffusive processes which such populations experience as a result of their planktonic existence. In this paper, these processes are reviewed in an effort to find out how far they are useful. This is followed by some remarks on those particular features of red tides which do not seem to be explicable on a purely physical basis, and for which strictly ecological mechanisms must be responsible.

CONCENTRATING MECHANISMS

i. *Thermally induced motion*

The static stability of a water column is usually defined by means of the criterion of Hesselberg:

$$E = \frac{1}{\rho}\left(\frac{\delta\rho}{dz}\right)$$

where ρ is the density and z the depth, and where $\delta\rho$ indicates that adiabatic temperature changes are accounted for. In shallow depths (less than 100 m), these can be ignored, so

$$E = \frac{1}{\rho}\left(\frac{d\rho}{dz}\right)$$

It is assumed here that there is no change in salinity with depth, i.e. $ds/dz = 0$. A relationship which incorporates salinity explicity is given by Defant (4). Negative values of E indicating static instability are observed in all parts of the Atlantic Ocean between 20°N and 50°S ('Meteor' pre-war data), and particularly high negative values between 15°S and 20°S. Defant suggests that negative values of E can be maintained by evaporation at the surface leading to the establishment of convection cells of the kind first described by Benard (3).

A schematic view of these is shown in Fig. 1. The diameter of the Benard cells is found experimentally to be 3 to 4 times the thickness of the liquid layer, so that if organisms are accumulated by the upwelling currents of such cells, concentrations of organisms may be expected to form bands at intervals of 6 to 8 times the depth of the bottom, or of the thermocline. In surface view, and in the absence of advection, these bands will form a polygonal pattern, but in a current the streamlines will be helical and the bands will take the form of sinuous streaks lying parallel to one another. Stommel (23) has summarized the theory of convection cells, and in a later paper (24) he gives an account of the effect such cells will have on passive particles with different sinking rates in a non-turbulent water column. Stommel defines regions of retention (R) as the ratio of the settling velocity (v) to an arbitrary stream function ψ . These regions then increase in size with decreasing values of R as shown in Fig. 1. The intervening regions lose their quota of particles either by sinking through the thermocline or by concentration into the regions of retention. The introduction of turbulence will of course work against this concentrating effect.

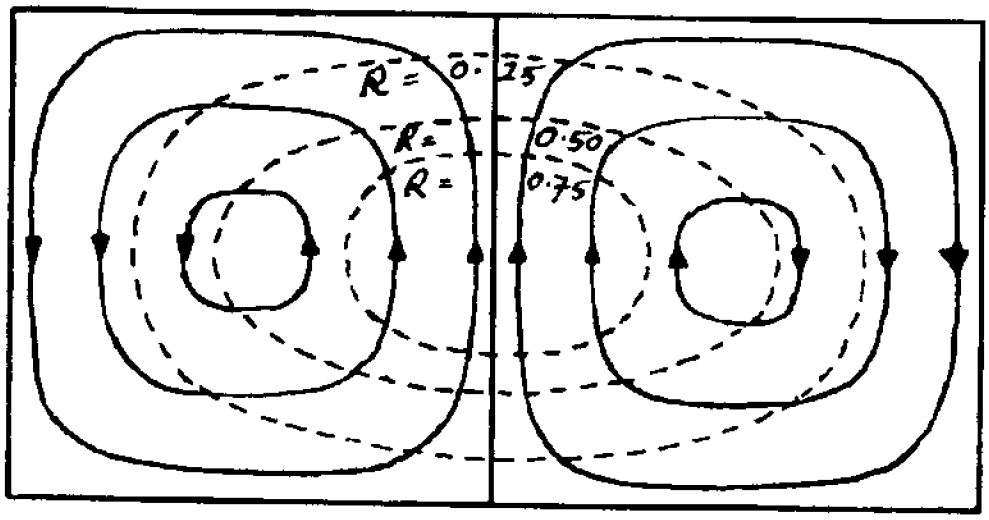

Figure 1. Diagram of streamlines in convection cells with boundaries of regions of retention for three values of R. (from Stommel 1949).

A system of this kind may be expected *a priori* to have a sorting effect on a phytoplankton population consisting of cells of different sizes. Bands of large cells, i.e. those with relatively rapid sinking rates, would be narrower than those of small cells in both the vertical and horizontal planes, and in positively buoyant cells, i.e. those with negative values of v, these relationships will be reversed and the regions of retention will be centered on the down-welling parts of the system. Motile positively phototactic cells would concentrate in the dotted regions marked Q in Fig. 2, provided that the downwelling current velocity was not greater than their own swimming speed. Neutrally buoyant cells would not be segregated. The effect of such convection cells on the

zooplankton will follow the same principles. These possibilities are shown in Fig. 2, and suggest why it is that lanes of plankton observed at the surface are much more frequently composed of motile organisms like dinoflagellates, or of buoyant organisms like *Trichodesmium* or *Sargassum*, and the beautiful float-bearing molluscs and siphonophores of the pneuston.

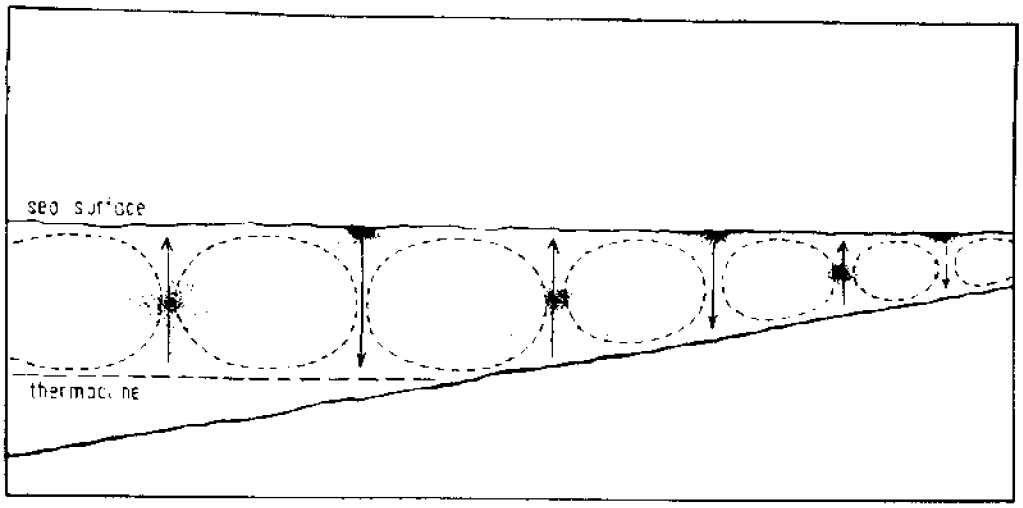

Figure 2. Diagram of convection cells over thermocline and shoaling bottom. Regions where organisms accumulate are dotted. P = non-motile organisms with positive sinking velocity; Q = motile or buoyant organisms.

WIND INDUCED MOTION

A mixing pattern similar to that of thermal convection cells is formed under the influence of wind, and is known as the Langmuir Circulation. Faller (6) thinks this may be the "primary mechanism by which the thermocline in the oceans is eroded during times of high wind". It is nearly always present, at least when wind velocities exceed 3 m sec $^{-1}$, and is sometimes indicated by surface slicks which lie 14^{o} to the right of the wind direction (7), and which re-orientate in 20 to 40 minutes following a change of wind direction (15). Fig. 3 shows the streamlines in surface view, with down-welling and upwelling regions indicated by full and broken lines respectively. The dimension of these cells is of the same order as that of thermal convection cells in shallow water, or where a thermocline exists near the surface; in deep water, however, it is related to the wind velocity, and as developed by Faller (7) the distance between slicks L = Wind Velocity x 4.8. Wind induced circulation patterns of this kind are not distinct from the convection cells already described as far as the plankton is concerned, and the remarks made in regard to the latter apply here too.

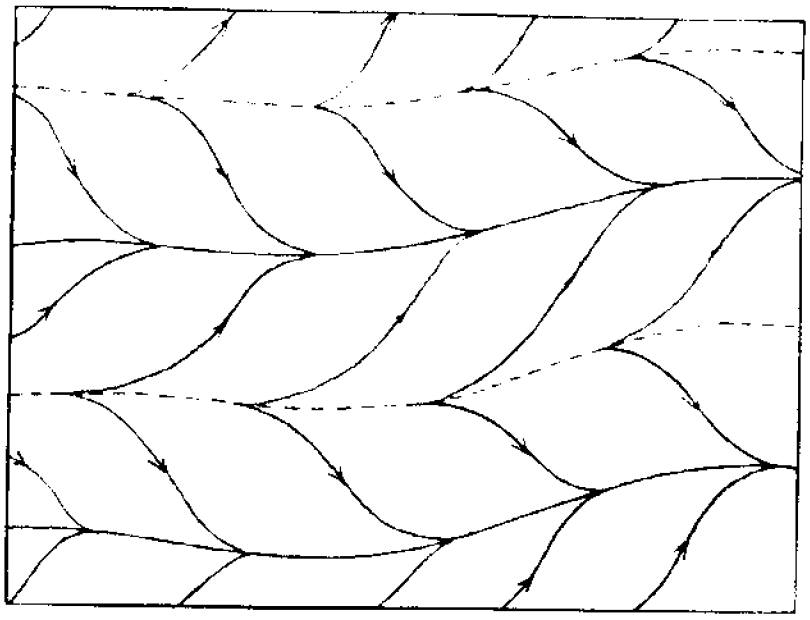

Figure 3. Surface view of streamlines of convection cells with lines of upwelling (divergence) shown as broken lines, and of downwelling (convergence) as full lines.

Bary (1) describes blooms of *Cyclotrichium meuneri* (= *Mesodinium rubrum*) in Wellington Harbour whose distributions seem to fulfill expectations based on this type of mechanism, though in some cases the lanes seem to be too far apart in view of the depth of ~20 m. The patterns are illustrated in Fig. 4 and are more confused in the restricted entrance to the harbour where tidal forces are amplified.

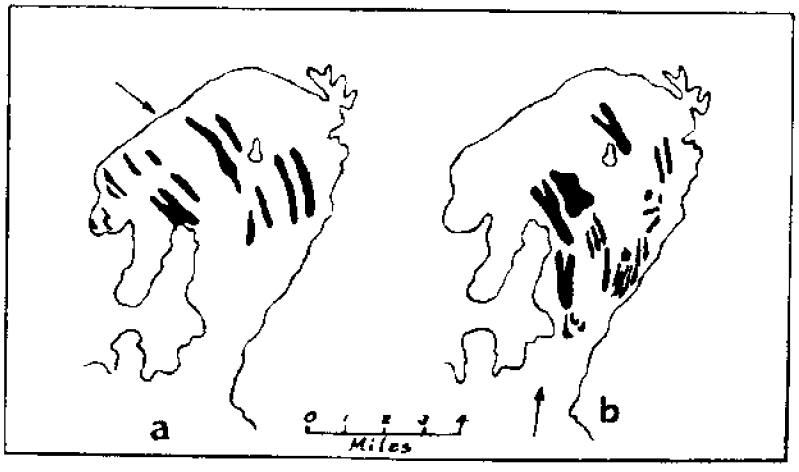

Figure 4. Distribution of patches and lanes of *Mesodinium rubrum* in Wellington Harbour, New Zealand. Wind vectors are shown:
a) March 24, 1949; wind N.W. force 5-6.
b) March 23, 1949; wind S.S.E. force 4-5. (after Bary 1953).

Stability in a turbulent medium is usually expressed in terms of R_i, the Richardson number, defined as

$$Ri = \frac{g}{\rho}\left(\frac{\partial \sigma}{\partial z}\right)\left(\frac{\partial u}{\partial z}\right)^{-2} \equiv gE\left(\frac{\partial u}{\partial z}\right)^{-2}$$

where $\partial u / \partial z$ is the vertical component of water movement, and E is as before. Large values of R_i indicate that turbulence is suppressed, and would further indicate that conditions were suitable for a generalized concentration of positively phototactic organisms at the surface, not necessarily in streaks. At somewhat lower, but still relatively high values of R_i (induced, for example, by an increase in wind stress), there would presumably be a tendency for such a generalized accumulation to form into streaks, indicating the establishment of convection cells. Such a change in the surface distribution pattern of plankton populations has in fact been observed following a number of outbreaks of red tide. One can also imagine a situation in which such a series of events occurs, and in which nutrient levels only become limiting after the formation of the streaks. The characteristic colour of the red tide might then appear to be associated with an increasing wind. This again has been observed. Unfortunately, data from which to calculate Richardson numbers are not often available.

The ubiquitous nature of convection cells suggests that they are an important force governing the evolution of plankton populations. In particular, it seems probable that the range of specific gravities which a species can adopt must be constrained by the normal values of the Richardson number experienced within its geographical distribution. If this range is genetically controlled, then in the event of unusually high R_i values, non-motile cells with positive sinking rates will not be able to maintain themselves in the water column. A mechanism of this kind could contribute further to the dominance of a motile species in our earlier model (31). Evolutionary trends in phytoplankton populations which may depend on convection cells are also likely to have both direct and indirect effects on organisms at higher trophic levels. A good illustration is provided by *Physalia* and *Velella*, whose sails are such that they move at a large angle to the wind. Woodcock (26) has suggested that this asymmetry is designed to exploit concentrations of plankton at divergences, and to avoid entrapment in convergences where they can become entangled with *Sargassum*. One imagines that nektonic animals have also learned to use convection cells, just as sea birds have the exactly analogous convection cells of the lower atmosphere. Some additional remarks on the evolutionary strategies of plankton in relation to turbulent processes are given by Wyatt (29).

FRONTS

The lines representing downwelling and upwelling regions in Fig. 2 are examples of singularities or fronts, and on a larger scale are well-known

features of the general oceanic circulation. They are usually caused by the meeting of water masses of different densities, and this provides a further mechanism for the concentration of plankton organisms. One of the most dramatic descriptions of such a front is given by Beebe (2), who, during the *Arcturus* expedition, at a position 400 miles southwest of Panama, encountered a greater concentration of organisms than he had ever seen before. "A few scoops with a hand net would collect a mass equal to a long haul through average ocean water, ... yet ten yards on either side of the central line of foam, the water was almost barren of life." This front was marked not only by accumulations of plankton, but by large concentrations of fish, dolphins and birds, and was followed by Beebe for a hundred miles. Analogous features, much smaller in scale, are found where estuarine and coastal waters meet. The circulation pattern observed at the interface between an estuarine wedge and coastal water is shown in Fig. 5 a, and two excellent examples of red tide outbreaks related to such wedges are illustrated in Fig. 5 b and c.

Intermediate in scale between the singularities of oceanographers, and those marking the offshore boundaries of estuaries, are those which mark the mixing regions between different water types in shallow coastal regions. A well

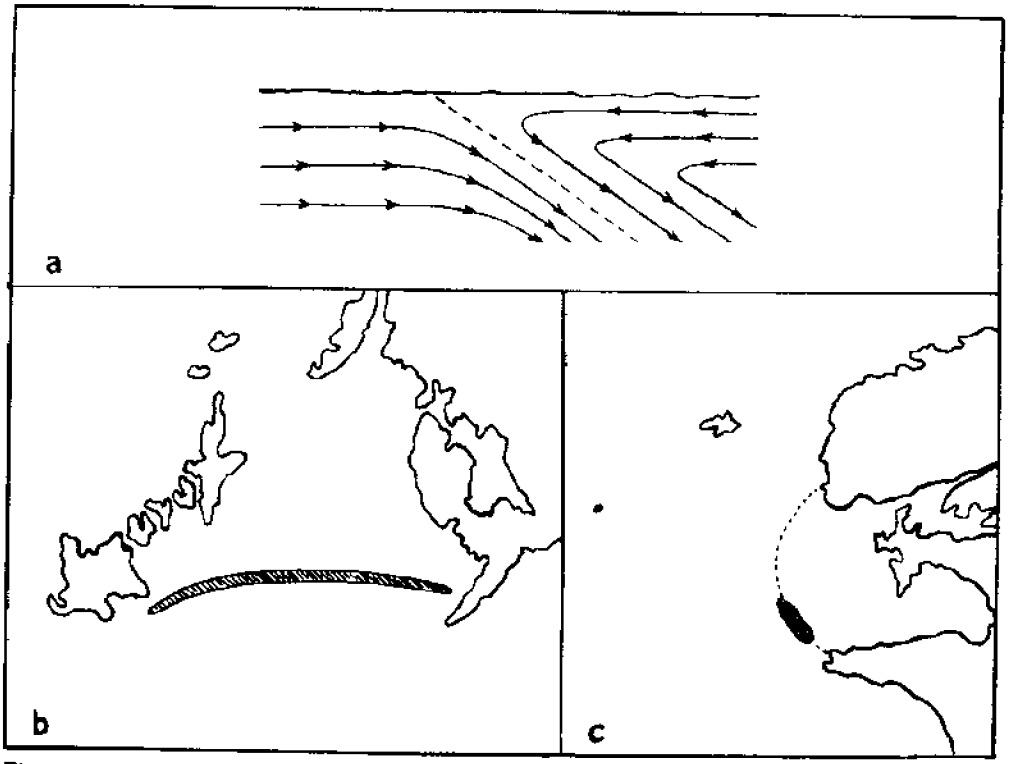

Figure 5. a. Streamlines associated with estuarine wedge giving rise to convergent front.
b. Goto Nada Bay in Nagasaki Prefecture, Japan, showing *Trichodesmium* distribution extending for 80 km along convergence (after Iizuka and Irie (12).
c. Ushant region, west coast of Brittany, showing similar distribution of *Noctiluca* along a convergence (after Le Fevre and Grall, 16).

known example is that found in the Sund between Sweden and Denmark where Baltic water of salinity 7‰ meets North Sea water of salinity 32‰. The change in salinity is not continuous as mean charts show, but in two 'jumps' (4). The local circulation patterns at fronts of this kind are again as in Fig. 5 a. Similar fronts of a more transient nature might be set up following hurricanes.

There is a further type of front in shelf seas. In some areas a thermocline is formed and in others it is not: formation depends on depth and tidal stream speed. Where these two sorts of area are contiguous a front is formed. An example in the English Channel has been described by Pingree, Forster and Morrison (19). Zooplankton in eight categories was concentrated on average about 30 times in this example.

INTERNAL WAVES

Surface slicks are found associated with internal waves when the wind speed is less than 3.5 m sec^{-1} (14), and are situated above the rising part of the thermocline. These slicks are generally visible as narrow lanes where the ripples are damped, and are caused by concentrations of surface-active materials. These substances are not retained in the slicks, but are drawn into an oncoming slick, held there for a time, and then expelled from the rear (5). There is no evidence of vertical water movement beneath these slicks sufficient to entrain buoyant material, and it does not seem likely that internal waves provide a direct mechanism through slick formation for the accumulation of plankton in lanes. But intuitively it seems probable that plankton is accumulated in shallow water by internal waves, where they are refracted and ultimately break, in the same way that surface waves throw up lines of floating debris on the beach. The bloom of *Aulacodiscus kittoni* recorded by Becking *et al.* in 1927, fide Rounsefell and Nelson (20) might have initially been concentrated in inshore waters at Copalis Bay by such a mechanism.

Some novel ideas concerning the ways in which plankton populations may interact with internal waves have been investigated from a theoretical standpoint by Kamykowski (13). He has considered these interactions for three cases: 1) passive phytoplankton, 2) motile phytoplankton which does not migrate through the thermocline, 3) motile phytoplankton which does migrate through the thermocline. All three cases lead to the possibility of contagious distributions of the phytoplankton, and to a sorting out of different species. The integrating effect of this mechanism is very dramatic, and field tests of Kamykowski's ideas might lead to some exciting results. Should such effects prove to be actually taking place in the sea, they will interact with the sorting effects of convection cells and, depending on the relative wavelengths of the two processes, either damping or amplification may be expected to occur. Such a combination of processes might then account for the fact that the spacing of lanes of *Trichodesmium* and other red tide species often seems at variance with that expected on the basis of convection cells alone. The wavelengths of internal waves vary, from 70 m moving at 15 cm sec^{-1} (14) to 60 km moving at 250 cm sec^{-1} (8), so that the possibilities are almost limitless.

LIMITATIONS OF PHYSICAL MODELS

All these various mechanisms under certain circumstances seem able to concentrate plankton into lanes or streaks, both at the surface and below it. An earlier review of some of them was given by Ryther (21). Their value in explaining the occurrence of such streaks must depend on detailed quantitative assessments of the water movements involved. Few relevant measurements are available. Sutcliffe *et al.* (25) give values for downwelling motion under slicks in the Bermuda area which range from 2.7 to 5.3 cm sec^{-1} at wind speeds of 6.4 to 13.0 knots, and Woodcock (27) reports a value of 5 cm sec^{-1} to a depth of several metres under windrows of *Sargassum*. The complementary upwelling motion must have about the same magnitude. These values are rather higher than the known swimming speeds of dinoflagellates, which do not exceed 1 cm sec^{-1} (18, 10), and point once again to the importance of calm conditions in red tide outbreaks. Only field work in which small-scale circulation patterns are studied in conjunction with the detailed distribution of plankton, both horizontally and vertically, can shed new light on these problems.

It is perhaps worth pointing out that although red tide is usually observed as a surface phenomenon, the maximum concentrations of cells are not necessarily found in the immediate surface layer. Iizuka & Irie (12) have shown the vertical distribution of cells in Omura Bay, Japan during a *Gymnodinium* bloom. The highest cell densities occurred about 5 m below the surface. A possible reason for this is discussed later.

Only one of these physical mechanisms directly allows patchy, as opposed to streaky, accumulations of plankton at or near the surface in offshore waters, namely the establishment of high Richardson numbers. The various ways in which this can occur are all frequently associated with red tides, as has been emphasized before (28). Streaks formed by convection cells might also be expected to spread laterally and join one another when the forces maintaining them decay. This would commonly lead to a patchy distribution in which individual patches would be of relatively small areal extent, say tens of metres in length; this is the typical pattern observed during *Noctiluca* blooms in temperate inshore regions. Such a situation would not necessarily be associated with high Richardson numbers, but would be much more persistent if it were. If very high concentrations of cells were present in the pre-existing lanes, lateral spreading could then lead to a very extended patch diversified by isolated regions of clear water. If the red colour is due to nutrient limitation, then a variety of different colour changes can be imagined in conjunction with these processes, depending on the flux rates of the nutrient in and between the lanes.

In shallow water, all the mechanisms discussed can lead to patchy distributions or at least to distributions which are not practically distinguishable as streaky. Seliger *et al.* (22) give a model based on convergence mechanisms which is used to account for the concentrations of *Pyrodinium bahamense* in Bahia Fosforescente, Puerto Rico. Kamykowski's

model might possibly offer an equally satisfying explanation of this curious phenomenon.

Finally, attention is drawn to some aspects of red tides which are not explicable in terms of the physical models reviewed here. In Japan, a distinction is made between red tides which occur in July, the rainy season, and those which occur in September. The former are surface phenomena found either in inland seas or close inshore, and do not occur in years of low rainfall. The latter are found at greater distances from the coast as well as inshore, and seem to be associated with anoxic bottom conditions. Different groups of species are involved in the two cases. A similar distinction can be made on the basis of published information concerning red tides in Indian coastal waters. Apart from the role which organic compounds may have in these outbreaks and which has puzzled Japanese scientists for so long (9), it would appear that the distinction made here must be related to successional events in the phytoplankton of regions concerned. Marine successions have been discussed elsewhere (17, 30), but briefly, though successional patterns are similar from one year to the next, the details vary a great deal depending on the exact history of the water mass in question. Food chain dynamics as well as the physiological preferences of individual species are important in this context. It is here that we must look for the reasons why the activities of one particular species give rise to a red tide, when there are several potential candidates.

Red tides caused by herbivorous organisms like *Noctiluca* and *Mesodinium* seem to present a special problem in that they would appear to need to be preceded by an algal bloom of red tide proportions. Yet algal and protozoan red tides have never been reported in close conjunction. At Lowestoft, we are attempting to simulate a system of this kind in such a way that a protozoan bloom is produced, without the algal bloom which precedes it reaching densities which we would judge to become visible. So far this has only been achieved by choice of a grazing function, for the existence of which evidence is at present restricted. It may of course be that in these instances physical concentrating mechanisms on their own are sufficent to account for the densities of organisms observed.

A third aspect of red tides has already been mentioned several times. This is the question of motility, which forms an essential constituent of all three models referred to (22, 31, 13). Almost all red tides, except those caused by *Trichodesmium*, are composed of motile organisms, and when this is not so, buoyancy seems to be a necessary attribute. Quantitative modelling of red tides therefore requires more detailed information on the swimming speeds and migratory habits of the organisms concerned.

In conclusion, it seems worth while to reiterate the fact that modelling techniques have played a relatively minor part in studies of red tides and related phenomena. Special explanations of them have often been suggested which have tended to set them apart from the mainstreams of plankton research. Yet there does not appear to be any convincing reason why they should be regarded in this way, as something more than an extreme product of

oceanographic and ecological forces. For at least two reasons it seems desirable that further efforts should be made to bring red tide studies into the general framework of theoretical marine ecology. In the present context it should lead to more soundly based field studies and to a better understanding of the events which give rise to red tides. This is particularly true now that modellers are turning their attention to the turbulent processes which have been reviewed here. Red tides may also have an important contribution to make to the problem of ecological stability. The situation they represent is just that which simulation studies seek to avoid. Persistent results of the kind which in nature would lead to a red tide usually result in the modeller tinkering about with his equations!

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FLORIDA DEPARTMENT OF NATURAL RESOURCES RED TIDE RESEARCH PROGRAM[1]

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ABSTRACT

The first reported fish kill accompanied by discolored water off Florida's west coast occurred in 1844. Since then, about 30 red tides have been reported, but research to define causative parameters was only activated in the early 1950's. Over the last 20 years, the Florida Department of Natural Resources (FDNR) has studied the following: 1) zone of outbreak of initiation, 2) nutrition of the causative organism, *Gymnodinium breve*, 3) hydrologic and meteorologic conditions and their importance in maintaining and transporting motile populations, 4) effects on offshore patch reef biota, 5) *G. breve* ecology in relation to shellfish toxicity, 6) taxonomy and ecology of associated phytoplankton, and 7) predictive methods.

Current FDNR research is concentrated on: 1) *G. breve* life cycle studies, 2) examining the possibility of seed beds 16 to 64 km (10 to 40 miles) offshore, 3) longevity of GB toxin in seawater and sediments, 4) susceptibility of various marine animals to GB toxin(s) in seawater or through the food-chain, 5) causes of death in affected marine animals, 6) fisheries repopulation studies, 7) further evaluation of land discharges in supporting red tides, and 8) developing methods to remove and utilize floating dead fish. This research is designed to aid in evaluating various predictive methods and to lessen economic impact. It has also become apparent that an information program is needed to educate the public on this natural phenomenon.

INTRODUCTION

Records of fish kills associated with discolored water along Florida's west coast exist from 1844. Since then, about 30 red tides have been documented (1); however, it was not until the 1946-47 red tide that scientists determined the cause (2). Florida red tides are caused by blooms of an unarmored, photosynthetic dinoflagellate, *Gymnodinium breve*. This microscopic organism produces a toxin which can kill fish and other marine animals. The toxin can also accumulate in exposed filter-feeding shellfish which then become toxic for human consumption. In the United States, *G. breve* red tides occur most frequently from the Anclote Keys to the Florida Keys, but they have occurred infrequently in the Gulf off Texas (3) and north Florida (4) as well as one incidence off the southeast coast of Florida (5). During the 1946-47 outbreak off southwest Florida, the U. S. Fish and Wildlife Service, University of Miami, and Woods Hole Oceanographic Institution initiated scientific studies to determine causal factors. Shortly thereafter, the Florida Board of Conservation (now the Florida Department of Natural Resources) became involved and since that time has actively been researching *G. breve* blooms.

PAST RESEARCH

The Florida Department of Natural Resources (FDNR) has performed or supported research in the following areas: *G. breve* morphology, nutrition of *G. breve*, biological, chemical, and physical factors involved in red tides, and prediction. The original approach, under the direction of Robert M. Ingle, past Chief of FDNR's Bureau of Marine Science and Technology, was to study coastal areas before, during, and after red tides. This was the same approach used by the U.S. Fish and Wildlife Service, Bureau of Commercial Fisheries (now the National Marine Fisheries Service).

G. breve Morphology

Since the description of *G. breve* by Davis (2), FDNR has supported further studies of its morphology for easy, accurate identification. Wilson (6) described morphological features of motile and immotile forms of *G. breve* in culture and was the first to suggest the possibility of a sexual cycle for this species. Additionally, Steidinger, Davis, and Williams (7) and Steidinger and Williams (8) described three significant morphological differences from Davis' original description.

Nutrition of G. breve

The importance of chelators in making seawater more suitable for *G. breve* growth was first demonstrated by Wilson (9). He attributed this to two possible factors: chelators 1) reduce toxicity of heavy metals and 2) make trace metals and minor nutrients more available to the organism. Following this, studies were conducted to determine distribution and concentration of naturally occurring chelators and trace metals in west coast Florida rivers and their possible correlation with red tide outbreaks (10, 11).

Gymnodinium breve is a unicellular planktonic alga and requires specific nutrients and growth factors. Two such requirements, phosphorus and vitamin B_{12}, were studied from a field and laboratory approach. At the time of our initial studies, phosphates were a major concern and some researchers suggested that excess discharge of phosphates might be a triggering mechanism. However, extensive culture analyses by Wilson (9) produced results which, when compared with field data, showed there were more than sufficient quantities of phosphates in nearshore waters to support a bloom year round and that increased phosphates merely supported a higher cell population per volume of seawater. Stewart, Wahlquist, and Burket (12) showed that over a seven-month period, inshore seawater samples collected from Tampa Bay to Cape Romano contained sufficient vitamin B_{12} to support a *G. breve* bloom; however, this species did not bloom during the sampling period. These results indicate that although *G. breve* requires phosphates and vitamin B_{12} for growth, sufficient quantities of each is not a triggering factor.

Biological, Chemical, and Physical Factors

Since 1955, FDNR sampling programs during red tide and non-red tide years have helped establish the distribution of *G. breve* and associated phytoplankton in the eastern Gulf and adjacent waters relative to basic oceanographic parameters. Much of this information is published (1, 8, 13, 14, 15, 16) and all of the raw data is on file. *Gymnodinium breve* is a coastal species and under normal conditions is restricted by low salinities from entering estuaries where shellfish are harvested. However, during droughts which cause higher salinity regimes in bays, *G. breve* red tides once transported inshore have a greater chance of survival and shellfish exposed to the organism or the toxin in seawater can become toxic (17, 18). Field data indicate that shellfish toxicity disappears one to two months after a red tide has terminated. Steidinger (19) described the probable sequence of red tides. *Gymnodinium breve* blooms are initiated 16 to 64 km offshore. Then favorable conditions, such as sufficient nutrients and growth factors and optimal salinities and temperatures, support the bloom which is maintained and concentrated by such physical mechanisms as winds, currents, and convergence areas. This offshore bloom may also be transported to inshore waters. In addition to transport, physical conditions may influence the severity of red tide effects. For example, during the unusual 1971 spring-summer red tide (18), some patch reef communities off Sarasota sustained heavy mortalities which were due indirectly to a red tide apparently confined to bottom water beneath a thermocline (20).

PREDICTION

Prediction or early detection of red tides would obviously be of assistance in forewarning public officials of potential public health hazards and needs for fish removal. Three methods have been suggested based on past research. Ingle and co-workers (21) suggested aerial surveillance to determine locations of discolored water and fish kills. Such information coupled with meteorological and current predictions could be sufficient in approximating where dead fish, irritation, and/or fish kills could be expected over the next few days. A second method is the iron index proposed by Ingle and Martin (22). Reviewing 25 years data, they found that major red tides occur off the Charlotte Harbor area when 235,000 pounds of iron are potentially delivered in Peace River discharge over a three-month period. They did not specify iron as a triggering factor, but considered it a measurable indicator of stimulatory factors in the water. A third method suggested was regular monitoring programs to detect coastal increases in *G. breve* populations (1). This method, based on actual cell counts, could give a two- to four-week forewarning of possible fish kills. However, cell counts alone cannot be used to determine duration or path of the bloom.

CURRENT RESEARCH

Present research basically covers eight projects in three areas: biology of the causative organism and zone of initiation, pathological and ecological effects of *G. breve*, and prediction and amelioration of Florida red tides. Our goal, then, is to determine why *G. breve* blooms occur, predict their path and duration, and lessen the economic impact to affected Florida communities.

Biology of G. breve and Initiation Zone

Electron microscopic studies of *G. breve* have begun and will be used to clarify its taxonomic position, characterize different life stages and determine the presence or absence of endosymbionts. Such studies may also assess why culture specimens are apparently more fragile than field specimens. Electron microscopy of cells exposed to different light qualities is planned as part of an experiment currently being performed to test the use of fluorescent bulbs other than cool white in culturing *G. breve*.

The major thrust biologically is to determine the life cycle of this dinoflagellate. Research by Wall and Dale (23) confirms the existence of benthic resting cysts for a variety of marine dinoflagellates, and von Stosch's work (24) on dinoflagellate sexual cycles suggests that such cysts may be benthic zygotes. Such stages may exist in the life cycle of *G. breve* (25), therefore, we have initiated a program to determine the presence or absence of benthic resting stages and, if they are found to exist, map their distribution. Pyle and Wallace (26) are examining the possibility of a relationship between the presence of underground marine springs and the location of initial *G. breve* blooms. Contractual arrangements are being made to determine if relationships do occur and emphasis will be placed on possible locations of *G. breve* resting or "seed" populations.

Pathological and Ecological Effects

Shellfish toxicity and aerosol irritation can occur even though no *G. breve* cells are apparent in water samples (17, FDNR unpublished data). Therefore, field samples were collected during and after the recent 1974 red tide. Mouse bioassays are being completed to determine relative longevity of neurotoxicity in inshore waters.

Also during the 1974 winter-spring red tide, necropsies of 13 species of affected fishes were performed by FDNR pathologists (27). Several abnormalities, particularly hematological, were associated with *G. breve*—killed fish. Consequently, laboratory and field studies are in progress to determine causes of marine animal mortalities and the degree of susceptibility among different animals. Standard pathological techniques will be performed as well as electron microscopy of tissues of animals exposed to *G. breve* toxin in nature and under laboratory conditions.

Red tides most certainly influence marine populations, but the overall results and consequences have not been evaluated. In years following red tides, increased catches of shrimp and crabs are frequently reported. These observations suggest a beneficial influence of red tides by reducing predators. Contrarily, isolated patch reef kills have been documented. Consequently, a survey project to assess the effect of red tides on various vertebrate and invertebrate populations is planned. Repopulation studies are extremely important to such an endeavor and one such project continues today. In the laboratory, we will evaluate the effect of *G. breve* toxin on various developmental stages of fishes and invertebrates.

Prediction and Amelioration

Methods developed over the last several years should be pursued along with new methods such as temperature patterns (28) and remote sensing. Coupled with prediction is amelioration of economic hardships imposed by red tides. When dead fish or potential fish kill areas are located and their direction of movement determined, municipalities advised to expect dead fish could remove them quickly. A better solution would be to remove floating dead fish before they are beached. With this object in view, contractual funds have been designated to design and build harvesting and processing equipment for dead fish. Blogoslawski and co-workers (29) have developed oxidation techniques to deactivate the toxin in contained seawater aquaria systems. Such developments are important to the continued operation of commercial marine aquataria and research laboratories using coastal or bay waters affected by red tides.

CONCLUSIONS

Red tides are recurring, natural phenomena. As such, they have been influencing west Florida shelf communities for decades. Isolated communities which have been almost annihilated by red tide appear to re-establish themselves in several years. Possibly, prevention of blooms could have serious consequences on overall community interrelationships. Presently, control is not feasible and would be very difficult. Once a red tide is established, treatment and retreatment of any location would be necessary. However, if methods of prevention or control were developed, we should seriously consider whether or not to use them. We should first assess how and to what extent *G. breve* blooms affect Gulf marine communities.

Undeniably, Florida red tides create hardships through loss of revenue from such industries as tourism and sports fisheries. The economic impact can be lessened by reducing or eliminating the undesirable results of red tides such as floating or beached dead fish along our shorelines. Predicting when and where problems may arise and being prepared to meet them will greatly ease economic difficulties.

Another aspect to consider is public health; red tides are a health hazard because people can suffer from respiratory irritation and mildly toxic shellfish. Potentially, these problems can be solved through further research. Efficient fish removal will be facilitated by early detection. Medical research is being considered by the Florida Department of Health and Rehabilitative Services and the National Institute of Environmental Health Services and could result in treatments to reduce respiratory irritation during red tides.

Red tide will probably not be controlled, although theoretically, the greatest chance of success for control would arise during the initiation stage before the red tide became so geographically extensive. Of course, this would be dependent on localized sites of initiation which have not been verified, but it emphasizes the need for basic biological studies of *G. breve*. Nevertheless, Florida red tides are a current problem and require immediate solutions. Therefore, we must approach the problem of red tide practically by promoting an extensive public education program and eliminating those factors which cause undesirable economic and public health difficulties.

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SYNOPTIC SIGHTINGS OF RED WATERS OF THE LOWER CHESAPEAKE BAY AND ITS TRIBUTARY RIVERS (MAY 1973 - SEPTEMBER 1974)[1]

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ABSTRACT

Visual sightings of extensive red water phytoplankton blooms in the lower Chesapeake Bay and its tributary rivers (James, York, Rappahannock, and Potomac) were made from low-flying aircraft.

With respect to the 16 synoptic surveys covering the months of May 1973 through September 1974, extensive blooms were observed most frequently on the Rappahannock (41/80), followed by the Potomac (17/48) and the York (15/48) Rivers.

These observations clearly indicate that the occurrence of red water phytoplankton blooms is not restricted to limited areas within the estuarine waters nor to seasonal spring and autumn pulses, but may potentially develop at any time when environmental conditions are favorable for the stimulation of specific dinoflagellates.

INTRODUCTION

In an attempt to document sporadic reports of red water phytoplankton (dinoflagellate) blooms in the lower Chesapeake Bay and its tributary rivers (James, York, Rappahannock, and Potomac), we have undertaken consistent synoptic visual surveillance from aircraft flying 152-200 meters above the waters' surface.

The hypothesis that persistent phytoplankton blooms contribute significantly to the periodic depletion of oxygen dissolved in the deeper estuarine waters in the summer needs to be tested. In order to develop a testable model on the dynamics of phytoplankton bloom development and dissipation, consistent observations on frequency of occurrence and duration are necessary. The data reported herein are a first attempt at obtaining these essential data. Field observations on meterology, hydrography, nutrients, chlorophyll *a*, primary productivity and heterotrophic potential (V_{max}, glucose) will be reported elsewhere (P. L. Zubkoff and J. E. Warinner, in preparation).

OBSERVATIONS

The areas observed are depicted in Figure 1. The sub-divisions into reaches are those reproduced from U.S. Coast and Geodetic Navigation charts (1): James (1-3), York (4-6), Rappahannock (7-11), Potomac (12-14) and Eastern Shore (15-17). Salinities ranged from less than 8 o/oo for the surface waters of the upper reaches of the rivers to 28 o/oo for those near the mouth of Chesapeake Bay. Water temperatures ranged from 4°C in February to 28°C in August.

Although the extent of the phytoplankton blooms were quite variable, the minimum areal dimension of a patch or streak was at least 100 meters in length and usually 10 meters in width. The maximum dimensions included the entire

observed reaches of the Rappahannock River (7-11), a linear distance of 60 nautical miles, or the entire York River (4-6), a linear distance of 28 nautical miles.

The frequency of red water occurrences is tabulated in Table 1: the occurrence of red waters was most frequent in the Rappahannock (0.5), followed by the Potomac (0.3) and the York (0.3), with fewer occurrences in the James (0.2) and the Eastern Shore (0.1).

On a calendar basis, the sightings for May of 1973 and 1974 were about the same: 7 and 9, respectively; however, those for July and September of 1974 were greater than for the same months of 1973. On the whole, the number of occurrences for 1974 (67 of a possible 170) was twice that for 1973 (21 of a possible 102). Undoubtedly a contributing factor to the greater frequency of occurrences in 1974 was the greater abundance of rainfall before stratification of the water column occurred; in comparison, the rainfall during 1973 was relatively sparse (2).

On selected occasions, the predominating species of dinoflagellates causing the blooms in the York River have been identified:

May 1973	*Prorocentrum minima* and *Peridinium triquetrum*
September 1973	*Cochlodinium heterolobatum*
October 1973	*Gymnodinium splendens*
May-June 1974	*Prorocentrum minima* and *Peridinium triquetrum*

DISCUSSION AND CONCLUSIONS

This particular surveillance is quite significant in identifying the (1) frequency, (2) magnitude, and (3) distribution of red water occurrence in the lower Chesapeake Bay estuaries. In addition, it has permitted us to hypothesize that at least 3 different conditions may contribute to the formation of dinoflagellate blooms after appreciable stabilization of the water column:

1. Red water formation in creeks and shallow waters after significant rainfall i.e., *Prorocentrum minima, Peridinium triquetrum*

2. Red water formation in the more saline bay waters at high temperature ($> 24^{\circ}C$) followed by intrusion into the deep channel of the York River i.e., *Cochlodinium heterolobatum*

3. Red water formation in the less saline waters of the deep channel of the York River, probably due to stratification and/or upwelling at the interface of the salt water wedge with subsequent development and migration of the bloom to the more saline waters.
   i.e., *Gymnodinium splendens*

Some of these conditions have been reported for other estuaries (3,4).

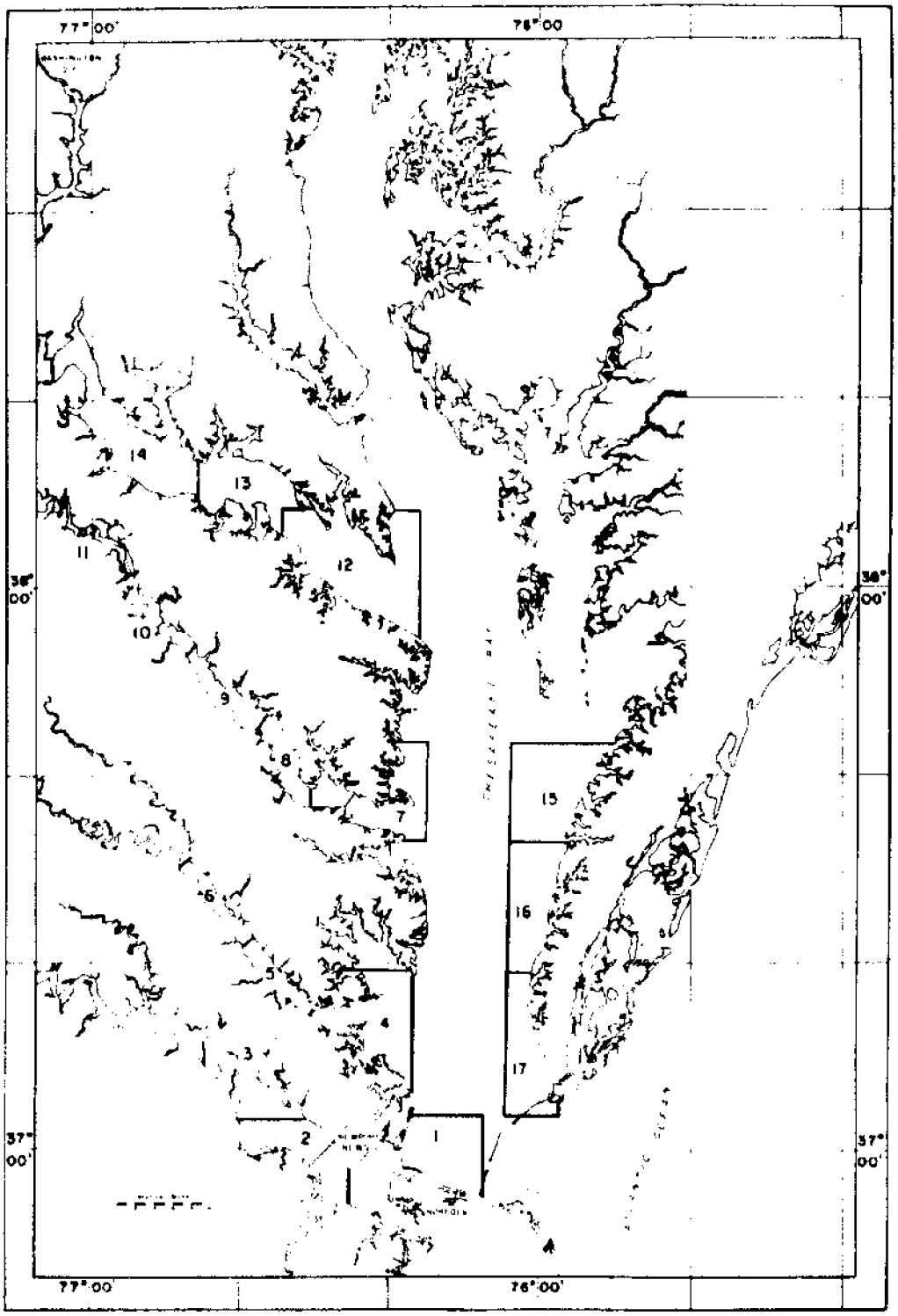

Figure 1: Reaches of lower Chesapeake Bay and its tributary rivers synoptically surveyed for red water blooms.

Table 1

Sightings of visible red water blooms during synoptic aerial surveys of the lower Chesapeake Bay and its tributary rivers.

Location	Reach	1973						1974									Total Sightings per Reach	Total Sightings per River	Frequency
		Month	5	5	7	9	9	10	2	3	3	4	5	5	6	7	8	9			
		Day	11	16	13	11	28	31	21	4	27	11	7	24	10	2	12	16			
James R.	1			+															1	10	
	2													+			+	+	3		0.2
	3					+						+		+	+		+	+	6		
York R.	4					+			+		+					+	+		5	15	
	5		+						+	+	+	+				+	+		7		0.3
	6										+	+					+		3		
Rappahannock R.	7		+			+	+	+				+			+	+	+		8	41	
	8		+	+			+		+			+			+		+	+	8		0.5
	9		+	+			+		+	+		+	+	+	+		+	+	11		
	10								+	+	+	+	+	+	+		+	+	9		
	11								+			+		+			+	+	5		
Potomac R.	12					+	+		+					+			+		5	17	
	13						+	+	+					+	+		+	+	7		0.3
	14						+		+						+	+	+		5		
Eastern Shore	15																	+	1	5	
	16																	+	1		0.1
	17					+	+				+								3		
Total Sightings																					
Per Survey			4	3	0	5	7	2	9	3	5	8	2	7	7	4	13	9			
Per Year						21								67							
Total Possible						102								170							

In the lower York River, Mackiernan (5) recognized the appearance of 118 separable species of dinoflagellates during a 14 month period, including 7 blooms of 5 dinoflagellates. During the present study period, 4 of these same organisms have appeared in bloom proportions. Unfortunately, the specific number of observations has been too restrictive for deeper interpretation.

This survey indicates that the multitude of red waters that develop in the lower Chesapeake Bay and its tributaries can be of massive proportions and possibly of long duration. In addition, the great frequency of dinoflagellate blooms in the Rappahannock, and possibly the lower reaches of the Potomac and the York, may lead to the development of persistent and continuous phytoplankton blooms throughout the year if the ecosystem is greatly perturbed, either through nutrient enrichment and/or other conditions leading to vertical stability of the water column.

RECOMMENDATIONS

We recommend the coordinated aerial surveillance and field observations for testing the hypothesized conditions leading to red water formation. Such measurements should include prior meterological and hydrographic analysis, nutrient balance, phytoplankton identification and enumeration, microbial activity, and grazing pressure. It is most likely that experimentation should be concentrated for selected short periods of time (i.e., measurements at several times daily for periods up to 2-3 weeks) rather than periodic measurements with great spans of time between.

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RED TIDES IN THE UPWELLING REGIONS

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ABSTRACT

The importance of dinoflagellate blooms to the productivity of upwelling areas is examined. The existing evidence suggests that, not only can dinoflagellates maintain themselves in turbulent ascending waters, but also, that as blooms, in the form of "red tides", these organisms contribute greatly to the productivity and must be considered in models of upwelling ecosystems.

INTRODUCTION

Until recently upwelling regions have been considered as ecosystems where diatoms were the major component of the phytoplankton population, and the presence of dinoflagellate blooms or "red tides", in these areas were considered as unusual phenomena. Probably the basis for this idea was that upwelling studies were directed towards evaluating the production of an area, a positive aspect; while the objective of red tides studies have been mainly to analyze a negative effect, the toxicity effect of the organisms on the system.

The object of this paper is to point out the high frequency of red water in the upwelling regions and to emphasize the photosynthetic capacity of some of these blooms. The fact that on certain occasions the dinoflagellates "over populate" sea water and endanger other marine organisms in the area, is not reason enough to exclude them from the primary producer community.

PRESENCE OF RED TIDES IN UPWELLING REGIONS

Past observations of high concentrations of photosynthetic dinoflagellates in two upwelling regions, Peru and Baja California, Blasco (1, 2), in press), together with the abundant references found in previous literature (Table 1), led me to consider the possibility that dinoflagellate blooms act as a normal component of the upwelling phytoplankton productivity, instead of an abnormal one. The discrepancies between the above hypothesis and the current knowledge on red tides are obviously apparent. The first difficulty rises when we combine the most accepted mechanisms for the origin of red tides, i.e., increase in the water stability, and the phenomena of upwelling; region of rapidly ascending water. Dissipation of red tides have been often related to an increase in the water motion, but no explicit information is available on what is the minimum value of the vertical mixing coefficient for the development and survival of a dinoflagellate bloom. On the other hand, it is well known that the intensity of the upwelling rather than being constant, is variable in both time and space. Based on these two facts we can suppose, that during certain periods of time, or in certain localities, the vertical upwelling velocity will be enough to enrich an area with significant amount of nutrients and at the same time, the hydrographic conditions will still be such that a swimming ability represents an advantage, allowing motile organisms to outcompete immotile organisms, see Wyatt and Horwood (16). The Baja California upwelling in March 1971 provided an example of this

situation (12). *Gonyaulax polyedra* was the dominant species during the 20 day cruise, although the vertical stability coefficient ($E = 10^{-3} \cdot \frac{\sigma t}{dz}$) was low, between 0 and -5; however, during a few days when the vertical stability coefficient dropped from -5 to -10, a diatom population did start to dominate (17). These results suggest that very small differences in the chemical and physical parameters regulate the success of one population or another, but based on the actual information, such small differences are still difficult to identify, and more research in this direction should be done.

PRODUCTION OF THE DINOFLAGELLATE BLOOMS

Another problem appears when we try to reconcile the positive idea of dinoflagellates as contributors to the primary production, and the negative one, based on their association with red tides and mass mortality of marine animals. The most general hypothesis attributes the mortality of the fish and other marine fauna, to the liberation of high concentration of toxin by the dinoflagellates or to lack of oxygen caused by the decay of these blooms. Both conditions occur only when the organisms accumulate in high densities, due to the migrational pattern of these organisms in a well defined and stratified mass of water. It is unlikely that such conditions occur frequently in an upwelling region, where an extreme high stability in the water column is a rare event. Confirming this assumption, is the fact that while the reported cell concentrations for red tides in closed environments, i.e., bays, estuaries, etc., are of the order of 5×10^3 cells/ml or higher; the maximum concentrations that

Table I.

Observations of Red Water in Upwelling Areas

AUTHOR	REGION	*ORGANISM*
Blasco (1971)	Peruvian Coast	*Gymnodinium splendens*
Hart and Currie (1960)	Benguela Current	*Peridinium triquetrum*
Brongersma and Sanders (1948)	South African Coast	*Gonyaulax spinifera*
Currie *et al.* (1973)	Arabian Sea	*Gonyaulax polyedra*
Braarud and Heimdal (1970)	Norwegian Coast	*Gyrodinium aureolum*
Torrey (1902)	California Coast	*Gonyaulax polyedra*
Allen (1941)	California Coast	*Prorocentrum micans*
Holmes *et al.* (1967)	California Coast	*Gymnodinium sp.*
Glendenning (1958)	California Coast	*Prorocentrum micans*
		Gonyaulax polyedra
Eppley *et al.* (1968)	California Coast	*Ceratium furca*
Walsh *et al.* (1974)	Baja California Coast	*Gonyaulax polyedra*
Gunther (1936)	Peruvian Coast	
Strickland *et al.* (1969)	Peruvian Coast	*Gymnodinium sp.*
Guillen *et al.* (1971)	Peruvian Coast	*Gymnodinium sp.*

I have observed in upwelling regions, is between 2×10^2 and 1×10^3 cells/ml. For the same reason it can be also assumed that, if a toxin is released by these organisms in an upwelling region, it will be diffused at a higher rate, and the chance of reaching a critical level is low.

Another view of the cause of death associated with red tide organisms is the ingestion of the organisms itself. To date, only a few species of the extensive group of dinoflagellates, have been positively established as primary sources of toxin and they have been rarely reported in upwelling areas. An additional point is the *Gymnodinium splendens* and *Gonyaulax polyedra*, the organisms numerically dominant in our observations, have been shown, in laboratory experiments, to be harmless when they are fed to a copepod population (18,19). The information on *Gonyaulax polyedra* is inconclusive since some authors describe it as a toxic organism (20) while others describe it as a potential food source (21).

Once the presence of "red tides" in upwelling areas is accepted, and that these blooms are not necessarily a danger for the marine life in those regions, the question remains as to whether this high concentration of dinoflagellates represent real growth and new production, or are just the result of the migration of these organisms into a given area. The mean assimilation index for the Baja California study, where *Gonyaulax polyedra* was the major component of the phytoplankton in terms of chlorophyll and C^{14} production (17), was 4.3 mg C/mg Chl *a* h^{-1}; and in Peru, it was of the order of 5.0 mg C/mg Chl *a* h^{-1}, in the stations where *Gymnodinium splendens* was the most abundant organism. These values compare well with the average values of the phytoplankton assimilatory capacity in the ocean noted by Ryther and Yentsch (22), and indicates an active growing population; but as one would expect, they are lower than those obtained by Curl and Small (23) for laboratory cultures of diatoms at their optimum growth conditions. Another piece of evidence that supports the idea of a net input of organic matter in the system by these organisms, are the significant nitrate reductase activity, 10 to 17 ng-at NO_2/μg Chl *a*/h^{-1}, and the N^{15} nitrate assimilation values, 0.241 to 0.402 gN m^{-2} day^{-1}, measured during the Baja California cruise, Blasco and Packard (24); Walsh *et al.*, (12).

CONCLUSIONS

Although dinoflagellates, as a class, are regarded as organisms with a lower productivity capacity than diatoms, their migration ability and the different physiological requirements enables them to exploit certain hydrographic conditions, and as long as these conditions last, we can assume that a dinoflagellate population is more productive than a diatom population. Example of these conditions occur when the vertical upwelling velocity is not strong enough for the nutrient-rich deep water to reach the surface, or when silica is the limiting nutrient in the upwelled water. If we accept then that dinoflagellates belong to the phytoplankton community of upwelling regions,

calculations of the overall production of these regions will increase, since it will be based on the sum of the diatoms production and the dinoflagellates production, instead of being based on the sum of different phases of diatoms production, from a range of maximum to a minimum. These two phytoplankton phases make upwelling regions more efficient as an ecosystem because it gives them the flexibility to exploit nutrient sources under a variety of environment conditions, and for the same reason makes upwelling regions easier to accept as ecosystems.

The consequences of the suggested hypothesis concern mainly future predictions of the potential food capacity of the upwelling regions. It demonstrates that both types of organisms must be considered, and their different strategies to exploit the system, their different chemical composition as well as their different predator-prey responses must be analyzed.

The idea that upwelling regions are systems where dinoflagellates and diatoms are the main primary producers, amplifies previous assumptions, but probably it is still a limited approach. Some observations of high concentrations of green flagellates by Beers *et al.*, (25); Margalef (26) (1973), and of the photosynthetic ciliate *Mesodinium rubrum*, in these areas, indicates that upwelling systems can respond to the enrichment processes of the surface water in a number of ways, and for a better understanding of the phenomena all these possibilities should be included.

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SESSION SUMMARY

THE ORGANISMS

Session Chairman

Luigi Provasoli

Haskins Laboratories
Yale University
New Haven, Connecticut

Co-Chairman

Alfred Loeblich, III

Biological Laboratories
Harvard University
Cambridge, Massachusetts

The limited range of topics presented in this section reflects the scarcity of scientists engaged in this phase of dinoflagellate research, which has suffered from the lack of support continuity. Short crash programs initiated during major outbreaks of red tides in Florida or when new localities were affected by the southward spreading of shellfish poisoning have proven to be too costly and wasteful for the results obtained. This policy of understandable political expediency was based on the fallacy that poisonous blooms are sporadic events. Major blooms, especially those of *G. breve* although sporadic, have been occurring more often; in addition, the fish kills and respiratory troubles caused by these blooms are now distressing large populations of tourists and retired citizens who have recently settled along the West Coast of Florida. The paralytic shellfish poisoning due to *G. catenella*, known and feared by the Indians of the US West Coast, can hardly be considered a transitory phenomenon; despite ups and downs, it is an ever-present hazard for shellfish-consumers and a threat to the stability of the shellfish industry. Shellfish poisoning due to *G. tamarensis* became a menace 20 years ago in Canada and has now spread to Maine and Massachusetts and may continue southward.

Clearly, as was concluded at the 1964 Red Tide Symposium, the problem caused by poisonous dinoflagellates requires *continued* support and attention by Federal and State Agencies. This change in policy is favored by the increased responsibility thrust upon the State Government by Federal cost sharing and by the pressure of electorates asking for accountability. The continued support by the Florida State Department of Marine Resources of in-house, and cooperative programs with local Institutions and Universities and the support by the Commonwealth of Massachusetts and by Federal and private sponsors for this conference indicate that we can expect a wiser approach to the problem.

Much can be expected by a steady, concerted attack because remarkable results have been obtained in the past 15 years by a few dedicated scientists who have profited well from the few spurts of abundant funding and persevered during the periods of penury. The various poisonous symptoms have been firmly attributed to the production of poison by dinoflagellates. The culprits have been identified, they have been cultured and the cultures have served to establish that the toxins of *G. breve* and other dinoflagellates are poisonous not only to fishes but to numerous invertebrates (worms, corals, etc.), affecting natural food chains and ecology; that other toxic dinoflagellates (*G. catenella* and *G. tamarensis*) do not harm, and are ingested by, clams and oysters, which accumulate the poison in the hepatopancreas, and to levels which cause paralysis and death in man and other vertebrates; dose-response relationships and a mouse-bioassay were perfected; correlations between numbers of dinoflagellates present in sea water and level of toxicity of molluscs were established permitting monitoring of dinoflagellate populations to forecast outbreaks of mollusc toxicity. It is precisely this basic information developed previously which has allowed the regulatory agencies of the

Commonwealth of Massachusetts to deal efficiently, without panic, with the invasion of their waters by *G. tamarensis*.

In accord with the times and the need of local agencies, the present emphasis is to eliminate as much as possible the obnoxious effect of the blooms. Forcasting, monitoring, and probable means of controlling blooms; measures for safe use of shellfish, detoxification and possibly antidotes, are some of the pressing problems for which we have to gather more basic knowledge to arrive at economical and effective measures. Previous knowledge has already been effectively applied and it has also served to define many areas where more detailed information is needed.

The papers of this section are a sample of this new trend. Modalities of growth in biology are the basics and are obviously the essence of algal bloom conditions—species predominance and large primary productivity being inherent characteristics of blooms. Indeed these might be the only similarities between blooms of the poisonous *Gymnodinium* and those of *Gonyaulax* species. Large blooms of diatoms and other algae are normal events during the growing season and being essential to marine productivity have been under intense scrutiny. More than a dozen parameters, and new ones are being tested, were found to be involved in bloom production. Most importantly, it was found that a different combination of parameters is responsible for the growth explosion of each specific organism. Likewise, it is most probable that the conditions favoring blooms of each poisonous dinoflagellate will be different and if so, each species may have special preferences. Yentsch, *et al.*, found that *G. tamarensis* responded to some ecological variables differently than other common phytoplankters and *Skeletonema costatum* which usually precedes or accompanies some dinoflagellate blooms. Hopefully, these idiosyncrasies of *G. tamarensis* may serve to pinpoint the factors leading to its temporary predominance over the other denizens. It is also interesting that NTA, a proposed substitute detergent, stimulates growth of *G. tamarensis* but in a narrow range of concentrations. With the same intent, the preferences of *G. catenella* in cross gradients of light intensity, salinity, and temperature were defined by Norris and Chew. Iwasaki, who unfortunately could not contribute to the Symposium, found interesting correlations between the *in vitro* physiology of several dinoflagellates and the ecological conditions prevailing when blooms of these species occurred in the Inland Sea of Japan. A detailed analysis of the oceanographic conditions accompanying or leading to recurrent blooms of several dinoflagellates in Chesapeake Bay was presented by Seliger, *et al.* Of particular interest are their perceptive observations on the participation of phototaxis, currents, windrows and water stratification in the accumulation mechanisms leading to blooms.

An interesting exploration of the relationship between population growth and toxicity of *G. breve* was reported by Wilson, *et al.* Eight large cultures, initiated under identical conditions and seeded with a common inoculum, showed three different patterns of population growth during the 5 month observation of the cultures. In spite of these differences, the toxicity and the

size of the accompanying bacterial flora were directly proportional to the levels of *G. breve* populations thus confirming the relationship toxicity: number of dinoflagellates and indicating the probability of a meaningful relationship between bacteria and dinoflagellate growth.

An analysis of the detailed studies on the ecology of *G. breve*, pursued for many years by the Florida Marine Research Laboratory stimulated Steidinger to postulate that cysts, sexual or asexual, and other periodical events of the life cycle of *G. breve* may be responsible for bloom initiation, the most critical phase for forecasting blooms. The brilliant research of Wall on dinoflagellate cysts (reported in part at this Symposium) and the revealing studies of von Stosch on dinoflagellate sexuality are the basis of her well-planned, long-range program of research. If cysts are involved, studies on encystment, excystment and cyst sedimentation may give new important leads.

The Loeblichs attack another basic aspect, speciation and taxonomy of *G. tamarensis*. This is not an excursion in, and resurrection of, a discipline fashionable years ago, i.e., giving the right name. They found that strains of *G. tamarensis* which are apparently identical morphologically, differ profoundly in their physiological attributes: i.e., some are toxic or non-toxic, luminescent or not. This is the tip of a huge iceberg, the beginning of an investigation on the inheritance of characters in dinoflagellates — a venture whose significance is enhanced by the peculiar chromosomal structure of the nucleus of the dinoflagellates and by the refinements of the modern research tools. If these physiological differences can be recognized easily by subtle but distinct morphological or biochemical characters, we could distinguish toxic from non-toxic strains of *G. tamarensis*, an important step in accurate forecasting. How does the biochemistry of these two strains differ? It is often mentioned that toxins may be a way to detoxify a metabolite dangerous for the producer; others consider toxins a valuable means for survival and competition. These questions can be approached by crossing toxic with non-toxic strains and by producing mutants, the classical tools for determining the pathways of biosynthesis.

These are exciting possibilities for advances in knowledge and in practical management offered by the papers presented. At a workshop, attended by the authors of this section and interested scientists, a number of suggestions on future research needs were formulated and are listed topically.

A culture collection of species and strains of poisonous dinoflagellates is essential as a repository and reference for species identity and preservation of physiological types. Cultures are essential to biologists for study leading to characterization of their physiology, nutrition, biosynthesis of toxins, to chemists for production of toxins; to ecologists and to toxicologists for effects on various marine animals and to vertebrates. The culture collection should be maintained on a *permanent* basis, and in duplicate for the most important species; too many losses have happened to many of us in maintaining key, unsubstitutable cultures! It may be part of a center devoted to maintaining marine algae and protozoa or a separately supported activity of a laboratory working on dinoflagellates.

Biology and life-history of the organisms

a) The need for detailed life cycle studies both in culture and in the field, has been mentioned. If cysts or resistant stages are formed and accumulate in sediments, our outlook on endemicity, locale and mode of initiation of blooms has to be revised by detailed field work and may offer new means of control. Some dinoflagellates are known also to have alternation of forms and generations which may be seasonal.

b) If cysts originate from a sexual union, the proposed studies on inheritance, mentioned above, acquire even more practical importance: field populations could be mixed and the presence of offspring endowed with new physiological abilities would explain adaptation to and colonization of new areas.

The identification of morphological and physiological species and varieties becomes imperative to characterize the ecological versatility and the toxicity of the population.

c) Variations in cell size during the exponential and other growth phases of the bloom have been reported often. Some consider that predominance of small cells in the population indicates presence of rapid cell division (i.e., a developing bloom) but others believe that predominance of large cells in *G. breve* populations indicate rapid growth (Wilson, *et al.*). The discrepancy may simply mean that we should not generalize, each species behaves differently. An indicator of increasing populations would be most useful to monitor blooms.

d) Autecological studies have been reported here. They need expansion and should be based on a multifactor design matrix and variable's levels representative of natural conditions. Most important is the determination of growth rates of the various poisonous dinoflagellates *in nature* and under different environmental conditions. It is not an easy task, and we may have to rely on approximations such as data acquired using large plastic bags *in situ*. Lack of division rate data hampers serious effort to predication, i.e., construction of models to assess the effects of grazing, physical accumulation, phototaxis and diurnal migration on bloom development.

e) Physical oceanographers need for their modeling data on motility *rates* in vertical and lateral motions of dinoflagellates and need to know whether downward movements in dinoflagellates are due to active swimming or sinking.

Physiological responses

Autecological studies directed at determining why an organism blooms (i.e., it is so fit to its environmental conditions as to out-compete and predominate) rely heavily on detection of idiosyncrasies and special or distinctive abilities in regard to: a) light utilization (intensity, spectrum, photoperiod), b) nutritional needs (sources of inorganic and organic P and N), c) needs for microheterotrophy (growth factors), macroheterotrophy (sources of energy

and building blocks), and possibly for phagotrophy, d) trace metals needs and tolerance (influence of natural - from soil and muds - and artificial chelators on trace metals availability). Quantitative studies, particularly data on *uptake rates* of needed nutrients at concentration levels mimicking oceanic or neritic conditions are essential to find whether or not dominance over other species depends upon an advantage in uptake rates. Equally revealing may be experiments to detect syntrophisms or inhibition between species common in the same environmental niches; a favorable preconditioning of the waters may lead to blooms. Other proposed or observed relationships may be relevant, the relationship of dinoflagellates with bacteria and predation by protozoa and crustacea.

Bioluminescence is an important attribute of dinoflagellates and could be exploited to monitor the seasonal fluctuations of endemic poisonous dinoflagellates. The sensitive photomultipliers used by biophysicists might be adapted to rugged field use and could serve to evaluate the size of dinoflagellate population. Such instrumentation could be automated and used in key locations to detect population increases and give a warning before the threshold known to cause poisonous shellfish is reached. Such monitoring would eliminate costly frequent sampling of waters and cell counts and/or laborious pigment analysis.

The wealth of suggestions received, attests that the new generation appreciates relevance and pragmation as an intellectual challenge and is ready to match the feats of Pasteur who wholehartedly accepted the challenge to cure the diseases of the silkworm and the mishaps in cheese and wine making and, in doing so, founded bacteriology and biochemistry.

GYMNODINIUM BREVE: POPULATION GROWTH AND DEVELOPMENT OF TOXICITY IN CULTURES

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ABSTRACT

The population growth and toxicity of eight 12-liter cultures of the dinoflagellate, *Gymnodinium breve*, were followed for five months. Although each culture was inoculated with a volume of *G. breve* culture calculated to provide between 50,000 to 60,000 organisms per liter, counts made shortly after inoculation varied from 3,000 to 9,000 per liter. During the first five weeks, 200 ml samples were removed at 2 to 4-day intervals for population estimates and bacterial enumeration. After the fifth week, 350 ml samples were taken at 7-day intervals to perform toxicity assays with the mosquito fish (*Gambusia affinis*). All experimental populations of *G. breve* increased uniformly by about three orders of magnitude and reached levels of five to nine million cells per liter (an average growth rate (K) of approximately 0.3/day) during the first five weeks. Afterwards, population levels differed considerably between cultures. At five months, culture population levels were from one-half to 24 million per liter. The eight cultures could be roughly classified as three types according to timing of peak growths:(1) early peak;(2) late peak; and (3) multiple small peaks. The causes for these differences between apparently replicate cultures are not known. The toxicity of the cultures and bacterial population levels varied directly with *G. breve* population levels. Electronic sizing of *G. breve* cells from populations of different ages and growth phases were performed with a Coulter Counter. Although there were shifts in the size distribution, these were not large and could not be related to age but the ratio of large: small cell volumes was higher in cultures with increasing population levels than in those with decreasing levels.

INTRODUCTION

Periodic mass kills of marine organisms, especially fish, in the Gulf of Mexico are often associated with "blooms" of the dinoflagellate, *Gymnodinium breve* (8, 6, 22, 23, 20, 18, 19).

The development of methods by Wilson and Collier (22) for culturing this organism has stimulated considerable research related to its growth requirements and toxin production under laboratory conditions. Ray and Wilson (12) working with bacteria-free cultures, clearly demonstrated the toxicity of *G. breve* to fish. McFarren *et al.* (10) and Eldred *et al.* (4) associated the occurrence of mild human illness, suggestive of paralytic shellfish poisoning, with the consumption of oysters taken from Sarasota Bay, Florida, during a "bloom" of *G. breve*. Extracts of the suspected batch of oysters were toxic to mice and kittens. Furthermore, McFarren *et al.* (10), Spikes *et al.* (16) and Cummins *et al.* (3) extracted materials from *G. breve* cultures that were toxic to mice. Shellfish poisoning was induced in chicks by feeding them oysters that had been exposed to *G. breve cultures* (13).

The present work with *G. breve* was designed to determine the possible relationships among age of culture, population density, and toxicity to fish. Another purpose was to compare population development, toxicity, and toxin stability in cultures of *G. breve* with another toxic dinoflagellate, *Gonyaulax monilata*, which occurs in the Gulf of Mexico.

The studies with *G. breve* parallel those conducted earlier with *G. monilata* by Aldrich *et al.* (1) *G. monilata* has been associated with fish kills on the east coast of Florida (9) and Offatts Bayou near Galveston (2). Gates and Wilson in 1960 (7) showed that unialgal cultures of this dinoflagellate were toxic to fish. In addition to fish, *G. monilata* is also toxic to some invertebrates, including annelids, crustaceans and molluscs according to Sievers (14). On the other hand, all of these invertebrates, except the annelid, *Neanthes succinea*, survived exposure to *G. breve* toxin.

MATERIALS AND METHODS

Eight unialgal cultures of *G. breve* were grown in an artificial sea water medium (7). These cultures with initial volumes of 12 liters were contained in 3.5 gallon Pyrex bottles. Each of the eight study cultures was inoculated with an equal volume of a culture containing about 22 million *G. breve* per liter. The inoculum was calculated to provide between 50,000 and 60,000 cells/l. However, visual counts of intact *G. breve* made shortly after inoculation varied from 3,000 to 9,000 cells/l. Each bottle contained a teflon-coated magnetic stirring bar. The cultures were held at 25±1°C and continuously illuminated with standard cool white fluorescent light at approximately 750 foot-candles.

At various intervals (2-7 days), each culture was thoroughly stirred for five minutes and a 200 ml sample was removed with a sterile pipette for population estimates, bacteria enumeration and toxicity assays. These samples were withdrawn in front of a laminar-flow sterile air device to prevent secondary contamination. During the first five weeks, sampling was done at 2 to 4-day intervals. After the fifth week, 350 ml samples were taken at 7-day intervals to include toxicity assay with the mosquito fish, *Gambusia affinis*. The volume reduction was similar for all cultures throughout the 5-month period of this study. Approximately six liters of culture remained in each bottle after the last sample was taken.

During the first 17 days, population estimates were based on cell counts at 12X and 36X with a stereoscopic microscope of replicate 1.0, 0.1, 0.05, or 0.01 ml subsamples, depending upon the population density. After this period, population levels were determined with a Model B Coulter Counter. This instrument was also used to determine the size-frequency distribution of the dinoflagellate population in two of the cultures at each sampling. The bacterial population was determined at each sampling with a haemocytometer and a phase-contrast microscope at 450X. The counts obtained were probably on the high side, since all particles that had the "appearance" of bacteria were enumerated.

At each sampling, 200 ml of culture removed from each bottle was immediately frozen (-15°C) and held for assaying at convenient times. On four occasions, after about the 5th, 9th and 13th weeks and at the end of the study (5 months), an additional 150 ml culture portion was removed from each bottle and held overnight under illumination for toxicity tests. The intact cell culture portions were tested along with the frozen materials for comparative toxicity.

The toxicity of each culture was assayed with *Gambusia affinis* (15 to 30 mm long), which were held in a large outdoor tank of fresh water for several weeks prior to the commencement of toxicity tests. Approximately 72 hours prior to each set of toxicity tests, test fish were acclimated as follows: (1) held for 24 hours in water from fish storage tank to permit them to acclimate to laboratory temperature (approximately 25°C); (2) 25% artificial sea-water medium (same as that used for growing *G. breve*) for about 9 hours; (3) 50% artificial medium for about 15 hours; (4) 75% artificial medium for about 9 hours; and (5) 100% artificial medium for 15 hours. Each sample was assayed with four fish. Each fish was held for a maximum of 24 hours in a small finger-bowl (3-1/2" diameter) containing 35 ml of the test culture. The test material in the finger-bowl, which was loosely covered with a plastic petri dish top, received no artificial aeration.

Four chronological samplings of the frozen cultures were assayed at one time to minimize the possible influence of fish condition variability on the assays. Furthermore, during each set of toxicity tests, 10 fish were assayed with a "reference standard" (three parts of fresh medium per part of a frozen culture which contained ca 1.9×10^7 *G. breve*/l) to check the uniformity of the assay fish. The mean death time of four test fish for seven individual standard tests varied from 2.3 hours to 4.9 hours. This variability was probably due to differences in fish sensitivity rather than loss of potency of the standard during freezer storage. Tests conducted at approximately the beginning, mid-point and at the end of the study each had mean death times of 2.3 hours.

The toxicity of *G. breve* and *G. monilata* to mosquito fish was compared. A frozen *G. breve* culture containing about 19 million cells per liter was diluted to prepare seven concentrations of culture that were calculated to be equivalent to 2.0×10^2, 5.0×10^2, 1.0×10^3, 2.0×10^3, 5.0×10^3, 1.0×10^4, and 5.0×10^4 cells per ml. *G. monilata* cells were obtained by continuous centrifugation (4600 g with a flow rate of 70-100 ml/min) of approximately 100 liters of culture containing about one million organisms per liter (1.12×10^8 cells harvested). These cultures grew in the same artificial medium that was employed for *G. breve*. The *G. monilata* cells were homogenized by several freezings and thawings and 2 minutes in a Virtis blender at 45,000 rpm with 50 ml of artificial sea water. From this homogenate, six quantities were removed and suspended in artificial sea water for the test. These quantities were calculated to be quivalent to approximately 1.4×10^3, 2.8×10^3, 5.6×10^3, 1.1×10^4, 2.3×10^4 and 4.6×10^4 cells per ml. Ten fish were used to test each concentration and in the uninoculated artificial sea-water control medium. All

test material in 35 ml amounts were held in a finger-bowl. During tests, fish response was determined by visual observation and elapsed times noted at the onset of equilibrium loss and at death. Death was defined as cessation of opercular movement.

The mean daily growth rates (K) of bacteria and *G. breve* were calculated using the formula of Smayda (15).

RESULTS

The eight cultures grew uniformly during the first five weeks. None exhibited a discernible lag phase. During the first 15 days the mean daily growth rate (K = 0.44 to 0.45) was more than twice the rate during the next 18 days (K = 0.18 to 0.20) (Fig. 1). The overall growth rate of the cultures during

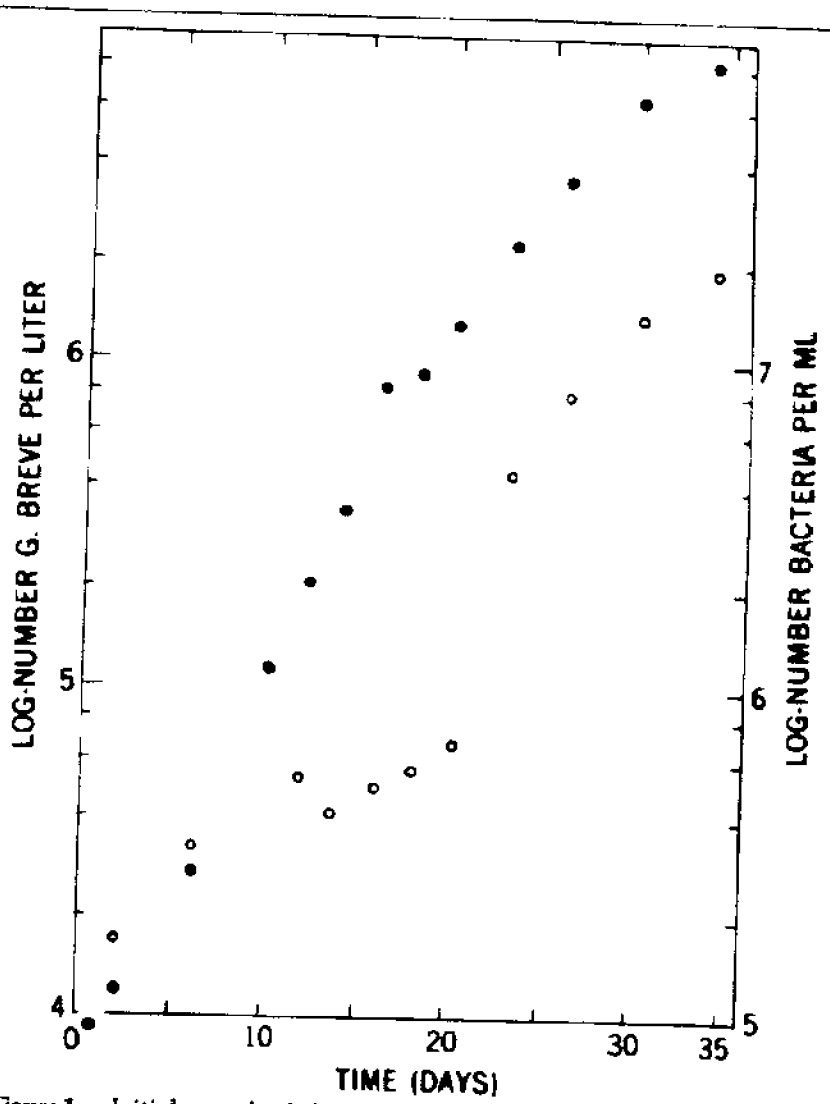

Figure 1 Initial growth of *G. breve* cultures and bacterial populations. ● = *G. breve*; O = bacteria

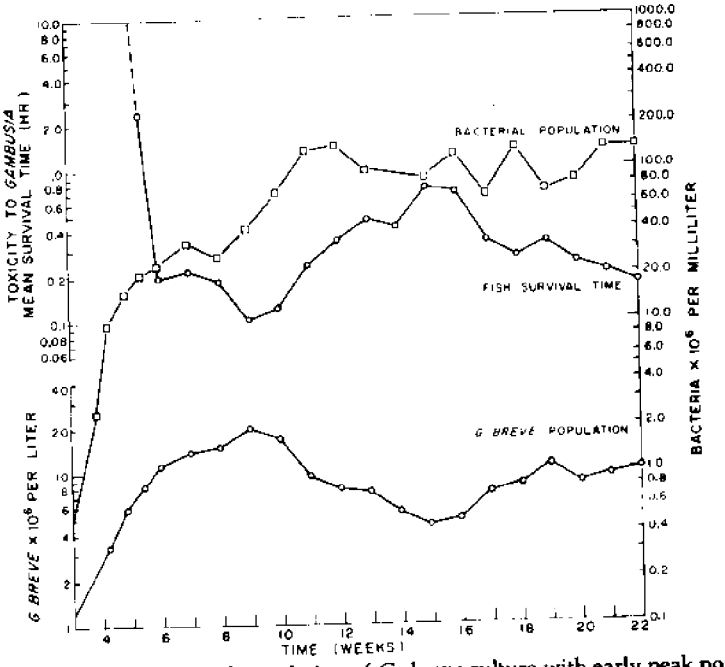

Figure 2. Toxicity and bacterial population of *G. breve* culture with early peak population development.

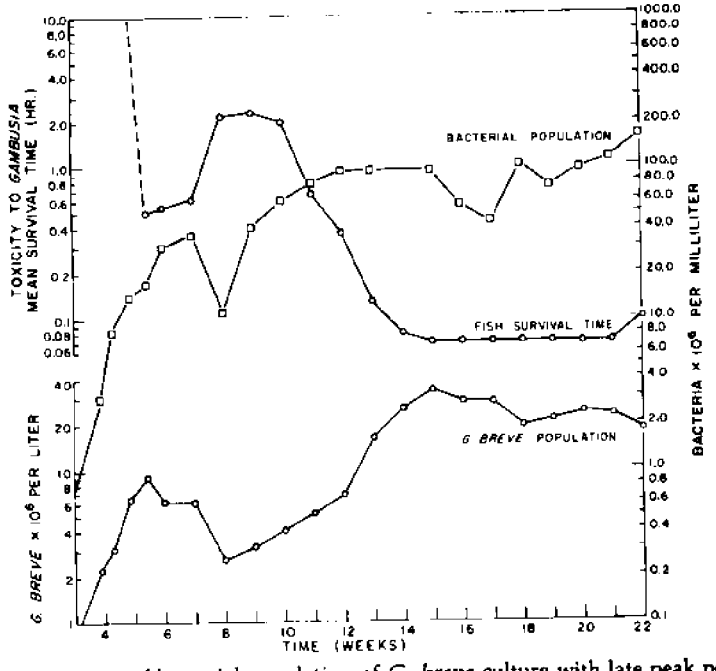

Figure 3. Toxicity and bacterial population of *G. breve* culture with late peak populati development.

the first five weeks (K=0.3) was faster than the rate during any comparable period during the next four months. At the end of this five-week period, the population levels were between 1×10^6 and 1×10^7 cells/l.

During the last four months of the study, the population levels of the cultures differed considerably. In one case, the population level decreased rapidly and subsequently increased at a slow irregular rate. The level of another culture continued to increase after the first five-week period at a reduced rate for two months, then decreased rapidly. The rise and fall of the population level of a third culture was cyclic with a period of approximately four weeks. Based on the growth patterns during the last four months of the study, the eight cultures could be roughly classified according to the time of peak growth as follows: (1) early peak (Fig. 2); (2) late peak (Fig. 3) and multiple small peaks (Fig. 4).

The cultures grew to population levels that were between 12 and 38 million cells/l at some time during the last four months of the study. Early peak populations were characteristic of three cultures. The peak population levels of these cultures were 19, 27 and 30 $\times 10^6$ cells/l and they occurred after 8 to 9 weeks growth. The growth rate of these cultures after the first 15 days growth declined more than 50%. The populations of the three cultures characterized by late maximum peaks reached levels of 8 to 13 $\times 10^6$ cells/l after five to seven weeks, decreased for periods of one to five weeks and subsequently

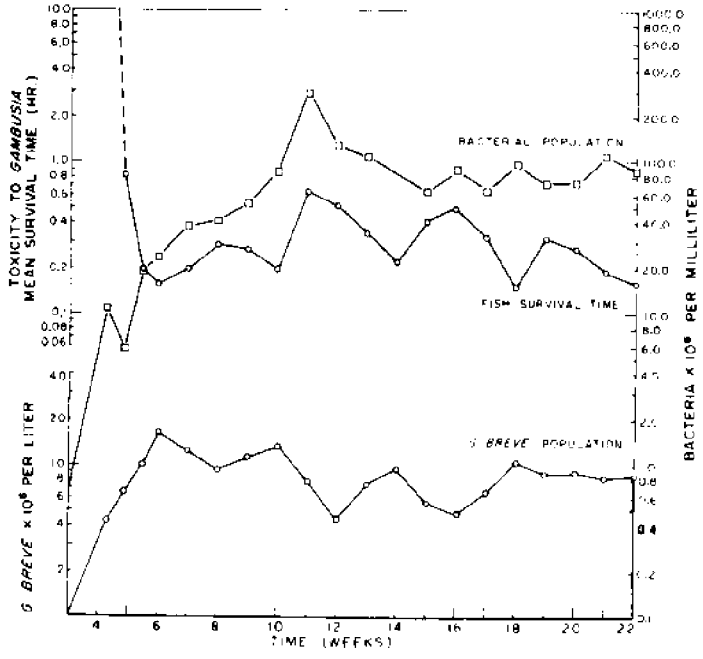

Figure 4. Toxicity and bacterial population of *G. breve* culture with multiple peak population development.

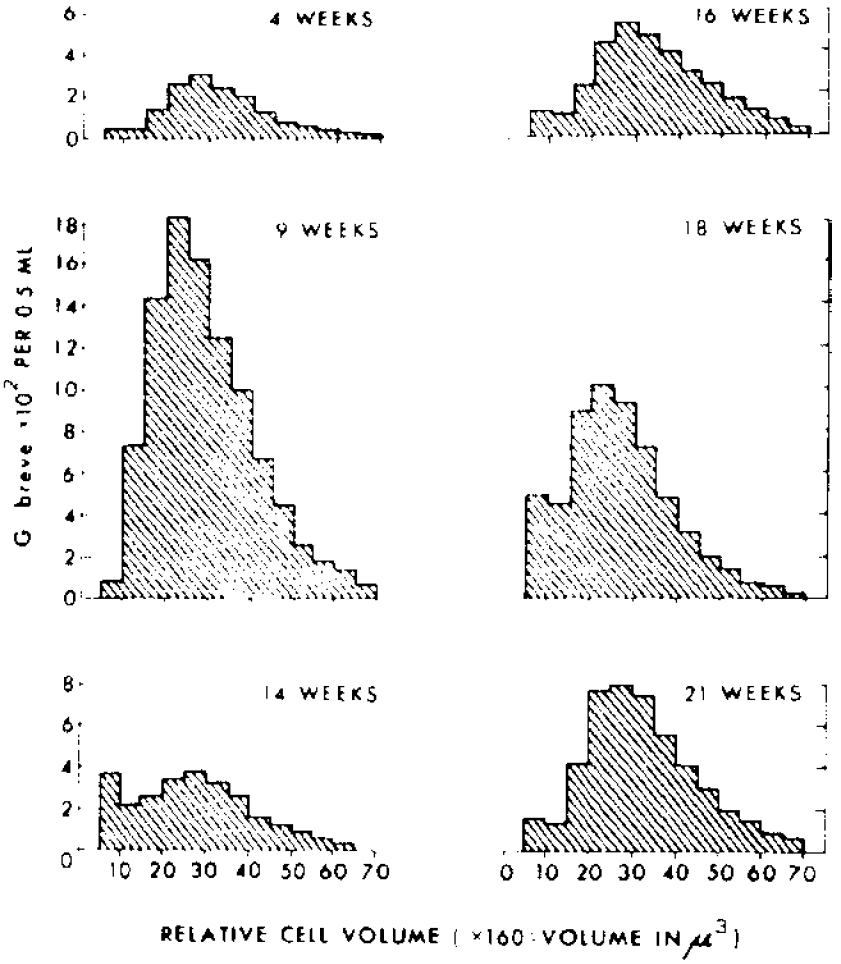

Figure 5. Size distributions of populations of a *G. breve* culture at various stages of development.

increased to levels of 22, 32 and 33 x 10^6 cells/l after 12 to 13 weeks of the study. The two cultures with multiple peak population levels declined after the initial five week growths and subsequently had three additional population cycles with periods of approximately four weeks. The maximum population levels (12.5 and 16.5 x 10^6 cells/l) of these cultures were lower than the maximum levels of the other six cultures, but both were at a level of ca. 10^6 cells/l at the end of the study. At the end of the 5-month study period, culture population levels were from 0.5 to 24 x 10^6 cells/l.

Observations of growing cultures often led us to think that cultures in the log-growth phase had a smaller average size than senescent cultures. Between 55% and 65% of the culture populations were always within the 3200 to 6400 μ^3 size range. Changes in the culture population levels were accompanied by similar changes within this range (Fig. 5). The population levels of organisms that were larger and smaller than this size range also changed on some

occasions. The relative changes in these cell sizes were not apparently related to the population levels of the cultures. On the other hand, if the culture population was increasing, the number of cells with a volume greater than 6400 μ^3 was higher than the number of cells with a volume less than 3200 μ^3. If the culture population was declining, the ratio of large-sized cells to small-sized cells was usually less than 1.

The bacterial populations in the cultures all exhibited a lag phase during the first three weeks of the study (Fig. 1). Although the bacterial populations increased from an initial level of approximately 1.5 x 10^6 bacteria/ml to 6.7 x 10^6 bacteria/ml, the rate of increase (K = 0.1) was not comparable to that of *G. breve* during this period. As the *G. breve* populations approached or passed the 1 x 10^6 cells/l level and the growth rates decreased, bacterial growth rates increased significantly. The bacteria growth rates at this time (K = 0.40 to 0.56) were similar to the *G. breve* growth rates during the first 15 days of the study. The bacterial populations continued to increase but at a slower rate until population levels of approximately 1 to 3 x 10^8 bacteria/ml were reached after approximately eight weeks. Subsequently, the bacterial populations, in general, varied directly with the *G. breve* population levels.

Toxicity of *G. breve* cultures to fish was not detected in any of the tests of the ten series of samples collected prior to the 31st day of the study. At this time the cultures contained between 4 and 9 x 10^6 cells/L. On the 27th day of the study and on the two previous sampling dates, the cultures contained between 1 and 4 x 10^6 cells/l. One of the ten test fish died that was exposed to one of the samples collected on the 27th day.

Table 1
Comparison of the Toxicity of Intact and Lysed *G. breve* Cultures at Various Population Levels to *Gambusia affinis*

G. breve Population Level	Toxicity to *Gambusia* Mean Survival Time (Hours)	
Cells/l x 10^6	Lysed Cells	Intact Cells
2.4 - 5.0	0.6 - 4.5	4.5 - >24
5.1 - 10.0	0.22 - 0.53	2.5 - 16.3
10.1 - 15.0	0.14 - 0.22	1.2 - 8.7
15.1 - 20.0	0.1 - 0.2	0.2 - 3.6
20.1 - 30.0	0.1 - 0.11	2.0
>30.0	0.09 - 01	1.3 - 1.7

After the cultures attained population levels of approximately 1 x 10^7 cells/l, the toxicity was directly related to the *G. breve* population level (Fig. 2, 3 and 4). The curves of the fish survival time approach being mirror images of the *G. breve* population curves. On the other hand, cultures that had the same population levels at two different times were not always equally toxic. On some occasions, a culture with an increasing population was more toxic than the same culture which on another date had declined to the same population level (Fig. 3, 7th and 12th week). On other occasions, a culture was more toxic during a population decline than during a population increase (Fig. 2, 5th and 12th weeks).

Frozen culture samples were consistently more toxic than their companion intact cell samples (Table 1). In addition, the toxicity of the intact cell sample was not as closely related to the culture population levels as was that of the frozen samples. In general, the toxicity of frozen samples and intact cell samples differed progressively less as the culture population levels increased.

Results of the experiment in which the toxicity of *G. breve* and *G. monilata* to the mosquito fish were compared indicated that the toxicity per cell of *G. breve* was three to four times that of *G. monilata* (Fig. 6). The difference in toxicity is greater if based on biomass. The cell volume of *G. breve* is approximately one-half the cell volume of *G. monilata*. On the other hand, the

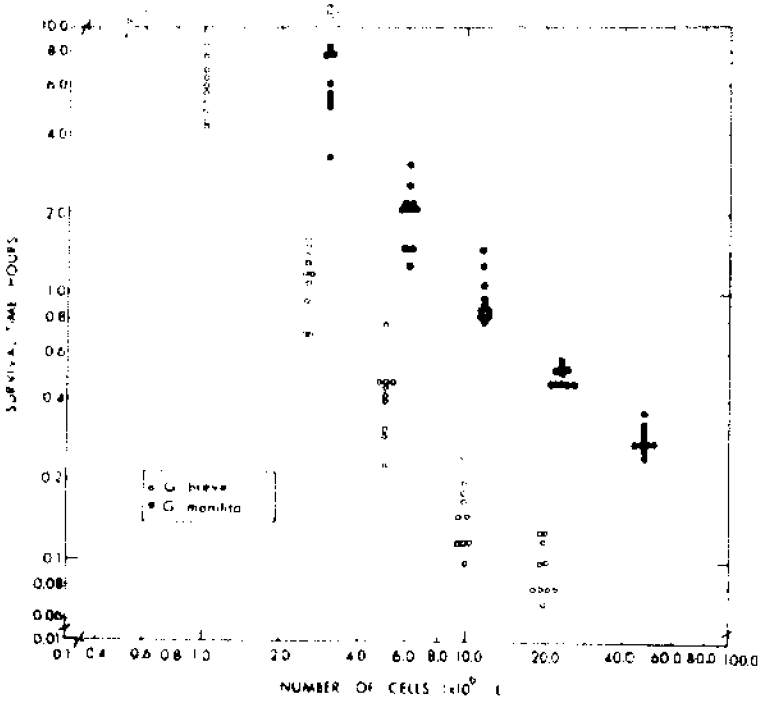

Figure 6. Survival of *Gambusia affinis* exposed to homogenized *G. monilata* cells and cell-free *G. breve* culture material (1, eight fish survived 24 hours; 2, two fish survived 24 hours).

G. breve test material was prepared by freezing and thawing the whole culture and *G. monilata* cells were concentrated by centrifugation and resuspended in fresh medium. This procedural difference may have caused the difference in the toxicity of the two organisms that was observed, at least, in part.

DISCUSSION

The maximum growth rate of *G. breve* that was observed during the first 15 days of the study was comparable to that previously reported but the apparent absence of a lag phase of 4 to 8 days is unusual (21). In the earlier study, the length of the lag phase was inversely related to inoculum size.We had noted (unpublished) that if the inoculum contained between 0.5 and 1.0x10 *G. breve*/l and the volume employed was ca 10% of the medium volume to be inoculated, the lag phase was not discernible if the enumeration interval was 24 hours. The inoculum volume used in this study was only 25.0 ml in 12 liters of medium. Fogg (5) stated that extracellular glycolate reduced or eliminated the lag phase of some algae. Cultures of *Chlorella* rapidly established an equilibrium between intra - and extracellular glycolate (11) and the lag phase of this organism was eliminated by additions of 1 mg/l of glycolic acid. The lag phase of cultures of *Ditylum brightwelli* was eliminated by the same addition. The absence of a lag phase in our study may indicate a "carryover" of an extracellular metabolite. On the other hand, this concentration of glycolic, palmitic or stearic acid was acutely toxic to *G. breve* (unpublished).

The presence of bacteria may not have contributed to the initial rapid growth of the cultures. Bacteria-free cultures of *G. breve* that grew on an artificial sea-water medium increased by ca. two orders of magnitude in ca. 25 days. This period probably includes a 4 to 8 day lag phase. The populations remained at this maximum level for ca. 10 to 60 days, then gradually declined within the next 30 days until they approached the inoculation level or died off completely (Wilson 1966). The bacterial growth in the eight cultures of the present study was slow during the period of rapid *G. breve* growth. The initiation of the maximum bacterial growth rate occurred after the period of maximum *G. breve* growth and after the *G. breve* population levels exceeded 1 x 10^6 cells/l. This feature suggests that, at least, the initial growth of the bacteria depends on the growth of *G. breve*. The dominant bacterium of these cultures was similar to the gram-negative bacillus that had been the dominant bacterium in *G. breve* cultures previously studied by Ray and Wilson in 1957 (12). The previous dominant bacterium grew much more in this medium if *G. breve* was present.

The continued, but reduced, *G. breve* growth in the cultures after the first 15 days and the previously mentioned lack of continued or secondary growth of bacteria-free cultures after they reached this approximate level suggests that bacteria contributed to secondary growth. This secondary growth continued for only about four weeks in some cultures before a population decrease occurred (Fig. 3) which was, in some cultures, followed by one or more

additional increases. The maximum population levels of the cultures always occurred at the end or after this secondary growth phase. In addition, bacteria-free *G. breve* cultures did not grow as consistently initially, nor attain population levels as high as those in cultures with bacteria (20).

The tendency for the *G. breve* population and bacterial population to fluctuate together after the initial *G. breve* growth phase indicates that either one was beneficial to the other, detrimental to the other or that they were mutually beneficial or detrimental. The longevity of this association in most of the cultures suggests a mutual benefit. This possibility is suggested by the previously mentioned secondary growth of *G. breve* and the initiation of the rapid growth of bacteria during the first four to six weeks of the study.

The reason for the large difference (0.5 to 24 x 10^6 cells/l)in the population levels between cultures at the end of the study is not known. It could have been caused by the cyclic growth of cultures with bacteria or differences in the bacterial floras of the cultures. Another possible cause of population declines may be a secondary bacterial or coccoid blue-green algae contamination. The latter were not observed in the cultures of this study, but they subsisted on the walls of our laboratory and they will grow in *G. breve* cultures that have reached maximum population levels. If so, the population of the cultures declines.

A question that is frequently asked regarding *G. breve* is whether or not it produces an exotoxin or endotoxin. Based on the experiments with frozen and intact cells, the toxin seems to be an endotoxin. Toxicity was directly related to population level only if the culture was frozen. Also, the fact that frozen material was consistently approximately 2 to 30 times more toxic (in time of survival of test fish) indicate it is an endotoxin. On the other hand, the reduced difference of toxicity of intact and lysed portions of a culture sample at high population levels indicated extracellular toxin may be produced.

The size distribution portion of the study suggests that the ratio of large cells (>6400 μ^3) to small cells (>3200 μ^3) may depend on stage of growth of the culture. If the culture population was increasing, the ratio of large: small cells was usually greater than 1.5. If the culture population was decreasing the ratio was less than 1.0. Cultures that were at a maximum or minimum population level had a ratio that was approximately 1.0 or higher, but less than 1.5. These distribution patterns may have been influenced by the increase in cell debris that apparently occurred in a declining population. This material increased the counts of the lower size class. Despite this aspect, there were relatively more larger cells in an increasing population.

A comparison of the toxicity of *G. monilata* cultures (Aldrich *et al.*, 1967) and *G. breve* cultures showed considerable differences in toxicity development and population levels. The uniformity of the relationship of the toxicity and population levels of *G. breve* cultures indicated that the toxin(s) were either unstable, reduced rapidly by bacteria or principally intracellular. The maximum toxicity of *G. monilata* cultures occurred at the time the population level had decreased by 50% or more. This apparent lag in toxicity indicated

that the toxin(s) were either stable, not reduced readily by bacteria, or possibly also principally intracellular. The *G. monilata* tests were conducted with live cultures. Thus, the coincidence of the occurrences of maximum toxicity and the decline in population levels may have been associated with cell lysis of the population. The toxicity of cultures of both organisms was consistently higher with lysed cells than with intact cells (Table 1). A comparison of chemical stability and/or bacterial reduction cannot be made unless bacteria-free, cell-free preparations of physiologically similar cultures are compared.

The per cell toxicity of *G. breve* and *G. monilata* may be similar. As previously mentioned the apparent difference we observed (Fig. 6) may have been caused by different methods of preparing the test material. The toxicity of *G. monilata* cultures increased, at least, two-fold after freezing and thawing as found by Gates and Wilson (7). Results of experiments conducted with frozen and thawed culture material assayed with *Cyprinodon variagatus* indicated that the per cell toxicity of these two organisms was similar as found by Sievers (14). If so, based on biomass, *G. breve* may be more toxic.

The toxicity per cell of cultures of both organisms was apparently less during the early growth phase than during a later phase. For example, cultures that had increased from the original inoculum level to a level of 1×10^6 cells/l were considerably less toxic than the same cultures which were at this population level subsequently.

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EFFECT OF ENVIRONMENTAL FACTORS ON GROWTH OF *GONYAULAX CATENELLA*[1]

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ABSTRACT

Gonyaulax catenella, the causative organism of paralytic shellfish poisoning on the Pacific coast of North America, has been grown in the laboratory in a series of experiments to determine the effects of variations of physical parameters and availability of both organic and inorganic nutrients on its growth.

Experiments using cross-gradients of salinity, temperature, and light intensity indicated that these parameters interact in their influence on growth. Extremes of either salinity or temperature reduced the tolerance range for the other factor. Tolerance ranges were shown to be 15 to at least 40°/00 salinity and 8 to 23°C. Growth occurred for all light intensities tested, from 50 to 500 f.c. Optimal ranges for these parameters were 20-37°/oo, 13-17°C, and 150-350 f.c.

This species can utilize a variety of nitrogen sources, growing equally well on nitrate and ammonium ions and urea at concentrations up to 100 µg-at N/1. Inhibition was caused by high levels of the latter two but not of nitrate. Asparagine supported good, but lesser growth. Glycine, serine and alanine supported slight growth.

Lactate, acetate, and glycerol did not significantly affect growth, but glucose reduced growth. None of these four carbon sources supported heterotrophic growth.

INTRODUCTION

Paralytic shellfish toxicity is likely to occur during each year along the State of Washington ocean coast and the Straits of Juan de Fuca, a shoreline of about 300 miles. Some of these beaches are affected almost annually; others have outbreaks less frequently. In addition to this portion of the coastline with fairly high probability of outbreaks of shellfish toxicity there are perhaps a thousand miles of shoreline in the labyrithine estuarine waters of the state. In the northern portion of these waters, between Vancouver Island and the mainland, shellfish toxicity is known to occur occasionally. In Puget Sound proper, *Gonyaulax catenella*, the causative organism, is not uncommon in summer at least as far south as Seattle, even though toxic shellfish have not been reported from this region. Thus, either toxic shellfish have occurred or the potential for an outbreak of shellfish toxicity exists in a high percentage of the marine waters of the state.

Nevertheless, there have been only three human deaths attributed to paralytic shellfish poisoning since the 1930's when the State Department of Health started studying shellfish toxicity. This low figure is due largely to regulations established in 1942 after those deaths occurred. The entire length of the open ocean coast and much of the Strait of Juan de Fuca shoreline are closed for the taking of shellfish other than razor clams between April 1 and October 31 of each year. Razor clams are excluded from the regulation,

because they retain the toxin in the digestive gland which is usually not eaten. In other areas the Department of Health maintains a surveillance program of certain commercial and sport shellfish areas.

The College of Fisheries at the University of Washington began its research program on the ecology of paralytic shellfish toxicity in 1961. Phytoplankton studies indicated that *Gonyaulax catenella* was abundant prior to outbreaks of shellfish toxicity and that no other dinoflagellate species was correlated with the periods of toxic shellfish (1,2). *Gonyaulax catenella* is the chain-forming dinoflagellate which has been shown by Sommer, et al. (3) to be the cause of the shellfish toxicity in California. Studies of the seasonal occurrence of shellfish toxicity showed that on the Straits of Juan de Fuca there were two periods each year when California mussels and Pacific oysters were toxic. A period of minor peaks of toxicity occurred in early summer; the major peaks occurred in late July to September. The abundance of *G. catenella* in Sequim Bay, Washington, was shown to be related to the surface water temperature, decline in diatom abundance, and amount of sunshine (1, 4). Laboratory studies with cultures of *G. catenella* showed that toxin content per cell varied inversely with growth rate and declined with time following the end of the log growth phase (4).

The response of shellfish to *G. catenella* was studied in both field and laboratory. Field work revealed differences in rates of uptake and loss of toxin among the four species of shellfish studied: butter clams, *Saxidomus giganteus;* Little-neck clams, *Protothaca staminea;* bay mussels, *Mytilus edulis;* and

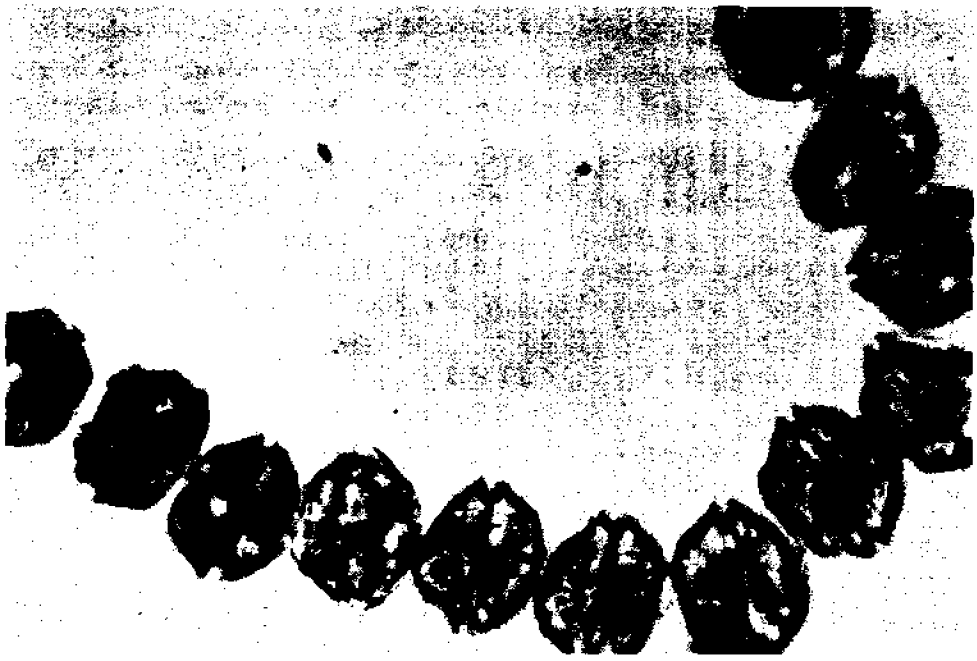

Figure 1. *Gonyaulax catenella*, culture Gc 71, isolated from Sequim Bay, Washington (500x).

Pacific oysters, *Crassostrea gigas* (5). In laboratory investigations, *Crassostrea gigas*, when fed *G. catenella*, reduced pumping rates at food concentrations as low as 20 cells/ml and stopped pumping at 220 cells/ml (4.)

The fate of saxitoxin in the food chain was investigated by a sampling program in areas along the Strait of Juan de Fuca where shellfish toxicity occurs annually. Despite the fact that levels of shellfish toxicity in the area were unusually low during the investigation, extracts of several species of animals caused mouse death reactions typical of paralytic shellfish poison: *Hemigrapsus nudus*, the purple shore crab; *Littorina sitkana*, a periwinkle; *Pagurus* sp., a hermit crab; and two species of snails, *Thais lamellosa* and *Thais lima* (= *T. emarginata*) (6).

CURRENT RESEARCH

Purpose

The major thrust of the more recent work has been a laboratory investigation of the effect of several environmental factors on growth of *G. catenella* in order to better understand the seasonal and geographic distribution and abundance of this organism. The additional hope, of course, is that a factor or a combination of factors might be identified which plays a major role in controlling the growth rate and that such information would be useful in the formulation of a procedure for prediction of toxic shellfish. Culture experiments have been conducted to study the effects of the physical parameters of temperature, light intensity, day length, and salinity, and nutritional factors, both organic and inorganic.

Methods

Our culture work with *G. catenella* has been carried out for the most part in 125 ml Erlenmeyer flasks containing 50 ml. of culture medium. These cultures have been grown in controlled environment chambers under conditions of temperature and light intensity determined by the nature of the experiments. Light was from cool white fluorescent tubes and was continuous in all experiments after early work indicated that reduced day length (either 12 or 18 hours) caused a reduction in growth below that of cultures in 24 hrs. of light.

The media used for maintenance of stock cultures were a soil extract medium[2] and an artificial seawater medium, ASP_7, modified by omission of silicate. A second artificial seawater medium, ASP_{12}, was tried several times without success. Both of these media were formulated by Provasoli (7). The

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[2] The soil extract medium was composed of: 950 ml. seawater (approx. 30°/oo), 50 ml. soil extract (supernatant of 1 kg. soil in 2l. water, autoclaved 1 hr. at 10 psi), 0.2 g. $NaNO_3$, 22.5 mg. $Na_2HPO_4.7H_20$, and 1 mg. Na_2EDTA.

ASP_7 medium was used for most of the experimental cultures and was modified as described later to fit the purpose of each experiment. In early studies of temperature and light tolerance, the soil extract medium was used.

Samples of experimental cultures were taken at frequent intervals and counted with an inverted plankton counting microscope.

The stock cultures of *G. catenella* used in these experiments were derived from three separate isolates made from Sequim Bay, Washington: Gw 64, isolated by Dupuy in 1964, and Gc 68 and Gc 71, isolated by Norris in 1968 and 1971, respectively. Axenic cultures were obtained by treatment with antibiotics, using the method described by Droop (8).

Results

Growth Responses to Varying Temperature, Light, and Salinity

Several experiments were carried out to determine the growth response to a variety of combinations of different temperature, salinity and light intensity values. To attain different salinity values, the ASP_7 medium was altered in one of two ways. One was to make up the medium in a large volume without NaCl and divide it into smaller volumes to each of which was added sufficient NaCl to attain the required salinities, thus changing the concentration of none of the ingredients except NaCl. The other was to make up a large volume adjusted to high salinity by the addition of NaCl, and to dilute portions to the salinities desired, thus changing the concentrations of all the ingredients. Three series of cross-gradient experiments were carried out, each using an inoculum derived from a different isolate of *G. catenella*.

The results of these experiments indicate a general agreement among the three isolates with regard to the tolerable values and optimal values of these parameters. They have been summarized in Table 1. In the course of these experiments growth was found to occur at temperatures from 8 to 23°C, at salinities from 15 to 40°/oo, and at light intensities from 50 to 500 f.c. Higher salinities and higher and lower light intensities were not tested. The optimal values for these parameters were in the ranges of 13 to 17°C and 150 to 350 f.c. for all three isolates. Growth of two isolates was optimal in the salinity range of 21-30°/oo while the third isolate grew best from 20-37°/oo. A possible explanation of this difference in salinity optima could lie in the fact that the culture which grew optimally at higher salinities had been maintained for several years in sea water from the open coast with salinity of 33°/oo, whereas the other two isolates had been cultured in estuarine water with salinity averaging around 30°/oo. All isolates were made from Sequim Bay in which the salinity was about 32°/oo. Another possible explanation for the differences in the ranges of salinity optima could lie in the difference in the methods used to adjust salinity.

Table 1.
Tolerance and Optimum Ranges
for Temperature, Salinity, and Light Intensity

Temperature		
Tolerance range	———	
Values tested	5 8 13 14 17 20 23 24 26	°C
Optimum range	———	
Salinity		
Tolerance range	———	
Values tested	15 17 21 25 29 31 35 37 40	°/oo
Optimum range	——— Cultures Gc 68 & Gc 71	
	——— Culture Gw 64	
Light Intensity		
Tolerance range	———	
Values tested	50 100 150 200 350 500	f.c.
Optimum range	———	

Culture experiments indicate that *Gonyaulax catenella* is a eurythermal, euryhaline dinoflagellate which grows best in cool temperatures and reduced light.

Table 2.
Response to Temperature and Salinity

	5	8	11	14	17	20	23	°C
40°/oo	d	d	d	ng	ng	d	d	
35	d	d	d	G	G	G	d	
29	d	d	G	G	G	G	G	
21	d	d	ng	G	G	G	ng	
15	d	d	ng	ng	G	G	d	

d = died; ng = no growth
G = growth (cell numbers at least double the initial level)
10 day culture period

Extremes of either temperature or salinity reduce the tolerance range for the other parameter.

The effect of extremes of temperature on the tolerance range for salinity and vice versa is shown in Table 2. At optimum salinity values, the organism can tolerate a temperature variation of a least 12 C. degrees, but at the extreme salinity of 40°/oo, the tolerable temperature variation is reduced to less than 9 C. degrees. Similarly, the wide range of salinities tolerable at optimal temperatures, i.e., from 15 to 40°/oo is reduced to a much narrower range of salinity values at suboptimal temperatures. Variation in light intensity from 100 to 350 f.c. had no significant effect on the tolerance ranges for temperature or salinity. Light intensities less than 100 and greater than 350 f.c. were not studied in cross-gradient experiments.

Growth in Response to Nitrogen Sources

The experiments to determine the effect of several nitrogen sources on growth of *G. catenella* were carried out in ASP_7 medium from which $NaNO_3$ was omitted. Various nitrogen compounds were added singly in the concentrations shown in Table 3. Two isolates were tested in separate experiments.

Although growth rates of the two isolates were not comparable, during the first 10 or 15 days growth rates for each isolate were approximately equal in $NaNO_3$, NH_4Cl, and urea at the following concentrations: NO_3^- from 10-40 µg-at N/l (up to 100 µg-at N/l in another experiment), and NH_4^+ from 10 to 100 µg-at N/l and urea from 25 to 80 µg-at N/l. At 200-1600 µg-at NO_3^--N/l, generation times were slightly longer. At higher concentrations of NH_4^+ (200-300 µg-at N/l) and urea (400-600 µg-at N/l) cell counts declined initially or at least did not double in the first week, but slow growth started thereafter. At even higher concentrations (800 µg-at NH_4^+-N/l and 1600 µg-at urea-N/l), there was no reversal in the initial decline in cell counts and cultures died rapidly (see Table 3).

Table 3
Growth Rates in Nitrogen Sources
(Generation times in hours.)
7-14 day period (logarithmic growth), GC 71.

	10	20	30	40	60	80	100	200	300	400	600	800	1000	1600	µg-at N/l
NO_3^-	48		53	53			62	68	71			65	61	68	
NH_4^+	53		46				46	ng-g	ng-g			d	d	d	
Urea		56			53	56		62		ng g	ng-g			d	

d = died
ng-g = no growth during 1st week, followed by growth

Growth rates are approximately equal at lower concentrations of NO_3^-, NH_4^+, and urea. The latter two are toxic at high concentrations.

Final cell counts were proportional to nitrogen concentration in NO_3^- and NH_4^+ at concentrations up to 100 µg-at N/l and in urea at concentrations up to at least 60 µg-at N/l. Final yields equivalent to those attained at 300-1600 µg-at N/l in nitrate medium were not attained in NH_4^+ or urea media because of their toxicity at those concentrations (see Table 4).

Table 4.
Final Growth (cells/ml) in Nitrogen Sources
(in excess of counts in controls)

	10	20	30	60	100	200	300	600	800	1600	µg-at N/l
NO_3^-	950		2790		8380		27,560		25,560	29,160	
NH_4^+	780		1900		7000		259		d	d	
Urea		720		3560		4370		2780		d	

d = died

Total growth in NO_3^-, NH_4^+, and urea is proportional to available nitrogen at concentrations up to 60-100 µg-at N/l.

Other nitrogen sources tested included: asparagine, which supported slow growth at concentrations up to 3200 µg-at N/l, at which concentration total growth was about 25% of maximum growth on NO_3^-; uric acid, glycine, serine, alanine, and glutamic acid, all of which supported growth at lower levels, ranging from 5-18% of maximum growth on NO_3^-; histidine and hydroxyproline, both of which retarded growth below the level of the controls without added nitrogen.

None of the 11 N sources mentioned above supported growth or permitted prolonged maintenance of cell numbers in the dark.

Growth in Response to Added Carbon Sources

Glucose, sodium acetate, sodium lactate, and glycerol were added singly to ASP_7 medium to determine their effect on growth in both light and dark. Concentrations from 1-20 mM were tested. Glucose reduced growth of both isolates at 5-20 mM concentrations. Glucose at 1-3 mM concentrations and acetate, lactate, and glycerol in concentrations from 1 to 20 mM had no significant effect on growth rate or total growth. Because glucose does not occur in marine waters at the relatively high concentrations required to inhibit growth, this finding is considered ecologically unimportant.

None of these 4 carbon compounds supported heterotrophic growth. However, although the data are inconclusive, they suggest that cultures of *G. catenella* die less rapidly in media containing added carbon sources than in controls.

CONCLUSIONS

Results of this experimental work indicate that *G. catenella* is a eurythermal, euryhaline organism which is capable of growth in relatively low concentrations of several nitrogen compounds.

The laboratory findings appear to be in agreement with field data. The salinities along the Strait of Juan de Fuca are always within the range of optimal salinity values determined by laboratory experiments (except for temporary localized lenses of freshwater runoff). *Gonyaulax catenella* was found in the plankton only in the months when the water temperature exceeded 8°C, which was shown to be the lowest tolerable temperature in the laboratory. The finding of a maximum growth rate even at low concentrations of nitrate is compatible with field data which indicate a general low level of NO_3 (0-10 ug-at N/l)[1] occurs during months of higher *G. catenella* populations. It may also help to explain the occurrence of *G. catenella* after the decline of diatom pulses which may have reduced the available nitrogen to low levels. Although growth in these experiments was better at light intensities below 500. f.c., this does not appear consistent with the fact that natural populations frequently occur in periods of bright sunshine. More definitive work on the effect of light intensity is needed. The lowest generation times in the laboratory have been in the range of 20-30 hours, i.e. approximately 1 division per day, a finding which is compatible with the rate of increase in natural populations observed in Sequim Bay.

The knowledge that growth of *G. catenella* is not closely restricted by physical parameters points out that the potential exists for occurrence of toxic shellfish over a long season in widespread areas of our marine waters. Information about tolerance and optimal values for temperature and salinity could be useful in delineating areas and seasons of closure of beaches for the taking of shellfish. However, closures based on these parameters alone would cover a far longer season and wider area than toxicity hazards would warrant.

Obviously, within these broad seasonal and geographic limits for potential growth of *G. catenella*, it would be highly desirable to be able to predict times and places of high probability of its occurrence in sufficient numbers to cause shellfish to become toxic. In order to do this, many additional physical and biological factors controlling its growth, survival, and toxin production must be considered. The fact that it can grow well on at least three nitrogen sources may be such a factor to be considered. Preliminary experiments indicate that concentrations of certain major inorganic nutrients and trace metals may also influence growth. Before it will be possible to predict shellfish toxicity on this basis, much additional work will be necessary to determine the significance of these and other factors in controlling populations of *G. catenella* in natural waters.

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BASIC FACTORS INFLUENCING RED TIDES[1]

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ABSTRACT

Ecologically, there are three aspects common to all toxic red tides. First, there is an increase in population size, called here initiation. Secondly, there is support, e.g., suitable salinity, temperature, nutrients and growth factors, and finally, in many red tides, the maintenance and transport of blooms by hydrologic and meteorologic forces. The first two aspects are often considered inseparable. However, with recent advances in dinoflagellate life cycle work, specifically, detection and description of sexual phases and viable benthic cysts, the possibility of benthic seed populations and the factors influencing their development should be primary research objectives. The locality of initiation is also critical. For example, *Gymnodinium breve* blooms are initiated about 16 to 64 km off southwest Florida in 12 to 37 m, and it is suggested that if a dormant stage exists, then seed populations or seed "beds" possibly can be delineated within this zone. Present data indicate gradual motile population increases, not sudden population "explosions" due to increased cell division rates. Once initial increases occur, specific chemical and physical conditions are necessary to support these blooms. Lastly, as evidenced by many dinoflagellate blooms, winds, currents, and organism migrations are important transport and concentrating mechanisms.

INTRODUCTION

Toxic dinoflagellate blooms are common in coastal and/or estuarine waters around the world, particularly temperate and subtropical regions. Ecologically, there are at least three aspects common to such blooms. First, there is an increase of initial motile populations above background levels, which in the case of *Gymnodinium breve* blooms, are not due to increased cell division rates. Secondly, water conditions must be optimal for support of such blooms either by land run-off, upwelling, submarine spring discharge, preceding biological conditioning, or possibly some other regulatory factor. The third progressional aspect in this proposed general concept concerns the maintenance and transport of blooms by meteorologic and hydrologic forces.

It is the first stage, initiation, that we think is critical to understanding red tides, or for that matter, many coastal and estuarine dinoflagellate blooms. This does not imply that subsequent stages are insignificant. On the contrary, they are essential to whether or not red tide conditions are established, how long they last, and what areas can be affected.

Data (1,2) suggest that *G. breve* blooms annually in Gulf of Mexico coastal waters (> 16 km offshore) and that probably 3/4 of these blooms terminate offshore without developing into major outbreaks. Others are transported to nearshore waters where they can become established and cause severe economic distress(3, 4). Once in nearshore waters, the offshore blooms which evidently seed nearshore blooms can continue (e.g. 1968) or subside (e.g. 1967). Occasionally, as in 1974, the nearshore blooms will subside but can be

reestablished sporadically by continuing offshore blooms of low to moderate concentrations. The point to be made here is that the zone of initiation and the status of the original "seed" population offshore should be evaluated to determine "triggering" factors. It is our contention that toxic dinoflagellate blooms are life cycle phenomena and to understand red tides, researchers need to concentrate on alternation of cytological and/or morphological generations and the environmental parameters which influence these stages.

The recent investigations of Dr. David Wall (Woods Hole Oceanographic Institution) and Dr. H. A. von Stosch (Phillips University, Marburg) on dinoflagellate life stages lends credence to the speculation that pelagic, toxic dinoflagellate blooms might originate from dormant stages and that these stages might be associated with certain bottom sediments. This then brings up the question, if benthic resting stages of certain dinoflagellates actually "seed" coastal red tides, are there localized areas of accumulation, or what we could call "seed beds"?

DISCUSSION

Sexuality and Resting Stages

There are at least ten dinoflagellates known to have sexual and asexual cycles involving cytological and/or morphological alternation of generations. These include the armored species *Ceratium cornutum*, *C. horridum*, *Glenodinium lubiniensiforme*, *Helgolandinium subglobosum*, and the unarmored species *Amphidinium carterae*, *Gymnodinium microadriaticum*, *G. pseudopalustre*, *Noctiluca scintillans*, *Oxyrrhis marina*, and *Woloszynskia apiculata*. (5,6,7,8,9). This list represents six marine and four freshwater species. The marine species are homothallic and either isogamous or anisogamous. The zygotes show a wide diversity ranging from resting cysts to motile cells bearing a close resemblance to their vegetative haplont counterpart. Although sexuality has been painstakingly described from laboratory experiments, the actual stages in fusion, zygote formation, meiosis, etc., can easily be missed *in situ* because of the time frame and the fact that very little cytological work has been done with field specimens. All dinoflagellate species known to have sexual cycles are haplontic with the exception of *Noctiluca*, which is diplontic.

The importance in discussing sexuality lies in the recent revelations that at least several benthic resting cysts (freshwater species) are actually hypnozygotes, and that sexual cycles could possibly account for seasonal appearances of certain bloom organisms. Braarud (10) speculated, "It would not seem unreasonable to assume that the seasonal distribution pattern of the meroplanktonic species may be influenced by a periodicity in sexual reproduction which may vary from one species to another."

Based on the work of Dr. Wall and others (11) there are at least 59 dinoflagellate taxa (38 marine, 21 freshwater) with described resting cysts isolated from sediments. Representatives have been documented for the armored genera *Cachonina*, *Ceratium*, *Diplopeltopsis*, *Diplopsalis*, *Ensiculi-*

fera, *Fragilidium*, *Gonyaulax*, *Helgolandinium*, *Heterocapsa*, *Peridinium*, *Pyrodinium*, *Pyrophacus*, *Scrippsiella*, and the unarmored genera *Gymnodinium*, *Gyrodinium* (?), and *Woloszynskia*. The majority of these cysts are thick-walled stages; however, there are several exceptions, including *Gonyaulax tamarensis*, which have thin walls and no ornamentation (12). Dormancy is known for several freshwater species, a few marine species, and is speculated for the remainder of those species which produce benthic cysts (8, 13, 14). This is an extremely important aspect of the life history which needs verification and experimentation to determine what exogenous and/or endogenous factors stimulate onset of or emergence from dormancy. Of the described cysts, at least ten represent bloom species including the toxic *Gonyaulax tamarensis*, *G. polyedra* and *Pyrodinium bahamense*. *Gonyaulax catenella* is said to produce cysts, but no descriptive work is published.

The hypothesis that such benthic cysts, whether they are zygotes or asexual stages, represent dormant "seed" populations in coastal and estuarine sediments is intriguing; however, two points should be stressed. First, not all zygotes are nonmotile benthic cysts; they can vary from nonmotile to motile pelagic stages. Secondly, benthic cysts have not been positively shown to function as seed populations in marine ecosystems, although it has been suggested by several researchers.

Zone of Initiation

Most toxic dinoflagellate blooms, e.g. *Gonyaulax tamarensis*, *G. polyedra*, *G. catenella*, *Pyrodinium bahamense*, and *Gymnodinium breve*, are coastal in origin (15, 16, 17, 18, 19), although some can become established in estuaries depending on hydrologic conditions. These species are characterized as neritic based on frequency of occurrence and distribution. Depending on continental shelf topography, neritic can mean 2 km to 160 km or more, and in the case of broad, shallow shelves, it becomes important to pinpoint origin of blooms to evaluate initiation versus support, particularly if initiation is "offshore".

Gymnodinium breve red tides along Florida's west coast have been assumed to originate close to shore near passes (3); however, monthly data from 1964-1965 along four transects up to 32 km and monthly data from 1965-1967 for transects up to 164 km indicate that *G. breve* blooms originate at greater than 16 km and are associated with an initiation zone 16 to 64 km offshore in depths of 12 to 37 m. Offshore blooms in these two instances had population increases above background levels (< 1,000 cells/liter) and involved counts up to several hundred thousand per liter, which during 1967 were associated with fish kills. Since motile *G. breve* are common to both inshore and offshore coastal waters at low background levels throughout non-red tide years, a sampling program to detect where initial population increases are occurring should extend far offshore. Inshore sampling programs in bays and nearshore waters would not give adequate forewarning of a red tide outbreak, whereas extended programs could detect red tides in their early stages.

Dragovich and Kelly (20) concluded from their 1964-1965 data representing stations out to 32 km, "...incidence of *G. breve* was highest at the 27.8-km (15-mile) stations and least at the 9.3-km (5-mile) stations." Summarizing unpublished data representing 28 monthly collections of 504 live samples up to 75 km and 16 live samples over 8 months at 139 and 164 km off two widely separated geographic points (Tampa Bay and Sanibel Island), Steidinger (11) showed that a 1967 red tide originated from blooms first detected 16 to 37 km off the Tampa Bay area with other sampling stations inshore and offshore of this detection area having less than 100 *G. breve*/liter. *Gymnodinium breve* (< 100/liter) was recorded only once at one of the two stations on the outermost shelf (164 km at 73 m) and this isolated occurrence was coincident with the 1967 red tide. These monthly data plus the fact that one station, 75 km offshore, was sampled biweekly rather than monthly, indicated that the 1967 red tide was not seeded from further offshore than the proposed zone of initiation 16 to 64 km. Data also suggest that initial blooms are localized.

Physical Factors

After initiation and support, currents can occasionally transport blooms long distances. In November 1972, the first *G. breve* red tide along the southeast Florida coast was attributed and documented to transport of bloom concentrations from off Sanibel Island through the Florida Keys into the Florida Straits and up the east coast via the Gulf Stream (21). Data from a Loop Current anomaly (22), *G. breve* counts, and satellite imagery suggested that an unusual current pattern set up in the lower eastern Gulf acted as an initial transport mechanism. Since *G. breve* can be transported by oceanic currents, this incidence has raised the question of whether or not *G. breve* is recruited from the Caribbean through the Yucatan Straits. However, in 34 live, 5-gallon water samples over a two year period (1967-1969) from 12 stations in the Yucatan Straits and open Gulf waters directly above the Straits, Ms. J. Williams (FDNR, personal communication) did not observe any motile *G. breve*.

Although currents, including tides, can transport blooms, the receiving waters must be suitable for survival. For example, data from two of the three red tides that have established in Tampa Bay, Florida, indicate that the normally low salinity barrier of the Tampa Bay System was nonexistent at these times because of drought conditions. Salinities in upper bay reaches, e.g. Old Tampa Bay, during the summer of 1971 were as high as 31°/oo when normally salinites of 25°/oo or less would be characteristic. In early months of 1974, the salinities were normal, up to 25°/oo in Old Tampa Bay. Consequently, high salinity conditions in 1971 allowed *G. breve* blooms, once in the estuary, to penetrate and survive in upper reaches in Old Tampa Bay while salinities in 1974 were at the lower limit for *G. breve* tolerance and blooms never became established in that part of the bay system, although blooms and fish kills were common in higher salinity waters of the lower bay reaches.

Gymnodinium breve during these two outbreaks originally gained access to Tampa Bay via the ship channel and cell counts showed it to be progressively moving up the bay. Once in Tampa Bay or the lower bays, e.g. Boca Ciega, winds and tides were instrumental in transporting and dispersing blooms. The importance of these mechanisms was obvious. On high tide in a back canal of Boca Ciega Bay with gentle winds from the SSW, surface samples from a distinctly discolored patch moving with the tide had 11 million *G. breve*/liter and 18" below the surface only 370,000/liter. The patch was oriented perpendicular to the wind direction. Five days later at the same time, same station, and with similar wind conditions, *G. breve* was 21 million/liter at the surface and 16.7 million/liter about 15" below. On the turn of the tide, the patch dissipated and counts at that station were 330,000/liter. It should be stressed however, that normally in coastal or estuarine waters, although there can be higher concentrations at the surface during daylight hours, fish-killing concentrations in the hundreds of thousands usually exist throughout the water column and mortality of fishes associated with bottom waters is common.

CONCLUSIONS

Gymnodinium breve red tides start offshore. The status of the seed population is not known, although there is a motile, planktonic population throughout the year at less than 1,000 cells/liter. No detailed cytological work has been attempted with this population, or even bloom populations, to determine whether specimens are haploid or diploid. If there is even the slightest possibility of toxic dinoflagellate blooms having their initiation from benthic resting cysts which may be hypnozygotes, it would seem that this avenue of research should have a high priority among phytoplankton systematists and ecologists.

The possibility of benthic seed populations and even seed beds for at least some *Gonyaulax* and *Pyrodinium* has a higher probability than for *Gymnodinium breve*. Again, however, no detailed cytological work has been attempted to determine whether these naturally occurring *Gonyaulax* and *Pyrodinium* cysts are zygotes. The only support for the possibility of *G. breve* also having a benthic stage is: 1) freshwater *Gymnodinium* and *Woloszynskia* have such stages and 2) Wall and Dale (23) described three marine benthic cysts, which on excystment produced a *Gymnodinium*, a *Gyrodinium* (?), and another member of the Gymnodiniales. Natural cycles in toxic as well as nontoxic dinoflagellate blooms should be clarified. This is an opportunity to look at the sediment/water interface, its community structure and regulating mechanisms in depths to about 46 m. Many coastal phytoplankters are said to be meroplanktonic (14, 24, 25), but evidence that the meroplanktonic cycle influences species succession, distribution, and abundance is minimal for open coastal waters.

The second aspect of blooms, nutritive support, also needs careful scrutiny because it is still not conclusive whether such planktonic populations are being supported by preceding biological conditioning, upwelling, or enrichment from land discharge, whether it be runoff from rivers (26) or discharge from submarine springs. (27). Plankton community metabolism *in situ* is difficult to assess, but much progress has been made in culture on basic growth requirements for individual species. Yet, little is known concerning community interactions. For example, what factors allow a single species, or a species complex now that hybridization is a possibility, to dominate plankton biomass to the exclusion of other plankters for long periods of time, e.g. months? Is it lack of natural predators, excretion of inhibitory substances, or competitive exclusion? Why, in other words, do we have monospecific dinoflagellate blooms covering vast areas of open coastal waters? I, for one, envision such coastal blooms as having occurred sporadically for centuries and consider them natural phenomena, an aspect of an ecosystem we do not fully understand rather than an imbalance of nature.

Modeling such phenomena becomes a precarious task, because we essentially do not know the regulatory mechanisms. Models based solely on nutrients or solely on hydrologic factors negate the interaction of the three proposed aspects, namely initiation, nutritive support, and physical maintenance and concentration. One such model stressed the importance of water column vertical stability over horizontal convection mechanisms (28). It is difficult to separate these two aspects because the influence of winds, vertical stability and organisms migrations are interrelated. Direction and intensity of winds play an extremely important role in horizontal transport and concentration of daytime, surface-concentrated *G. breve* blooms, either by surface currents, tidal penetration of bays, or convection mechanisms (1). This is not to say that vertical stability is not an influencing parameter in dinoflagellate blooms. Rather, vertical stability is only one factor and should be considered along with temperature, salinity, winds, currents, nutrients and growth factors, tropism, light, metabolic requirements and efficiencies, life cycles, community interactions, etc. Red tides are an interdisciplinary problem and require an interdisciplinary approach by biologists, chemists, physicists, and geologists.

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SOME OF THE GROWTH CHARACTERISTICS OF *GONYAULAX TAMARENSIS* ISOLATED FROM THE GULF OF MAINE

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ABSTRACT

Various growth characteristics of axenic and non-axenic cultures were measured in an attempt to decipher what might give *Gonyaulax tamarensis* a selective advantage over other common marine phytoplankton species found in the Gulf of Maine. Characteristics found to be similar with *Gonyaulax* and other phytoplankters were: photosynthesis/ light relationships; temperature preference; salinity tolerance; nitrate utilization; production of alkaline phosphatase; and ammonium enhancement of dark carbon dioxide fixation when nitrogen deficient. Several other growth characteristics were found to differ markedly between *Gonyaulax* and other common marine phytoplankters. These include: stimulation of growth and photosynthesis by the chelator NTA; subtrate assimilation; percentage of photosynthetic product incorporated into protein; and response to daylength regimes.

INTRODUCTION

There are several general theories as to the cause of large abundances of red tide organisms. The first is that physical forcing motions of water cause high concentrations, i.e. accumulations; the second is that blooms occur as the result of enrichment. Proponents of the latter are further divided into those supporting enrichment by a) run-off b) thermocline breakdown and c) upwelling of nutrient-rich water.

There is ample evidence that both are important factors. That is, the appearance of bloom proportions cannot be explained without both interpretations; yet one of the most startling features of the red tide blooms observed by us is that the bloom is almost unialgal. This enigma has posed the prime question of our research which simply is: How is *Gonyaulax tamarensis* different from other phytoplankton? Specifically, are its requirements for light, temperature, nutrients different from other phytoplankton?

Our approach has been one of laboratory and field experimentation. During the years of 1972 and 1974 we were able to conduct experiments in the field on these dinoflagellate blooms as well as measure hydrographic parameters associated with the bloom. In the laboratory, we have isolated the organism in unialgal and axenic cultures, and tested the role of external factors on growth rate.

The data presented are a part of an ongoing study and as such are incomplete.

Field Observations: September 1972

In 1972, *G. tamarensis* was present as early as mid-July from the mouth of the Merrimack River to Boston Harbor (7). Visibly red patches were seen by fishermen and yachtsmen between September 4 to September 10 about 5 miles northwest of Cape Ann and near the Isle of Shoals. Some of these were

identified as species of *Ceratium*. Others were not identified. Also outbreaks of the non-toxic dinoflagellate *Peridinium* sp. were reported in near-shore waters.

In Ipswich Bay we observed maximum numbers of *G. tamarensis* in water samples on September 14. Chlorophyll values exceeded 30 mg/m^3 around much of Cape Ann and concentrations in some patches were in excess of 100 mg/m^3 with cell counts of 40 million cells per liter. Aircraft overfly and ship observations placed the distribution of *G. tamarensis* from Plum Island south and around the entire Cape Ann area. The dimensions of the patches were approximately 500 meters by 2 kilometers long oriented north-south.

Prior to the outbreak, maximum water temperatures of 18°C occurred at the end of August. In early September a rapid decrease in water temperaure occurred to 15°C. The water column was mixing vertically. This mixing process was accentuated by Hurricane Carrie which struck the New England coast with winds of 40 to 50 mph on September 3, 1972. This brought three inches of rainfall. The vertical mixing was enriching the water column with 3 µgAt/1 nitrogen (NO_3) and 0.8µg At /1 phosphorus (PO_4) until the bloom occurred. Then both were depleted.

Tests for the physiological state of the cells were made on the organisms over the time period prior, during and after the bloom. Assimilation coefficients for carbon-fixation were 8.0 on September 8 and 3.5 on September 14 when the chlorophyll concentration was maximum. The cells were never found to be phosphate-deficient as measured by the alkaline phosphatase test (11). The cells were found to be nitrogen-deficient only during the peak of the bloom, i.e. September 14, and the bloom declined rapidly thereafter. Nitrogen deficiency was measured by the ammonium enhancement of dark-fixation of carbon dioxide (6).

Two additional enrichment sources were associated with patches of red tide around Cape Ann. These were 1) an area where dredge materials were being dumped, and 2) near a sewage outfall.

Field Observations: September 1974

On September 8 a red patch was noted some five miles off Monhegan Island; the organism was identified as *G. tamarensis*. Our research vessel the R. V. BIGELOW encountered a patch some 600 x 1500 meters in size oriented northwest-southeast three miles southwest of Pemaquid Point on September 9. The patch was sampled vertically and horizontally. The water temperatures within the patch were in excess of 15.5°C while to the seaward (the most discrete edge of the patch) the temperatures were one-half to one degree lower. Salinities were between 29.5 and 31.0 parts per thousand, with no apparent pattern. The water column was isothermal, suggesting that it was completely mixed. The patch contained *G. tamarensis* at 5.2 million cells per liter at the surface, with chlorophylls at 45 mg/m^3. Associated with the patch were the copepods *Calanus finnmarchicus* and *Pseudocalanus minutus* and small fish. Birds, especially Petrels were observed to be feeding in the patch. In addition

to a red appearance, the patch appeared as a slick and contained debris and foam. Chlorophyll contents in the waters surrounding the patch were quite normal, about 1.6 mg/m^3 and nutrient levels were normal for the late summer. Data from a drifting drogue revealed that the patch studied was moving in a southeasterly direction at speeds near one knot.

Physiological data show the cells were near-expended. Assimilation coefficients for carbon-fixation were below one. The cells were presumed to be nutrient-deficient (vs. nitrogen-deficient) in that the ammonium-enhancement tests were negative and the assimilation coefficients low. A summary of field observations appears in Table I.

Initiation of Gonyaulax tamarensis laboratory culture

Dr. C. Martin isolated cells of *G. tamarensis* from the waters of the Gulf of Maine in 1972 and succeeded in culturing these in the *Gonyaulax* medium of

Table I
Summary of Field Observations:

	1972	1974
peak dates	September 14	September 8, 9, 10
location of observations	Cape Ann, Mass.	Boothbay Harbor, Me.
general description	wide spread bloom superimposed with patches	patches only
environmental conditions	data from 9-14 dock	data from patch 9-9 Sta. 5 OM
water temperature (°C)	17.5	15.5
mixed water column	yes	yes
salinity (°/oo)	30.63	30.00
light	hazy; overcast	bright full sun
nutrients (µg At/l)		
NO_3	0.14	0.40
NO_2	0.15	0.09
NH_3	0.44	2.66
PO_4	0.24	0.70
Si	6.37	7.05
biological conditions		
cell count (cells per liter)		
G. tamarensis (50µ)	1.78×10^6	5.2×10^6
S. costatum (5µ)	1.87×10^6	8.2×10^4
chlorophyll (mg/m^3)		
in patch	60-100	45
outside patch	31.0	< 2.0
assimilation number	3.5	< 1.0
nitrogen-deficiency (D+/D-)	3.0 yes	1.0 no
phosphate-deficiency	no	-
maximum PSP (source, J. Hurst		
clams *Mya* (µg/100g tissue)	2,854 (Wells, 9-20)	1,733 (York, 9-3)
mussels *Mytilus*	10,092 (York, 9-21)	23,055 (Monhegan, 9-9)

Fogel and Hastings (3). These unialgal cultures were used to evaluate the principal factors favoring growth. Our standard growth conditions are: 15-18°C, light intensity of 10,000 lux and a 12/12 hour light/dark cycle. Growth is reasonably good following a rather long lag phase. The first doubling is some 6 to 8 days after inoculation which is in contrast to the common 1 to 3 day lag phase for other phytoplankton species in culture. Maximum growth is reached after 18 to 21 days, with chlorophyll levels around 150 to 300 mg/m^3 — notably not substantially in excess of the levels measured in nature during the outbreak situations. The growth rate is commonly one doubling per three days.

Photosynthesis/Light Relationships

The photosynthetic rate at different intensities was measured using neutral density filters which give intensities of 0, 400, 700, 2200, 4300, 5600, 7500, and 9500 lux. Photosynthetic carbon-14 fixation was measured over four-hour periods at these intensities. Although maximum carbon fixed varied from species to species, normalized photosynthesis/light intensity curves are shown in Figure 1, and it can be concluded that the photosynthetic response to light intensity by *G. tamarensis* is similar to other species of marine phytoplankton. To insure that this was no artifact of culturing, similar experiments were conducted using natural populations collected from a bloom. These finding agree with the culture experiments.

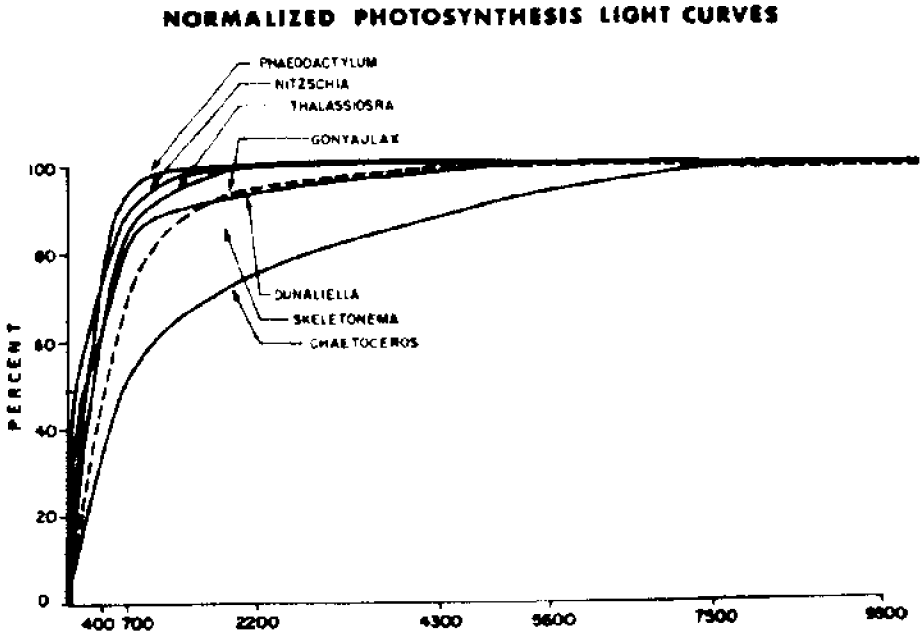

Figure 1: Normalized photosynthesis light (as lux) curves for various non-axenic cultures of marine phytoplankton species including *G. tamarensis* (interrupted line).

Salinity

Salinity preference was tested using *Gonyaulax* medium with a salinity range of 20 to 28 parts per thousand. Salinity was altered prior to enrichment. Near equal tolerance to these salinities was observed, with change between maximum and minimum growth over this range being about 20 percent. The tolerance is common to many other marine phytoplankton species.

Temperature

Cultures grown in *Gonyaulax* medium were held in water baths at approximately 0-5°, 10°, 15-20°, 19-24°C under 10,000 lux of constant illumination (figure 2). Below 5°C, cyst formation was observed, with no

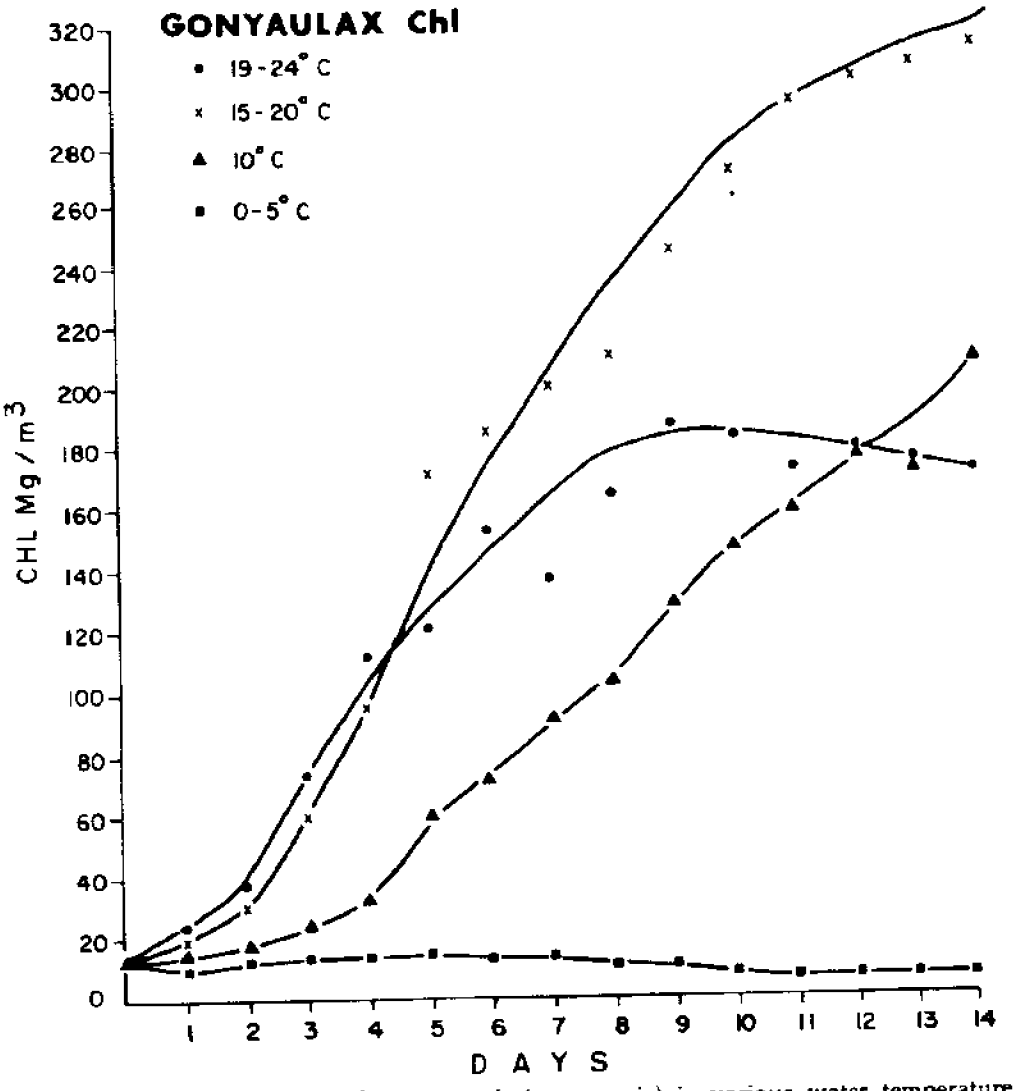

Figure 2: Growth curves for *G. tamarensis* (non-axenic) in various water temperature baths.

increase in chlorophyll over the 20-day growth period. Optimal growth was achieved at 15-20° with highest division rates being a doubling per 3 days. This optimal growth response at 15 to 20°C temperatures is common to many species and natural populations of phytoplankton (13).

Initial studies of nitrogen metabolism

The rate of removal of nitrate-nitrogen, nitrite-nitrogen and ammonium-nitrogen from the culture medium by *G. tamarensis* was monitored in unialgal cultures. Growth requirements of *G. tamarensis* could be satisfied by using nitrate-nitrogen as a source. If consumption rates are used as an index of nitrate reduction, then *G. tamarensis* appears to grow as well as diatoms on nitrate as the sole source. (1).

We are presently attempting to measure the affinity (half-saturation rate) of *G. tamarensis* for different forms of nitrogen.

Axenic cultures

The initial nitrate consumption data were very erratic suggesting interference by bacteria in these non-axenic cultures.

Axenic cultures were obtained as follows: Fifty ml. of *G. tamarensis* were inoculated into 500 ml. of the *Gonyaulax* medium. A 400 mg/1 penicillin and

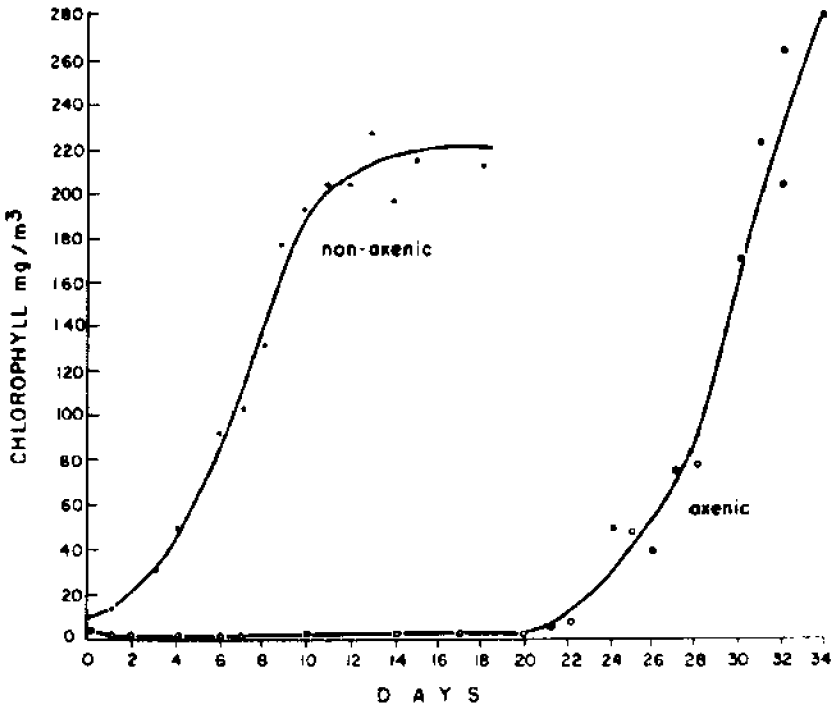

Figure 3: Comparison of growth curves of non-axenic and axenic cultures of *G. tamarensis*. Inoculum was not necessarily in log phase for either or both cultures. Of note is that the chlorophylls in the axenic cultures eventually are greater than in the non-axenic cultures.

200 mg/1 streptomycin sulfate mixture was added for a "contact time" of 6 hours. Then an inoculum of this "antibiotic" sample was returned to fresh *Gonyaulax* medium. Purity of axenic cultures was checked frequently in the same medium with 1 gram per liter of bacto-peptone. Positive contamination is indicated by increased turbidity in the test medium after 48-72 hours at 20°C. Of note is the extended lag phase with axenic cultures. (figure 3).

Role of micro-nutrients, definition of basal medium

The contents of the Fogel and Hastings *Gonyaulax* medium differs from most enriched seawater mixtures in the following ways: 1) a tremendously complex mixture of vitamins, 2) unusually high levels of chelators, EDTA and NTA and 3) the addition of phosphate only in the organic form of sodium glycerophosphate. (See Table II).

We tested the vitamin requirement of our unialgal cultures by set-ups of total *Gonyaulax* medium minus one each of the 16 vitamins; no vitamins; and total vitamins. Growth was monitored daily by chlorophylls. There was no significant difference in the growth of the variables. We transferred the no-vitamin and total-vitamin cultures for three months to counter the argument that the cells might have had enough vitamin reserve to grow for several generations before showing deficiency. Again we found no significant difference. Vitamin additions do not appear to be essential for the culturing of this isolate of *G. tamarensis*.

Our data are in contrast to the reports by Prakash and colleagues that vitamin B_{12}, thiamin, and biotin were essential for his isolates of *G. tamarensis* (8). Two experimental limitations of both our work and Prakash's should be mentioned. 1) Cultures were non-axenic and bacteria are known to produce vitamins essential for growth and 2) the level of vitamins in the filtered seawater used for the medium was not known.

Media commonly have chelators at a level of 10 mg/1 or less. The *Gonyaulax* medium has a final concentration of 41.5 mg/1 of EDTA and 80 mg/1 of NTA (nitrilotriacetic acid, $N(CH_2COOH)_3$). As is well known, NTA is a leading replacement candidate for phosphate in detergents, and is presently in use as such in Canada and some other countries. The summaries of the environmental acceptability of NTA (10) and (4) indicate that NTA can stimulate some algal species and not others. Doig and Martin (2) show no biostimulation by additions of NTA to *Gymnodinium breve*, the red tide organism of the Florida coast. Our observations with *G. tamarensis* however show definite biostimulation.

For these studies, we used both measurement of growth by chlorophyll and measurement of photosynthesis by carbon-14. Experiments were performed under standard growth condition but using the media revisions as noted.

In tests using cultures of *G. tamarensis*, growth can be achieved by inoculating the organism into filtered seawater with no enrichment. Increased growth is achieved with additions of low levels of NTA (10 ppm) while levels

Table II

Gonyaulax Medium (Fogel & Hastings, 1971)

Final Preparation (contents per liter)

750.0	ml filtered seawater
2.31	ml NTA mix (solution A)
6.0	ml PII metal mix (solution B)
0.1	ml vitamin mix (solution C)
243.0	ml deionized/distilled water

← concentrations per liter in final preparation →

solution A: NTA mix

80.15 mg	NTA
231.29 mg	KNO_3
70.07 mg	Na_2 glycerophosphate
11.45 mg	EDTA
3.32 mg	$FeCl_3 \cdot H_2O$

(2N NaOH added to about pH 7.8 to dissolve NTA)

solution B: PII metal mix

30.0	mg EDTA
34.2	mg H_3BO_3
1.44	mg $FeCl_3 \cdot 6H_2O$
4.92	mg $MnSO_4 \cdot H_2O$
0.66	mg $ZnSO_4 \cdot 7H_2O$
0.014	mg $CoSO_4 \cdot H_2O$

solution C: Vitamin mix

.060	mg thiamine · HCl
.010	mg pyridoxamine 2 HCl
.004	mg riboflavin
.030	mg Ca panthothenate
.040	mg nicotinic acid
.0025	mg folic acid
.0001	mg biotin
.00001	mg B_{12}
.0002	mg citrovorum facto leucovorin Ca · $5H_2O$ (Lederle)
.004	mg putrescine · 2HCl
.001	mg para-amino-benzoic acid
.1	mg choline
.1	mg inositol
.1	mg thymine
.03	mg orotic acid
.033	mg protogen (DL thiotic or DL lipoic)

of 50 ppm and above were found to be inhibitory to growth when added to filtered seawater. In Figure 4, the open bars represent the chlorophyll levels initially, and the stippled bars represent chlorophylls after a period of 14 days. Figure 5 compares various other natural phytoplankton with *G. tamarensis*. Note that no additional nitrogen or phosphorus was added to any of the above.

The apparent contradiction here of our various growth experiments where in one, 50 ppm is toxic and yet in the *Gonyaulax* medium there is 80 ppm NTA and good growth is achieved, is likely due to the situation when NTA is functioning as as chelator. The amount of "added" chelator is misleading. It would be more reasonable that the biologically significant parameter would be: a) the amount of chelator metal complex; b) the number of equivalents of chelator in excess of complexed chelator metal; or c) the number of equivalents

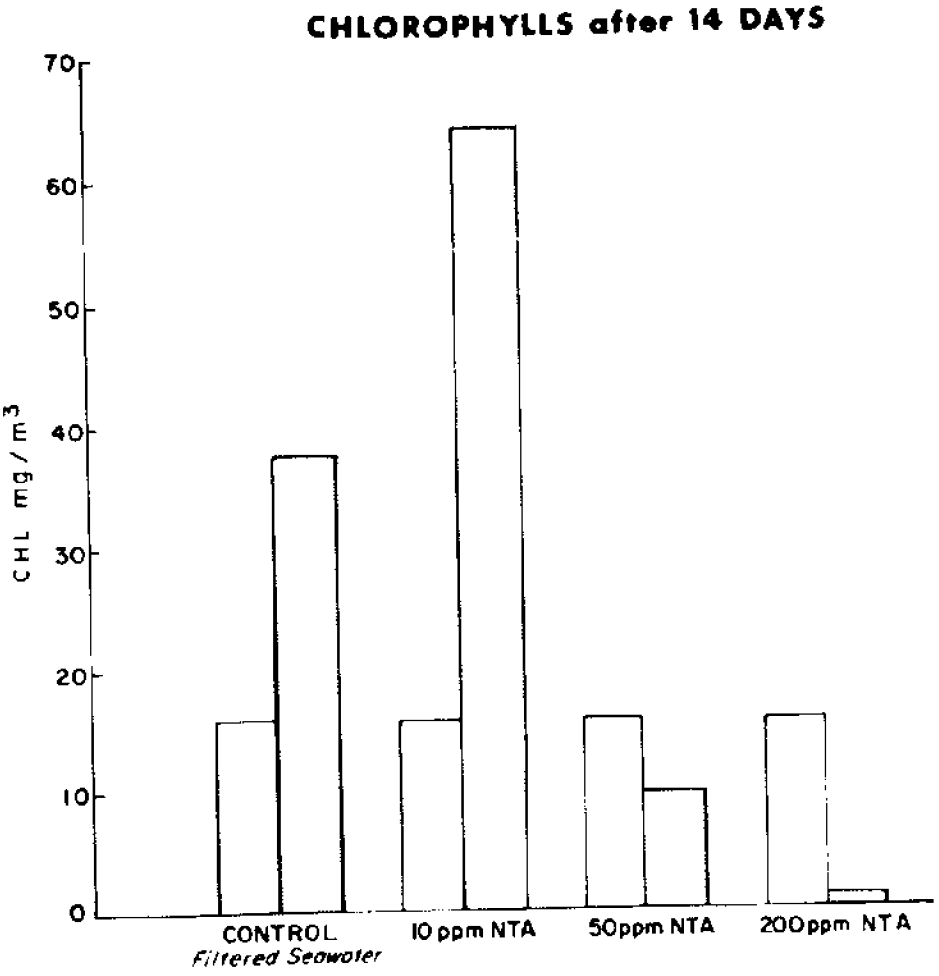

Figure 4: Chlorophylls after 14 days of growth when non-axenic *G. tamarensis* is inoculated into filtered seawater (control) as compared to filtered seawater plus various concentrations of NTA. This suggests that a reasonable medium could be merely filtered seawater plus 10 ppm NTA.

of metal in excess of complexed chelator metal. "Added" levels also do not reflect the rapid photodegradation of NTA Fe^{+3} to IDA Fe^{+3} (Richard Stolzberg, personal communication) (12).

We have also observed stimulation of photosynthesis by added NTA. Between 1 to 200 ppm NTA and EDTA were added to unialgal cultures grown in *Gonyaulax* medium. Cultures were incubated with sodium bicarbonate carbon-14 for four hours. Enhancement of the photosynthetic rate are shown in Table III. It should be emphasized that we have found considerable variability in the effectiveness of identical "added" amounts of NTA to dissimilar cultures of *G. tamarensis*, likely reflecting the remaining metal content of the medium and the physiological state of the cells. We did not get stimulation of photosynthesis with any other species in culture, nor with natural populations where *Skeletonema costatum* was the dominant species.

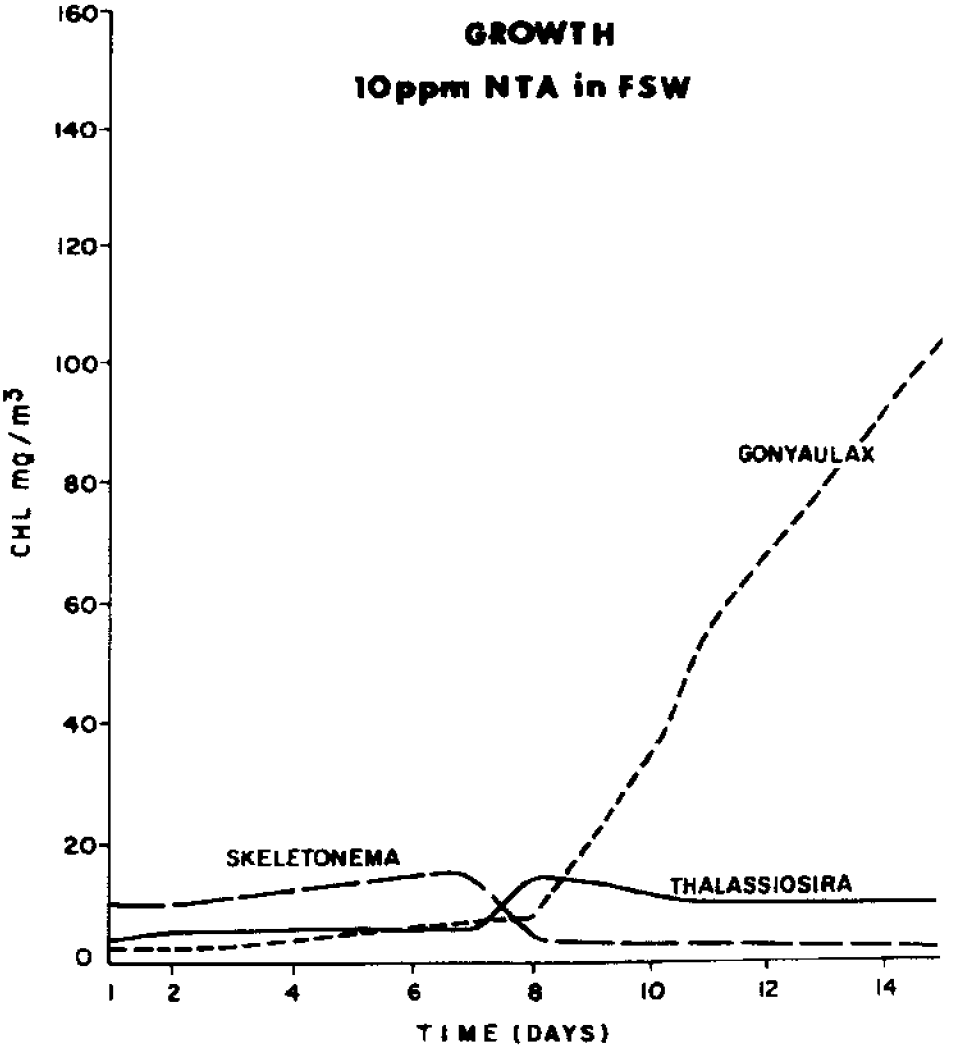

Figure 5: Growth of *G. tamarensis* and lack of growth of *Thalassiosira* sp. and *Skeletonema costatum* in medium of merely filtered seawater plus 10 ppm NTA.

Table III

Stimulation of Carbon Fixation

Gonyaulax tamarensis - 22 day old cultures - 10,000 lux illumination

Additives during incubation	average cpm	average cpm control (no additives)	percent increase over control
10 ppm NTA	80,891	75,175	7
40 ppm NTA	112,977	75,174	50
200 ppm NTA	247,513	75,174	229

We then incubated *G. tamarensis* with trace levels of NTA-C^{14} which was randomly labelled. The NTA-C^{14} was taken up by non-axenic cultures of *G. tamarensis* at similar rates in both the light and the dark. We did not find uptake in either light or dark with other species, also non-axenic. This led us to suspect substrate assimilation (heterotrophy), which is not uncommon with dinoflagellates. We have measured similar uptake rates in light and dark with trace levels of radioactive NTA, EDTA, glucose, and glycollic acid, all as C^{14} (Table IV). Fractionation of fixed NTA-C^{14} resulted in recognizable assimilation into the amino acids, simple sugars, and fatty acid fraction; the protein fraction and the polysaccharide fraction.

Attempts to grow axenic cultures of *G. tamarensis* in the dark on the sole substrates of glucose, NTA, IDA, glycine, EDTA, glycollic acid and acetate were fruitless.

More pertinent to the environment is the testing of the naturally abundant chelators such as humic substances, amino acids and algal excretion products, also shown to enhance growth at low levels (5).

The influence of daylength on growth

By altering our standard growth conditions in another way, we have been comparing growth rates (as assessed by chlorophyll) of several common marine phytoplankton species subjected to 8/16, 10/14, 12/12, 14/10 and 16/8 hours of light/dark cycles. By way of comparison, diatoms such as *Phaeodactylum tricornutum* and *Skeletonema costatum*, the dominant species of the coastal waters in the Gulf of Maine were used. Both *Phaeodactylum* and *Skeletonema* have environmental requirements, i.e. temperature, salinity, similar to *G. tamarensis* however the reaction of *Phaeodactylum* to daylength is opposite (Figure 6). Growth rate of *Skeletonema* as *Phaeodactylum* shown in Figure 7 increases when the number of daylight hours is reduced. Growth rate of *G. tamarensis* on the other hand hastens when the number of daylight hours is increased. In the experiments we performed, the growth rate of *G. tamarensis* supercedes the growth rate of *Phaeodactylum* at approximately a

Table IV

Carbon 14 assimilated by laboratory cultures
(cpm minus cpm of zero time)

as sodium bicarbonate - 10 μCi/ml for 4 hours

Gonyaulax tamarensis

Light - 57,601
Dark- 1,426

Skeletonema costatum

Light - 165,389
Dark - 4,519

as NTA (carbon-14 randomly distributed on acetate groups)
8.3 μCi/ml for 4 hours

Gonyaulax tamarensis

Light - 46,508
Dark - 40,920

Skeletonema costatum

Light - 0
Dark - 0

as glucose - .63 μCi/ml for 2 hours

Gonyaulax tamarensis

Light - 25,847
Dark - 29,819

as glycollic acid - .5 μCi/ml for 2 hours

Gonyaulax tamarensis

Light - 37,138
Dark - 37,126

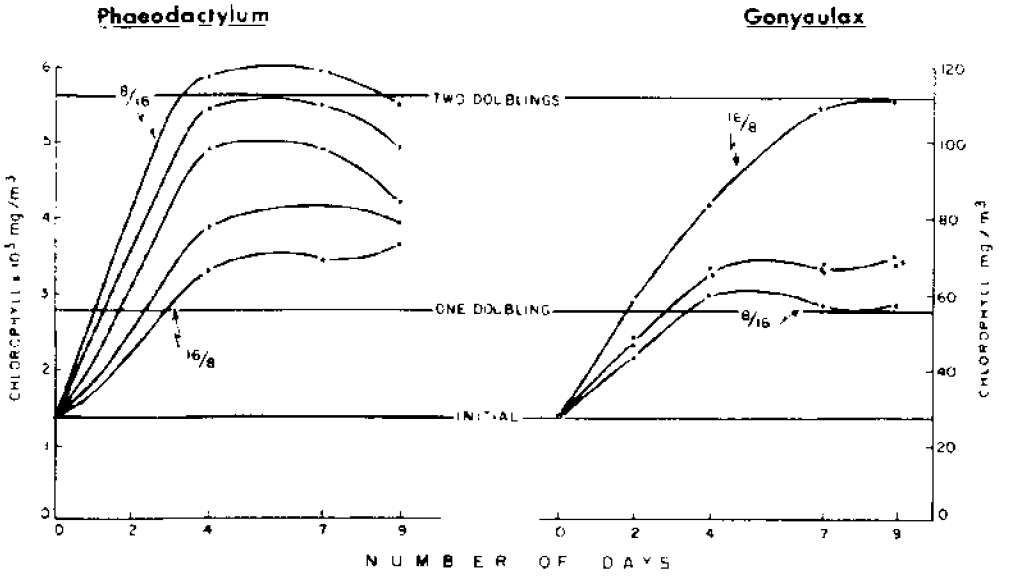

Figure 6: Growth response of *Phaeodactylum tricornutum* and *G. tamarensis* when subjected to various lengths of day i.e. 8/16, 10/14, 12/12, 14/10 and 16/8 light/dark cycles. Growth response is opposite.

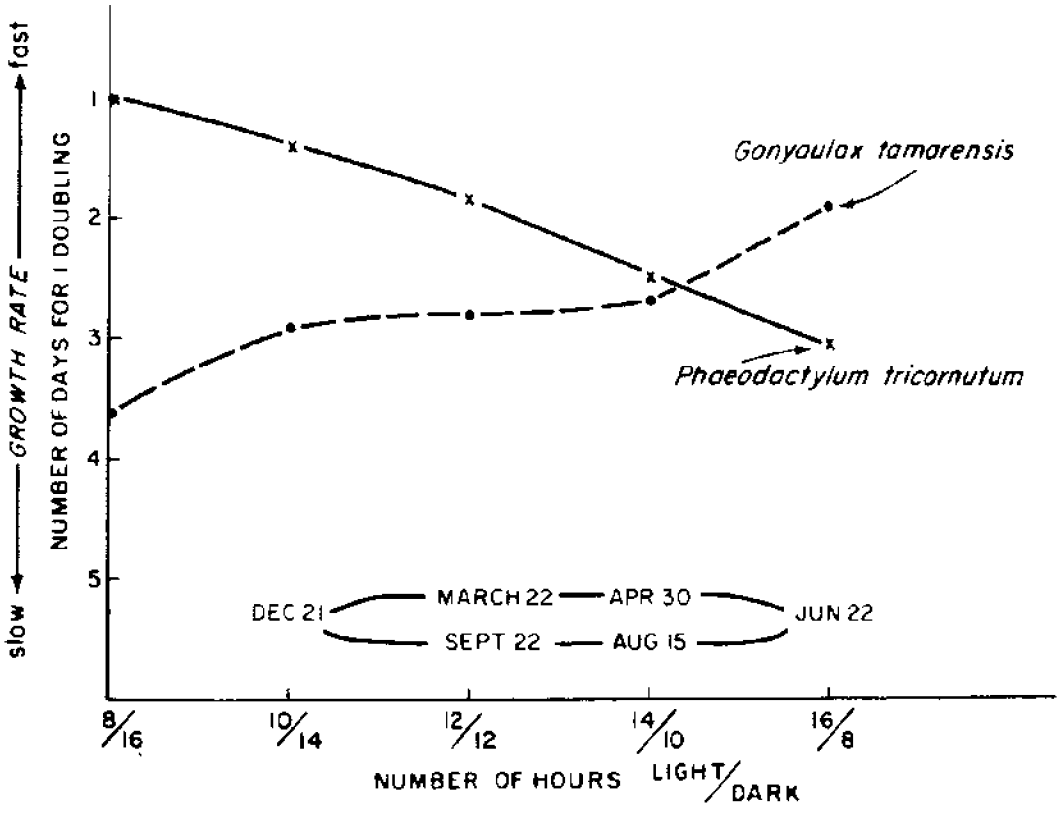

Figure 7: Comparison of growth rate of *Phaeodactylum tricornutum* and *G. tamarensis* as calculated from data of figure 6.

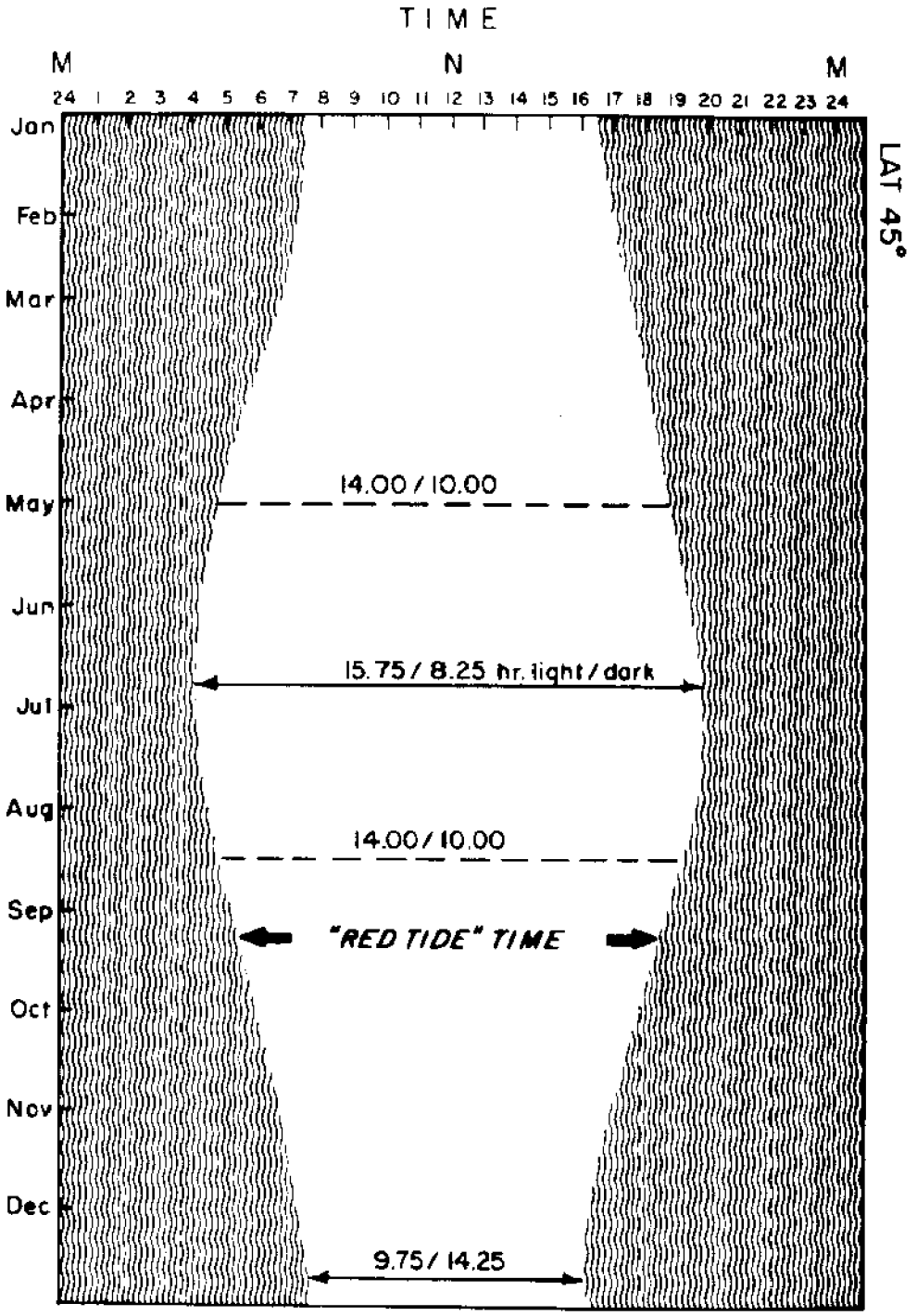

Figure 8: Yearly daylength variations at latitude 45° indicating that 14/10 light/dark or greater light occurs from April 30 to August 15. Based on the limited experimental data of figure 6, *G. tamarensis* could theoretically outcompete a diatom such as *Phaeodactylum tricornutum* during that period of time.

14/10 light/dark regime. At latitude 45° such would be the case from approximately April 30 through August 15 (Figure 8).

One obvious limitation of this experimental approach is that optimum growth conditions yielding optimum growth rates is essential for calculations and predictions of this type. In this case favorable conditions, however not necessarily optimum, were employed.

Implications of studies of environmental physiology to date

The observation of a single species bloom of phytoplankton characteristically poses the question as to why one species alone is present when "normal" populations are generally composed of many species.

It is tempting to rationalize in terms of the degree of enrichment for growth — where oligotrophic conditions generally promote many species as opposed to few species in eutrophic environments. Probably the most classic example of single species of dominance due to nutrient enrichment is that in Great South Bay (11). Here one species, *Nannochloris* sp. overgrew all others as the result of nitrogen enrichment in the form of urea derived from duck wastes washed into the bay. The "normal" members of the phytoplankton community apparently could not use this nitrogen source for growth.

In our research, we hope to isolate some factors analogous to the Great South Bay situation and nitrogen metabolism of the organism may be the important area. However, much of our research at the moment is in progress.

Some factors we have found which singly can promote dominance by *G. tamarensis* are: 1) the dependence of this organism on a chelator such as NTA, 2) the possibility of active heterotrophy and 3) the growth response to daylength. It is interesting to note that our experiments demonstrate that favorable situations for the above factors do not promote "wildfire growth" or anything close to it. What these factors do is to *maintain a tolerable division rate* for this organism. This maintenance when placed against a background of declining or poor growth for other species works to favor dominance. This coupled wtih a physical means for concentration, i.e. convergences, etc. can account for the accumulation of large numbers of this organism.

ACKNOWLEDGEMENTS

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DINOFLAGELLATE ACCUMULATIONS IN CHESAPEAKE BAY

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ABSTRACT

In the tributary estuaries of the Chesapeake Bay and in the central bay proper between 80-90 percent of the phytoplankton primary productivity and production is carried out by nannoplankton less than 10 microns in linear dimension. Stabilities of the water column and turbidities are such that the well-mixed surface waters encompass the entire euphotic zone. Superimposed on this background which averages 10-20 micrograms per liter of extractable chlorophyll *a* is a seasonal procession of the larger, strongly phototactic dinoflagellates *Prorocentrum minimum, Gymnodinium nelsoni, G. splendens* and *Katodinium rotundatum* in the upper bay and in addition *Ceratium furca* in the lower bay. These dinoflagellates increase in concentration subsequent to an initial cycle of nannoplankton production and rotifer and tintinnid predation on the nannoplankton. There appears to be a correlation between the conversion of dissolved inorganic nitrogen and phosphorus into organic forms and the appearance of these species of dinoflagellates. The strong positive phototaxis (which can overcome mixing forces) of these dinoflagellates gives rise to disproportionate surface and near-surface concentrations in the water column. This upward vector, also periodic with a diel frequency, can occasionally phase with the two layer tidal exchange in the estuary and produce a reduced down-bay horizontal displacement for the dinoflagellates relative to the nannoplankton. This reduction in flushing rate is in effect an accumulation mechanism. It operates in conjunction with the windrow accumulation mechanism and the mechanism of differential horizontal movement of water masses of different densities which have been shown to be operative in tropical bioluminescent bays where the dinoflagellate *Pyrodinium bahamense* is the dominant phytoplankter. These accumulation mechanisms result in elongated surface patches ranging in area from tens of hectares to many hundreds of hectares. These surface patches have been observed to contain chlorophyll *a* concentrations higher than 1000 micrograms per liter. The strong relative absorption of blue light by accessory pigments and chlorophyll gives a brownish-red visible color to these patches. High bacterial concentrations have been associated with many of these patches. Since the bacteria possess no obvious accumulation mechanism it may be assumed that they are stimulated to rapid growth within the dense dinoflagellate patches. It is possible therefore that fish kills in the Chesapeake Bay, which have been coincident with "mahogany tides", are initiated by the bacteria within the patches. The accumulation of a major biomass of floating dead fish within the same windrows as the dinoflagellates produces an "explosion" in bacterial population, an auto-catastrophic event.

INTRODUCTION

A water mass may, by virtue of land run-off following a period of heavy rainfall (23) or by virtue of coastal upwelling (1), be provided with sufficient nutrients to support high growth rates of phytoplankton and their immediate

herbivores. This turnover of inorganic nitrogen and phosphorus into organic nutrients appears to stimulate the growth of the larger, strongly positively phototactic dinoflagellates. These dinoflagellates, under proper conditions of insolation and wind vectors (18), form dense, strongly light-absorbing, discontinuous but sometimes extensive surface water patches which are called blooms or red or brown or yellow tides, depending on the absorption by the accessory photosynthetic pigments of the particular dinoflagellate species.

Most dinoflagellate species exhibit positive phototaxis and can migrate at speeds *ca.* 1 m hr^{-1} (8, 5). Some of these, *Gonyaulax polyedra, Prorocentrum micans, Ceratium furca, Cachonina niei,* (ibid.), show a marked diurnal migration, actively swimming downward at night as deep as 15 m. Others, *Gymnodinium breve, Noctiluca miliaris* (18), remain at the surface during the night.

The combinations of positive phototaxis and
a) prevailing onshore winds (windrows),
b) convergence lines of water masses of different densities,
c) wind-driven convection cells (Langmuir circulation) leading to surface patches of organisms have been described by Ryther (18).

Pomeroy *et al.* (17) have invoked weak convergences between wind-driven convection cells to account for *Amphidinium fusiforme* and *Gymnodinium splendens* concentrations in Delaware Bay (1952-1954), and onshore winds for *Gymnodinium sp.* concentrations at Sapelo Island, Georgia. Conover (4) has proposed southerly sea breezes which reduced the water exchange in New Haven Harbor to permit increased standing crops of *Gonyaulax africana* (?). Holmes *et al.* (9) have proposed a diurnal migration of *Gymnodinium spp., Cochlodinium spp., Prorocentrum micans* and *Gonyaulax poleydra* in La Jolla Bay in order to account for nutrient utilization within the entire euphotic zone. This was later demonstrated more directly by Eppley (5) and put forward as a competitive advantage for dinoflagellates over non-motile species.

In the case of *Pyrodinium bahamense* in Bahía Fosforescente, Puerto Rico (22), the prevailing easterly winds reduce the effective exchange rate of the positively phototactic dinoflagellates. The surrounding mangrove swamps apparently furnish a specific organic nutrient requirement. In Oyster Bay, Jamaica, West Indies, there is a unique case of positive phototaxis and differential motion by wind-driven water layers of different salinities (Seliger *et al.*, (21)) which accounts for the accumulations of surface patches of *P. bahamense* up to 10^7 per liter (700 μg chl *a* per liter).

In the present paper we describe the seasonal and spatial processions of dinoflagellate accumulations in the Chesapeake Bay and propose a number of accumulation and lateral migration mechanisms for the major dinoflagellates *Prorocentrum minimum, Gymnodinium nelsoni, Katodinium rotundatum* and *Ceratium furca*. The relationship between bacterial growth and dinoflagellate growth (Collier, (3)) is extended and a specific train of events is proposed to explain massive fish kills triggered by non-toxic dinoflagellates.

EXPERIMENTAL

The application of the *in vivo* fluorescence technique (Lorenzen, (14) Flemer, (7)) to large scale areal surveys has been described in detail (Loftus *et al.* (13); Loftus and Seliger, (10). Salinity measurements, nutrient analysis, chlorophyll assays, and primary productivity measurements are referenced in Seliger and Loftus (20). Bacterial counts were made using Millipore Type total bacteria plates.

There is some uncertainty in the species identification of some of the smaller dinoflagellates. We believe that the *Prorocentrum sp.* identified in the Rhode River in 1970 and 1971 as *triangulatum* is really *minimum*; the *Amphidinium sp.* observed in 1970 and 1971 is really *Katodinium rotundatum.*

A major portion of our phytoplankton ecology program has been centered in the northern Chesapeake Bay at the Rhode River, a small tributary estuary on the western Chesapeake Bay 5 miles south of Annapolis on the Severn River. A major concern of our research program has been to study the natural phytoplankton community in a subestuary. We assume that the quantitative relationships among nutrients and nutrient turnover, salinity, temperature, turbidity, species selection and succession, predation and exchange with the bay can be determined. From these quantitative relationships it follows that specific parameters will emerge which can serve as diagnostic indicators of the physiological state and of the previous history and permit the prognosis of the stability of the phytoplankton community. These relationships should permit the prediction of the direction of changes in the community in response to proposed nutrient, sediment or heat loading.

The Rhode River is a special case of an estuary with two-layer flow; strong vertical mixing (Bowden, (2)) due to tidal currents and wind gives rise to vertical and lateral homogeneity. The vertical salinity profiles show monotonic increases from top to close-to-bottom of *ca.* 0.1 to 0.3%, with increases in the bottom 0.5 - 1 m of 0.5 to 1%.

Because of the small volume of the tidal section of Rhode River, rainfall produces relatively large excursions in the upper limit of the estuary. There is a negligible "river section" associated with the Rhode River. The land runoff into Rhode River consists of drainage directly into the tidal section of the sub-estuary. Winds play an important part in maintaining the well-mixed essentially isohaline character of this shallow subestuary. Under proper conditions, a strong northwest wind will rapidly exchange the estuary section of the Rhode River and its plankton populations with the bay.

The delivery of nutrients to the estuary sections of the Rhode River is the result of tidal action (flushing of the marshes, remixing of soluble nutrients from interstitial water, and resuspension of interstitial sediments) and exchange with the Chesapeake Bay across the mouth of the river. However, subsequent to heavy rains the transition zone is subject to major changes in phytoplankton relative species compositions, coinciding with large increases in standing crops of chlorophyll *a*.

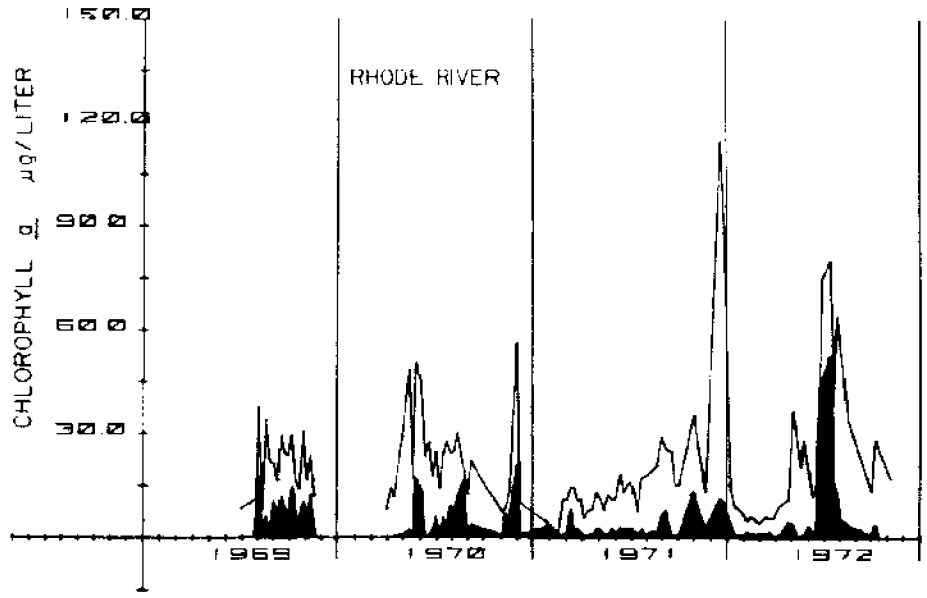


Figure 1a and 1b: Standing crops of total chlorophyll *a* (unshaded) and the chlorophyll *a* contained in a size fraction greater than 20 µ (darkened areas) for the time period 1969-1972 for a) Rhode River, and b) Chesapeake Bay. The blank area enclosed between the solid lines and the darkened areas represents the nannoplankton.

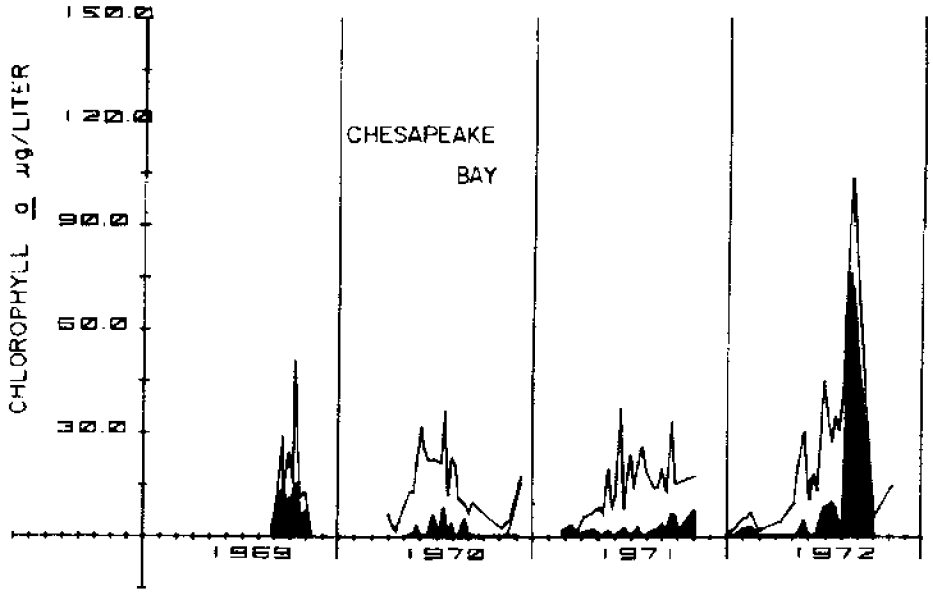
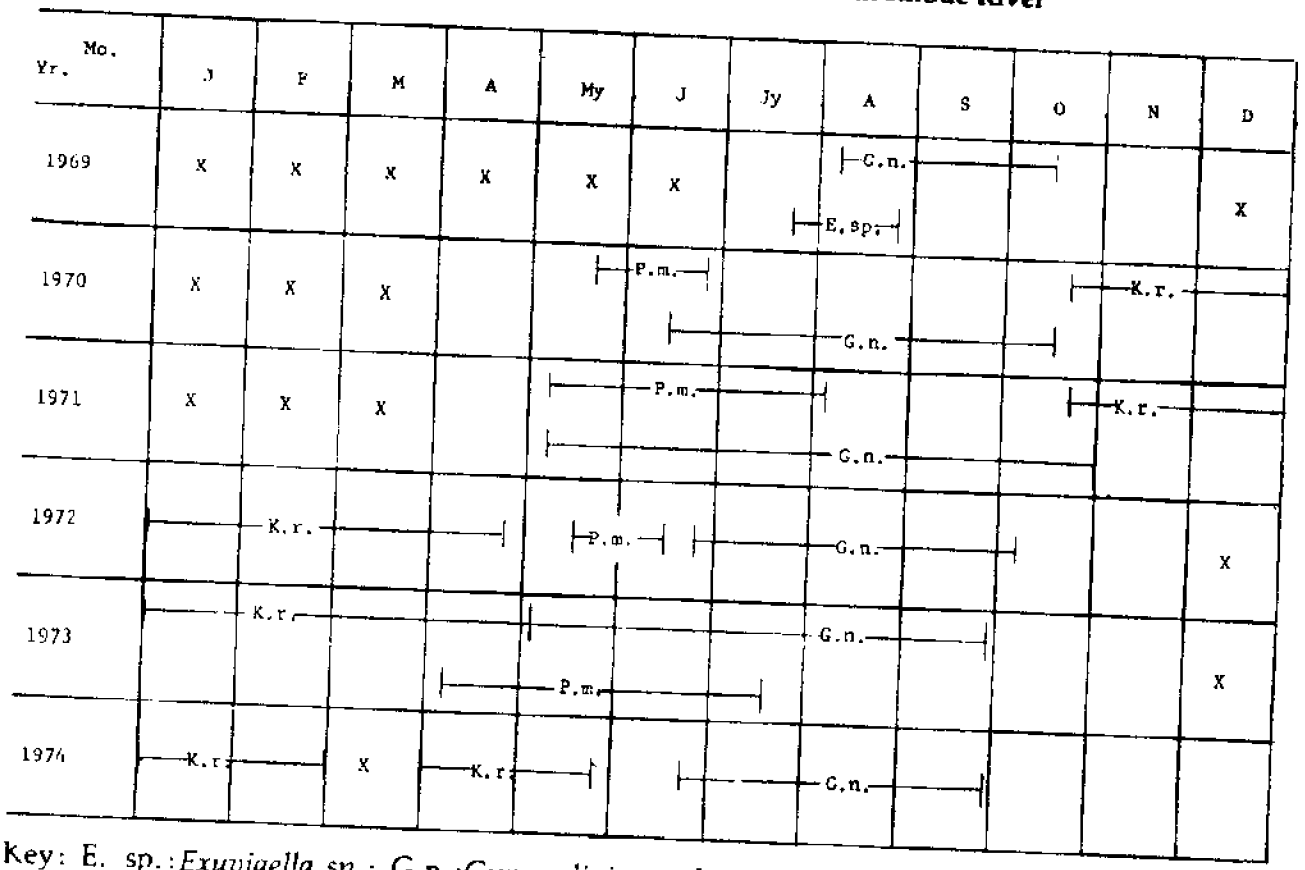


Table 1 **Dinoflagellate Procession in Rhode River**



Key: E. sp.: *Exuviaella sp.*; G.n.: *Gymnodinium nelsoni*; K.r.: *Katodinium rotundatum* P.m.: *Prorocentrum minimum*. Months during which no observations were made are marked X.

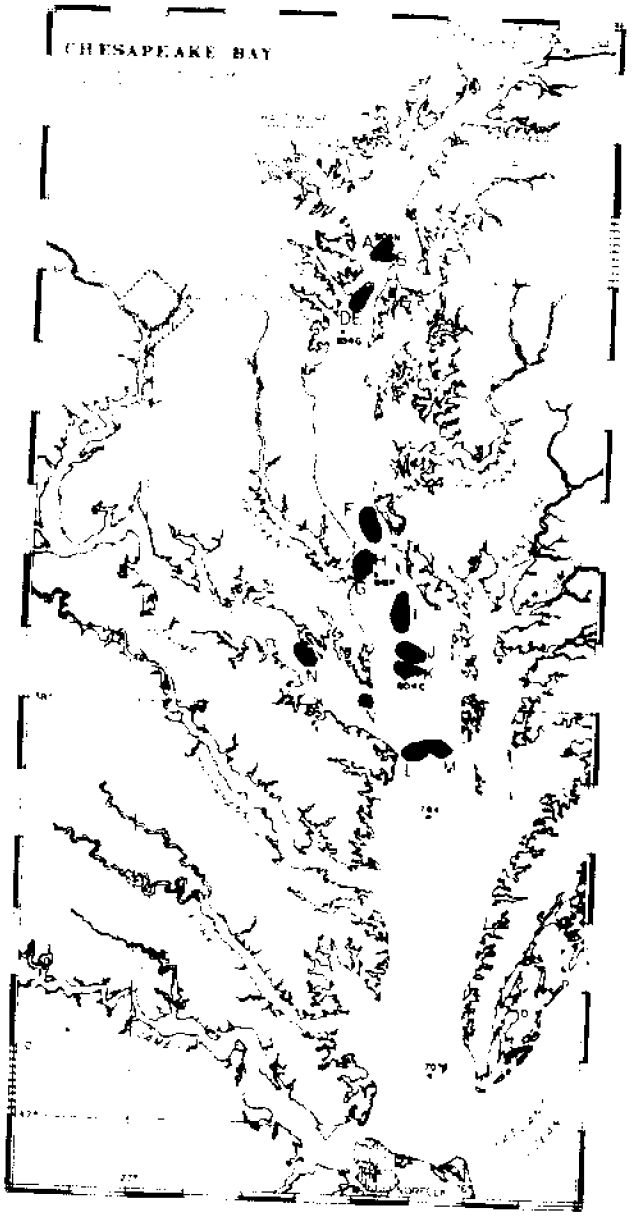


Figure 2: Chart of the Chesapeake Bay showing approximate locations and areal extents of surface water dinoflagellate concentrations exceeding 50 micrograms per liter of extractable chlorophyll *a*. These data represent our own direct observations and are therefore representative but not a complete summary. Key: A: *G. nelsoni*, August 1973; B: *G. nelsoni*, August 1974; C: *G. nelsoni*, August, 1973; D: *P. minimum*, June 1970; E: *P. minimum*, June 1971; F: mixed small dinoflagellates, August 1973; G: mixed small dinoflagellates, August 1973; H. mixed small dinoflagellates, August 1974; I: *C. furca*, August 1974; J: *C. furca*, April 1972; K: *C. furca*, August 1973; L: *C. furca*, August 1973; M: *C. furca*, October 1973; N: *G. nelsoni*, July 1972.

RESULTS

The mean standing crops of total chlorophyll *a* in the Rhode River due west of Station 854G in Fig. 2 and the adjacent Chesapeake Bay for the period 1969 through 1972 are shown by the solid lines in Fig. 1a) and b). In these graphs the solid lines have been drawn through the actual weekly data points. Each point represents an average value of total chlorophyll *a* liter^{-1} for a complete transect. Several points can be made:

1) except for a trend toward high standing crops in summer and low standing crops in winter, there existed no apparent reproducibility of standing crops of phytoplankton from one year to the next.

2) Despite the averaging involved in the individual sampling transects the weekly measurements of standing crops showed large oscillations.

3) The solid black portions in the figures show the contributions to the total chlorophyll *a* pigments in the surface waters, of dinoflagellates greater than 20 μ in cross sectional linear dimension. As can be seen, the major contribution to chlorophyll pigments is due to phytoplankton which pass through a 20 μ mesh net (Seliger, (19) ; Loftus *et al.*, (13); McCarthy *et al.*, (16)).

Superimposed on the nannoplankton populations in the Rhode River is a seasonal procession of the larger, positively phototactic dinoflagellates. This temporal sequence is shown in Table 1 for the 6 year period from 1969 through 1974, for *G. nelsoni*, *Katodinium rotundatum*, and *P. minimum*.

The locations and approximate areal extents in the Chesapeake Bay of surface water dinoflagellate concentrations exceeding 50 micrograms per liter of extractable chlorophyll *a* are shown in Fig. 2. The measurements reported include those made on a series of Procon cruises of the Chesapeake Bay Institute during 1972-1974. *P. minimum* and *G. nelsoni* were present in the central bay as well as the subestuary, while *C. furca* was present only in the high salinity waters of the southern bay. *K. rotundatum* in not shown in Fig. 2; we have only recently observed it in the southern bay.

During June, 1970, brown-colored surface patches of *P. minimum* were observed initially (June 9, 1970) north of Station 854G (see Fig. 2) and within the week extended into the South, Rhode and West Rivers with surface water concentrations greater than 50 μg L^{-1}. In subsequent surveys carried out between 17-25 June 1970 extensive brownish-red patches were visible mainly in the area between the mouth of the South River and Station 854G. On 25 June 1970 the dense visible patches were only of the order of tens of meters long and up to 10 meters wide. The time sequences of *Prorocentrum* concentrations and extractable chlorophyll *a* for 1970 and 1971 are given in Table 2.

On 17, 19 and 25 June 1970 surface concentrations (*ca.* 10 cm depth) were compared with concentrations averaged between the surface and the depth of disappearance of a Secchi disc in order to demonstrate the positive phototaxis exhibited by *Prorocentrum* in the dense patches. The ratios of surface : average concentration for these three days were 1.6, 7 and 2, respectively, indicating a variable but significant phototactic accumulation.

Table 2

Surface Concentrations of *P. minimum* During 1970, 1971

Date	Day No.	Location	10^6 Cells/l	chl *a* µg/l
		1970		
9 June	160	Bay Transect[a]	4	30-40
17 June	168	" "	4-7	42
19 June	170	" "	7-48	200
22 June	173	" "	1.5	22
25 June	176		1	19
		1971		
2 June	153	Bay Transect	4	
9 June	160	" "	11.2	20
11 June	162	" "	67	37
11 June	162	West River	27	13
11 June	162	Rhode River	43	17
16 June	167	Bay Transect	13	
16 June	167	Rhode River	13	84
18 June	169	Bay Transect	3.3	8
18 June	169	N. of Bay Bridge	1	
18 June	169	S. of Bay Bridge	101	
18 June	169	1.5 Km S. of Bridge	116	
18 June	169	Opposite Severn R.	62	
18 June	169	Station 854G	3.3	
30 June	181	Bay Transect	1	

[a] Mouth of South River to Station 854G.

Prorocentrum concentrations were low just north of the Bay Bridge (*ca.* 39° in Fig. 2) and were high just south of the Bay Bridge, the change by almost an order of magnitude occurring within 20-30 meters. This very sharp north-south discontinuity appeared to be specific for *Prorocentrum* and was not observed for other accumulations in this area (Loftus *et al.* (13); see Fig. 2).

Table 3 shows the results of productivity measurements made on surface water captured samples of *P. minimum* incubated *in situ* and irradiated at light saturation in a Lucite incubation tank. Surface water samples of *P. minimum* were also isolated and brought into unialgal culture. The results of productivity measurements with log phase laboratory cultures are also shown

Table 3

Date (Day of Year)	Winkler Technique O_2 *in situ*		Carbon Uptake $H^{14}CO_3^-$
	Z	P/R	Z
	μgC/μg chl *a*-hr		μgC/μg chl *a*-hr
1970			
17 June (168)	5	7	
19 June (170)	4	4	
22 June (173)	12	4	
25 June (175)	4	2	
1971			
9 June (160)	10[a]	6	7[b]
11 June (162)			12
11 June (162)			16
16 June (167)	8[c]	7	5
18 June (169)			10

	LIGHT SATURATION	
	Z	P/R
	μgC/μg chl *a*-hr	
1970		
17 June (168)	12	10
19 June (170)	4	5
22 June (173)	13	4
25 June (175)	13	6

	UNIALGAL CULTURE [d]		800 Ft. c.
	Z	P/R	Z
	μgC/μg chl *a*-hr		μgC/μg chl *a*-hr
	4	6	3

[a] Mean of 5 replicates
[b] Mean of 6 replicates
[c] Extrapolated value at zero time of incubation
[d] 10^8 cells L^{-1}; 586 μg L^{-1} chl *a*

Z = Assimilation Ratio $\mu gC/\mu g\ chl_a\text{-}hr$
P/R = Photosynthesis to Respiration Ratio

in Table 3. Assimilation ratios and P/R (gross photosynthesis to respiration) ratios were not significantly different for all cases.

This apparent annual appearance of *Prorocentrum* patches during June did not occur in 1972 and 1974. However in 1973, subsequent to the 1972 Tropical Storm Agnes, high concentrations of *P. minimum* were delivered to the Rhode River as early as May.

There appears to be a relation between the appearance of high concentrations of dissolved organic phosphate (DOP) and the appearance of *P. minimum* accumulations. These DOP levels disappeared at the temporal peak of the accumulation (19). The high DOP prior to the *Prorocentrum* accumulation could have originated from zooplankton excretion (15). The decrease during the accumulation was then due to assimilation of DOP by *Prorocentrum*. Similar observations were made by Martin (15) on *P. redfieldii* in Narragansett Bay.

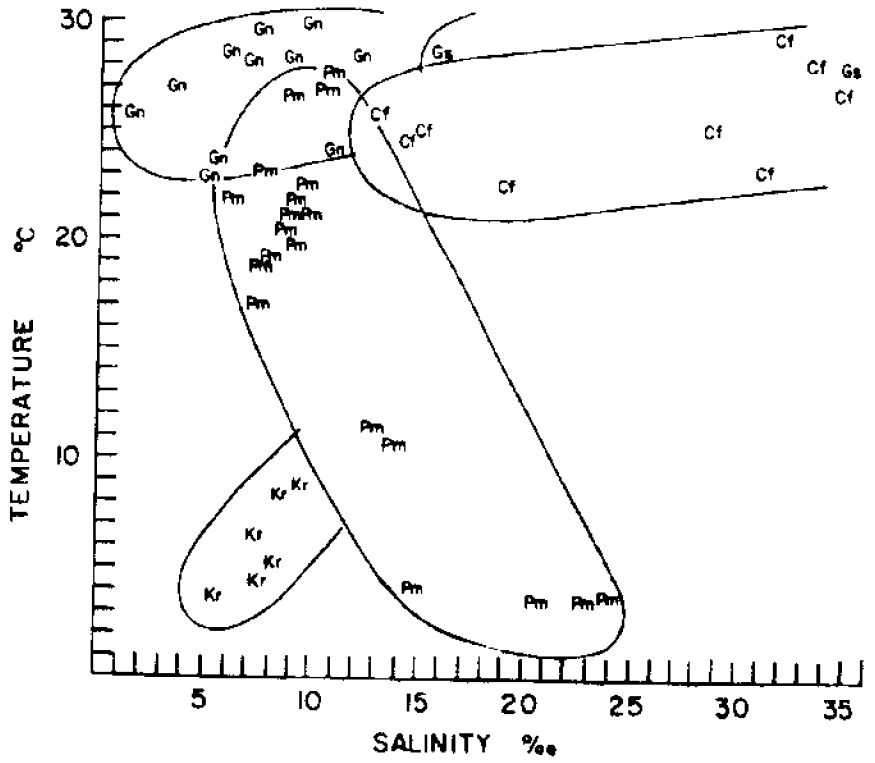


Figure 3: Temperature-Salinity profile through the multidimensional niche surface for the larger dinoflagellates of the Chesapeake Bay. The abbreviations for the dinoflagellate names are entered at the specific temperature-salinity coordinates observed during our surveys in 1970-1974. The entries for salinities greater than 25 °/oo are based on observations in Bahía Fosforescente, a bioluminescent bay on the southwest coast of Puerto Rico. Key: G_n, *Gymnodinium nelsoni*; G_s, *Gymnodinium splendens*; P_m, *Prorocentrum minimum*; C_f, *Ceratium furca*; K_r, *Katodinium rotundatum*.

Aside from specific nutrient requirements or predation pressure, temperature and salinity are major factors in the selection of phytoplankton species. Figure 3 represents a summary of our observations of temperature and salinity associated with dinoflagellate concentrations $> 50\ \mu g\ L^{-1}$ in the Chesapeake Bay over the past 5 years. For the sake of completeness coordinates for *C. furca* and *G. splendens* for salinities greater than 25% for Puerto Rico are also included. The two-dimensional partial profile of the T-S niche characteristics is consistent with the seasonal procession shown in Table 1.

We have found strong circumstantial evidence that the *Prorocentrum* appearing in the upper bay (north of Station 854G) are delivered by bottom

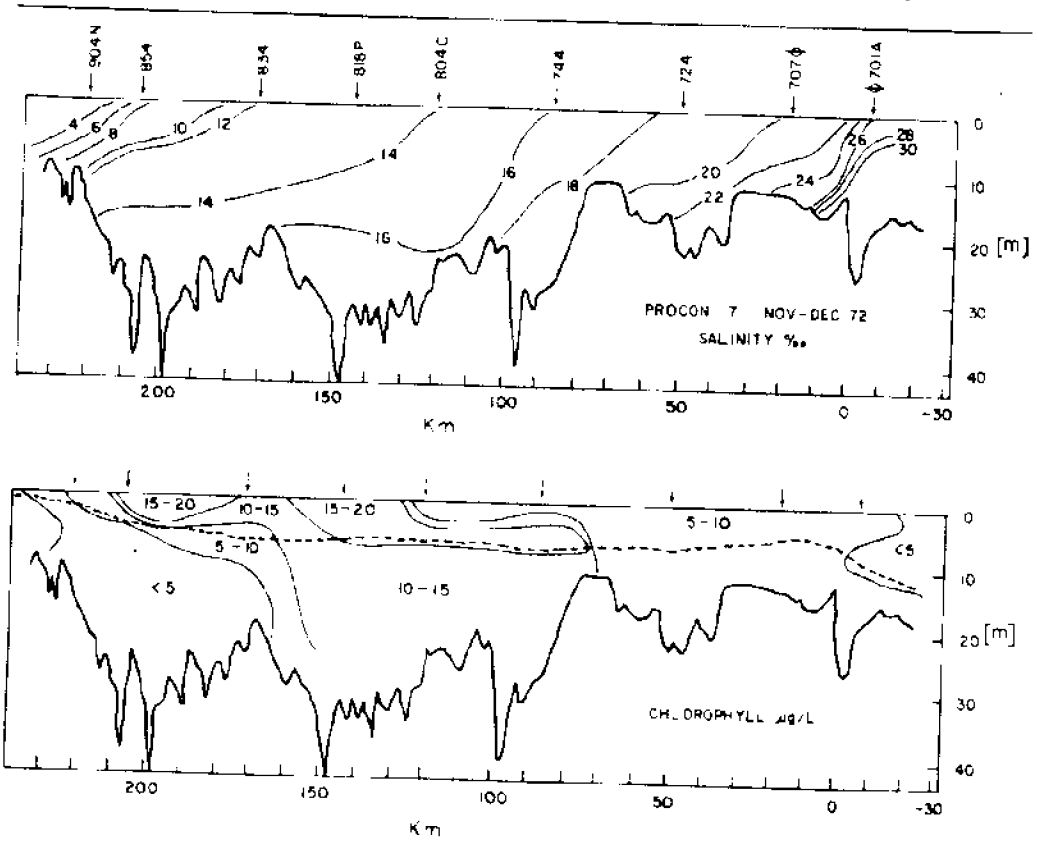


Figure 4: Vertical section along the spine of the Chesapeake Bay from the mouth of the bay at 0 miles to Station 904N north of Annapolis and the Severn River, a distance of 225 Km. Data were obtained over the period November 28 to December 8, 1972 during the Procon 7 cruise of the Chesapeake Bay Institute subsequent to Tropical Storm Agnes. Upper diagram: Salinity profiles. Lower diagram: chlorophyll *a* profiles by *in vivo* fluorescence. The depths at which the underwater downwelling light intensities were 1% of the surface values are plotted as the dashed line. The higher surface water concentrations of chlorophyll *a* at Stations 854 and 818 correspond with the Severn and Patuxent Rivers, respectively.

waters from the southern bay. This annual south-to-north migration begins around February in the bottom waters near the mouth of the bay and reaches the Bay Bridge area in late May—early June. At this point there is an upwelling phenomenon owing to a steep rise where, within the linear distance of 15 Km the bottom contours change from a mean depth of 30 m to a mean depth of 10 m. A partial sequence of this migration is shown in Figures 4 through 7 obtained on the Procon 7, 8 and 9 cruises of the Chesapeake Bay Institute. In all figures the bottom contours of a vertical section through the spine of the Chesapeake Bay extending from the bay mouth to a distance of 225 Km north are shown. The steep rise in bottom contour can be seen around 215 Km from the mouth of the bay (Station 854G to Station 904N). In the upper diagrams are shown

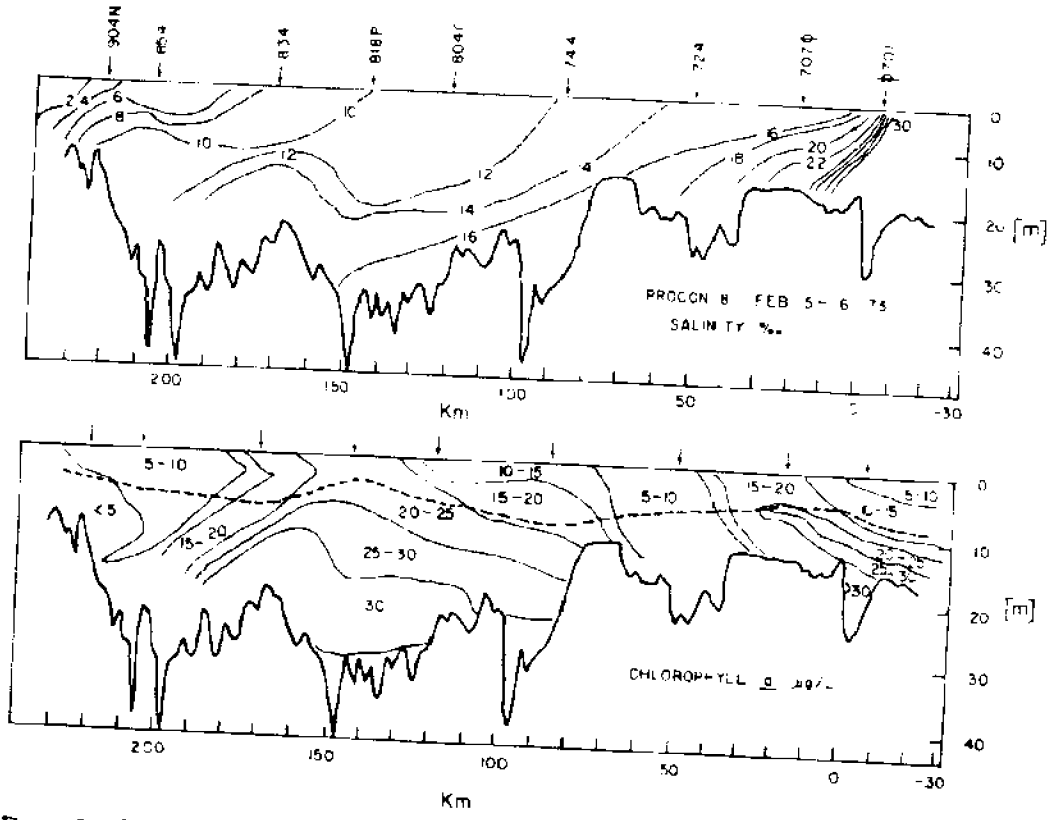


Figure 5: Vertical section along the spine of the Chesapeake Bay from the mouth of the bay at 0 miles to Station 904N, north of Annapolis and the Severn River, a distance of 225 Km. Data were obtained over the period February 5-16, 1973 during the Procon 8 cruise of the Chesapeake Bay Institute. Upper diagram: Salinity profiles. Lower diagram: Chlorophyll *a* profiles by *in vivo* fluorescence. The depths at which the underwater downwelling light intensities were 1% of the surface values are plotted as the dashed line. In these profiles the major dinoflagellate concentrations are distinctly below the 1% light level and below the sharp pyncnocline which defines the upper mixed layer of the water column.

the salinity contours. Since the density is dominated by salinity the data also represent the density layering in the bay. In the lower diagrams are shown the concentrations of chlorophyll *a* obtained by *in vivo* fluorescence. These isocontours separate concentrations in steps of 5 $\mu g\ L^{-1}$. Also shown in the lower diagrams are the depths at which the surface water ambient light intensities have been reduced to 1%. These points are connected by a horizontal dashed line.

During the Procon 7 cruise, Nov. 28-Dec. 8, 1972 subsequent to Tropical Storm Agnes (Fig. 4) the bay is seen to have essentially recovered its salinity pattern. The higher surface water concentrations around Stations 854 and 818P represent the delivery of organisms from the Severn and the Patuxent Rivers, respectively. The sequence of dinoflagellate growth in the subestuaries and the *subsequent* growth in the bay as the result of Agnes can be seen in Fig. 1a and 1b for 1972. The major pulse in chlorophyll *a* standing crops in Rhode River (Fig. 1a) immediately followed the storm (July), while in the adjacent

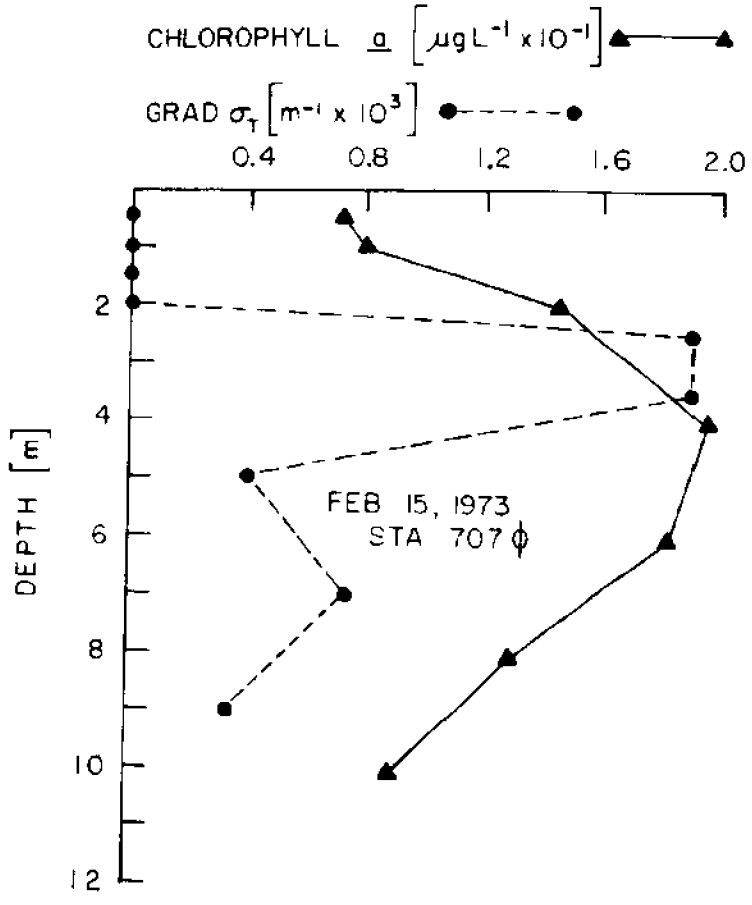


Figure 6: Vertical profiles of extractable chlorophyll *a* and grad σ_T for February 15, 1973 at Station 707ϕ. The major chlorophyll *a* peak, identified microscopically as *P. minimum*, appears below the high values of grad σ_T.

bay (Fig. 1b) the peak was not reached until August when the high concentrations of *G. nelsoni* in the Rhode River had been mainly dissipated. At this time the dinoflagellates were concentrated within and above the 1 % light level.

This was not observed in February, 1973 during the Procon 8 cruise. In Fig. 5 it is seen that major distributions of chlorophyll *a* (living phytoplankton) are *below* the 1% ambient light intensity level, in bottom waters separated from the upper mixed layer by a strong pycnocline. These bottom concentrations have been identified as *P. minimum* at concentrations up to 1.7×10^6 cells L^{-1} (*ca.*17μg L^{-1} chlorophyll *a*.). Depth profiles of chlorophyll *a* concentrations and the gradient of water density (grad σ_T) are shown in Fig. 6 for Station

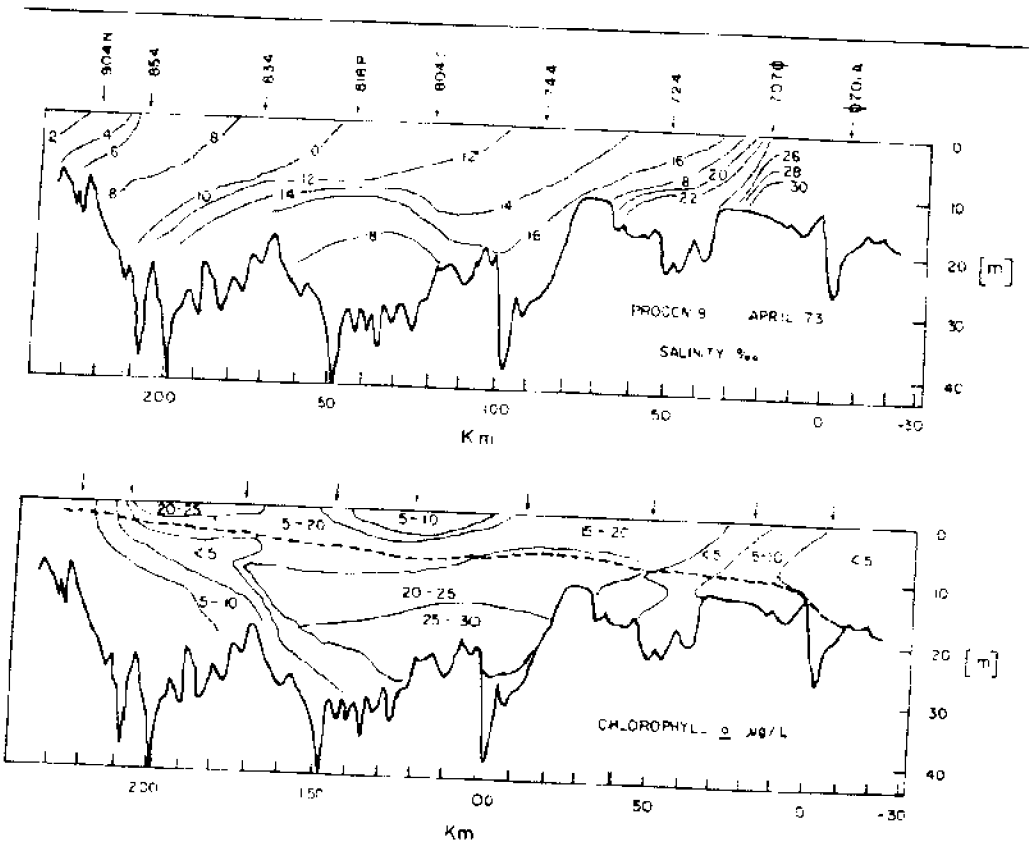


Figure 7: Vertical section along the spine of the Chesapeake Bay from the mouth of the bay at 0 miles to Station 904N, north of Annapolis and the Severn River, a distance of 225 Km. Data were obtained in late April, 1973, during the Procon 9 cruise of the Chesapeake Bay Institute. Upper diagram: Salinity profiles. Lower diagram: Chlorophyll *a* profiles by *in vivo* fluorescence. The depths at which the underwater downwelling light intensities were 1% of the surface values are plotted as the dashed line. In these profiles the major dinoflagellate concentrations are below the 1% light level and below the sharp pycnocline which defines the upper mixed layer of the water column.

707Φ, near the bay mouth. The sharp pyncnocline is evident from the grad σ_T values. The concentration of *P. minimum* is below the upper mixed water layer and mainly below the 1% light level (see Fig. 5). The direction of movement of the bottom waters is north. During this same period there were no *Prorocentrum* in surface waters at Stations 804, 834 and 854. However on February 15-16 during rough weather where turbulent mixing could disturb the pyncnocline significant concentrations (*ca.* 10^5 L^{-1}) of *P. minimum* were observed in the surface waters at Stations 707ϕ and 724.

The general progression can be inferred from Fig. 7, obtained during the Procon 9 cruise in late April, 1973. The concentrations around Station 707ϕ, near the mouth of the bay, have disappeared. The pyncnocline is quite pronounced and the major dinoflagellate concentrations are below the upper mixed layer and below the 1% light level.

In 1973 *P. minimum* formed very extensive surface patches and was a major dinoflagellate in Rhode River during May, a month earlier than the 1970, 1971

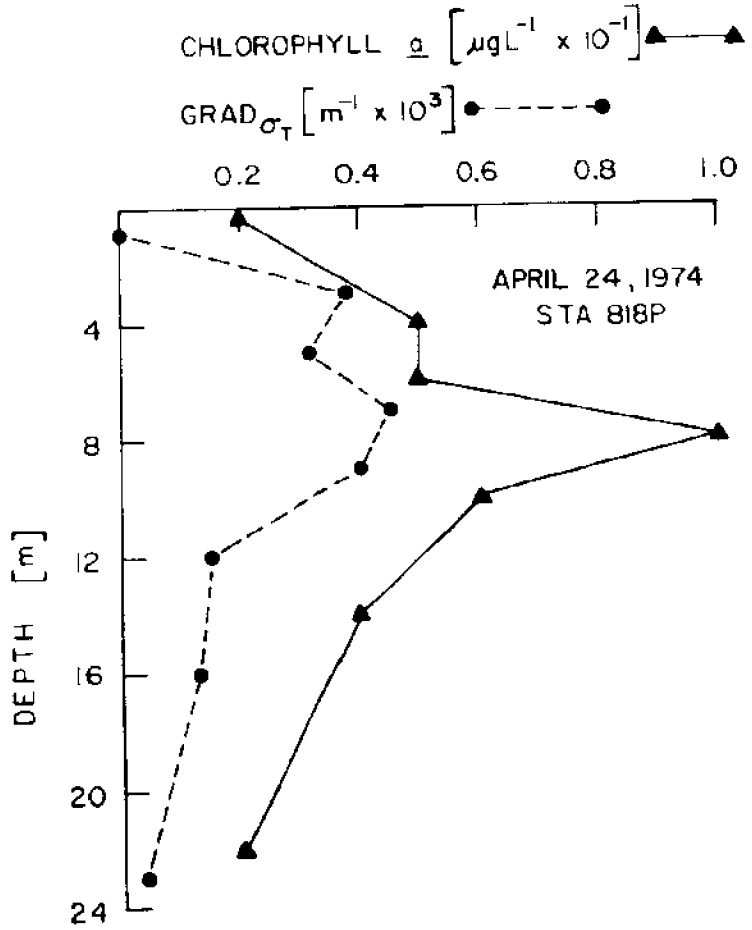


Figure 8: Vertical profiles of extractable chlorophyll *a* and grad σ_T for April 24, 1974 at Station 818P. The major chlorophyll *a* peak, identified microscopically as *P. minimum*, appears below the high values of grad σ_T.

sequences (see Table 1). We attributed this to the extensive nutrient delivery by Tropical Storm Agnes in June, 1972 and its subsequent effects on dissolved organic nutrients which extended through 1973 Loftus and Seliger, (12).

The cruise schedule of the Chesapeake Bay Institute in 1974 did not permit the verification of the 1973 pattern. However we were able to make a sequence of observations which are consistent with the proposed south-to-north migration of *Prorocentrum*.

a) 8 April 1974, Station 854: no *P. minimum* in surface waters. High concentrations of amino acids ($> 2\,\mu M$) below 3 m depth---—that is below the 1% light intensity level.

b) 24 April 1974, Station 818: Fig. 8 shows the depth profiles of grad σT and chlorophyll *a* concentrations. Again the peak *Prorocentrum* concentrations are below the upper mixed layer and below the 1% light intensity level (see Fig. 7). Chlorophyll *a* concentrations below the pyncnocline were identified as *P. minimum*.

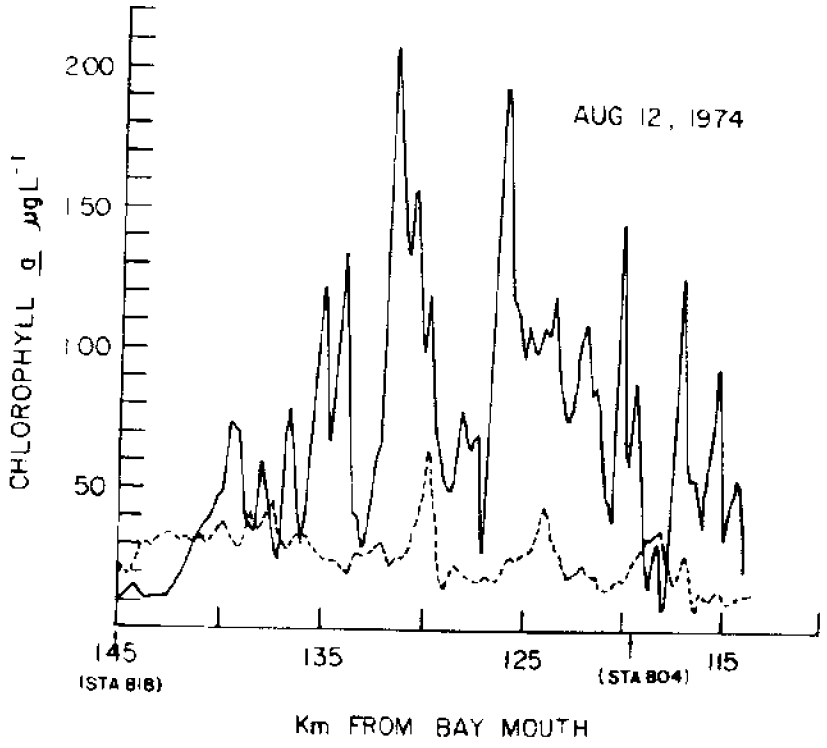


Figure 9: North-south surface water transects of *in vivo* fluorescence of chlorophyll *a* at a depth of 1 m for *C. furca* on August 12, 1974. The dashed curve is the 1 m transect during the morning (0850-1050) in the region south of the Patuxent River (Sta. 818). The solid curve is the same transect during the afternoon of the same day (1430-1730). Since the transect extends over the entire accumulation area the ratio of the integrated transects is a measure of the upward migration of the *C. furca*.

c) 22 May—25 May 1974, Bay Bridge: *P. minimum* was present in surface waters. Averaged 2-6 x $10^6 L^{-1}$ at the surface and 6-9 x $10^6 L^{-1}$ at 4 m. *Prorocentrum* had not yet been delivered to the South or Rhode Rivers.

d) 3 June 1974, Bay Bridge: *P. minimum* had dropped to $10^4 L^{-1}$ at all depths. It was not found at all in the South or Rhode Rivers.

e) 10 June—19 June 1974: No *Prorocentrum* patches observable north of Station 854 by *in vivo* fluorescence. We have no explanation for this disappearance.

An example of the vertical migration of dinoflagellates into surface waters subsequent to sunrise and also of the extreme patchiness of dinoflagellate accumulations in the Chesapeake Bay is shown in Fig. 9. In August 1974 a dense accumulation of *G. furca* extended for approximately 15 Km, south of the Patuxent River (Station 818). The lower dashed curve represents a 1 m depth *in vivo* chlorophyll *a* fluorescence transect in the morning (0850-1050) on August 12, 1974 and the upper solid line curve is the same 1 m depth fluorescence transect, returning in the afternoon (1430-1730). The ratio of the means of the afternoon to morning transects is 3:1. In relation to the general sampling protocols in the bay, Fig. 9 illustrates that the lateral extent of the patches (full width at half maximum) is around 1Km. It follows therefore that station sampling or spot sampling can be subject to large errors if they are not corroborated by coincident lateral transects as well as vertical profiles of *in vivo* fluorescence.

The center of gravity of the *C. furca* patches remained at the same relative position in the bay over the next two days of chlorophyll *a* fluorescence transects. Unfortunately ship scheduling did not permit any further observations in this area.

A relationship between the depth of the upper mixed layer in the bay (defined by the pyncnocline) and the euphotic zone (defined arbitrarily by the 1% level of ambient surface water light intensity) in areas where there are no significant surface accumulations or patches of dinoflagellates is shown in Fig. 10. These data were obtained during the August cruise of the Chesapeake Bay Institute as part of our program for measuring absolute scalar irradiance spectra (6). In August there was an extremely significant correlation ($R^2 = 0.94$) between these two parameters. The numbers beside each entry are the station numbers shown in Fig. 2. The data represent a major section of the Chesapeake Bay, approximately 110 Km from the Potomac River north to the Magothy River (Sta. 904) and cover the range of 1% light intensity depths normally encountered in other years.

DISCUSSION

1. *"Filling" the Euphotic Zone*

The strong correlation between upper mixed layer depth and euphotic zone depth has very interesting ecological ramifications. The particulate plant

pigments appear to account for the major absorption of light in the eutrophic estuary. The presence of sediment scatterers (turbidity) increases the effective geometrical pathlength for downwelling light. In late August, following a period of minimum rainfall and high insolation, the turbidity in the bay would be at a minimum. The phytoplankton in the *mixed* upper layer would tend to grow to fill the available "space". In this case growth has occurred to utilize "all" of the available light. This self-absorption limit for the standing crops of phytoplankton is dependent on nutrient availability and represents a steady-state which the system approaches according to the following arguments:

a) At low nutrient levels (or turnover) the growth rate constant for the phytoplankton will be low. The herbivore population which follows the rate of growth of the phytoplankton will adjust partially to this low rate of growth and the depth of the 1% light intensity will be greater than the depth of the mixed layer.

b) As the nutrient level (or turnover) increases the phytoplankton growth rate constant will increase, stimulating an increase in predation rate. Thus initially the delivery of nutrients (including sediment) by run-off from the land will push the level of the 1% light intensity *above* the depth of the mixed layer. Under these conditions the system is light-limited, as contrasted with nutrient-limited. As predation increases, the *most efficient* utilization of light is approached (Fig. 10) i.e., the entire mixed upper layer, through which the

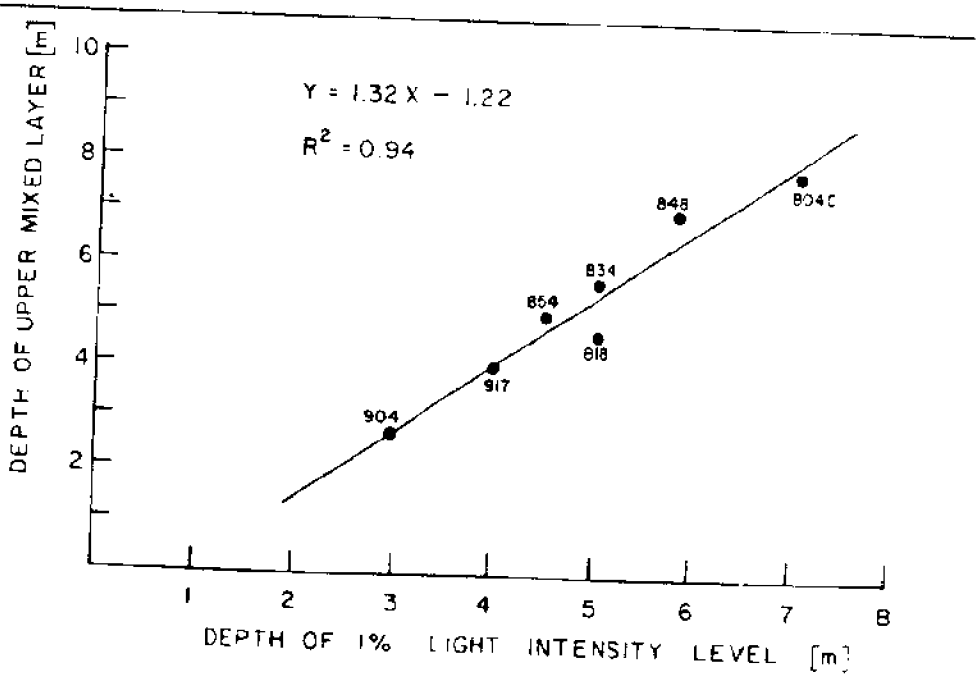


Figure 10: Relation between the depth of the upper mixed layer in the Chesapeake Bay and the depth of the euphotic zone (1% of ambient light intensity), obtained during the August cruise of the Chesapeake Bay Institute. The numbers associated with each entry are the station numbers (see Fig. 2).

phytoplankton are transported by the energy delivered by sun and wind, absorbs the available light energy for photosynthesis. Under these conditions the smaller relatively non-motile nannoplankton have no need for positive phototaxis.

c) Eventually the inorganic nutrient pool is utilized, dissolved organic nutrients are released and other species, presumably the positively phototactic dinoflagellates, are selected for. The strong positive phototaxis originates in response to other selection pressures and is specifically related to the development of surface patches.

2. *Diurnal Vertical Migration*

In the Chesapeake estuary there is a gradient in nutrient concentrations, from the creeks to the rivers to the central bay and then south to the ocean. Therefore in a two-layered system where the surface waters were moving generally south and the bottom waters were moving generally north, there would be a major advantage accruing to any organism which could periodically migrate into the upper surface waters and down into the bottom waters. In proper phase with the tide such an organism by a periodic *vertical migration could be subjected to a net lateral displacement*, north or south or zero. Since phytoplankton are photosynthetic it would be reasonable to suppose a solar phase photoperiodic rhythm, a positive phototaxis (upward vector) by day and a negative vector (geo- or chemi-taxis) 12 hours later. Only organisms able to swim around 1 m hr^{-1}, in order to take advantage within reasonable times of the currents in the upper and lower water layers of the two layer system, could be successful.

A diurnal migration has been reported for *C. furca* (5). This would readily explain our observations relative to the temporal stability of the *C. furca* accumulation of Fig 9. In this same paper, Eppley *et al.* (5) have proposed "The combination of vertical migration and the ability to assimilate nitrogenous nutrients in darkness appear to be the chief factors in providing the dinoflagellate bloom organisms with a competitive advantage over other species..." presumably the non-motile plankton.

We have already measured, for *G. nelsoni* in the Rhode River, a downward night time migration into interstitial waters and an upward daytime migration into surface waters (A. Place, private communication).

The combination of diurnal upward and downward migration to

a) take advantage at night of higher nutrient concentrations in interstitial waters or in bottom waters.

b) reduce the exchange rate of the dinoflagellates relative to the uniformly distributed nannoplankton in the water column can produce much higher *G. nelsoni* populations than would be predicted on the basis of the average soluble nutrient concentrations in the upper layer waters.

3. *Bacterial Accumulations*

The excretion by *G. nelsoni* of organic exudates permits the extremely rapid accompaniment, in these accumulations, of large bacterial populations. Surface water samples of viable *G. nelsoni* in the Rhode River and in Chesapeake Bay have contained up to 10^9-10^{10} bacteria per liter in 1973. So far as we have observed this has not resulted in any specific fish kills in the Rhode River, although in the shallower sections of the river the oxygen demand is such that occasionally the water column goes anaerobic during the night (R. Cory, personal communication).

The bacteria are essentially non-motile. However their extremely small generation times and the relatively high concentrations of organic exudates in the high concentration surface patches of dinoflagellates permit a rapid growth even in the short space of 6-8 hours. Collier (3) has proposed that *G. breve* has specitic bacterial strains associated with it, possibly in a symbiotic relationship, although this is not necessary.

It is therefore *possible* that in the Chesapeake Bay non-toxic dinoflagellate accumulations may indirectly precipitate an autocatastrophic fish kill by the following sequence of events:

a) Formation of dense, relatively stable surface patches by the diurnal migration mechanism described above.

b) Alignment of patches into windrows or convergence lines by prevailing winds.

c) Rapid growth, in warm weather, of specific fish-pathogenic bacterial populations to high concentrations within the surface patches. The bacteria can use the organic exudates of the dinoflagellates (3) as a food source.

d) At these high bacterial concentrations and at high temperatures juvenile fish passing through the patch can ingest potentially fatal inocula of the pathogens.

e) When these fish die and float to the surface (the upward vector of flotation is equivalent to a constant positive phototaxis), the physical forces which produce the windrows will accumulate the dead fish in the dinoflagellate patches.

f) This large biomass influx can produce a second "explosion" of bacterial populations, resulting in a positive feedback pathogenic system for killing fish.

g) Eventually the oxygen demand rate due to the dead fish will exceed the rate of supply of oxygen by the phytoplankton as well as by exchange mixing with adjacent oxygen-containing waters and a massive kill including bottom organisms will ensue.

4. *Prorocentrum Accumulations in the Upper Chesapeake Bay*

The circumstantial evidence indicates that the *P. minimum* accumulations observed in the upper Chesapeake Bay in June have migrated northward in bottom waters. The migration is over a distance of approximately 200 Km in

four months; a net movement of 1.7 Km day^{-1}. The bottom waters appear to be high in organic nutrients. Therefore if *P. minimum* were partially heterotrophic it could compensate for the very low rates of photosynthesis to which it must be constrained in these bottom waters. All photoautotrophs which become trapped below the pycnocline die and sink to the bottom.

The upwelling of *Prorocentrum* at the steep rise in bottom contour in the neighborhood of the Bay Bridge (north of 854G) and the ensuing photosynthesis and phototaxis result in extensive surface patches in this region. *P. minimum*, while positively phototactic, does not appear to exhibit a nocturnal downward migration, since the patches appear to move southward at the same net rate as the surface waters (1.6 Km day^{-1}). The organisms are delivered to the South and Rhode Rivers by the mechanism described by Seliger and Loftus (20) in which there is transport of near surface bay waters to deeper layers in the rivers. In these cases there will be an increased net delivery of these dinoflagellates to the surface waters of the rivers, since the organisms migrate to the surface while the water in which they arrived sinks in the river to its appropriate density layer. This river accumulation mechanism and the low exchange rate of river waters with the bay combine to keep *P. minimum* in the rivers for some time after the patches in the central bay have been dissipated by exchange. However because they apparently lack the nocturnal downward migration (or for some other unknown reason, *P. minimum* is gradually lost from the rivers as the surface waters are exchanged with those of the bay. The duration of *P. minimum* concentrations in the Rhode River is qualitatively proportional to the density and duration of the surface patches produced by the original upwelling phenomenon (see Table 1).

5. *Gymnodinium Accumulations*

From our observations in the Severn, South, Rhode and West Rivers it appears that *G. nelsoni* grows up to high concentrations in the shallow portions of the river, maintains its lateral position and obtains nutrients by virtue of the diurnal upward and downward migration described above. When the solar rhythm passes out of phase with the tidal rhythm, *G. nelsoni* patches are washed out into the bay. Once outside of the rivers the rhythms start to go into phase again. The surface patches now appear in windrows or convergences and remain relatively stable in geographical extent. This *reduction* in exchange rate relative to the uniformly mixed nannoplankton, in conjunction with the windrow and convergence accumulation mechanisms serves to extend the duration of the *G. nelsoni* in the central bay beyond that predicted on the basis of dye studies of homogeneous water exchange.

6. *Katodinium Accumulations*

K. rotundatum appears to be the cold-weather counterpart of *G. nelsoni* (see Table 1 and Fig. 3). It has been a consistent winter dinoflagellate in the Rhode

River during the past 5 years of observations. Peak concentrations can be greater than 10^8 L^{-1} (>100 µg chlorophyll *a* L^{-1}).

CONCLUSIONS

Dinoflagellate accumulation mechanisms in the Chesapeake Bay appear to make use of the biological effects:

a) positive phototaxis
b) diurnal upward and nocturnal downward taxes
c) partial heterotrophy

and the physical effects:

d) wind-driven convection forming windrows or Langmuir convection cells
e) convergence of water masses of different densities
f) differential motion of surface and bottom waters in a two-layer estuarine system.

Prorocentrum migrates to the upper bay making use of f) and c). After upwelling, a) and d) form surface patches. It is delivered to the rivers by e) and a).

Gymnodinium originates in the river sections making use of b) and f) for nutrients and to maintain its position in the river. When it loses its phase it is washed out into the bay where again b) and f) act to delay the dissipation.

Ceratium also appears to maintain its relative position in the bay by virtue of b) and f).

It is possible that non-toxic dinoflagellate accumulations can serve as nutrient sources for fish-pathogenic bacteria and thus precipitate a self-regenerative massive fish kill.

ACKNOWLEDGMENTS

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THE ORGANISM CAUSING NEW ENGLAND RED TIDES: *GONYAULAX EXCAVATA*

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ABSTRACT

Using scanning electron microscopy, we compared the thecal morphology of the New England red tide organism (isolate 426 from the 1972 red tide in Massachusetts), two isolates (173, 173a) identified as *Gonyaulax tamarensis* from its type locality (Tamar River Estuary region, England) and preserved *Gonyaulax acatenella* cells from the June 10, 1965 red tide in Malaspina Inlet, B.C., Canada. The tabulation of the major body plates is identical in all our specimens but differs from Lebour's original description of *G. tamarensis*. Her epithecal tabulation is a mirror image of the tabulation we observe and is interpreted as erroneous. *Gonyaulax acatenella* is morphologically distinguishable, having pronounced and numerous flanges on the hypothecal plates. All of the organisms have U-shaped nuclei. Isolate 173 is considered to be the typical *G. tamarensis*. It is non-bioluminescent, non-toxic and has ventral pore. Isolate 173a is bioluminescent and lacks a ventral pore. It is not conspecific with isolate 173 and requires further study. The New England red tide organism is bioluminescent, toxic and lacks a ventral pore. It is considered to be *Gonyaulax excavata* as circumscribed by Braarud when he described *G. tamarensis* var. *excavata* and not as circumscribed by Balech to include a small, tropical form with a ventral pore. *Pyrodinium phoneus* is considered to be a *Gonyaulax* in view of its tabulation, toxicity and plastid appearance.

INTRODUCTION

Since 1972 there have been outbreaks of red tides and occurrences of shellfish toxicity along the coast of New England. The organism responsible was identified by a biologist at the University of Massachusetts, Marine Laboratory, Gloucester, Massachusetts (1) as *Gonyaulax tamarensis* Lebour, 1925. To our knowledge, no illustrations of the New England red tide organism have been published.

We are studying the biology of the New England red tide organism. Prior to our physiological studies we decided to check the identification of our isolates. Lebour (2) originally described *G. tamarensis* from the Tamar River Estuary, England and stated that it did not occur outside of the estuary. Her original figures are reproduced in Figs. 1A-D. Braarud (3), observing Norwegian *G. tamarensis* in the light microscope, found differences in the epithecal tabulation, occurrence of spines and cell shape (compare Figs. 1A-D with 1K-N). For these reasons he established two new varieties: *G. tamarensis* var. *excavata* Braarud, 1945, (Figs. 1K, L) a form occurring in both east Canadian and Norwegian waters and *G. tamarensis* var. *globosa* Braarud, 1945, (Figs. 1M,N) a Norwegian form. These differences have not been adequately documented as further examination of thecal tabulation of *G. tamarensis* type specimens is not possible since the holotypes were not preserved. However, two isolates (173, 173a) from the type locality identified as *G. tamarensis* exist. Before an accurate identification of our isolates could be made, we felt it

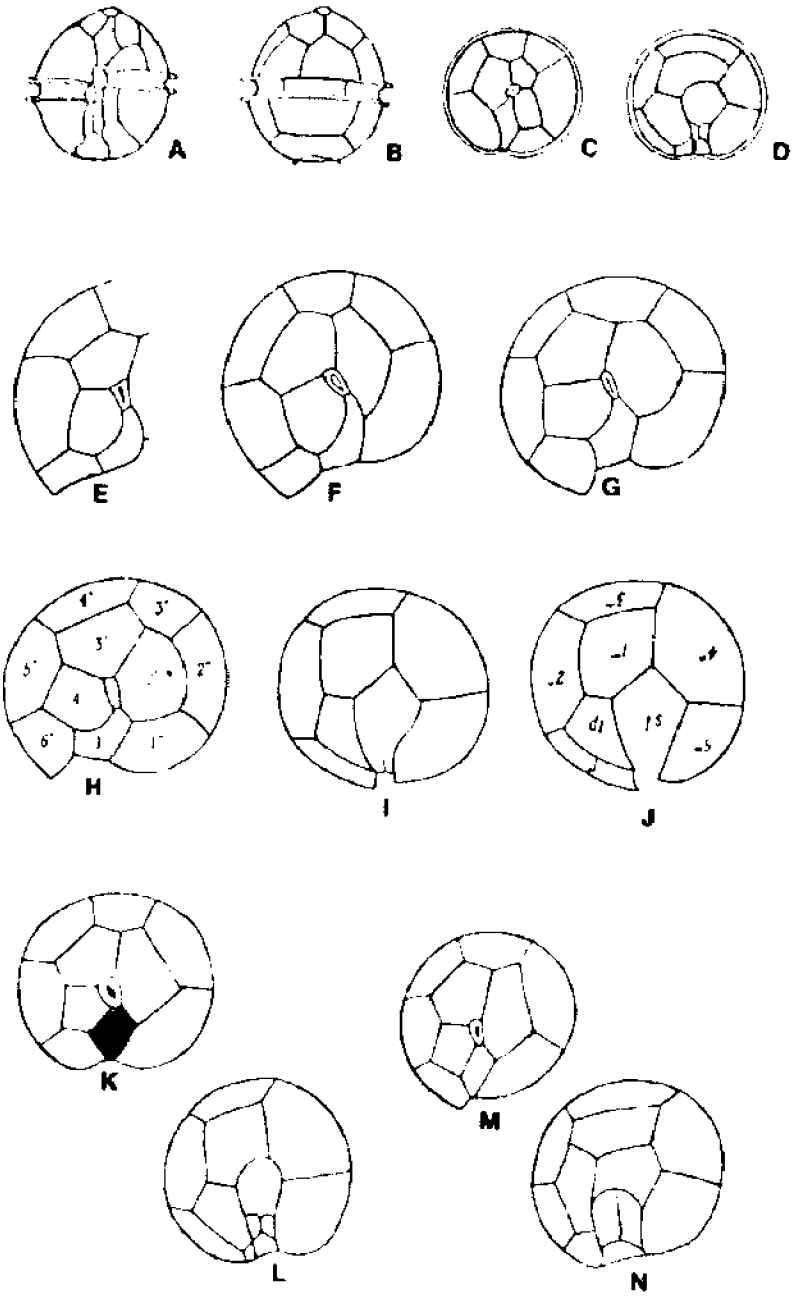


Figure 1: Figures of holotypes. A-D, *Gonyaulax tamarensis* from Lebour, 1925; E-J, *Pyrodinium phoneus* from Woloszyńska and Conrad, 1939; K, L, *G. tamarensis* var. *excavata* from Braarud, 1945; M, N, *G. tamarensis* var. *globosa* from Braarud, 1945. Note mirror image of epithecal plates in C. See Table 1 for cell dimensions.

necessary to compare them with the isolates from the type locality of *G. tamarensis*. Additionally, preserved material of *Gonyaulax acatenella* Whedon and Kofoid, 1936, (Figs. 2A-F) was examined in view of the remarks of Whedon and Kofoid (4) as to its similarity to *G. tamarensis*. Comparisons were also made to published descriptions of *Pyrodinium phoneus* Woloszyńska and Conrad, 1939, and *Gonyaulax excavata* (Braarud) Balech, 1971, since these appear to be related (Figs. 1E-J, 2G-L).

The New England red tide organism is highly toxic (1) containing two neurotoxins (5, 6). The paralytic shellfish poison isolated from the scallop, *Pecten grandis* Solander, 1786, from the Bay of Fundy is not similar in charge to saxitoxin (7). Toxic mussels, *Mytilus edulis* Linné, 1758, from the northeast coast of England near Holy Island contain two neurotoxins (8). The minor component resembled saxitoxin. The major component was dissimilar in charge to saxitoxin, however, it had similar neurobiological activity. It appears that the scallops and mussels had fed upon a dinoflagellate (7, 9) whose major toxin was not saxitoxin but had two toxins similar to the New England red tide organism (5, 6). On the basis of these toxin and morphological similarities, it is widely assumed that the New England form is identical to: 1) the Canadian Bay of Fundy - Gulf of St. Lawrence organism (10) (Lebour in Medcof *et al.* identified material from Head Harbor, Bay of Fundy, New Brunswick, Canada as *G. tamarensis* (11)), 2) the northeastern England organism (determined to be *G. tamarensis* (9)) and 3) Norway *G. tamarensis* (12).

Bioluminexcence commonly accompanies outbreaks of toxic dinoflagellates (9) but is often not documented.

The Eastern Canadian form was the most toxic of three *Gonyaulax* species studied (13). Ballantine in Seaton (14) and Prakash (15) were unable to detect any toxicity in the Plymouth isolate 173 of *G. tamarensis*. A culture of *G. tamarensis* isolated from a "bloom" (1×10^6 cells per liter) over the oyster beds in the Tamar River in 1967 was found to be non-toxic to mice by Barrow in Seaton (14).

In view of 1) the differences in published descriptions of organisms determined to be *G. tamarensis* and 2) the occurrence of toxic and non-toxic strains of *G. tamarensis*, it seems likely that more than one species is involved. Balech (16) elevated the varieties of *G. tamarensis* described by Braarud (3) to the level of species, however, he did not reexamine the typical variety from the type locality. It is the purpose of our paper 1) to examine the New England red tide organism and establish its identity, 2) to present some observations on the two isolates from the type locality of *G. tamarensis*, 3) to compare the morphology of these organisms with *G. acatenella* and 4) to suggest future lines of research that could further clarify the systematics of these and related organisms.

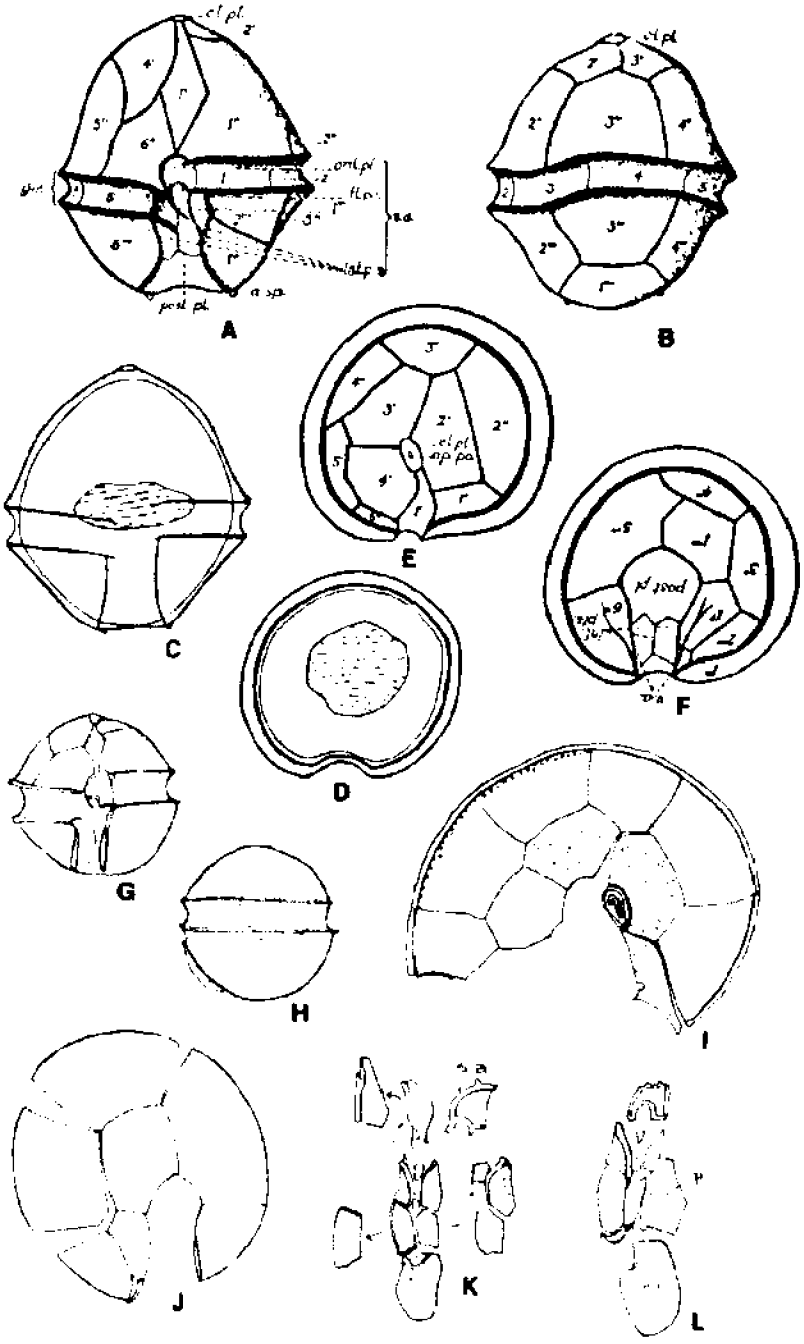


Figure 2: A-F, Holotype of *Gonyaulax acatenella* from Whedon and Kofoid, 1936; note nucleus in C, D; G-L, Figures of *Gonyaulax excavata* from Balech, 1971. Note nucleus in G, H and ventral pore on margin of first apical in 1. See Table I for cell dimensions.

MATERIALS AND METHODS

Organisms

Strain 426 of the New England red tide organism was isolated by A. Loeblich from a sample of the 1972 red tide collected by Edward Gilfillan from Lanesville Station, Gloucester, Massachusetts. Isolate 429 from the same outbreak was isolated by C. Martin, Gloucester, Marine Station, Massachusetts and was made axenic by L. Loeblich using penicillin G and streptomycin sulfate. Plymouth isolates 173 and 173a previously identified as *Gonyaulax tamarensis* were obtained from J. C. Green, Marine Biological Laboratory, Plymouth, England. Both of these isolates were obtained from the estuarine regions known as the Tamar River Estuary and were collected within approximately three miles of each other. Isolate 173 was isolated by Irene Adams from the Lynher River (position approximately 50°23 1/2'N, 4°13'W) from material collected June 24, 1957. Isolate 173a was isolated by Rosemary Jowett from the Estuary of the Tamar River at the mouth of the Lynher River (position approximately 50°22 1/2' N, 4°16' W, close to Beggars Island) from material collected May 3, 1967. All of these organisms are grown in medium GPM (17), at a light intensity of about 400 ft - candles. Isolates 426 and 429 are maintained at 12°C; isolates 173 and 173a are maintained at 21°C. A preserved sample of phytoplankton from the June 10, 1965 *Gonyaulax acatenella* red tide in Malaspina Inlet, B.C., Canada (18) was obtained from N. Bourne, Fisheries Research Board of Canada, Nanaimo B.C., Canada. *Gonyaulax polyedra* Stein, 1878, isolate GP 52 was obtained from J. W. Hastings, Harvard University, Cambridge, Massachusetts.

Scanning electron microscopy

Cells from a 50 ml culture were concentrated by gravity filtration using an 8 μ SCWP Millipore filter. When less than 3 ml of the cell suspension remained on the filter about 20 ml of fixative at 25°C was added, consisting of 0.05M cacodylic acid (pH 7), 0.15 M sucrose and 4% glutaraldehyde. When nearly all of this solution had passed through the filter, the cells were washed three times with the cacodylate buffer without glutaraldehyde, each wash containing a decreased concentration of sucrose (0.1, 0.075 and 0.04 M). Cells were rinsed off the filter, transferred to a centrifuge tube, and dehydrated in an ethanol series (25%, 50%, 75%, 95%, 100%, 100%). The ethanol was serially replaced by Freon 113 (25%, 50%, 80%, 100%, 100%, 100%). The cells were critical point dried in aluminum foil "boats" employing the Freon 13 procedure (19) in an apparatus made available by Y. Zeevi, Harvard University. Dried cells were coated (200 Å) with carbon and then with a gold-palladium mixture. Scanning electron micrographs were taken on an AMR 1000 and a JEOL JSM-35.

Light microscopy

Unfixed cells were measured at 400X. Nuclei were stained using the acetocarmine technique (20).

Bioluminescence

One liter cultures growing in a light-dark cycle (12hr:12hr) were examined in the middle of the dark period after the investigators' eyes were dark adapted. The cultures were agitated and observed. This procedure was repeated on a subsequent day.

For a quantitative analysis of bioluminescence a photomultiplier photometer was used (21). One ml aliquots were removed from cultures grown on a light-dark cycle (12hr:12hr) and placed in vials. The aliquots were returned to the light-dark cycle and left undisturbed for 20 hours until analysis, which was in the eighth hour of the dark period. The vials were placed in the photometer chamber with minimal agitation and 1.0 ml of 60 mM acetic acid was added. The recorded response was calibrated against the emission of a standard solution of hexadecane-1-C^{14} in a scintillation fluid (22). From these measurements and cell counts, quanta emitted per cell were calculated.

RESULTS AND DISCUSSION

Cell size

Table I relates our cell measurements to previously published values. The isolates we have in culture cannot be distinguished on the basis of size. Most cells were slightly longer than wide. There is a large variation in cell size (Table I) in both exponentially growing and stationary phase cultures. Variation in size in exponentially growing cultures is due to small, recently divided cells and larger cells which presumably will shortly divide. In stationary phase cultures cells were seen attached side by side rather than the usual head to tail arrangement of recently divided cells. These laterally attached pairs were interpreted to be fusing gametes. This interpretation may explain the size variation in stationary phase cultures.

Scanning electron microscopy

Scanning electron micrographs of isolates 426, 173, 173a and preserved *G. acatenella* were compared. All specimens examined have a plate tabulation of the major body plates similar to that first illustrated for *G. acatenella* (4). However, the epithecal tabulation of all our specimens (Figs. 3, 11, 12, 18) is a mirror image of the epithecal tabulation given in the original description of *G. tamarensis* (Fig. 1C). We conclude that the epithecal illustration is erroneous.

Table I

Size of *Gonyaulax tamarensis* and related species

Organism	Size in µm Length	Width	Source
Gonyaulax tamarensis	36	-	2
G. tamarensis	36	-	10
G. phoneus comb. nov.	36 - 44	32 - 36	23
G. tamarensis var. *excavata*	35 - 53	-	3
G. tamarensis var. *globosa*	25 - 45	-	3
G. tamarensis	36	-	11
G. tamarensis	15 - 36 (most commonly 23 - 28)	-	24
G. tamarensis	30 - 40[1]		15
G. tamarensis	28 - 38[1]		13
G. excavata	24.5 - 28	20 - 23.5	16
New England Red Tide			
Isolate 429[2]	27.5-55.0 (37.7)	25.0-52.5 (35.9)	This paper
Plymouth			
Isolate 173[2]	27.5-50.0 (38.6)	27.5-47.5 (36.8)	This paper
Plymouth			
Isolate 173a[2]	30.0-43.8 (36.6)	30.0-41.3 (36.0)	This paper

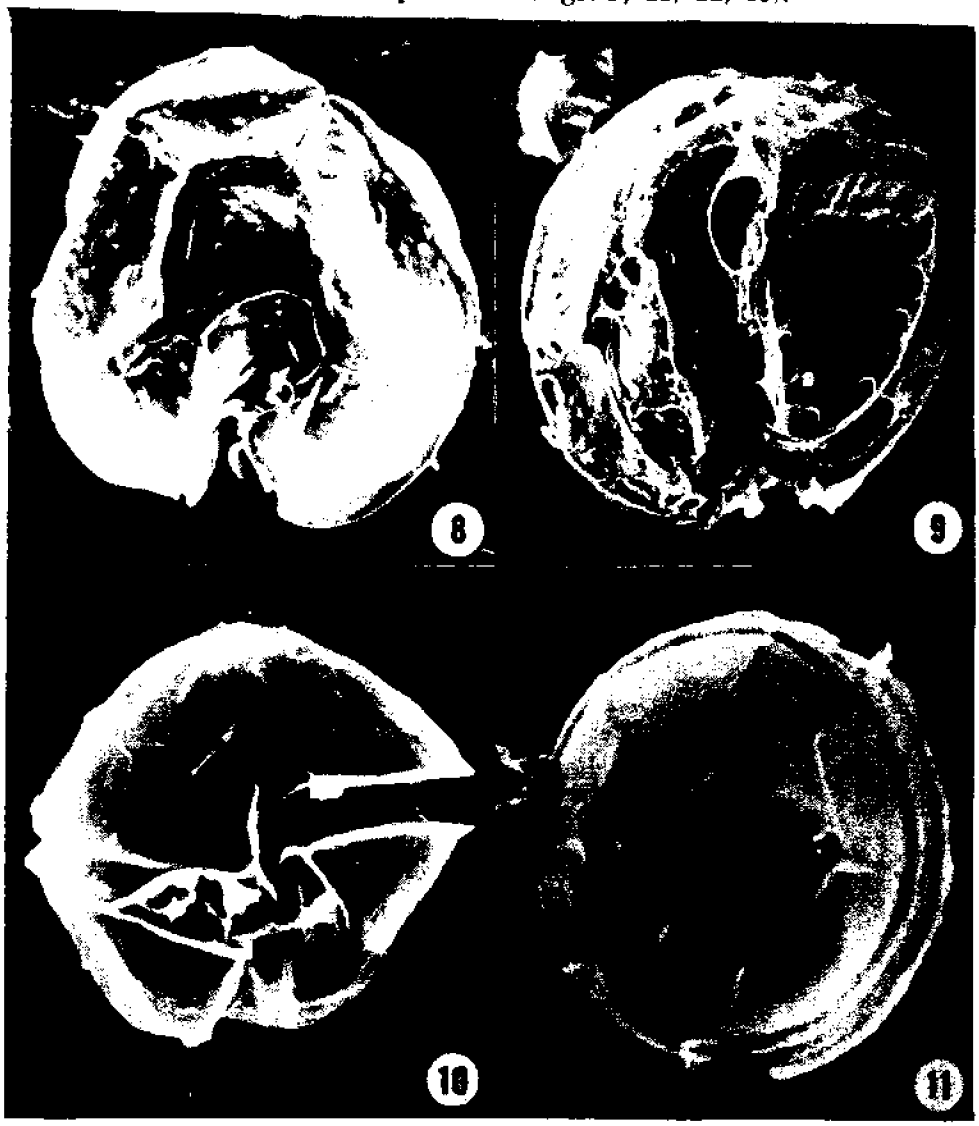
[1]Values not differentiated as to length or width.

[2]For each isolate 20 living cells from an exponentially growing culture were measured. Values in parentheses are the means.

The spines reported for *G. tamarensis* (2) and for *G. acatenella* (4) are in reality flanges arising from plate 6''' and 1p margins (Figs. 10, 13, 15, 17, 19). *Gonyaulax acatenella* has several pronounced flanges parallel to but recessed from the plate sutures (Figs. 15, 19).

Figures 3-7: New England red tide organism. Figs. 3-6, Isolate 426. Fig. 7, Isolate 429. Fig. 3, Scanning electron micrograph (SEM) of epitheca and apical pore, X2550. Note that the tabulation is a mirror image to that in Fig. 1C. Fig. 4, SEM of ventral surface, X1880. Fig. 5, SEM of upper sulcal area. Note that the first postcingular is in the sulcal groove and that the trichocyst pores are frequently paired, X7220. Fig. 6, SEM of ventral surface. Note wrinkled pellicular layer visible between separated epithecal plates, X1870. Fig. 7, Apical view of acetocarmine stained U-shaped nucleus with the arms pointing toward the ventral surface.

A ventral pore along the margin of plates 1′ and 4′ was detected on specimens of isolate 173 (Figs. 12, 13). It was absent in specimens of 173a, 426 and *G. acatenella*. The trichocyst pores on all our specimens were similar in distribution and morphology. They were often paired (Figs. 5, 11, 14, 18). The apical pore was similar in all specimens (Figs. 3, 11, 12, 18).

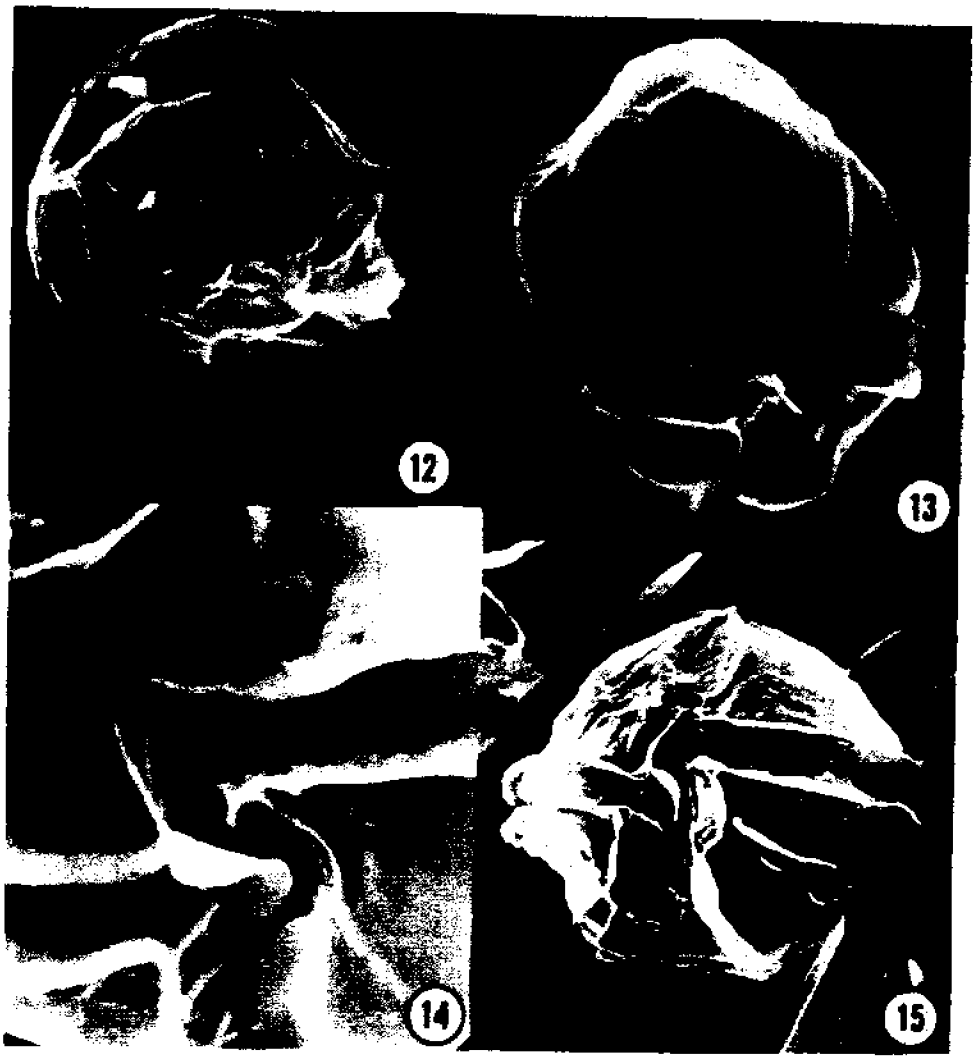


Figures 8-11:

Scanning electron micrographs of New England red tide isolate 426 (Figs. 8,9) and Plymouth isolate 173a (Figs. 10,11). Fig. 8, View of hypotheca. Note the flanges between 1p and 6″ and the sulcal area. X2420. Fig. 9, Hypothecal view of cell with damaged outer and exterior thecal membranes obscuring the tabulation. Note large reniform-shaped opening in membranes revealing a portion of the posterior sulcal plate; this may not be an artifact but the site of attachment of the posterior daughter cell during chain formation. X2110. Fig. 10, Ventral view. Note absence of ventral pore on margin of plate 1′ and raised flange along margin of plate 6″ and sulcal region. X2230. Fig. 11, Epithecal view. Note the apical pore and that the tabulation is a mirror image to that in Fig. 1C. X2560.

Chain formation

Chains of individuals occur in the English isolates. Chains of two or four cells were seen in strain 429. The cells are attached "head to tail" and not "tail to tail" as figured earlier (25). No antapical pore in the thecal layer was present. Such an attachment pore has been described on *Gonyaulax catenella* Whedon and Kofoid, 1936, which forms chains many cells long (4). However,



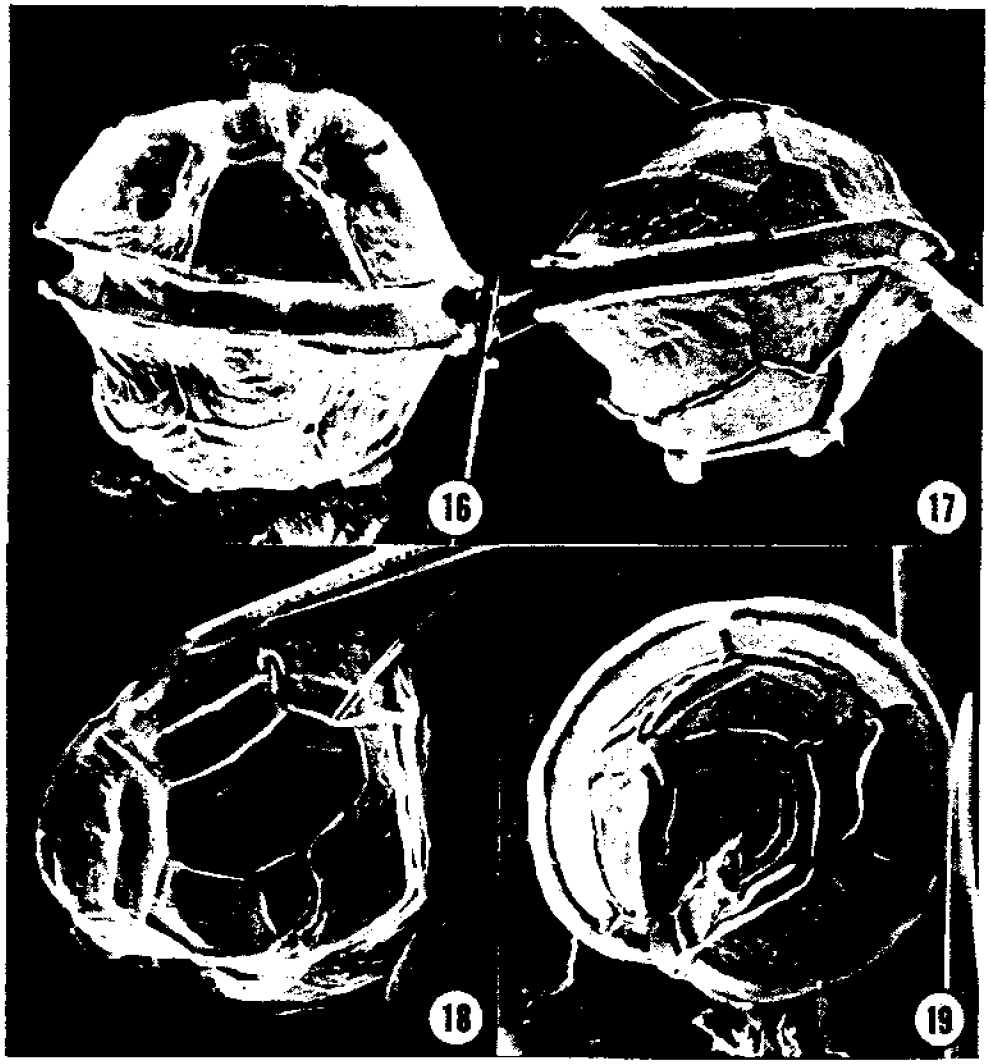
Figures 12-15:

Scanning electron micrographs of Plymouth isolate 173 (Figs. 12-14) and *Gonyaulax acatenella* (Fig. 15). Fig. 12, View of epitheca and apical pore. Arrow points to ventral pore between plates 1' and 4', X1550. Fig. 13, View of ventral surface. Arrow points to ventral pore between plates 1' and 4'. Note flange visible along right margin of sulcal groove, X1550. Fig. 14, View of ventral area. Note flange on left side of ventral area, X3250. Fig. 15, Ventral view. Note flanges on both sides of ventral area as well as others along plate margins on hypotheca. No ventral pore visible, X1350.

Fig. 9, a specimen of isolate 426, shows a reniform-shaped opening in the antapical membranes covering the thecal layer. We believe this area may be the point of attachment to the apical pore of another cell; the apical pore (Fig. 3) and the reniform opening (Fig. 9) are similar in shape and orientation.

Nucleus

Figure 7 shows the U-shaped nucleus of isolate 429. The English and American isolates and *G. acatenella* have nuclei of similar morphology and



Figures 16-19:

Scanning electron micrographs of *Gonyaulax acatenella*. Fig. 16, Dorsal view of older cell with intercalary growth evident. Note plate 3'' shows intercalary growth along two of its faces on the organism's left side, X1410. Fig. 17, Dorsal view of younger cell showing no intercalary growth along margins of plate 3''. Note hypothecal flanges along plate margins, X1530. Fig. 18, View of epitheca and apical pore, X1390. Fig. 19, View of hypotheca. Note flanges along plate margins especially near sulcal region, X1400.

position. The open end of the U is directed toward the sulcus. The U-shaped nucleus is in the same plane as the cingular groove and not as figured earlier (25). These data are in contrast to the figure of a flattened sphere (Figs. 2C,D) given originally for *G. acatenella* (4).

The appearance of the plastids, plate tabulation (Figs 1E-J) and toxicity of *Pyrodinium phoneus* (23) are characters in common with isolate 426. For these reasons we believe it is a species of *Gonyaulax* related to the *G. acatenella-G. tamarensis* group. The nucleus of *P. phoneus* was figured as a small sphere, located slightly below the cingulum; it appears to be out of proportion for a dinoflagellate cell. We believe that *P. phoneus* may possess a U-shaped nucleus.

Temperature

The three cultures were tested at three temperatures: 11, 16 and 21°C. Strains 173a and 429 grew well at all three temperatures with the shortest generation time, 3 days, at 16°C. Strain 173 did not grow at 11°C and grew best at 16°C with a generation time of 6.5 days. Forms assumed by others to be identical to *G. tamarensis* var. *excavata* occur in tropical waters of about 27 - 28°C and 35.5 - 35.9 °/oo salinity (16,26) and are therefore different from the strains studied by us.

Bioluminescence

By visual observation isolates 429 and 173a are bioluminescent and isolate 173 is not.

These results were confirmed and quantified using a photomultiplier photometer. The following values of bioluminescence expressed in quanta/cell were obtained: 3.1×10^7 for *Gonyaulax polyedra*, 8.3×10^5 for isolate 429 and 4.0×10^5 for isolate 173a. No bioluminescence was detected in isolate 173 at a cell density of 3563 cells/ml and at the most sensitive settings of the instrument. Under these conditions bioluminescence would have been detected if cells of isolate 173 emitted as little as one ten thousandth the bioluminescence of *G. polyedra* cells.

CONCLUSIONS

The obvious question to ask is: do isolates 173, 173a and 426 represent previously described species or new forms?

English isolates

Two isolates (173, 173a) both previously determined to be *G. tamarensis* from its type locality region are distinguishable on the basis of presence or absence of a ventral pore, temperature optima and bioluminescence. We feel they represent separate species. On the basis of a sketchy original description

and lacking any physiological data (2), we cannot know whether 173 or 173a more closely resembles the typical *G. tamarensis*. Morphologically isolate 173 is distinguishable from 173a by the presence of a ventral pore. Lebour (2) could have overlooked such a pore. In view of the absence of a history of paralytic shellfish poisoning in the Tamar River Estuary (14) it is reasonable to assume that the form Lebour described was non-toxic. As toxicity has not been associated with isolate 173 we propose to emend the description of *Gonyaulax tamarensis* to encompass the characters of isolate 173. Isolate 173 has been the subject of several publications in which it was assumed to be *G. tamarensis* thus making it a more suitable choice than 173a in our opinion. To determine the identity of isolate 173a will require further studies. It would be most interesting to know if isolate 173a is toxic.

American forms

All available evidence including bioluminescence, the presence of two toxin fractions, and morphological similarities, suggests that the Eastern Canadian, New England and northeastern English toxic *Gonyaulax* are identical. Braarud (3) included the Gulf of Maine-Bay of Fundy material in the circumscription of *Gonyaulax tamarensis* var. *excavata*. Isolate 426 conforms to the description of *G. tamarensis* var. *excavata* (3). For these reasons we believe Braarud's variety represents the New England form. We do not consider it to be conspecific with typical *G. tamarensis* (here considered to be represented by isolate 173) because it is toxic, bioluminescent, has a lower temperature tolerance and lacks a ventral pore. *G. tamarensis* var. *excavata* Braarud was elevated to specific status (16). We agree with this and consider the New England form to be *Gonyaulax excavata;* however we do not agree with Balech's (16) circumscription of *G. excavata* for the following reasons. Balech's illustrations (Figs 2G-L) and description do not conform to those of Braarud (3) (Figs. 1K,L). Balech's specimens have the nucleus in the epitheca, possess a ventral pore, are smaller in size (Table I) and come from high salinity, tropical waters (27.3-28.1°C; 35.6-35.9 o/oo (16). A similar form called *G. tamarensis* f. *excavata* was reported off Florida (27-28°C; 35.5 o/oo) (26). We believe that the specimens figured by Steidinger and Williams (26) and Balech (16) represent an undescribed tropical species which is not conspecific with temperate *G. tamarensis* var. *excavata*. We conclude that the Eastern Canadian, New England and northeastern English toxic dinoflagellate is *Gonyaulax excavata* (Braarud) Balech, 1971, as originally described by Braarud (3). A similar conclusion has been reached for the northeastern English toxic organism (14).

Western Canadian form

G. acatenella is readily distinguishable from *G. tamarensis* and *G excavata* by the possession of numerous and pronounced flanges on the hypotheca (Figs. 15, 19) and chain lengths of 4-6 cells (18). Isolates 173, 173a, 426, 429 have

not been observed to form chains of six cells. A major dinoflagellate toxin dissimilar to saxitoxin has never been reported from Western Canadian material although such a dissimilar toxin has been reported for Eastern Canadian material and New England material (5, 6). The above morphological and physiological differences lead us to the conclusion that *G. acatenella* is a valid species easily distinguishable from *G. excavata* and *G. tamarensis*.

Belgian form

On the basis of the appearance of plastids, plate tabulation and toxicity we propose to transfer *Pyrodinium phoneus* to the genus *Gonyaulax* [*Gonyaulax phoneus* (Wołoszyńska and Conrad) comb. nov. basionym: *Pyrodinium phoneus* Wołoszyńska and Conrad, 1939, Bull, Mus. Roy. Hist. Nat. Belg. 15 (46): 3-5, text-figs. 1-12]. This form has been considered to be synonymous with *G. tamarensis* var. *excavata* (27), however, we believe that the description of *G. phoneus* comb. nov. is insufficient to draw a conclusion.

Future studies

Most of the isolates we have studied appear morphologically similar, but they possess distinct physiological differences. We believe that further investigations will reveal more morphological and physiological differences between the isolates. Demonstration of genetic or mating barriers would aid in defining species. Our observations on fusing cells and the recent detection of genetic recombination in dinoflagellates (28) indicate that a genetic analysis may be possible. The technique of DNA-DNA hybridization applied to these isolates is perhaps more feasible and could be used to determine relatedness.

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RED TIDES I HAVE KNOWN

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ABSTRACT

Quantitative data for the species composition of dinoflagellate red tides which I have observed during the last 25 years in southern California and elsewhere are presented. One species is usually markedly more plentiful than any other. *Gonyaulax polyedra* and *Prorocentrum micans* have been the dinoflagellates responsible for red water along the coast of southern California. Near Madang, in New Guinea, I observed red tides of *Gonyaulax polygramma*, *Pyrodinium bahamense* f. *compressa* Bohm, and the blue-green alga *Trichodesmium*, and at Oyster Bay, Jamaica, red water caused by *Pyrodinium bahamense*.

Red water typically is restricted in its distribution, both vertically and laterally. Water collected within a red patch containing large numbers of *Prorocentrum* was shown to have growth-promoting properties for this species in culture, while water from outside this patch did not. The vitamin B_{12} content of both samples by *Euglena* bioassay was identical, 0.02 mμg ml^{-1}, and *Prorocentrum* cells from these water samples both contained 0.7 mμg mg^{-1} dry weight. Sea water collected at a time when there was no red tide contained much smaller amounts of vitamin B_{12}, as did *Prorocentrum* cultured in medium made up with this sea water. Thus *Prorocentrum* may accumulate this and perhaps other organic micronutrients in excess of immediate requirements. No evidence was obtained for toxicity of *Gonyaulax polyedra*, either in red water or in culture.

INTRODUCTION

"Red tides" are spectacular phenomena when the sea turns the color of tomato soup because of the presence of large numbers of organisms, often dinoflagellates,containing orange or red pigment. When the species responsible produces a toxin, aquatic animals may be killed. The longest series of observations of red tides in the world is probably that at La Jolla, California. Between 1924 and 1945, Allen (1-5) documented 5 red tides there, two caused by *Prorocentrum micans* in 1924 and 1933, and three caused by *Gonyaulax polyedra* in 1938, 1942 and 1945. My observations of this interesting phenomenon at La Jolla began in 1949 and continued until 1961. I have also been privileged to see red tides in Oyster Bay, Jamaica and near Madang in New Guinea, and, since 1967, in the vicinity of Santa Barbara, California. After my departure from Scripps Institution of Oceanography, others continued observations of red tides there (9).

QUANTITATIVE DATA WITH REGARD TO DATE, LOCATION AND SPECIES COMPOSITION

Cell counts of the red tides which I have observed personally (Table I) show clearly that only a single dinoflagellate is responsible for the discoloration of the sea at any one time. The only exception to this generalization was in 1961.

Table I

Red tides observed by the author

Date	Location	Organism Responsible	Number of cells ml^{-1} in Surface Water		
			Most Plentiful Dinoflagellate	Other Dinoflagellates	Others
7/11/52	La Jolla, Ca.	*Prorocentrum micans*	2470	36	34
3/9/54	"	"	12000	40	-
4/26/54	"	"	2080	12	-
4/21/55	"	"	1430	330	-
6/17/58	Ensenada, B.C.	*Gonyaulax polyedra*	15400	25	-
7/4/58	"	"	1900	82	2
7/21/58	Imperial Beach, Ca.	"	7510	-	-
8/15/58	La Jolla, Ca.	"	22200	43	-
4/13/61	"	*Prorocentrum micans*	880	122(95 *Gonyaulax*)	-
4/28/61	"	*Gonyaulax polyedra*	3780	1710 (1350 *Prorocentrum*)	-
1/13/66	Oyster Bay, Jamaica	*Pyrodinium bahamense*	590	16	-
10/13/69	Bostrem Bay, New Guinea	*Gonyaulax polygramma*	3440	-	-
10/4/70	Santa Monica, Ca.	*Gonyaulax polyedra*	13180	200	-
7/30/71	"	"	20000	0	-
8/4/71	Rincon, Ca	"	8060	435	-
8/7/71	"	"	12610	75	-
8/16/71	Goleta Bay, Ca.	"	9330	0	-
8/18/71	"	"	5310	125	-
9/8/72	"	"	5160	0	1
9/15/72	"	"	7000	63	-
10/22/73	"	"	1440	1	-
8/2/74	Long Beach, Ca.	"	7540	9	-
8/10/74	Ventura, Ca.	"	3720	13	25
8/19/74	Rincon, Ca.	"	11200	28	3

The dominant organism was accompanied by a mixture of other dinoflagellates, relatively few in number. It is remarkable that only two dinoflagellates, *Gonyaulax polyedra* and *Prorocentrum micans*, have been responsible for every red tide I have seen off Southern California, yet the nutritional requirements of photosynthetic dinoflagellates, as determined in culture, appear to be similar, and no special features distinguish either *Gonyaulax polyedra* or *Prorocentrum micans*, as far as we know. Indeed, accompanying organisms are almost exclusively dinoflagellates (Table I), in California a mixture of *Ceratium dens, C. furca, C. fusus, Gymnodinium splendens* and a number of species of *Peridinium*, which suggests that conditions are generally favorable for the growth of dinoflagellates at times when a red tide develops.

Although there have been exceptions (9), red tides typically occur in the summer in S. California, *Prorocentrum* red tides somewhat earlier than those in which *Gonyaulax* is the dominant organism. In Oyster Bay, Jamaica, red water persists during the entire year, and the organism responsible for the discoloration of the water, and incidentally for brilliant displays of bioluminescence, is *Pyrodinium bahamense*. In New Guinea on Kranket Island near Madang, large numbers of a similar *Pyrodinium bahamense* f. *compressa* Bohm[1] were seen in October, 1969, during the "Alpha Helix" expedition to New Guinea. The more open Bostrem Bay near by developed a deep orange color at the same time, but here *Gonyaulax polygramma* was responsible. In the open sea off Madang, a third red tide was observed, due to large numbers of *Trichodesmium* sp. This organism is a red blue-green alga, not a dinoflagellate. All the red tides observed in California and in New Guinea (Table I) occurred during the dry season, so run off from the land does not seem to be an important factor in the development of a red tide.

In 1958, 1971 and 1974, red water appeared first in the southernmost part of the California coast, as far south as Ensenada, Baja California in 1958, and progressed northwestward up the coast over a period of several months. Although water generally moves north along the Southern California coast, it is not known whether the organisms or only the favorable conditions moved northward. However, in La Jolla in 1952, plankton samples were collected from Scripps pier every day. Samples for each week were combined and the number of *Prorocentrum* in these samples was counted after a red tide of *Prorocentrum* occurred in July (Fig. I). The increase in the number of this dinoflagellate between May 26 and July 10th could be accounted for by a growth rate of 0.3 divisions day $^{-1}$ (broken line, Fig. 1), in good agreement with growth rates measured in culture. Thus it is not impossible that the red tide developed by cell division of the local *Prorocentrum* population.

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[1] The author is indebted to Dr. Enrique Balech for the identification of the *Pyrodinium* and *Gonyaulax* from New Guinea.

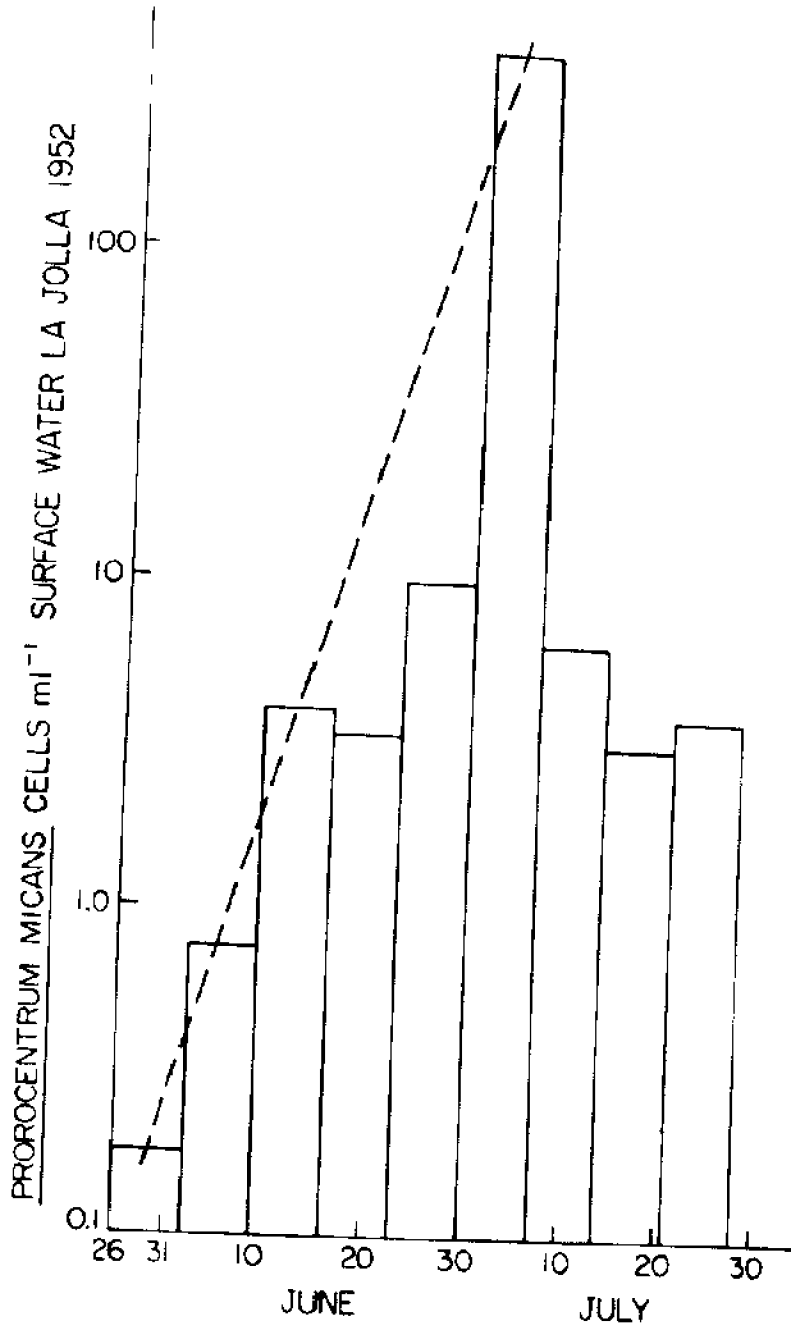

Figure 1: The number of *Prorocentrum micans* in water samples collected from the pier at Scripps Institution of Oceanography in May-July, 1952. Samples were collected daily. One ml samples for each day of a week were pooled and counted to give the average number of cells for that week. Red water was observed on July 7-11, 1952. The dotted line is the calculated growth rate of 0.3 divisions day^{-1}.

The organsims collected during red tides were motile and appeared to be in good condition. The number of bacterial colonies growing on plates inoculated with red water collected aseptically by Donald Lear at La Jolla, 7/26/54, was not higher than usual: less than 50 bacteria ml^{-1}. During the last few days of red water on several occasions in Santa Barbara, large numbers of resting spores of *Gonyaulax* were observed.

DISTRIBUTION

Red water is usually restricted to discrete patches, the edges of which are so distinct that it is possible to locate them even from a small boat. The same organism is found outside as well as inside these patches but in much lower numbers (Table II). On three occasions when samples were collected from different depths in a red patch during the day, large numbers of the organism responsible for the discoloration of the water were only found in the top few meters (Fig. 2). Dinoflagellates other than the most common species were evenly distributed with depth, down to 10-15 m. In laboratory cultures, *Prorocentrum micans* and *Gonyaulax polyedra* are only weakly phototactic, but there is evidence for their vertical migration in the sea (7, 8). Red patches have been observed to disperse at night and reform in the morning (9), possibly through phototaxis. However, the sharp horizontal boundaries cannot be explained by phototaxis and may mark the separation of two distinct water masses.

Table II

Prorocentrum micans, cells ml^{-1} in surface water inside and outside red water patches, La Jolla, California

Date	Cells ml^{-1} Inside red patch	Cells ml^{-1} Outside red patch
7/10/52	1350	240
7/11/52	2456	440
7/21/52	800	260
7/26/54	2080	150

BIOLOGICAL PROPERTIES OF RED WATER

During the red tide of 1952, water samples from inside and outside a red patch were tested for promotion of the growth of *Prorocentrum micans* in culture. Both sea water samples were filter-sterilized and then added as a supplement to mineral-enriched aged sea water medium. Water collected

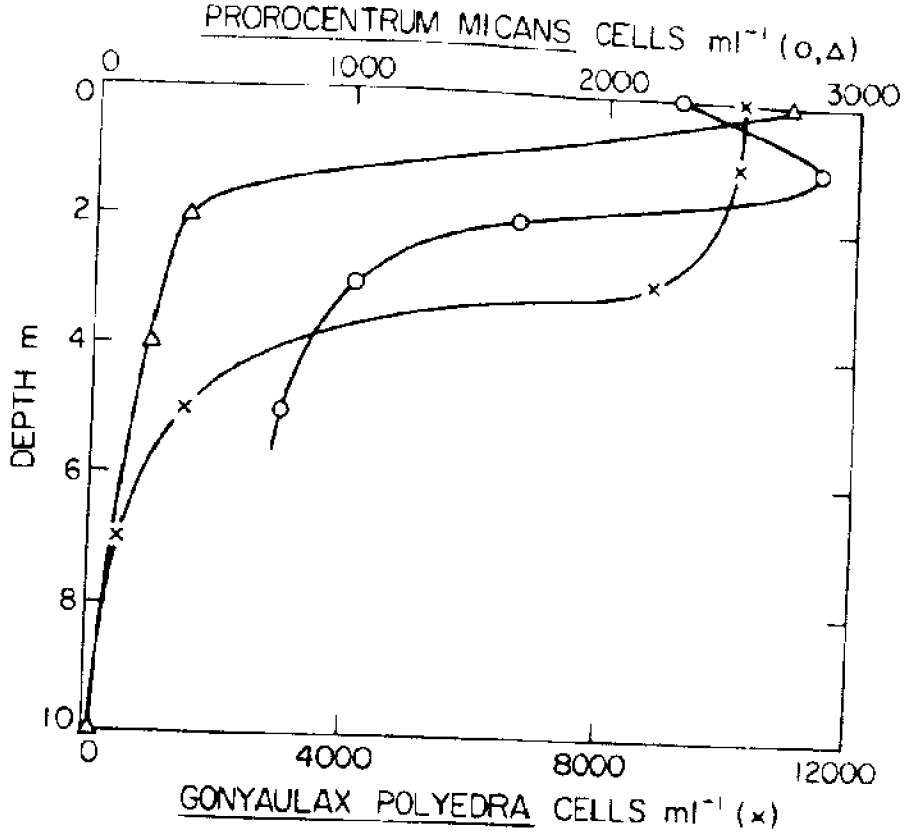

Figure 2: The distribution of red tide organisms with depth: *Gonyaulax polyedra*, Ensenada, B.C. 6/18/58 (X); *Prorocentrum micans*, La Jolla, Ca. 7/10/52 (0), and 7/11/52 ( ). Accompanying dinoflagellates: on 7/10/52 *Ceratium dens*, 100 ml^{-1}, *C. furca*, 100 ml^{-1}, *Peridinium* sp, 100 ml^{-1}; on 7/11/52, *C. dens*, 10 ml^{-1}, *C. furca*, 10 ml^{-1}, *Peridinium* sp, 10 ml^{-1} at all depths. Samples were collected during the day.

inside a red patch promoted the growth of the *Prorocentrum*, while water from outside this patch was only slightly growth-promoting (Table III). Boiling the water samples abolished all activity.

Since dinoflagellates require an external source of vitamin B_{12}, the possibility that this vitamin was responsible for the growth-promoting activity of water from the red patch was explored. Vitamin B_{12} was detected by *Euglena* bioassay in sea water from both inside and outside the red patch, but both samples contained the same amount of vitamin B_{12}, 0.02 mμg ml^{-1}. *Prorocentrum* cells, removed by filtration from the two water samples, were also similar with respect to their content of vitamin B_{12}, 0.7 mμg mg^{-1} dry weight or about 3 mμg in 10^6 cells. This value was kindly corroborated by independent assay carried out by Dr. David Hendlin of Merck and Co. No vitamin B_{12} could be detected in the aged sea water collected before the red tide, and very little in bacteria-free *Prorocentrum micans* grown in medium

Table III

Growth promotion of *Prorocentrum micans* in culture by filter-sterilized sea water from inside and outside a red water patch, 7/10/52.

Basal medium: aged sea water supplemented with nitrate, 2 mM, phosphate, 0.2 mM, $FeCl_3$, 0.05 mM, EDTA, 1 mg. l^{-1} and micronutrients (Arnon's A_4B_7). Natural illumination, 20°C. Inoculum: 0.05 ml; medium : 5 ml.

Supplement:	0	4% soil extract	5% sea water from *inside* red patch	5% sea water from *outside* red patch	5% sea water from *inside* red patch, *boiled* 1 min	5% sea water from *outside* red patch *boiled* 1 min.
Yield, cells ml^{-1}	0	19670	16000	150	200	0

made up using this sea water, 0.13 mμg in 10^6 cells in one culture (assayed 3/31/53) and none in another (assayed 2/9/53).

Considered together, these observations point to the existence of a growth-promoting substance or substances, present in the red patch and absent outside it. It is unlikely that inorganic macro- or micronutrients were responsible for the growth promotion observed, since the assay medium contained P, N, Fe and micronutrients. Vitamin B_{12} does not seem a likely candidate, since there was no difference with respect to this vitamin either in water samples or cells from inside and outside a red patch. However the higher vitamin B_{12} content of *Prorocentrum* from the red tide, as compared with the same species in culture, suggests that these cells can accumulate an excess of this and perhaps also of other organic micronutrients. The accumulation of growth factors in excess of immediate needs and the observed progression of red water northward along the coast of California raise the possibility that the initiation of a red tide may be distant, both in time and in space, from its realization.

THE QUESTION OF TOXICITY

Since *Gonyaulax catenella* is known to produce saxitoxin (6, 10, 11), the possibility that *Gonyaulax polyedra* might be toxic was examined, although no mass mortality of any kind was observed during any of the red tides listed in Table I. Samples (1 1/2 or 2 liters) of the *Gonyaulax* red tide in June and July, 1958, were transferred to Fernbach flasks in the laboratory at Scripps

Institution of Oceanography, and aerated gently. Sea water collected during the previous winter and laboratory cultures of *Gonyaulax polyedra* served as controls. Two anchovies or two red crabs [2] (*Pleuroncodes planipes*) were introduced into each flask, and the behavior and survival of these animals were observed for several days. Neither the naturally occurring nor the cultured *Gonyaulax polyedra* showed any evidence of toxicity under these conditions.

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[2] The author wishes to acknowledge the collaboration of Carl Boyd in these experiments.

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DINOFLAGELLATE BIOLUMINESCENCE: MOLECULAR MECHANISMS AND CIRCADIAN CONTROL

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ABSTRACT

The so-called "phosphorescence" of the sea is due in very large measure to the bioluminescence of dinoflagellates, which are virtually ubiquitous in the oceans of the earth. Many of the red tide species of dinoflagellates are luminous, but not all. This is also true for dinoflagellates generally; perhaps one half are bioluminescent. Many dinoflagellates exhibit circadian (daily) rhythmicity in bioluminescence demonstrated in both laboratory and field studies. Many other physiological systems are subjected to circadian control, a fact of significance with respect to dinoflagellate physiology in general. Light emission by individual cells is typically exhibited as a brief (0.1 sec) bright (10^7 to 10^{10}photons)flash, which occurs upon mechanical stimulation, mediated by a propagated cell membrane action potential. In extracts subcellular particles can be isolated which are capable of emitting a similar flash following a pH jump from 8 to 5.7. It is postulated that the *in vivo* flash is the result of a transient change in permeability to H^+ ions in the membrane of the luminescent organelle or element.

INTRODUCTION

Awak'd before the rushing prow,
The mimic fires of ocean glow,
  Those lightnings of the wave;
Wild sparkles crest the broken tides,
And flashing round, the vessel's sides
  With elfish lustre lave;
While far behind, their livid light
To the dark billows of the night
  A blooming splendour gave.[1]

Anyone who has gone swimming or boating at night in the ocean will have seen the beautiful sparkling luminescence, the so-called phosphorescence of the ocean which occurs after the water is disturbed. The true image of its sometime brilliant and even spectacular appearance is difficult to capture, but it has been well represented in poetry[1], prose[2], and painting. Collecting from red tides off Southern California, one sees the undulating luminescent pattern left behind by fleeing fish, darting away as the boat approaches. World War II aviators based on carriers in the South Pacific tell of the ease with which they could relocate their ship after a mission: a luminescent wake is seen for many miles behind a ship as a consequence of the persistent wake which stimulates the luminescence of the cells. A submarine or a diver under water may be equally easily located by the luminescent track. More poetic prose is found in the record of the "Challenger" as she passed southeast of the Cape Verde Islands[2]:

[1]Sir Walter Scott, *Lord of the Isles*, i, 21 (1815).

[2]C.W. Thomson, Voyage of the Challenger, 2:85 (1877)

"On the night of the 14th the sea was most gloriously phosphorescent to a degree unrivalled in our experience. A fresh wind was blowing and every wave and wavelet, as far as one could see from the ship on all sides to the distant horizon flashed brightly as they broke while above the horizon hung a faint but visible white light. Astern of the ship, deep down where the keel cut the water, glowed a broad band of blue, emerald green light, from which came streaming up, or floated to the surface, myriads of yellow sparks, which glittered and sparkled against the brilliant cloud light below, until both mingled and died out astern far away in our wake. Ahead of the ship, where the old bluff bows of the "Challenger" went ploughing and churning through the sea, there was enough light to read the smallest print with ease. It was as if the "Milky Way" as seen through a telescope, "scattered in millions like glittering dust" had dropped down into the ocean, and we were sailing through it."

For many years the origin and nature of the "phosphorescence" of the ocean was debated (20). Descartes thought that when a wave hits an obstacle, the agitation imparted to the particles of salt caused them to separate from the particles of water and to "generate sparks" similar to those emitted by flint. Over the years, many other ideas and variations thereupon were put forward, including theories involving electricity, phosphorus and putrefaction. Although many serious students were concerned with the question, including Robert Boyle, J. J. D. de Mairan and Benjamin Franklin, it is significant that none of the great microscopists of the period[3] recorded any interest or observations concerning the phenomenon. It was thus not until the late part of the eighteenth century that luminescence of the sea was definitely attributed to living organisms, and even then dispute continued, both as to whether or not all cases could be so explained, and how living organisms are capable of emitting light.

The first definitive demonstration that dinoflagellates are luminous is attributed to G. A. Michaelis in 1830; the fact that these dinoflagellates are clearly responsible for the sparkling luminescence of the sea can be credited to him and to C. G. Ehrenberg (20).

ORGANISMS: LUMINESCENCE AND RED TIDES

Although it was suggested by Kofoid (as quoted in Harvey (19), p. 124) that all dinoflagellate species might be luminescent, this is definitely not the case. Sweeney (44) reported that only about one half of the species which she isolated and/or cultured in La Jolla were luminous. Similar proportions were found in observations which included additional species (32); (33); (48); all of these are summarized by Schmitter (36), by Esaias (14), and by Tett and Kelly

[3]Marcello Malpighi (1628-1694), Jan Swammerdam (1629-1682), Antony van Leeuwenhoek (1632-1723), Robert Hooke (1635-1702), and Nehemiah Grew (1614-1712).

(49). From this it can be seen that many dinoflagellate genera comprise both luminous and non-luminous species. In the genus *Peridinium* there are many luminous and only a few non-luminous species, while in *Ceratium* the converse pertains. In *Gonyaulax* all species which have been studied are recorded as luminous [4], while in some genera (*Gyrodinium*) there are no reported luminous species. *Noctiluca miliaris* includes both luminous and non-luminous varieties (10), although it might be that the two should be classified as different species.

Although several of the red tide organisms are also bioluminescent, this ability is not associated with all red tide species. Along the southern part of the U. S. West Coast, from Santa Barbara southward, *Gonyaulax polyedra*, which is brightly luminescent, has been responsible for many of the red tides. But *Prorocentrum micans*, recorded by Sweeney (44) as non-luminescent, has also bloomed. Farther north, *Gonyaulax catenella*, also a luminous species, appears to be the one most frequently involved. On the East Coast in the Gulf of Maine, another luminous *Gonyaulax* species, *G. tamarensis*[4] is involved, while along the west coast of Florida the non-luminous *Gymnodinium breve* is the red tide species. The many apparently sympatric dinoflagellate species which occur in the same regions must have physiological and/or nutritional features which distinguish them with regard to their growth and behavior in giving rise to red tide blooms, but these features have not yet been elucidated. All in all, however, there have been very few studies of the fundamental biological and physiological properties of dinoflagellates.

FUNCTION OF BIOLUMINESCENCE IN DINOFLAGELLATES

There is no generally accepted explanation for the role of bioluminescence in dinoflagellates. The question is brought into focus by the fact that there are closely related, including some presumably sympatric species, some possessing and others lacking the ability to luminesce. A reasonable hypothesis is that the sparkling luminescence of the water confers a selective advantage on the population as a whole, and that selection towards a greater percentage of luminous members is not so strong once the luminescence of the water is above a certain level. A specific suggestion along these lines is the burglar alarm hypothesis: the presence of a herbivore grazing on the dinoflagellates would be revealed by their luminescence, thus giving an advantage to the predators of the herbivore and thereby contributing to the survival of all the dinoflagellates, luminous or not (3). However, Esaias and Curl (15) presented experimental evidence showing that there was less grazing on more brightly luminous members of a population of a given species, both being derived from the same clone. Brighter and dimmer members were obtained by mixing labeled populations obtained from different times of the day, or by inhibiting the ability to luminescence of one part of a population.

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[4]However, see the contribution by Loeblich and Loeblich in this volume.

IN VIVO LUMINESCENCE

Red tides of *Gonyaulax polyedra* often involve the formation of distinct patches, an accumulation of the organisms in high numbers - up to 10^6 or 10^7 cells per liter. This is perhaps one hundred times greater than the population density in the water outside the patch, only a few meters away. At night in a boat one may in fact be startled by the sudden increase in luminescence from the wake as one crosses into such an area. Several field studies of bioluminescence and dinoflagellate populations have been reported which deal with various aspects of this matter, such as day-night and seasonal patterns of luminescence, mapping, and distributrion of organisms as well as accumulation mechanisms (37, 38, 39, 40, 41, 42, 4, 43, 32).

The luminescence of the individual cell (Fig. 1) commonly occurs as a discrete flash, which occurs upon stimulation and lasts only a fraction of a second (21, 25). Depending on species, between 10^7 and 10^{10} quanta per cell are emitted per flash (1, 39, 49). In *G. polyedra* at 9° the half decay time is about 50 msec (Fig. 1). The temperature coefficient is about 20

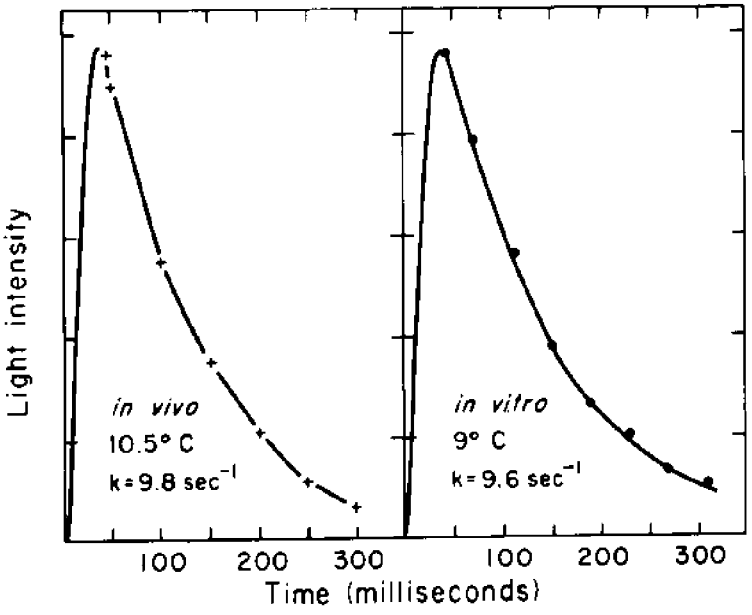

Figure 1: Comparison of *in vivo* and *in vitro* flashes from *Gonyaulax polyedra* at about 10°C. Ordinate: Light intensity in arbitrary units, the two having been normalized. Abscissa: time in milliseconds. The decay of light intensity is accurately exponential in both cases, with first-order rate constants of 9.8 and 9.6 sec^{-1} respectively. The *in vivo* flash was recorded from a culture placed in front of a phototube, stimulated mechanically by tapping lightly on the housing. The *in vitro* flash was obtained by mixing, in the stopped-flow apparatus, scintillons suspended in assay buffer (pH 8.2) from one syringe and 0.03 N acetic acid from a second syringe. Rise times were also the same - about 40 msec to the peak.

kcal, so that the half decay time is about 20 msec at 20° (6, 8). The color of the light is a deep blue, peaking at about 475 nm (2, 30)(Fig. 2).

Individual cells will give additional flashes upon repeated stimulation. Extensive physiological studies with isolated individual cells have been carried out by Eckert and colleagues (9, 11, 12) using *Noctiluca miliaris*, the giant naked dinoflagellate. They were able to stimulate electrically, to demonstrate the existence of a conducted action potential which serves to trigger the bioluminescent flash, and to show that the light comes from discrete and

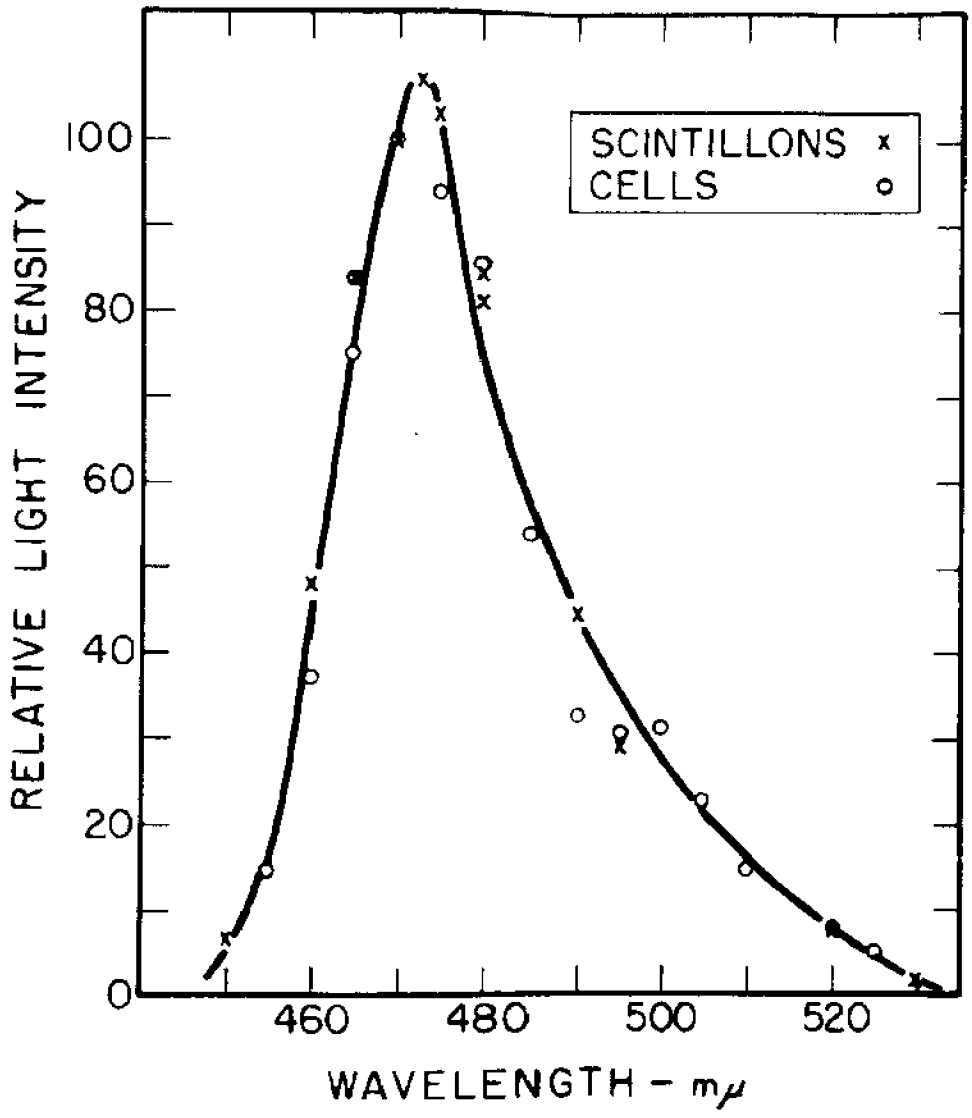

Figure 2: This figure illustrates the similarity between the color of light emitted by isolated scintillons and by living *Gonyaulax* cells. The spectra were obtained using a small Bausch and Lomb monochromator with a second photomultiplier at the entrance slit to monitor the intensity of the incident light. No corrections for phototube sensitivity and monochromator efficiency have been made.

microscopically resolvable spots, which they referred to as "microsources". In *Gonyaulax* an excellent experimental analysis of the sensitivity of individual cells to mechanical stimulation, which is of course the natural mode for triggering bioluminescence, has been provided in the paper of Christianson and Sweeney (5).

Dinoflagellates also emit light even if they are not stimulated (Sweeney and Hastings (47). If a cell suspension is left undisturbed in front of a sensitive photomultiplier, a record such as shown in Figure 3 is obtained. The closely spaced vertical lines are due to flashing, which may result from cells bumping into one another or into the side of the tube. It is not due to external stimulation (building vibrations, etc.), but it may be truly spontaneous, or related to swimming and flagellar movement.

A second form of bioluminescence emission is also evident. A gradual rise and fall of the baseline on the record results from a steady light emission or glow. The cause for this is not yet known; quantitatively it represents only a small fraction of the number of quanta which the cell is capable of emitting.

CIRCADIAN RHYTHMS OF LUMINESCENCE

The record of Figure 3 was made under constant conditions, the culture being maintained all the while in the dark within the phototube housing at 23°. The second glow peak recurred about 24 hours after the first. A similar rhythm in flashing frequency can also be seen in the record.

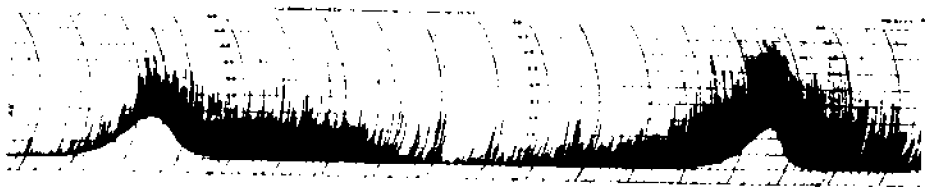

Figure 3: The spontaneous bioluminescence of *Gonyaulax polyedra*, measured from vial of cells (approximately 10^4 cells ml^{-1}) kept in the phototube housing in the dark in front of the phototube at 23°. Ordinate light intensity, baseline was offset: abscissa, time one division equals 20 min. Span of record, about 34 hours. The smooth rise and fall is due to a steady glow, the peaks being about 24 hours apart. The vertical traces are due to flashing.

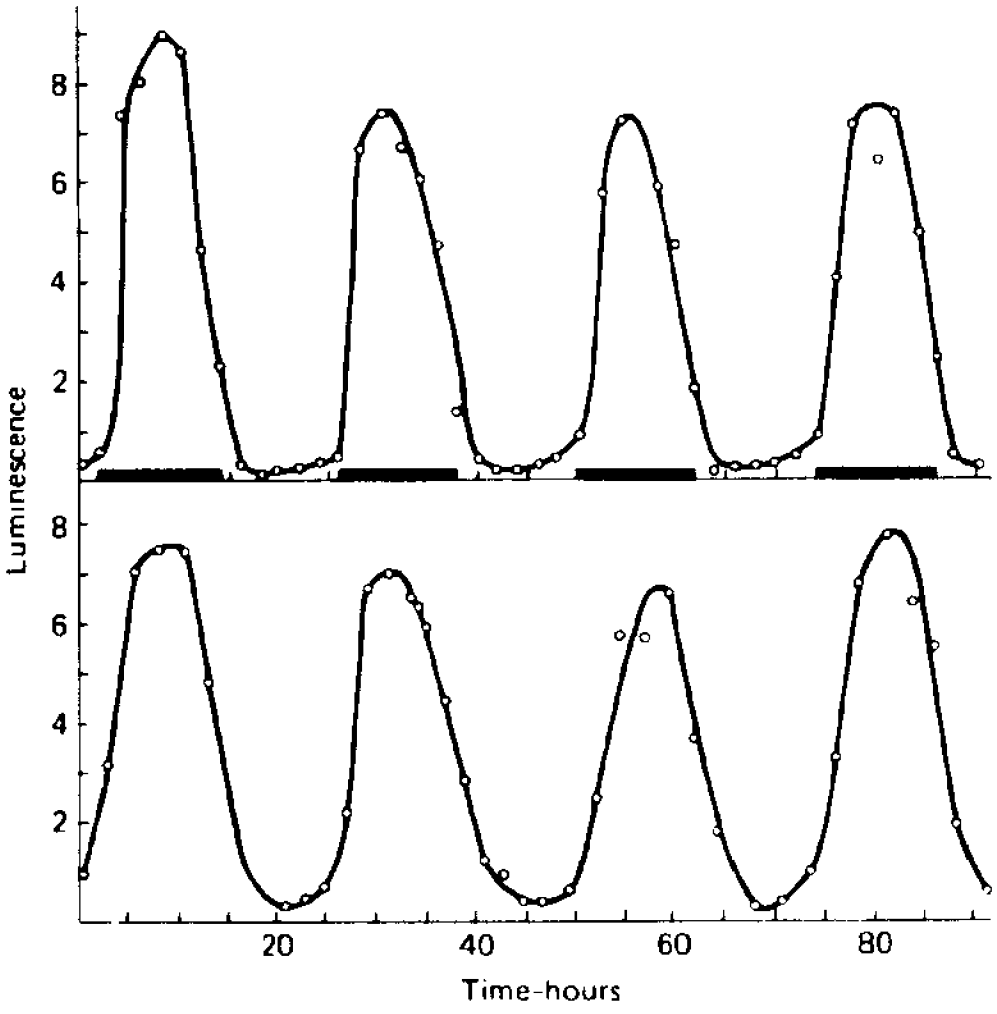

Figure 4: Persistent circadian rhythm of stimulated luminescence of *Gonyaulax*, first under 24 hour light dark cycle (top) followed by conditions of constant light intensity (100 foot candles) and constant temperature (21°C), where the period was about 24 1/2 hours. Ordinate, total luminescence obtained upon mechanical stimulation of an aliquot. Abscissa, time.

The biology of the circadian rhythm in *Gonyaulax polyedra* has been extensively studied utilizing both the "glow" rhythm and the rhythm of "stimulated" bioluminescence, i.e. the toal amount of light which can be obtained from a culture by mechanically stimulating to exhaustion (Fig. 4).

These studies (47, 26, 22) have served to establish and illustrate the basic clock-like properties of circadian rhythms:

1. *Persistence*: the rhythm continues for many days under constant conditions with a period close to but not exactly equal to 24 hours (Fig. 4).
2. *Temperature compensation*: the period or frequency is not greatly different at different temperatures within the physiological range.
3. *Phase lability*: the phase of the rhythm may be shifted under the influence of environment periodicities, illustrated in Figure 4 for a change in light intensity.

In most attempts to dissect and understand the mechanisms involved in circadian rhythms it has been useful to distinguish the timing *mechanism* and the controlled physiological *processes*: the clock and its hands. In *Gonyaulax* and probably other dinoflagellates there are aspects of the cell's physiology other than luminescence which are rhythmic, notably cell division (47) and photosynthesis (27, 28). Any fundamental investigation of the physiology and biochemistry of dinoflagellates involved in red tides must be done with full cognizance of these rhythmic aspects of the cell.

The nature of the rhythmic mechanism itself has eluded description, even in most general terms. (29, 24). Most investigators believe that circadian rhythms are generated by a cellular biochemical system and that, though the details may vary from species to species, some fundamental mechanism is common to all. Two conceptually different approaches have emerged. One conceives of the mechanism as a "tape" with discrete biochemical events occurring in a strict sequence. In molecular terms this has been interpreted as sequential gene expression: transcription of DNA proceeds, one gene at a time, at a controlled rate, as described most explicitly in the chronon model of Ehret and Trucco (13). Another approach conceives of the rhythmic mechanism as a biochemical network with self-sustained oscillations arising from feedback within the biochemical system (35). A recent model (34) proposes that these oscillations involve membranes where ion concentrations across membranes and membrane ion transport channels function as a feedback system to generate self-sustained circadian oscillations in ion concentrations, which in turn control the physiological rhythms.

IN VITRO LUMINESCENCE: THE pH TRIGGER

The biochemical basis for dinoflagellate bioluminescence is also dual in nature. In parallel with the glow and the flash of the living cell, extracts also have two comparable emission modes. Extracts in buffer at pH 8 thus contain two classes of elements. Let us consider first those responsible for a "glow". Following high speed centrifugation, the soluble supernatant contains an enzyme (*Gonyaulax* luciferase) and a substrate (*Gonyaulax* luciferin) which when mixed with oxygen at pH 6 to 6.6 will give rise to a long-lasting light

emission whose half decay time may typically be between 2 and 20 minutes. This reaction is somewhat unusual in its dependence on pH; it cuts off sharply at pH 7 and is virtually zero above 7.5. The substrate also exhibits unusual behavior as a function of pH. An estimation of its molecular weight by gel filtration gives a value of about 100,000 even though the partially purified luciferin, which is fully active in the assay at pH 6.6, has a molecular weight of about 500. The explanation is that there is a substrate binding protein which binds the substrate tightly at pH 8 but not at 6.6 (16). The pH activity profile is thus attributable to the concerted action of the two proteins. When the pH of an extract is adjusted from 8 to 6.6, the luciferin, released from its binding protein, reacts with luciferase, which is now active at this pH.

The sedimented particulate matter from the extract referred to above possesses the potential for bioluminescent flashing. The active particles are referred to as scintillons (7), but earlier indication that they are associated with guanine crystals (30, 8) has now been shown to be incorrect (18). However, a definitive identity of the subcellular structures involved is not yet available (36) even though they can be purified by sucrose isodensity gradient centrifugation.

The activity of scintillons can be evoked by a pH jump from 8 to 5.7; this results in a flash which closely mimics the flash of the living cell (Fig. 1). Although the molecular elements of scintillons are not lost by dilution or by washing, treatment with high salt or with detergents results in the differential removal of the luciferin-binding-protein or the luciferase respectively (31). Also, scintillons which have been "discharged" by a pH jump can be "recharged" by readjusting the pH to 8, adding purified (free) luciferin, and allowing a time for it to be taken up by the scintillons. A second pH jump results in a second flash very similar to the first (17).

The model for *in vivo* luminescent flashing (31) thus envisions a poised state in some particle or vesicle in which luciferase is localized adjacent to a substrate carrier protein, both elements having highly asymetric pH profiles. A membrane change which occurs as a consequence of stimulation results in a transient pH change; substrate is released and each luciferase molecule is charged with a single substrate molecule, the ensemble providing a flash derived from the concerted reaction of many or all luciferases operating, in effect, in synchrony. The duration and kinetics of the flash are determined by and identical with the turnover time, i.e. a single catalytic cycle which, it should be noted, is relatively long. The model explains how a luciferin-luciferase reaction which in solution produces a glow can be integrated in a subcellular fraction which then can be triggered to flash with rapid and specific kinetic properties.

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TAXONOMY AND CYSTS OF RED-TIDE DINOFLAGELLATES

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ABSTRACT

Many estuarine and neritic dinoflagellates, including species that cause red-tides, incorporate an encysted stage in their life cycles. These cysts are morphologically distinctive and differ from the planktonic flagellated stage. They provide the taxonomist with a supplemental set of characters with which to evaluate the classification of dinoflagellates. Initial results of theca-cyst studies indicate that important taxa such as *Gonyaulax* Diesing 1866 are genetically heterogeneous and therefore of limited systematic significance. Species-complexes within *Gonyaulax* are more meaningful as genetic groupings of closely related organisms and more effort should be devoted to defining them and examining the life cycles of their constituent species in connection with red-tides.

INTRODUCTION

Red-tide and toxicity phenomena that are associated with the dinoflagellates are caused by the motile biflagellated stage in the life history and most of our knowledge of this group, inclusive of its taxonomy, is derived from this stage in the plankton. However, many dinoflagellates that produce red-tides, including the toxic ones, are coastal neritic species and their life cycles incorporate an encysted stage. This encysted stage is not directly responsible for the red-tide, nor are the cysts known to be toxic, but they play a significant role in the total ecology of these neritic species by serving several important functions. First, being non-motile, the cysts settle to the sediment substrate to over-winter and thereby ensure repopulation of the same area where the plankton bloom occurred. Second, the cysts become dormant and have a ripening period before "germination" (excystment) and so they act as timing devices that control the time of repopulation. Third, many cysts probably are associated with reproduction of the dinoflagellate species and facilitate genetic recombination although this is not well documented as yet. Fourth, because encysted stages are dormant and resistant to damage and predation, they aid in dispersal and migration of the species. Fifth, being encysted probably enables the organism to survive in any one location over the relatively long time periods of several months and perhaps even several years through conditions that would destroy the corresponding motile state. Consequently, encysted stages potentially contribute to red-tides by enabling the causative species to repeatedly occur in the same local region year after year (even if the flagellated stage is temporarily absent in plankton for several months), to re-appear at approximately the same time of year, to invade previously unaffected areas by cyst dispersal and may contribute to the intensity of initial stages of a bloom dependent on whether the cysts are abundant in sediments or scarce in the region.

Despite these seemingly important considerations, the encystment-excystment cycle in dinoflagellates has not been expressly studied in relation to

any specific red-tides or related instances of paralytic shellfish poisoning. Details of the cyst cycle have been accumulated independently of red-tide research however, and presumably they are applicable to this problem and will stimulate investigation of red-tide and toxic species along similar lines. Recently some advances have been made in understanding the genetic nature of dinoflagellate cysts and, in the field of systematics, efforts have been made to identify cyst-producing species, determine their cyst morphology and reassess some taxonomy of dinoflagellates from this relatively new viewpoint. These are briefly discussed here.

Nature and ubiquity of dinoflagellate cysts

Encysted dinoflagellates were discovered in the late 1800's in freshwater species but whether they function asexually or sexually still is not known for the majority of species. Recently, von Stosch (13, 14) clearly demonstrated the cysts of *Ceratium cornutum* (Ehrenberg) Claperede and Lachmann, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* von Stosch are hypnozygotes (thick-walled diploid sexual cysts) that develop from relatively long-lived planozygotes (a flagellated diploid stage). These observations on freshwater species suggest rather pointedly that the more numerous but morphologically comparable cysts of marine species likewise are hypnozygotes but sexual processes have not been demonstrated in marine meroplanktonic species and it is necessary to refer to them by the non-committal terms "resting cysts" or "spores".

Efforts to identify the cysts of living species were inspired by the realization that fossil dinoflagellates, which are very useful in petroleum exploration and biostratigraphy, are themselves cysts (4). Detailed studies of marine cysts were carried out using a method of cyst incubation to produce the motile stage in culture from cysts that were isolated from marine and freshwater sediments (11). Cysts were identified in over 40 species included in the genera *Gonyaulax* Diesing, *Protoceratium* Bergh, *Pyrodinium* Plate, *Pyrophacus* Stein, *Peridinium* Ehrenberg *Diplopsalis* Bergh, *Diplopsalopsis* Meunier, *Diplopeltopsis* Pavillard, *Scrippsiella* Balech and *Ensiculifera* Balech (6,7,8,9,10,12). Other studies (2) demonstrated that cysts of *Gonyaulax* and *Protoceratium* are abundant on marine continental shelf and inshore sediments and reference other work on this subject. Davey (*loc. cit.*) found cyst densities in the order of 10^4 per gram dry weight of sediment in the region of southern South Africa and comparable abundances of cysts apparently are common in many other regions too. (These values are determined by palynological methods and do not distinguish between viable and dead cysts, i.e. empty cyst cases are included).

The cyst walls of many marine and some freshwater species are composed of a sporopollenin-like substance. Sporopollenin is an oxidative polymer of carotenoids and is a substance that has great stability and resistance to decay (1). Others, like the cyst walls of freshwater *Ceratium hirundinella* are cellulose,

and more rarely the outer cyst wall is composed of calcite (12). The sporopollenin cyst wall may be single or double layered. Usually in bilayered cysts the two layers are virtually inseparable, but occasionally inner and outer layers enclose a pronounced cavity. All the sporopollenin cysts have a germinal aperture which is termed an archeopyle (3) and they often develop spines, ridges, granulation and other modes of ornamentation. The site and shape of the archeopyle and the arrangement of ornamentation often follow a pattern that is clearly similar to that of the tabulation or plate arrangement of the corresponding motile thecate stage. Hence similar procedures can be used in cyst descriptions and taxonomy. It should be noted that the term "cysts" is used for a variety of non-thecate, non-flagellated dinoflagellate cells. Many of these are the immediate products of thecal ecdysis and these "sporangial" cysts undoubtedly are only of temporary durability. Other cysts are coated by a mucilaginous compound (for example, *Pyrophacus horologium* Stein, *Gonyaulax tamarensis* Lebour) and appear to have minor amounts of sporopollenin associated with cellulose in their walls and they may be over-wintering cells. These "agglutinous" cysts are very poorly known.

Systematic issues

Cyst morphology within the dinoflagellates is diverse and as far as present knowledge extends is species-specific. Cysts therefore represent a new set of morphologic criteria to add to those already utilized in taxonomy. Often they raise questions about the validity or usefulness of existing taxa and force re-examination of criteria that were used to define these taxa many years ago (most dinoflagellate species and genera were designated over 40 years ago and have never been updated as new information accumulated). The following is a viewpoint that has developed as a result of theca-cyst studies. The remarks are confined to the genus *Gonyaulax* Diesing 1866 emend. Kofoid (5) because it is most often implicated in red-tides and has toxic species.

1. *Gonyaulax* is a genetically/morphologically heterogenous taxon that merely represents a wide range of dinoflagellates with general similarity because they possess the same approximate number of thecal plates in the same general (but not specific) pattern of arrangement. They have the general plate-formular 3-4', 0-3a, 6", 6c, 6‴, lp, 1⁗ but at the same time, other dinoflagellates with this formula are (or were) excluded from *Gonyaulax*, (for example, *Pyrodinium* Plate, *Protoceratium* Bergh). It is arbitrary what comprises a protistan genus, but probably most systematists would agree that *Gonyaulax* is too broadly defined to have critical taxonomic value at this time.

2. Within the genus *Gonyaulax* there are groups of species (species complexes) that are internally consistent in sharing virtually identical plate-patterns and other characteristics but at the same time differ from "neighboring" groups. Kofoid (5) originally listed six such groups but at least one more (the *G. tamarensis*-group) must be added and a revision of others with respect to species content is desirable.

3. Within dinoflagellate species-complexes there are dinoflagellates that produce contrasting types of spores (i.e. different spore morphotypes) although they have remarkably similar if not identical flagellated stages (6). This means that genetically different dinoflagellates (species, subspecies, races, varieties etc.) sometimes are extremely difficult to identify by reference to the thecate stage alone and yet these dinoflagellates may be physiologically and ecologically distinct. The best known example of this phenomenon is the *Gonyaulax spinifera* complex, (6, 7, 9) wherein the cysts are so distinctive that using the methods employed in micropaleontology (where cysts are classified without regard to any other stage), they are referable to approximately six different genera. There is every reason to believe such species complexes are numerous among the dinoflagellates and it is very advisable that future efforts are directed towards identifying these complexes, defining how and what species are constituent to them and studying their cyst cycles.

Species complexes and the toxic dinoflagellates

Many of the toxic dinoflagellates belong to one species complex, namely, the *G. tamarensis* group. These are *G. tamarensis* Lebour, *G. catenella* Whedon and Kofoid, *G. acatenella* Whedon and Kofoid, and *G. monilata* Howell. It is very characteristic that in the hypotheca of these species, the posterior sulcal platelet contacts the fifth postcingular plate (5‴), and in general, their tabulation is more similar to that of *Pyrodinium bahamense* Plate (8) than it is to that of *Gonyaulax spinifera*, the type species of *Gonyaulax*. It is thus noteworthy that there is a recent report of toxicity in *Pyrodinium* in the South Pacific. *Gonyaulax polyedra*, another toxic species, also may in reality represent a small species complex. Cyst studies show that there are at least three morphotypes developed and *G. polyedra* possibly is "multispecific". Thus the toxic dinoflagellates of the genus *Gonyaulax* are not randomly distributed throughout its numerous species, but belong to just two species-complexes out of seven or more of its complexes. Future work on the taxonomy and life histories of species of *Gonyaulax* should help to provide information that is relevant to a better understanding of the roles that dinoflagellates play in red-tide and paralytic shellfish poisoning.

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MORTALITY OF MARINE ORGANISMS ASSOCIATED WITH OFFSHORE SUMMER BLOOMS OF THE TOXIC DINOFLAGELLATE *GONYAULAX MONILATA* HOWELL AT GALVESTON, TEXAS

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ABSTRACT

The present report documents the occurrence of blooms of the toxic dinoflagellate *Gonyaulax monilata* in the offshore coastal waters of Texas at Galveston. In 1971, the first *G. monilata* bloom was sampled on August 25, and counts of up to 1,200,000 cells/liter were recorded. Unusual numbers of dead and moribund marine organisms were collected subsequently from the beachfront. In 1972, a *G. monilata* bloom was first sampled on August 12 and a peak cell count of 1,880,000 cells/liter was recorded on the following day, coincident with the deposition of many dead and moribund marine organisms on the beach. Cell counts then declined to 280,000 cells/liter until August 16, at which time the deposition of organisms on the beach ceased.

The types and quantities of organisms noted during the 1971 and 1972 blooms were quite similar. They consisted of 29 species, including coelenterates, annelids, molluscs, crustaceans, echinoderms and fishes, all of which were either sessile, sedentary or weakly motile, suggesting that the more mobile species were able to avoid the area of the bloom before accumulating lethal amounts of toxin. The numerically dominant species were the snails, *Polinices duplicata* and *Thais haemastoma*, the hermit crab *Clibanarius vittatus*, and the brittle star *Micropholis atra*.

Despite extensive monitoring in late July and August of 1973, no *G. monilata* were noted in the offshore area of Galveston Island. It is suggested that the absence of the red tides during these years is related to relatively low salinities and temperatures in the Galveston Bay drainage area during the month of August.

INTRODUCTION

Red tides due to blooms of *Gonyaulax monilata* are a common phenomenon in the inshore coastal waters of the Gulf of Mexico. Connell and Cross (1) reported the occurrences of red waters in Offats Bayou, Galveston, Texas, and identified the causative organism as an unidentified species of the chain-forming dinoflagellate genus *Gonyaulax*. In 1952, Howell examined a red water sample from Offats Bayou. He identified (3) this red tide-causing dinoflagellate as *Gonyaulax monilata*, which he previously had found and described in Eastern Florida waters. Subsequent researchers Gates and Wilson (2); Marvin (5); Marvin and Proctor (5); Ray and Aldrich (6) show that inshore summer blooms of *G. monilata* are common in the Galveston area. Counts of up to 1,000 cells per ml from Offats Bayou in 1955 were reported by Gates and Wilson (2).

Williams and Ingle (7) reported the first documented occurrence of summer blooms of *Gonyaulax monilata* in offshore coastal waters. This occurred in 1966 and the resulting red tide extended from Anna Maria Island to Cape Romano, Florida. Fish kills were reported in conjunction with this bloom.

MATERIALS AND METHODS

Water samples were taken from the surface in a 2-1/2 gallon plastic bucket and returned immediately to the laboratory. Samples were then thoroughly agitated to ensure uniform distribution of phytoplankton, and two successive 2.5 ml subsamples were removed and examined at 20x under a dissecting microscope. If no *G. monilata* cells were observed, the sample was considered negative. If *G. monilata* cells were present, five 0.1 ml aliquots were examined as before and counts in cells per liter were thus extrapolated from the number of cells contained in 0.5 ml of water.

RESULTS AND CONCLUSIONS

On August 25, 1971, an offshore bloom of *Gonyaulax monilata* was first recorded along the beachfront at Galveston, Texas. The bloom was observed and sampled for three days (Table 1) and a maximum count of 1,200,000 *G. monilata* cells per liter was obtained. Concurrent with this bloom, numerous macroinvertebrates and bottom-dwelling fishes were collected dead and moribund from the strand line of the beachfront. These are listed in Table 2. Next year (1972) on the 11 of August, another bloom of *G. monilata* was detected in the offshore beachfront waters along Galveston Island, and on August 12, a peak cell count of 1,880,000 cells per liter (Table 1) was obtained. This is the highest naturally-occurring count of *G. monilata* recorded based on published records. At this time, many dead and moribund macroorganisms began to wash up on the strand line of the beach, and these are recorded in Table 2. Cell counts then declined to 280,000 cells/liter until August 16, at which time the deposition of organisms on the beach ceased.

Water temperature and salinities recorded throughout the period of the 1972 bloom remained uniformly high (Table 1). Unfortunately, these data were not recorded during the 1971 red tide. In the following year (1973), an early water-monitoring program was initiated in late July in order to record the onset of the bloom; however, no *G. monilata* were detected. The procedure was repeated again in 1974, but no *G. monilata* were detected either. In 1973, water temperatures were not taken, and salinities fluctuated between 18 and 33 ppt. In 1974, temperatures varied from 26.7 to 30.4 C and salinities from 22 to 33 ppt (Figure 1).

Figure 1 shows the temporal relationship of *G. monilata* cell counts and salinity values for the four years of observations of offshore red tides in Galveston. Data from the Western Florida offshore *G. monilata* bloom as recorded by Williams and Ingle (7) are also included for comparison. In years when offshore *G. monilata* blooms were observed both in Florida and in Texas, salinities were substantially above 32 ppt during the peak days of the blooms. Temperatures during these periods were generally above 29°C. In years in which no red tide occurred, salinities remained generally below 32 ppt and temperatures generally below 29°C.

TABLE 1

Counts (Cells/Liter) of *Gonyaulax monilata* at Stations along Galveston Beach (1971-1972)

Date	Time	Count/Liter	Location	Temperature (°C)	Salinity (ppt)
1971					
* 8-25	1200	270,000	61st Street Pier	-	-
	2200	1,200,000	91st Street Pier	-	-
8-26	1200	10,000	61st Street Pier	-	-
8-27	1200	negative	61st Street Pier	-	-
	1200	340,000	East Lagoon		
1972					
8-11	1700	126,000	53rd Street Beach	32	33.5
	1800	372,000	61st Street mid pier	30	33.5
	1800	720,000	61st Street end pier	30	33.5
* 8-12	0900	196,000	61st Street Pier	31.5	33.5
	1500	1,880,000	25th Street Flagship pier	31	33
	1600	1,067,000	61st Street Pier	31	34
	1700	1,773,000	91st Street Pier	32	34
* 8-13	0900	1,367,000	25th Street Pier	32	34
	1200	1,247,000	25th Street Pier	32	34
	1800	860,000	61st Street Pier	31	34
	1800	1,000	East Lagoon	30	30
* 8-14	1000	367,000	25th Street Pier	28	33
	1100	1,483,000	East Beach (30 yds off)	29	33
	1500	190,000	25th Street Pier	29	-
* 8-15	1000	320,000	25th Street Pier	29	33
	1100	50,000	East Beach (30 yds off)	29	33
* 8-16	1000	280,000	25th Street Pier	29	32
	1100	100,000	East Beach (30 yds off)	29	32
	1200	33,000	25th Street Pier	30	31
8-17	1000	200,000	25th Street Pier	30.5	32
	1600	340,000	25th Street Pier	30	32
	1600	310,000	91st Street Pier	29	32
8-18	1000	133,000	91st Street Pier	31	32
	1000	70,000	25th Street Pier	31	32
	1200	230,000	91st Street Pier	31	32
		(*Trichodesmium*)			
	1300	840,000	25th Street Pier	30	32
8-21	1000	3,000	25th Street Pier	30	31
	1100	7,000	91st Street Pier	30	31

* mass mortality noted

** not recorded

Table 2

Moribund Macroorganisms Found on Galveston Beach in Association with Blooms of *Gonyaulax monilata*

A = Abundant
C = Common
R = Rare

1971	1972
Coelenterata	
Bunodosoma cavernata (C)	*Bunodosoma cavernata* (C)
Annelida	*Onuphis magna* (C)
Onuphis magna (C)	*Nereis* sp. (C)
Mollusca	
Polinices duplicata (A)	*Polinices duplicata* (A)
Thais haemastoma (A)	*Thais haemastoma* (A)
Terebra cinerea (C)	*Terebra cinerea* (C)
Anadara ovalis (R)	*Oliva sayana* (C)
Anadara brasiliana (R)	*Siphonaria pectinata* (R)
Donax variabilis (C)	*Crassostrea virginica* (C)
Spisula solidissima (R)	
Arthropoda	
Emerita benedicti (C)	*Emerita benedicti* (C)
Arenaeus cribarius (C)	*Aranaeus cribarius* (C)
Clibanarius vittatus (A)	*Clibanarius vittatus* (A)
Isocheles wurdemanni (C)	*Petrolisthes armatus* (R)
Hepatus epheliticus (R)	*Callinectes similis* (C)
Porcellana sayana (R)	*Porcellana sayana* (R)
Callinectes sapidus (C)	*Callinectes sapidus* (C)
Menippe mercenaria (C)	*Menippe mercenaria* (C)
Echinodermata	
Micropholis atra (A)	*Micropholis atra* (A)
Mellita quinquesperforata (C)	*Mellita quinquesperforata* (C)
"Holothuroids" (C)	"Holothuroids" (C)
Chordata	
Hypleurochilus geminatus (C)	*Gobiesox punctulatus* (R)
Bascanichthys scuticaris (C)	

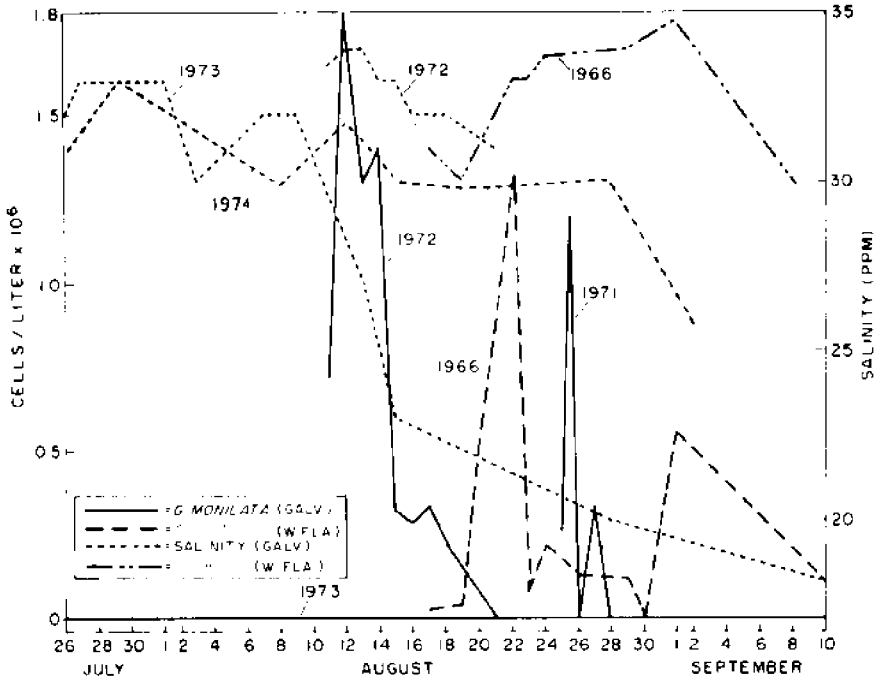
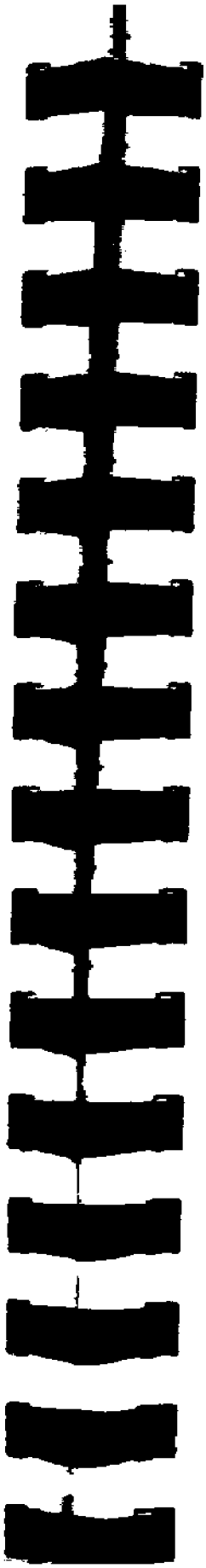


Figure 1. Comparison of *G. monilata* blooms from Texas and Western Florida.

The types of organisms washed ashore during the 1971 and 1972 blooms were quite similar in type and quantity. Twenty-nine species of coelenterates, annelids, molluscs, crustaceans, echinoderms and fishes were collected, and the numerically dominant species both years were the snails, *Polinices duplicata* and *Thais haemastoma*, the hermit crab *Clibanarius vittatus*, and the brittle star *Micropholis atra*. All organisms collected were either sessile, sedentary or weakly motile, suggesting that the more mobile species were able to avoid the area of the bloom before accumulating lethal amounts of toxin. This is in contrast to the mortality data given by Williams and Ingle (7) who reported kills of relatively mobile fishes ("jacks, needlefish, whiting, carangids, pinfish") as well as relatively slow-moving invertebrates and bottom fishes. This difference might possibly be explained by considering differences in coastal morphology. The west Florida coast in the vicinity of the 1966 *G. monilata* bloom is relatively indented and provided with numerous barrier islands, whereas the coast at Galveston is relatively straight with few barrier islands, affording little opportunity for mobile fishes to be physically trapped in channels or passes transected by toxic waters.



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SESSION SUMMARY

CHEMISTRY

Session Chairman

Robert Gagosian

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The isolation, purification and structural elucidation of the toxins produced by dinoflagellate blooms has proven to be one of the most difficult problems to solve for marine natural products chemists and biochemists. In addition to being concerned with changing or possible total loss of biological activity through structural rearrangements during isolation, the investigator must concern himself with the decomposition of bioactive metal or protein bound molecular complexes.

The complication that several active toxins in varying concentrations may be present must also be considered. Certain organic compounds may have zero toxic activity when isolated individually, but may be extremely toxic in the presence of other organic constituents in the organisms. Therefore, synergistic effects may complicate the problem even further.

Contaminants from both the marine environment and the laboratory are always a problem in the isolation of extremely small quantities of biologically active material. The contaminants may not only interfere with the isolation of the active constituents, e.g. by co-chromatography with the active toxic compounds, but the contaminants may also be biologically active themselves, thus leading to incorrect structural assignments for the true bioactive substances.

In the papers presented in this session the investigators have dealt with various aspects of all these problems and discussed their relative importance. Several new results are reported concerning the isolation of new toxins from various species of marine fauna and flora, structure-reactivity relationships, and the presentation of a revised chemical structure for the dinoflagellate toxin, saxitoxin.

PARALYTIC POISONS FROM MARINE DINOFLAGELLATES *

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ABSTRACT

Certain species of *Gonyaulax* produce deadly paralytic poisons that cause shellfish to become toxic. *G. catenella* is the predominant poisonous dinoflagellate along the northwest Pacific coast of North America and *G. tamarensis* is the predominant poisonous dinoflagellate along the northeast Atlantic coasts of North America and England. There are several species of dinoflagellates found in various parts of the world that produce other poisons. This paper describes the purification and properties of the paralytic poisons produced by *G. catenella* and *G. tamarensis* and their interrelationship. The poison produced by *G. catenella* in axenic culture has been purified. This poison, known as saxitoxin, is a 3, 4, 6 trialkyl tetrahydropurine with a molecular weight of 372 as the dihydrochloride salt. It is a strong base; one pK_a is at 11.5 and another at 8.2. The poison produced in axenic culture is identical to that found in the California sea mussel and the Alaska butter clam and there is no evidence that *G. catenella* produces more than this one poison. *G. tamarensis*, however, produces at least two different poisons. The one produced in smaller amount has been characterized as saxitoxin. The one produced in larger amount appears to be a neutral substance and has not been purified sufficiently for structural studies. Its physiological action appears identical to that of saxitoxin. There has been some speculation that this poison may convert to saxitoxin on aging in acid solution. Saxitoxin appears to be produced by other species such as some blue-green algae. Also it is found in some crabs in Japan, probably as a result of some organisms consumed by the crabs. The origin of saxitoxin in the Alaska butter clam is still not definitely known.

INTRODUCTION

Marine organisms produce many toxic substances. Halstead (4) in a compilation of the poisonous and venomous marine animals of the world has listed hundreds of species in this category. Among these species are the marine flagellates and in particular the dinoflagellates that produce many of the poisonous substances man encounters through food poisoning as a result of eating shellfish that have consumed the poisonous organisms.

The paralytic poisons, which are the subject of this paper, are produced by certain species of *Gonyaulax*. The important ones known to date are *G. catenella* (16), the predominant poisonous dinoflagellate along the northwest Pacific coast of North America, and *G. tamarensis*[1](9), the predominant one along the northeast coast of North America and the coasts of the countries along the North Sea. Another poisonous species, *G. acatenella* (10), has been reported along the coast of British Columbia. Blooms of these organisms do not occur commonly but when environmental conditions are right, and these conditions are not well understood, the blooms often reach red tide proportions of 20,000 organisms or more per ml. Such blooms cause

[1]It is proposed by Loeblich and Loeblich (these proceedings) that this organism be called *G. excavata*.

plankton-consuming shellfish to become very poisonous resulting in a definite public health hazard to people that eat the shellfish at the time these blooms are present.

Symptoms of paralytic shellfish poisoning in people begin with a numbness of the lips, tongue and finger tips that may be apparent within a few minutes after eating contaminated shellfish. This feeling is followed by numbness of the arms, legs and neck and by general muscular incoordination with respiratory distress. As the illness progresses, respiratory distress becomes more marked with death from respiratory paralysis within 2 to 24 hours depending on the magnitude of the dose. If one survives 24 hours the prognosis is good and there appears to be no lasting effects of the ordeal. From accidental cases of shellfish poisoning in people, it is estimated that the amount of poison to cause sickness and death is 3000 to 5000 mouse units or one-half to one milligram by oral route. A mouse unit (MU) is defined as the amount that will kill a 20 gram white mouse in 15 min.

NATURE OF THE PARALYTIC POISONS

The purification of the poison from California mussels (*Mytilus californianus*) and Alaska butter clams (*Saxidomas giganteus*) was accomplished by ion exchange chromatography on carboxylic acid resins, followed by chromatography on acid washed alumina (11). By this procedure a white hygroscopic product was obtained that had a potency of 5,500 MU/mg solids. The purified poison, saxitoxin, is a dibasic salt with a pK_a at 8.2 and another at 11.5, and is very soluble in water. Its molecular formula as a hydrochloride salt is $C_{10}H_{17}N_7O_4 \cdot 2$ HCl (mol. wt. 372) (11). It has no ultraviolet absorption, gives positive Benedict-Behre and Jaffe tests, and is completely detoxified by mild catalytic reduction with the uptake of 1 mol hydrogen per mol of poison at atmospheric pressure (12).

The poison was also isolated in pure form from axenic cultures of *G. catenella* obtained through the courtesy of Dr. Luigi Provasoli, Haskins Laboratory. This organism was cultured in sterile seawater for 17 days at 13°C. When the cell count reached about 30,000/ml, the cells were filtered from the medium and lysed with dilute HCl, pH 2-3, to remove the poison. The poison from the extract of the cells was purified in exactly the same manner that the poison was isolated from shellfish. A study of its chemical and physical properties showed that it is identical to the poison from clams and mussels (14). Table 1 lists some of the properties of these poisons. A structure was reported by Wong *et al.* (17) in 1971. However this structure has been subjected to some criticism because it does not fit all properties of the toxin. Recently in our work at the University of Wisconsin-Madison, we obtained a crystalline derivative with p-bromobenzene sulfonic acid. The needle-like crystals were formed with one mole of saxitoxin and two moles of p-bromobenzene sulfonic acid. These crystals were subjected to crystallographic analyses at Iowa State University and the following structure of saxitoxin was established.

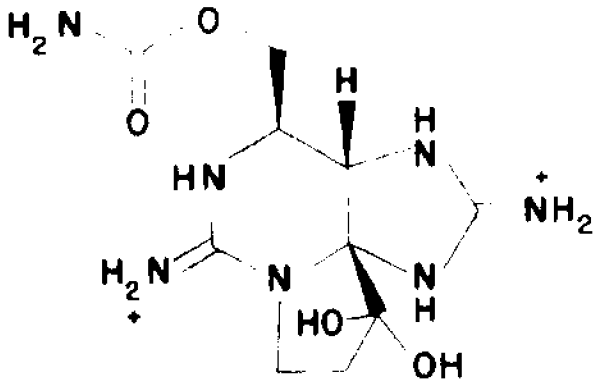

Table 1
Comparison of Properties of Poison from Cultured *Gonyaulax catenella* Cells with Poison from Mussels and Clams[a]

Property	Clam Poison	Mussel Poison	*G. catenella* Poison
Bioassay (MU/mg)[b]	5,200	5,300	5,100
Specific optical rotation	+128°	+130°	+128°
pK_a	8.3; 11.5	8.3; 11.5	8.2; 11.5
Diffusion coefficient	4.9×10^{-6}	4.9×10^{-6}	4.8×10^{-6}
Adsorption in ultraviolet and visible[c]	None	None	None
N content (Kjeldahl)	26.8	26.1	26.3
Sakaguchi	-	-	-
Benedict-Behre	+	+	+
Jaffe	+	+	+
Reduction with H_2	Dihydro derivative, nontoxic	Dihydro derivative, nontoxic	Dihydro derivative, nontoxic

[a]Reproduced from Schantz, et al. (14), courtesy American Chemical Society.

[b]All bioassay values are within experimental error of the value 5,500 ± 500 MU/mg solids reported previously for clam and mussel poisons.

[c]Infrared adsorption of *G. catenella* poison was identical with that of clam and mussel poisons (14).

This structure appears to be compatible with all known properties of the molecule. Details of the structure are being presented for publication in the near future (15).

Attempts at purification of the paralytic poisons found in scallops that fed on *G. tamarensis* or that produced in axenic cultures of *G. tamarensis* have not met with complete success. It appears that this organism produces at least two poisons. One occurring in relatively small amount (5 to 10%) is a basic substance and can be separated from the remainder of the poison because it binds to cation exchange resins such as Amberlite XE-64 or CG-50. The other poison or poisons pass through the resin and appear to be neutral or very weakly basic. Even on electrodialysis they are not pulled to the cathode. Evans (2), Kao (6) and recently Narahashi *et al.* (7) have shown that the poison from axenic cultures of *G. tamarensis* that passes through the cation exchange resins has essentially the same physiological action as saxitoxin and inhibits the passage of sodium ions into a nerve or muscle cell.

Recently in our laboratory, we have identified the basic component found in the poison from scallops, that had fed on *G. tamarensis*, to be saxitoxin (3). The purification was accomplished by chromatography on the sodium form of Amberlite CG-50 followed by chromatography on the hydrogen form of CG-50 using 0.1 M acetic acid as the eluting solvent in much the same manner as used for the purification of saxitoxin in past work. Instead of chromatography on acid washed alumina, as used for the purification of saxitoxin, we used chromatography on silicic acid with absolute ethanol as the eluting solvent followed by chromatography on Sephadex LH-20 with methanol as the solvent. The specific toxicity of the best fractions at this point was 5150 MU per mg. Further chromatography did not increase the specific toxicity which is well within the specific toxicity of saxitoxin, 5500 MU per mg ± 10%. Thin layer chromatography in six different solvent systems showed the prepraration to have identical R_f values with saxitoxin in each case (3).

COMPARISON OF PARALYTIC POISONS FROM DIFFERENT SOURCES

Past work has definitely established that the poison produced by *G. catenella* is bound by the dark gland of the California sea mussel and can be extracted with acid without a change in its chemical structure. Also there is no evidence that *G. catenella* produces more than the one poison. The fact that the same poison is produced in axenic cultures of *G. catenella* indicates too that the poison is a metabolic product of the organism itself and not due to some symbiotic effect (1). When we discovered that the poison produced by cultures of *G. catenella* had the identical chemical structure as the poison from Alaska butter clams, it gave support to the belief that its original source was in *G. catenella*. The occurrence of the poison in the Alaska butter clam is difficult to explain (13). In 1948 to 1950 one of us in cooperative studies with the United States Department of the Interior and the Alaska Experimental Commission found that the occurrence of poisonous butter clams in Southeastern Alaska was quite spotty. One clam bed might contain very poisonous clams while

another bed one-tenth mile away, and apparently washed by the same water because of the heavy tide flow between the islands, would contain no poisonous clams. If the poisonous organisms were evenly distributed in the water, both beds should contain poisonous clams. Attempts to determine if *G. catenella* was in the waters was very difficult because of the many organisms of other species in the water, but occasionally an organism was found that answered the description of *G. catenella*. Mussels (*Mytilus edulis*) in areas where clams were toxic showed very little if any poison in the dark gland by mouse assay. Acid extracts of the gross plankton growth in the water collected with nets showed no toxicity to mice. The butter clam retains the poison in the siphon for a year or more while the mussels excrete or destroy the poison in the dark gland within a week or two. It seemed reasonable therefore that as few as 10 to 20 *G. catenella* cells per ml in the water, which would be difficult to detect and count under the conditions, would account for the toxicity in the clams if the poison accumulated over a period of several months. Transplanting poisonous clams into beds where the indigenous clams were not poisonous resulted in a detectable loss within a year and gradual loss thereafter. Transplanting nonpoisonous clams into beds where the indigenous clams were poisonous resulted in the transplanted clams becoming equally toxic within a few months (13). Because the source of the poison in the clams is not definitely known, sources other than *G. catenella* should not be overlooked. Saxitoxin has been reported to be produced by a blue green alga, *Aphanizomenon flos-aquae* (5), and has been found in crabs in Japan (8) probably as a result of some poisonous organisms consumed by the crabs.

It is well established that *G. tamarensis* produces at least two poisons, the minor one being identified as saxitoxin (3). Cultures of *G. acatenella* supplied by Dr. Prakash also produce at least two-poisons that have a physiological action in mice similar to saxitoxin. This species has morphological characteristics common to both *G. catenella* and *G. tamarensis*. The poison(s) produced by *G. tamarensis* in axenic culture that passes through columns of carboxylic acid exchange resins appears to be a neutral or very weakly basic substance. This substance has not been highly purified and has not been characterized sufficiently to know if it has a markedly different chemical structure than saxitoxin. The fact that it has the same physiological action as saxitoxin can be no indication of similarity of structure because tetrodotoxin, an entirely different structure, has at least one property of saxitoxin, that of inhibiting the passage of sodium ions into a nerve or muscle cell. Our observations on the amount of basic substance in aged extracts containing poison from scallops have led us to hold the possibility that the neutral or weakly basic substance may convert to saxitoxin (3). Certain amine oxides, common in marine organisms will convert to the free amine which is distinctly basic.

Much is still be be learned about the structure of these poisons and modified structures in relation to the physiological activity. Because they have an action somewhat similar to some of our local anesthetics, they may become valuable

in medicine. The elucidation of the chemical structure of saxitoxin should help in the development of a chemical assay and possibly an antidote to the poison. An antidote would be of great assistance to the medical profession for treating poisoned persons and preventing death from shellfish poisoning.

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PURIFICATION AND PARTIAL CHARACTERIZATION OF TOXINS FROM POISONOUS CLAMS

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ABSTRACT

Toxic clams, collected during the outbreaks of "red-tide" in September, 1972, and the summer of 1974, were used in an attempt to isolate, purify, and characterize the toxin(s) responsible for the paralytic shellfish poisoning. The method involved extraction of the hepatopancreas with water, and chromatography of the concentrated extract on Amberlite IRC-50, CG-50, Sephadex G-25, G-15, and G-10. Preliminary results indicate the presence of at least two different toxins, one of which does not behave like saxitoxin. The marine dinoflagellate *Gonyaulax tamarensis* which has been implicated with PSP along the Atlantic coast was cultured under well defined conditions, and provided material for the comparison of the toxins from the cells and clams. The method of extraction and purification of the toxins from *G. tamarensis* and from clams will be discussed.

INTRODUCTION

Paralytic shellfish poisoning (PSP) caused by the dinoflagellate *Gonyaulax tamarensis* has been noticed in the Atlantic coast of Canada (4) and the Northern coast of New England. The occurrence was sporadic and in limited scale. However, during September, 1972, there was a large outbreak of the red-tide on the Massachusetts coast which had a damaging effect on the New England seafood industry. As the result of the outbreak, 33 persons (three rather seriously) were poisoned (3). A similar incident was reported during May, 1968, off the Northeast coast of Britain, and the organism was also identified as *G. tamarensis* (8).

The nature of the poisoning is very similar to the west coast PSP which is caused by *G. catenella*. In fact, the toxicity test and the other government control procedures are all based upon the knowledge of the west coast PSP, whose toxin (saxitoxin) was used as the standard. Although saxitoxin has been a target of extensive pharmacological and chemical studies, the nature of the toxin of *G. tamarensis* has not been clarified.

Schantz (6) reported that the toxin from scallops collected in the Bay of Fundy was not adsorbed on the Na^+ form of Amberlite IRC-50 as saxitoxin. Schantz speculated that the failure might be attributed either to the difference of the nature of the toxin (eg. pKa) or the presence of the interfering substances in the extract. The latter possibility was examined by the co-chromatography with saxitoxin. The results rather indicated that the toxin from the scallop was different from the saxitoxin. Later Schantz (7) re-stated the result adding that the toxin isolated from *G. tamarensis* culture also resisted the isolation method.

Evans (1) using *Mytilus edulis* infested in the 1968 outbreak in Britain tried the same isolation method (5). He observed that about one-third of the toxin was adsorbed on the Amberlite IRC-50 Na^+ form and was purified like saxitoxin, whereas the rest was only weakly bound to the resin. Further

purification of the major toxin resulted in a loss of about 98 percent of the toxin. He reported that, pharmacologically, the minor toxin resembles saxitoxin, while the major toxin is similar to tetrodotoxin, but not identical.

In this paper, we report and discuss some of the results of our attempts to purify the toxin extracted from soft-shell clams *Mya arenaria* collected during the 1972 and 1974 outbreak, and from a culture of *G. tamarensis*.

MATERIALS AND METHODS:

Parts of the toxic clams *Mya arenaria* collected at the north shore of Cape Ann in September, 1972, (average toxicity 2,000 MU/100 g): June, 1974, (average toxicity 200 MU/100 g); and in September, 1974, (average toxicity 2,000 MU/100 g) were used in this experiment.

For the extraction the hepatopancreas of the clams were dissected out and minced with Celite. Various extraction media including dilute HCl and water were tried. About 80 percent of the total toxicity was easily extracted with either of the media. It was discovered very effective to precipitate inactive mostly proteinaceous substances by adding equal volume of ethanol to the vacuum concentrated extract.

Culture of *G. tamarensis* was achieved by Guillard "F" medium (2) at 10± 2°C under controlled fluorescent light. The seed cultures were obtained from Dr. P. Hargraves (Graduate School of Oceanography, U.R.I., Kingston) and Dr. Chris Martin (Marine Station, University of Massachusetts, Gloucester, Mass.). When the cell-number reached 36± 2 million per liter, the organisms were isolated, lyophilized and extracted with hot ethanol.

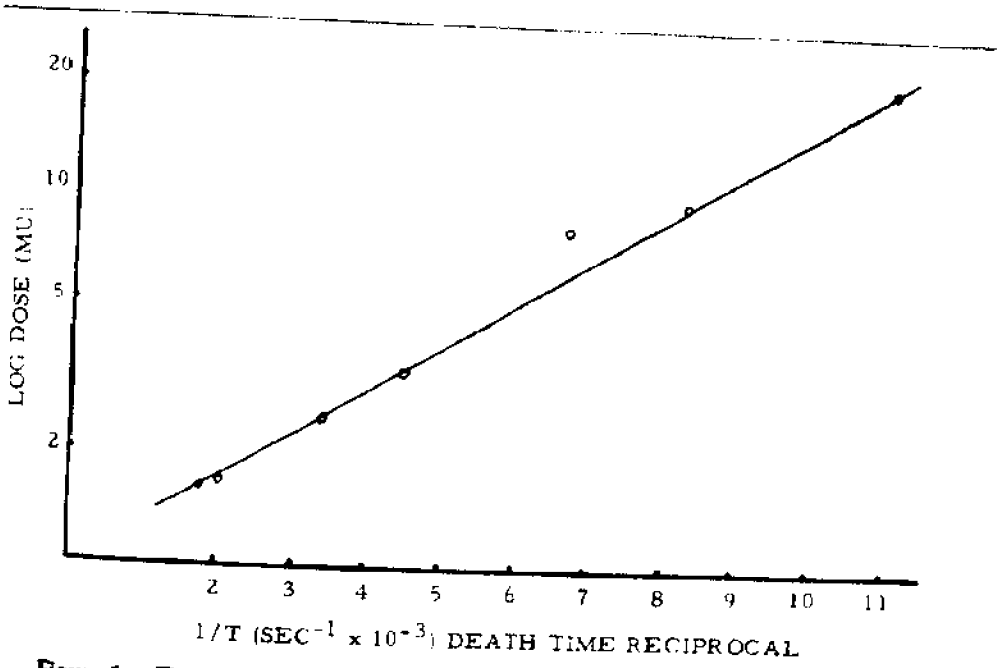

Figure 1: Dose-death time relation of partially purified *G. tamarensis* toxin.

Table 1

Chromatography of Toxic Extract from *Mya arenaria* on Amberlite IRC-50 H^+ *

		SOLVENT	VOLUME (ML)	TOTAL TOXICITY (MU)	ELUATE pH
STARTING EXTRACT		H_2O	200	46,000	5.5
FRAC.	1	H_2O	500	20,000	
	2	H_2O	200	2,700	
	3	H_2O	200	No death	
	4	H_2O	200	No death	7.2
	5	0.5M HOAc	450	1,200	5.0
	6	0.5M HOAc	400	2,400	3.9
	7	0.5M HOAc	400	4,500	3.4
	8	0.5M HOAc	400	7,200	3.0
	9	0.5M HOAc	300	2,700	2.8
	10	0.5M HOAc	150	900	2.8
	11	0.5M HOAc	230	No death	2.8

* Bed volume 500 ml.

Assays were done according to the USPHS method originally described by Sommer and Schantz (5). Death-Time/Dose curve used in this work was prepared using partially purified toxin from the above-mentioned clams (See Fig. 1).

RESULTS

Several attempts to isolate the toxin by Schantz's procedure (5) using Amberlite IRC-50 Na^+ form resulted in the elution of the major part of the toxicity with retention of a part of the toxicity on the column as described by Schantz (5) and Evans (1). It was also found that the part eluted with sodium acetate buffer was destroyed quickly due to the strong basicity of the eluate, which resulted in loss of a large portion of the toxicity in the process. It was avoided by passing the extract adjusted to pH 5.5 through a IRC-50 H^+ form column of a large bed volume. A typical example is shown in Table 1. The toxin was roughly separated into two portions. The part eluted by washing the column with water was treated with IRC-50 $\hat{N}H_4$ form prepared with a pH 5.0 ammonium acetate buffer and washed with water. As shown in Table 2, the major portion of the toxicity was adsorbed on the column and later eluted with 0.5 M acetic acid in the range of the eluate pH 4.9-2.7. In this process a good

Table 2

Chromatography of the Unadsorbed Toxic Fractions on Amberlite IRC-50 N^+H_4 *

		SOLVENT	VOLUME (ML)	TOTAL TOXICITY (MU)	ELUATE pH
STARTING SOLUTION		H_2O	100	20,000	5.0
FRACT.	1	H_2O	200	no death	4.9
	2	H_2O	200	no death	4.8
	3	H_2O	200	*ca.* 1000	4.8
	4	H_2O	200	*ca.* 1700	4.9
	5	H_2O	200	no death	5.0
	6	H_2O	1,200	no death	6-7
	7	0.5N AcOH	600	no death	5.9-5.2
	8	0.5N AcOH	200	1,000	4.9
	9	0.5N AcOH	200	1,400	4.4
	10	0.5N AcOH	200	1,400	3.7
	11	0.5N AcOH	200	1,300	3.7
	12	0.5N AcOH	200	1,200	3.7
	13	0.5N AcOH	200	1,000	3.5
	14	0.5N AcOH	200	1,200	3.3
	15	0.5N AcOH	200	1,200	3.0
	16	0.5N AcOH	200	1,000	2.7
	17	0.5N AcOH	200	700	2.7

*Bed volume 700 ml.

part of the residue was removed, and the fraction consisted mostly of ammonium acetate salt. Rechromatography of the combined toxic fraction was done on Amberlite CG-50 H^+ form (Table 3). Elution with 0.1 M acetic acid fractionated the toxin into two separate bands; out of the total 10,200 MU charged on the column, 3,200 MU were in the eluate pH range 4.5-4.8 and *ca.* 4,000 MU in the range 3.9-3.0. The latter fraction was further rechromatographed on Amberlite CG-50 H^+ form column, in which the toxic fraction appeared with an almost invisible residue.

In another attempt to fractionate the toxin, use of gel filtration was extensively investigated. Crude toxin (clam extract) was placed on a Sephadex G-25 column which was then washed with water. Fractions of 200 ml were collected. Fig. 2 shows a typical example of the Sephadex G-25 chromatography. The toxic fractions were combined, concentrated *in vacuo* to

Table 3

Re-chromatography of the Unadsorbed Toxic Fractions on Amberlite CG-50 Column

		SOLVENT	VOLUME (ML)	TOTAL TOXICITY (MU)	ELUATE pH
STARTING SOLUTION		H_2O	50	10,200	
FRAC.	1-7	H_2O	1,400	no death	7.1-7.8
	8	0.1N AcOH	175	---	5.4
	9	0.1N AcOH	175	---	5.2
	10	0.1N AcOH	175	---	4.9
	11	0.1N AcOH	175	780	4.5
	12	0.1N AcOH	175	1,080	4.5
	13	0.1N AcOH	175	800	4.7
	14	0.1N AcOH	175	480	4.8
	15	0.1N AcOH	175	no death	4.6
	16-18	0.1N AcOH	500	---	4.6-4.4
	19	0.1N AcOH	170	2,040	3.9
	20	0.1N AcOH	170	952	3.3
	21	0.1N AcOH	170	680	3.1
	22	0.1N AcOH	170	400	3.0
	23	0.1N AcOH	170	---	2.8

about 15 ml volume. Out of 9,300 MU placed on the column 7,000 MU were recovered. The combined toxic fraction was then placed on a Sephadex G-15 column, which was washed with water. The elution pattern of toxin from Sephadex G-15 column is shown in Fig. 3. Total recovery of toxicity from Sephadex G-15 column was 66 percent (charged 1250 MU, recovered 830 MU). Erratic results were obtained when large volume of toxin were placed on the column. Repeated Sephadex G-15 chromatography did not result in an increase in the purity of the toxins.

In one attempt the toxic fractions from Sephadex G-15 chromatography were placed on an Amberlite CG-50 H^+ form, which was washed with water, followed by 0.125 M acetic acid. The elution pattern of toxic fractions from Amberlite CG-50 H^+ form column is shown in Fig. 4. The total recovery was 85 percent (charged 5,300 MU, recovered 4,450 MU). Apparently, at least three toxins were separated on Amberlite column, one between pH 2.2-3.5, which was not adsorbed on the column. The second (major) toxin was eluted between pH 3.9-4.2 with 0.125 M acetic acid, and the third was eluted only with 0.1 M HCl at a very low pH.

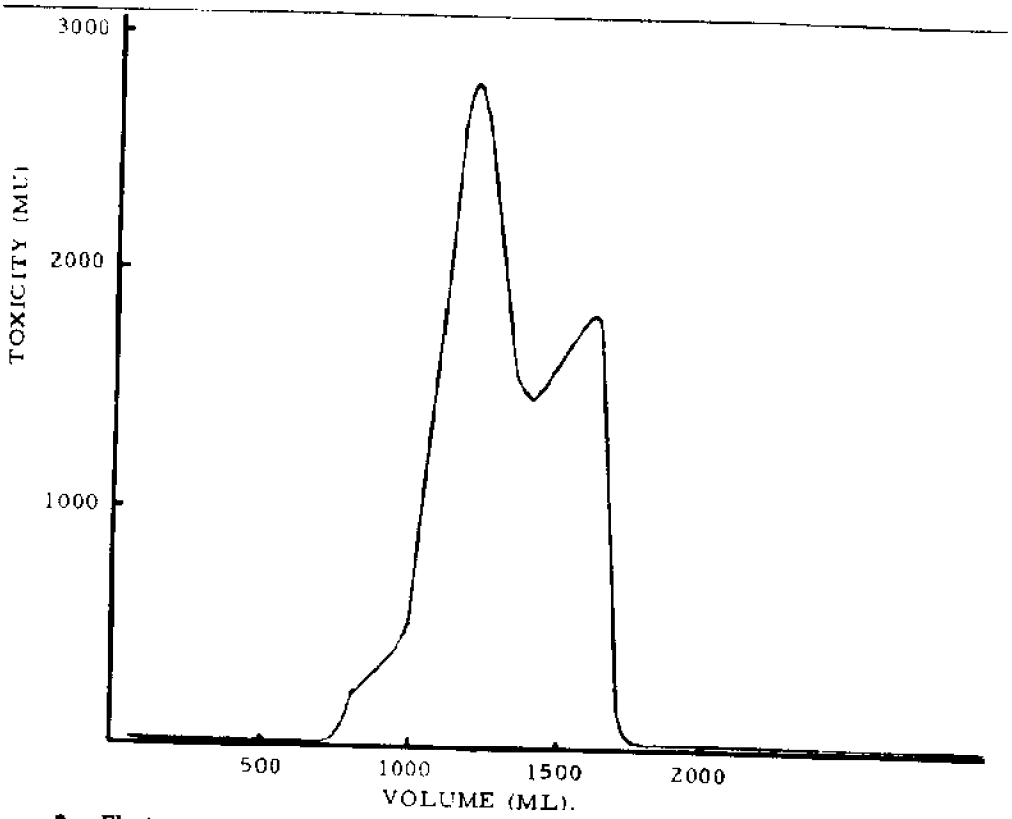

Figure 2: Elution pattern from a Sephadex G-25 Column. Charged 9300 MU; recovered 7000 MU.

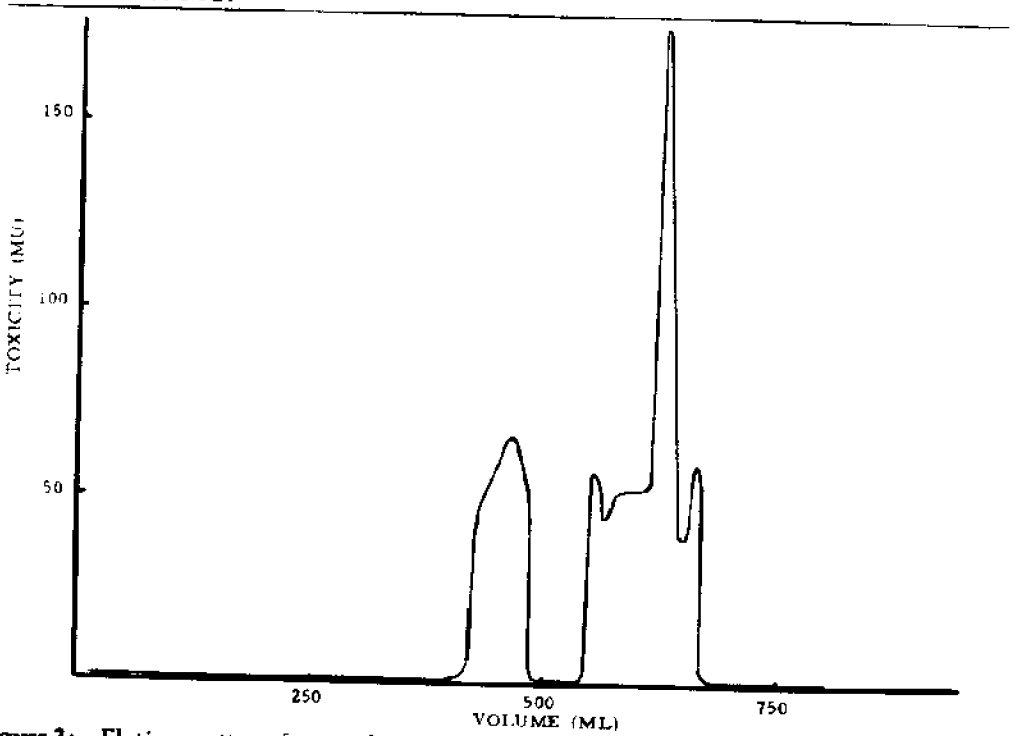

Figure 3: Elution pattern from a Sephadex G-15 Column. Charged 1250 MU; recovered 830 MU.

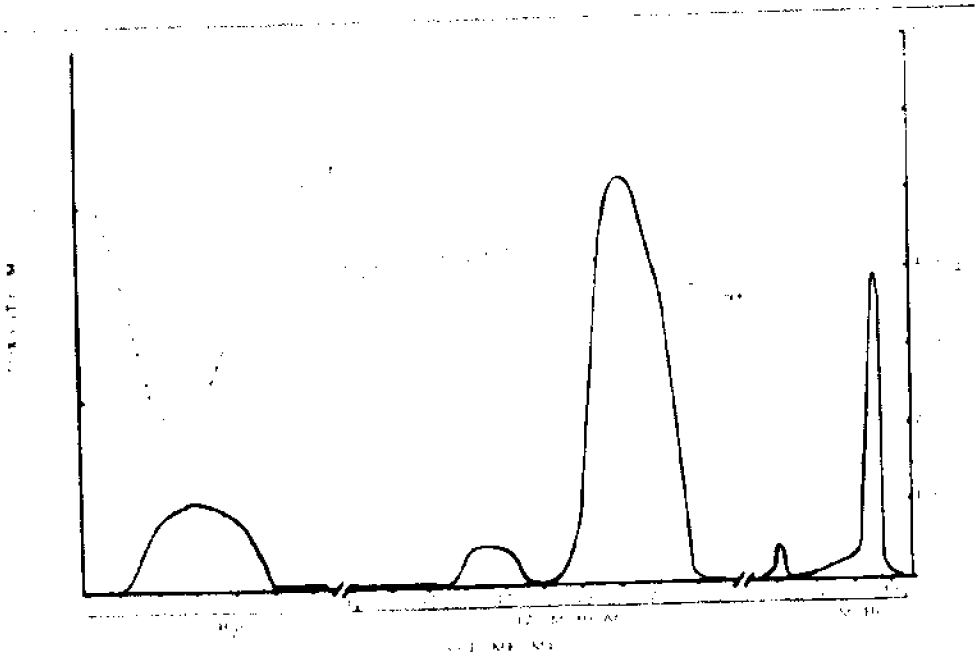

Figure 4: Elution pattern from an Amberlite CG-50 [H⁺ form] Column.
———— Toxicity, ·····pH
Charged 5300 MU, recovered 4450 MU.

Extraction of G. tamarensis toxin from culture

Growth curve of *G. tamarensis* is shown in Fig. 5. The cells (42×10^6) were collected by centrifugation and the residue (5.8 g) was extracted with hot ethanol. The ethanol extract was partitioned between equal volumes of chloroform and water (pH 5.0). The aqueous layer was separated, concentrated *in vacuo* and lyophilized to 1.23 g of residue. The toxicity of the residue was 2.8 MU/mg. Attempts to purify the cell toxin by gel filtration resulted in the loss of a large portion of the toxicity probably due to the adsorption. Attempts to purify the toxin using a combination of gel filtration and ion exchange resin chromatography is in progress.

DISCUSSION

Our experimental results of the study on the toxin extract from softshell clams, *Mya arenaria* infested with *G. tamarensis* are in good agreement with the observations Schantz (6) and Evans (1) made previously on scallop, *G. tamarensis* and *Mytilus edulis*. However, it still does not warrant that the toxin eluted unadsorbed and the toxin retained on the Amberlite IRC-50 column are different. Possible interaction with other molecules such as binding with proteins can change the adsorbability of the toxin on the resin. In fact when the eluted toxin was recharged on the identical resin column a small portion of the toxin was again adsorbed on the column.

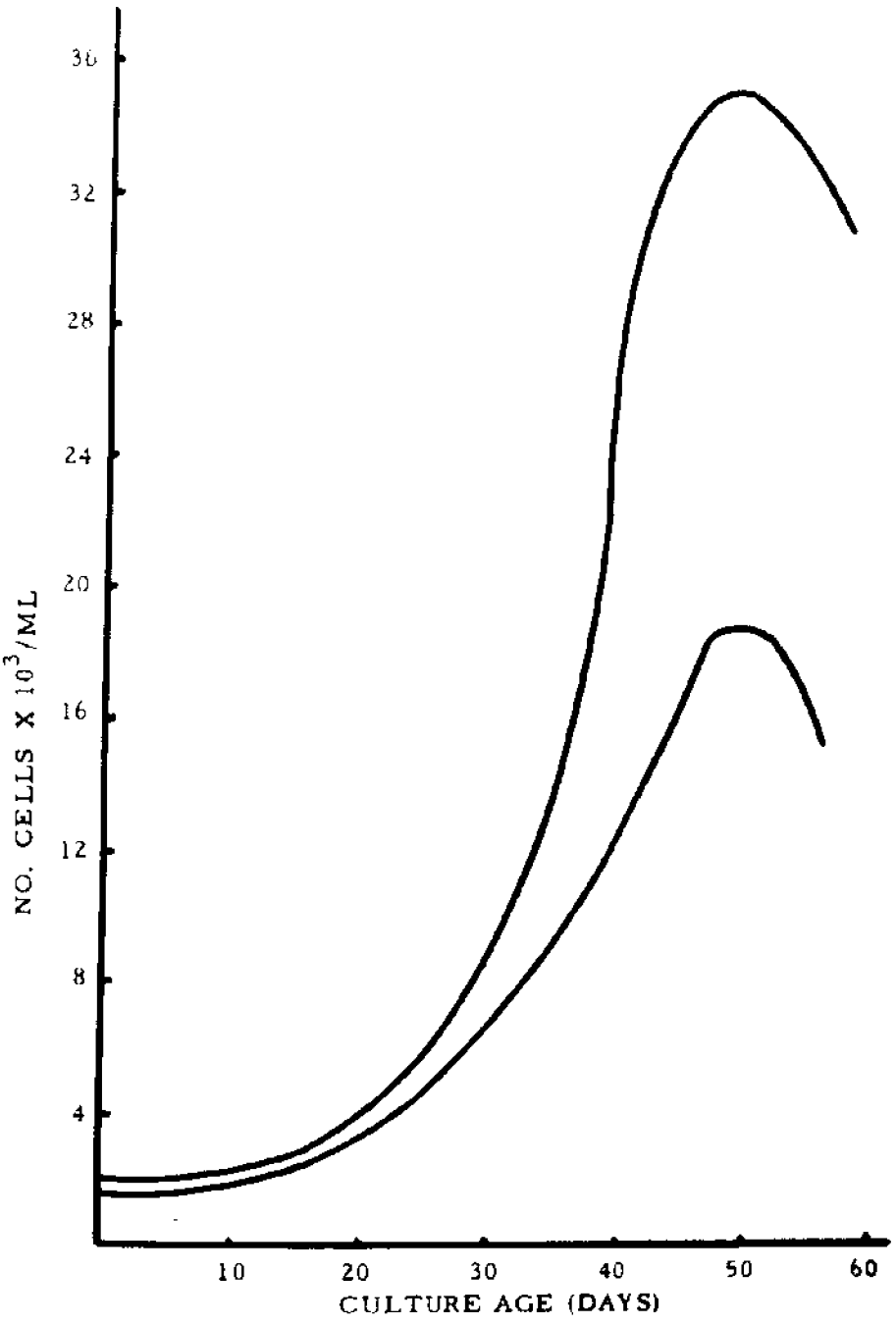

Figure 5: Growth curve of *G. tamarensis* cultures.

The unadsorbed toxin was adsorbed very well on the pH 5.0 buffered ammonium form of the carboxylate resin. This behavior is similar to one observed with tetrodotoxin (pK_a 8.3). It is also important to note that the toxin was eluted at the eluate pH range of 4.9-2.7. In the rechromatography of the toxic fractions resulted again in the elution of the major portion around pH 3.9 with a smaller portion eluted at pH 4.5. Although we are very much interested in the identity of the adsorbed and unadsorbed toxin, we have not been able to draw a conclusion on the matter, and our major efforts are now directed toward the removal of the minor contaminants (mostly ammonium acetate) from the preparation.

Our attempts to purify the clam toxin by gel filtration met with some success. It is, however, clear from the Fig. 2 that the toxin(s) was eluted as a single large band from Sephadex G-25 column. The combined toxic fraction was found to have a toxicity of about 1.2 MU/mg. Using G-25, we were able to separate salts from the toxin. It is conceivable that there are two toxins which can be separated on Sephadex G-25 (Fig. 2). It was found that Sephadex G-15 was a better medium, and rechromatography of the combined toxic fraction from the Sephadex G-25 column gave at least two toxic fractions. It was observed that the fractions between the toxic fractions always contain a large quantity of yellowish proteinaceous material which was non-toxic to mice. At the present time we believe that the loss of toxicity and poor recovery from Sephadex G-15 column is due to the adsorption to the gel and decomposition there. There was two-fold increase in toxicity at this stage. Although our attempts to purify the toxin(s) further by gel filtration on Sephadex G-15 and G-10 alone were successful, when combined fractions from G-15 chromatography were placed on an Amberlite CG-50 H^+ form column, the toxin(s) can be fractionated into at least three toxic fractions (Fig. 4). Again, it is open to question whether the fractionation is due to the presence of a number of toxins or interaction with other molecules.

ACKNOWLEDGMENTS:

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STUDIES OF TOXINS FROM FLORIDA RED-TIDE OUTBREAKS

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The factors affecting the growth and development of the unarmoured dinoflagellate *Gymnodinium breve* have been of interest since *G. breve* was identified twenty-five years ago as the organism responsible for mass mortalities of marine organisms in west Florida coastal waters. The toxin(s) elaborated when cultures of *G. breve* and bloom samples are acidified and extracted with chloroform are considered. The material is an ichthyotoxin, and hemolytic activity has been demonstrated for one fraction. Elemental analysis is also consistent with another property, viz., anticoagulation. The properties of material isolated from culture or bloom appear to be identical. The purification schemes and means of characterizing activity and structure-reactivity are considered. The environment in which the toxin is produced has been studied, and the rate of growth of *G. breve* as a function of salinity has been compared with the rates of DNA and toxin production. The possibility of reducing the toxic activity has been considered and the physical (light, temperatures) and biochemical experiments (action of enzymes) are reviewed. Finally, the possibility of limitation of *G. breve* by means of a naturally-occurring biochemical agent and contemporary developments are briefly summarized.

INTRODUCTION

Catastrophic fish kills along the west coast of Florida have long been associated with the unarmored dinoflagellate *Gymnodinium breve* Davis. It appears that in addition to causing mortalities of marine animals, these outbreaks have a significant economic effect, and that the air-born aerosols from outbreaks also cause respiratory discomfort in some persons, unless minimum precautions are taken (remaining within an air-conditioned room or car or using a surgical mask, for example).

There appear to be three major areas that deserve considerable attention: prediction, amelioration and control (or limitation or management).

Considerable efforts have been made to predict the occurrence of these blooms, and the trends that assist the predictive capabilities have been reviewed by Rounsefell and Nelson (22), Martin and Martin (14), and Steidinger (26).

The possibility of a similar causality of outbreaks of *Gonyaulax catenella* and *Gymnodinium breve* has been considered, for example, in terms of the role of chelate - and metal-chelate compounds.

Though predictability is a significant goal, more needs to be done about the amelioration of red tides in Florida coastal waters. Amelioration would cover a range of activities: removal and utilization of dead fish before they reached the beaches, assistance of persons who may be indirectly affected by some primary or secondary aspect of the red tide outbreak, and mitigation of the effects of the toxin.

Clearly, to be able to mitigate the effects of the toxin elaborated by *Gymnodinium breve*, it is necessary to know much about the properties (chemical, physical, and physiological) of the toxin(s), and the present paper summarizes the pertinent findings from our laboratories and others.

Finally, the possibility of direct management of the organism has been considered and deserves review inasmuch as understanding the role of bio-control in nature or the absence of it may be a key point in controlling, limiting or managing a red tide outbreak.

TOXIN ISOLATION

Toxic materials have been isolated from cultures of *Gymnodinium breve*, from blooms of *Gymnodinium breve*, and from oysters taken from blooms. The procedure used in our laboratories has been acidification to a pH of 4 followed by extraction with chloroform (12). Sasner and co-workers (22, 23) have extracted with ether, and Cummins and co-workers (2, 3), have used ethyl ether and a liquid extractor while Trieff and co-workers (27), have also reported using ether for extraction. Martin and Chatterjee (12) noted the similarity of material isolated from bloom and from culture, and using different techniques Alam and co-workers (1) also noted the similarity of material isolated from the two sources.

TOXIN PURIFICATION

Typically the toxin(s) have been purified by thin-layer and/or column chromatography, using silica gel (27, 12, 2). Trieff and co-workers (27) reported the interesting use of dry-column chromatography, using silica gel.

Workers in our laboratories have used silica-gel column chromatography, followed by separation using other columns. Sephadex LH-20 was used with choloroform as eluent (12) and a single toxic fraction was obtained that was an ichthyotoxin, though it had no hemolytic activity. The fraction that is associated with hemolytic activity apparently is removed only by elution with methanol-chloroform (1:2) from Sephadex LH-20 (19); B. C. Abbott, personal comm. Finally, Doig (4) used a series of columns to effect an enhanced toxicity (cf. Table 1).

ICHTHYOTOXICITY

Doig and Martin (5) defined a toxin unit as the amount of toxin in methanol required to kill a 1.0-2.5 g fish (*Poecilia sphenops*, 4-6 cm) in 20 ± 4 minutes under standard conditions in (50 ml of fresh water, pH 7, 23°C). Controls with methanol were run concurrently.

Fraction	Yield	Specific Activity	Total Activity	Relative Activity	Recovered Activity
Crude Toxic Extract	270	4.43	12090	1.0	100
Methanol Eluent from Silica Gel	112	9.76	1090	2.2	91
LH-20 Fractions (4-7)	51	17.3	882	3.9	74
DEAE-Cellulose Fractions (3-9)	7	121.8	853	27.3	71

* cf. Doig (4)

HEMOLYTIC ASSAY

A number of techniques have been used to measure hemolytic activity of marine bioactive agents, and seem to fall into two groups: (1) measurement of amount of hemoglobin released following incubation and (2) direct measurements of the kinetics of hemolysis. A detailed description of the methods has been provided by Padilla and Martin (17).

EFFECT OF ENZYMES

The effect of various enzymes on the hemolytic activity of chloroform extracts of *G. breve* toxin was studied. The extracts were lyophilized and the residue redissolved in methanol. Vials containing blood buffer (4.9 ml; cf.) Padilla and Martin, (17) were warmed to 37° in a gyrorotatory bath. Enzyme stock solution was added (0.1 ml in blood buffer), followed by methanol (0.1 ml) or methanolic solution of toxin (0.1 ml). The vials were incubated for 1 hour at 37° in the shaker bath, then rabbit blood suspension in blood buffer (15 ml) was added and the mixture incubated for an additional hour at 37°. Samples were counted electronically using a Coulter Counter Model B, equipped with a C-1000 channelyzer for enhanced sensitivity. The cell number N was obtained for the threshold range (3-20) for 0.5 ml sample. Typical data are summarized in Table 2. β-glucosidase is an enzyme that does not appear to cause hemolysis, but it does appear to limit hemolytic activity of *G. breve* toxin(s). For example in the presence of enzyme or methanol control the observed peak was at 7-8 threshold units and the relative value of $N_{3\text{-}20}$ was 0.97-1.05. In the presence of toxin, however, the peak was shifted to 27-29 threshold units, due to lysis and agglomeration. The number of red cells was reduced about 87%.

It would appear that an enzyme can function as an inhibitor of the activity of this toxin. In order to define the effectiveness of the enzyme and perhaps gain an insight into the mode of action of the enzyme, we have chosen to define a relative constant k_{rel} as the ratio of cell number in the presence of

$$k_{rel} = N_t / N_{t+e} \qquad \text{(eqn. 1)}$$

toxin (N_t) and the cell number in the presence of toxin and enzyme (N_{t+e}). If the enzyme is without effect k_{rel} should be equal to unity and if the hemolytic activity of the toxin is completely suppressed by enzyme, k_{rel} should approach but not equal zero. The inhibition parameter, I.P., is defined as before as being equal to $(1 - k_{rel})/k_{rel}$ (cf. Martin and Padilla (16).

If the model developed before holds (16, 18), a plot of enzyme concentration as a function of inhibitor parameter should yield one of three diagnostic relations depending upon the nature of the enzyme, toxin interaction. (1) hyperbola concave downward, should be diagnostic of the reduction in effective concentration of lysing agent; (2) a linear relationship, should

Table 2

Effect of β-Glucosidase on Hemolytic Activity of G. breve Toxin

Enzyme mg	Enzyme units	Toxin	$N_{3-20} \times 10^{-3}$	$(N_{3-20})_{rel}$	Peak	I.P.
1.02	5.2		69.5 ± 0.3	1.05	7	7.56
1.02	5.2		68.2 ± 0.7	1.03	7	7.42
1.02	5.2	0.1	70.5 ± 0.8	1.06	8	7.70
1.02	5.2	0.1	70.4 ± 0.09	1.06	9	7.69
0.51	2.6	0.1	64.6 ± 0.7	$.97_4$	10	6.98
0.51	2.6	0.1	68.7 ± 0.3	1.04	9	7.48
0.51	2.6	0.1	64.5 ± 0.2	$.97_3$	10	6.96
0.51	2.6	0.1	67.8 ± 1.3	1.02	9	7.37
			66.0 ± 0.2	1.00	7	7.15
			66.6 ± 1.2	1.00	7	7.22
		0.1	8.5 ± 0.3	0.13	27	+0.05
		0.1	7.7 ± 0.2	0.12	28-38	-0.05
		0.1	8.2 ± 0.9	0.12	29	+0.01

* Inhibition parameter = $k_{rel}/(1-k_{rel})$

indicate the formation of an enzyme-toxin complex that interacts with rec blood cells in competition with the toxin; and (3) a curve concave upward, should indicate the enzyme is behaving as a competitive lysing agent and is displacing the toxin from sites of attack on the erythrocytes. Several enzymes examined to date indicate the first pattern of behavior, and some indicate a total absence of interaction with toxin or red blood cells.

The possibility of mitigation of the effects of toxins of *G. breve* by enzyme action is remote, but the action of enzymes will provide useful information concerning the functional groups responsible for hemolytic activity and/or ichthyotoxicity.

The role of hemolytic activity of the toxin in actual outbreaks has been indicated by two observations. Kim and co-workers (7) reported that the susceptibility of mullet blood to *G. breve* toxin is considerably greater than that of rabbit blood. Quick and Henderson (20) have presented evidence of severe hematological degradation by the 1973-1974 red tide in fishes, particularly catfish, mullet, and sheephead. Their data suggest two different, though not exclusive mechanisms of death: neurointoxication and fatal hemopathy.

TOXIN CHARACTERIZATION

Structural features of the toxins are reviewed elsewhere by Padilla *et al.*, in these proceedings, and little additional comment is required here. The presence of a carbonyl group was noted by Martin (12), and Doig and Martin (5) noted the existence of sulfated polysaccharide in the purified toxin that was consistent with observed anticoagulant properties. Available microanalytical data have indicated the presence of carbon, hydrogen, nitrogen, phosphorus and sulfur in toxins that have been purified in our laboratories, (cf. Table 3).

It appears that unique substances have not been isolated and purified from *Gymnodinium breve* cultures, though the results obtained by various workers over the years have been fairly consistent. These appear to be due to the components responsible for hemolytic versus those responsible for neurotoxic properties. The material described by Martin and Chatterjee (12) did not exhibit anticholinestrase activity, nor did the material similarly isolated by Trieff and co-workers (27). On the other hand, the material isolated by Sasner *et al.* (24), had a much higher nitrogen content and did exhibit anticholonestrase activity.

The source of the toxin and the mode of formation are two points of interest. The former has been indicated, though not as unequivocally as might be wished. Martin and Chatterjee (12) concluded as others had previously that the material was an endotoxin. Doig (4) provided more quantitative assessment of this point. Culture of *G. breve* (10 liters representing about 50 toxin units) was subjected to continuous flow centrifugation, and 40 toxin units appeared in the pellet, 10 in the supernatant. When a similar 10 liters of culture was acidified and stirred, then subjected to continuous flow

Table 3

Elemental Analyses of Purified Gymnodinium breve Toxin(s)

Element	Present Study*	Trieff et al., (1972)
C	67.95	70.92
H	9.94	10.10
N	0.50	-
P	0.20	1.99
S	2.08	-
O	19.33	17.54

* cf. Doig (1973)

centrifugation, 100% of the toxic material was found in the centrifugate none in the pellet. It appears that the toxin is present in the cytoplasmic fluid or that it is released from particulate cell material during the lysing procedure.

The problem of two artifacts is recognized. First, some toxin was found in the supernatant fluid during the first procedure, probably because of cell rupture of the unarmored dinoflagellate. Second, it is possible that toxin is associated with particulate cell material that is not sedimented under the conditions of the experiment (12,500 x g). The results do suggest, however, that the material is a degradation product of the cell wall or the cytoplasmic fluid.

Kim and Martin (8) have measured the effects of salinity on the synthesis of DNA, acidic polysaccharide and ichthyotoxin of *G. breve*. The variation of growth constant with salinity was similar to previous observations in field and laboratory studies. The rate of toxin synthesis showed a linear decrease with the rate of DNA or polysaccharide synthesis. Specifically,

$$\text{Rate of toxin synthesis} = 0.21 \pm 0.03 - 0.021 \pm 0.004\ R'_{DNA}$$
$$0.13 \pm 0.01 - 0.0362 \pm R'_{poly}$$

where R′ DNA and R'_{poly} refer to the rates of DNA synthesis and polysaccharide, respectively (µg/ 10^6 cells/day), and the rates of toxin synthesis are expressed as mg/10^6 cells/day.

In contrast, no significant differences in amounts of hemolytically active material were observed at a significantly different salinities. This would suggest that the modes of synthesis of ichthyotoxin and hemolytically active material are different. The implications of these observations were considered by Kim and Martin (8).

RED TIDE LIMITATION OR CONTROL

The suggestion that red tides might be limited or controlled on a limited basis must be made with considerable caution. As Martin (11) noted, the areas to be treated are vast, and were chemical treatment to be suggested, the problems, economic and ecological, might be enormous and far reaching. An earlier assessment of the use of copper sulfate in controlling red tide near Florida has been reviewed by Rounsefell and Evans (21), and the limitations have been noted. Admittedly, copper sulfate might not be the chemical of choice, because, Martin and Olander (15) noted a stimulatory effect of this element at low concentrations in laboratory cultures, and though the ability to extrapolate these results may be in question, the implications give pause for consideration.

The use of bio-control, on the other hand, deserves consideration, because it seems likely that such control may well be a factor in the disappearance of red tide on occasion. Two possibilities of bio-control exist: through predation and through limitation of red tide organisms by bio-chemicals that are released as secondary plant substances cf. Sieburth (25) by phytoplankters, usually blue-green algae. It appears that the role of both possibilities in the natural red tide spread and disappearance deserves consideration and research for possible application. The possibility of limitation by predators was raised earlier by Martin *et al.* (13), and the possibility of bio-control has been raised recently by Kutt and Martin (9, 10).

One such biochemical, provisionally called aponin, has been isolated from a blue-green alga found in the red tide outbreak area. Kutt and Martin (10) have found that this material is cytolytic toward *G. breve* cells. The properties and structure of this material are under active investigation, and it appears that the role of such biochemicals to natural outbreaks should be actively investigated.

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SEPARATION AND ANALYSIS OF TOXINS ISOLATED FROM A RED-TIDE SAMPLE OF *GYMNODINIUM BREVE*

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ABSTRACT

Three toxic fractions have been separated from a naturally occurring red tide sample of *Gymnodinium breve* by a combination of column and thin layer chromatography. They appear to be relatively polar lipid compounds. Infrared, visible, and ultraviolet spectroscopy suggest the toxins have aromatic and carbonyl groups. Although distinct from undefined non-toxic phthalate-like compounds, on the basis of their potent ichthyotoxicity, the infrared spectrum and mass spectroscopic analysis show that the toxins have some similarity to this class of compounds.

INTRODUCTION

The isolation and chemical characterization of toxins derived from red tide organisms such as *G. breve* constitute a laborious and drawn-out process. Not only is the starting material available in limited quantities, but there is no "standard of purity" for the fractions being isolated. Investigators must, in most cases, rely on a potency assay to follow the fate of the active component being taken through a purification procedure. Thus, progress in this field has followed the divergent interest of the two disciplines: chemistry and physiology, and as a result, the chemical and structural properties of red tide toxin(s), specifically those derived from the dinoflagellate, *Gymnodinium breve*, remain unresolved (1-4).

The present investigation is an extension of the studies initiated at the University of South Florida on the physico-chemical properties of *Gymnodinium breve* toxin(s) (5-6). We have used a simpler and more direct method of isolation of *G. breve* toxin by the use of mixed organic solvents in conjunction with column and preparative thin layer chromatography (PTLC). We report here the separation of toxin fractions from *G. breve* and their analysis by a variety of physico-chemical methods such as UV and IR spectroscopy, gas-liquid chromatography, and mass spectrometry. Suggestions regarding the chemical nature of the toxic principles and their similarity to some alkyl phthalates are also presented.

EXPERIMENTAL PROCEDURES AND RESULTS

Extraction and Preparative Thin Layer Chromatography [PTLC]

Sea water samples (50-60 liters) were collected during a recent red tide outbreak (January-February, 1974, in the St. Petersburg area on the west coast of Florida). The sea water samples, containing *G. breve* organisms, were acidified to pH 4 by addition of concentrated hydrochloric acid and the toxin was extracted with 15 ml of chloroform/liter of sample. After mixing for 15-20 min, the chloroform layer was removed, centrifuged, and evaporated under vacuum. The residue was redissolved in a small amount of chloroform under a nitrogen atmosphere. This chloroform solution (∿ 1 ml) was passed through a

silica gel column (2.5x40 cm) and eluted with chloroform at a flow rate of about 2 ml/min. The silica gel column was prepared as follows: activated silica gel (100-200 mesh) was mixed with celite (2:1 weight ratio) and washed with chloroform-methanol (4:1) mixture. The gel suspension was filtered through a medium porosity sintered glass funnel, resuspended in fresh chloroform-methanol solvent, and poured into the column to form a ∿ 30 cm bed. The column was eluted with chloroform, resulting in the separation of a dark green band. This is called fraction III. Since several diffused colored bands still appeared in the column, which were not easily separated, they were eluted together with a chloroform-methanol mixture (93:7). The next fraction (fraction II) is the eluate between fraction III and I (the last fraction to be eluted). Fraction II was found to be non-ichthyotoxic and was not analyzed any further. Fractions I and III were further separated by PTLC and tested for ichthyotoxicity using the procedure described by Martin and Chatterjee (5). Spectrometric examinations were carried out on these subfractions. The separated fractions were concentrated under vacuum and kept in the cold under nitrogen atmosphere until needed. We also retained an aliquot of the original red-tide sample which was carefully extracted in an all-glass system. This crude toxin was later analyzed as will be discussed.

The solvents used in the PTLC separation of fraction I (Table I) were chloroform:methanol (98:2). Fraction I_A which remained close to the origin, was dark green in color, ichthyotoxic (weaker than I_C) and had a fishy smell. Fraction I_B was light green in color but there was not enough material to test for ichthyotoxicity. Fraction I_C was the most toxic and had an orange-yellow

Table I
PREPARATIVE THIN LAYER CHROMATOGRAPHY OF *G. BREVE* TOXINS

Column Fraction I		Column Fraction III	
Fraction	R_f at 22°C	Fraction	R_f at 22°C
I_A	0	III_a	0.3
I_B	0.03	III_b	0.65
I_C	0.06	III_c	0.80
I_D	0.10		

Chloroform extracts were applied to preparative thin layer plates (E. Merck, Silica gel 60, F-254, 2 mm layers) using a Desaga Wide-Band Streaking Pipet (Brinkmann, Inc.). The plates were subjected to ascending chromatography at room temperature for approximately 90 min. The separations were visualized by UV light under dark.

color. Fraction I_D was also toxic but less than I_A and had an orange-brown color. As mentioned above, fraction II from the column was not ichthyotoxic and had a light green color and a strong dusty smell.

As shown in Table I, fraction III from the column was further separated by PTLC into three subfractions by using a toluene:ether:ethyl acetate:acetic acid (80:10:10:0.2) solvent system. It was resolved into three distinct bands. Fraction III_a was a greasy, green colored material while fraction III_b was dark green. The residue from fraciton III_c was a light yellow, oily material. All these subfractions were non-toxic. Since fraction III_c was suspected of containing phthalates, either as contaminants from the laboratory or from the natural source (sea water), it was analyzed further along with the toxic fractions. As will be discussed later, we also examined the infrared and mass spectra of a chloroform extract of tygon tubing, which was not ichthyotoxic.

Spectroscopic Analyses of Toxic Fractions

Ultraviolet, Visible and Infrared Spectrophotometry: The UV absorption spectra in the range of 190-240 nm for fractions I_A, I_C, and I_D are almost identical but the peak shape was sharper and narrower for fraction I_D (Fig. Ia). There was no significant absorption in the visible range (600-500 nm) except a slight absorption at 533 nm for fraction I_A which as noted before had a dark green color (Fig. 1b). The instrument used in these measurements was a double

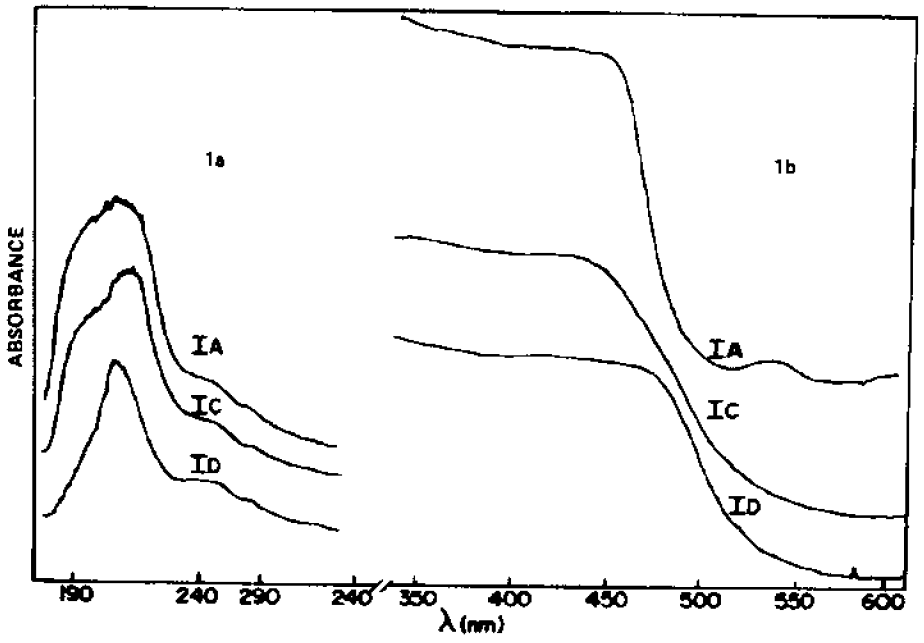

Fig. 1. Ultraviolet and visible spectra of *G. breve* toxins. Abscissa: wave length (nm); Ordinate: absorbance. a) Left side = UV absorption spectra, b) Right side = visible absorption spectra. I_A = toxic fraction I_A, I_C = toxic fraction I_C, I_D = toxic fraction I_D.

beam Beckman Spectrophotometer (ACTA II) with a 1.0 cm light path quartz cells linked to a strip chart recorder (Esterline Angus). The samples were dissolved in methanol for these measurements.

The IR spectrum of the toxic fractions was obtained with a Grating IR Spectrophotometer (Perkin-Elmer, Model 337). The toxic fractions were dissolved in chloroform and analyzed with 0.1 mm light path NaCl cells. The spectra of the various fractions are shown in Fig. 2.

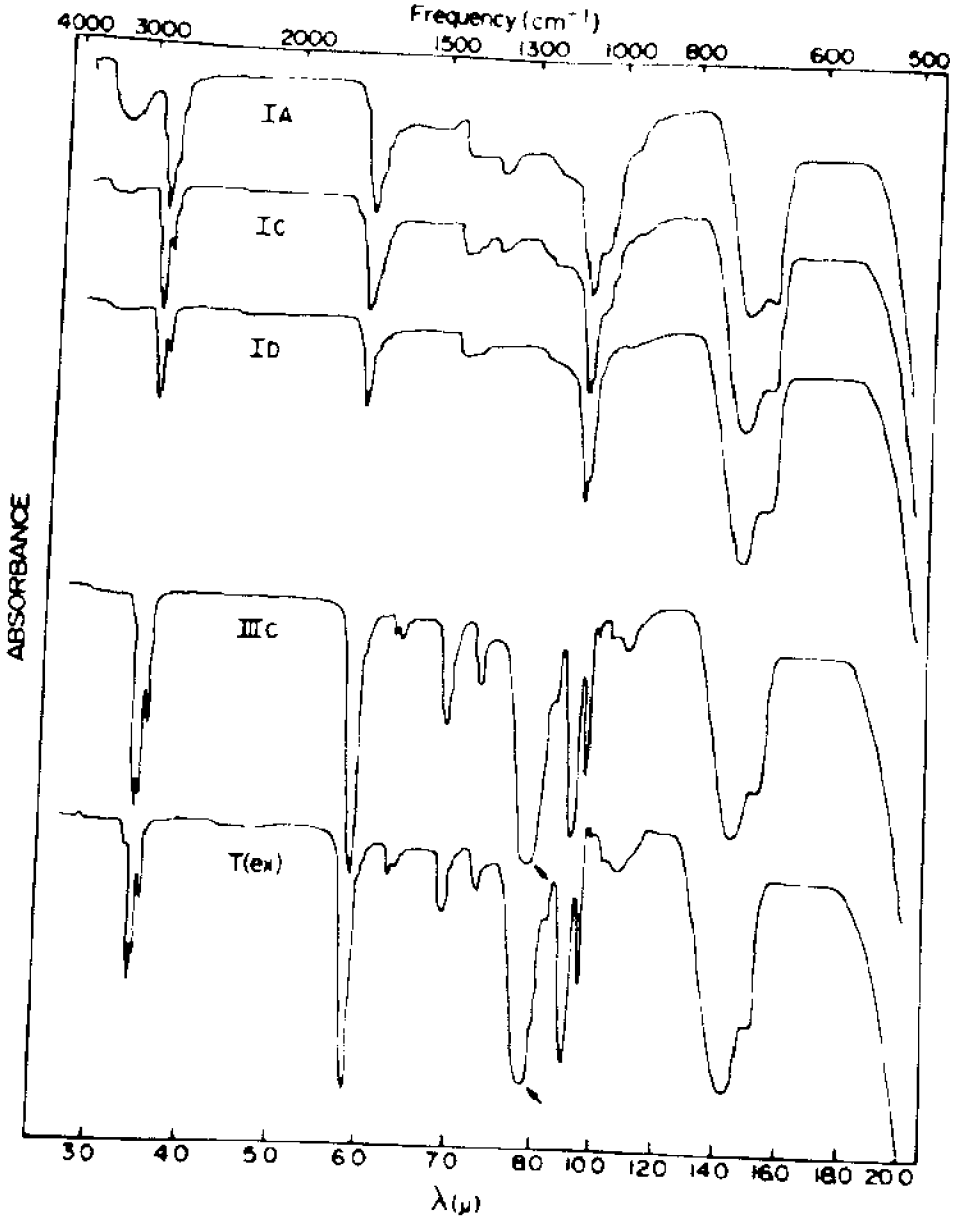

Fig. 2. Infrared spectra of *G. breve* toxins and tygon tubing extract. Upper abscissa: frequency (cm^{-1}); lower abscissa: wave length (μ = microns); ordinate: absorbance. I_A = toxic fraction I_A, I_C = toxic fraction I_C, I_D = toxic fraction I_D, III_c = non-toxic fraction III_c, $T_{(ex)}$ = tygon tubing extract.

Gas-Liquid Chromatography

Analyses were performed with a Beckman GC-45 dual column gas chromatograph. The results and conditions of analysis are summarized in Table II. Two peaks were obtained from the tygon extract, one of these peaks had the same retention time as the single peaks obtained with crude toxin (glass extracted) and non-toxic fraction III_c. The purified toxin, fraction I_c, had the same retention time with the 1% SP 1000 column but somewhat lower retention times with the other columns (Table II). The purified toxic fraction I_c may be completely different from the non-toxic fractions and some phthalate-like compounds extracted from natural red-tide sample.

Table II

SUMMARY OF GAS-LIQUID CHROMATOGRAPHY

Column Temperature Flow rate	1% SP 1000 230°C 35 ml/min		1% SP 2401 (OV 210) 230°C 35 ml/min		3% SP 2100 (SE 30) 230°C 20 ml/min	
	Retention Time (min)					
Purified toxin fraction I_C	1.5	-	-	1.4	-	6.2
Crude toxin	1.5	-	-	1.6	-	8.2
Non-toxic fraction III_c	1.5	-	-	1.7	-	8.0
Tygon extract	1.5	2.5	1.1	1.7	3.7	7.9

Flame ionization Detector, Temp = 300°C; N_2 = 30 psi; Air = 300 psi; H_2 = 50 psi.

Columns: 6 ft x 2 mm I.D., glass, pre-packed (Supelco, Inc., Bellefonte, Pa.).

DISCUSSION

The isolation and purification procedures of *G. breve* toxin seem to have developed as a matter of choice by various investigators. As a result, there is no standard procedure. A comparison between the various toxins is impossible and it is not surprising that different results are obtained with these preparations and that a degree of artifactuality may have been introduced. This seems to be particularly true with samples derived from naturally occurring red tides. Not only may similar compounds be extracted from a mixed population of plankton, one may be inadvertently concentrating toxic compounds, arising not from the red-tide organisms but also from man-made

pollutants. This problem is no longer a remote one in view of the reported wide occurrence of lipophilic organic compounds (7), tars and plastics (8), insecticides (9), and phthalates (10). The latter class of compounds are particularly troublesome in view of their wide distribution in the environment particularly in river effluents (11-13).

In the course of the present investigation, we have attempted to simplify procedures for the isolation of *G. breve* toxins as to reduce the possibility of introducing artifacts such as the contamination of the isolates with materials extracted from plastics in the laboratory (e.g. tygon tubing) or the inadvertent breakdown of the parent toxic principle.

First of all, the toxic material from *G. breve* is almost surely a lipid compound. For this reason, we used the chloroform extraction which is widely used for lipid isolation from biological samples. Although polar and nonpolar compounds extracted by chloroform can be further separated by activated silica gel column chromatography, we did not obtain a clear separation of the various bands on the column. The bands were diffused and merged as the elution continued.

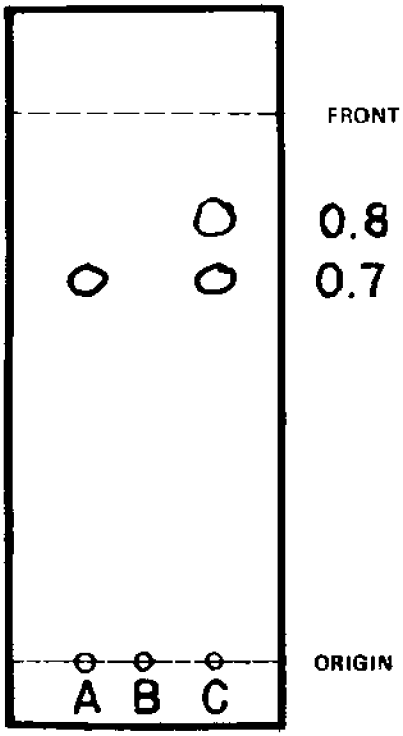

Fig. 3. TLC analysis of crude *G. breve* toxin, fraction I_C and tygon tubing extract. TLC plate: silica gel 60 F-254 (E. Merck). Solvent system: toluene:ether:ethyl acetate: acetic acid (80:10:10:0.2). A: crude toxin (extracted in an all-glass system from red-tide sample), B: purified toxic fraction I_C, C: tygon tubing extract.

Thin layer chromatography was done on (A) a separate sample of crude toxin which was extracted and separated under carefully controlled conditions to assure that it never came into contact with any plastic tubing, etc., (B) fraction I_C, and (C) material extracted from tygon tubing by overnight exposure to chloroform. The results, shown in Fig. 3, indicate that the crude toxin had a component with an R_f value of 0.7 (in common with the tygon tubing extract) in addition to material which remained at the origin. The purified fraction I_C on the other hand, did not migrate but remained at the origin. The tygon extract was completely resolved into two components with R_f values shown in Fig. 3. Thus, we tentatively conclude that alkyl-phthalates were originally present in the red-tide sample and did not arise during the extraction procedures in the laboratory.

The UV spectrum showed that all toxic fractions had strong absorptions between 190-240 nm although peak shape became sharper and narrower for fractions I_C and I_D. This suggests that all toxic fractions seem to have substituted aromatic structures (Fig. 1a). There was no significant absorption in the visible range, except for a small absorption peak at 533 nm for fraction I_A which may be due to the dark green color (Fig. 1b).

The strong and wide absorption in the 14-15 μ region in the IR spectrum (Fig. 2) also indicates the possibility of aromatic ring structures in all toxic fractions. Fraction I_A had a peak at 3.0 μ which may show the presence of -OH (or -NH) groups. This peak was not prevalent in fractions I_C and I_D. All the fractions have a relatively strong absorption at 5.8 μ which may indicate the presence of carbonyl groups (-C = O).

The three toxic fractions have a basically similar pattern in their IR spectra but this pattern is also similar to those of aliphatic phthalate compounds (14) which might have been extracted from plastic material (i.e tygon tubing). We have included an IR spectrum of this material (T_{ex}) in the lower part of Fig. 2 together with the non-toxic fraction III_c. Note, however, that these spectra have a strong absorption peak at 7.8-7.9 μ (arrows) which may indicate the presence of .-C-O-C structure. Unlike the toxic fractions they show no absorption at 3.0 μ. These results seem to agree with TLC analysis above.

Sasner *et al* (1) performed similar kinds of analyses and found that their preparation had two peaks of absorption at 410 and 210 nm. They suggested that the peak in the visible range was due to pigment contamination but did not comment on the UV absorption. Their IR spectrum did not indicate the presence of aromatic and conjugated double bond systems.

We also analyzed fraction I_C and the tygon extract with a mass spectrometer (AEI, 902, with electron excitation) and obtained ion spectra similar to those found with aliphatic phthalates, particularly, di-2-ethylhexyl phthalate as reported by Sato *et al.* (12). The diagnostic fragments have m/e values of 279, 167, and 149. They were present in fraction I_C which in addition had additional fragments in the range of 185-263. As noted by Hites (13), however, closely related phthalates will have additional fragments in this region. This analysis suggests that (a) the toxic fraction we have isolated is similar to

dialkyl phthalate compounds or (b) the phthalates are contaminants of fraction I_C which are more readily volatilized during the mass spectrometric analysis than the toxins themselves which may be higher molecular weight compounds as suggested by Trieff *et al.* (4).

It should be noted that generally speaking phthalates do not show molecular ion peaks in their mass spectra. If the parent *G. breve* toxin behaves similarly, the molecular weight determinations must be performed by alternate physical methods. In view of the ubiquitous nature of plastics in the environment, it is imperative to conduct investigations on the chemical nature of *G. breve* toxins on laboratory grown cultures. The results of the present investigation emphasize this need.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Mr. E. Rauckman of the Chemistry Department, Duke University, for performing the initial mass spectrometric analyses, and Dr. D. Rosenthal of the Mass Spectroscopy Laboratory, Research Triangle Institute, Research Triangle Park, North Carolina, for the high resolution mass spectrometric analyses. This investigation was supported by Grant #FD00120, FDA.

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ISOLATION, PHYSICO-CHEMICAL, AND TOXICOLOGIC CHARACTERIZATION OF TOXINS FROM *GYMNODINIUM BREVE* DAVIS

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ABSTRACT

At least five toxins have been isolated from a crude acidified ether extract of unialgal cultures of the dinoflagellate *Gymnodinium breve* Davis. Techniques of column, liquid and thin layer chromatography (TLC) have been employed for the isolation and purification of these toxins. The toxins were monitored by mouse toxicity. Some of the toxins appear to have neuro-toxic effects. No acetylcholinesterase inhibitory properties have been detected in any of the toxins studied.

The method of isolation of the toxins is a modification of Alam *et al.* (2). The fractions were initially separated in the dark on a silicic acid column using eluting solvents of 2:1(v/v) benzene:ethyl acetate (B:E), 1:1(v/v) B:E and methanol. Subsequently, 2-dimensional TLC was performed in the dark on the previously separated fractions, the spots being eluted with ether. Physico-chemical techniques of UV, IR and mass spectrometry have been utilized for characterization.

T_1(R_f 0.42-0.44), possesses hemolytic activity; T_2 (R_f 0.35) studied to the greatest extent, has a molecular weight of 725 and an empirical formula $C_{41}H_{59}NO_{10}$, possessing unsaturation and a lactone ring. T_3(R_f 0.24-0.30) and T_4 (R_f 0.16-0.19) have not been prepared in sufficient amounts to be studied further; T_5 (R_f 0-0.08) is probably a complex mixture of toxins and is a severe respiratory irritant.

Differences in the batches of crude toxin may account for differences in the results of the present work as compared with that of Alam *et al.* (2). Ethanol was found to degrade T_5, producing multiple fractions of higher R_f's, the time of contact with ethanol affecting the results.

INTRODUCTION

The dinoflagellate *Gymnodinium breve* Davis (*G. breve*), has caused numerous fish-kills during the time of its blooms, called the "red-tide" occurring periodically along the west coast of Florida and the Gulf of Mexico. *G. breve* is also responsible for numerous cases of poisoning of humans who have eaten shell-fish contaminated with the dinoflagellate (10) and for respiratory irritation of individuals who have inhaled the spray carrying the organism during a "red-tide" (11). The dinoflagellate produces endotoxins, shown by McFarren *et al.* (9), Ray and Wilson (13), Halstead (6), Spikes *et al.* (21), and Sievers (18) to be toxic to marine and laboratory animals. Various workers [Cummins *et al.* (4), Martin and Chatterjee (7), Paster and Abbott (12), Trieff *et al.* (22), Sasner *et al.* (16), Spiegelstein *et al.* (19) and Alam *et al.* (2)] have reported the isolation and partial characterization of toxins(s) from *G. breve*. The physiologic or pharmacologic actions of the crude and partially purified toxins(s) have been reported by a number of workers [Sasner (15), Sasner *et al.* (16), Paster and Abbott (12), Abbott and Paster (1), and Spikes *et al.* (20, 21)]. It is important to note that most investigators report that their

toxin(s) was contaminated with carotenoid(s) pigments. It is also significant that no two workers have reported the isolation of the same toxin from *G. breve*.

Studies on *G. breve* toxin(s) have been going on for the past few years in our laboratory. We wish to report the present status of our work on *G. breve* toxin(s) and offer a partial explanation for the diversity of results among the different laboratories.

EXPERIMENTAL

G. breve[1] was cultured in modified NH-15 media by the method of Gates and Wilson (5) and extracted by the method of Spikes (20). The ether extract was evaporated to dryness *in vacuo* in a rotating evaporator and the residue redissolved in a small volume of ether. Suspended particles were removed by centrifugation and pouring off the supernatant which was evaporated to dryness. The residue was dissolved in 3 ml of 2:1 (v/v) benzene:ethyl acetate (B:E).

Column Chromatography

The *G. breve* extract dissolved in 2:1 (v/v)B:E. was placed on a column (7.5 cm x 7.5 cm) prepared by packing with a silicic acid[2] slurry in 2:1 (v/v) B:E.

Eluting solvents were 2:1 (v/v) B:E (360 ml, fractions 1-9), followed by 1:1 (v/v) B:E 360 ml, fractions 10-18), and finally with methanol (200 ml, fractions 19-23). In all, 23 fractions (40 ml each) were collected from column chromatography. After concentration of each fraction separately to 3-4 ml *in vacuo* on a rotating evaporator, each fraction was spotted on a TLC plate and its R_f values determined on two solvent systems (see below). On the basis of the R_f value, fractions were combined to give a total of 9 fractions. All column chromatography was carried out in darkness to avoid photochemical transformation which was suspected.

Thin Layer Chromatography

Glass plates (20 x 20 cm), coated with silicic acid[3] (300 and 500 μ thickness) were used for qualitative and preparative work respectively. Plates were activated for 45 minutes at 110°C just before use. TLC was carried out in the dark to avoid possible photochemical change. The solvent systems used were

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1. The unialgal starter culture was obtained from the culture collection of Dr. W. B. Wilson, Moody College of Marine Sciences and Maritime Resources, Texas A&M University, Galveston, Texas 77550.
2. SilicAR 7GF, for TLC; Mallinckrodt Chemical Works, St. Louis, Mo.
3. Silica gel for TLC, D-5, Camag, Inc., Wisconsin.

I. 2:1(v/v) B:E and II 1:1(v/v) B:E. Location of the spots was performed by using iodine vapor followed by spraying with 50% H_2SO_4 and heating for one half hour at 110°C. In previous work, 1% $KMnO_4$ and Dragendorff's reagent[4] were also used. Two-dimensional TLC was employed for final separation of the components using Solvent System II. A sample chromatogram is included in Figure 1. In preliminary experiments the components were located in the manner described above, but for preparative work no location reagents were used and the R_f values were used to locate the spots. The spots were scraped off, eluted with ether and evaporated to dryness *in vacuo*. The procedure was repeated until optimal purification was achieved.

Monitoring of Toxicity

The particular component was dissolved in a known volume of ether. An aliquot of this solution was taken to provide 200 µg of material. To the ether aliquot was added 1 drop of polysorbate-80 and the ether evaporated. To the residue, 1.0 ml of isotonic saline was added and the two-phase system shaken until it was homogeneous. Then, the entire 1.0 ml was injected in a white Swiss yale mouse weighing approximately 20 g. The mouse was examined for 72 hours and the symptoms observed. Death, pain or difficulty in respiration

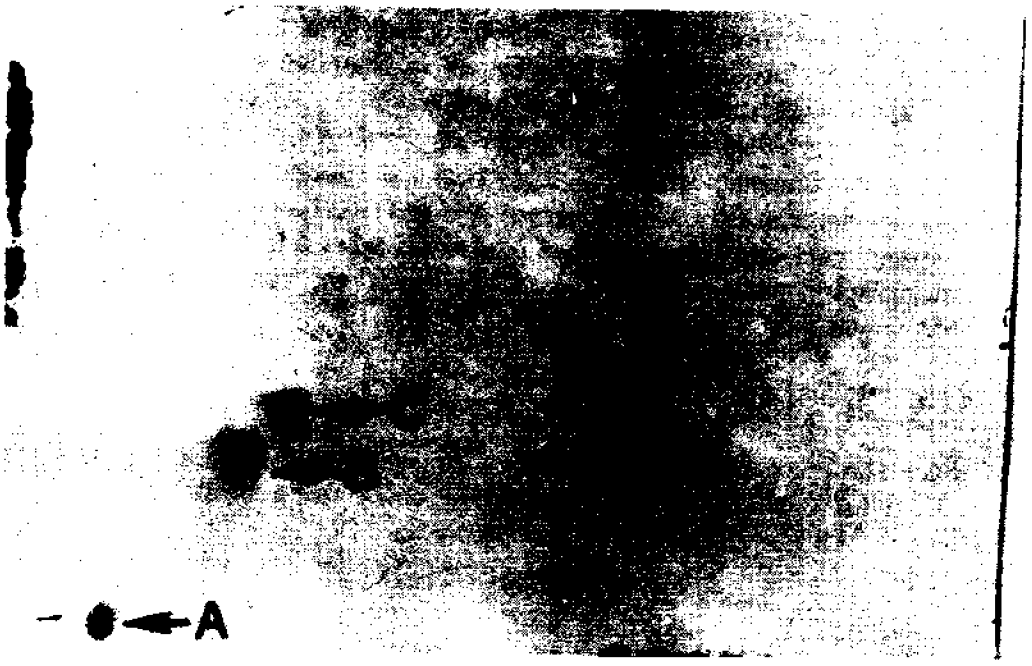

Figure 1. Two dimensional TLC of fraction 9 on silica gel coated plates (500 µ thickness) using 1:1(v/v) B:E solvent after iodination and charring with 50% H_2SO_4. A = T_{5_0} + T_{5_1}; B = T_4; and C = T_3.

4. Dragendorff's reagent type QSR-D, Quantum Industries, Fairfield, N.J.

were noted. If there was any ambiguity, the experiment was repeated. If a fraction had either an LD100 of greater than 10 mg/kg or elicited no noticeable discomfort, it was considered non-toxic.

Liquid Chromatography

Liquid chromatography was carried out on several fractions in an effort to effect further purification. A Merck pre-packed silica column (310 mm length x 25 mm i.d.) and basic equipment for liquid chromatography[5] were employed using an LKB 4912A peristaltic pump and Research Specialties Co. fraction collector with drop counting unit. Two ml samples were applied with ethanol which was also the eluting solvent. Forty fractions (5 ml each) were collected.

Physiologic and Physico-chemical Characterizations

Visible and UV spectra were recorded in methanol solutions on a Perkin-Elmer Coleman 124 spectrophotometer. IR spectra were recorded in carbon tetrachloride (0.05 g/ml) on a Perkin-Elmer 337 grating IR Spectrophotometer. A mass spectrum was recorded on toxin T_2 using a CEC 21-110B double focusing instrument; the temperature was 380°C and the resolution 8000 M/ΔM. Elemental analysis was performed by Huffman Laboratories, Inc., Wheat Ridge, Colorado. Phosphorous was determined by the method of Mason *et al.* (8) and Bartlett (3). The effect of crude toxin, various fractions, and purified toxins on human blood serum cholinesterase activity was determined by spectrophotometry[6]. Hemolytic activities of the crude, purified toxins T_1 and T_2 and various fractions were determined by the method of Reich *et al.* (14).

Degradative Studies

Acid hydrolysis of toxin T_2 (9.0 mg) was carried out by refluxing in 50 ml of 2N HCl in methanol at 70°C for 9 hours. After hydrolysis, the methanol was removed by distillation and the hydrolysate partitioned between equal volumes of water and ether. The ether layer was separated and evaporated to dryness *in vacuo*. The residue was spotted on a TLC plate, developed in solvent system II.

Degradative studies to demonstrate the effect of ethanol, if any, on various toxic fractions were also performed. Various contact times and temperatures were utilized and details are given under the "Results" section.

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5. From EM Laboratories Inc., 500 Executive Boulevard, Elmsford, N.Y. 10523

6. Sigma Technical Bulletin No. 420, Signa Chemical Co., St. Louis, Mo.

TABLE 1

Weight, R_f Values, Toxicity and Physiological Properties of the Toxins from *G. breve* Davis [Alam *et al.* (2)]

		Fraction number									Purified Toxin	
		Crude	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5	Fr. 6	Fr. 7	Fr. 8	Fr. 9	T-1	T-2
1.	Column chromatography eluting solvent		← B:E 2:1(v/v) →					← B:E 1:1(v/v) →			Methanol		
2.	Weight of toxin(s) mgs.+	353.7	168.0	25.0	4.3	5.46	6.0	25.4	5.07	16.6	97.1	2.3	4.3
3.	Phosphorus % of weight	0.025	0.010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.062	0.00	0.00
4.	TLC: R_f x 100 in system I	-	96.90, 84,71	90,84, 71	84,71	71,60	71,60, 43	43,25, 12	11,8, 5	8-5	less than 5	8-5	8-5
5.	TLC: Rf x 100 in system II	-	-	-	-	-	92,85 74	85,74 70	60,50 42,35	42,35 26,8, 4,0	26,20	42	35
6.	Acetylcholinesterase activity	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	**	**	(-)ve	(-)ve	(-)ve
7.	Hemolytic activity (H_{50})	100	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	**	**	(-)ve	25	(-)ve
8.	Mouse toxicity: LD 100 (mg/kg)	5.9	> 10	> 10	> 10	> 10	> 10	> 10	.0.30	0.49	0.20	0.30	0.25

* Hemolytic activity (H_{50} in μgs) Concentration of the toxin (in μgs) which will produce 50% hemolysis of rabbit erythrocytes under the experimental conditions.

\+ Average of 15 column chromatography fractionations.

** Was not determined as fraction 7 and 8 had both toxins T_1 and T_2.

RESULTS

Chromatography and Properties of Various Fractions

Table 1 shows the results of Alam *et al.* (2) describing the chromatography and properties of the various fractions. Only fractions 7, 8 and 9 were found toxic to mice. By repeated preparation TLC of fractions 7 and 8, Alam *et al.* (2) obtained purified toxins T_1 and T_2, having R_f values of 0.42 and 0.35 respectively. The presence of a third crude toxin was observed in fraction 9.

In a recent study using two additional batches of crude *G. breve* ether extract, by further 2-dimensional TLC of fractions 7 we separated Alam's T_3 into two toxins with R_f's of 0.24-0.30 (designated as T_3) and 0.16-0.19 (designated as T_4). Another toxin (designated as T_5) was obtained mainly from the methanol-eluted fraction of column chromatography. It consisted, on 2-dimensional TLC with solvent system II, of two spots, one with Rf=O and the other with Rf 0.04-0.08. This corresponds exactly with what Alam *et al.* (2) had found,by starting with different batches. A comparison of the two studies is presented in Table 2.

Table 2: A comparison of R_f Values by TLC in 1:1 (v/v) B:E Solvent Starting from Different Batches of Crude *G. Breve* Toxin.

	Designation	R_f	
		Present Study	Alam *et al.* (2)
Toxic Fractions	Toxin T_5	0	0
		0.06	0.04
			0.08
	Toxin T_4	0.16 - 0.19	0.20 *
	Toxin T_3	0.24 -0.30	0.26
	Toxin T_2	--- **	0.35
	Toxin T_1	0.44 ***	0.42
Non-toxic Fractions		0.50	0.50
		0.63	0.60
		0.75	0.70 - 0.74
		0.81 - 0.88	0.85
		0.91	0.92

* This crude toxin was designated by *Alam et al.* (2) as toxin T_3

** Indicates that the band was not observed.

*** Indicates that the band was found non-toxic to mice ($LD_{100} > 10$ mg/kg.)

Degradation Studies on Toxin T_5.

Alam *et al.* (2) purified toxins T_3 and T_4 (designated previously as T_3) by column chromatography on silicic acid and eluting with 5% methanol in chloroform. It was found contaminated with small amounts of pigment(s) and not further characterized.

As noted in Table 2, we observed toxin T_5 to be a complex of two components, one with $R_f = 0 (T_{5_0})$ and the other with R_f 0.06 (T_{5_1}). This mixture was injected into mice and found toxic, causing severe respiratory distress, although it did not cause death at the dosage empolyed. The mixture, T_5 was purified by liquid chromatography with ethanol as eluting solvent and separated into fractions. Results were the same both in the presence of and absence of light. The forty tubes were combined into eight fractions, evaporated *in vacuo* at 50-60°C and tested on TLC with solvent system II. Curiously, the R_f's were altered to a marked extent compared with the original and were found to vary between 0.72 and 0.90, and all fractions were found non-toxic.

To ascertain whether the liquid chromatographic process or the ethanol caused the degradation and loss of toxicity, T_5 was eluted with ethanol, evaporated *in vacuo* at 50-60°C and rechromatographed on TLC with solvent system II. The R_f of T_{5_0} was altered from 0 to 0.94, while that of T_{5_1} hanged from 0.06 to 0.84. In a further experiment the T_5 was eluted with ether and evaporated *in vacuo*. The residue was dissolved in ethanol and immediately spotted on a TLC plate. This time, four different fractions were observed with R_f's of 0.36, 0.46, 0.55, 0.70. The results of these experiments are summarized in Figure 2.

Physico-chemical Data on Toxin T_2.

Some of these data have already been reported [(Alam *et al.* (2)]. Toxin T_2, a white amorphous powder, obtained by lyophilization of a benzene extract after TLC purification, was found by mass spectrometry to possess a molecular ion with m/e of 725. From the mass spectrometry the most probable molecular formula was found to be $C_{41}H_{59}NO_{10}$. The IR spectrum of T_2 has bands at 3000 cm^{-1} (shoulder, -C-H unsaturated) 2895 cm^{-1} (-C-H saturated), 1740 cm^{-1}, and 1710 cm^{-1} (carbonyl) and a strong absorption band between 1120 cm^{-1} and 1025 cm^{-1} (-C-O-C-or tertiary amine). The absence of any band in the region of 3700-3200 cm^{-1} indicates the absence of hydroxyl and primary or secondary amino groups. A UV spectrum of T_2 gives absorption maxima at 260 nm ($\epsilon_M = 5.0 \times 10^3$), 267 nm and 270 nm ($\epsilon_M = 3.8 \times 10^3$). Both the IR spectrum and the fact that the toxin decolorizes potassium permanganate indicate unsaturation.

The ether-soluble hydrolytic product of T_2 gave a single spot (0.67) in solvent system II and the IR spectrum had a strong absorption band at 3300 cm^{-1} (-OH or N-H) and weak absorption at 1750 cm^{-1} (-C=O). On the basis

of its IR spectrum, the ether-soluble hydrolytic product has at least two of th ten original oxygens in T_2 present in a lactone ring or an ester bond.

DISCUSSION

Table 2 provides evidence for the existence of at least five toxins from the G *breve*. Depending on the batches used, there is variability in the amount of th different fractions. In the present study no band was found at 0.35. We hav no satisfactory explanation for the fact that Alam *et al.* (2) found toxin T_1 t be toxic while in the present study toxin T_1 was found non-toxic. In the stud of Alam *et al.* (2), no toxicity measurements were made on toxin T_5.

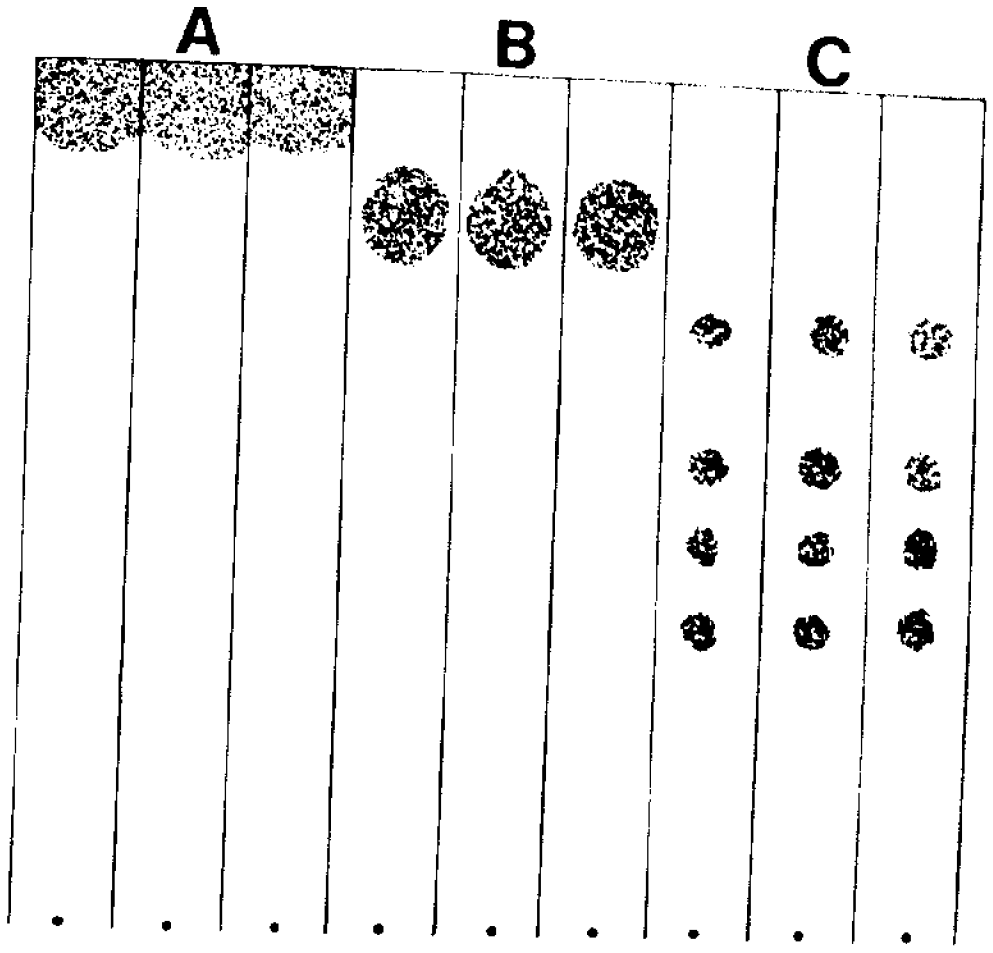

Figure 2. Degradation studies of T_5 with ethanol. A results when T_{5_0}, previously separated by 2-dimensional TLC and elution with ether, followed by hydrolysis with ethanol, heating to 50°-60°C, and evaporation, is chromatographed: $R_f = 0.94$. Similarly B is T_{5_1} after using the above procedure; $R_f = 0.84$. C results after T_5 is dissolved in ether and evaporated. Solution in ethanol and TLC results in the 4 fraction observed with R_f's = 0.36, 0.46, 0.55 and 0.70.

This batch-to-batch variation in the concentrations of the different components was confirmed by performing TLC on three different batches of crude *G. breve* the results of which clearly indicated that the components of different batches differ markedly in both qualitative and quantitative aspects (See Figure 3).

The experiments showing the effects of ethanol on toxin T_5 (Figure 2) clearly indicate that ethanol is both degrading and detoxifying the toxic material. The R_f values in solvent system II of toxin T_5 increase further, the longer the exposure to ethanol and the higher the temperature. Thus, for a short exposure at room temperature the R_f's are a continuum from 0.36 to 0.70, while for a longer exposure at higher temperatures (50-60°C) the R_f's are 0.84 and 0.94. The latter appear to be final decomposition products. The fact that the R_f's are increasing indicates that the decomposition is causing the original molecules to become more non-polar. It is pertinent to point out that in earlier work [Trieff *et al.* (23)],we noted that degradation and detoxification of a partially purified *G. breve* toxin occurs in the solvent system $CHCl_3$, CH_3OH, $6NNH_3$ (90:9.5:0.5) as well as in 0.1 N NaOH with heating.

Alam *et al.* (2) have reported that our toxin T_2 is similar to toxin T_2 of Spiegelstein *et al.* (19) as well as to substance II of Martin and Chatterjee (7) with regard to its lack of hemolytic activity. With regard to acetyl

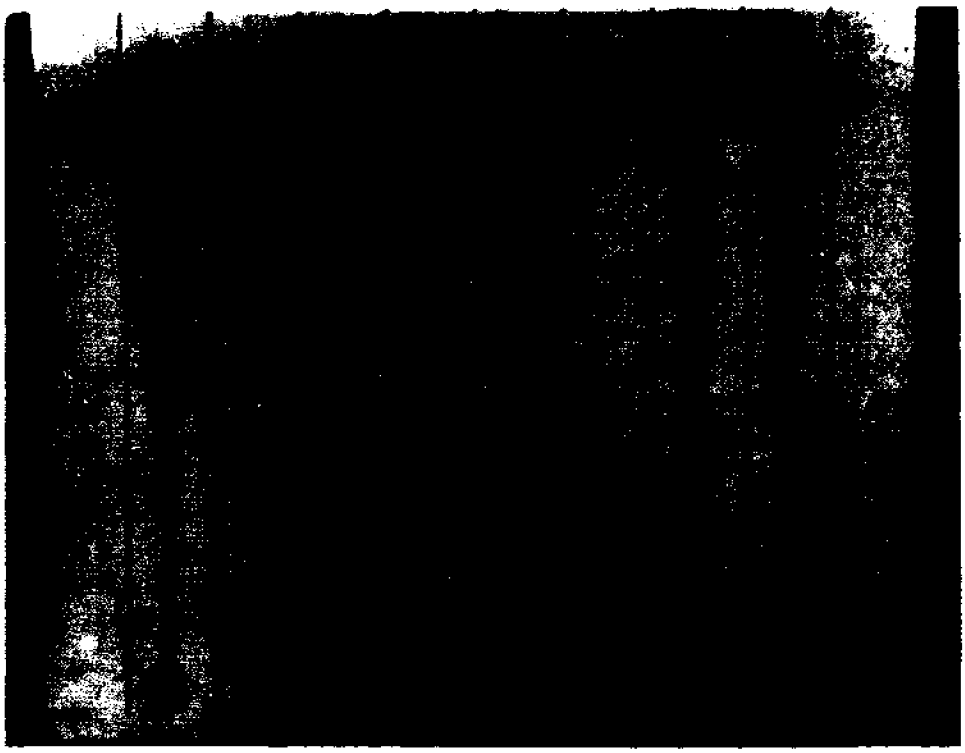

Figure 3. TLC of three different batches of crude *G. breve* toxin obtained at different times. TLC utilized silica gel coated plates (500μ) and 1:1(v/v) B:E as solvent. Columns 2,3 = batch 1;5,6 = batch 2;8,9 = batch 3.

cholinesterase inhibitory activity, it is different from toxin T_2 of Spiegelstein *et al.* (19) but similar to substance II of Martin and Chatterjee, who also reported the absence of anti-acetyl cholinesterase activity in their major toxin; however, toxin T_2 differs from substance II of Martin and Chatterjee (7) in that it has no phosphorous. The physicochemical parameters of our toxin T_2 bear some similarity to those of ciguatoxin as described by Scheuer *et al.* (17) in terms of its IR and UV spectra, lack of phosphorus and a Dragendorff-positive nitrogen. There is a possibility of some metabolic relationship between toxin T_2 and ciguatoxin (2).

CONCLUSIONS

At least five toxins have been isolated from a crude acidified ether extract of *G. breve* using a modification of the method of Alam *et al.* (2), employing column, thin layer, and liquid chromatography. Toxicity was monitored by mouse bio-assay. Each of the toxins displayed neuro-toxic effects although no inhibition of acetylcholinesterase activity was noted.

One of the toxins, T_2, previously studied in detail by Alam *et al.* (2) was not isolated by us from two batches of crude toxin, and one of the toxins, T_1, isolated in the present study, was not found to be toxic. There was complete agreement on the R_f's of the non-toxic fractions. Significant qualitative and quantitative differences in the components of the batches of the crude toxin and degradation that results from exposure of some components to polar solvents such as ethanol or methanol may account for the observed differences.

ACKNOWLEDGMENT

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CHEMICAL AND PHYSIOLOGICAL STUDIES ON THE MARINE DINOFLAGELLATE *AMPHIDINIUM CARTERAE*

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ABSTRACT

Species of the genus *Amphidinium* (Family Gymnodiniidae) have been suspect as toxin-producing dinoflagellates. Recently, cells of *A. carterae* have been shown to be toxic to fish and mice, and the effects of crude extracts of the organism on molluscan, crustacean, and amphibian heart activity and on mouse intestine contractions indicated the presence of choline-like substances. Cells of *A. carterae* contain 0.4% total choline chloride equivalents dry wt. Upward of 75% of the choline is non-lipoidal in nature and occurs mainly in three water soluble compounds. These have been purified by preparative paper and thin layer chromatography. One of the compounds has been identified as acrylylcholine on the basis of hydrolysis to acrylic acid and choline and by comparison with the synthetic product. One compound has been shown to be choline O-sulfate. The third compound, present in smallest amount, is also a choline ester, probably also esterified with a small molecular weight organic acid, but has not been identified. Acrylylcholine exhibited 1:4,000 the activity of acetylcholine in decreasing both the amplitude and frequency of beat of isolated myogenic *Mercenaria mercenaria* heart, and, as with acetylcholine, the activity could be blocked with Mytolon. Choline O-sulfate exhibited 1:20,000 the activity of acetylcholine on isolated *Mercenaria* heart. Acrylylcholine also caused an increase in frequency of beat of *in vivo* crustacean heart and an increase in frequency and amplitude of contractions of isolated mouse intestine. All these effects could be reversed by washing.

INTRODUCTION

The genus *Amphidinium* is included in the family Gymnodiniidae. The annulus or girdle is near the anterior end whereas in the genus *Gymnodinium* the girdle is located near the center. Red tides in the Delaware Bay have been attributed to *Amphidinium* species (14) and high concentrations have been reported to discolor sands in subtidal areas (6,7). Halstead (5) includes the genus *Amphidinium* in his list of toxic dinoflagellates. In 1957 McLaughlin and Provasoli (15) reported that supernates from cultures of *A. klebsii* and *A. rhynchocephalum* were toxic to fish.

A. carterae is a temperate water marine dinoflagellate. It was described from the Cape Cod area of Massachusetts, U.S.A., by Hulbert (9). Recently Thurberg and Sasner (21) showed that cells of *A. carterae* were toxic to fish and to mice. They showed that crude cell extracts of the organism increase tension development in crustacean heart and mouse intestine and inhibit molluscan and amphibian heart activity and that pretreatment of molluscan heart with Mytolon and amphibian heart with atropine block the inhibitory effects of the extracts. These physiological tests indicated the presence of acetylcholine-like substances in the extracts of *A. carterae*. Wangersky and Guillard (22) have reported that an acetylcholine analog may be present in the organism.

This report summarizes the further chemical and physiological studies which have recently been carried out on substances produced by *A. carterae*.

EXPERIMENTAL PROCEDURES

Organism and Culturing

A culture of *A. carterae* obtained from R. L. Guillard, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, was used in this study. The organism was grown in 20 liter carboys in artificial sea water medium at 24± 2°C under constant artificial illumination and aeration and the cells harvested by centrifugation (21).

Choline Assays

Choline assays were carried out using a choline-requiring mutant of the mold *Neurospora crassa* (strain 485, Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire) and Choline Assay Medium (Difco Laboratories, Detroit, Michigan) by the method of Horowitz and Beadle (8). Samples were first hydrolyzed with HCl to liberate free choline (10). In the assay N-methylethanolamine and N, N-dimethylethanolamine are equally active as choline on a molar basis, and intact choline esters show almost zero to almost full acetylcholine activity, when compared on an equimolar basis, depending upon the acyl group (10).

Physiological Methods

Acetylcholine activity was measured using isolated *Mercenaria mercenaria* heart according to the method of Florey (4) and effects on *in vivo* crustacean heart and isolated mouse intestine measured by previously described methods (18). Heart beats and intestinal contractions were measured using Grass FTO3C force displacement transducers and the signals were recorded on a Grass Model 5 Polygraph.

Gas-Liquid Chromatography

Gas-liquid chromatography for volatile products resulting from the hydrolysis of choline-containing compounds was carried out on a Barber-Colman Model 5000 Gas Chromatograph equipped with a hydrogen flame ionization detector. The column consisted of a 6 ft x 4 mm (i.d.) glass U-tube column packed with 100-120 mesh Porapack Q (Waters Associates, Inc., Milford, Massachusetts). Details for the hydrolysis of the samples and preparation of the volatile hydrolysis products for analysis as well as details of the analytical procedure are given elsewhere (19,20). The retention times of a variety of short chain acids, alcohols, aldehydes, ketones, and lactones using this system have been recorded (19).

Paper and Thin-Layer Chromatography

Whatman No. 1 paper and precoated silica gel (E. Merck) and alumina (Macherey, Nagel) glass thin-layer plates (Brinkman Instruments, Westbury, New York) were used. The choline compounds were detected by exposing the paper or plates to iodine vapor or by spraying with Dragendorff's Reagent. Silica gel plates were also sprayed with 50% sulfuric acid and charred. The paper and thin-layer chromatographic properties of a number of choline derivatives in a variety of solvent systems have been recorded (10).

Isolation of Choline Compounds

The following procedure was developed for the isolation of the non-lipoidal choline fraction from *A. carteri* cells (20). One g samples of dry cells were extracted first with 250 ml of 95% ethanol, centrifuged, then extracted with 250 ml of 90% ethanol and again centrifuged. The ethanol extracts were combined and concentrated to dryness *in vacuo*. The residue was suspended in 100 ml of water and extracted with 100 ml n-butanol. The resulting aqueous phase, which contained the non-lipoidal choline compounds, was then concentrated *in vacuo* to dryness. By means of preparative paper chromatography using Whatman No. 1 paper and the solvent system 2-propanol: pyridine: water (6:4:3 v/v), three choline-containing compounds, designated 1, 2, and 3, were separated from each other (10,20).

CHEMICAL STUDIES

Choline Content of A. carterae

Hydrolysis and assay of *A. carterae* whole cells showed that they contained 0.36-0.41% choline chloride equivalents on a dry weight basis (10). When this value was compared with values found in other algal divisions (10, 11), namely, < 0.001-0.015% for blue-green algae, 0.01-0.12% for red algae, < 0.001-0.07% for brown algae, 0.08% for a euglenid, and 0.002-0.18% for green algae, it was seen that it was significantly higher. An investigation was therefore undertaken to determine the nature of the choline compounds present in the organism.

Nature of the Choline Compounds in A. carterae

Choline in eucaryotic cells occurs generally in the form of the lipid phosphatidylcholine. However, by the procedure described in the previous section it became apparent that approximately 75% of the choline present in the cells was water-soluble and non-lipoidal in nature (10). The non-lipoidal choline-containing fraction was separated into three choline-containing compounds by preparative paper chromatography (10). When the chroma-

tography was carried out in 2-propanol: pyridine: water (6:4:3, v/v), the choline-containing fraction was resolved into a slowest moving, a faster moving, and a fastest moving fraction, which were designated as Compounds 1, 2, and 3, respectively.

Identification of the Choline Compounds

Compound 1 was further purified by a second preparative paper chromatographic step and treatment with 95% ethanol to remove ethanol insoluble impurities (20). Compound 1 was extremely hygroscopic. It gave a positive hydroxylamine test, indicating it to be an ester. On hydrolysis it yielded choline chloride and a volatile component which was identified as acrylic acid by a gas-liquid chromatographic comparison with authentic redistilled acrylic acid. This indicated that Compound 1 was acrylylcholine. A comparison between Compound 1 and synthetic acrylylcholine in 14 paper chromatographic and 8 thin-layer chromatographic systems indicated that the two compounds were identical (20). Both Compound 1 and acrylylcholine also give the same bright orange color typical of choline esters when chromatograms were sprayed with Dragendorff's Reagent.

Compound 2, which occurred in the smallest amount of the three, was purified further by a second paper chromatographic system (20). It also gave a positive hydroxylamine test and an orange Dragendorff color on chromatograms, thus indicating that it too was an ester. Upon HCl hydrolysis it yielded choline chloride and a volatile acidic product which had a retention time on gas-liquid chromatography midway between that of acetic acid and acrylic acid (19). Propionic acid has a retention time near that of acrylic acid. The retention time of the volatile hydrolysis product of Compound 2 did not correspond to any two or three carbon atom acid, alcohol, lactone, aldehyde, or ketone tested (19). At this point it can only be concluded that Compound 2 appears to be a choline ester with a low molecular weight acid.

Compound 3 was further purified by precipitation from an aqueous solution by the addition of absolute ethanol and obtained as a crystalline precipitate (20). The material melted at 309-310°C in agreement with the reported melting point of choline O-sulfate and showed no melting point depression when mixed with a sample of authentic choline O-sulfate. Both Compound 3 and choline O-sulfate gave identical ultraviolet spectra, showing a peak at 203 nm with a shoulder at 256 nm. In 14 paper and 8 thin-layer chromatographic systems Compound 3 and choline O-sulfate showed identical behavior (20). It was therefore established that Compound 3 was indeed choline O-sulfate.

PHYSIOLOGICAL STUDIES

Compound 1 was tested on isolated myogenic *Mercenaria mercenaria* heart, neurogenic *in vivo* crustacean *Carcinus maenus* and *Cancer irroratus* hearts and on isolated mouse intestine (20). At a concentration of 2.2×10^{-5} g/ml it

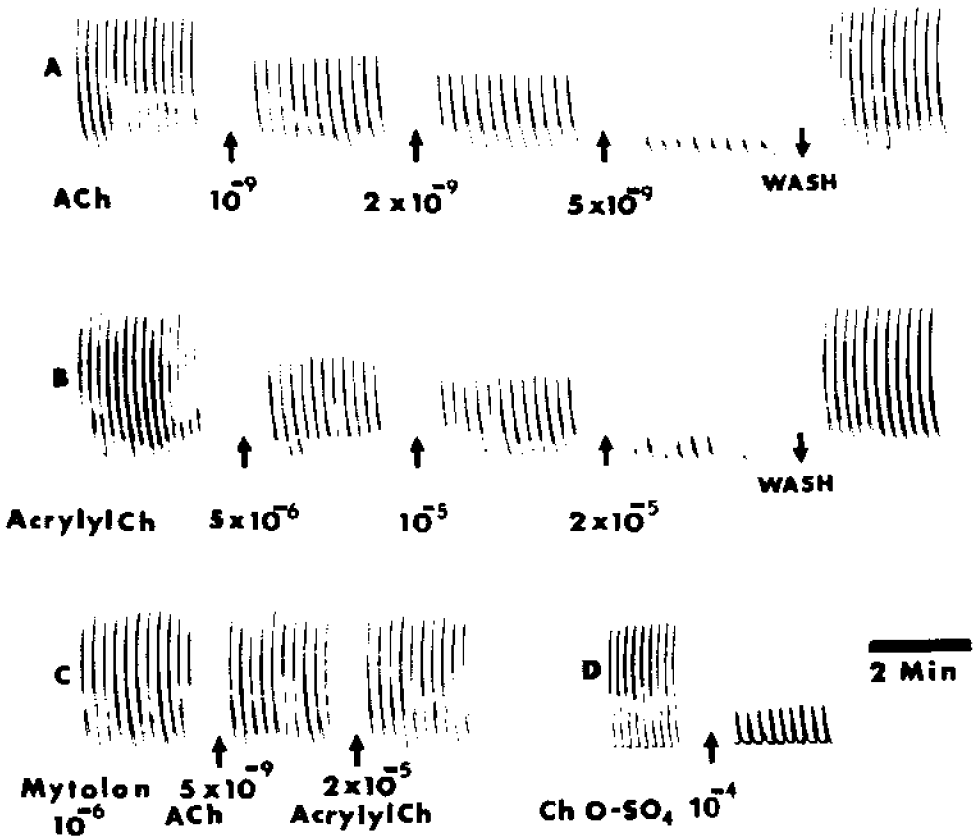

Figure 1: Effect of acetylcholine (A), acrylylcholine (B), Mytolon pretreatment (C), and choline O-sulfate (D) on isolated *Mercenaria mercenaria* heart. Concentrations given in g/ml. In (C) system washed between ACh and AcrylylCh and pretreated again with Mytolon.

caused a decrease in both beat frequency and amplitude of *Mercenaria* heart similar to that produced by 10^{-9} to 10^{-7} g/ml of acetylcholine. Synthetic acrylylcholine showed results similar to Compound 1 (20) (Fig. 1) thus confirming the identity of the two compounds. Synthetic acrylylcholine showed about 1/4,000 the activity of acetylcholine. This is less than the 1/10 activity of acrylylcholine reported by Phillis (17) using the heart of the molluscan *Tapes*. As in the case of acetylcholine the activity of both Compound 1 and synthetic acrylylcholine was blocked by pretreatment of the *Mercenaria* heart with Mytolon. Dropwise treatment of *in vivo* crustacean heart with 2.2×10^{-4} g/ml of Compound 1 caused a 2-fold increase in frequency. Actylcholine is reported to stimulate crustacean heart at levels as low as 10^{-9} g/ml (23). Compound 1 at 1.25×10^{-4} g/ml stimulated a two-fold increase in the frequency of contractions of mouse intestine in 5 minutes as well as a 50% increase in amplitude. These effects were comparable to those produced by acetylcholine at 10^{-6} g/ml. In all the above systems the effects of Compound 1 could be reversed by washing. All the effects observed above are

specific for choline esters. It has been observed, however, that generally much higher concentrations of other esters are required to elicit a response comparable to that given by acetylcholine (4, 17, 24).

Choline O-sulfate when tested on isolated *Mercenaria* heart was found to have about 1/20,000 the activity of acetylcholine (Fig. 1). Choline O-phosphate showed no activity at 10^{-4} g/ml and therefore has about 1/100,000 or less the activity of acetylcholine.

DISCUSSION AND CONCLUSIONS

Although choline esters serve as neurotransmitting substances at synaptic junctions in higher animals, their role in dinoflagellate metabolism remains obscure. Wangersky and Guillard (22) suggested that some analog of acetylcholine may be released by *A. carteri* either as a waste product or as a protective agent against zooplankton. For choline O-sulfate a role as a sulfate transport agent has been suggested since active transport mechanisms for the compound have been demonstrated in fungi (1) and plants (16), and choline O-sulfate was the only form of choline taken up by the bacterium *Lactobacillus plantarum* (12). An osmoregulatory role for choline O-sulfate has also been suggested since it has been found that mangrove plants which excrete 2 M NaCl at their leaf surfaces also excrete large amounts of choline O-sulfate with the salt (2). Since choline O-sulfate is zwitterionic, it could be involved in the transport of excess ion pairs, such as Na^+ and Cl^-, out of the cell. Therefore, the role of choline O-sulfate in the dinoflagellate may be as a sulfate transport agent and osmoregulator.

Several unsaturated choline esters have been isolated from various marine invertebrate sources. Thus acrylylcholine (13), senecioylcholine (β, β-dimethlacrylylcholine) (13, 25), and urocanylcholine (murexine, β-imidazol-4-ylacrylylcholine) (3, 13, 25) have been found to occur in the secretions of gastropods and it has been postulated that they act as toxins during the normal hunting and feeding activities of these organisms. With the finding of acrylylcholine and other choline esters in a dinoflagellate several interesting questions are raised. Are other choline compounds, such as senecioylcholine and urocanylcholine, also produced by dinoflagellates and do these compounds persist in the food chain and become incorporated into other organisms? An important question is also how widespread is the occurrence of choline esters in dinoflagellates? These compounds may contribute significantly to the toxic effects of red tide blooms.

ACKNOWLEDGEMENTS

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SESSION SUMMARY

PHARMACOLOGY

Session Chairperson

Betty Twarog

Tufts University
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Co-Chairman

Edward Gilfillan

Bigelow Laboratory for Ocean Sciences
McKown Point
West Boothbay Harbor, Maine

This session served to bring together workers interested in the pharmacology of dinoflagellate toxins, not only to share technical details, but to identify problems and exchange information concerning progress in areas of interest outside of their respective specialities. The conference as a whole has enabled specialists to become acquainted with problems which confront those who must determine management policies during toxic dinoflagellate blooms. Four major considerations formed the basis of the session on pharmacology.

I. Nature of the Toxins and Aspects of Toxin Release by Dinoflagellates

Results of pharmacological studies of paralytic shellfish toxins are consistent with the conclusion that the principal toxic component of the West Coast species, *Gonyaulax catenella* is saxitoxin (STX); while the East Coast *Gonyaulax* (one or more species closely related to *Gonyaulax tamarensis*) contains a toxic principle in addition to STX. The toxic components of *Gymnodinium breve* have not yet been well characterized by chemists although the pharmacology is rapidly being elucidated. It was pointed out that the fragility of *Gymnodinium* to some extent explains the fact that its toxin is released into the medium containing the organism; while toxins of the structurally more robust *Gonyaulax* species are not so released.

The studies reported in this session give rise to further inquiries. It will be necessary to identify chemically all the *Gonyaulax* toxins, especially the "*Gonyaulax tamarensis* toxin "(GTTX)." The same holds for the *Gymnodinium* toxins. In addition, one must ask why the active *Gymnodinium* toxin is found in culture media and in seawater in the absence of intact organisms, while the *Gonyaulax* toxin is only found in association with the organisms or particulate matter. It may well be that the *Gonyaulax* toxins are stable only when adsorbed on particulate matter.

II. Effects of Toxins on Organisms

Major clinical aspects of paralytic shellfish poisoning have been described in detail in this session: Differences between effects produced by STX and related, uncharacterized *Gonyaulax* toxin were reported. Clinical implications of toxic symptoms, (both respiratory paralysis and cardiovascular disturbances) were discussed. Fewer details were available concerning the action of the toxins on lower vertebrate animals and on marine invertebrates, including shellfish. Preliminary studies of the action of paralytic shellfish toxins on shellfish showed that filter feeding diminished as toxin accumulated.

In this category a number of interesting problems should be investigated: Work analyzing the effects of *Gonyaulax* toxin on shellfish has only begun and should be vigorously pursued. The detailed mode of action of the *Gymnodinium* toxins on fish should be carefully worked out since these animals perish in large numbers during blooms. Virtually no work has yet been accomplished which throws light on the action of *Gymnodinium* toxins on marine invertebrates and such knowledge is needed.

III. Resistance to Toxins

Two aspects of physiological resistance to toxin action were discussed in this session. In fish, toxin resistance is to some extent correlated with accumulation in these species of a toxin related closely in its mode of action to STX. The resistance to STX found in a number of bivalve species is predictive of the amount of toxin which the species can accumulate; and therefore, its potential toxicity to man. To the extent that a shellfish is sensitive to the toxin, it shows symptoms indicating intoxication. Some shellfish were shown to have evolved behavioral mechanisms which protect them from ingesting toxic organisms.

The resistance to tetrodotoxin noted in lower vertebrates may be related to the TTX and STX resistance characteristically observed in certain specialized excitable membranes as well as in developing muscle fibers and in muscle which has been denervated. This resistance to toxins is not at all well understood, but is clearly related to modification of physiological function, rather than protection against exposure to toxin. Understanding the general mechanisms responsible for resistance may well shed light on how resistance evolved in response to exposure to toxins. In shellfish, further understanding will be required of toxic symptoms and the implication of these symptoms: for instance, it will be useful to recognize symptoms of poisoning in bivalves under field conditions and to be able to estimate the extent of toxin accumulation. Also resistance of bivalves may be linked not only to rate of uptake of toxin, but to rate of detoxification after a bloom.

IV. Physiological and Molecular Bases of the Action of Dinoflagellate Toxins

Compelling evidence was presented that TTX, STX, and GTTX specifically block the sodium channels of the excitable cell membrane, that is, they block the generation of nerve impulses. In man, lower vertebrates, and in shellfish, this is the mode of action. Within recent years, a component of nerve membranes has been isolated which appears to be identical with the specific binding site of these toxins in the intact nerve. The mode of action of the *Gymnodinium* toxins is less completely known. A hemolytic factor is involved and there is also a neurotoxin.

With respect to the *Gonyaulax* toxins, it is clear that considerable progress has been made in elucidating their actions. Continued studies along the lines already undertaken should be pursued, not only because investigation of the binding sites of these toxins contributes to knowledge of the molecular nature of the sodium channel, the basis of all nerve impulses, but also because knowledge of the conditions under which these toxins bind to nerve membranes will give clues as to how to counteract them, both in the clinic and conceivably in fisheries. There have been fewer fundamental studies of *Gymnodinium* toxins compared to *Gonyaulax*, in part because the individual toxins have not yet been chemically purified and characterized at the

molecular level. In view of this fact the chemistry and pharmacology of *Gymnodinium* toxins need to be more intensively investigated.

It became clear during this conference that complete studies of the pharmacology of many dinoflagellate toxins have been recently undertaken. It will be welcome in future conferences to discuss more detailed information from chemists and pharmacologists concerning the *Gymnodinium* toxins, their nature, and their action on man, lower vertebrates, and shellfish. It is hoped that far more information will be available concerning *Gonyaulax* species and the chemistry of the now uncharacterized toxic constituent called GTTX. It is also hoped that studies will have been completed on lower vertebrates and shellfish. Particularly important will be information relating on one hand to mechanisms of toxin resistance, and on the other, to the molecular basis of the toxicity. These last considerations should be primary foci of attention at succeeding conferences.

SAXITOXIN AND RELATED POISONS: THEIR ACTIONS ON MAN AND OTHER MAMMALS

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ABSTRACT

Outbreaks of paralytic shellfish poisoning along the Pacific coast of North America appear to be caused by saxitoxin derived from *Gonyaulax catenella*. Saxitoxin causes paralysis in man and mammals by a specific and direct action on nerve and skeletal muscle, in which it prevents initiation and propagation of action potentials, by blocking the sodium channels of the excitable cell membrane. The probably cause of death is the failure of the movements of respiration, resulting from this peripheral paralysis. There does not appear to be any significant paralysis of the central nervous system, except in experimental animals which have had saxitoxin administered directly into the cerebrospinal fluid.

Paralysis associated with blooms of *G. tamarensis* appears to be caused by a poison which resembles saxitoxin, though it is not identical. Large amounts of this unknown poison were present in the mussels responsible for a serious outbreak of poisoning along the North-East coast of Britain in 1968. It could not be purified by means of the ion-exchange techniques which work efficiently with saxitoxin. It was partially purified by gel filtration, and found to be pharmacologically similar to saxitoxin. It too caused death of experimental animals through a peripheral paralysis of the respiratory muscles. There were, however, differences between this poison and saxitoxin in the details of some of their effects on test preparations.

INTRODUCTION

Paralytic shellfish poisoning is a well recognized clinical condition. Halstead in 1965 (7) listed 85 separate outbreaks, between the years 1689 and 1962, with a total estimate of over 959 victims and more than 222 deaths. A more recent estimate by Prakash *et al.* in 1971 (19) puts the world-wide total at about 1600 cases. The signs and symptoms that victims present to a clinician have been described in detail by Meyer *et al.* (16), Meyer (15), Seven (24), Gemmill & Manderson (6), McCollum *et al.* (12) and by many others.

All the clinical accounts agree that the first symptom is a paraesthesia which is variously described as a tingling, prickling, stinging or burning sensation. It is first felt by the patient in the oral and circumoral area and soon afterwards appears in the fingers and perhaps in the toes. This first symptom occurs within 5 - 30 minutes after eating the poisonous bivalves, the speed of onset being related to the ultimate severity of the intoxication. It is soon replaced by a numbness spreading slowly from the mouth and the extremities eventually to affect arms, legs and neck. In this numbness, tactile and proprioceptive sensibility is impaired or lost, but pain appreciation appears usually to be retained. Other symptoms develop later, including dizzyness and a feeling of lightness or floating. Ataxia, incoordination and muscular weakness in the limbs and neck are then noticeable. Serious cases develop dysarthria or even aphonia and respiration becomes impaired. The tendon reflexes are not usually

altered, except as a consequence of the generalized muscular weakness. As long as the victims are conscious they are mentally alert and rational, though choking sensations cause anxiety. When consciousness is lost it is probably because of respiratory inadequacy, and most authorities consider death to be due to respiratory failure.

Symptoms of poisoning are not usually felt by adults unless at least 2000 mouse units (m.u. of poison = approximately 360 micrograms of saxitoxin) have been ingested. Serious cases will have consumed 5,000 - 20,000 m.u. (about 0.9 - 3.6 mg), though some individuals have ingested even greater quantities with minimal or even no effects (1,12). Individual susceptibility to the poison is so variable that one cannot yet give a reliable figure for the average human lethal dose.

Following the work of Sommer, Meyer and their colleagues, it has become accepted that the outbreaks of paralytic shellfish poisoning in California and other parts of the Pacific coast are caused by saxitoxin, acquired by the bivalves as a result of feeding on blooms of the dinoflagellate *Gonyaulax catenella*. The outbreaks on either side of the Atlantic seem to be correlated with blooms of a different dinoflagellate, *G. tamarensis*, according to Needler (17), Robinson (20) and Prakash *et al.* (19). Because there is evidence that this organism (or *G. excavata*) produces a different poison, it is of interest to compare the clinical descriptions of the signs and symptoms encountered in cases from these two main areas.

A study of clinicians' reports has failed to reveal any essential difference between the neurological manifestations of paralytic shellfish poisoning seen on the Pacific coast and those seen on Atlantic coasts. The typical case history outlined above applies as accurately to outbreaks associated with *G. tamarensis* as it does to those which follow blooms of *G. catenella*. However, there is one non-neurological effect that seems to be consistently different: during outbreaks along the Pacific coast, nausea, vomiting and other gastro-intestinal disturbance have rarely been reported. These effects are seen in quite a high percentage of the Atlantic coast cases (21, 26, 1, 12).

LABORATORY INVESTIGATIONS

Following a major outbreak of paralytic shellfish poisoning in England at the end of May 1968 (12) an attempt was made to extract saxitoxin from some of the poisonous mussels, *Mytilus edulis* (5). It was found that when a crude extract was injected into mice, following the assay technique developed by McFarren (13) from Sommer & Meyer (25) the dose-survival time curve was significantly different from that obtained when pure saxitoxin was injected.

At about the same time it became clear that these mussels had acquired their poison from a bloom of *G. tamarensis* (8, 20), so this finding gave support to an earlier suggestion by Schantz (22) that the poison of *G. tamarensis* might be different from saxitoxin.

Attempts were then made to purify the crude extracts. The first attempt made use of the ion-exchange technique used successfully by Schantz *et al.* (23)

in purifying saxitoxin. However, only 18% of the total toxicity present in the crude extract was recovered by means of their technique. About 45% of the toxicity ran off the column during the preliminary wash with buffer, having failed to bind to the resin. It was later partially purified by gel filtration in columns of Sephadex G.10 and G.25. All the rest of the original toxic material was irrecoverably lost, and losses of the major toxic fraction were high throughout the subsequent stages of purification. The specific toxicity of this material was eventually raised to about 270 m.u./mg. Because at least 90% of the impurity was found to be NaCl, it was decided that this was sufficiently pure to be used in preliminary pharmacological tests. The other poison that did bind to the resin was quite easily purified by following the preliminary stages of the process for saxitoxin (23), reaching about 1,550 m.u./mg (5). Pure saxitoxin assays at about 5,500 m.u./mg.

Neuropharmacological actions of saxitoxin and the G. tamarensis poisons.

The material which formed 18% of the total mussel toxicity, and which behaved like saxitoxin during the stages of purification, was pharmacologically indistinguishable from pure saxitoxin. It blocked the conduction of nerve impulses in filaments from the rat cauda equina and in desheathed frog sciatic nerve. In each case the degree of block was dose dependent, and equal to the blocking effect of a comparable concentration of saxitoxin, expressed as m.u./l. Figure 1 shows an example of the compound action potential becoming reduced by (a): saxitoxin at 20 µg/l and (b): the saxitoxin-like poison

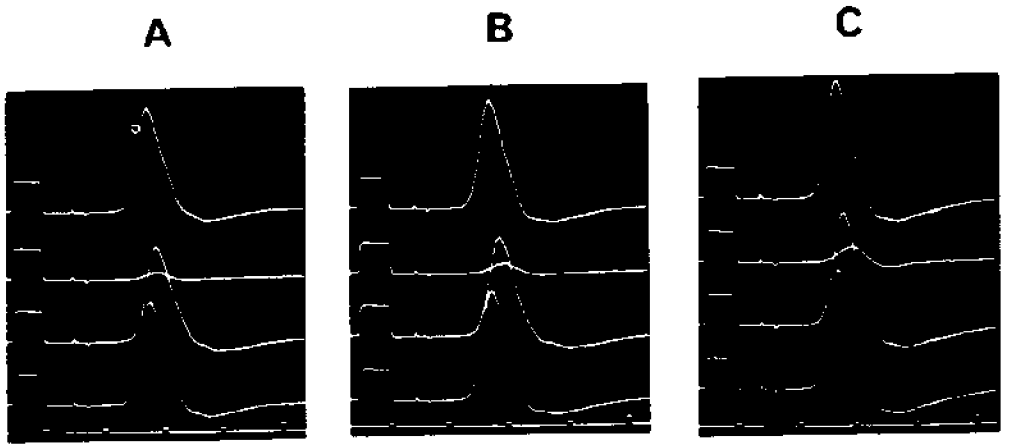

Figure 1. Compound action potentials recorded from a nerve, showing the effects of shellfish toxins. Each photograph shows 4 recordings; top - control, second - after 3 min. in poison, third - after washing for 3 min. fourth - after washing for 15 min. The 3 photographs show the actions of: (A) saxitoxin 20 µg l., (B) minor saxitoxin-like component from *Mytilus edulis* 100 m.u./l., (C) major unknown components 100 m.u./l. Each trace has a 1mV calibration pulse on the left and the timing pips at the bottom of the photographs show 1 mSec intervals.

at 100 m.u./l. At this concentration the poison washed out easily and the illustrations show the quick recovery of the action potentials after a few minutes wash.

Saxitoxin, unlike tetrodotoxin, causes a progressive diminution in the amplitude of the end-plate potential in frog sartorius muscle (4,11) and the saxitoxin-like material also had this effect.

Like saxitoxin, this minor component of the poisonous extract also had a direct paralyzing action on skeletal muscle, preventing generation of an action potential without any depolarizing effect on the resting potential. *In vitro* this paralysis was easily reversed by washing out the poison.

When saxitoxin is administered to an experimental animal by slow intravenous infusion, it does not affect the spinal cord reflexes until the dose has become sufficient to start blocking conduction in nerve fibers (3). In an anaesthetized animal saxitoxin brings about a progressing hypotension and diminishes the respiratory movements. When a lethal dose is given, respiration becomes paralyzed because of the peripheral action of the poison on nerve conduction and muscle contraction, and death is directly due to this respiratory failure. There is no central failure of respiration, the medullary respiratory centers continuing to function for a time after tidal volume has fallen to zero (2). If the animal is artificially ventilated before the terminal collapse, the heart continues to beat and the animal may eliminate sufficient poison, after an hour or longer, to be able to resume unaided respiration. Only if the saxitoxin is injected into the cerebrospinal fluid in the lateral cerebral ventricles does it cause a direct failure of the respiratory centers (Jaggard & Evans, in the press).

The saxitoxin-like material from the poisonous mussels has identical effects on anaesthetized rabbits. When infused slowly intravenously, it brought about a progressive hypotension and diminution of tidal volume. The phrenic nerve continued to discharge impulses generated by the medullary respiratory centers for a short time after the movements of respiration had ceased altogether. Therefore, like saxitoxin, this material had no depressant effect on the CNS when given intravenously, but brought about the effects through its peripheral actions on nerve and muscle.

The major toxic component extracted from these poisonous mussels, and presumably derived from *G. tamarensis*, was a different poison. As mentioned above, it did not have the same chemical properties as saxitoxin and could not be purified by means of the same techniques. It resembled saxitoxin in most, but not all, of its pharmacological effects. Like saxitoxin, and the saxitoxin-like component of the mussel extract, it blocked nerve conduction in mammalian and frog nerves. Figure 1c illustrates an experiment in which this poison, in a concentration of 100 m.u./l , reversibly depressed the compound action potential. The extent of the block is similar to that produced by equivalent concentrations of the other two poisons. When saxitoxin and the two poisons derived from *G. tamarensis* were bio-assayed on a desheathed frog nerve, at concentrations containing equal numbers of m.u./l, the rates and extents of depression of the compound action potential were indistinguishable. The

unknown poison also had a direct paralyzing action on skeletal muscle, without depolarization of the resting potential, and the paralysis could be reversed by washing. When given to anaesthetized rabbits by slow intravenous infusion it had similar effects to saxitoxin, causing the hypotension and peripheral respiratory failure as described above, without any detectable effect on the medullary respiratory centers.

One test preparation clearly and consistently responded differently to this unknown poison. When it was applied to the frog sartorius neuromuscular junction this poison always caused the end-plate potential to fail abruptly after a latent period. Recovery occurred after rather prolonged washout, when the end-plate potential re-appeared suddenly. In these respects the unknown poison behaved more like tetrodotoxin, the poison from puffer fish and newts, than like saxitoxin. Tetrodotoxin appears to cause these effects at the neuromuscular junction by blocking nerve conduction proximal to the motor terminals, whereas saxitoxin progressively acts on the terminals so as to reduce transmitter release (4).

The unknown poison was not tetrodotoxin, however. They were distinguished from each other by testing on the desheathed nerve of a *Taricha torosa* newt, which is known to be relatively immune to the nerve-blocking action of the tetrodotoxin (10). Conduction in the nerve of this species was blocked by pure saxitoxin, by the saxitoxin-like component, and by the major unknown component of the mussel extract, all in comparable concentrations. Tetrodotoxin did not block the action potential, confirming the findings of Kao & Fuhrman (10). Another distinction from tetrodotoxin was the observation that none of the mussel poisons lost toxicity when heated in acid solution. Like pure saxitoxin, they were stable at pH 1, whereas tetrodotoxin is rapidly inactivated under these conditions (27).

CONCLUSIONS

The laboratory experiments described above confirm the suggestion made by Schantz in 1960 (22) that shellfish toxicity derived from *G. tamarensis* is not due to the presence of saxitoxin, but to some other compound with similar neuro-pharmacological effects. It is true that some material indistinguishable from saxitoxin was extracted from the mussels responsible for the 1968 outbreak in England, but it accounted for less than 20% of the total toxicity. Most of the toxicity was due to another poison, chemically distinct from saxitoxin, with similar though not identical pharmacological effects. One cannot yet say whether this unknown poison causes the nausea, vomiting and other gastro-intestinal effects that are commonly seen when paralytic shellfish poisoning follows a bloom of *G. tamarensis*. It is equally possible for these effects to be due to some other irritant produced by this organism but not by *G. catenella*.

Kao in 1966 (9) stressed that one of the dangers associated with blooms of *G. catenella* was due to the heat stability of saxitoxin. Cooking poisonous Pacific coast shellfish lessens their toxicity only slightly. Both the poisons extracted

from the poisonous English mussels were found to be heat-stable at pH 1, but nevertheless there were unexplained and heavy losses of the major unknown component throughout the stages of purification. It may therefore be less stable, in general, than saxitoxin is. Various accounts of outbreaks along Atlantic coasts have suggested that the toxicity of these shellfish is lowered considerably by cooking (18, 14, 12). A reduction of toxicity by cooking has been thought to explain the absence of fatalities during the 1968 outbreak in England, when the raw mussels had very high levels of toxicity. Nevertheless, many fatalities have occurred during other Atlantic coast outbreaks, and the overall fatality rates are not noticeably different between the Pacific and Atlantic areas.

When death occurs, it is almost certainly due to respiratory paralysis caused by the action of these poisons on the respiratory muscles and their peripheral motor innervation. The poisons seem unable to pass through the blood-brain barrier, so the respiratory centers in the CNS are not affected. Therefore, administration of central stimulants and analeptics will not be beneficial.

All authorities agree that artificial ventilation is the measure most likely to prevent death of a badly poisoned victim. However, it seems that very few cases have been treated in this way, perhaps because in such grave cases the respiration fails before skilled medical assistance can be obtained. Wider public training in such 'first aid' measures as mouth-to-mouth artificial ventilation might enable victims to be kept alive long enough to receive professional medical care.

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CARDIOVASCULAR ACTIONS OF SAXITOXIN

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ABSTRACT

In whole animals, saxitoxin causes a marked fall in the arterial blood pressure which is frequently followed by a compensatory pressor response of moderate degree. The hypotensive action is due to a lowering of the peripheral resistance and not to any selective action of saxitoxin on the central vasomotor mechanism or on the heart. The peripheral action is due to a combination of direct relaxant effect on the vascular smooth muscle at low doses, and of a release of vasomotor tone in higher doses which blocked the adrenergic vasoconstrictor nerves. The late compensatory pressor phase is due to released catecholamines. In clinical cases of paralytic shellfish poisoning, the initial hypotensive phase is almost never seen, but the late pressor phase may be a presenting sign.

INTRODUCTION

Saxitoxin, like tetrodotoxin, is among the most deadly poisons known, the minimal lethal dose determined in mice being about 8 μg/kg body weight. Assuming homogeneous distribution throughout body water, the lethal concentration is probably about 10nM. Although the fundamental action of saxitoxin is a highly selective blockage of an increase in sodium permeability in many excitable membranes, interfering with the generation of action potentials (5), there are important actions on the cardiovascular system in whole animals. (For more references, see 1, 2 and 3).

Site of hypotensive action

Except in very low doses (less than 1 μg/kg intravenously), the cardiovascular actions of saxitoxin are manifested as a marked fall in the arterial blood pressure (Fig. 1). Although there was some old information

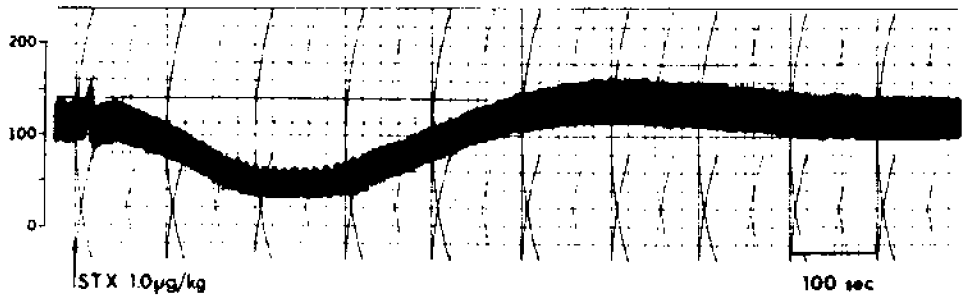


Figure 1. Effect of saxitoxin (STX) on systemic arterial pressure. Cat, ♀, 3.8 kg. The systemic arterial pressure was originally 138/98 mm Hg, visible at the left end of the record. STX injection is indicated by arrow. Note that the pressure began to fall within 40 seconds, and reached its lowest at 62/40 mm Hg in 200 sec. Note that the pressure returned to pretoxin level in about 8 min., and was followed by a phase of pressor response (from ref. 10).

which suggested that the hypotension was due to some action of saxitoxin (o rather an impure extract of contaminated mussels and clams) on centra vasomotor centers (for ref., see 1 and 2), recent experiments using head-body cross perfusion technique showed that the primary site for the hypotensive actions could not be in any central structures. Briefly, the experiments involved perfusing the head of one animal (the recipient, either a cat or a dog) with the blood from another animal (the donor), while the nervous innervation from the recipient's head to its own body was left intact. Application of saxitoxin to the recipient's head alone did not cause hypotension in the recipient's body, but application of saxitoxin to the recipient's body reproduced all the cardiovascular effects observed when saxitoxin was introduced intravenously to single animals (7). Saxitoxin is known not to have marked effects on the heart. The observations are: (a) the contractile force of the isolated myocardium is not affected until very high concentrations are applied; (b) the cardiac rate is not significantly affected when moderate vasodepression is present. These observations locate the probable site of the hypotensive action peripheral to the heart, at either the sympathetic nerves responsible for normal vasomotor tone, or at the vascular smooth muscles responsible for peripheral resistance.

Mechanism of peripheral vasodilation

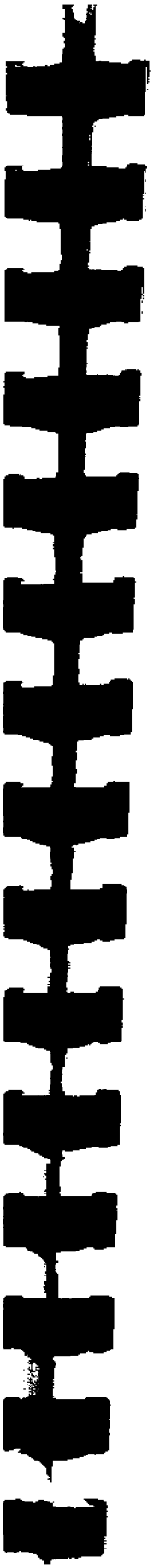
To understand the mechanism of the hypotensive action, experiments using a technique of regional perfusion was necessary. In this technique, a vascular bed with relatively homogeneous pharmacological responses was isolated from the circulation of the rest of the body. In the cat and in the dog, the vasculature of skeletal muscles was chosen; in the cat, a whole hind leg, appropriately prepared, was used (6, 10), and in the dog, the gracilis muscle was used (8). The circulation to the muscle bed was then provided via a constant volume pump from either the animal's own body, or from a separate donor animal. Since the flow into the perfused muscular bed is constant, changes in the peripheral vascular resistance become manifested as changes in the pressure required to perfuse the vasculature. As in the head-body cross-perfusion studies, the nervous innervation between the perfused regions and the rest of the body was left intact.

When saxitoxin was injected intravenously into the body of an animal, the systemic arterial blood pressure fell rapidly, but in the perfused region, the immediate response to the hypotension in the body was a rise in blood pressure (Fig. 2). In other words, instead of a fall in peripheral resistance, there was actually an increase in peripheral resistance in the perfused region, a vasoconstriction. If the circulation to the perfused region was obtained from the same animal, then this pressor response was followed by a depressor response. If the circulation to the perfused region was supplied by a donor animal whose blood was not poisoned by saxitoxin, then the pressor response in the perfusion pressure remained, and only gradually subsided coincident

with a progressive recovery from hypotension in the systemic arterial blood pressure of the recipient (to whose body saxitoxin was given) or as neural blockade became severe (Fig. 2) (10).

The explanation of the above observations are as follows. Because of the systemic hypotension, there developed a reflex vasoconstriction in the perfused muscular bed. The proof of this conclusion is that the pressor response was abolished by severance of the innervating nerve. When the contaminated blood from the body was delivered to the perfused muscle by the pump, then a direct vasodilatory response was produced to account for the depressor response in the perfusion pressure. The proof for this conclusion is twofold: (a) the timing of this depressor response was coincident with the lag introduced by the pump circuit, (b) when no contaminated blood was delivered to the muscular bed, as in the case of cross-perfusion from a donor animal, no depressor response occurred (Fig. 2). From these experiments, a conclusion was reached that the hypotensive action of saxitoxin can be attributed in part to a direct vasodilatory effect on the vascular smooth muscle.

Figure 2. Effect of saxitoxin (STX) in a cross-perfusion experiment. Donor cat, ♂, 4.2 Kg, recipient cat, ♂, 3.9 Kg. One hind leg of the recipient was perfused, with pump assistance, by blood from donor. Neural elements between recipient body and perfused leg were intact. Bold line below bottom trace indicates periods of electrical stimulation of lumbar sympathetic chain. Stimulation caused vasoconstrictor responses in perfused leg as well as recipient body. STX was given into a brachial vein of the recipient, became distributed throughout the body of the recipient, but could not gain access to the perfused leg. Note that the systemic pressure in the recipient's body fell, while the perfusion pressure of the recipient's leg rose. These changes indicate that one aspect of the hypotension cannot be attributed to blockade of vasomotor nerves, but is attributed to a direct relaxant effect of STX on the vascular smooth muscle. The increase in the steady perfusion pressure is due to reflex vasoconstriction in the perfused leg in response to the systemic hypotension. Some blockade of vasomotor nerves did occur, as indicated by the progressively smaller vasoconstrictor responses elicited by electrical stimulation of the lumbar sympathetic chain. The progressive fall of the steady perfusion pressure is also consistent with a developing blockade of the vasomotor nerves (from ref. 10).



The full hypotensive action of saxitoxin, however, is slightly more complex because it is dose-dependent. The events described above and the conclusion reached are valid when doses of saxitoxin less than 1 to 1.5 μg kg were used. When the dose was increased to above about 1.5 μg kg, the responses in the perfused muscular vasculature were as follows: Coincident with the systemic hypotension, there was a fall in the perfusion pressure, indicating a fall in peripheral resistance, or a vasodilatory response. A later depressor response, attributable to the direct action of saxitoxin on the vasculature, was similar to that in cases with low doses. The first depressor response was due to a blockade of sympathetic vasomotor nerves which provided the normal vasomotor tone. The proof for this conclusion is that vasoconstrictor responses in the muscular vasculature normally elicited by electrical stimulation of the sympathetic nerves were markedly reduced, or abolished, after such saxitoxin treatment. Therefore, a more complete picture of the hypotensive action of saxitoxin is that in low doses there is a direct vasodilatory action on the vascular smooth muscle, and in high doses there is an additional release of vasomotor tone. Other evidence which permitted the conclusion of a direct vasodilatory response is that the effect is present even after the vascular smooth muscle has been blocked completely with alpha- and beta-adrenergic blocking agents (phentolamine and propranolol) and with a cholinergic blocking agent (atropine). By analogy with the lack of effect of low doses of tetrodotoxin on the release of norepinephrine from the splenic nerve (4), it may be assumed that low doses of saxitoxin which caused reflex vasoconstriction did not interfere with release of norepinephrine.

Differences between the cardiovascular actions of saxitoxin and tetrodotoxin

The biological actions of saxitoxin are very similar to those of tetrodotoxin, even though they are different chemically. A few minor differences between the cardiovascular actions of these two toxins are present, and may explain some of the seemingly basic differences in the clinical symptomatology in cases of paralytic shellfish poisoning (due to saxitoxin) and of tetrodon fish poisoning (due to tetrodotoxin) (10). In very low doses (less than 1 μg kg), saxitoxin can produce neuromuscular paralysis without appreciable hypotension (5). With tetrodotoxin, effects on these two systems are inseparable, occurring together, and in parallel degrees, with all doses. Saxitoxin is slightly less potent than tetrodotoxin in its hypotensive effects, causing less of a fall in blood pressure, and the effect is shorter lasting. Saxitoxin has a greater selectivity for adrenergic nerves than cholinergic nerves as compared with tetrodotoxin. This selectivity is manifested by the more frequent occurrence of nerve-elicited vasodilatory response in the skeletal muscle vascular bed, a response which is known to be mediated by some postganglionic cholinergic sympathetic nerves. Lastly, there is greater tendency, in the case of saxitoxin hypotension, for a late phase of pressor response to appear. This late pressor response is caused by catecholamine released either from the adrenal medulla

or locally from nerve endings, because hexamethonium, acute adrenalectomy, and pretreatment with reserpine all abolished this late pressor phase.

Possible relevance to clinical paralytic shellfish poisoning in man

A possible clinical relevance of this last observation is that whereas, in cases of tetrodon poisoning, hypotension is an invariable part of the symptomatology, in paralytic shellfish poisoning no case of hypotension has appeared in the clinical literature (2). My guess is that in paralytic shellfish poisoning the concentration of saxitoxin in the body water is not very high, in part because of the need for absorption of the toxin through the gastrointestinal tract. The hypotensive episode, therefore, may be rather fleeting, not detectable at the time of medical attention. On the contrary, the initial physical finding might occur during the compensatory pressor phase due to released catecholamines. That this interpretation is plausible is supported by the finding in three patients during the 1968 outbreak of paralytic shellfish poisoning off the Northumberland Coast (Britain). In all three victims, the blood pressure on first admission to the hospital was slightly higher than it was two weeks later when recovery had occurred (9).

The differences between the cardiovascular actions of saxitoxin and tetrodotoxin may also be the explanation for the enormous difference between the mortality rates in poisonings by the two toxins. In paralytic shellfish poisoning, death is relatively rare; in tetrodon poisoning, the mortality rate is about 50% (see 2).

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TOXINS FROM THE BLOOMS OF *GYMNODINIUM BREVE*

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ABSTRACT

The dinoflagellate *Gymnodinium breve*, which is the major organism of the ichthyotoxic red tides in the Gulf of Mexico, is a naked dinoflagellate which generates the toxin metabolically. In contrast to the hardy armored dinoflagellates related to paralytic shellfish toxin, these naked cells are very fragile. They are ruptured by passage through the gill processes of fish, releasing their contents containing toxin which readily passes through the gill surfaces with lethal effect if the red tide cell density is sufficiently high. Fish which swim into an area of red tide will continue actively for a while, then will suddenly lose balance, gasping at the surface, and then will be passive on the bottom before a terminal struggle. Death occurs with no pathologic lesions. Within the surf zone, the cells are similarly ruptured, and the spray can irritate the mouths eyes, and noses of exposed humans.

Extracts of the cells can be toxic on injection to all vertebrates. Purification of the extract has produced evidence of a hemolytic factor of high molecular weight and a smaller neurotoxic factor. At the membrane level, the neurotoxin induces spontaneous bursts of discharges in nonmedullated nerve membranes. At low concentrations, spontaneous subthreshold oscillatory variation of the membrane potential can appear and may build up to give spikes in a way that closely resemble the phenomena that are observed when the membrane is placed in low Ca^{++} solution. The effect of the neurotoxin in producing spontaneous discharges in nonmedullated frog axon is counteracted by a tenfold increase of Ca^{++} in the bathing solution.

The Action of Toxins From the Blooms of Gymnodinium Breve: a review

The red tides caused by blooms of *Gymnodinium breve* off the west Florida coast are characterized by massive mortalities of fish. Their death is best described in the words of Walker (18):

"On leaving Clear Water, November 20, I sailed south through Boca Ciega Bay and encountered the first dead fish floating on the water near Bird Key, a little southeast of Pass A'Trilla. These were mullet, and as we progressed to the south and east I began to encounter toadfish, eels, puff-fish and cow-fish, in immense numbers...I saw many fish in every stage of sickness, from the first attack to the end. All were affected in nearly the same manner. The fish, apparently active and healthy, would be swimming along, when suddenly it would turn on its sides and shoot up to the top of the water, gasping as though out of the water, apparently unable to control its motions, often lying on its side on the bottom for five or ten minutes motionless, then suddenly shooting hither and thither without aim or object, and finally ending the struggle on the surface and floating off dead. Whole schools of mullet would suddenly stand upright on their tails, spouting water and die in five minutes. Gars would run for a long time with their snouts above the water, and then lie motionless, as if dead, for ten or fifteen minutes. These generally lived an hour or more after being attacked."

It was not until sixty years later that Davis (4) described the dinoflagellate *Gymnodinium breve* as the major organism in such red tides. In contract to the sturdy armored *Gonyaulax* dinoflagellates associated with paralytic shellfish poisoning, these *Gymnodinium* cells are naked and fragile and rupture at the slightest mechanical shock. This makes them difficult to culture but probably explains how they leak their cytoplasm during passage through the gill processes of fishes enabling the lipid-soluble neurotoxins to enter probably across the gill membranes into the blood stream. If the red tide is sufficiently dense, enough toxin can enter the fish to produce the syndrome described above leading to death with no evidence of pathologic lesions.

The same fragility of the cells can produce discomfort to humans at the shore line. Ingle (5) states that "odorless, colorless gases, irritating to the nose and eyes commonly occur in conjunction with outbreaks of the red tide." He quotes from Hardin Taylor (16) that:

"While on the beach I felt a slight tendency to sneeze and cough: shortly afterwards my attention was called to the action of the dog which was sneezing violently and seemed to be in acute asphyxiation. I carried him back and the same thing happened again. I then noticed that my lungs were feeling sore and that my breathing was labored in much the same manner as when I board ships after fumigation, except that I noticed no odor..."

Ingle summarizes that the irritant effects are present only when red tide occurs and even then do not appear unless wind driven waves with associated vapor and droplets exist. The effects do not usually go far inland beyond open beaches.

The identification of *G. breve* was followed by studies on sample of red tide (2), and efforts were made to isolate and identify the toxic factors. A suggestion made by Bein (1) that bacteria of the *Flavobacterium* species isolated from the red tide water produced the lethal factor appears not to have been followed up because of the demonstration that axenic cultures of *G. breve* (19) contained a potent toxin (9, 14).

It is perhaps unfortunate that the extraction methods used to isolate, concentrate and purify the toxic fractions have not been uniform (8, 3, 17, 12 13), although most have employed a preliminary phase separation followed by column chromatography separation and a final TLC step.

There is no doubt that even the crude toxin is lethal to all vertebrates if it enters the blood stream at above a certain very low concentration. Below that lethal threshold the effects are transitory. For example, in cats following intravenous injection of toxin, there is a rapid reduction in heart rate, accentuated respiration and muscle fibrillation which eventually die away. Intense irritation of human mouth tissues has been experienced in our laboratory when the dried powder of our extract T1 is transferred during weighing procedures, and Steidinger *et al.* (15) have provided a detailed account of the reactions by humans to exposure to the toxins. Similarly, mice exhibit considerable facial discomfort as indicated by pawing of the mouth if exposed to a weak spray of water which contains toxin.

Although the majority of studies have been made on culture extracts, concentration of toxic fractions occur when pelecypods are maintained in an environment containing *G. breve* cells. The molluscan tissues are poisonous if fed to chickens (10) and appear, under the correct conditions, to produce a ciguatera like effect on test vertebrates (7).

Our method of extraction has consisted essentially of a phase separation with a 2:1 chloroform methanol mixture against water. The organic phase is lyophilized, and the resultant crude powder is run on a Sephadex LH20 column with 2:1 chloroform methanol. Two biologically active components could be separated (13). The first one labelled GT in tubes 8-12 (5ml fractions) exhibits neurotoxic and lethal actions on fish. The second active component, tubes 38-63, exhibits hemolytic effects in vitro using rabbit erythrocytes but shows little or now neurotoxic activity. Purification by several groups of the neurotoxic fraction of the early tubes with higher molecular weight has produced different empirical formulae (8, 3, 11). We have proceeded only to the stage of purification with TLC to use two neurotoxic fractions labelled T1 and T2. These fractions are very toxic to vertebrates because even the cruder fraction GT is lethal to mice at 150 ng/g of animal. The mode of action of the *G. breve* toxin has been investigated by Sasner (12) who has presented reasonable evidence that his fraction IV has a depolarizing post-synaptic action. His main evidence was obtained from a frog nerve-muscle preparation. Exposure of the junctional region produces fibrillation of the muscle followed by blockage of the junction as well as motor nerve and muscle if concentration of the toxin in high enough. The fibrillation effect can be blocked by curare.

We have made observations which offer another explanation. Our experiments were very similar to those of Sasner, also using a frog sartorius nerve muscle preparation. However, careful observation of the spontaneous movements which occurred when low doses of toxin were added to the fluid bathing region of the nerve muscle junction showed that the movements were fasciculations and not fibrillations. It was clear that numbers of fibers throughout the muscle were contracting synchronously. The effect could be seen even on the mechanical records, and this suggests that individual motor units are contracting as entire entities. If the various fibers of a motor unit fire together, then the presynaptic endings must also be discharging simultaneously. In contemplating the implied firing of the motor nerve common to the motor unit family of fibers, we realized that a fifth alternative can be added to Sasner's four possibilities (12, p. 160): spontaneous spikes in the nonmedullated termination of the motor nerve. Let us note the evidence:

1. 0.5 FTU/ml of our extract T1, after TLC was used in frog sartorius nerve-muscle studies. (Fish Toxic Unit, FTU, is defined in Spiegelstein *et al.*, 1973, as that amount of toxin per ml which just kills 3 test fish in 3 hours under standard conditions).
2. At this concentration, spontaneous brief series of contractions occurred (Fig. 1), but there was no loss of response to nerve stimulation.

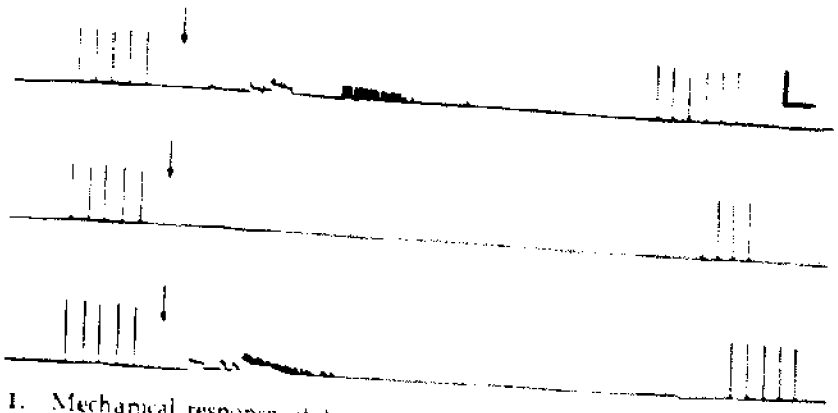

Figure 1. Mechanical response of frog nerve-sartorius preparation. Indirect stimulation tested once every five seconds before and after addition of toxic fraction T_1 at 0.5 FTU/ml. Note that at this dose there is no loss of indirect response and that the spontaneous contractions are of a fascicular nature. Calibration: see the bars above (upper right) vertical bar = 10 gm, horizontal bar = 10 sec.

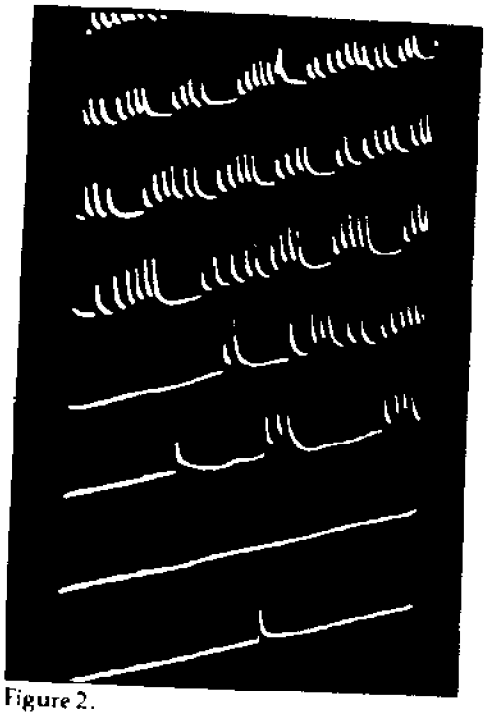

Figure 2.

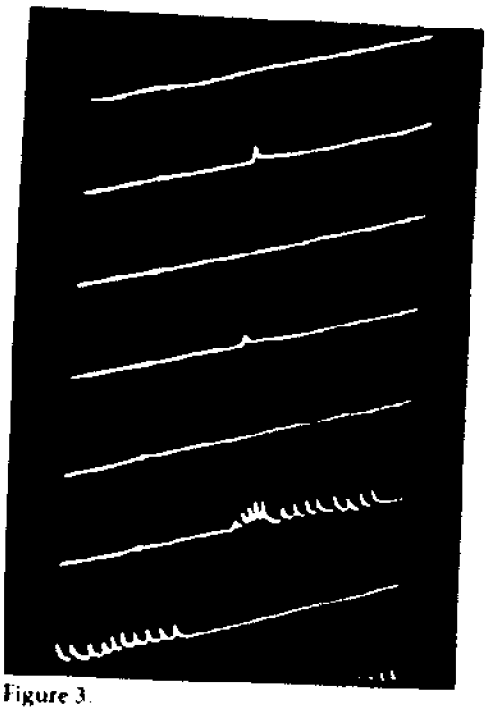

Figure 3.

Figure 2. Endplate potentials in Frog nerve-sartorius muscle preparation soaked in toxin fraction T1 at 4 TU/ml in addition to 2.5×10^{-6} d tubocurarine to eliminate mechanical responses to indirect stimulation. One evoked EPP is imposed once per second (indicated by small circle over the response). The remaining EPP's are spontaneous. Calibration: 0.5 sec time sweep, with vertical rise of 9 mV per sweep.

Figure 3. Continuation of Figure 2, end of first burst.

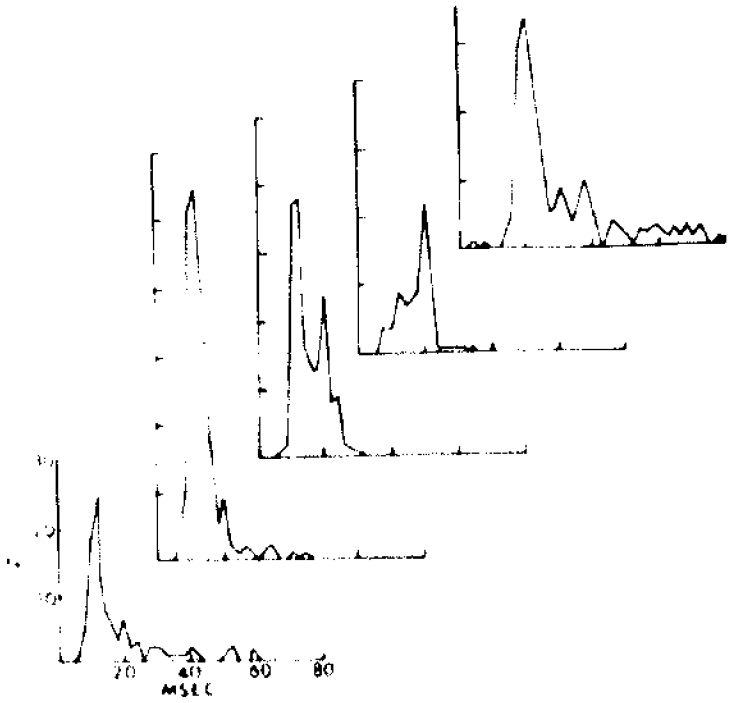

Figure 4. Histogram summary of spontaneous EPPs produced in frog sartorius nerve-muscle preparation in presence of 4 FTU/ml *C. breve* toxin fraction T1 and 2.5×10^{-6} d tubocurarine. Sampling made of 2.5 sec segments all throughout the spontaneous bursts of the experiment of Fig. 2.

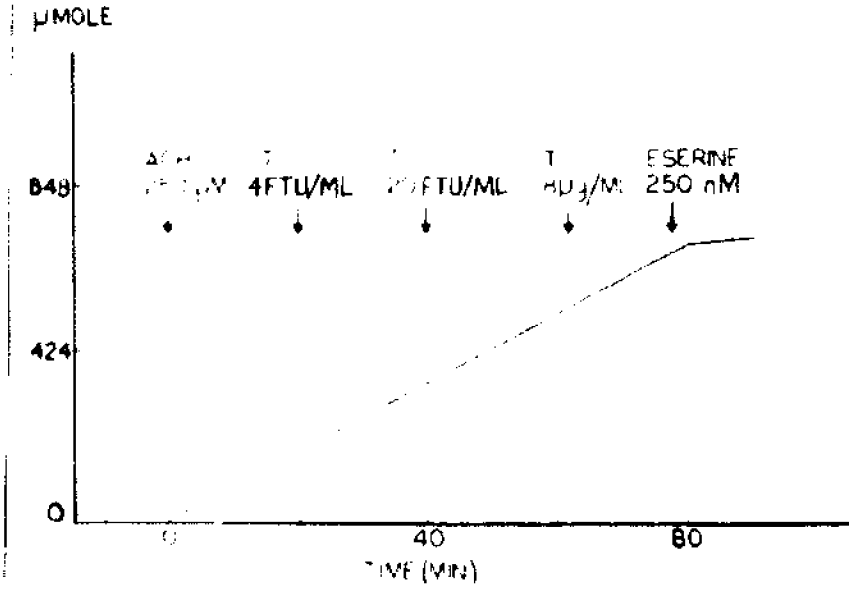

Figure 5. Hydrolysis of acetylcholine (ACH) by cholinesterase in blood; the effect of various concentrations of toxin up to 8 μg/ml of fraction GT (equivalent to 160 FTU/ml) followed by addition of Eserine.

3. The rise of tension in these spontaneous tetani could be blocked by curare.
4. No drop in the resting potential across the muscle membrane was detectable.
5. There was no change in the shape, amplitude or rate of the miniature endplate potentials (MEPP).
6. In the presence of 4 FTU/ml of extract T1 with 2.5×10^{-6} w/v curare, the mechanical responses were blocked, but trains of spontaneous end plate potentials (EPP) appear. These are seen in Fig. 2 recorded with intracellular electrodes. The traces run successively upwards with 0.5 sec sweep and 9 mv steps per sweep. Facilitation can be seen in each burst and the beginning of depression later in the burst. The first burst shown lasted more than 10 sec and the end as shown in Fig. 3. After a period of silence further bursts occur (Fig. 3).
   An interval histogram (Fig. 4) shows that the impulses are not random. In the first burst there is a 10 msec interval which later (second and third histograms) shifts to a 20 msec interval together with higher multiples.
7. In our neurotoxic extract, we find no anticholinesterase activity as measured by the Jensen-Holm method (6) even at 4000 times the mechanical threshold (Fig. 5).

We believe that this evidence supports the proposition that at low concentrations the extract T_1 produces bursts of spontaneous discharges in the terminal nonmyelinated region of the motor axon in a fashion similar to the effect of bathing the nerve in low Ca^{++} medium. This possibility was strongly supported by the demonstration that when the Ca^{++} in the Ringers solution was increased ten fold (to 10mM) the excitation action of the low level of toxin was immediately blocked.

Finally, a comparable action of the extract T_1 on squid axon has been demonstrated with the measurement of membrane potential by an intracellular electrode. It is known that the natural accommodation factor of axons varies widely between specimens, and this is recognized in the response to 4 TU/ml toxin. And indeed a variety of responses was observed including a single pulse followed by a damped oscillation, small subthreshold oscillations which build up to a train of spikes and spike trains of constant interval which trail off into damped oscillations (Fig. 6, 7, 8). There were also instances of very small sub-threshold oscillations of the membrane potential evoked by a brief pulse which died away without initiating a train. Such depolarizations of the membrane are very small and may be difficult to identify (Fig. 9).

ACKNOWLEDGEMENT

Appreciation is expressed to the hard work and devotion of Jane Spitzer to this project. Support for the research has been through contracts from the Office of Naval Research and the USC Sea Grant Program. Grateful acknowledgements for cultures are made to Dr. S. M. Ray and to Dr. D. F. Martin.

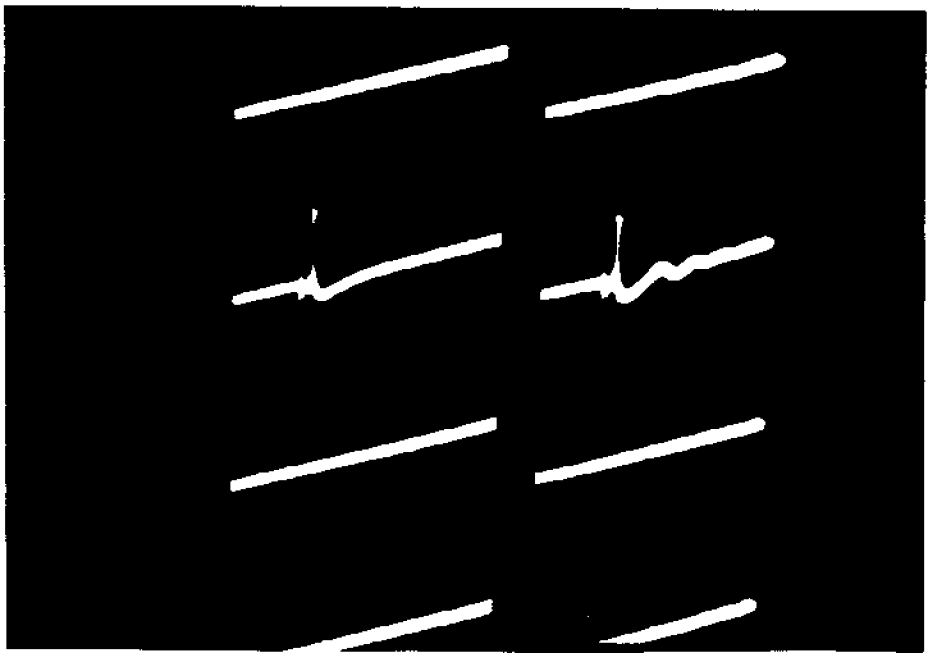

Figure 6. Squid axon bathed in *G. breve* toxin at 4 FTU/ml. A variety of responses is seen in the next four figures; shown in Fig. 6 is an evoked action potential followed by subthreshold ringing of the membrane potential.

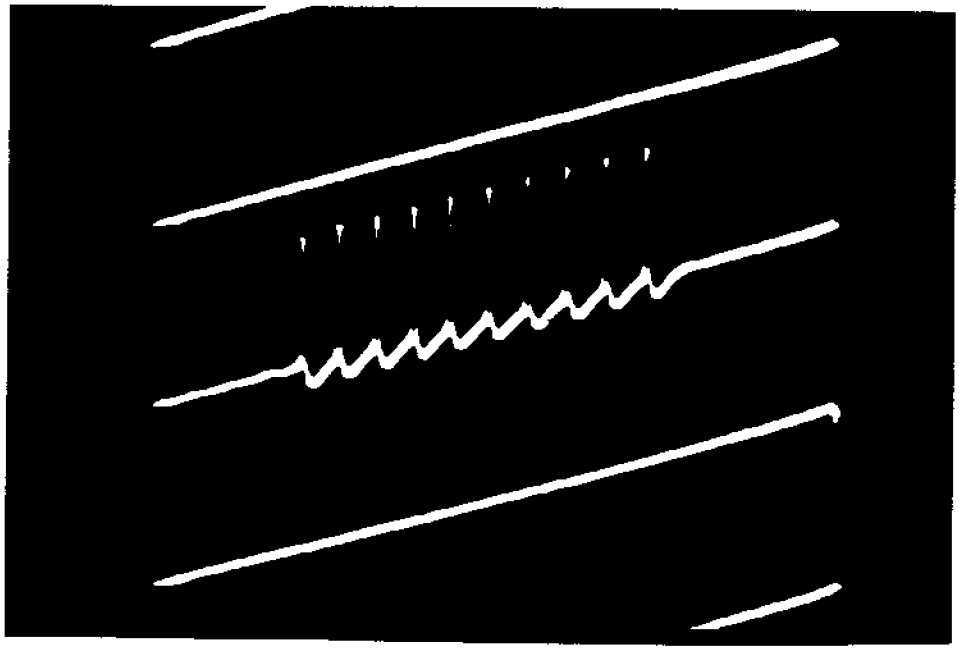

Figure 7. Squid axon: single impulse followed by very small subthreshold oscillations leading to a train of spikes. Toxin 4 FTU/ml.

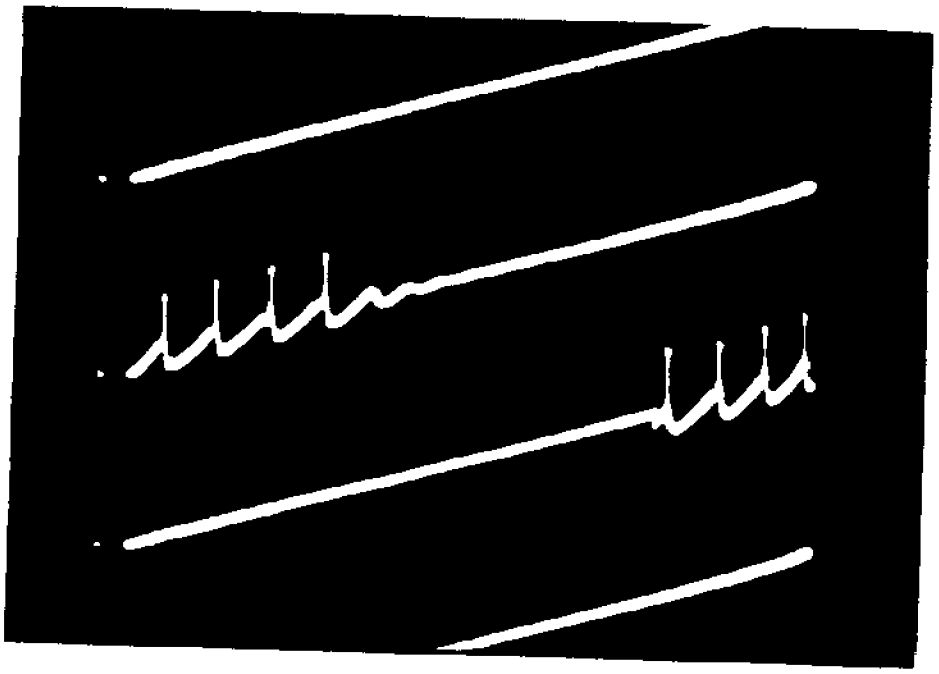

Figure 8. Squid axon: train evoked by a single pulse ending in membrane oscillation. Toxin 4 PTU/ml.

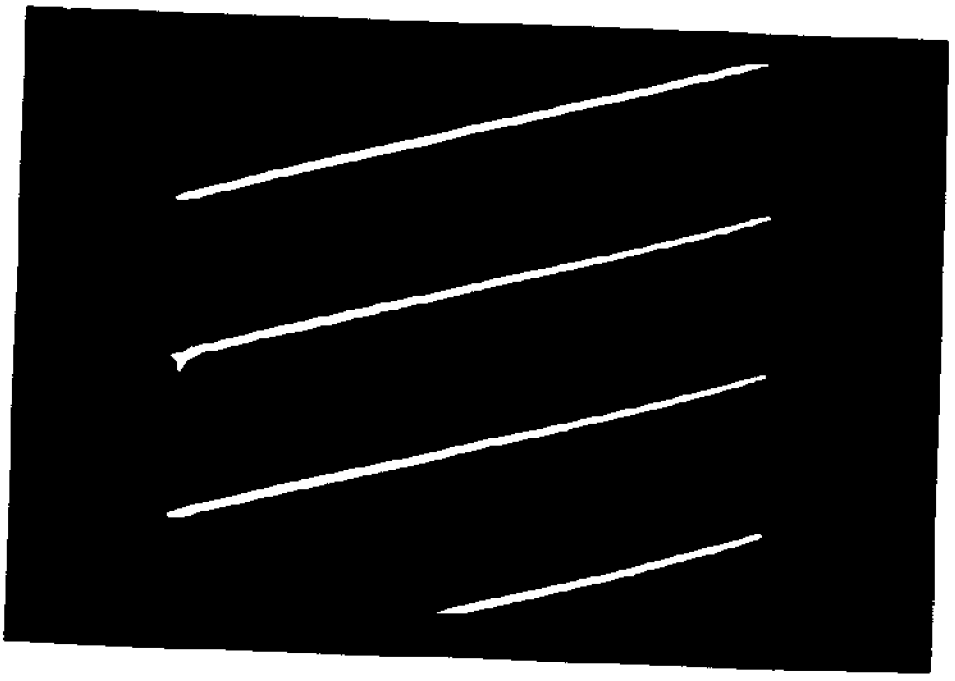

Figure 9 Squid axon: Spike which induces small damped oscillations only. Toxin 4 PTU/ml.

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EFFECTS OF PARALYTIC SHELLFISH POISONING TOXIN ON THE BEHAVIOR AND PHYSIOLOGY OF MARINE INVERTEBRATES

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ABSTRACT

Paralytic shellfish poisoning toxin can affect marine invertebrates in a variety of ways. It appears that the most striking effects were caused by the toxin effects on the animals nerves. In filter feeding species filtration rates may be greatly depressed by PSP toxin.

INTRODUCTION

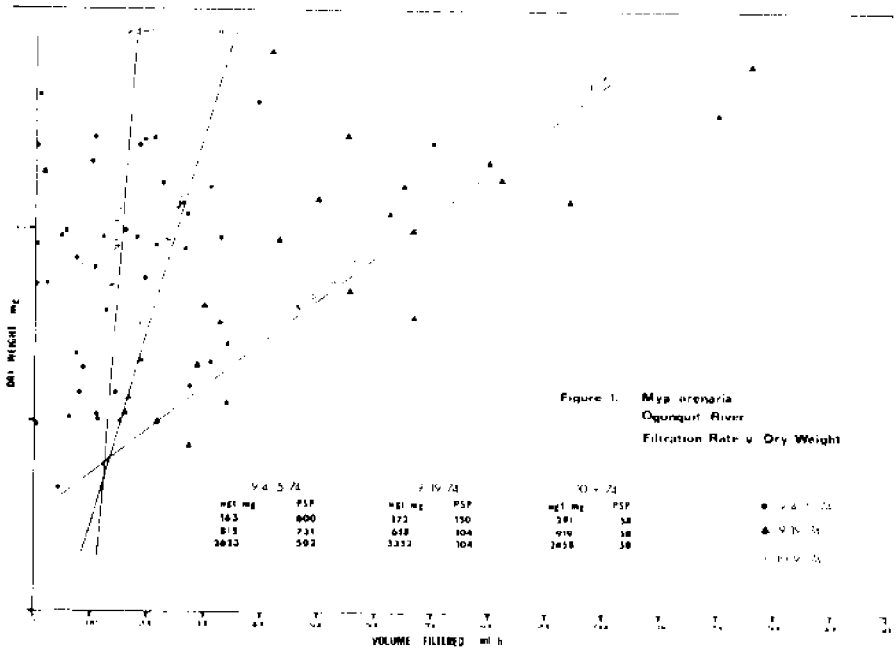
One of the problems in describing the effects of paralytic shellfish poisoning (PSP) toxin on marine invertebrates is that there is very little published work that bears directly on the subject. Perhaps the best way to approach the subject is to consider the factors such as selective feeding, varying filtration rates and susceptibility of nerves to PSP toxin which affect the toxicity of filter-feeding organisms exposed to a toxic dinoflagellate. The questions of which species of invertebrates become toxic, as well as which species of dinoflagellates are toxic, is covered exhaustively in a number of recent reviews (9, 14, 15).

EFFECTS OF SELECTIVE FEEDING

It appears to be a general feature of PSP testing that different species of animals exposed to roughly similar amounts of dinoflagellates will frequently accumulate quite different amounts of toxin. Once explanation for this is that while some species may feed on toxic dinoflagellates, others may find them unpalatable. Both of the above situations have been reported. For example, Buley (3) observed that the mussel *Mytilis californianus* fed selectively on dinoflagellates even when they only accounted for some 2% of the phytoplanktonic community. Dupuy and Sparks (6) report that the Pacific oyster, *Crassostrea gigas*, does not readily accept the dinoflagellate *Gonyaulax washingtonensis* as food. Other than these reports there appear to be no other studies in the literature. Selective feeding needs further research.

EFFECTS OF VARYING FILTRATION RATES

Another possible source of the variations in toxicity between different species of filter-feeding animals would be a difference in filtration rates for specific species. Comparative data of this sort is difficult to find. One such set of data is available for a population of mussels, *Mytilus edulis*, from Casco Bay, Maine, and a population of soft shell clams, *Mya arenaria* from Ogunquit. During July and August 1973 100 mg *M. edulis* from Casco Bay filtered an average of 1875 ml/h; during this same period 100 mg *M. arenaria* from Ogunquit filtered an average of 1100 ml/h (Gilfillan, unpublished data). Over this period mussels of comparable size filtered approximately 1.63 times as much water as clams. If one assumes equal efficiencies of filtration, then mussels should gain toxin at 1.6 times the rate seen in clams. This being the



case, if the rates of toxin excretion are not markedly different for the two species, mussels should be about twice as toxic as clams of comparable size for the same exposure. On the basis of data collected in 1972 mussels appear to detoxify more quickly than clams, yet during a rise in PSP level, mussels are always very considerably more toxic than clams: usually by a factor closer to 10 than 2 (John Hurst, Dept. Marine Resources, West Boothbay Harbor, Maine, pers. comm.). Certainly such a situation existed in Ogunquit in early September of this year when *M. edulis* were more than 6 times as toxic as *M. arenaria* of comparable weight. (Figs. 1 and 2).

EFFECTS OF SENSITIVITY OF NERVES TO PSP TOXIN

Investigations carried out by Twarog, *et al.* (16), suggest that the relative toxicities attained by a group of filter-feeding mollusks may be more nearly a result of differing sensitivities of each of the species' nerves to saxitoxin (STX) than anything else. Without exception, those species which Twarog, *et al.* have shown to be more resistant to STX accumulate toxin to a greater extent in any given area than those which are less resistant. The one exception is the case of *Mercenaria mercenaria*, the quahog, whose nerves are considerably more resistant to STX than those of *Mya arenaria*, the soft shell clam. In Massachusetts during the 1972 bloom of *Gonyaulax tamarensis*[1] quahogs from Ipswich did not accumulate nearly as much toxin as did soft shell clams from nearby areas. Whether this situation resulted from selective feeding by the quahogs or from a patchy distribution of *G. tamarensis* can only be answered by further work.

[1]The name *Gonyaulax excavata* has been suggested for this species but not officially adopted.

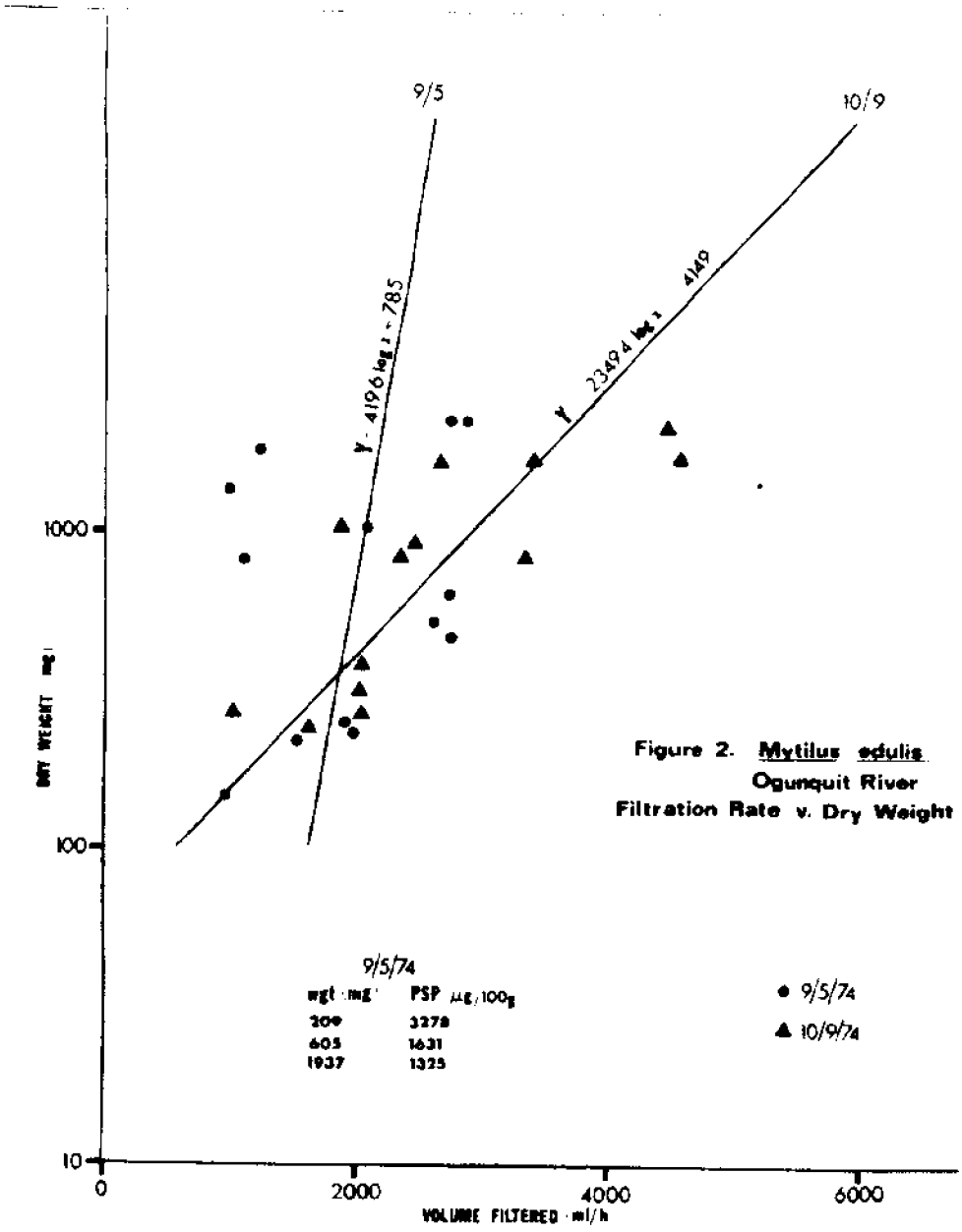


Figure 2. Mytilus edulis
Ogunquit River
Filtration Rate v. Dry Weight

RECENT EXPERIMENTAL RESULTS

Work carried out with *M. arenaria* and *M. edulis* from Ogunquit, Maine during the September 1974 bloom of *Gonyaulax tamarensis* may shed some light on the relation of the resistance of a species nerves to STX to the species' physiology.

Mya arenaria, soft shell clams and *Mytilus edulis*, blue mussels, were collected from the Oqunquit River, Maine. Clams were collected on 4, 5, 19 September and 9 October; mussels were collected on 5 September and 9 October.

Table 1

Temperatures at which filtration rates were determined for animals from Ogunquit.

Date	T (°C)
4-5 September	15.0
9 September	14.3
10 September	11.5

Filtration rates were determined for as wide a variety of sizes of animal as possible on each date. Filtration rates were determined in full strength sea water (30 °/oo) using methods described in Gilfillan (7). Temperatures at which the filtration rates were determined are shown in Table 1. PSP content was determined for three size ranges within each species. The PSP assays were carried out according to the methods described in Prakash, *et al.* (14). Results of PSP assays for each size range are shown in Figures 1 and 2. Results are shown in Figures 1 and 2 as plots of filtration rate against dry weight. Straight lines shown on the plots are linear regressions of filtraiton rate on the common logarithm of dry weight. In normal clams and mussels this relation approximates a straight line.

Points to note on Figure 1 are that on 4-5 September there appears to be very little relation between filtration rate and dry weight. The regression is non-significant ($r = 0.1046$, $t = 0.6896$). It should also be noted that on 4 - 5 September all animals filtered low volumes of water. Nine animals did not filter at all; five of these were large animals weighing more than 1000 mg. Only 1 out of 50 animals filtered more than 400 ml/h. These are very low filtration rates for the time of year. In late August 1973 100 mg clams from Ogunquit River filtered about 6 times as much as in September 1974. PSP content did not appear to vary with size.

On September 19 a highly significant regression ($r=0.613$, $t=3.33$) of filtration rate on log dry weight was obtained. Control data from 1973 are available only for small animals weighing about 100 mg. For animals of this size filtration rates obtained on 19 September appeared normal. By 19 September PSP content had fallen to about 20% of the values observed on 4 - 5 September.

On 9 October a non-significant regression was obtained ($r=0.361$, $t=1.397$). Filtration rates observed for small animals (100 mg) are comparable with those observed the previous fall (1973) in October. These animals filtered less water on the whole than those collected on 19 September. The primary reason for this is that by 9 October the water temperature had dropped to 11.5°C (Table 1) and the clams were preparing to enter their winter period of dormancy. What effect these physiological changes have on the relation between filtration rate and dry weight is unknown. By 9 October the animals were essentially clean of PSP.

Figure 2 shows results obtained with *Mytilus edulis* on 5 September and 9 October. On 5 September a non-significant regression of filtration rate on log dry weight was obtained ($r=0.265$, $t=0.91$); larger animals (> 600 mg) tended to filter less water per unit dry weight than smaller animals.

Looking at the plot shown in Figure 2 it appears that filtration approximates a linear function of log body weight up to a weight of about 600 mg. This trend appears to be reflected in the distribution of PSP in these animals. Animals weighing 600 mg or more tend to have about half the PSP content of smaller animals. Compared with control mussels from Casco Bay in September 1973 small mussels' (100 mg) filtration rate was reduced by *ca.* 50%.

On 9 October the PSP content of the mussels was < 58 and they appeared in all respects healthy and normal. A highly significant regression of filtration rate on log dry weight was obtained ($r=0.783$, $t=4.18$). Smaller animals filtered about the same amount of water as on 5 September, larger animals (> 600 mg) filtered more water than on 5 September, all this notwithstanding that the temperature on 9 October was nearly 5°C cooler. Small (100 mg) mussels were filtering at about the rate observed in Casco Bay, 17 October 1973.

Detailed comparison of these sets of data is complicated by both the lack of control data for all size ranges and by the over-riding influence of autumnal cooling. However, it seems clear that both the clams and the mussels were adversely affected by intoxication with PSP toxin.

The clams appeared to be more severely affected at lower PSP levels than the mussels. This is in keeping with the findings of Twarog, *et al.* (16) who found that *Mya arenaria* nerves were completely blocked by a concentration of STX two orders of magnitude lower than that which had no effect on *Mytilus edulis* nerves.

On 4 - 5 September *M. arenaria* in the flats were observed to be very sluggish and to behave as though they were partially paralyzed. Similarly apparently paralyzed soft shell clams were reported from both Western Maine and Massachusetts in 1972. Apparently no such paralysis of soft shell clams has ever been reported from Eastern Maine and Atlantic Canada. (Prakash *et al.* (14)).

Neither McFarren *et al.* (12), Quayle (15), nor Halstead (9) make any mention of detrimental effects of PSP toxin on the affected animals. The only reference in the literature to deleterious effects of PSP toxin appears to have risen from the 1968 bloom of *G. tamarensis* off the coast of England (10, 1). Both morbidity and mortality of shellfish were associated with the 1968 bloom.

Figure 3 shows a plot of filtration rate *v* dry weight for *Mya arenaria* from Black's Harbor, New Brunswick. These animals were collected on 25 July 1974; filtration rates were determined on 28 July 1974. On 25 July these animals were assayed at 1400 μg PSP/100 g. shellfish meat, yet when their filtration rates were determined they appeared healthy *i. e.* a significant regression of filtration rate on log dry weight was obtained ($r=0.693$,

t = 2.88). No control data for non-toxic clams from this location is available, but these clams filtered much more water per unit time than clams of equivalent weight and half the toxicity from Ogunquit. They did not appear sluggish as did the Ogunquit clams having half the toxicity.

The above results pose a lot more questions than they answer. For example, are Canadian *Mya arenaria* more resistant to PSP because they have been chronically exposed to it? These results clearly indicate that much further research is needed to clarify the relation between PSP and the physiology of filter feeding molluscs.

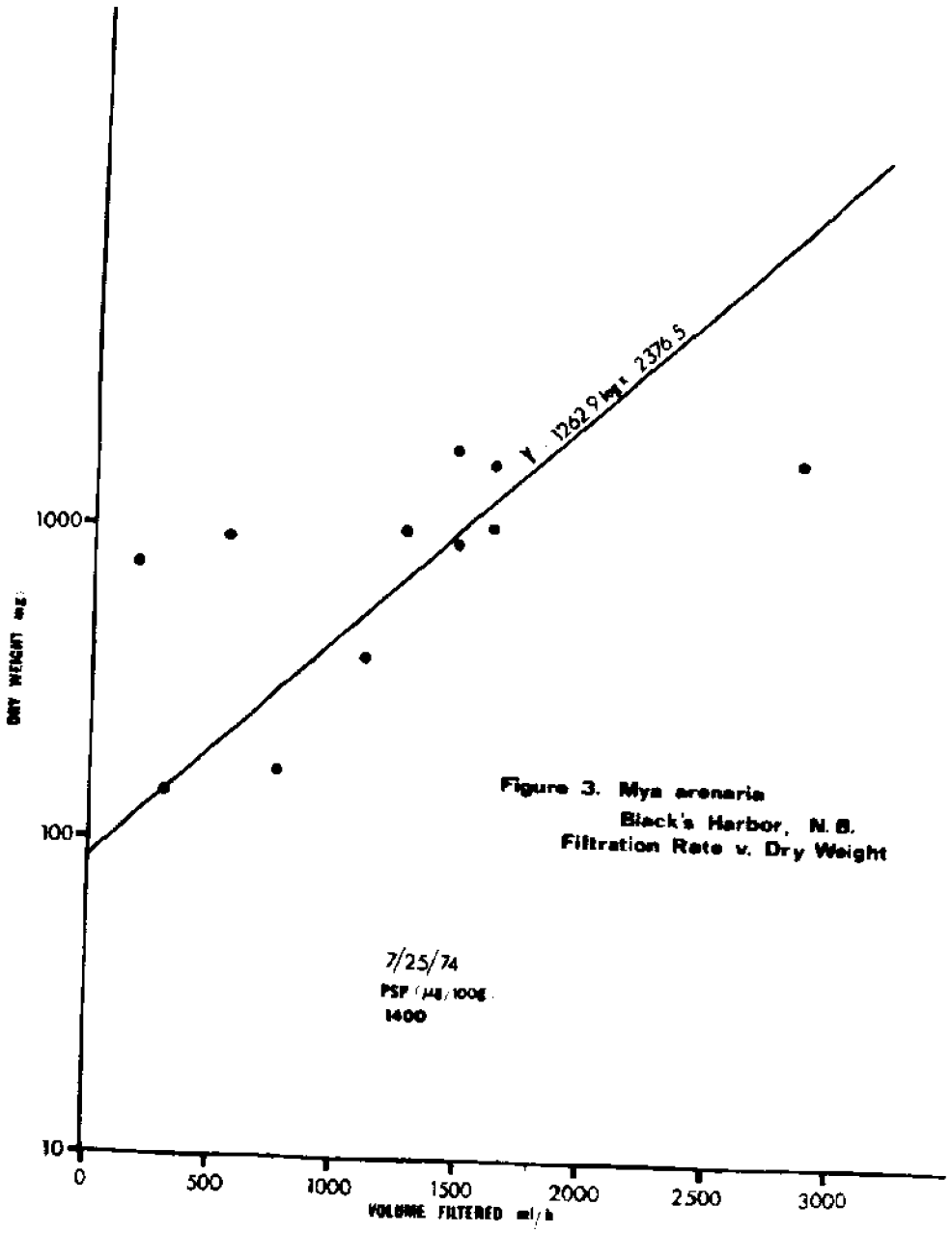


Figure 3. Mya arenaria
Black's Harbor, N. B.
Filtration Rate v. Dry Weight

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PHYLOGENETIC GRADATION OF RESISTANCE TO TETRODOTOXIN AND SAXITOXIN IN PUFFERFISHES AND RELATED FISHES

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The potent nerve poisons, tetrodotoxin (TTX) and saxitoxin (STX) have been shown to be extremely powerful, specific blockers of the early inward Na^+ current of action potentials in excitable membranes. Studies of the kinetics of this blocking action by Hille (7) and by Cuervo and Adelman (1) have revealed that it is a simple adsorption phenomenon, following the Langmuir adsorption isotherm. Other types of ionic conductance channels are not affected, eg. the Ca^{++}channels of many invertebrate tissues (4), or are very little affected, eg. the depolarizing channels of mechanosensory terminals (11, 12), chemosensory synaptic membranes (3), and the Na^+ - conductance channel of vertebrate cardiac action potentials (4). Most interesting is the observation that Na^+ spikes in the animals that produce TTX, pufferfishes and certain newts, are resistant to TTX but can be blocked by STX (9, 8, 5).

The molecular basis for this resistance is not known, but might provide important insight into the mechanism of action of TTX and of the channel itself. Further interest is added by the observation that normally sensitive Na-channels in mammalian muscle can become TTX and STX resistant as ACh-sensitivity spreads across the surface of a denervated or damaged muscle fiber (13, 6). This has been interpreted as a conversion of some channels from sensitive to insensitive form.

Thus there are several questions posed by the known instances of TTX and STX insensitivity that we felt were worth pursuing. For example, is the resistance all-or-none, or graded for any given channel. If graded, can this gradation be correlated with toxin concentrations in an animal's tissues, and is this phylogenetically governed? Is the resistance due to differences in toxin binding, or in its effect on conductance through a given channel after binding? Does TTX interfere competitively with STX binding? Can the resistance be attributed in any given instance to the appearance of a new kind of conductance channel, eg. to Ca^{++}? These and other questions were addressed, at least in part, during a recent Alpha Helix Expedition to the Great Barrier Reef, where TTX and STX resistance were examined in several species of pufferfishes and their relatives (eg. porcupine fish, boxfish, triggerfish). This work has been reported in detail elsewhere (10, 2). Briefly summarized, our findings were as follows:

The action potentials of muscle fibers in the pufferfishes examined were insensitive to 3×10^{-5} M TTX, the highest concentration tried. These action potentials were found to be Na-dependent, with no apparent Ca^{++} -component. On the other hand, TTX blocked spikes by 50% in three unrelated control species at a concentration of 3 to 5×10^{-8} M. Pufferfish relatives were less sensitive than were control fish, but nevertheless were susceptible to the toxin. 50% blocking concentrations were in the range of 1 to 5×10^{-7} M. The shellfish toxin, saxitoxin (STX), which blocks electrical activity in the same manner as TTX, was also tried. Pufferfish muscle fibers were insensitive to STX, but other fishes showed action potential block at approximately the same concentrations as with TTX. Pufferfish supramedullary cells (SMC) had Na^+ spikes which were not blocked by TTX or

STX, in contrast to unrelated fish or the related triggerfish. Both pufferfish and triggerfish SMCs showed prolonged Ca^{++}- dependent action potentials in the absence of external Na^{+}.

The variable degree of resistance to TTX shows a phylogenetic dependence, and reflects differences in the ability of the toxin to bind to a receptor site rather than in the degree of TTX effectiveness following binding. The ability of TTX at sufficient concentration to completely block action potentials in pufferfish relatives indicates that there are not some sensitive channels, some insensitive ones. The fact that adaptations imparting resistance to TTX also provide immunity to STX indicates that the two toxins bind in similar fashion to the same receptor, and are similarly affected by charges in the membrane environment.

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RESISTANCE TO PARALYTIC SHELLFISH TOXINS IN BIVALVE MOLLUSCS

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ABSTRACT

Nerves of *Mytilus edulis, L.* and certain other bivalves are resistant to the blocking effects of saxitoxin (STX), an active principle of paralytic shellfish toxin, and tetrodotoxin (TTX), a toxin derived from pufferfish. It has been shown that species resistant to STX accumulate levels of paralytic shellfish toxin dangerous to man. Resistance to STX and TTX is a property of individual nerve fibers, and is not due to a protective sheath around the fibers. Sodium deficiency reduces and blocks the action potential, so the resistance does not depend on development of a non-sodium spike-generating mechanism.

Physiological, biochemical, ecological, and behavioral problems with respect to toxin resistance and accumulation will be discussed.

Saxitoxin (STX): Mode of action and structural relationship to tetrodotoxin (TTX).

Saxitoxin (STX), a powerful neurotoxin, is a major constituent of paralytic shellfish toxin. It was first isolated from the Pacific butter clam, *Saxidomus nuttalli* (Conrad) and later identified in *Gonyaulax catenella* by Schantz and associates (16). *G. tamarensis* contains STX, and in addition, an unidentified toxin perhaps chemically related to STX (Evans, 7) Narahashi (personal communication) has called this unidentified toxin *Gonyaulax tamarensis* toxin (GTTX). The mode of action of STX and GTTX are similar to that of tetrodotoxin (TTX), the active principle of the toxin of pufferfish (*Sphaeroides*) and the California newt *Taricha torosa*: Both STX and TTX block conduction of the nerve impulse by reversibly interfering with the early voltage-dependent increase in sodium ion conductance which generates the nerve action potential, (13). Conduction block leads to paralysis. This paralysis superficially resembles curare poisoning but is fundamentally different since curare selectively blocks neuromuscular and synaptic transmission and does not affect conduction in nerve fibres. STX and TTX do not interfere with synaptic transmission.

The molecular structure of STX has been deduced by Wong and associates (20). Further information on the chemical structure of STX and GTTX is presented in this symposium by Schantz.

Comparative studies of sensitivity to STX and TTX in mollusc nerve.

In view of the fact that some molluscs are exposed to large amounts of paralytic toxin and nevertheless survive, it was of interest to analyze the effects of the toxin on their neuromuscular systems. It had been found previously that TTX does not block the action potential in the anterior byssus retractor muscle of *Mytilus edulis* (Linnaeus); and it had been suggested that the spike-generating mechanism in this muscle depends on increased conductance

to calcium rather than sodium ions (17). Subsequent evidence supported this suggestion (19).

It was also observed that nerve fibers in *Mytilus* were resistant to TTX. It was therefore of interest to find that in contrast to muscle, the spike-generating mechanism is dependent on sodium ions. The possibility that the neural sheath may protect the nerve fibers was excluded in experiments which showed that "naked" nerve fibers, with neural sheath stripped off, are as insensitive to TTX as normally sheathed fibers. Since the puffer fish, which secretes and accumulates TTX, has nerve fibers which are resistant to the toxin, it seemed possible that TTX resistance in *Mytilus* nerve might be related to the ability of *Mytilus* to feed on *G. catenella* and *G. tamarensis* and to accumulate large quantities of paralytic shellfish toxin, containing STX, which resembles TTX in structure and mode of action. The sensitivity to STX was tested and indeed, *Mytilus* nerve was resistant. Since Kao and Fuhrman (12) had found that the pufferfish is selectively immune to TTX but sensitive to STX, it was surprising to find *Mytilus* resistant to both.

Presumably a bivalve mollusc would be exposed only to STX under normal conditions. Does cross resistance to STX and TTX develop in molluscs, in contrast to the pufferfish, on the basis of similar chemical structure of STX and TTX? Alternatively, is *Mytilus* resistant to a toxic principle very similar to TTX which remains unidentified in paralytic shellfish toxin, that is, GTTX (Narahashi and Evans in these proceedings). In order to answer some of these questions, TTX and STX sensitivity were compared in eight species of bivalve molluscs. These data are discussed along with preliminary observations on toxin accumulation and behavior in some of these species during the September, 1972 outbreak of paralytic shellfish poisoning in Massachusetts.

A comparison of sensitivity to STX and TTX in bivalve molluscs.

The marine species tested were *Mytilus edulis* (Linnaeus), the bay mussel or edible mussel; *Modiolus demissus* (Dillwyn), the ribbed mussel; *Mya arenaria* (Linnaeus), the softshell clam, steamer; *Mercenaria mercenaria* (Linnaeus), the quahog or cherrystone clam; *Placopecten magellanicus* (Gmelin), the sea scallop; *Pecten irradians* (Lamarck), the bay scallop, and *Crassostrea virginica* (Gmelin), the edible oyster. These were collected either at Nahant or Woods Hole, Massachusetts. The freshwater clam, *Elliptio complanata* (Lightfoot) was obtained from the Connecticut Valley Supply House in Massachusetts. The experimental method has been described elsewhere (18).

Effects of STX and TTX on conduction of the nerve impulse.

Figure 1 shows records of compound action potentials in desheathed visceral connectives of *Mytilus* and *Placopecten*, two species highly resistant to STX. After ten minutes in 10^{-4} g per ml STX, the action potential is normal. No block was observed even after thirty minutes.

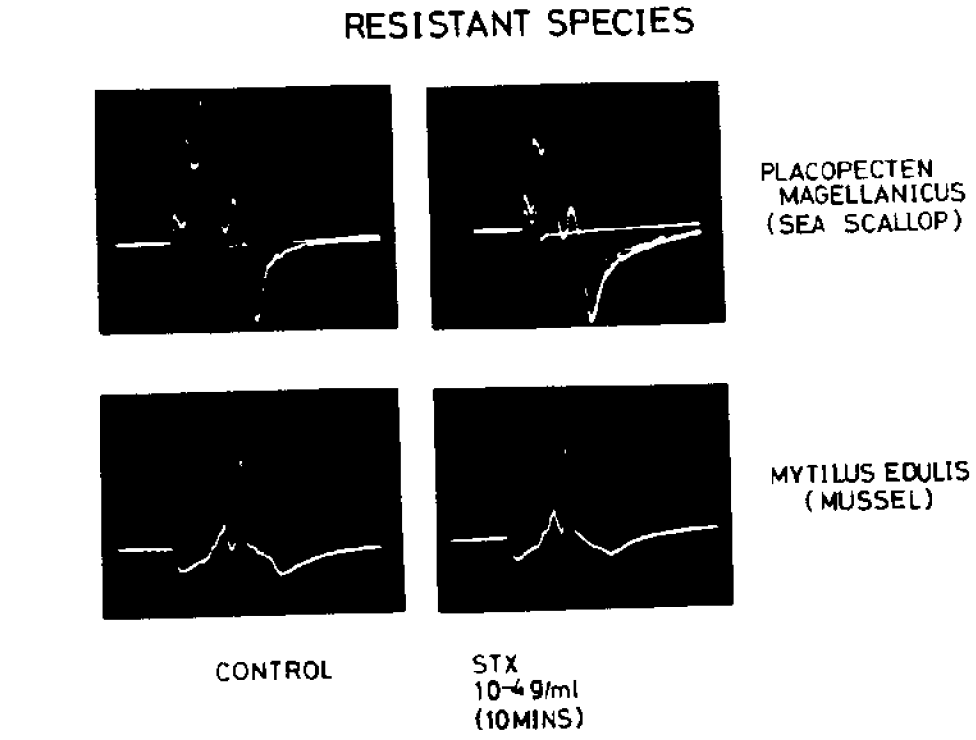

Figure 1. The effect of STX on compound action potentials in nerves of resistant species. Note that action potentials in 10^{-4} g per ml STX do not differ from the control.

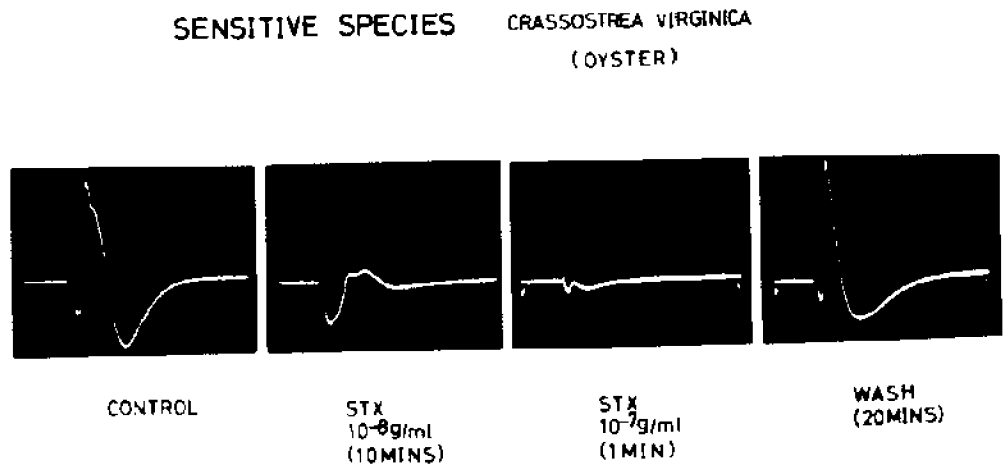

Figure 2. The effect of STX on compound action potentials in nerves of a sensitive species. Note that block is complete at 10^{-7} g per ml.

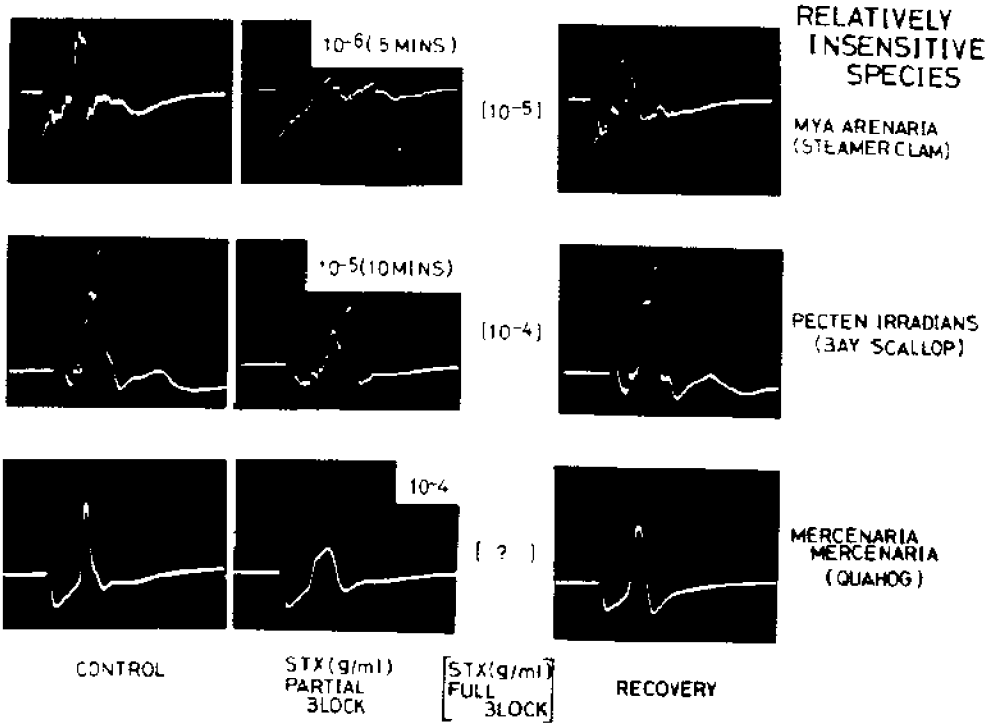

Figure 3. The effect of STX on compound action potentials in nerves of relatively insensitive species. Records of partial block are shown, at concentrations indicated. Full block occurred at the concentrations indicated in parenthesis: at 10^{-5} in *Mya*, 10^{-4} in *Pecten*, and above 10^{-4} g per ml in *Mercenaria*.

Figure 2 displays results obtained with the highly sensitive *Crassostrea*. The action potential is almost completely blocked at 10^{-8}, and fully blocked at 10^{-7} g per ml STX. Similar results were obtained with *Elliptio*.

The remaining species resisted full block by STX until concentrations of 10^{-5} g per ml or more were applied. Fig. 3 shows records obtained with *Mercenaria*, *Pecten*, and *Mya*, which are all considered relatively insensitive, although not fully resistant to STX.

Similar experiments were performed with TTX.

In all cases, it is apparent that the blocking effect is reversed after washing off STX or TTX.

Since it seemed possible that resistance in some species might be due to limitation of TTX or STX diffusion by a protective covering around the nerve fibers, all the experiments were done using nerve fibers from which the neural sheath had been removed. In some experiments, resistance was studied before and after removal of the neural sheath. No significant difference was noted. Thus, resistance to TTX and STX is a property of the nerve fibers, not the sheath.

Table I

Block of action potential by saxitoxin (STX)

Species	10^{-8}	10^{-7}	10^{-6} (g/ml)	10^{-5}	10^{-4}
Mytilus edulis	-	-	-	-	-
Placopecten magellanicus	-	-	-	-	-
Mercenaria mercenaria	-	-	-	-	-
Modiolus demissus	-	-	-	-	+
Pecten irradians	-	-	-	-	+
Mya arenaria	-	-	-	+	+
Crassostrea virginica	-	+	+	+	+
Elliptio complanata	-	+	+	+	+

The species are listed in order of increasing sensitivity to STX.
+ = Full Block.

If the action potential of the resistant species is not generated by an increase in conductance to sodium ions, then resistance to TTX or STX could easily be explained, since these substances specifically block the early sodium current (13). The desheathed nerves of all resistant and sensitive species were exposed to sodium deficient and sodium free solutions. In every case sodium deficiency reduced and blocked the action potential within minutes.

Relative blocking potencies of STX and TTX.

Tables 1 and 2 indicate the concentrations of STX and TTX required fully to block the compound action potential in various species. Resistance to STX roughly parallels TTX resistance. *Mytilus* and *Placopecten* are resistant to both toxins; *Crassostrea* and *Elliptio* are highly sensitive. Among the species relatively insensitive to STX, the correlation is less striking:

STX resistance, *Mercenaria* > *Modiolus* > *Pecten* > *Mya*

TTX resistance, *Mya* » *Modiolus* > *Mercenaria* » *Pecten*.

With respect to TTX, *Mya* should be classed as resistant and *Pecten* as sensitive.

Table 2

Block of action potential by tetrodotoxin (TTX)

Species	10^{-8}	10^{-7}	10^{-6} (g /ml)	10^{-5}
Mytilus edulis	–	–	–	–
Placopecten magellanicus	–	–	–	–
Mya arenaria	–	–	–	–
Modiolus demissus	–	–	–	+
Mercenaria mercenaria	–	–	–	+
Pecten irradians	–	–	+	+
Crassostrea virginica	–	+	+	+
Elliptio complanata	–	+	+	+

The species are listed in order of increasing sensitivity to TTX.
+ = Full Block.

Table 3

Whole Body Accumulation of Paralytic Shellfish Toxin

Species	Location	STX content µg per 100 g *	Maximum STX g per ml
Mytilus edulis (edible mussel)	York Harbor, Me.	10,092	10^{-4}
	Merrimack River estuary	7,392	7×10^{-5}
	Essex, Mass.	7,200	7×10^{-5}
	Eastham, Mass.	0 to "high"	0
Mercenaria mercenaria (Quahog, cherrystone clam)	Eastham, Mass.	0	0
Pecten irradians (bay scallop)	Eastham, Mass.	2,040	2×10^{-5}
Mya arenaria (steamer, littleneck clam)	York Harbor, Me.	1,726	2×10^{-5}
	**Merrimack River estuary	9,600	10^{-4}
	**Hampton Beach, N.H.	6,000	6×10^{-5}
	**Essex, Mass.	3,500	3.5×10^{-5}
Crassostrea virginica (oyster)	Eastham, Mass.	0	0
	Great Bay, N. H.	56	6×10^{-7}

* Quarantine level: 80 µg per 100 g

** Many *Mya* were "weak"; siphons were flaccid.

Effects of accumulation of paralytic shellfish toxin on the molluscs themselves.

During the Red Tide of September, 1972, along the Northeast coast of the United States, some observations were recorded on the relationship between whole body accumulation of paralytic shellfish toxin and behavioral symptoms in the molluscs (5).

Table 3 summarizes the observations. The level of toxin is calculated as micrograms of STX per 100 g of wet tissue (μg per 100 g), on the basis of measurements of mouse-toxicity of extracts of whole molluscs. It is probably that toxins other than STX are being detected, causing an overestimate of STX. The maximum concentrations of STX in the whole volume of soft parts are calculated from the data, on the assumption that the toxin is uniformly distributed throughout the body of the mollusc. This assumption is convenient, but questionable. Since a single measurement (in *Pecten*) showed that the concentration of toxin in muscle was about one-tenth of the whole animal concentration, it is probable that the actual concentration in muscle and in nerve is less than the concentration in the whole organism.

Resistance to STX and TTX as related to paralytic shellfish poisoning.

Species shown in the present study to have STX and TTX-resistant spike mechanisms have all been implicated in outbreaks of paralytic shellfish poisoning in humans. Halstead (8) and Bourne (4) indicated that species implicated in mass outbreaks of paralytic shellfish poisoning involving fatalities include *M. edulis*, *Mya arenaria*, and *Placopecten magellanicus*. Of these, *Mytilus* and *Placopecten* are resistant to STX and TTX; *Mya* is relatively insensitive to STX and resistant to TTX. In contrast *Crassostrea*, an edible species highly sensitive to both toxins, has never been implicated in paralytic shellfish poisoning.

We suggested earlier (18) that species resistant to STX and TTX can accumulate these toxins without harm while sensitive species cannot without themselves being poisoned (unless the toxin can be prevented from reaching the nervous system in an active form). This idea is supported by the field data, which show that *Mytilus* accumulates the highest levels of toxin of all species studied, yet shows no overtly abnormal symptoms whatsoever; whereas, when samples of *Mya* showed levels of toxin about 2000 μg per 100 g, many clams were obviously abnormal, with siphons flaccid. That the *Mya* were indeed displaying paralytic symptoms due to STX is supported by observations that the clams recovered within hours when restored to toxin-free seawater. Rapid complete reversal would be expected after nerve block by STX, but not if the clams suffered from a more general metabolic poison. In no case did the amount of toxin accumulated by the sensitive *Crassostrea* exceed 56 μg per 100 g even though it was surely exposed to the Red Tide. It is interesting that *Mercenaria*, although resistant, also did not accumulate toxin; perhaps it escaped exposure to *Gonyaulax*.

Mechanisms of Resistance to STX and TTX.

A. The physiological basis of resistance.

In the species studied, resistance is not conferred by an impermeable neural sheath, nor does it depend on abandoning a sodium mechanism for the generation of the action potential. One mechanism could be specific resistance of the sodium channel to the toxins. Kao and Fuhrman (12) found that the pufferfish and the newt have nerves which are highly resistant to TTX but sensitive to STX. In our observations, on the contrary, the resistance of several species to TTX seems to parallel resistance to STX. There could be a cross-resistance to the closely related molecules of STX and TTX in molluscs. Resistance to GTTX, the third toxin, has not yet been studied. Further research may reveal that the sodium channels of the nerves of resistant species are associated with a substance or structure that impedes access and prevents binding of the large toxin molecules. Alternatively, the molecular characteristics of the channels may be specifically altered to preclude interaction with STX or TTX.

This last question can best be approached by biochemical studies of the site(s) of attachment of the toxins to the nerve membrane. Methods for doing so have recently been developed. Isolation, from species sensitive to TTX and STX, of protein and/or lipoprotein fractions of nerve membranes that specifically bind these toxins has recently been described by Henderson and Wang (9); Benzer and Raftery, (2, 3); Barnola *et al.* (1) and Henderson, *et al.* (10). Also Benzer and Raftery (2, 3); have studied the susceptibility of the TTX-binding component of garfish olfactory nerve membrane to a number of enzymes. Their data suggest that there exists some phospholipid component, as well as protein, in the specific binding site of TTX (and presumably STX) to the sodium channels of garfish olfactory nerve.

In some species toxins may be stored in such a way as to reduce the levels of toxin in the circulation, thus protecting the nervous system from exposure. Such a mechanism has been described by Price and Lee (14,15) in *Saxidomus*, where the toxin is sequestered in pigmented granules in the siphon. Norris and Chew in this conference have cited another example of this mechanism, the accumulation of toxin selectively in the digestive gland of the West Coast razor clam. The muscular tissues contain no toxin, and thus the clam is safe to eat once cleaned of the digestive gland.

B. Genetic or acquired resistance?

Possibly species which are periodically exposed to dinoflagellate blooms have evolved mechanisms permitting them to exploit the toxic organisms as food. It would be interesting to know if resistance to TTX and STX is acquired on exposure to the toxins or is inherited. It seems likely that exposure to

dinoflagellate blooms means exposure to STX, not TTX; many of the species studied never encounter TTX in their natural habitat. However exposure to a TTX-like substance is not ruled out. GTTX as reported by Narahashi in this conference may be identical to the neurotoxin detected by Evans (7) in poisonous *Mytilus*. The neurotoxin described by Evans is not identical with TTX, and blocks action potentials in *Taricha torosa*, a species resistant to TTX but sensitive to STX. Isolation and identification of this substance may clarify the problem. It would be of interest to study *Mytilus* from an area which is known to be free of Red Tides. Unless the resistance is a genetic characteristic of the species, one would expect to find many sensitive individuals. In a previous study (18) my associates and I found no correlation between systematic relationship and resistance to STX or TTX. Some sensitivity to TTX was noted among individual *Mytilus* from Maine in contrast to those from California and Massachusetts, but no data were found in the literature on relative exposure to Red Tides.

Ecological and behavioral factors controlling accumulation of paralytic toxin.

Among the sensitive species, ecological isolation, that is, lack of exposure to these toxins, would permit survival. *Gonyaulax* is found only in a marine habitat. The fresh water species are very sensitive to STX and TTX. During blooms the alga *Aphanizomenon flos-aquae*, in fresh water habitats was found by Jackim & Gentile (11) to release a toxin similar to STX. However, we found no reports concerning whether or not fresh water molluscs die during these algal blooms, and no toxicity data are available since these molluscs are not considered edible.

Crassostrea in very sensitive to STX and TTX, and is surely exposed to blooms of *Gonyaulax*. It is probable that a behavioral response protects this species. *Crassostrea gigas* has been shown by Dupuy & Sparks (6) to cease filtering when exposed to *Gonyaulax*. The oyster shuts its valves tightly and does not resume filtering until the water is clear. However, after prolonged exposure the oyster filters minimally. If *Mytilus* and *Crassostrea* are both exposed to the toxic dinoflagellates, *Mytilus* accumulates more than four times as much toxin as does *Crassostrea*. Protective behavioral responses may well be found in other sensitive marine species.

During the 1972 Red Tide in Massachusetts, *Mercenaria* was never found to accumulate toxin, even in heavily infested areas (i.e. localities where high toxin levels were found in *Mytilus* and *Mya*). Since *Mercenaria* is resistant, it could theoretically accumulate toxin without harm to itself. It is possible that *Mercenaria* is protected from exposure by its habitat, which is deeper than that of *Mya* and/or by its feeding habits; unlike *Mya* and *Pecten*, it may not be a surface feeder or it may reject detritus. This interesting problem deserves further exploration.

An important observation was made during the 1972 Red Tide emphasizing that not only habitat but behavior (feeding habits) determine toxin

accumulation: A number of species collected at a depth of 35 feet contained more than 1000 μg per 100 g toxin. Since *Gonuaylax* is not known to penetrate to this depth, it seems possible that these species filtered detritus to which the toxin was absorbed. Deepwater species which do not filter detritus could not be exposed.

CONCLUSION

An investigation of resistance to STX and TTX in bivalve molluscs has contributed to our understanding of accumulation of paralytic shellfish toxin during Red Tides. Continued studies of the neurophysiology, ecology, and behavior of molluscs and other marine organisms during exposure to Red Tide should prove to be of considerable practical value and theoretical interest.

ACKNOWLEDGEMENT

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MODE OF ACTION OF DINOFLAGELLATE TOXINS ON NERVE MEMBRANES

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ABSTRACT

Certain dinoflagellates produce highly toxic substances. *Gonyaulax catenella* produces saxitoxin (STX) and *Gonyaulax tamarensis* produces another toxin (GTTX). The mechanism of action of these two toxins on nerve membranes has been studied in comparison to that of tetrodotoxin (TTX). All of them exert the same effect on membrane ionic permeability.

These toxins block impulse conduction of squid giant axons at very low concentrations without any effect on the resting membrane potential. The blockade is due to a specific inhibition of the mechanism whereby the nerve membrane undergoes a regenerative increase in permeability to sodium ions. The effect is reversible upon washing with toxin-free medium. The toxins have no effect when applied directly to the internal membrane surface, indicating that the site of action is located on or near the external surface. TTX exists in two cation forms and a zwitterion form, and the former is responsible for the block of sodium current.

Further electrophysiological analyses have unveiled several important features of the blockade of sodium current by these toxins. They have a strong affinity for the membrane sodium channel *per se*, and the number of sodium channels as estimated from the TTX binding to the nerve membrane is extremely small. These and several other characteristics of the action of TTX, STX and GTTX give promise of using these toxins as important and powerful tools for the study of neurophysiology and neuropharmacology.

INTRODUCTION

Certain dinoflagellates produce highly toxic substances. *Gonyaulax catenella* produces saxitoxin (STX) and *Gonyaulax tamarensis* produces another toxin (GTTX). Since these *Gonyaulax* toxins are potent neuropoisons, their mechanism of action on nerve membranes has been studied by means of a variety of advanced electrophysiological techniques. These toxins exert almost identical actions on nerve membranes as tetrodotoxin (TTX), the toxin isolated from the ovary and liver of pufferfish. The present paper gives an up-to-date summary of these studies.

Rationale and Methods

The membrane is the site of nerve excitation. At rest, the nerve membrane is permeable to potassium ions, but poorly permeable to sodium and other ions. Since potassium concentration is much higher in the axoplasm than in the external medium (serum), and sodium and chloride concentrations are much higher in the external medium than in the axoplasm, a potential difference is established across the nerve membrane with the inside negative with respect to the outside, and the value approaches the potassium equilibrium potential defined by the Nernst equation. This potential is called the resting membrane potential.

When the nerve is stimulated by a brief outward current across the membrane, the membrane permeability to sodium ions increases in a regenerative manner, so that the membrane becomes almost exclusively permeable to sodium ions. Thus the membrane potential is changed toward the equilibrium potential for sodium, forming the rising phase of the action potential. The increased sodium permeability starts decreasing soon, and the potassium permeability starts increasing beyond the resting level, thus bringing the membrane potential back to the original resting level. These changes in membrane potential and membrane permeabilities occur in a matter of milliseconds.

Thus, in order to study the mechanism of action of a toxin on nerve excitability, one will have to measure these permeabilities as affected by the toxin. The ionic permeabilities can be measured as ionic conductances by a method called voltage clamp. Since conductance is given by the ratio current/potential, if we can measure the current carried by a specific ion and the electromotive force for the ion, we should be able to estimate the ionic conductance. This can be accomplished by clamping the membrane potential at various levels by using a feedback circuit, and by measuring the current necessary to hold the membrane potential at those levels. Since the time course of sodium and potassium conductance changes are different, each ionic current can be identified and measured separately. Isolated preparations of squid and lobster giant axons and frog skeletal muscle fibers were used as material.

Effects on Resting Potential, Action Potential and Ionic Conductances.

Since the effects of STX, GTTX and TTX on nerve membranes are the same, the results of experiments will be discussed together. The resting potential is not affected by the toxins, while the action potential is drastically reduced in amplitude (15). This suggests that the sodium conductance increase (sodium activation) responsible for the rising phase of the action potential is inhibited by the toxins. Voltage clamp experiments have clearly demonstrated that this is actually the case (see 19, 16, 14). An example of such an experiment for TTX is illustrated in Figure 1. While the peak transient sodium current is completely suppressed by TTX, the steady-state potassium current remains unaffected. It should be noted that the effect is exerted at very low concentrations, the apparent dissociation constant of TTX in blocking the sodium current of squid axons being estimated to be 3nM by Cuervo and Adelman (5).

Further experiments have been carried out to characterize the action of TTX on the sodium channel, the conceptual site in the nerve membrane where sodium ions are transported during activity. TTX has no affinity for sodium ions *per se*, but blocks the sodium channel regardless of the direction (outward or inward) of ionic current or regardless of the kind of ions involved (9). It should be noted that not only sodium but also lithium, potassium, rubidium and cesium are transported through the sodium channel to varying extents (3).

Site of Action in the Membrane

In contrast to the very high potency of TTX, STX and GTTX as applied externally to nerve preparations, they are totally ineffective when applied internally. The axoplasm of the squid giant axon can be squeezed out by means of a small roller and perfused internally with artificial solutions without impairing excitability. No effect on action potential or sodium current is observed by internal perfusion of these toxins at very high concentrations. For example, TTX has no effect internally at a concentration of 1×10^{-5}M, 3000 times higher than the concentration needed to block sodium current 50% by

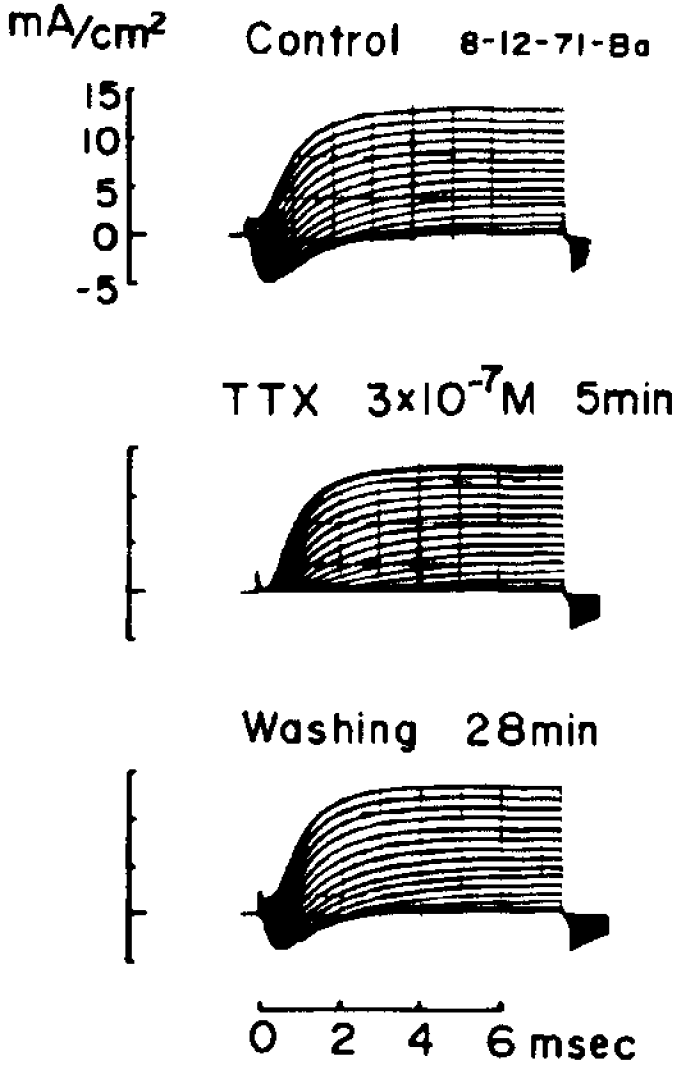

Figure 1. Families of membrane currents associated with step depolarizations (10mV steps) in a squid giant axon before and during external applications of 3×10^{-7} M tetrodotoxin (TTX) and after washing with toxin-free medium. Note that TTX blocks transient sodium currents without any effect on steady-state potassium currents

external application (13). Similar results are obtained with STX and GTTX (4, 9). Since TTX and STX are poorly soluble in organic solvents, they presumably penetrate lipid membranes very poorly. Thus, the site of action of these toxins should be located on or near the external surface of the nerve membrane.

Active Form

TTX exists in two cation forms and a zwitterion form with a pK_a of 8.8. Therefore, a question arises as to which form is responsible for the sodium conductance block. This problem can be studied by comparing the blocking potency at different pH values. The results of such experiments clearly indicate that the cation forms are active (2, 7, 17, 20).

Structure-activity Relationship

The chemical structure of TTX is characterized by a guanidinium group, hemilactal link and several hydroxy groups. The study of the structure-activity relationship has encountered great difficulty due to possible contaminations of derivative samples with TTX. TTX is so potent that a small contamination with it to the extent that cannot be detected by an chemical or physical means will significantly distort the potency measurement. For this reason, the only reliable conclusions we have been able to draw is that deoxytetrodotoxin and tetrodonic acid (Fig. 2) are totally ineffective in blocking the sodium

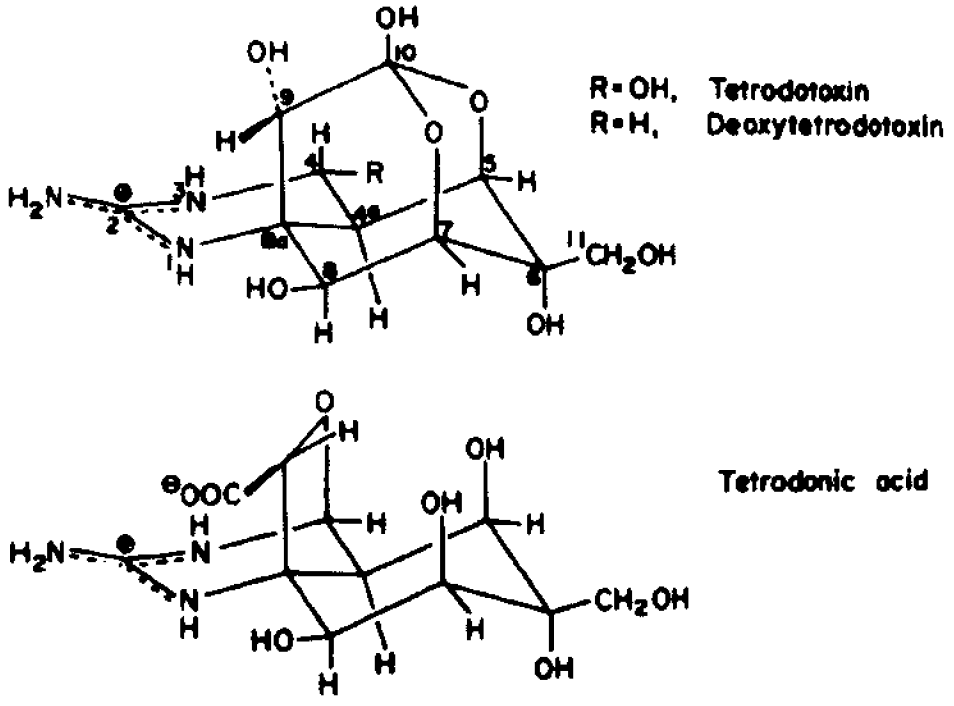

Figure 2. Structures of tetrodotoxin, deoxytetrodotoxin and tetrodonic acid (18)

activation (8). The chemical structure of deoxytetrodotoxin is different from that of TTX in carbon 4 position; the hydroxy group of TTX is reduced to a hydrogen to form deoxytetrodotoxin. It is remarkable that such a small change in structure completely abolishes the physiological activity.

The structure of STX is considerably different from that of TTX, and contains two guanidinium groups (see Wong, Oesterlin and Rapoport, 21). The structure of GTTX has not been identified yet, but there is evidence that it is different from that of TTX or STX (E. Schantz, personal communication). Thus it is of particular interest that these three toxins, which are chemically different, exert the same physiological effect on nerve membranes. When the structure of GTTX is completely identified, the common denominator of the three toxins will be a useful parameter to identify the topography of the receptor and sodium channel.

Binding to Nerve Membranes

It has been demonstrated that TTX and STX bind to the receptor in the nerve membrane on a one-to-one stoichiometric basis (5, 7). Thus we should be able to count the number of receptors from the study of binding. Our initial bioassay experiments with the walking leg nerve of the lobster have resulted in 13 binding sites per square micron of the membrane as an upper limit (10). Similar measurements have since been repeated by either bioassay or tritiated TTX using various nerve preparations (1, 4, 6, 8). The values range from 3 per square micron of membrane in the garfish olfactory nerve to 75 per square micron in the rabbit vagus nerve. If we assume that one receptor controls or is part of one sodium channel, then these numbers give the density of the sodium channels in the membrane. TTX is now being used as a tool to isolate and identify the sodium channel *in vitro* (12).

CONCLUSION

Tetrodotoxin, saxitoxin and *Gonyaulax tamarensis* toxin exert the same effect on nerve membranes. They inhibit the mechanism whereby the nerve membrane undergoes a conductance-increase to sodium upon stimulation, thereby blocking nervous conduction. This effect is exerted at very low concentrations (nanomolar) and only from outside of the membrane. The toxin and receptor interact on a one-to-one stoichiometric basis. It should be noted that these three toxins have different structures yet exhibit the same physiological effect. These toxins are extremely useful probes to characterize and identify the sodium channel in the nerve membrane.

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THE BINDING OF SAXITOXIN TO NERVE TISSUE AND ITS ANTAGONISM BY VARIOUS AGENTS

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ABSTRACT

We have studied the binding of radioactively labelled saxitoxin to nerve membranes and to receptors from those membranes which were solubilized by detergents. The binding has two aspects; relatively few sites on the nerve are saturated by very low (10^{-8} M) toxin concentrations (specific binding) while the nerve also continues to take up toxin in proportion to its bath concentration, showing no saturation up to 10^{-6} M toxin concentrations (non-specific binding). The specific binding sites have the affinity and kinetic constants for toxin binding reactions as would be predicted from the physiological effects of the toxin (Henderson, Ritchie & Strichartz, (9, 10), so we conclude that specific binding corresponds to the physiological, nerve blocking activity of saxitoxin..

Several agents strongly antagonize the specific binding. Among these are polyvalent metal cations (La^{3+}:K_i = 1 mM; Ca^{2+}:K_i = 30 mM), thallous ion (K_i = 20 mM) and H^+ ($pK_a \sim 5,7$). Like the toxin binding itself, all these inhibitory reactions are reversible. However, all the inhibitory agents are quite toxic at their effective concentrations. Preliminary experiments suggest that specific binding of saxitoxin may be antagonized by thiamine (5 mM).

The binding of labelled saxitoxin to animal tissue is useful as a tool for measuring the surface density of molecular structures responsible for electrical excitability, for studying the changes in such sites during development and under pathological conditions.

INTRODUCTION

Saxitoxin is a potent inhibitor of the electrical activity of nerves and muscles. Action potentials in these tissues usually rely on an increase in the permeability of the cell membrane to sodium ions. The currently accepted paradigm states that this membrane permeability increase arises from the opening of discrete sites, the so-called "sodium channels", which are sparsely distributed in the nerve membrane. Saxitoxin (STX) and the analogous poison tetrodotoxin (TTX) block action potentials by preventing the permeability increase to sodium (12, 17, 21). We examined the binding of radioactively labelled STX and TTX to nerve membranes to determine just how these toxins interacted with the membranes and their sodium channels. We also examined a number of agents known to interact with sodium channels to see if they would effect STX binding.

METHODS

Both STX and TTX were labelled with tritium (^3H) by exposing them to tritium gas during an electric discharge (9). They were then purified by high-voltage paper electrophoresis. The specific activities were usually about 100 mc/mmole 300 toxin: this is equivalent to having about one tritium atom

in 300 toxin molecules. Binding was measured in two systems: (a) intact nerves and (b) receptors for toxin which had been liberated by solubilizing the membranes with non-ionic detergents (8). Nerves were removed from various animals - rabbits, lobsters, and garfish, but the results of binding studies usually agreed, regardless of the source of nerve tissue. Solubilized receptors come from the garfish olfactory nerve which has an exceedingly high ratio of nerve cell membrane area to non-excitable cell membrane area (6). The details of the experimental procedures are contained in the references (9, 10). Previous studies with TTX had shown its binding behavior to be very similar to that of STX (3), so in later experiments we used the toxins interchangeably.

In experiments where we wished to study competitive binding we compared the toxin uptake by a control tissue to the uptake by tissue in the presence of the potentially competitive agent. The results were corrected for non-saturable toxin binding (see below) and expressed as the percentage of the toxin uptake by the control sample. The resulting values are expressed as mean percentages ± the standard error of the mean.

RESULTS

Radioactively labelled saxitoxin binds to nerves in two modes. One is a binding which is first-order (one STX binds to one receptor), has a very low dissociation constant ($K_D \sim 3 \times 10^{-9}$ Molar, the toxin concentration at which

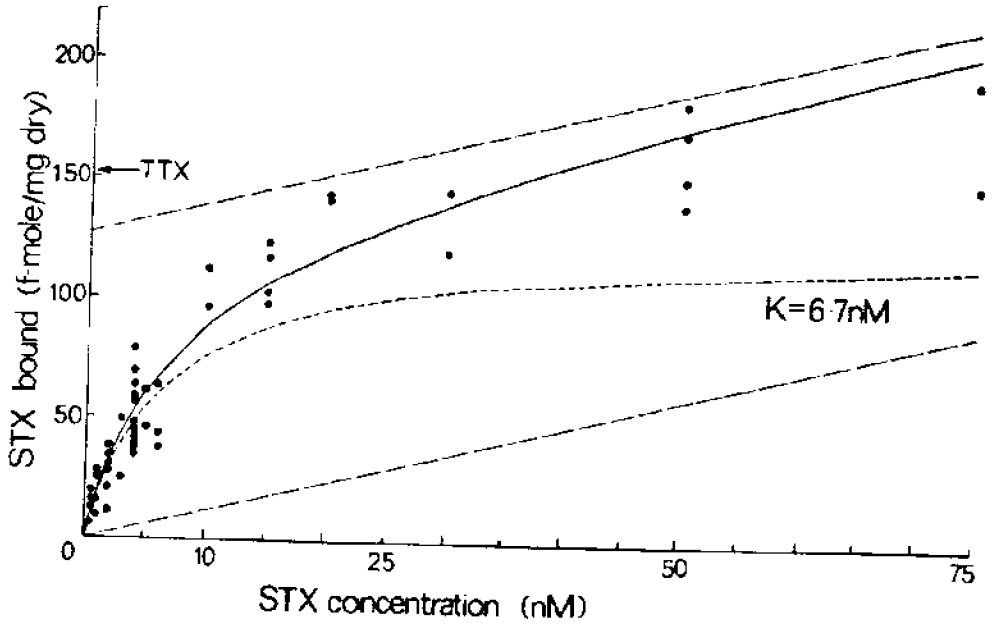

Figure 1. Uptake of tritium-labelled STX by rabbit vagus nerves as a function of STX concentration. The solid line is a computer-fitted (least squares) sum of a saturable, Langmuir binding term (short dashed line) and a non-saturable term which is linear with concentration (broken line). (Reprinted from Henderson, *et al.* 1973 with permission of the Journal of Physiology).

Table I

TTX and STX Binding Parameters

	TTX		
	Binding to intact nerve	Binding to solubilized receptor from gar nerve	Physiological effects
$K_{diss.}$ (nM)[E]	3.0 ± 0.4[A,I] 10.1 ± 1.3[I,B] 8.3 - [II,B]	6 - 15[VII] 2.5 - [IX]	3-5[A,III] 3.60 ± 17[D,V] 12 ± 6[B,IV]
k_{off}	———	0.95 min^{-1} (20°C)[VII]	0.35 min^{-1} (12°C)[C,VI] 0.82 min^{-1} (20°C)[D,V]
k_{on}	———	1.6 x 10^8 $M^{-1}Min^{-1}$ (20°C)[VIII]	1.5 x 10^8 $M^{-1}min^{-1}$ (12°C) [C,IV] 1.7 x 10^8 $M^{-1}min$ (20°C) [D,V]
	STX		
$K_{diss.}$ (nM)	6.7 ± 3.0[A] VIII	6.3 VIII	6 - 3[B,IV]
k_{off}	———	3.2 min^{-1} (20°C) VIII	1.5 min^{-1} (12°C)[C,VI]
k_{on}	———	5 x 10^8 $M^{-1}min^{-1}$ (20°C)[VIII]	5.7 x 10^8 $M^{-1}min^{-1}$ (12°C)[CVI]

(from Strichartz, 1975)

Footnotes for Table I

A. rabbit vagus nerve

B. garfish olfactory nerve

C. *Rana pipiens*, node of Ranvier: inhibition of I_{Na} during voltage-clamp

D. *Xenopus laevis* node of Ranvier: inhibition of I_{Na} during voltage-clamp

E. All measurements done at 20°C unless otherwise noted.

I Colquhoun *et al*., 1972

II Benzer and Raftery, 1972

III Colquhoun and Ritchie, 1972.

IV R. Henderson and G. Strichartz (1974)

V Schwarz *et al*., 1973.

VI B. Hille, personal communication.

VII Henderson and Wang, 1972

VIII Henderson *et al*., 1973

IX Benzer and Raftery, 1973

half the saturable sites are bound with STX) and is inhibited by > 100-fold concentrations of non-radioactive saxitoxin or tetrodotoxin. This is the saturable, or specific binding activity. The other mode of binding is an uptake by the nerve which never saturates, even at toxin concentrations up to 10^{-6} M, and is not affected by high concentrations of unlabelled toxin. The sum of these two binding modes is shown in Fig. 1. The saturable uptake indicates that the surface density of STX binding sites is $\sim$ 1-50 /μ^2 in many non-myelinated nerves, but this number may be several hundred /μ^2 in giant axons of squid (Strichartz, unpublished observation).

STX and TTX are bound only in the saturable mode by solubilized receptors. The equilibrium binding parameters are like those in intact nerves (Table I). Using these receptors we were able to measure the rate at which STX and TTX bound to the membrane specifically and to compare the rate measurements with those from electrophysiological experiments. All the data of this type are collected in Table I, along with that from equilibrium uptake studies to whole nerves. Table I shows clearly that the rate constants and equilibrium dissociation constants which were measured in binding experiments compare most favorably to the values derived from electrophysiological experiments. This leaves little doubt that we were studying the physiologically important binding and not some artifact.

Competition Experiments

Table II summarizes the results from the competition studies. Agents which block action potentials by binding in the sodium channels near the outside of the nerve membrane were able to block STX uptake. Among these were

Table II

Specific binding of toxins to nerves in the presence of neuro-active agents.

Agent	Concentration	% Control binding
H^+	3×10^{-6} M	57 ± 5
Thallous (Tl^{+1})	0.1 M	22 ± 6
Li^+	0.154 M	60 ± 10
Ca^{2+}	0.88 M	45 ± 2 (TTX)
Ca^{2+}	0.88 M	23 ± 2 (STX)
Veratrine	50 μg/ml	80 ± 7
Lidocaine	10^{-3} M	103 ± 5
Batrachotoxin	5×10^{-7} M	98 ± 4

All studies done at pH 7.0, 20°C - except for the H^+ effect. The means are the results of at least 6 experiments on each compound. Toxins at 3-4 nM.

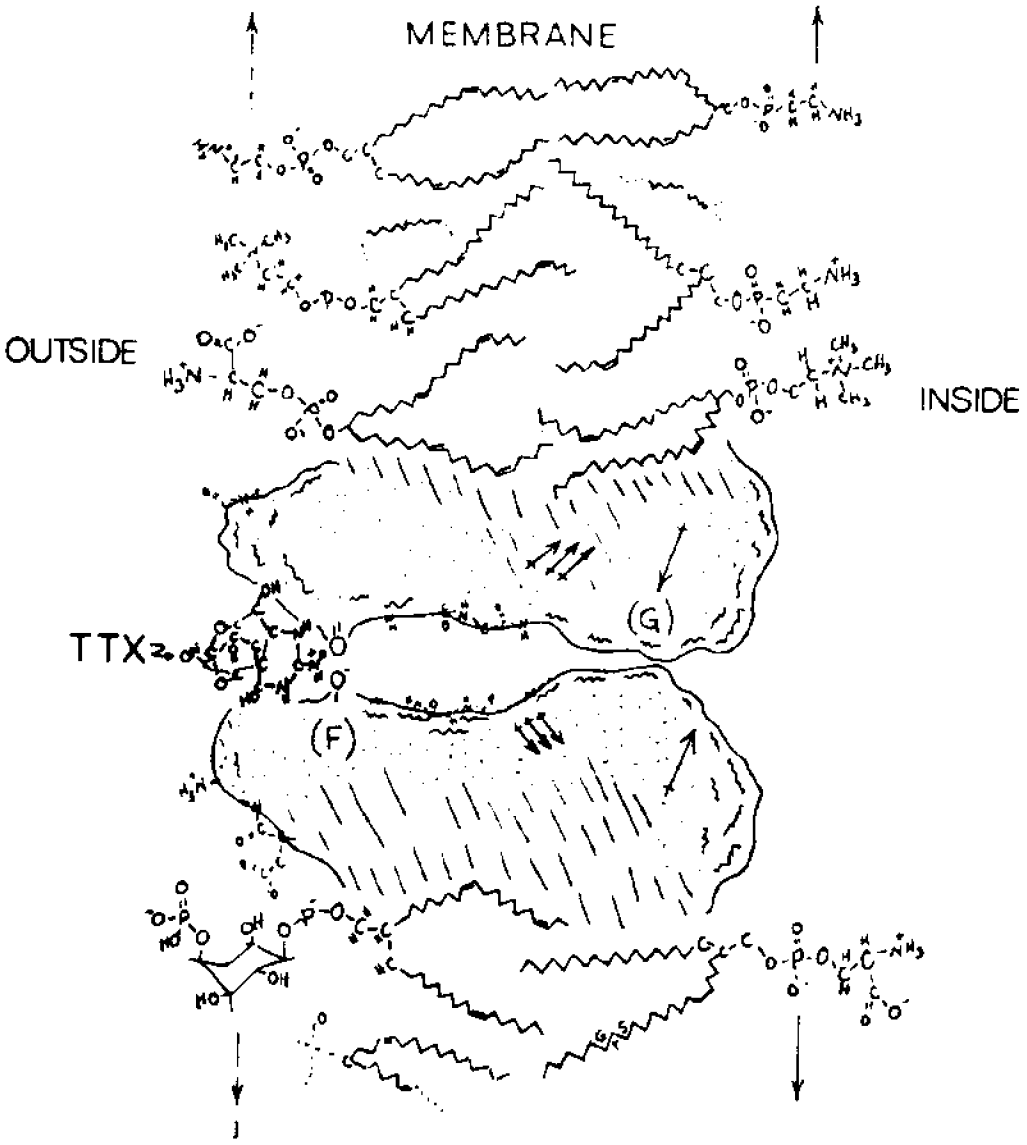

Figure 2. Artist's rendering of the sodium channel spanning a nerve membrane. The receptor for saxitoxin and tetrodotoxin is the outer opening of the sodium channel near or at the ion-selectivity filter (F) which includes a negatively charged group of apparent $pK_a = 5.6 \pm 0.3$. The voltage-dependent gates which modulate the flux of ions during a nerve impulse (G) are located near the axoplasmic side of the channel, and are near a hydrophobic milieu in the membrane which is the locus of action of local anesthetics and veratridine. Note that the STX receptor is a rather rigid structure which maintains its conformation regardless of the movement of the gates.

calcium and protons (26), lithium, thallium (14) and trivalent metal cations. Agents which affect sodium conductance at the inside of the nerve membrane, such as local anesthetics (15, 20, 23) and veratridine, do not depress STX or TTX binding. This is consistent with the observation that TTX (and presumably STX) act only from outside nerve membranes having no effect if present within the cells (19). Our results indicate that TTX and STX bind directly to the outside of the sodium channels. We think that the quanidinium portion of the toxin molecules sits in the opening of the channel and that the numerous hydroxyl and amino groups on the toxins are important in the formation of bonds between toxin and sodium channels. Kao and Nishiyama (18) and Hille (13) have previously speculated about such an interaction. In this configuration the ionic bond between the positively charged quanidinium moiety on the toxins and the negative site known to exist in the sodium channel is also important (26).

We present Fig. 2 as a very liberal visualization of our working hypothesis about STX interaction with sodium channels.

All of the substances listed in Table II which antagonize toxin binding are themselves toxic. Hence, unfortuantely, they are clinically useless. However, we have some preliminary results from competition experiments with thiamine (Vitamin B_1) and thiamine antimetabolites which provide evidence for their modulation of STX and TTX binding. Thiamine is released from nerves following stimulation (27, 5), and by TTX exposure (16) and its anti-metabolites have a pronounced effect on the shape of the nerve impulse (7). Our early results (Table III), while not conclusive, do indicate a possible role for thiamine as a TTX and STX antidote.

Table III

Specific binding of STX to nerves in the presence of thiamine and pyrithiamine.

Agent	Number of experiments	% Control STX binding
Thiamine - 5 mM	4	68 ± 8
Purithiamine - 5 mM	5	129 ± 10

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EVIDENCES OF NEW ICHTHYOINTOXICATIVE PHENOMENA IN *GYMNODINIUM BREVE* RED TIDES[1]

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A red tide caused by the toxic dinoflagellate *Gymnodinium breve* Davis occurred intermittently along the west coast of peninsular Florida from October 1973 through June 1974. Distress behavior of fishes in the red tide area was observed and 129 severely distressed or freshly dead specimens were collected and subjected to immediate necroptic examination. The 16 consistently observed pathologies suggest that many fishes die in red tides from chronic tissue damage rather than by previously recognized neurointoxication. Some species seem to succumb to neurointoxication under the same conditions that produce lethal hemopathy and histopathology in others. The observed sign complexes are indicative of dehydration, hemolysis, and interferences in the blood clotting mechanisms.

INTRODUCTION

Substantial red tides caused by blooms of the unarmored dinoflagellate *Gymnodinium breve* Davis occur periodically along the west coast of Florida (1, 2, 3). In addition to causing toxicity in shellfish, *G. breve* red tides directly kill large numbers of fish and some marine invertebrates, produce a highly irritating aerosol in coastal areas, and can rarely cause contact dermatitis (4, 5, 6, 7, 8, 9). As a result of these effects, such red tides have serious adverse economic impact, particularly on the tourist industry, in addition to being a public health problem.

Considerable scientific study has been made of *G. breve* red tides, particularly since the discovery and description of the causative dinoflagellate by Davis in 1948. Early laboratory studies indicated that fish were killed by a neurotoxin as evidenced by speed of action, agonal behavior, and lack of other patent signs or lesions (10, 11). These observations were repeatedly confirmed by numerous experimental exposures of fishes to natural and cultured *G. breve*, to cell-free filtrates, and to extracted and fractionated toxins (11, 12, 13, 14, 15). Consequently, *G. breve* red tides became accepted as a neurointoxicative phenomenon.

Detailed studies of toxins extracted from *G. breve* in recent years have provided evidences of other biological activity. In addition to potent neurotoxic activity, *in vitro* hemolytic activity was detected in crude toxin extracts (16, 17, 18, 19, 20, 21, 22, 23) and in a purified non-neurotoxic fraction (15). Hemolytic activity was considered mild until Kim, Linton, and Martin (23) tested fish blood (mullet) in addition to the mammalian blood (usually rabbit) utilized by other workers. They found the piscine blood to be some 300 times more sensitive. They suggested that hemolytic activity could be a factor in red tide fish kills.

Doig and Martin (24) further discovered mild anticoagulant activity in toxin extracted from a natural red tide. Trieff *et al.* (25) isolated five toxins from *G. breve* cultures, one of which was a heretofore unreported, severe respiratory

irritant upon either intraperitoneal injection or inhalation. All five of their toxins had neurotoxic activity and one was also hemolytic.

Despite the findings of additional biological activities, the neurotoxic activity of *G. breve* has still been generally considered to be the only consequential ichthyointoxicative mechanism. This assumption was supported by the few reports of premortal fish behavior in natural red tides (5, 26, 27) which indicated, as laboratory studies had suggested, that death usually came rapidly upon exposure to *G. breve* red tide.

Beginning in October 1973, we collected dead and dying fishes and other organisms from red tide areas and subjected them to detailed pathological analyses to determine mechanisms of death. Preliminary results were presented by Quick and Henderson (28). Further results and interpretations are presented herein. These studies are the first pathological examinations made of red tide exposed animals from the field or laboratory since the discovery of the causative agent over 25 years ago. In fact, a few brief comments by Taylor (29) concerning darkened gills and slow blood clotting in dissected fishes seem to represent the only prior published observation of organic changes caused by red tide.

METHODS AND MATERIALS

Beginning in October 1973, a red tide developed offshore of the west central coast of Florida in the Gulf of Mexico and moved shoreward. After a short absence, it reappeared in January 1974 and moved inshore along some 270 linear km (168 miles) of coastline and penetrated Boca Ciega Bay, Sarasota Bay, Tampa Bay, and Charlotte Harbor. Severe, widespread fish kills resulted along with respiratory irritation and toxic shellfish.

In late October 1973, we began collecting distressed or freshly dead fishes from the red tide areas. Collections were made with aid of SCUBA, with nets, and/or by hand. Every effort was made to observe any distress behavior. Water samples were collected at or near fish sampling locations and analyzed quantitatively for *G. breve* (cell counts) in addition to the usual hydrographic parameters.

Specimens and samples were rushed without refrigeration back to our main laboratory in St. Petersburg. Most analyses were begun within 45 minutes of collection or death and none after more than 6 hours. Fishes were subjected to a detailed necroptic analysis. After initial observations and measurements (color, slime, length, weight, etc.) blood samples were taken from one or more of the sinuses or major veins just afferent to the heart. Blood was subjected to standard hematological analyses including cell counts, clotting, and chemistry. Careful necroptic dissection followed. Tissue samples were excised and fixed for later histological technique processing and for preparation for electron microscopy. Procedures are detailed in Quick and Henderson (28).

Following the 1973-74 red tide, several others occurred from which additional specimens were collected and examined. In August 1974, a red tide

developed in the Gulf off the Tampa Bay area and moved shoreward. Many sharks appeared inshore, apparently driven ahead of the advancing dinoflagellate bloom. These sharks, principally young spinner and black tip species (*Carcharinus maculipinnis* and *C. limbarus*), became progressively lethargic and died. Several specimens were netted in distress and subjected to necropsy. As is usual with sharks, none floated after death and the mortality was relatively inobvious.

The following month, red tide appeared in north Florida near Panama City and moved westward for several weeks, finally subsiding near Pensacola, Florida. Widespread fish kills occurred and irritating aerosols were severe. Several cusk eels (*Ophidion holbrooki*) were collected in *rigor mortis* and necropsied.

So far, 129 fishes of 15 species and genera representing 13 families, 8 orders, and 2 classes have been subjected to detailed pathological examination.

RESULTS

Analyses are still incomplete, but initial findings already indicate that *G. breve* red tide ichthyointoxication may be substantially different from the processes previously thought to occur.

Sixteen identifiable pathologies occur repeatedly in red tide exposed fishes and seem to be due, directly or indirectly, to *G. breve* toxins: 1) anoxic premortal distress behavior, 2) cyanemia and branchial cyanosis, 3) severe achromic normoblastosis, 4) moderate to severe normochromic normacytic anemia, 5) marked hemal hyperviscosity, 6) increased whole blood clotting time (WBCT), incipient and total, 7) increased packed cell volume (PCV), 8) increased total blood count (TBC), 9) increased mean cell volume (MCV), 10) thrombocytopenia, 11) leucocytopenia, 12) plasma debris, 13) manifest plasma hemoglobin, 14) absent or reduced recent feeding, 15) splenomegally, and 16) excessive hepatic vascular endothelial hemosiderin deposition. Each of these pathologies is described in some detail in Quick and Henderson (28).

Observed pathologies tended to vary somewhat from one individual specimen to the next, particularly quantitatively. Since all cases were natural exposures, we can only infer the level of toxin(s) each may have encountered from our knowledge of *G. breve* concentrations in the collection area at and before the time of collection. One would, therefore, expect the effects on the fishes to differ somewhat from place to place and time to time in different collections. Some differences were seen, however, between specimens of the same species from the same collection and presumably from the same school. Since these individuals probably had the same red tide exposure history, we must conclude that there is some variation in individual response to *G. breve* under similar conditions. This variation may be due to individual genetic or physiological postures.

A much greater diversity of pathological responses was seen between species than between individuals of a single species. In fact, the two species for which we had the most cases, the striped mullet (*Mugil cephalus*) and the ladyfish (*Elops saurus*) showed almost completely opposite responses to a red tide intoxication.

CONCLUSIONS

As we said earlier, acute, non species specific neurointoxication is the rule in laboratory studies of *G. breve* toxin and has been inferred from limited field observations. The results of our studies of naturally exposed fishes, however, lead us to propose two additional mechanisms of death in red tides: 1) chronic neurointoxication and 2) chronic lethal hemopathy. Unlike the acute neurointoxication which seems to result in death of any fish exposed to sufficient toxin, the two chronic intoxications seem to be species specific, the ladyfish being a good example of the former and mullet of the latter. Many fishes show elements of both.

In chronic neurointoxication, exposed fishes seem to act almost normally during many hours or days of exposure to red tide. A neuromuscular crisis then occurs causing extreme, uncontrolled, but somewhat coordinated, hyperactivity. Rapid swimming in large circles and leaping from the water is typical. This degenerates to an uncoordinated vibrating and tumbling and death ensues rapidly, usually in 15 to 30 seconds after the beginning of the agonal distress period. Terminal cardiac arrest was directly observed in small transparent anchovies (*Anchoa mitchelli*). At necropsy, chronically neurointoxicated fishes show little pathology aside from slight precipate hemolysis as evidenced by corpuscular ghosts, plasma debris, and patent hemoglobinemia, and frequent cerebrovascular congestion. The hemolysis is consistent with that seen in cases of lactic acid buildup in the blood due to capture stress and struggle (30). It is therefore likely that it results from the fatal agony rather than directly from red tide effects. It may be significant that ladyfish and anchovies seem to be exceptionally susceptible to lethal hemopathic acidosis from capture stress under non red tide conditions.

Individual fishes of the same species seem to vary in their sensitivity to the red tide toxins. Typically, in a large school under continuous exposure, individual fish randomly but suddenly enter the premortal frenzy and expire. The majority continue to act normally until each, in turn, is affected.

In chronic hemopathic intoxication, exposed fishes seem to show a progressive general hypoesthesia, particularly ocular hypoesthesia and hypacusis, and hypokinesia. Balance and coordination do not seem to be affected and once a fright response is elicited, it appears normal.

As before, premortal distress seems to overtake individual fish suddenly, frequently following a fright response or other exertion. Unlike the previous hyperactivity, fishes in terminal hemopathic distress undergo a slightly

hypoactive, anoxic behavior mimicking the surface breathing and gulping seen in fishes immersed in oxygen deficient waters. Progressive weakening follows and the fish begin to sink for short periods before swimming back to the surface. These periods of sinking toward the bottom increase in length as the time at the surface decreases. Swimming becomes increasingly encumbered as coordination is lost and death comes uneventfully. Sometimes, there is a moment of uncoordinated hyperactivity just premortal. Time from initiation of terminal distress to death was usually 15 to 30 minutes.

At necropsy, these fish typically showed most if not all of the 16 pathologies listed previously. The fish apparently ate little or nothing in their hypoesthetic-hypokinetic state.

Hemolysis also seems to occur chronically during this period of almost asymptomatic exposure to red tide. The finding of well developed normacytic anemia with replacement normoblastosis is evidence of selective hemocatheresis of some duration. The splenomegally and pronounced hepatic hemosiderosis, common results of such chronic hemoclasis, further support this interpretation.

However, findings of patent plasma hemoglobin, plasma debris, and corpuscular ghosts are indicative of an acute hemolytic crisis. The degree of hemoglobinemia, often approaching the corpuscular hemoglobin, far exceeds that found in cases of distress mediated lacticemia. It would seem that some unusual rapid hemolysis occurs during terminal distress in addition to the long term preexisting chronic hemolysis. The observation of the rapid darkening of the gills of fishes upon laboratory exposure to *G. breve* (Doig, pers. comm.) and of the ears and feet of injected mice (Trieff, pers. comm.) may be a manifestation of similar acute hemoglobinemia in these animals. The observed cyanemia could have resulted from the hemoglobinemia except that it was nonreversible in air.

The markedly elevated hematocrit (PCV) is indicative of dehydration as is the increased TBC. Hyperviscosity would also be expected under these conditions, although the observed level greatly exceeds that seen in previous cases of dehydration. The indicated level of hemoconcentration is much greater than that resulting from periods of exercise (31). Teleosts can be quite susceptible to dehydration due to their dependence on active transport mechanisms in gills to maintain their hypotonicity relative to sea water. Thus, agents that disrupt this or associated physiological mechanisms can cause rapid dehydration. Our hypothesis of dehydration of teleosts by red tide is further supported by the fact that elasmobranchs, normally nearly isotonic to sea water, showed little or no increase in hematocrit, TBC, or viscosity. Although there were no direct indications of the duration of the hemoconcentration, experience suggests that such a severely increased viscosity (whether or not due to dehydration) was sufficiently pronounced to have been rapidly lethal by spontaneous capillary hemostasis. The hepatic vasoconstriction due to endothelial hemosiderosis would be expected to aggravate this mechanism.

The markedly increased incipient WBCT and similarly increased or infinite total WBCT indicate severe disruption of the normal clotting mechanisms. The frequent findings of polymerized short fibrin fibers (soft clot) suspended in the serum suggest some diffuse intravascular coagulation may be active. These fibers did not anastomose nor contract upon standing and, in most cases were not adhesive to erythrocytes. This presence of suspended fibrin fibers may act synergistically with the increased PCV to produce the observed hemal hyperviscosity. The thrombocytopenia is further evidence of clotting abnormalities. The few thrombocytes present did seem to show normal coagulative activity, however.

These proposed intoxicative mechanisms plus other observations including pronounced cyanemia invite the question of what is the actual cause of death in fishes that have hemopathic response to red tide. At least four of the conditions seem to be potentially lethal: dehydration, hemal hyperviscosity, cyanemia, and hemocatheresis. Some clue may be provided by the anoxic premortal behavior of affected fishes. It would be expected that replacement of erythrocytes by achromic normoblasts would greatly decrease the oxygen transport capacity of the blood. The irreversible cyanemia is further evidence of loss of availability of hemoglobin for oxygen transport. Any decrease in blood flow due to hyperviscosity would further aggravate this situation. It may thus be that fish susceptible to red tide hemopathy succumb by histologic anoxia even though sufficient oxygen may be present in the surrounding water. Such a mechanism also suggests a synergism with depressed sea water oxygen concentrations. Several unusual fish kills associated with red tides, such as those reported on offshore reefs by Smith (32) may have been accentuated or caused by such a synergism.

This work is still continuing with particular emphasis on clarifying some of the processes and mechanisms hypothesized in the foregoing section and to determine the significance of other conditions such as leucocytopenia and cyanemia that remain unexplained. Further studies will be made through laboratory exposures of selected marine animals to controlled, quantitated *G. breve* cultures.

DIAGNOSTIC APPLICATIONS

One of the important and most difficult activities of marine environmental biologists is the determination of cause in fish kills and other mortalities, epizootics, and epiphytotics. The finding of an identifiable, unique sign complex in at least some species of fish commonly killed in *G. breve* red tides provides a diagnostic tool of considerable value.

Heretofore, diagnosis of red tide has only been possible if a properly collected, carefully transported, fresh, water sample was available for analysis. Now, at least with some species of fish, such a diagnosis can be made without water samples. Fortunately, the species that show the strongest hemopathic signs are also those that die earliest and most commonly in *G. breve* red tides.

Already, diagnosis of red tide has been made by examination of fish in several cases and later supported by water collections: in two mullet kills thought to have resulted from culls and losses from nets of fishermen; in an offshore kill of grunts in which the actual red tide was concealed below the thermocline; and in a shark kill in which low oxygen had been suspected. In a reverse situation where cusk eels were thought to have been killed by *G. breve* as confirmed by water samples, necroptic analysis indicated anoxia to be the primary cause of death. In one surprising case, freshwater goldfish showed a particularly well developed red tide sign complex. It appears from this and other evidence that the *G. breve* toxins must have been transported as aerosols from the nearby red tide affected bay to the pools containing the hypersensitive fish. In another case, an extensive, massive duck kill occurred in a red tide area. Analyses showed mild red tide hemopathy in the ducks. Other data, however, did not indicate the red tide to be causative of the bird mortality and the hemopathic syndrome was judged nonlethal.

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PURIFICATION OF TWO *GONYAULAX TAMARENSIS* TOXINS FROM CLAMS (*MYA ARENARIA*) AND THE IDENTIFICATION OF SAXITOXIN

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ABSTRACT

Two toxins have been purified from clams (*Mya arenaria*) which had become highly toxic during the *Gonyaulax tamarensis* red tide which affected the central New England coast in September 1972. The toxins were extracted from the clams with dilute acidic ethanol and isolated by chromatography on weak acid cation exchange resin columns and on gel filtration resins. A minor toxin, purified to a potency of 2,800 mouse units per mg is shown to be identical to saxitoxin in its thin-layer chromatographic behavior, its color reactions with various chromatographic spray reagents, and its effects on mice. A major toxin, purified to a potency of 2,310 mouse units per mg behaved differently from saxitoxin and the minor toxin on weak cation exchange resin columns, thin-layer chromatography, and in its color reactions with various spray reagents.

INTRODUCTION

Outbreaks of paralytic shellfish poisoning (PSP) occur sporadically along the coasts of countries bordering on the North Atlantic and Pacific Oceans. On the Pacific Coast of North America, outbreaks of PSP have been associated with blooms of the toxic dinoflagellate *Gonyaulax catenella*. Saxitoxin (STX), a powerful neurotoxin, has been isolated from axenic cultures of *G. catenella* (8) and from extracts of California sea mussels *Mytilus californianus* and Alaska butterclams *Saxidomus giganteus* (9). STX has been purified to a specific activity of 5,500 ± 500 mouse units per mg and its chemical structure determined (10). On the Atlantic Coast of North America, outbreaks of PSP have been associated with blooms of *Gonyaulax tamarensis* (5). Attempts to isolate the poison from cultures of *G. tamarensis* failed since the bulk of the toxin was not held by weak acid cation exchange resins used in the isolation of STX (7). At least two toxic compounds appear to be present in extracts of shellfish exposed to blooms of *G. tamarensis* (2). From extracts of mussels *Mytilus edulis* two toxin fractions were obtained. A minor toxic fraction, purified to a specific activity of 1,550 MU/mg, was shown to resemble STX in its biological effects and behavior on weak acid cation exchange resin. The majority of the toxicity was only weakly bound to the resin and could not be separated from inorganic salts which were present in large amounts. Repeated chromatography on Sephadex improved the toxicity to 270 MU/mg. Recently only one toxin was reported to be present in ten year old extracts of scallop hepatopancreas which had become toxic during a *G. tamarensis* bloom (3). The toxin was purified to a specific activity of 5,150 MU/mg, and on the basis of its biological activity and thin layer chromatographic behavior, the toxin was identified as STX.

We wish to report here on the purification of two toxic components from clams (*Mya arenaria*) which became toxic during the *G. tamarensis* bloom that occurred in New England in September, 1972.

MATERIALS AND METHODS

Source of Toxin

The clams (*Mya arenaria*) used in this study were collected by the State of New Hampshire Fish and Game Department and the Parker River National Wildlife Refuge, Plum Island, Massachusetts, during the height of the *G. tamarensis* red tide that occurred along the central New England coast in September, 1972. The clams had scores of 2,000-4,000 ug of poison (as STX) per 100 g of meat. The clams were stored whole in the frozen state and thawed out just prior to processing.

Toxin Bioassay

The toxicity of aqueous solutions was determined by the IP injection of one ml of solution into mice weighing approximately 20 g. The death time in minutes was converted to Mouse Units (MU) using the tables of Sommer (see ref. 4), where one MU is defined as the amount of toxin required to kill a 20 g mouse in 15 minutes. Toxicity is expressed as MU/mg of the dry material.

Extraction and Initial Purification of the Toxins

The extraction and preliminary steps in the purification of the toxins were carried out using the procedure described by Schantz, *et al.* (9), with minor modifications. Whole clams (23 kg) were thawed and shucked to yield 11 kg of meat and juice. The meat and juice were ground together in a blender, mixed with Celite, and the mixture extracted with 15% ethanol (pH 2-3). The extract was concentrated *in vacuo* on a rotary evaporator and the concentrate heated to 90°C, cooled rapidly, and centrifuged at 23,000 x g for 20 minutes. This additional heating step resulted in the removal of a large amount of inactive insoluble material with no loss of activity. The supernate was adjusted to pH 5.5 with NaOH and again centrifuged. The supernate (11.2 l), containing 786 g of solids and with an activity of 42 MU/ml, was applied to an Amberlite IRC-50 (Mallinckrodt Chemical Works, St. Louis, Missouri) column (4 x 100 cm) in the sodium form (9). The supernate was applied until the activity in the effluent rose to 20 MU/ml. At this point the addition of supernate was stopped, and the column was rinsed with distilled water until no appreciable activity remained in the effluent. The column was then eluted with 1 M acetic acid buffered to pH 4 with a saturated sodium acetate solution. This removed the *major toxic fraction* from the column. Elution with the pH 4 buffer was continued until no more activity appeared in the effluent. The column was again rinsed with water and eluted with 0.5 M acetic acid. This removed the *minor toxic fraction* from the column. The sodium form of the resin was then regenerated and the remainder of the supernate applied and the major and minor toxic fractions isolated by repeating the process.

Purification of the Minor Toxin

The minor toxic fraction was purified by two passages through an Amberlite CG-50 (200-400 mesh) column (2 x 43 cm) in the hydrogen form according to the procedure of Schantz *et al.* (9) for STX to yield the *minor toxin*.

Purification of the Major Toxin

The major toxic fraction was divided into four equal batches (1.4 l each) and each batch processed in the following manner. The fraction was concentrated on a rotary evaporator to about 200 ml, the pH brought down to 5 with conc. hydrochloric acid (about 25 ml), and the fraction taken completely to dryness. The residue was dried further for 48 hours in a vacuum desiccator containing calcium chloride lumps, sodium hydroxide pellets, and conc. sulfuric acid in separate dishes. A pale yellow crystalline solid was obtained which was extracted with 100 ml of cold ethanol. The mixture was centrifuged at 12,000 x g for 20 minutes. The pellet remaining after pouring off the supernate was re-extracted with 50 ml of cold ethanol and the mixture again centrifuged. The supernates were combined, taken to dryness *in vacuo*, and the residue desiccated for 24 hours. The residue was extracted with 30 ml of cold ethanol and the mixture centrifuged. The supernate was concentrated *in vacuo* to dryness and the residue taken up in 10 ml of water to give the *ethanol soluble major toxic fraction* (7-8 g of solids containing 20,000-25,000 total MU).

The ethanol-soluble major toxic fraction was placed on a Bio-Gel column prepared by pouring 400 g of Bio-Gel P-2 (200-400 mesh) (Bio-Rad Laboratories,Richmond, California) as a slurry into a 4 x 104 cm column and washing with 6l of deionized distilled water. The column was eluted with deionized water at a flow rate of 1.4 ml per minute and fractions having a toxicity of over 50 MU/ml were pooled and concentrated to give the *major toxin*. Variable amounts of toxicity appeared to bind to the column. This could be eluted with 0.1 M acetic acid.

Thin-Layer Chromatography (TLC)

TLC was run on precoated silica gel G plates (EM Reagent), activated at 110° C for 10 minutes, in the following solvent systems: (A) *n*-butanol:acetic acid:water (50:25:25); (B) *tert*-butanol:acetic acid:water (50:25:25); (C) ethanol:pyridine:water:acetic acid (60:40:20:10); (D) ethanol:water:acetic acid (100:40:25). All solutions for TLC were made up to a toxicity of about 11,000 MU/ml in water. The STX standard solution (2 mg/ml) was prepared from a sample of pure saxitoxin kindly supplied by Dr. E.J. Schantz. The major toxin used for TLC had an activity of 2,100 MU/mg or greater, and the minor toxin used had 1,383 MU/mg. Five ul of each toxin solution were applied to the plates. Spots were detected by spraying the plates with conc. sulfuric acid and heating them at 130°C for 4-8 minutes.

In order to locate toxicity on TLC plates, three 5 ul spots of each toxin were applied to a plate and the plate run in solvent B until the front had traveled 10 cm from the origin. The entire chromatogram from one of the spots was divided into ten 1 cm bands, and each band was scraped off the plate and extracted with 2 ml of water acidified to pH 4. The extracts were centrifuged and 1:2 dilutions of the supernates injected into mice. The chromatogram from one of the companion spots was sprayed with sulfuric acid and that from the third spot sprayed with Weber Reagent.

RESULTS

A summary of the weights and activities of the fractions at different stages of the isolation procedure is shown in Table I. From 23 kg of clams, 11 kg of meat and shell liquor were obtained after shucking. These yielded a total of 44 l of acid alcoholic extract which had a total acitivity of 470,000 MU. Because of the limited size of the Amberlite IRC-50 column, the concentrated supernate from the extract had to be applied in two runs. The greater part of the activity was

Table I

Summary of the Isolation of the Toxins from Toxic Clams

Fraction	Weight	Total Activity (MU)	Specific Activity (MU/mg)
Whole clams	23 kg		
Meat & juice	11 kg		
Supernate from second centrifugation	786 g	470,000	0.6
Major toxic fraction	282 g	150,000	0.44
Ethanol-soluble major toxic fraction	26 g	86,000	3.3
Major toxin	34 mg	64,000	1,400-2,310
Minor toxic fraction	9 g	106,000	11.5
Minor toxin after one run on Amberlite CG-50	736 mg	140,000	192
Minor toxin after 2nd run on Amberlite CG-50	65 mg	92,000	1,250-2,800

eluted from the Amberlite IRC-50 column with pH 4 acetate buffer and is therefore referred to as the *major toxic fraction*. Acetic acid (0.5 M) eluted a second toxic fraction, which is referred to as the *minor toxic fraction*.

The *major toxic fraction* (0.44 MU/mg) was purified to a level of 3.3 MU/mg by a series of ethanol extractions which removed the greater portion of the salts. Passage of the *ethanol-soluble major toxic fraction* through a Bio-Gel column yielded the *major toxin* with an activity of 1,400-2,310 MU/mg. It was observed that when purified major toxin was passed through a second Biogel column, most of the activity remained on the column. This could be eluted from the column with 0.1 M acetic acid, but the resulting toxin did not show any increase in potency. The removal of salts from the toxin appears to affect its behavior on Bio-Gel columns. That the separation is not strictly a gel filtration process is indicated by the fact that the salts were eluted from the column before the toxin. The binding of certain organic groups to polyacrylamide gels has been previously reported (1, 6). The binding appears to be affected by the ionic strength of the medium.

The *minor toxic fraction* (11.5 MU/mg) was purified by two passages through an Amberlite CG-50 column to give the minor toxin with an activity of 1,250-2,800 MU/mg.

TLC of STX and the *G. tamarensis* major and minor toxins in four different solvent systems is shown in Fig. 1. The minor toxin and STX showed identical

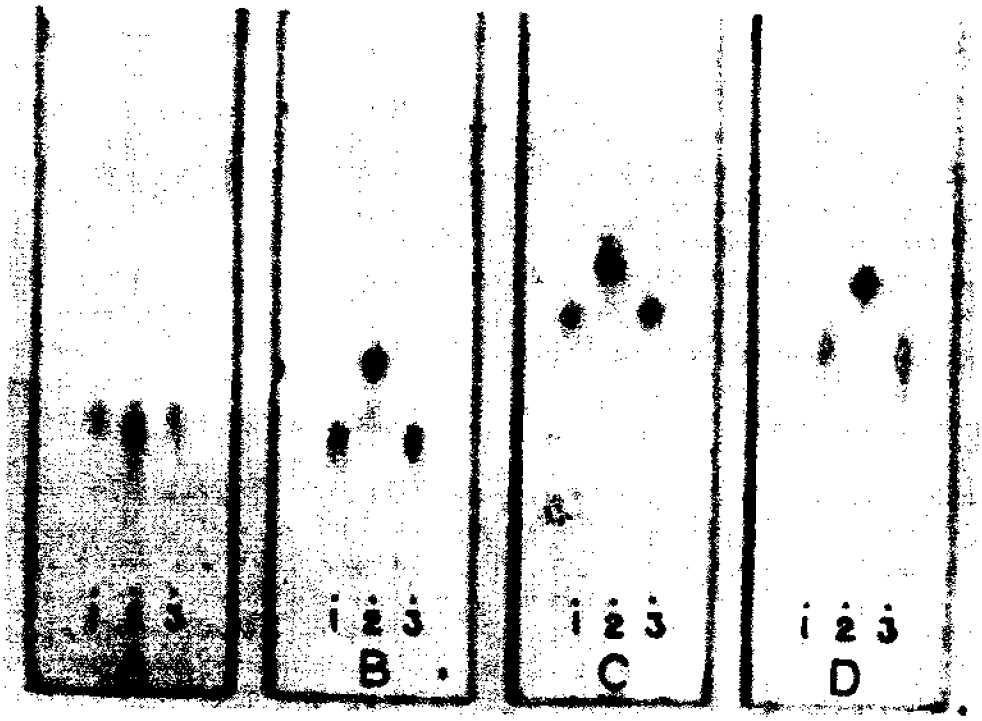

Figure 1 Thin-layer chromatography of STX and the *G. tamarensis* toxins on silica gel plates. A, B, C, D indicate solvent systems (see text). 1, 2, 3 indicate STX, major toxin, and minor toxin, respectively. Plates were sprayed with sulfuric acid and charred.

Rf values and gave the same blue-black spot after spraying with sulfuric acid and heating. Under the same conditions the major toxin appeared as an intense green spot. In solvent systems A, B, and C, the major toxin showed a much less intense yellow spot just below the green spot with the sulfuric acid spray.

The color reactions given by STX and the major and minor toxins after TLC in solvent system A and spraying with different reagents are listed in Table 2. In each case the colored spot corresponded to the main sulfuric acid positive spot and in each case the minor toxin and STX gave the same color.

When both the major and minor toxins were chromatographed in solvent B and the chromatograms scraped off in sections and each section tested in the mouse bioassay, it was found that in both cases the activity coincided with the sulfuric acid-positive and Weber-positive spot.

DISCUSSION

The behavior of the *G. tamarensis* toxins on sodium Amberlite columns and TLC clearly demonstrate that at least two chemically distinct toxins are present in frozen stored *Mya arenaria*. The minor toxin was purified to a potency of up to 2,800 MU/mg using the procedure of Schantz, *et al.*, (9) for the isolation of STX. Although the potency of our minor toxin was less than that of pure STX, its TLC properties and its color reactions with various spray reagents corresponded exactly with those of STX. In addition, its symptoms on mice

Table 2

Color Reactions of STX and the *G. Tamarensis* Major and Minor Toxins with Different TLC Spray Reagents.*

Spray	STX	Major toxin	Minor toxin
Jaffe (picric acid)	Orange	Neg.	Orange
Benedict-Behre (3, 5-dinitrobenzoic acid)	Purple	Neg.	Purple
Weber (ferricyanide-nitroprusside)	Pink	Pink	Pink
Sakaguchi	Neg.	Light yellow	Neg
Diacetyl- α-naphthol	Blue	Neg.	Blue
Ninhydrin	Yellow	Neg.	Yellow

* Chromatograms were run in solvent A on precoated silica gel glass plates

were those of STX, namely, nervousness, ataxia, convulsions, respiratory distress, and paralysis. It therefore appears that the *G. tamarensis* minor toxin is identical with saxitoxin. These results confirm the recent findings of Ghazarossian, *et al*. (3), who identified STX as the toxin present in ten year old extracts of scallops which had been exposed to *G. tamarensis*.

However, as previously reported by Schantz, *et al*., (7) and Evans, (2), the majority of the toxicity present in our extracts was also unbound or weakly bound to sodium Amberlite columns. After purification to a potency of 2,310 MU/mg the major toxin was shown to differ significantly from STX or the minor toxin in its TLC behavior and its color reactions with various spray reagents. Its effects on mice, however, were similar to those of STX and the minor toxin. Ghazarossian, *et al*., (3) have speculated that STX may result from the breakdown of a less basic molecule, perhaps an amine oxide. Whether the *G. tamarensis* major toxin is such a compound remains to be seen.

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COMPARATIVE STUDIES ON TOXINS OF MICROORGANISMS

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ABSTRACT

Standard resting potential, action potential and muscle mechanical activity measurements were made on preparations challenged with active materials from several toxic microorganisms. Associated preparative work included either mass cultivation or field collection of cells, harvesting, fractionation and purification, and the bioassay of active extracts. Thus far, we have tested the toxic effects of material from 3 marine dinoflagellates (*Gymnodinium breve*, *Amphidinium carterae* and *Gonyaulax tamarensis*) and one freshwater blue-green alga (*Aphanizomenon flos-aquae*). The latter was included because of its chemical and physiological similarities when compared to saxitoxin from *Gonyaulax catenella*.

The objective is to determine the site and mode of action of the marine biotoxins at the cell and tissue level. Three general types of effects were recorded: 1) The destruction of excitability by membrane depolarization (crude *G. breve* toxin). 2) The destruction of excitability with no depolarization (characteristic of toxins from *G. tamarensis* and *A. flos-aquae* that may involve blockage of specific ion conductance pathways, e.g. saxitoxin and tetrodotoxin). 3) The alteration of transmission between excitable cells (*A. carterae* and *G. breve* fractions display motor end plate effects and alter mechanical activity in cholinergic systems).

INTRODUCTION

Toxic materials of biological origin attract attention from researchers because of their specificity, potency, and potential utility as physiological or pharmacological "tools". Since many microorganisms elaborate one or sometimes several toxins, a comparative approach utilizing appropriate extraction and testing procedures should point out meaningful similarities and differences in the effect of the bioactive materials. It may be important also to study the harmful effects on aquatic macroorganisms such as shellfish and crustaceans that may experience potent toxic concentrations during bloom conditions. Some shellfish species are apparently not affected by several dinoflagellate toxins. This may suggest that mechanisms for maintaining electrogenic membrane systems differ in bivalves and vertebrates. Preliminary investigations have revealed the involvement of neuro-muscular systems with the general site of action of a wide variety of aquatic microorganisms toxins. It is appropriate that their effects be studied at the membrane level on excitable tissue where a more specific mode of action can be determined. Such an approach has public health implications as well as providing potentially useful compounds with which to challenge neuro-muscular preparations in the course of studying their normal physiology. General reviews (4, 5, 9, 17, 20) reveal the paucity of information on the chemical and physiological characteristics of compounds elaborated by marine organisms. Only tetrodotoxin, saxitoxin and ciguatera - poisons have been studied in great detail. Investigations currently

underway at the University of New Hampshire have resulted in the purification of several toxins and the determination of their physiological effects. These studies have included the marine dinoflagellates *Gymnodinium breve*, *Amphidinium carterae*, *Gonyaulax tamarensis*, and the freshwater blue-green alga *Aphanizomenon flos-aquae*.

ACCUMULATION OF TOXIC MATERIALS

Although it is important to prove the existance of a particular biotoxin in nature, laboratory mass cultivation may provide much of the material for physiological testing. We have successfully cultured several thousand liters of *Gymnodinium breve* and *Amphidinium carterae*, two marine dinoflagellate species. Both were grown under completely defined conditions (temperature 24°C; 14 hour daily light period; 500 foot-candles). The medium employed was the NH-15 described by Gates and Wilson (7, 25). Axenic cultures of both species contained potent activity when tested on whole animals and isolated neuro-muscular preparations. Unialgal, but not bacteria free, cultures were conveniently raised in either 2 liter quantities (*G. breve*) or 10-12 liter quantities (*A. carterae*). Bacteriarized, but unialgal, cultivation favored more rapid growth and therefore greater toxin yield. Growth curves were constructed from cell counts made with a Model F Coulter Counter and provided a regular check on the efficiency of our mass culture techniques (16, 23). New batches were inoculated with high cell numbers to minimize an initial lag phase of slow growth. Extraction of the active materials was done during or shortly after the phase of maximum growth. *G. breve* cultures reached peak cell density in 14-16 days with counts of 19×10^6 cells/liter and had a doubling time of approximately 2.5 days. *A. carterae* reached peak cell density in 8-10 days with counts of 100×10^6 cells/liter.

For chemical and physiological comparisons, harvesting from natural blooms can be important and must be done to establish the potency of biotoxins in nature. During the 1971 *G. breve* red tide bloom in Tampa Bay, Florida, approximately 100 liters of sea water were collected and processed. These samples showed the presence of the same toxic material with the same physiological properties as we had previously found in laboratory cultures of this species (3, 16). The toxic blue-green alga *Aphanizomenon flos-aquae* has been harvested from several New Hampshire lakes during bloom conditions (4.0×10^8 cells/liter) that caused water coloration and musty odor. We used DeLaval separators at lakeside to spin the cells from hundreds of liters of water/day. Cell residues were frozen until processed in the laboratory (2, 19, 22). *Gonyaulax tamarensis* toxins were accumulated from harvested shellfish (*Mya arenaria* and *Mytilus edulis*) that were collected during the 1972 and 1974 red tides at Hampton, New Hampshire (18).

EXTRACTION, PURIFICATION AND BIOASSAY

Gymnodinium breve

The removal of *G. breve* toxin from cultures or natural bloom samples was done by acidifying the medium with HCl (pH 5.5) and extracting with ether before evaporation to dryness. An average dry weight of 5.4 ± 0.4 mg was obtained per 10^6 cells. The potency of the dried residue was 2-7 MU/mg. Purification of the toxin was effected by extraction first with ethanol, followed by chloroform. Chromatography of the ethanol and chloroform soluble fraction on a silicic acid column resulted in the isolation of a toxic material that showed a single spot on TLC and had a potency of 67 MU/mg (16).

Amphidinium carterae and Gonyaulax tamarensis

Extraction, purification and bioassay data on these two toxic species are presented elsewhere in these proceedings. For information on *A. carterae* see (21). Buckley *et al.* (1974 conference proceedings) have recently verified the presence of saxitoxin as one toxic component from *G. tamarensis* as presented by Ghazarossian *et al.* (8).

Aphanizomenon flos-aquae

The thick algal suspensions obtained from centrifugation were acidified (pH 3.0), heated to 80-90°C, chilled and then centrifuged at 10,000 X g for 15 minutes to remove solids. This procedure was repeated 3 times to ensure maximum yield. The combined supernatants were neutralized, dried and then extracted 3 times with 95% ethanol and dried again. The resulting residue was dissolved in water, acidified to pH 3 with HCl and extracted with chloroform and the aqueous layer dried again. The residue was purified by high voltage electrophoresis, ion-exchange column chromatography and thin layer chromatography. The toxicity was increased from 2.5 to 745 MU/mg during the purification procedure. The material obtained is much less potent than saxitoxin, which has 5500 MU/mg (2).

PHYSIOLOGICAL TESTING

Striated, smooth and cardiac muscle contractions or tension development were recorded using Grass FT-03C transducers or RCA 5734 electro-mechanical transducers with signals displayed on a Model 5 Grass Polygraph or Beckman RP Dynograph. Extracellular action potentials were recorded with Ag-AgCl electrodes. Intracellular measurements employed 3 M KCl filled glass microcapillaries with a tip resistance between 10-20 MΩ and a low tip potential. Action potentials from spontaneously active tissues (hearts) were recorded with a "hanging microelectrode" (26). All resting and activity

potentials were displayed on a Tektronix 502A oscilloscope preceded by a Keithley Model 605 electrometer. Appropriate Ringer solutions acted as a vehicle for the various toxins used in these studies. Control solutions were prepared by extracting uninoculated culture medium and suspending the residue in appropriate saline solutions.

COMPARATIVE PHYSIOLOGICAL EFFECTS

When excitable systems are challenged with extracts or purified components from toxic microorganisms, the acute sensitivity of neuromuscular systems and the potency of the active materials is evident. Table I lists the range of effects of several biotoxins and generally summarizes their mode of action and site of action in several physiological preparations. Three general types of responses were recorded using standard nerve-muscle preparations.

1) The first involves destruction of excitability by membrane depolarization. This is characteristic of rather crude, unpurified extracts from the dinoflagellates *Gymnodinium breve* and *G. veneficum*. The effects of skeletal muscle using the former species was irreversible and was accompanied by violent muscle fibrillations prior to blockage of electrical and mechanical activity (15, 16). The latter species, however, showed effects that were reversible and not accompanied by enhanced spontaneous activity prior to blockage (1). End plate activity also differed between the two depolarizing toxins. The apparent difference between the activity of the *Gymnodinium* toxins may be explained by the discovery of several toxic components in *G. breve* that mask the effects of the depolarizing fraction. Erythrocyte hemolysis (22), ciguatera-like activity (12), and anticholinesterase-like effects (16) have been described for purified fractions from this red tide causing species. In nature, it appears that the toxins from *G. breve* may produce massive fish mortalities (fast acting neurotoxin) and also toxicity in resident shellfish (slow acting ciguatera-like toxin). This species, then, defies the general rule that dinoflagellates producing one type of toxicity were different than those causing the other. Dinoflagellates from the genus *Gonyaulax* appear to support this generality in that they are only implicated with paralytic shellfish poisoning (PSP).

2) The second general type of response involves the destruction of the conduction properties of excitable cells with no transmembrane depolarization. This is characteristic of the active materials from *G. breve* (Fraction IVa), *Aphanizomenon flos-aquae*, *Gonyaulax catenella* (STX) and the minor toxin from *Gonyaulax tamarensis* (STX). The latter two species evidently produce identical toxins that specifically block Na^+ conductance pathways in membrane systems (8). *Aphanizomenon flos-aquae* causes the same kind of effects as the *Gonyaulax* toxins. However, it is chemically different and may block activity in Ca^{++} dependent membrane systems in addition (2, 19).

Table I — Physiological Effects On Neuromuscular Systems

Species	Dosage Range	Electrical Potentials: Resting	Electrical Potentials: Activity	Muscle Mechanical Activity
Gymnodinium breve	crude 100-200 µg/ml Fraction IVa 0.5-10 µg/ml	Depolarization with crude No Effect	AP red. to block; initial 10 fold incr. in EPP freq. & amp., then red. to total block; Heart AP freq. red., blocked by Atropine.	*Striated*: Initial fibrill. during incr. EPP activity; tension loss via indir. (nerve)-then dir. stim. *Smooth*: Incr. freq. & tension; ACh-like effect. *Cardiac*: Vert. - slow to diastolic arrest, blocked by Atropine: Crustacean - incr. freq. to systolic arrest, blocked by 2-PAM: Mollusc - no effect at incr. dose levels (100X).
Gymnodinium veneficum	crude neutral extract	Depolarization	AP blocked in desheathed & non myel. nerves & striated muscle. EPP decr. & blocked.	*Striated*: Decr. tension level to dir. & indir. stim., follow same time course, no fibrill. *Smooth*: Decr. freq., slow relax. *Cardiac*: Vert. & Mollusc - systolic arrest, reversible. Crustacean - rate slowed.
Gonyaulax catenella *G. tamarensis* (minor toxin)	.01-3 µg/ml	No Effect	AP blocked in desheathed nerves and striated muscle, reversible.	*Striated*: Decr. mech. response to indir. and dir. stim., simultaneous block., no fibrill. *Cardiac*: Vert. - rate slows, EKG altered. Crustacean - rate slows to diastolic arrest. Mollusc - No effect at incr. doses.
Aphanizomenon flos-aquae	5-100 µg/ml	No Effect	AP blocked in desheathed nerves & striated muscle; Heart AP-reduced amp.	*Striated*: Mech. activity blocked to ind. then dir. stim., reversible. *Cardiac*: Vert. & Crustacean - rate slowed to blockage, reversible. Mollusc - no effect at incr. doses.
Amphidinium carterae	crude 1-10 mg/ml Acrylyl Ch 5-20 µg/ml Ch O-SO_4 1mg/ml		Incr. motor EP activity	*Smooth*: Incr. freq. & amp., similar to 3×10^{-7} g/ml ACh. *Cardiac*: Vert. & Mollusc. - decr. freq. to diastolic arrest. Effects blocked by Atropine & Mytolon, respectively.

3) The third general type of response involves the transmission properties between excitable cells. *G. breve* (Fraction IVa) and *Amphidinium carterae* directly affect end plate phenomena in cholinergic systems. For testing we have employed the neurogenic and myogenic hearts of vertebrate and invertebrate animals. Fraction IVa from *G. breve* slows the frog heart to subsequent diastolic arrest. This effect is blocked by Atropine. The neurogenic crustacean heart responds by an increase in frequency, irregular tension development and systolic arrest. This effect is reduced or blocked by pyridine -2 aldoxime methiodide (2-PAM) - a substance that reverses the effects of organophosphate insecticides and nerve gases that have anticholinesterase activity (22). *Amphidinium carterae* is the source of two choline compounds (acrylylcholine and choline O-SO_4). Their physiological effects are discussed by M. Ikawa elsewhere in these proceedings (21).

Table II provides more general information on the species under consideration and source material from the literature.

CONCLUSIONS

As one might expect, the information on aquatic microorganisms' toxins compiled to date has provided some answers but also stimulates more questions. Biotoxins may be useful in determining the similarities and differences in the normal functioning of electrogenic and neuro-transmitter systems in a wide range of preparations. However, before toxins can be used as biological tools their specific site and mode of action must be determined. The increased frequency of microorganisms blooms, the establishment of nutritional requirements and productive culture techniques and the increasing interest in marine biology should accelerate the work accomplished in this area.

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Table II

Characteristics of Seclected Toxic Microorganisms

Species	Location	Chemical Characteristics	Other Effects	Biological Characteristics	References
Gymnodinium breve	Gulf of Mexico	Ether + $CHCl_3$ sol; low mol. wt. 279-694.	RBC hemolysis with crude; Red. in human serum Ch-ase activity; no RBC hemolysis with Fraction IVa.	Depol. membranes-crude; anti ACh ase-like; Ciguatera-like	3, 12, 13, 14, 15, 16, 24.
Gymnodinium veneficum	British Waters	H_2O + alc. sol; ether and $CHCl_3$ insol; non-dial., high mol. wt., >1000.	Frog skin: Tox. inside-RP decr. & reverses polarity; Tox. outside-slow RP decrease. Muscle resp. rates incr.	Depol. membranes; perm. incr. to Na^+	1, 17.
Gonyaulax catenella	Cent.-N Pac. Coast U. S. & Can.	H_2O sol; mol. wt. 372 Tetrahydropurine Saxitoxin (STX)	No effect on Ca^{++} dep. membrane sys; Similar to TTX.	Blocks mem. Na^+ conductance. PSP.	6,9, 10,11.
Gonyaulax tamarensis	North Atlantic	At least 2 toxins; 1 identical to STX.	Same as STX	Same as STX	8,18
Aphanizomenon flos-aquae	temperate lakes of U. S. & Can.	Similar but not ident. to STX; H_2O sol.	Unlike STX, Ca^{++} dep. sys. affected; Crustacean DEAM & DEAL	Similar to STX	2, 19, 22
Amphidinium carterae	Atlantic temp. H_2O	At least 2 choline cmpds: acrylyl Ch and Ch O-SO_4	Affects cholinergic systems	ACh-like activity	21, 22, 23.

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SESSION SUMMARY

MANAGEMENT

Session Chairman

John C. Collins

Director - Division of Environmental Heal
Boston, Massachusetts

Co-Chairman

Frank Grice

Division of Marine Fisheries
Boston, Massachusetts

On November 6, 1974 a special PSP management committee met following the PSP management session. The purpose of the workshop was to review the information presented during the session and discuss management problems associated with toxic dinoflagellate blooms and to develop recommendations towards their more effective control. The committee limited their discussion to public health management problems. The following recommendations are submitted in summary of the activities and papers of this session.

A. Communication Between Researchers and Administrators:

Research efforts directed at various aspects of toxic dinoflagellate blooms are generally fragmented and short term. Historically, this problem has commanded a low priority on the research dollar. In order to more effectively study the toxic dinoflagellate bloom phenomenon, the Committee recommends that there be improved communications between the research community and the program administrator. This could be achieved through development of research coordination committees to provide a forum where researchers and administrators could discuss concerns and develop mutually agreeable priorities. Such coordination could perhaps be more easily initiated at government level.

B. Public Education:

Historically, the major public health problem associated with toxic dinoflagellate blooms has been the consumption of toxic shellfish by individuals who harvest for their own use. Management programs, on the other hand, are normally directed towards controlling commercial harvesting and distribution of toxic clams. The committee recommends that concerted efforts be directed towards the development of public educational materials aimed at increasing the public's general understanding of the health dangers that can be associated with toxic dinoflagellate blooms. Such educational materials should clearly differentiate algal blooms which present potential health problems from those that do not, and differentiate foods that can be potentially contaminated from those that are not. The Committee felt that the media's use of general terms such as "red tides" and "shellfish" or sea food lacks the necessary specificity to enable the consumer to properly determine what type of marine foods may or may not be implicated in PSP outbreaks.

C. PSP Control Versus Market Standards:

Management programs are currently using the U.S. National Shellfish Sanitation Program's PSP guideline of 80 µg/100g shellfish meats as both a control standard to close shellfish growing and harvesting areas, and as a market standard to embargo shellfish on the wholesale and retail markets. The committee agrees that there is sound epidemiological and administrative considerations which makes the 80 µg/100g meats a valid control standard. However, less is known about the 80 µg/100g guideline in its application as a market standard. The Committee recommends that research efforts be directed towards developing toxicological and epidemiological studies necessary to determine the validity of the use of this guideline as a market standard.

D. Other Environmental Indicators:

Toxic dinoflagellate blooms can have a wide and varying effect on the marine and coastal biota. The biota affected can include both primary algae feeders, such as clams, as well as higher predators, such as ducks. The Committee recommends that research efforts be directed at defining general bio-environmental indicators that can be used to help identify the development and presence of toxic dinoflagellate blooms. Such indicators are needed to supplement present monitoring programs relying exclusively on shellfish sampling. Once such indicators are developed, efforts are needed to disseminate this information to governmental, research, and academic personnel working along the coastlines.

THE FIRST "RED TIDE" IN RECORDED MASSACHUSETTS HISTORY

Managing an Acute and Unexpected Public Health Emergency

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ABSTRACT

On September 14, 1972 a toxic dinoflagellate bloom was identified in the northern coastal waters off Massachusetts. The sudden, almost accidental discovery of the first "red tide" in recorded Massachusetts history precipitated a public health emergency in the state. Faced with a potentially lethal problem, and with frustratingly limited information, the Massachusetts Department of Public Health had to assume the worst — that contaminated product had already reached the markets — and to gauge its response accordingly. An intense consumer education campaign through the mass media was instituted and a sweeping embargo placed on all shellfish products in order to minimize ambiguity. Harvesting area, market, and clinical surveillance programs, developed and instituted within two days of the emergency's onset, began producing data on which to base modifications of the initial assumption. Within a week, the danger had been pinpointed in softshell clams and mussels, and restrictions on other species of shellfish removed. Markets were cleared of the suspect species and then reopened. Ultimately some 2800 acres of Massachusetts shellfish harvesting shoreline were found to be contaminated by the marine dinoflagellate. Twenty-six cases of paralytic shellfish poisoning were confirmed, two classified as severe, and no deaths.

During the latter part of September and early October 1972, the emergency control effort was phased into a routine surveillance program. Prospective control of PSP since the unanticipated and virtually unanticipatable outbreak in 1972 has enabled the state to protect not only the consumer, whose life and health are of paramount concern, but also the seafood industry.

INTRODUCTION

Suddenly, and largely by accident, on September 14, 1972 the first "red tide" in recorded Massachusetts history was identified in waters off the North Shore. The events during the early days of the ensuing public health emergency—particularly when contrasted to control of subsequent "red tides" in Massachusetts waters—provide an object lesson in the problems and principles involved in managing a distinct type of public health threat—one that is sudden, unanticipated, and virtually unanticipatable.

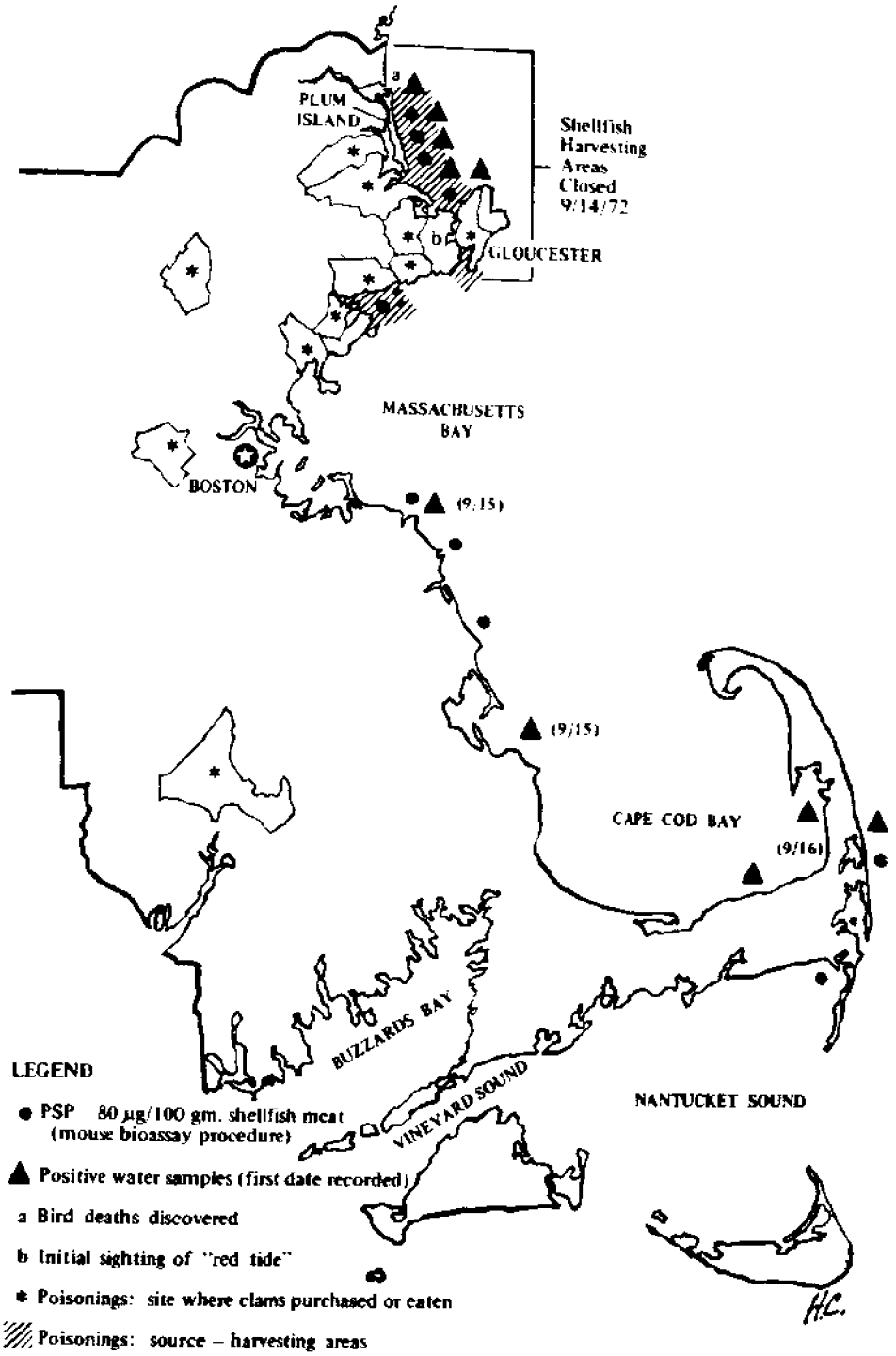
ONSET OF THE EMERGENCY

The first evidence of a toxic dinoflagellate bloom appeared in the morning of September 14, 1972 in two independent reports to the Department of Public Health's Division of Environmental Health. A partol warden attached to the U.S. Government Parker Wildlife Reservation at Plum Island (Figure 1) reported that during the preceding 24 hours, 95 dead birds — seagulls and black ducks — had been retrieved from the surface of the Merrimack River estuary. Initially ascribed to aerial spraying of pesticides, the bird deaths had

Figure 1

GONYAULAX TAMARENSIS AND PARALYTIC SHELLFISH POISONING IN MASSACHUSETTS

SEPTEMBER, 1972



been traced by the time of the report to recent ingestion of clams. The second report — from 12 miles south of Plum Island — came from a marine biologist with the University of Massachusetts marine laboratory in Gloucester (Figure 1) who had observed a reddish-brown mass in the water, had identified a one-celled dinoflagellate and then had distilled extracts from the algae and injected them into two mice, killing the mice within eight minutes. The organism was identified tentatively as *Gonyaulax tamarensis*.

On these grounds, under routine authority of the Department of Public Health*, the clam beds north of Boston Harbor — from Gloucester to the New Hampshire line — were closed to the harvesting of shellfish. The action was taken at 2:00 on the afternoon of the 14th and was relayed to the appropriate enforcement officials in Massachusetts and to shellfish officials in New Hampshire and Maine. A meeting of state and federal health officials, chaired by the Commissioner of Public Health at 10:00 the following morning, assessed the magnitude of the problem and defined resources needed to combat it.

A quick literature search conducted on the evening of the 14th had yielded two review articles (1, 2). Summarized at the outset of Friday morning's meeting, the articles suggested that the danger posed by the "red tide" was potentially very great**and also revealed that *G. tamarensis* had been infesting eastern Canadian waters regularly for the past ten years.

In view of Canada's experience, a telephone call was placed to the Director of Inspections for the Canadian Department of Fisheries in Ottawa. In a 45-minute conference telephone call, he and the man directly responsible for shellfish poisoning control provided essential information on the background, the epidemiology, the biochemisty, and the practical public health management of paralytic shellfish poisoning (PSP). It was agreed during the conversation that the Canadians would arrange to have several copies of their control program manual (3) flown to Boston that afternoon.

DATA UNCERTAIN: WORST CASE ASSUMPTION

With that background information on *G. tamarensis* and paralytic shellfish poisoning, and knowing only: (a) that a problem of unknown magnitude existed on the North Shore and (b) that it was Friday, traditionally the day of

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* hereafter, "the Department".

** Paralytic shellfish poison, acquired by shellfish from feeding on certain species of marine dinoflagellates, is 50 times as potent as the paralytic poison curare. About 30 minutes after eating contaminated shellfish, PSP victims experience gastrointestinal distress, burning sensations, and, in severe cases, ataxia, respiratory paralysis, and cardiovascular collapse. Death can occur within 12 to 24 hours. No antidote to the toxin is known; treatment is largely symptomatic. Cooking of shellfish meat only partially reduces its toxicity. A lethal dose of poison can be contained in as few as three fried clams (3).

FIGURE 2
PARALYTIC SHELLFISH POISONING
IN MASSACHUSETTS — SEPTEMBER 14-17, 1972

TIME LINE SHOWING AVAILABILITY OF INFORMATION FOR DECISION MAKING

SEPTEMBER	REPORTS	DECISIONS	ONSET OF ILLNESS*
A.M. 14 P.M.	"RED TIDE" OBSERVED IDENTIFIED (TENTATIVELY) 95 BIRD DEATHS REPORTED	CLOSE NORTH SHORE CLAM BEDS	6 PSP CASES
A.M. 15 P.M.	7 PSP CASES REPORTED	TOTAL EMBARGO ON ALL HARVESTING & MARKETING OF SHELL FISH MASSIVE CONSUMER EDUCATION	15 PSP CASES
A.M. 16 P.M.	10 PSP CASES REPORTED		
A.M. 17 P.M.	6 PSP CASES REPORTED		2 PSP CASES
A.M. 18 P.M.	8 PSP CASES REPORTED	ORDER DESTRUCTION OF ALL FRESH SOFT-SHELL STOCK IN RETAIL STORES AND RESTAURANTS	

*Cases and other relevant information
**Reporting of cases lagged behind actual onset of illness by one to four days

greatest seafood consumption in the state, an appropriate response had to be framed. It was not known at this time whether toxic shellfish had penetrated the markets; the capacity to make such a determination quickly enough did not exist. The information on which to base a decision was frustratingly limited (Figure 2). The assumption that was made at the time — and that has to be made in such situations — was a "worst case" assumption. Not knowing for certain whether there was market penetration but knowing that if there were it could be extremely dangerous, there was no choice but to assume that there was penetration and to take a protective stance toward the public health. On the limited information available — largely because of the lethal potential of the problem — it was necessary to take rather sweeping actions.

Emergency situations are characterized frequently by limitations not only in knowledge and understanding of the problem, but also in the capacity of the organizational structure to respond. Initially, the state apparatus in Massachusetts was ill-suited to emergency control of PSP. One immediate question was whether the Department possessed the authority to take the necessary actions. As the control program was mobilized, the Governor, at the request of the Commissioner and Public Health Council,* conferred emergency

* The Public Health Council, chaired by the Commissioner, is a quasi-independent body in the executive branch of state government which, by law, together with the Commissioner, is the Department of Public Health, and as such sets overall policy, through the formal promulgation of regulations.

powers on the Department by declaring a public health emergency on September 15 and thus effectively removing any ambiguity from the state's authority to act.

EMERGENCY RECOURSE TO MASS PUBLIC EDUCATION

After making the assumption that certain shellfish products had reached the retail level and were probably hazardous, it was necessary immediately to stop the consumption of all shellfish. The method chosen was an extensive public education campaign with the full aid of the communications media. The embargo decision was reached at noontime of Friday the 15th, and a press conference was called for 2:00 that afternoon. Through the electronic and print media, the public was advised of the emergency situation and instructed not to purchase or consume any shellfish until further notice. As a result of that broadside educational approach — initiated on Friday afternoon, the traditional fish-eating day — shellfish consumption virtually ceased within a very few hours' time. That result was critical, signal to averting a potential epidemic.

Of the 26 confirmed cases of PSP reported in 1972 (Figure 2), only two — both involving people who were isolated at a campsite and who privately harvested some softshell clams — occurred after Friday, September 15 at the time the embargo decision was reached. In retrospect, judging from the toxin levels subsequently found in shellfish harvesting areas along the North Shore (Table *I*) and on the estimate that one can expect about 20 per cent mortality with PSP (3), the evidence strongly suggests that the successful public education of 1972 may have prevented hundreds of paralytic shellfish poisonings and a number of fatalities (4, 5). The final toll was 26 illnesses, two classified as severe, and no deaths.

SWEEPING EMBARGO TO MINIMIZE AMBIGUITY

Having made the worst case assumption — that contaminated shellfish of unknown quantities had penetrated both wholesale and retail markets — and the determination that the first task was to prevent their consumption, the Department began to encounter taxonomic problems.

Seafood taxonomy is complex. There is little agreement within the industry itself on nomenclature, public discrimination among different species of shellfish is limited, and the best informed consumer can find it difficult to identify the original type of shellfish as it appears in a finished product (a fried clam, for instance). Since both data and time were in short supply, the only sure way to prevent consumption of suspect shellfish was to forestall consumption of all shellfish until the suspect product could be identified and isolated. To insure consumer protection, the Department decided to institute a total embargo, proscribing the harvesting and marketing (sale, importation, and exportation) of all fresh and frozen shellfish and shellfish products at wholesale and retail levels. Careful consideration was given the alternative of

Table I

Paralytic Shellfish Poisoning in Massachusetts

	1972	1973		1974	
	September	June	Aug.	May/June	Aug./Sept.
Toxin Levels (Peak) [1]	7266[3] (9/19)[4]	105 (6/5)	111 (8/23)	870[3] (5/30)	5400[3,6] (9/3)
A. Harvesting Area Samples[2]	5460 (9/25)			345 (5/30)	3800 (9/3)
B. Wholesale Samples	9600 (9/15)				190 (8/29) 345[5] (9/12)
C. Retail Samples	1900 (9/15)				350[5] (9/11)
Clinical Cases	26	0		0[7]	
Prospective Control Program?	NO	YES		YES	
A. Harvesting Control?	YES	NO	YES	YES	YES
B. Consumption Control?	YES	NO	NO	NO	NO

1 Toxin levels expressed in micrograms of poison present in 100 grams of shellfish meat. Readings surpasing 80µg/100gm are considered potentially dangerous for human consumption.

2 All peak harvesting area samples were taken from North Shore harvesting areas.

3 Mussels, all other readings are softshell clams.

4 Numbers in parentheses are dates of test results.

5 Point of origin was Maine.

6 A reading of 1103 was found in a sea clam evidently washed ashore in a storm and dug on 11/14 from a North Shore beach by an inspector. Though not a true "harvesting area" sample, the reading does illustrate the need for surveillance for toxic sea clams, particularly during and after storms.

7 Four cases, three very mild, resulted from consumption of softshell clams dug in Rye, N.H., and are not recorded as Massachusetts cases.

calling a selective embargo instead, for example, allowing importation of products known to be safe. But advocates of a complete embargo prevailed on grounds that it offered the best chance during the worst of the crisis to minimize ambiguity and confusion in consumers' minds and to facilitate implementation of a control program. It was felt that control problems might be greatly exacerbated if the denominator — the total amount of shellfish in the Massachusetts seafood market — were allowed to increase during the process of market analysis and cleansing. In order to simplify and expedite that process, no new shellfish products were allowed to enter the market until after all hazardous products had been removed from commercial channels.

As more and better data were accumulated, it became possible to begin relaxing the sweeping embargo to accommodate mitigating factors. For example, the importation ban could be lifted after three criteria could be satisfied: wholesale and retail markets had to be free of contaminated product; results of laboratory tests from the point of origin had to be available;* and a means of verifying the safety of incoming stock had to be established. As soon as these were met the ban was lifted.

HARVESTING AREA, MARKET AND CLINICAL SURVEILLANCE

At the same time the sweeping embargo was being imposed and publicized, the groundwork was being laid to enable the Department to begin relaxing it as quickly as possible. Data were needed to assess quickly and accurately the initial assumption which had been the basis for the embargo decision. As of September 14, Massachusetts had no established program monitoring shellfish harvesting areas for evidence of *G. tamarensis* and no laboratory capacity to bioassay for toxin in shellfish. Within the next two days, an intensive monitoring program of all shellfish growing areas had been established, and five laboratories had been equipped and were functioning. Based first on microscopic examination of water samples, then, as soon as the necessary laboratory capacity was established, on the preferred procedure — bioassays of shellfish samples — the program sought to determine where and which varieties of shellfish were affected. Ultimately, some 2,800 acres of Massachusetts shellfish harvesting shoreline were found to be contaminated by the marine dinoflagellate.

In addition to the harvesting area samples tested, bioassays were performed on shellfish stock in markets, restaurants, and wholesale outlets. Clinical surveillance was assigned to a team of four physicians, who conducted epidemiologic investigations of all reported cases, including field studies where appropriate.

By Sunday, September 17, it was clear that the danger was limited to softshell clams and mussels — that no scallops, oysters, or hardshell clams (quahogs) on the markets had been harvested from infested waters.

* In the Chesapeake Bay area, for example, there was, at the time, shellfish contaminations unrelated to the red tide.

Accordingly, the ban on *sale* of Cape scallops and hardshell clams was released. Digging of all shellfish from all Massachusetts coastal waters continued to be proscribed, awaiting complete market clearance. Enforcement of harvesting bans was carried out by local shellfish wardens as well as state Division of Natural Resources officers. Patrols were intensive, around the clock, with officers and wardens on foot, in boats, cars, and searchlight equipped helicopters for nocturnal surveillance.

Subsequent days brought improved data and further modifications of certain restrictions based on new geographical, species-specific, or commercial information. On the 22nd, the importation ban was relaxed to allow shellfish except softshell clams and mussels from south of Massachusetts into the state; as of the 25th, commercial harvesting, and as of the 28th, leisure digging of hardshell clams in selected areas of the Southeastern Massachusetts coast were permitted. But this gradual easing of restrictions was possible only after the market had been cleansed, hazardous harvesting areas had been identified and were well-patrolled, and the Department could be certain that remaining stock was safe. The ban on importation, distribution, and marketing of softshell clams and mussels was not lifted until October 18 when the toxin levels had dropped sufficiently low that even a breach of the harvesting ban would almost certainly not lead to a fatality. Inexperience with "red tide" and lack of complete confidence in the harvesting control efforts necessitated this conservative posture in 1972.

MARKET CLEARANCE

The early assumption of the existence of serious market penetration was rapidly verified (Table I) and it was also learned that wholesalers and retailers frequently mix separate lots of softshell stock, obliterating evidence of the point of origin. By Saturday, September 16, the control program was directed at surveying the markets (wholesale and retail) for possibly-contaminated shellfish — specifically, softshell clams and mussels. On September 18, wholesale dealers were ordered to destroy, by burning or burying, all fresh softshell stock; to hold and inventory by date, source, and quantity, all fresh and frozen shuck stock; and to hold all other frozen clam stock. Retail stores and restaurants were ordered to destroy fresh softshell stock, and restaurants were again reminded that they were forbidden to serve softshell clams or any dish with the word "clam" in its description.

Paradoxically, the decision to confiscate and destroy fresh shellfish stock was economically the least onerous alternative. A survey taken of the retail and wholesale markets had revealed that the quantities of stock-on-hand, fortuitously, were unusually small. The decision reduced to a trade-off for the industry: the Department could either hold the markets closed and continue the slow testing process, or it could call for the destruction of all fresh softshell clams and mussels and then immediately open the markets. The second alternative was the lesser of evils. Before the end of September, the

Department was confident that no softshell stock remained in the marketplace and was able, from that point on, to advise the public: "If you can buy it, you can safely eat it."

PHASING IN OF A PROSPECTIVE PROGRAM

With market clearance achieved, the acute phase of the emergency was weathered and the strategy shifted to one more closely analogous to a prospective program. Control of consumption was no longer necessary. The Department was able to say with complete assurance that everything in the markets was safe, and was able to stress that message in an attempt to assist the industry. A broadside approach, during a sudden, acute episode, offends the industry and does ripple over into sales of unaffected seafood. But aggressive initial control is the only safe recourse when a lethal product may have reached the marketplace. Resulting animosity may be an unfortunate but unavoidable cost. In an unanticipated and acute public health emergency of this type, it is impossible to protect the industry and the consumer simultaneously. The first concern has to be the life and health of the consumer.

As the problem evolves from one of a first experience to a recurring one — which PSP in Massachusetts has done since the first episode in 1972 — government can reduce the damage to the industry by instituting an effective prospective control program. In the early days of the 1972 bloom, the state had no organized system for collecting and analyzing data. But during the latter part of September and early October, the emergency control effort was phased gradually into a routine surveillance program. Based on the Canadian model (3), tailored to Massachusetts needs (4), the program relies on well-defined geographical areas (24 to 36 "key stations"), monitored constantly, so that any proliferation of *G. tamarensis* can be detected, and the infested harvesting area closed before a public health threat is posed. The use of predetermined sampling areas permits systematic definition of the extent of a particular bloom, and allows selective opening and closing of shellfish harvesting areas. With an area's closing comes the implementation of a multifaceted program to warn unintentional violators, including targeted news releases and helicopter monitoring to supplement warning signs posted along the shore and patrolling by shellfish wardens on foot and in boats.

The Massachusetts experience since the initial toxic dinoflagellate bloom in 1972 has tended to validate the efficacy of a prospective control program. Table I shows that in 1973 *G. tamarensis* contamination of shellfish rose to marginally dangerous levels, and, in 1974, to very high levels, with some contaminated product reaching wholesale and retail outlets in September of 1974. But through market surveillance, the Department was able to identify, isolate and remove contaminated shellfish from commercial channels at the wholesale level and prevent wide dissemination to retail outlets and restaurants. The retail market penetration in 1974 was by clams imported from the state of Maine and was limited to a single batch in one restaurant. No

PSP-contaminated shellfish harvested from Massachusetts waters have reached retail markets since the 1972 episode.

Table I illustrates clearly the importance of an integrated control program. Surveillance of the harvesting areas is the first line of defense. It triggers control measures in the event of any build-up of toxicity and helps to identify unusual dangers, for example, the sea clam contamination discovered in 1974. Market surveillance at the wholesale and retail levels serves to back-up harvesting area surveillance and also to monitor shellfish harvested outside of the state. PSP, of course, does not respect territorial boundaries; complete control is impossible without interstate communication and cooperation. A great deal of the seafood consumed in Massachusetts is imported, much of it from Maine, and, as Table I indicates, the peak wholesale and retail readings in 1974 were both taken from samples originating in Maine. Clinical surveillance comes into play only when there is evidence of market penetration.

Since the first experience with "red tide" in 1972, intensive surveillance, yielding accurate and comprehensive data, has permitted the Department to confine its control efforts to harvesting beds and markets, avoiding the consumption control program directed at consumers that is so damaging to the industry. Fortunately there has not been another Massachusetts case of paralytic shellfish poisoning since September of 1972.

CONCLUSION

A sweeping embargo and consumer-oriented consumption control were effective and necessary steps in the initial 1972 Massachusetts occurrence of paralytic shellfish poisoing. Prospective harvesting area and market surveillance in subsequent years have satisfied the dual objective of consumer and industry protection.

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MANAGEMENT OF THE PARALYTIC SHELLFISH POISON PROBLEM IN THE UNITED STATES

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ABSTRACT

The development of United States public health control measures for paralytic shellfish poison (PSP) had their beginnings in California. Management plans have evolved slowly, as the exact cause of PSP eluded scientists, until the fundamental work of H. Sommer and associates, in 1937, associated toxic mussels with the occurrence of the dinoflagellate, *Gonyaulax catenella*. A standardized bioassay procedure for detection of PSP in shellfish was successfully collaborated and adopted by the Association of Official Analytical Chemists (AOAC) in 1958.

The present generally accepted quarantine level for PSP was based upon Canadian epidemiological studies in the 1950's and put into practice in the United States in 1958. The States of California, Washington, and Maine have the longest history of operating PSP surveillance programs. This paper attempts to characterize these state PSP management plans to arrive at a common guide for managing this problem. Admittedly, there are legal and administrative shortcomings to the surveillance schemes that have been developed; however, improvements await some key research developments which will then provide state administrators newer management practices which, hopefully, will be fiscally and administratively feasible. The area of greatest need for management is a reliable and rapid forecasting technique which detects the approach of toxic dinoflagellates.

INTRODUCTION

The first recognized outbreak of paralytic shellfish poison to occur in the United States was in 1903 at Timber Cove, California. However, an Eskimo legend noted that 100 Aleut hunters died within two hours after consuming mussels near Peril Way, Alaska in 1799 (5). Public health officials did not become sufficiently concerned about PSP until 1927 when a series of explosive outbreaks occurred in the vicinity of San Francisco involving 102 cases of mussel poisoning resulting in six deaths. This outbreak led California public health officials to issue a warning against the use of mussels during the summer of each year, usually the months of July and August. At the national level, the adequacy of shellfish sanitary control measures was being discussed.

NATIONAL SHELLFISH SANITATION PROGRAM (NSSP)

From 1900 to 1925 health officials became increasingly concerned about the rising incidence of shellfish associated typhoid fever outbreaks. Then, during the winter of 1924-25, a sensational typhoid fever outbreak occurred that involved in excess of 500 persons who consumed oysters in Washington, D.C. Chicago, New York, and ten other cities (6). The shellfish industry, by this time, was without markets because of the adverse publicity and state health and conservation officers appealed to the U. S. Public Health Service for more adequate controls. After an advisory committee report that considered many

possible solutions, including a ban on raw shellfish consumption, the Surgeon General decided the best way to assure the safety of shellfish was by a cooperative program involving the health and conservation agencies in shellfish-producing states. This cooperative program is now known as the National Shellfish Sanitation Program (NSSP) and administered at the federal level by the United States Food and Drug Administration. The early NSSP did not recognize shellfish marine biotoxins as a serious food control problem. Today, as in the early years of the NSSP, the major public health problem associated with the consumption of raw shellfish is the prevention and control of shellfish borne infectious diseases.

HISTORICAL DEVELOPMENT OF PSP CONTROL IN THE UNITED STATES

The earliest reported control program in North America designed to protect the public from PSP was established by certain West Coast Indian tribes before the arrival of Russian or European trappers. Sentries were posted on high points along the beach to look for bio-luminescence which they believed signaled the approach of a toxic red tide. Harvesting in affected areas would then be prohibited.

California

The earliest modern public health control of paralyt.c shellfish poison in the United States apparently began in California. Mussels were quarantined in 1927, and in 1929 health officials recognized that clams could cause intoxication (11).

As California gave more attention to the epidemiological patterns of PSP cases, the seasonal aspects became more apparent. Thus, in 1939, California authorities established a mussel quarantine order extending from May 1 to October 31 of each year (2). They further recommended that the dark parts of the clam should be discarded and the remaining portions thoroughly washed before eating.

Washington State

The Washington State Health Department initiated a paralytic shellfish poison surveillance program in 1932 and continued it until 1946 (15). Presumably, the lack of any toxic shellfish samples and human intoxications indicated that there was no longer a need for the surveillance program after 1946.

Federal Activity

The first federal attempt to develop guidelines for the control of toxic shellfish was initiated at a 1943 conference sponsored by the United States Fish

and Wildlife Service. Before this conference, there was a general concern over the problem which resulted in many discussions between health and fishery agencies in Canada and the United States. The first mention of PSP control measures in the NSSP was made in the September 1, 1943 draft of "A Manual of Recommended Practice for the Sanitary Control of the Shellfish Industry." The section on PSP control was brief and was entitled "Examination of Mussels and Clams for Presence of Poison". The requirement of this section read:

> "...A representative number of samples of mussels and clams shall be collected from growing areas suspected of containing poisonous shellfish and shall be examined for the presence of poison before mussels and clams intended for human consumption are taken from such growing areas..."

It was acknowledged that the problem was mainly confined to the Pacific Coast and was attributed to the consumption of the toxic sea mussel, *Mytilus californianus*.

Territory of Alaska

During World War II the demand for seafood products expanded considerably, which prompted the development of a significant butter clam (*Saxidomus giganteus*) industry in Southeastern Alaska. During the 1945-46 season the production of canned and frozen clams reached a peak of 260,000 pounds (16). Lots from that season's production were examined by the Food and Drug Administration (FDA) and found to be adulterated with paralytic shellfish poison. The lots were seized and the butter clam industry was devastated. Fisheries officials in the Territory of Alaska and the Fish and Wildlife Service then worked for twelve years to develop an acceptable quality control and area management program.

By 1950, the shellfish industry realized that new regulatory control requirements for PSP in butter clams harvested from Alaskan waters would prohibit further exploitation of that resource. The cost of a quality control program necessary to assure that FDA tolerance limits would not be exceeded plus the high risk of having to discard large portions of the packs proved to be unsurmountable obstacles for the industry.

The outlook for the Alaska butter clam remains the same today and for that reason Alaska does not now have any shellfish certified for interstate shipment. The situation for the Alaskan razor clam, *Siligna patula*, is different and the status of public health control for this species will be discussed later.

U. S. Public Health Service Conferences

In 1955 and 1957 the U. S. Public Health Service (13, 14) sponsored conferences to further develop a standard bioassay procedure, determine the maximum allowable concentrations of the shellfish toxin, and develop a

program for utilization of the Alaskan butter clam. These conferences stimulated the development of the current recommended methods, procedures, and standards used for the control of PSP by the NSSP. With the availability of a purified source of the PSP reported by Dr. E. J. Schantz and associates, a reliable and reproducible bioassay procedure using mice for the detection of PSP was developed. A quarantine limit based upon Canadian epidemiological investigations of 1945, 1954, and 1957 human poisonings substantiated the 400 mouse unit per 100 grams of edible shellfish, as suggested by Dr. Medcof in 1947. The 1958 NSSP National Workshop, with participation from the Canadian Department of Fisheries, recommended acceptance of the 400 mouse unit quarantine limit which was equivalent in Canadian laboratories to 64 µg. of PSP per 100 grams of the edible portions of shellfish meats. Later collaborative studies showed that the Conversion Factor (CF) more nearly averages 0.2 for most strains of mice than the CF value of 0.16 used by the Canadian laboratories, and the quarantine limit was rounded off to 80 µ g. (i.e., 400 m.u. x 0.2=80 µg).

The changes that have occurred since the 1946 edition of the NSSP Manual of Operations have been the universal acceptance of the 80 µg quarantine limit and a good standard bioassay method which was finally adopted by AOAC in 1959. These technical developments are the latest management requirements that are specified by the NSSP (16).

Maine

Up until 1958, the states on the East Coast had no routine PSP sampling programs. After receiving reports of very high toxicity levels in Canadian shellfish and 33 nonfatal cases of paralytic shellfish poisoning in 1957 from New Brunswick, the Maine Department of Sea and Shore Fisheries initiated a PSP surveillance program. Their efforts were concentrated near the shellfish waters adjoining the Canadian-United States border. In 1958, low levels of PSP were found in samples of mussels and clams taken from the area around Quoddy Bar. Following this disclosure, routine monitoring of a limited number of stations was conducted after May 1 and PSP results from the Canadian Bay of Fundy stations were closely followed. Eventually, other key stations were established and monitored on a routine basis during the hazardous season.

It should also be noted that the Washington State Department of Health reactivated their PSP surveillance program in the spring of 1957 after eleven years of no activity. This was prompted by an outbreak involving approximately 50 persons who ate oysters in British Columbia. Washington State now has a routine PSP closure between April 1 and October 31 of each year for areas adjoining the Straits of Juan de Fuca and certain ocean beaches. Razor clams are excluded from these closures.

This has been a brief historical review of the development of United States PSP public health controls. Thus, prior to 1972, only California, Washington, and Maine had organized PSP surveillance programs while the Alaska butter clam was the subject of intensive research work.

Current PSP Management Programs

Current management programs have evolved, with minor modifications, into an overall operational pattern. These programs are based upon local geographic, hydrographic, and meteorological data, as well as historic information regarding occurrence of PSP and, in general, operate as follows:

1. Key shellfish sampling stations are located
2. Seasonal sampling plans are developed
3. Most sensitive species are used as indicator, usually mussels
4. As PSP levels rise at key stations, satellite stations are established and monitored
5. Areas exceeding quarantine levels are closed to harvesting, posted, and patrolled
6. Market sampling may be conducted as warranted, and affected shellfish embargoed.
7. Public education measures are taken to alert recreational harvesters.
8. Areas are reopened to harvesting when PSP levels in shellfish meats fall consistently below quarantine levels.

Visual "red slicks", dead ducks or gulls, and "weakened shellfish" have more recently been recognized on the East Coast as early warning indicators. During extended periods of freedom from toxicity, surveillance tends to decrease resulting in an increased health hazard when massive unexpected blooms occur.

Surveillance Activity

The three states that have had routine PSP surveillance programs generally follow this pattern. The state control officials have selected *key* sampling stations that are located on beaches of wide straits near open water or the seaward side of inlets. These key stations provide the early warning sites which detect the approach of dangerous levels of toxic dinoflagellates. In most regions, a knowledge about the hydrography of the coastal waters was helpful in the selection of these key sampling stations.

The type of shellfish sought for sampling at key stations is the mussel which is known to accumulate PSP to the highest levels, but it also depletes the toxin at a relatively rapid rate. It is not always possible to find mussels at the desired locations so other shellfish which are naturally abundant are used. Clams would be the next most common species samples, with the oyster being the least frequent species collected for PSP analysis. Another accepted practice is to gather the shellfish nearest the lowest tide mark since most studies show a significant difference between those on the upper beach areas from those near the low tide mark.

A question that usually arises on sampling is how much variation in levels of PSP exists among individual animals of the same species collected from the

same beach. Work by Magnusson and associates (7) has shown, with five or more animals grouped in a bioassay sample that the variation was within an acceptable range. However, PSP levels have been known to vary by more than a factor of two in shellfish harvested from separate stations on the same beach.

The state control officials have generally arrived at seasonal sampling plans with the PSP levels peaking in the months of July through October, when more frequent and intensive surveillance samples are collected. Unexpected peaks have been known to occur in late fall. During the expected toxic season, routine biweekly samples are collected at the key sampling stations and if a sample above 80 μg is detected, either more extensive and more frequent sampling is initiated or the area is closed. Typically, California averages about 150 samples per year, Washington about 175, and Maine, until 1972, about 50, when the whole coast of Maine was closed because of PSP. Since 1972, Maine and Massachusetts have conducted extensive sampling programs.

Analytical Support

It is common practice for a state or county official to collect the sample and either mail or directly transport the sample to a centralized state laboratory. The AOAC standard bioassay is conducted using three mice. The estimated present-day cost for collection and analysis at one of these centralized labs is $40.00 per sample. The results of these tests are usually available to state shellfish control officials from one to five days after collection. If the results indicate levels requiring closures or warnings, the administrative action may be immediate or as long as two days to effect. This time lag can be critical when we know that toxic dinoflagellates can move and multiply rapidly in a matter of a few tidal cycles. The present management schemes require very prompt responses, and in some instances, the time lag for decision making is too long.

Administrative Actions

The administrative action taken by state officials once it has been determined that an area is above quarantine level is to notify all commercial harvesters. Phone contact is used to promptly notify those commercial harvesters known to gather shellfish from the affected area. Recreational harvesters are advised of the toxic shellfish by posting the area with placards, by newspaper and radio broadcasts, and by posting notices in sport stores, marinas, and public access facilities (Figures 1 and 2). In spite of these educational measures, the predominance of PSP poisonings have involved persons gathering toxic shellfish from posted areas during the quarantine periods. Effective and convincing communications to reach the recreational shellfish harvesters have been the most difficult PSP management problem.

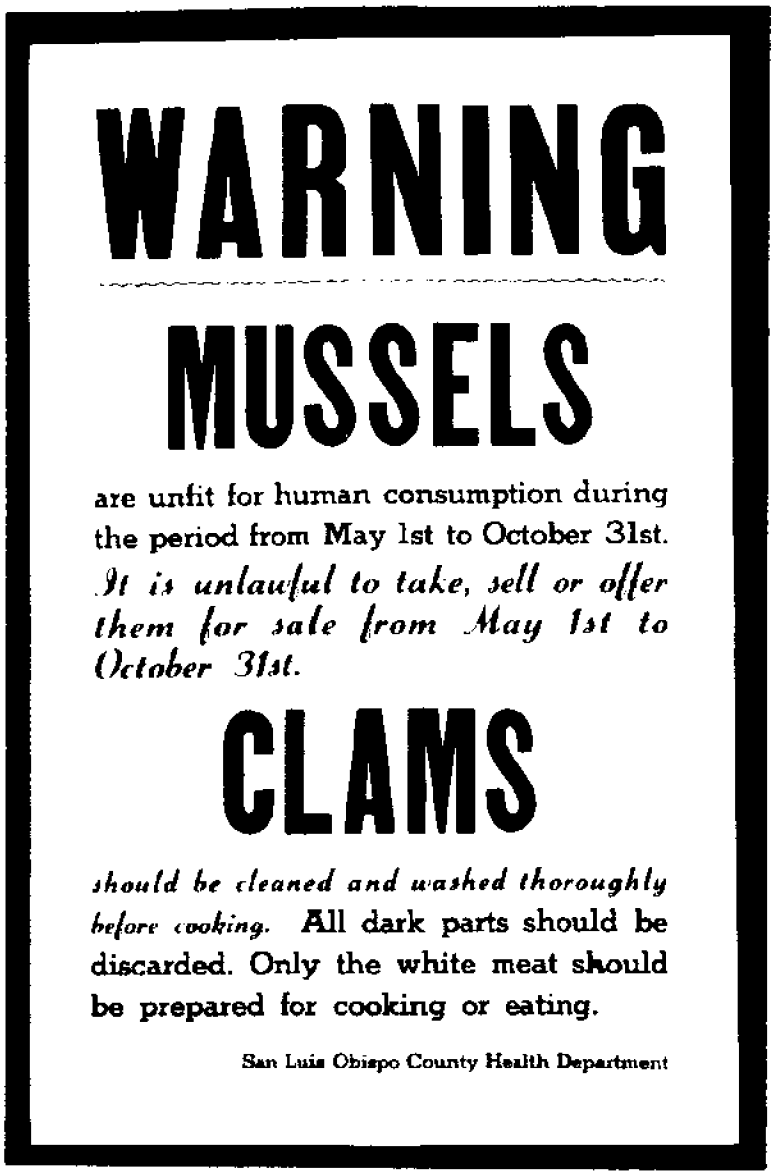


Figure 1: PSP placard which is routinely posted along the California coast during the annual quarantine period.

To dispel many erroneous notions about mussel poisonings, the Manual for the Control of Communicable Disease (3), published by the State of California, advises that the following points should be emphasized in public educational programs:

1. Paralytic shellfish poison is not a post-mortem product,
2. Temporary exposure to the sun does not harm living mussels, nor does it make them poisonous,
3. Mussels below the tide line are, if anything, more poisonous than those above the water,

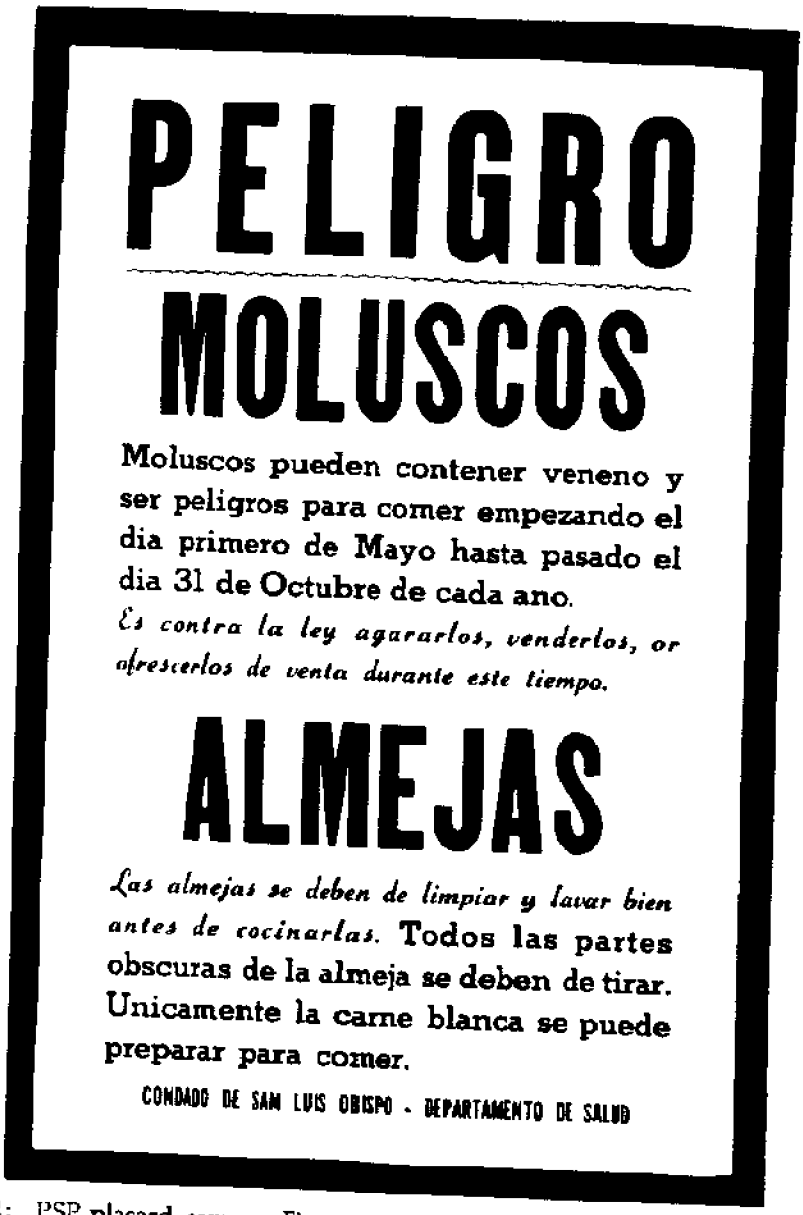


Figure 2: PSP placard, same as Figure 1, in Spanish because of many Spanish-speaking California natives who do not read English.

4. Copper in the rocks, oil on the beaches, and stagnation or pollution of the water are in no way connected with mussel poisoning,
5. Toxic mussels or clams cannot be distinguished from normal ones without laboratory tests, and
6. Discoloration of a piece of garlic or of a silver spoon in the pot are not indicators for poisonous mussels.

(Of course, these points are applicable to other shellfish.) The publication explains that the PSP phenomenon should be described as a natural food chain

condition caused by toxic marine dinoflagellates being consumed by shellfish which are, in turn, eaten by man. This simple explanation should be comprehensible to most people and help eliminate this type of food poisoning.

New PSP Efforts

More recently, Alaska has nearly completed a PSP management plan for the commercial harvesting of razor clams (*Silignas patula*) in the Kodiak - Cook Inlet area. It is anticipated that the eviscerated razor clam will be certified in accordance with the NSSP in the near future. This program has developed after several years of cooperative study by the Food and Drug Administration and Alaska State officials.

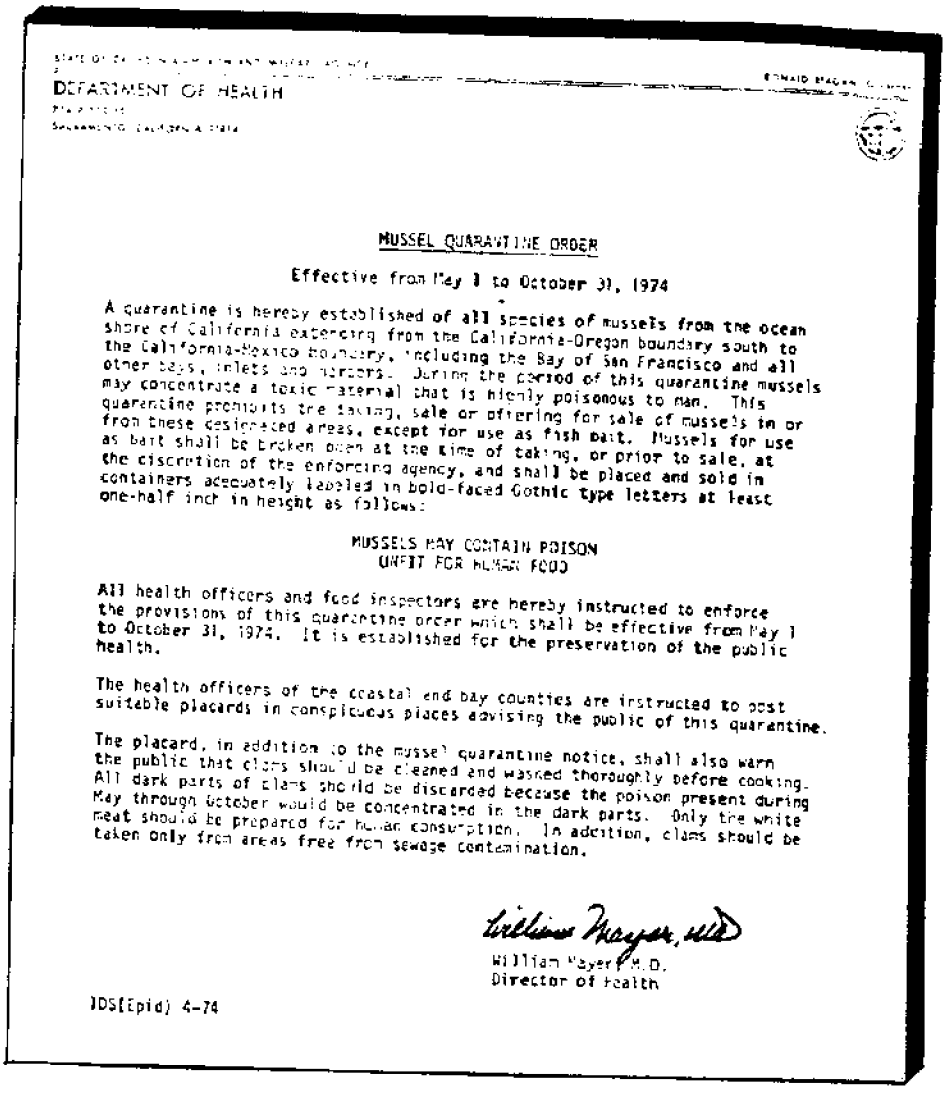
It is also understood that Oregon and New Hampshire are developing PSP surveillance programs which they expect to initiate in the near future. The state of Massachusetts began its PSP surveillance program in 1972 after the infamous PSP outbreak in September of that year. The previous speaker has already described the action taken by Massachusetts after that unfortunate and unprecendented episode.

Evaluation of PSP Management

One of the responsibilities of the Food and Drug Administration in the cooperative NSSP is to evaluate the effectiveness of state shellfish sanitary control programs. This responsibility also includes foreign countries that have signed bilateral agreements on the terms that they will only export shellfish to the United States in accordance with the provisions of the NSSP. FDA has such an agreement with Canada, Japan, and Korea. An agreement is now under negotiation with Mexico. Only Canada has a recognized need for a PSP surveillance program. Needless to say, the Canadians are managing their PSP program on an equivalent basis to the accepted standard of the NSSP.

The adequacy of state PSP surveillance programs has been overall generally satisfactory in view of the resources committed, quarantine measures (Figure 3), administrative actions taken, scientific knowledge, and the relatively rare occurrence of shellfish poisonings. The quarantine limit of 80 μg still is considered a sound limit and the AOAC bioassay procedure is a reliable method. The toxins from both *Gonyaulax catenella* and *G. tamarensis* have been shown to have identical toxic effects on mice so the quarantine limit applies for either dinoflagellate (10).

There are a few areas in which additional information would be decidedly helpful and lead to more effective and efficient management of PSP control programs. A simple field test to detect the presence of PSP is believed to have promise. FDA has funded a research contract with the University of Alaska to look into such a possible test. However, a more important need is to discover a reliable detectable precursor to the occurrence of the toxic dinoflagellates. If such a precursor could be defined and effectively monitored, PSP management schemes could be measureable improved.



DEPARTMENT OF HEALTH

MUSSEL QUARANTINE ORDER

Effective from May 1 to October 31, 1974

A quarantine is hereby established of all species of mussels from the ocean shore of California extending from the California-Oregon boundary south to the California-Mexico boundary, including the Bay of San Francisco and all other bays, inlets and waters. During the period of this quarantine mussels may concentrate a toxic material that is highly poisonous to man. This quarantine prohibits the taking, sale or offering for sale of mussels in or from these designated areas, except for use as fish bait. Mussels for use as bait shall be broken open at the time of taking, or prior to sale, at the discretion of the enforcing agency, and shall be placed and sold in containers adequately labeled in bold-faced Gothic type letters at least one-half inch in height as follows:

MUSSELS MAY CONTAIN POISON
UNFIT FOR HUMAN FOOD

All health officers and food inspectors are hereby instructed to enforce the provisions of this quarantine order which shall be effective from May 1 to October 31, 1974. It is established for the preservation of the public health.

The health officers of the coastal and bay counties are instructed to post suitable placards in conspicuous places advising the public of this quarantine.

The placard, in addition to the mussel quarantine notice, shall also warn the public that clams should be cleaned and washed thoroughly before cooking. All dark parts of clams should be discarded because the poison present during May through October would be concentrated in the dark parts. Only the white meat should be prepared for human consumption. In addition, clams should be taken only from areas free from sewage contamination.

William Mayer, M.D.
Director of Health

JDS(Epid) 4-74

Figure 3: Typical shellfish PSP quarantine order issued by state health authority.

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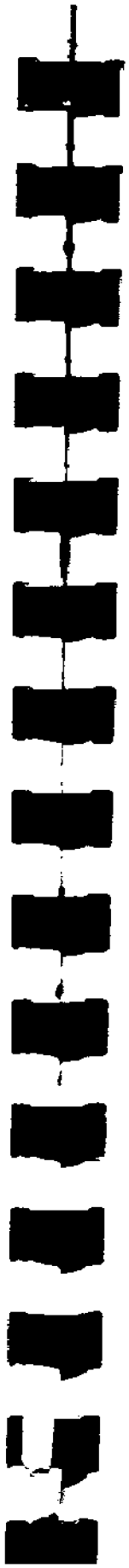

MANAGEMENT OF PSP IN CANADA

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ABSTRACT

Paralytic shellfish poisoning occurs regularly in three areas of Canada: the lower Bay of Fundy regions of Nova Scotia and New Brunswick, the St. Lawrence Region of Quebec and along the coast of British Columbia. On the East Coast 14 species of shellfish are potential public health risks and 8 West Coast species accumulate sufficient toxin to be a problem. The Bay of Fundy shellfish area classification and management system is outlined as are the control mechanisms utilized in Quebec and British Columbia. Reference is made to the source of the poison, seasonal variations, selection of sampling stations, effects of processing methods on toxicity, standards applied and problems encountered in managing PSP.

INTRODUCTION

There is ample evidence in historical records to indicate a public health risk from molluscan shellfish in both eastern and western Canada. Deaths and illnesses from PSP had occurred in British Columbia in 1793 (14), in the St. Lawrence Region of Quebec in 1880 (13) and in the Fundy Region of New Brunswick in 1889 (2).

Attention was focused on the East Coast problems in 1936 when 35 to 45 people became ill and two died in the Digby Neck area of Nova Scotia (5). The public health significance of the problem was not well understood until Sommer and Meyer reported on Californian poisonings and bioassay methodology in 1937. The methodology made it possible to evaluate the potential scope of the problem and studies on both the east and west coasts of Canada were published in 1939 (3, 10). However, it took several fatalities in British Columbia in 1942 (11) and a wartime interest in canning mussels from the Fundy Region before harvesting and processing controls were instituted. It required yet another outbreak in Nova Scotia and New Brunswick in 1945 (4) to gain full support for research and management programs. A comprehensive management scheme has been in operation in the Fundy Region since 1945; PSP control in one form or another has been in effect in British Columbia since 1942 and in Quebec since 1950.

The story of PSP in British Columbia to 1969 has been summarized by Quayle (11) and an excellent review of PSP in eastern Canada was published by Prakash *et al.* in 1971. The following sections deal only with those aspects of the problem relevant to understanding and operating management schemes and control regimes. PSP content is determined by mouse bio-assay and is expressed in micrograms per 100 grams of wet weight of shellfish. For details on terminology and methodology, the reader is referred to (9).

Source of PSP in Shellfish

Plankton studies in the Bay of Fundy indicated that the origin of PSP in shellfish was the dinoflagellate *Gonyaulax tamarensis* [1] (6). It was shown that

[1] Loeblich and Loeblich (these proceedings) have indicated that the name of this species should be changed to *G. excavata*.

shellfish became toxic as soon as *Gonyaulax* appeared in the water, that peak in the numbers of *Gonyaulax* were reflected by peaks in the poison content o shellfish and that poison content declined when the dinoflagellate disappearec from the surface water (7). A similar relationship between abundance of *G. tamarensis* and toxicity in shellfish was found to exist in the St. Lawrence Region of Quebec. Prakash *et al.* (9) provided conclusive proof by testing extracts of mass cultures of *G. tamarensis* on mice and by demonstrating through feeding experiments that rises in toxicity of clams were associated with the number of cells consumed.

In British Columbia the causative organism was believed to be *Gonyaulax catenella*, although it had not actually been recorded in coastal waters. Prakash and Taylor (8) identified *G. catenella* as the causative organism in a 1965 PSP outbreak in Malaspina Inlet, B.C. In the latter case the waters in the inlet were maroon-coloured.

Much publicity has been given to the red tide phenomenon and there has been a tendency to link red tides and PSP in molluscan shellfish. It should be noted that PSP occurs every year in east coast Canadian shellfish, and red water blooms have never been related to rises in shellfish toxicity. The only bloom linked with PSP outbreak or rise in toxicity in Canada was the previously mentioned Malaspina incident.

Species Affected, Geographic Distribution and Seasonal Variation

In areas known to have produced toxic shellfish any filter feeding mollusc is potentially dangerous as are some carniverous snails such as the rough whelk (*Buccinum undatum*). On the East Coast six species of clams (*Mya arenaria, Spisula solidissima, Mercenaria mercenaria, Mesodisma arctatum, Ensis directus* and *Arctica islandica*) two of mussels (*Mytilus edulis, Volsella modiolus*) five of whelks, moonshells and winkles (*Buccinum undatum, Colus stimpsoni, Neptunea decemcostota, Lunatia heros* and *Thais lapillus*) and the rims and gonads of scallops (*Placopecten magellanicus*) have been found to be toxic. PSP has been recorded on the West Coast in six species of clams (*Saxidomus giganteus, Venerupis japonica, Protothaca staminea, Tresers capax, Lucinoma annulata* and *Mya arenaria*) two of mussels (*Mytilus edulis, M. californianus*) the moon snail (*Polynices Lewisi*) cockles (*Clinocardium nuttalli*) the body of the rock scallop (*Hinnites multirugosus*) and in oysters (*Crassostrea gigas*). However, based on reported cases of PSP, the most hazardous shellfish on both coasts are clams and mussels of various types although whelks have caused poisonings in Quebec and oysters and cockles have been implicated in British Columbian outbreaks (11, 9).

In the Bay of Fundy, PSP generally peaks in July, August or September but the peak may be as early as June or as late as October. Generally during the winter and spring most shellfish are at safe levels. The area affected on the Nova Scotia side covers about 200 miles from Briar Island to Minas Channel. On the New Brunswick side the danger zone extends about 100 miles just south

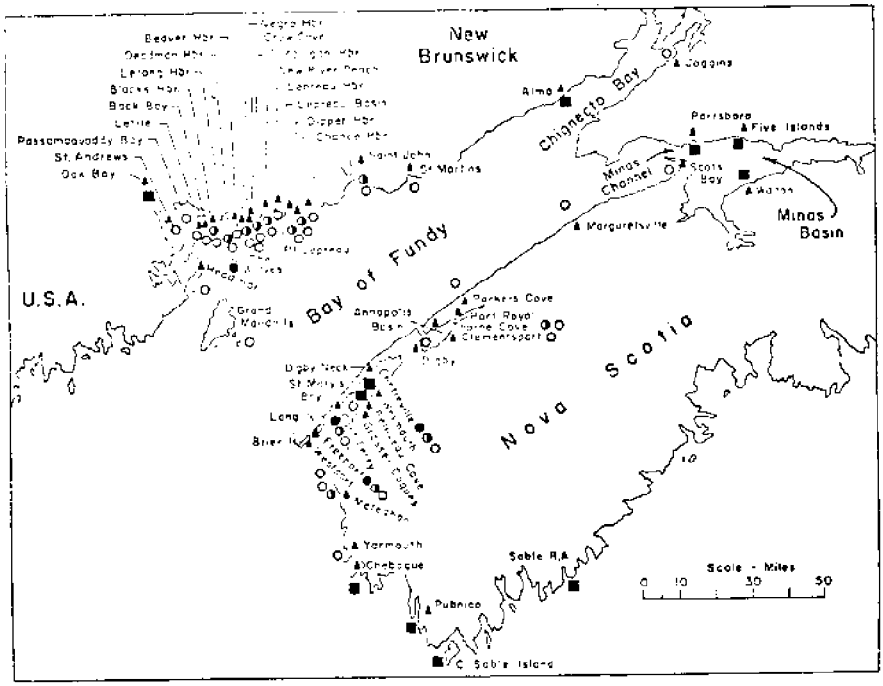

Figure 1. Geographic distributions of toxic shellfish and poisonings in the Bay of Fundy region, 1889-1969 (■, shellfish always poison free; O, shellfish sometimes toxic; ◑, nonfatal poisonings; ●, fatal poisonings). (From Prakash *et al.* (9)).

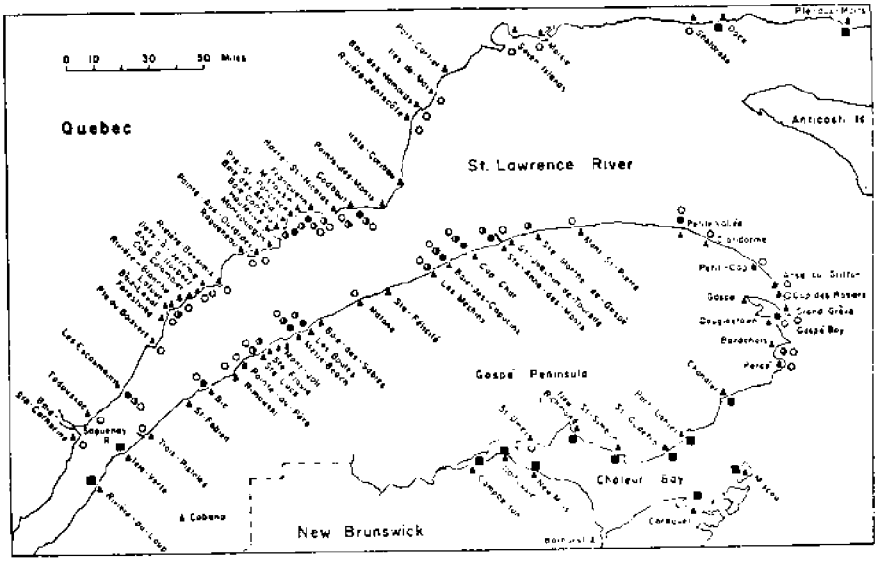

Figure 2. Geographic distributions of toxic shellfish and poisonings in the St. Lawrence region, 1880-1969 (■ shellfish always poison free; O, shellfish sometimes toxic; ◑, nonfatal poisonings; ●, fatal poisonings). (From Prakash *et al.* (9)).

of St. John to the Canada-U.S. boundary and includes Deer, Campobello and Grand Manan Islands (Figure 1).

There is no relationship between levels of toxicity in the Fundy and St. Lawrence Regions. In the St. Lawrence the typical pattern of a July, August or September peak is observed in many areas, but their year-to-year variations are much more irregular than in the Fundy, and winter carry-over of relatively high levels is not uncommon in many areas. The toxic area on the south side of the St. Lawrence River includes the entire Gaspe Penninsula, some 300 miles, from Trois-Pistoles to St. Omer in the Bay of Chaleur. On the north shore, PSP has been found along the 300 mile coastline from the mouth of the Saguenay River to Sheldrake, about opposite the tip of Anticosti Island (Figure 2).

If British Columbia (Figure 3), toxic shellfish are known to have occurred along the whole coastline, although in any one year distribution of PSP tends to be highly localized. PSP may peak at any time between May and November and while butter clams may retain PSP in their siphons over winter, other molluscan shellfish are poison-free during this period.

Effect of Processing and Standards Applied

The most common processing methods are shucking and canning. In the case of scallops, only the adductor muscle, which is poison-free, is retained for marketing so that commercial shucking eliminates all risk. Conversly for soft shell clams, the only parts discarded during shucking (siphon cover and tips) are nontoxic and this tends to increase the risk of poisoning. Trimming off the siphons of butter clams and the digestive gland of whelks reduces but does not eliminate the risk of poisoning. Ordinary cooking also reduces PSP content, but since practically all recorded poisonings have been caused by cooked shellfish, cooking can hardly be recommended as a safety measure.

With respect to canning it has been shown that poisonfree packs are consistently obtained if the scores of raw shellfish are below 200 µg/100 g and the pack is heat processed at 250°F for 45 minutes (4, 1).

The standard currently applied in Canada is 80 µg/100 g for raw shellfish that are marketed shucked or in the shell. Digging under special permit for canning purposes is permitted in areas where the scores are between 80 and 160 µg and the canned packs are tested before release for sale.

Selection of Sampling Stations

In any area where toxicity occurs the PSP content of the shellfish will be highest, and show up earliest, at seaward locations in an inlet. In addition, toxicities will be highest at the low tide level on a beach. Toxicities of individual shellfish taken at the same time in the same location vary somewhat, but composite samples of ten clams each give reasonably consistent results.

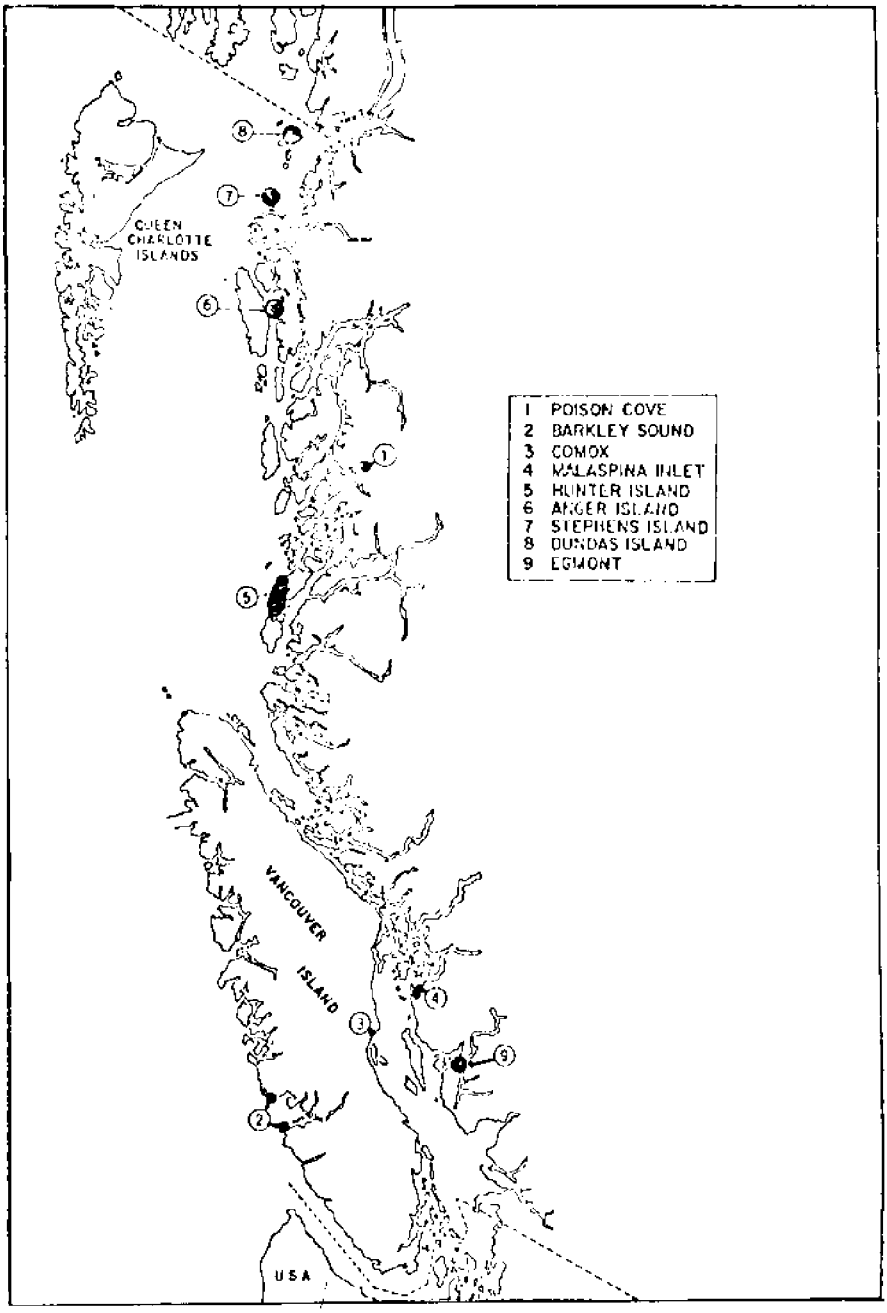

Figure 3. Map of the British Columbia coastline to show areas where outbreaks of P.S.P. have occurred. (From Quayle (11)).

Table I

Bay of Fundy PSP Management Program

AREA CLASSIFICATION	SAMPLING PROGRAM	CLOSURE REQUIREMENTS
Key Stations	Twice monthly Nov. 1 - May 1; weekly for balance of year	
Class I - shellfish rarely if ever toxic	Monitored when Class II areas are closed	closed if a single sample exceeds 80 μg*
Class II - shellfish free from PSP for long periods each year	weekly from June 1 to Nov. 1	closed if a single sample exceeds 80 μg*
	balance of year - any time two consecutive counts at key stations exceed 160 μg.	closed if two consecutive samples at the same location exceed 80 μg *
Class III - shellfish potentially dangerous all year	weekly from June 1 to Nov. 1	open for canning under permit; closed to canners if a single sample exceeds 160 μg
	balance of year - weekly in any area open to digging under permit for canning	closed to canners if two consecutive samples at the same location exceed 160 μg

* Open to canners under permit when score exceeds 80 μg, but is less than 160. Closed to canners if one sample exceeds 160 μg.

Sampling locations for long-term monitoring should be chosen carefully and marked in some way so that samplers may return to the same location each time.

Bay of Fundy Management Program

All shellfish areas in the danger zone of the Fundy have been classified and three locations, referred to as "key stations," have been selected. Experience has shown that the key stations begin accumulating poison about a week earlier than shellfish in other areas. The Bay of Fundy Management Program is summarized in Table 1.

Once an area has been closed it must be demonstrated to be consistently below 80 μg before reopening. For this purpose we require a minimum of two samples, one week apart, accompanied by a clear reduction in levels at the key station.

St. Lawrence Region Management Regime

The Quebec program is not as well defined as in the Fundy and the areas have not been fully classified. At present two areas are closed all year and

some 50 locations are sampled on a weekly, twice per month or monthly basis depending on the potential danger as established in previous years. A single sample from any area in excess of 80 μg results in closure of the area to all harvesting except for canning. A score in excess of 160 μg closes an area for all purposes. Openings are based on three consecutive samples below the specified limits.

British Columbia Management Program

The north coast of British Columbia from about the tip of Vancouver Island to the Alaska border has been closed for the past 10 years to the harvesting of mulluscan shellfish (except razor clams) because of PSP. In addition, a large section of the west coast of Vancouver Island is closed. Because of the length of coastline and the localized nature of PSP rises in shellfish, it was virtually impossible to develop an effective sampling program. This was amply demonstrated in 1972 when manila clams from Vancouver Island caused several poisonings in the United States and nine in Canada. While we are still striving to detect patterns and establish an effective area control program, we have had to take special precautions to allow continuation of the commercial fishery. Packers are required to notify the nearest Inspection Branch office of intent to ship so that shipments may be sampled (sometimes en route) and subjected to a mouse screening test. Since the result is available in a matter of hours, effective action can be taken where necessary to withhold nonconforming shellfish from the market.

Enforcement of Controls

The program is enforced by regulations under the Fisheries and Fish Inspection Acts. The federal Department of the Environment and the Department of Industry and Commerce in Quebec are responsible for instituting and lifting closures, advising the public and industry, posting warning notices, policing closures, and obtaining and extracting shellfish samples.

All extracts are shipped by air to Health and Welfare Canada laboratories in Ottawa, where one group of technicians carry out all bioassays and recommend closures or openings on the basis of the management plans.

Effectiveness and Problems of Control

Since control programs have been instituted there have been 236 illnesses and 10 deaths from PSP in Canada. Quebec has accounted for 107 illnesses and 9 deaths; British Columbia 82 and 1; and in the Fundy Region 47 illnesses and no deaths.

Less than 5% of the illnesses and none of the deaths have been caused by commercially produced shellfish. We have been much less successful in protecting picnickers and local residents who dig for their own use. The most

recent outbreak which occurred in Quebec this past summer is typical. There were 44 illnesses and one death. Investigation showed that all shellfish were taken from closed, posted areas and, in addition, affidavits have been obtained from local residents who warned some of the sufferers that the shellfish were dangerous. This is a familiar story to investigators. Despite their best efforts in publicizing danger areas in newspapers and over television and radio, the information is not reaching or impressing the individual who occasionally goes to the beach and digs his own shellfish.

The management methods described provide a short-range warning and we very badly need better forecasting methods. Long-range forecasting for the Funday Region was tried (7) using meteorological and hydrographic data. However, these and other biological factors interacted with such complexity that useful results were not obtained. Hopefully with increasing knowledge of factors affecting the abundance of *Gonyaulax*, long-range predictions will be possible.

This in itself will not solve the problem of the casual digger, and every avenue for publicizing the problem during danger periods must be utilized to the fullest.

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MANAGEMENT OF FLORIDA RED TIDES REGARDING SHELLFISH HARVESTING

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ABSTRACT

A management program regarding shellfish harvesting areas was established after the 1962 reported human illnesses associated with consumption of oysters in the Sarasota Bay area of Florida. These shellfish had become toxic during the penetration of *Gymnodinium breve* into this estuary.

Shellfish, as defined in the Florida Administrative Code, includes oysters, clams, mussels and scallops of edible species.

This management program includes a constant monitoring of shellfish harvesting areas, a surveillance program and control guidelines.

There are ten permanent monitoring stations that are sampled at least monthly, and more often if necessary. There are six additional monitoring stations sampled in the event a "red tide" alert is published. Surveillance is accomplished via reports from sports and commercial fishermen, county health department personnel, marine patrolmen, research laboratory personnel and private citizens. These people are informants regarding visible water discoloration, fish and bird kills. At first evidence of a "red tide bloom", or fish-bird kills, water samples are collected at sea, near shore and at the passages or inlets into the estuaries. If a "red tide" seems evident, an alert is published while the bloom is well offshore. If conditions, weather, tides, winds etc., indicate the "bloom" may move inshore, all waters in the affected area are closed to the harvesting of shellfish and shellfish meats are collected and analyzed for toxin content.

If routine shellfish meat analysis indicates *any* detectable level of toxin in accordance with our present protocol, the area is immediately closed to the harvesting of shellfish. The Florida Marine Patrol enforces this closure and sampling frequency is intensified in the area. The public is informed via newspaper, radio and television, and commercial shellfishermen are informed by official letter. When water samples are negative for toxin, the affected area is reopened for shellfish harvesting.

INTRODUCTION

Red tides or discolored waters have been documented throughout recorded history in virtually all parts of the world. The major cause of Florida's red tides was identified as *G. breve* by Dr. C. C. Davis (2) during the 1946-47 occurrence of red tide off the Southwest Coast of Florida. Evidence associating *G. breve* "red tides" with human illnesses was not accumulated until December, 1962, when several persons became ill after eating oysters and clams harvested from Sarasota Bay, Florida (4). Mr. E. F. McFarren analyzed nine oyster samples harvested from Sarasota and Lemon Bays for Ciguatera poison due to the similarity of Ciguatera poison and the poison found in the Sarasota-Lemon Bay oysters. These nine samples recorded poison ranging from four to 30 mouse units. The method of determining Ciguatera-like toxin(s) in shellfish presently utilized by the Florida Division of Health is incorporated in the Laboratory Manual published by the American Public Health Association (8).

FIGURE 1

A management program for Florida's historical "red tide" zone (Figure 1) was instituted in the summer of 1963. Since 1963, red tides of public health significance have occurred in areas outside the historical zone. The lower East Coast of Florida experienced a red tide during November, 1972 (5) as reported by Stanley I. Music *et al.* in a 1973 publication. Several complaints of eye and upper respiratory irritations were received. Shellfish, oysters and clams, collected from Lake Worth and St. Lucie Inlet were positive for Ciguatera-like toxin(s); however, these areas are permanently closed to shellfish harvesting. The Northwest Coast (Panhandle) of Florida experienced a "red tide" bloom the early part of September, 1974, necessitating the closure of these waters for shellfish harvesting. (1)

The management of Florida's red tide by the Florida Division of Health is directed toward "human health" rather than "resource health". The objective has been to prevent harvesting of shellfish during red tide outbreaks; however, this concept is not without problems. The vast areas affected and public disregard for warnings of danger make patrol of these areas very difficult. The human cases that occurred during the 1973 outbreak were associated with shellfish harvested from areas that are permanently closed to shellfish harvesting or areas that were officially closed during November, 1973 (3).

Routine Monitoring

Oyster and/or clam samples are collected and analyzed from ten permanent monitoring stations. Nine of these stations are located in the historical red tide zone and one is located in the Apalachicola Bay area. Samples are collected monthly during the open harvest season, September through May. In the event a "red tide" alert is published, six additional stations are sampled. These six additional stations are located within the historical zone. It is felt that this continuous monitoring program is necessary in that toxin may occur in shellfish in the presence of undetectable or low numbers of *G. breve* due to the ability of the shellfish to accumulate these organisms as reported by Ray and Aldrich (7).

Surveillance

All state and county health department personnel, Florida Marine Patrol, Research Laboratory personnel, private as well as Governmental, sports and commercial fishermen and private citizens act as sources of information regarding visible water discoloration or fish-bird kills. At the first evidence of a red tide bloom or fish-bird kills, water samples are collected for *G. breve* identification-cell count. Sampling points are at sea, near inshore and at passages or inlets into the estuarine waters. If analyses of these waters indicate a "red tide" is probable, an alert is published. This alert is published when the "bloom" is well offshore. Tides, wind direction, temperature and rainfall are closely monitored and water samples are analyzed daily. If weather conditions indicate that the bloom may move inshore, all waters in the affected area are closed to the harvesting of shellfish and shellfish meats are collected and analyzed for Ciguatera-like toxin(s).

Control

If routine analyses of shellfish meat indicate *any* detectable level of toxin per 100 grams of meat, the area is immediately closed to the harvesting of shellfish, regardless of presence or absence of "red tide".

The Florida Marine Patrol enforces this closure and the sampling frequency is intensified in the area, as well as waters contiguous to the affected area. The public is informed of the closure via newspaper, radio, television and word-of-mouth. Commercial shellfish harvesters-plant operators are informed by official letter or hand-delivered notice.

The Marine Research and Technology Laboratory of the Florida Department of Natural Resources continuously monitors the affected area for the presence of *G. breve* and the Mote Marine Laboratory also monitors waters within the historical "red tide" zone. Data from the Mote Laboratory is also made available to state agencies.

The closed area is normally reopened to shellfish harvesting when water samples are negative for *G. breve* cells and two consecutive shellfish meat samples are negative for Ciguatera-like toxin(s).

Approximately 600,000 acres of water are located within the historical red tide zone which has been closed to the harvesting of shellfish since November, 1973. Approximately 280,000 acres of water were closed to shellfish harvesting during the September, 1974, red tide occurrence in the Northwest section of Florida.

The control and management of Florida's shellfish harvesting areas regarding shellfish toxins is in compliance with the National Shellfish Sanitation Program (6) and has been so evaluated by the Federal agency having responsibility in this program since its inception in 1963.

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THE PUBLIC HEALTH IMPLICATIONS OF *GYMNODINIUM BREVE* RED TIDES, A REVIEW OF THE LITERATURE AND RECENT EVENTS

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ABSTRACT

The world's scientific literature provides virtually no discussion of *Gymnodinium breve* and related public health problems. The recent 1973-74 *Gymnodinium breve* red tide affecting the southwestern coast of Florida has provided experience which highlights three definable areas of health hazard posed by such red tide exposure. These include neurotoxic shellfish poisoning, respiratory irritation and contact irritation. A fourth possible red tide-related effect, namely hematologic pathology, has also been considered.

NEUROTOXIC SHELLFISH POISONING

The year 1844 marks the earliest written documentation of a Florida Red Tide (1). However, it was not until 1880-81 that the poisonous nature of shellfish collected during a red tide was noted (2). Excepting a report in the 1954 lay press (3), more than 80 years transpired without scientific discussion of neurotoxic shellfish poisoning. The silence was broken in 1965 when McFarren *et al* (4) provided the first substantial evidence supporting the suspected relationship between *G. breve* red tides and human illness. They reported five well-documented cases and 14 less well documented cases of human neurotoxic shellfish poisoning. The described symptom complex consisted primarily of numbness and tingling about the face, hands, and feet. A standardized mouse bioassay system using shellfish extracts was developed and a very limited attempt to correlate symptoms with doses of ingested toxin was made (Table 1).

On the basis of solubility properties and the observed reactions of cats and mice fed or injected with the extract, these investigators concluded that this toxin and ciguatera toxin were very similar. Subsequently, *G. breve* neurotoxin has been described as "ciguatera-like". It is now clear that there are some aspects of ciguatera toxin and intoxication that are quite unlike those of *G. breve*. For instance, in ciguatera poisoning the neurotoxin is present and assayable in the flesh of poisoned fish. Conversely, no fish or fish parts exposed to or killed by a Florida red tide have had detectable levels of neurotoxin when mouse bioassay studies have been performed (5). Secondly, a striking skin eruption with generalized pruritis is common with ciguatera intoxication. This has not been observed in *G. breve* neurotoxic shellfish poisoning. And finally, the convalescent phase from ciguatera toxin may be as long as several months to years which is in marked contrast to the recovery period observed in the syndrome described by McFarren or in more recently observed cases described below.

In addition to ciguatera poisoning, *G. breve* toxin effects have been confused with those effects and the toxin produced by another dinoflagellate genus, *Gonyaulax*. Species of *Gonyaulax* produce a toxin which is physically, chemically and pharmacologically distinct from *G. breve* toxin and is responsible for causing what is commonly called paralytic shellfish poisoning

Table 1

Correlation of Human Illness
With Toxicity to Mice[1]

Human Cases	Number shellfish eaten	Toxicity in mouse units		Severity of illness
		per 100 g	Total eaten	
A,B,C & D	15-20	135	405-540	mild
E	4-5 doz.	100	960-1200	mild
F	7	65	91	---
G	----	65	---	---

[1]from: McFarren *et al.* (4).

(PSP). PSP-causing *Gonyaulax* species are not found in Florida's coastal waters. Furthermore, the *Gymnodinium* toxin seems far less potent than the *Gonyaulax* toxin responsible for PSP, the latter having caused nearly two thousand recorded human PSP cases with a 15 to 20% mortality (6, 7).

1973-74 CASES OF NEUROTOXIC SHELLFISH POISONING

The 1973-74 Florida red tide was associated with three episodes of human neurotoxic shellfish poisoning. A total of 11 people were exposed, of which six developed symptomatology. The first episode occurred in November 1973 when two young boys, ages 10 and 12, consumed steamed Atlantic surf clams (*Spisula solidissma raveneli*) from the Gulf side of Siesta Key near Sarasota, Florida. Shortly after their meal the boys began experiencing mild stomach ache, headache and paresthesias which were described as feeling like "cold rain". Both boys eventually developed unsteadiness on their feet and the youngest boy's symptoms progressed to include convulsions, coma, decerebrate posturing and finally respiratory arrest requiring mechanical ventilatory assistance in an intensive care unit.

The second episode also occurred in November 1973 when steamed southern quahog clams (*Mercenaria campechiensis*) which had been harvested in Sarasota Bay were consumed by five people at a family meal. Three individuals developed symptoms ranging from tingling toes in one individual to pronounced total body paresthesias, severe abdominal cramps, loss of balance and a sensation of paralysis in another individual who was hospitalized.

The third episode occurred in March 1974 when four individuals consumed well cooked southern quahog clams which had been harvested in estuarine waters near Englewood, Florida. One individual developed a strange "plastic" sensation in his face a few hours after the meal followed by severe crampy pain in his abdomen and legs. His symptom complex was obscured by previous consumption of a quantity of beer, smoking marijuana and the development of hyperventilation syndrome. This person was also hospitalized.

All 11 exposed individuals were interviewed and the three hospital records were studied in detail. There was no clinical evidence of anticholinesterase activity and no unusual cardiovascular effects were observed. Specifically, bradycardias, arrhythmias and blood pressure abnormalities were not noted. All recovered without residual effects.

After all three episodes, clams were either obtained from the lots used in meal preparation or harvested at the original site of harvest and analyzed for the presence of neurotoxin at the Florida State Division of Health Laboratories.

Given the individuals' weight, number of shellfish consumed and shellfish toxicity expressed in mouse units, one can roughly determine the dose of toxin per kg of body weight as seen in Table 2. Cases A & B represent the two young

Table 2

Data Table

Case	Weight in Kg	Number shellfish eaten	Toxicity in Mouse Units*		Dose MU/Kg
			per 100 g	Total eaten	
A	30	5-7	75	94-131	3.1-4.4
B	36.4	5-7	75	94-131	2.6-3.6
C	70.5	6-8 doz.	96	691-922	9.8-13.1
D	54.5	3-4 doz.	96	346-461	6.3-8.5
E	68.2	3-4 doz.	96	346-461	5.1-6.8
F	65.9	2 doz.	96	230	3.5
G	59.1	4-5	96	38-48	0.6-0.8
H	78.2	2 doz.	118	470-708	6.0-9.1
I	77.3	1	118	20-30	0.3-0.4
J	61.4	1	118	20-30	0.3-0.5
K	81.8	10	118	196-295	2.4-3.6

* Determined by the Bureau of Laboratories, Florida State Division of Health.

boys. C,D,E,F and G are those people exposed at the family meals. H,I,J and K are those people exposed in the third episode described. These data suffer from the following limitations:

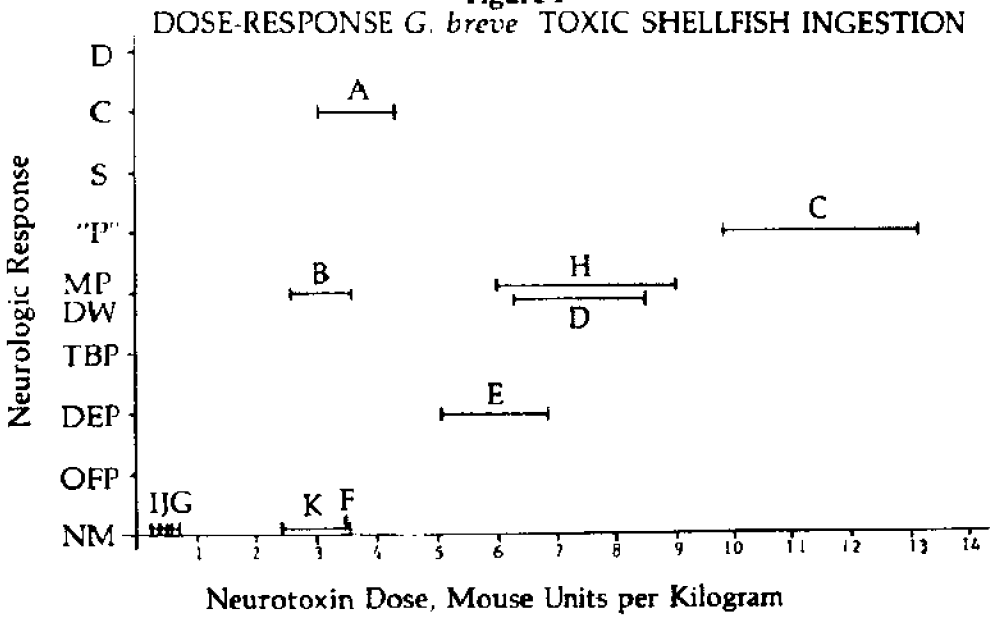
1) The reported number of shellfish ingested came from recollection.
2) In some of the initial assays the number of clams comprising 100 gms of assayed flesh was not precisely determined.
3) For the first episode involving the young boys, since they had consumed all of the clams they had collected, the only clams available for assay were collected two days after the poisoning episode at the original site of the boys' harvest.

However, accepting these criticisms and using an arbitrarily defined graduation of neurologic response as seen in Table 3, it is possible to construct a rough dose-response curve as illustrated in Figure 1. In this Figure, wherever

Table 3.
Neurologic Responses
by
Increasing Severity

NO MANIFESTATIONS	NM
ORAL FACIAL PARESTHESIA	OFP
DISTAL EXTREMITY PARESTHESIA	DEP
TOTAL BODY PARESTHESIA	TBP
MUSCLE PAIN/DIFFICULTY WALKING	MP/DW
"PARALYSIS"	"P"
SEIZURE	S
COMA	C
DEATH	D

Figure 1



a case is represented that case usually experienced all of the symptoms of lesser severity listed below it. For example, case C not only experienced a sensation of being paralyzed, but complained also of difficulty walking, muscle pain and paresthesias. "Paralysis" is set in quotation marks because it is doubtful that actual paralysis occurred, but instead that the person felt paralyzed, his sensory input having been impaired by the neurotoxin.

A pattern is clear if one excludes cases A and B. Such an exclusion might be justified by the following:

1) The ingested dose calculation in this exposure is the weakest calculation of all since clams prepared for the meal were not available for assay. The calculation was based on assayed clams collected at the original harvest site two days after the episode.
2) In contrast to the other case, these two individuals ate only clams with no side dishes approximately ten hours after their previous meal. These factors may have served to enhance toxin absorption.
3) These two cases were the only children involved in any of the episodes.
4) The implicated clams in this episode were Gulf of Mexico species as opposed to estuarine species.

As for the boy who nearly died, perhaps there was something unusual about one of the clams he consumed or perhaps this represented an idiosyncratic reaction for this particular individual. It is possible that he had some hidden predisposing factors which, taken in combination with *G. breve* toxin, triggered his severe life-threatening affliction.

RESPIRATORY IRRITANT

The first written report of respiratory irritation associated with exposure to a Florida Red Tide was made in 1917 (8), and subsequently confirmed by others (3, 9, 10, 11, 12). It is postulated that when a *G. breve* bloom comes close to the shore and is caught in a high energy surf situation, that the easily disintegrated *G. breve* organisms may be aerosolized and swept through the air by prevailing winds to the beach. Only one very limited scientific attempt has been made at characterization of the irritant (11).

A unique set of meteorologic and hydrographic circumstances led to the unusual presence of a *G. breve* red tide off the southeastern coast of Florida in the fall of 1972. The respiratory irritation was well described (12) and agrees with the 1973-74 red tide experience. Literally thousands of people on or near beach areas have been affected. The irritant appears to be virtually odorless and the attack is initiated by paroxysmal coughing with tearing and rhinorrhea from irritated eyes and nasal passages. Resolution occurs almost immediately upon leaving the beach area, entering an air conditioned enclosure or by filtering the inhaled air through a surgical mask or bandana. The clinical reaction would not appear to be an allergic phenomenon but is more like an immediate contact/chemical irritation. No chronic or permanent disability has been documented.

There has been virtually no basic research or controlled laboratory studies directed at this problem. Galtsoff summed it up in 1948 by stating, "Of greatest interest is, of course, the question of whether the poison that kills fish is identical with the airborne substance causing coughing and other respiratory difficulties in humans. The public health aspects of the red water problem fully justify an extensive investigation along these lines (8)." It is ironic that we know the least about that aspect of the Florida red tide problem which poses the greatest public health hazard in terms of number of people affected.

CONTACT IRRITATION

There have been many anecdotal reports over the years of people suffering from contract dermatitis or conjunctivitis following exposure to *G. breve* affected waters. The skin irritation is real; however, there is considerable variation from individual to individual. Most people have no symptoms at all while the majority of those with symptoms describe a mild pruritis. No one has been reported as having been seriously affected by a dermatitis. The severity of the conjunctivitis can be striking and appears to be clearly related to the intensity of the exposure. Swimmers, skin divers, surfers and people wading and living along the beach have been questioned and no one describes any permanent disabling or chronic effects.

POSSIBLE HEMATOLOGIC ABNORMALITIES

Apparent coagulation abnormalities were noted in fish affected by Florida red tide as early as 1917 (8). More recently there have been reports of hemolytic activity present in *G. breve* extracts (13, 14). Others have attributed an anticoagulant property to *G. breve* toxin (15). During the spring of 1974 marine pathologists at the Florida Department of Natural Resources Marine Research Laboratory, while performing necropsies on dead fish and ducks, discovered hematologic abnormalities consistent with a chronic hemolytic anemia and consumptive coagulopathy (16).

Recognizing the importance of these findings an assessment was made of the possibility of a similar abnormality being present in human beings chronically exposed to the *G. breve* elements. Five individuals who had been significantly exposed for prolonged periods of time to the red tide aerosol in Pinellas County were bled. All of these people had normal complete blood counts, reticulocyte counts, platelet counts, and coagulation studies. Furthermore, the hospitalized victims of neurotoxic shellfish poisoning described above also had normal complete blood counts.

SUMMARY

The public health problems associated with *G. breve* red tides are of no small magnitude. Many known effects are very poorly understood and the possibility of other heretofore undiscovered significant metabolic and physiologic effects remains.

Gymnodinium breve red tides in Florida's coastal waters are apparently here to stay, and represent a natural phenomenon which remains equally as undisciplined as hurricanes and earthquakes. The posed public health questions will remain and loom ever larger as Florida's human population continues to increase along the southwestern coast. The technical wherewithal to get at these problems is well developed. Is not now the time to ply our trade and eliminate the unknown elements of *G. breve* toxin exposure?

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A PRELIMINARY INVESTIGATION OF THE ECONOMIC EFFECTS OF THE RED TIDE OF 1973-1974 ON THE WEST COAST OF FLORIDA

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ABSTRACT

The bloom of the toxic dinoflagellate, *Gymnodinium breve*, which persisted from 1973 to 1974 inflicted extensive economic losses on seven counties on the west coast of Florida. An approximate assessment of the damage can be made by an evaluation of information from many diverse sources. The reports published by Florida's Departments of Commerce, Business Regulation, and Revenue contain pertinent data. The monthly statements "Trends of Business of Florida Hotels and Motor Hotels", issued by Laventhol, Krekstein, Horwath and Horwath, provide an excellent measure of the fluctuation in numbers of tourists. Equally valuable are personal interviews with shore-based businessmen, commercial fishermen, and members of Chambers of Commerce of the cities involved. The authors' former publication (5) has served as a standard for comparison.

During the course of the 1973-1974 red tide, two factors, a shortage of gasoline and increasingly high interest rates, played havoc with the economy of the west coast and masked losses attributable to the red tide alone. The east coast of Florida suffered from both the gasoline shortage and high interest rates but NOT from the red tide and, to a limited degree, has served as a control to give a better perspective to the assessment of the damage due solely to the red tide on Florida's west coast.

The data collected so far indicate that the Florida red tide of 1973-1974 inflicted on the tourist industry alone a loss conservatively set at $15,000,000. Complete data are not yet available on the losses the red tide imposed on commercial fishing, real estate, and construction. Current information indicates that these losses could equal if not exceed those suffered by the tourist industry.

INTRODUCTION

Since 1971 the west coast of Florida has experienced two red tides. The causative agent, *Gymnodinium breve*, unlike some dinoflagellates, is only rarely implicated in shellfish poisoning (1). Other direct effects are, however, so unpleasant that people shun the beaches. The air becomes laden with toxic particles which irritate the eyes and respiratory membranes and the shore becomes covered with rotting fish. Indirect, and far more deleterious, effects are experienced by the economies of the areas involved (2). Tourism decreases sharply, boating and water sports practically cease, both commercial and sports fishing come to a virtual standstill, real estate sales diminish, and construction is hurt. In addition, the counties must pay the costs of beach cleanup.

METHODS

To put a dollar value on these damages, data were collected from many sources, including the publications of several departments of the state of Florida (2, 3, 4). Reliable facts were received from a certified public accountant firm which services the hotel/motel industry in Florida. Most informative have been personal interviews with many in the affected areas - businessmen, officials of local governments, and members of the Chambers of Commerce. Our recent publication dealing with 1971 red tide (5) has served as a basis for comparison for the present investigation of the 1973-1974 red tide's economic effects. The final estimate of damages, however, must await the publication of annual statistics. The two red tides differed considerably both in the time and extent of occurrence as well as in the kind and extent of damages to the economy.

THE 1971 RED TIDE

The 1971 red tide was of moderate severity, short duration, and widespread publicity. Progressing from south to north it successively struck seven counties on the west coast of Florida in rather contained but overlapping time frames. Those counties were Collier, Lee, Charlotte, Sarasota, Manatee, Pinellas, and Hillsborough. It started in early June, was completely over in early August, but unfortunately, the media which gave it such intense and extended coverage, neglected to publicize its termination and consequently adverse economic effects continued for many weeks after it was over.

The 1971 red tide occurred during the summer months when the usual hotel/motel occupancy is 66% of capacity, when rates are 50% less than in winter, when many gift shops are closed or open fewer hours, and when the whole economy is geared to a slower pace (2). We estimated that about 7000 units were vacant in the summer of '71 because of the Red Tide and this resulted in a loss of approximately $6,000,000 for hotels and motels. The cost of living quarters is only 33% of the average tourist's total outlay, according to a 1970 survey by the Florida Department of Commerce(3). About two-thirds is spent on all his other expenditures: food and drink in restaurants and in retail stores, amusements, clothing and footwear, gas, pharmaceuticals, gifts, tobacco, photo supplies, etc. Since the $6,000,000 loss to landlords represented only one-third, the total loss to the tourist industry was about three times $6,000,000 or $18,000,000. In actuality we found it was $18,500,000. One and one-half million dollars more were added in losses to the purveyors of hotel/motel supplies and commercial fisheries (7) and in the expense of beach cleanup which brought the total costs of the 1971 Florida Red Tide to $20,000,000.

THE 1973-1974 RED TIDE

The Red Tide of 1973-1974 on the west coast of Florida was almost open-ended, breaking out sporadically and capriciously, widespread in time

and place. It was first noticed on October 22, 1973, 8 - 10 miles offshore between St. Petersburg and Longboat Key. The first shoreline involvement was a massive fish kill at Boca Grande on October 29, 1973. Nearly a year later in September of 1974 four fish kills occurred on the northwest Florida coast between Panama City and Pensacola. While generally moderate, some excessive counts of *Gymnodinium breve* occurred in small patches. Twenty-two million/liter were reported at Boca Ciega Canal near Redington Beach on April 3. Warm Gulf waters were blamed for its continual reappearance through the winter. In contrast to the red tide of 1971 this one occurred predominantly in the winter months when tourism is normally at its peak. Fortunately national publicity was minimal so the economic damage wrought is due solely to its prolonged severity.

HEALTH PROBLEMS

Health problems attributable to the red tide toxins have been both more frequent and more acute than in 1971. Complaints of nose and throat irritations were common. The principal of the Anna Maria Elementary School blamed higher absenteeism on allergic reactions to the red tide. Shellfish poisoning from high concentrations of *Gymnodinium breve* in clams nearly took the life of a ten-year-old Sarasota boy and hospitalized several others. Dr. John McGarry, then Sarasota County Health Officer, speculated that the prolonged exposure of shellfish to *Gymnodinium breve* might cause more serious problems than previously thought possible. In contrast health problems stemming from the '71 red tide were chiefly in the nuisance category.

CLEANUP COSTS

Cleanup costs will undoubtedly exceed those of 1971. From newspaper reports we learned that in the city of St. Petersburg alone from February 18 to March 18 as many as 150 men worked in 8-hour shifts to clean up the beaches. On April 16 a St. Petersburg official estimated the city had spent $160,000 on beach cleanup.

FISHING LOSSES

Both commercial and sports fishermen were seriously affected by this red tide. Sportsfishing was off 50% at Indian Rocks Beach. Charter boat captains in Clearwater reported on April 2 that their business was off 100%.

The Clearwater harbormaster said on April 2 that commercial fishing piers were going unused. On the same day John's Pass Seafood Company reported that both wholesale and retail sales were hurt. In restaurants very few customers ordered fish. Ironically all through this period fishing offshore was excellent and the fish served in restaurants was as safe and delicious as ever.

CHANGES SINCE 1971

Not only did the two red tides differ markedly, but the west coast of Florida had itself undergone considerable changes. Building had increased immensely between 1971 and 1974. The number of hotel/motel units was up 25%. Condominiums and shorebased apartments had mushroomed. Construction and the real estate business, both non-factors in the survey of the 1971 red tide, will be major factors in this one.

MASKING FACTORS

New elements threatened the economy. Rumors of an energy crisis which started in the summer of 1973 erupted in a severe gasoline shortage in early 1974. Interest rates increased steeply and had a marked effect on the economy. Neither of these figured in the 1971 red tide survey.

LOSSES TO THE TOURIST INDUSTRY

Thanks to monthly statements of hotel/motel trends (6), a preliminary and very tentative assessment was made of the losses incurred by the tourist industry during the three months, January, February, and March of this past winter.

The gasoline shortage made its greatest impact on hotels and motels located in central Florida and the northeastern and northwestern coasts of Florida, according to the trends in business reports. Tourists normally arrive in these areas by car while hotel/motel patrons of Florida's west coast and the Miami Beach and Keys areas more often arrive by airplane (5). The gasoline shortage evidently affected both equally. Both were also equally affected by high interest rates and the resultant economic malaise, so the big difference in the two areas in the winter of 1974 was the red tide, present only on the west coast of Florida. When monthly hotel/motel occupancies were compared with the prior year, both areas were down in January, -8% for Florida's west coast, -9% for Miami Beach and the Keys. The west coast continued down in monthly vacancies and stayed down almost on the same plateau, -7% for February, -8% for March but the Miami Beach and Keys areas recovered to -2% in February and +8% in March (5).

In early 1974 there were approximately 40,000 shore-based hotel/motel units in the seven counties on the west coast of Florida. We conservatively estimated that 5% or 2000 of these were vacant because of the Red Tide. The average rate per day was $28 so the daily loss was 2000 times $28 or $56,000/day which over a 90-day or 3-month period brought the loss to about $5,000,000. Since the cost of lodging is about 1/3 the total expenditure of the average tourist, the total loss to the tourist industry was three times $5,000,000 or $15,000,000 for the 3-month period. This is only one part and perhaps a minor part of the losses due to the 1973-1974 red tide on the west coast of Florida.

REAL ESTATE AND CONSTRUCTION LOSSES

From private interviews we learned that real estate losses will unquestionably be very high. Many residents, recent retirees, have experienced two Red Tides and are moving away from the west coast of Florida. Initially they had chosen this area as their permanent homes but now "want out" for reasons of health, discomfort, or lessened ability to rent where extra income is needed. A salesman of condominiums in a large complex of shore-based apartments remarked that he had returned deposits to over 30% of the buyers who had bought in the fall of 1973 and returned in February or March of 1974 to check on construction. When they encountered the noxious fumes and decaying fish of the red tide they concluded they had made a mistake and demanded their money back. If the average condominium sells for $35,000 - $40,000, every 100 non-sales means there is 3 1/2 to 4 million dollars worth of unsold real estate.

Along the beaches expansion has been vertical. Skyscrapers have made it possible to make living units out of space formerly occupied only by the gulls. In many areas there are 100 or more units along every 1/2 mile of beach frontage. Undoubtedly high interest rates are the major villain in losses to construction and real estate but if the red tide is responsible for even 5% of the unsold property, it could result in figures which would dwarf the losses of the tourist industry.

CONCLUSION

The extensive losses to the economy wrought by the red tide suggest that money spent in red tide research will prove to be a very sound investment.

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THE ECONOMIC HALO OF A RED TIDE *

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ABSTRACT

The impact of a "red tide" is not always restricted to the immediate area of the outbreak. The New England red tide caused by *Gonyaulax tamarensis* in September, 1972 is a case in point. The presence of this organism created havoc in the New England fishing industry. As a public health safety measure, shellfish from New England waters, including soft clams, hard clams, mussels, and bay scallops were removed from the market. But the halo effect of the outbreak hurt the seafood industry in other states, including New York. Although *G. tamarensis* did not bloom in New York waters, the publicity from New England created buyer resistance in New York to hard clams and other shellfish. The halo effect touched almost all seafood as the buying public over-reacted. Consumers avoided hard clams, both bay and sea scallops, lobsters, and finfish. The paper describes in detail the halo effect of red tide outbreaks, the economic implications to the seafood industry, and alleviating programs established by regulatory agencies.

INTRODUCTION

"Food scares" generated by botulism poisoning, pesticide residues, heavy metals, additives, and gross contaminants, frequently affect foods other than those directly concerned. Nowhere is the scare phenomenon more prevalent than in the seafood industry, particularly that segment which deals with shellfish. Stories linking shellfish and contaminated waters are sometimes blown out of proportion by rumors that are intensified by press headlines and proclamations issued by people who overreact to the situation. When there is genuine reason to be concerned about the safety and wholesomeness of a shellfish product, a "halo effect" often touches seafood items remotely associated with the product in question. The 1972 red tide and the occurrence of paralytic shellfish poisoning (PSP) in New England and its affect on New York's shellfish industry is an excellent illustration of this.

NEW ENGLAND RED TIDE - 1972

About the middle of September 1972, a bloom of the dinoflagellate *Gonyaulax tamarensis* was reported in coastal waters from Maine to Massachusetts. Since this organism has been implicated in PSP, officials in the affected states banned the harvest and sale of soft clams (*Mya arenaria*), hard clams (*Mercenaria mercenaria*), and mussels (*Mytilus edulis*), shellfish found to be carriers of the toxin. Bay scallops (*Aequipecten irradians*) were briefly included in the ban but later were restored to the market. Connecticut and New York, as a prophylactic measure, halted the importation and sale of shellfish from the affected states.

This red tide was unusual in several ways. It evidently began, and the heaviest bloom was found, between Cape Ann, Mass., and mid-coast Maine. Traces of the bloom were found as far south as Chatham, Mass., on Cape

Cod. (Blooms of *G. tamarensis* and PSP usually occur in the coastal waters of northern Maine and the Canadian Maritimes.)

The fortuitous collection of oceanographic and meteorologic data for another purpose in 1972 led to conclusions about the causative factors of the bloom. It was believed to have resulted from: (1) the upwelling of nutrients which triggered the bloom (2) dry August weather which allowed the resident population to multiply in the upwelled water, and (3) heavy rainfall with resulting nutrient-laden runoff. The offshore populations of the dinoflagellate then were moved by the upwelling water into the estuaries where they were ingested by the clams and other shellfish.

Effect on New England Shellfisheries

The National Shellfish Sanitation Program of the U.S. Public Health Service requires that state agencies shall close shellfish grounds when the PSP toxin in shellfish reaches 80 micrograms (μg) per 100 grams (g) of the edible portions of raw shellfish meat. On September 19, 1972, clams gathered north of Cape Ann, Mass., were found to contain up to 9000 μg per 100 g of meat.

The shellfish ban varied in length in the different New England states from about 2 weeks in Maine to over a month in Massachusetts. During the time that soft clam flats in Maine were closed, the diggers were forced to draw on savings to support themselves. Later, the red tide was termed a disaster, which made the diggers eligible for $47 per week unemployment insurance. (In contrast, a good digger could earn about $27 to $36 per day harvesting soft clams during the season.) Dealers were forced to destroy stocks on hand and in some instances this amounted to several thousand dollars worth of shellfish.

Although the ban was imposed on soft clams, mussels and hard clams, public reaction to the situation caused consumers to avoid fish, lobsters (*Homarus americanus*), sea scallops (*Placopecten magellanicus*), and northern shrimp (*Pandalus borealis*). None of these organisms has been linked with PSP. Loss to the fisherman was estimated by the National Marine Fisheries Service (NMFS) to be more than $1 million because of adverse publicity that depressed the market. The Shellfish Institute of North America (SINA), reported that news releases issued by the U. S. Food and Drug Administration (FDA) were not always properly quoted by the news media. For example, species unaffected by the red tide were not included in published news articles although they were named in the original release.

PSP Cases

The first evidence of PSP was the occurrence of dying gulls and other birds on September 14. The cause of the deaths at first was thought to be pesticide poisoning but PSP also was suspected. Symptomatology details of PSP were immediately distributed to area hospitals and the first human case was reported that same day. The total number of humans suffering from PSP has been estimated between 13 and 30; probably some victims were never reported. There were no fatalities.

NEW YORK'S SHELLFISHERIES AND THE RED TIDE

In 1973, New York's commercial fishing industry landed 16,384 metric tons of a variety of fresh fishery products worth $21.8 million at the dock. The industry is geared to producing high-quality items for the retail and restaurant trade. The shellfish portion of the industry produces slightly less than half the volume but nearly 80 percent of the landed value of the total. About 50 percent of the hard clams produced in the United States are harvested in New York's marine waters. Thus, the entire seafood industry is particularly sensitive to factors that influence the market not only in the northeast region but, with shellfish, also in the nation.

Reaction to the New England Red Tide

When the New England states banned the harvest and shipment of shellfish because of the threat of PSP, New York quickly placed an embargo on the shipment of shellfish from the affected states. (The State adheres to the National Shellfish Sanitation Program.) The embargo was made under the provision of Section 13-0309 of the N.Y. Environmental Conservation Law. In addition, high level officials of the N.Y. State Departments of Environmental Conservation and Health maintained frequent telephone communication with their counterparts in Massachusetts to keep abreast of the situation.

Despite the fact that there was no evidence of a bloom of *G. tamarensis* or PSP in N.Y. waters, public reaction to the news from New England was marked by an avoidance of several fishery products, particularly hard clams. A small survey of local businessmen who buy or sell seafood elicited the following reactions:

1. Some lobster fishermen were unable to sell their catches.

2. Wholesalers of shellfish and finfish reported business decreases of 25 percent to 50 percent.

3. A restaurant supplier said that business in Long Island seafood restaurants was off by up to 50 percent.

4. The wholesale price in Fulton Fish Market (the major wholesale outlet for fishery products located in New York City) for clams from Long Island and other unaffected areas was down about 25 percent.

These comments admittedly are quite subjective and when viewed in the light of the data available, the true situation does not appear to have been that bleak. Figure 1 (data in Table 1) is a comparison of landings of hard clams from New York waters for 1972 and 1973 with the 5-year average for 1967-1971. The landings for 1972 were uniformly higher than the average and

suggest a banner year was in store for the industry. The sharp decline from the landings in August to the landings for September is coincidental with the PSP cases in New England. There usually is a decrease during this period which reflects a reduction in effort as young people leave the fishery to return to schools and colleges. However, the very sharp decline in that period in 1972 suggests that it was more than just the usual drop in effort and that probably the PSP publicity contributed to a reduction in consumer demand and a resulting drop in landings to satisfy the shrunken market.

The trend of the 1973 landings is the same as the 5-year average but at a generally lower rate. There had been some local reports and newspaper articles in July and August that year about "red tides" in Long Island waters which may account for the departure from the average landings during that period. Investigations by the author and his staff revealed no blooms of toxic dinoflagellates in the reported 1973 red tides.

Red Tides in New York Waters

Phytoplankton studies by several investigators in New York waters failed to find *G. tamarensis* in the marine flora. It is characterized as a coldwater species and probably it could not bloom in the local waters which at most reach 22° C in Long Island Sound in August and higher in the bays and harbors where shellfish are harvested. From time to time, red tides are reported in New York

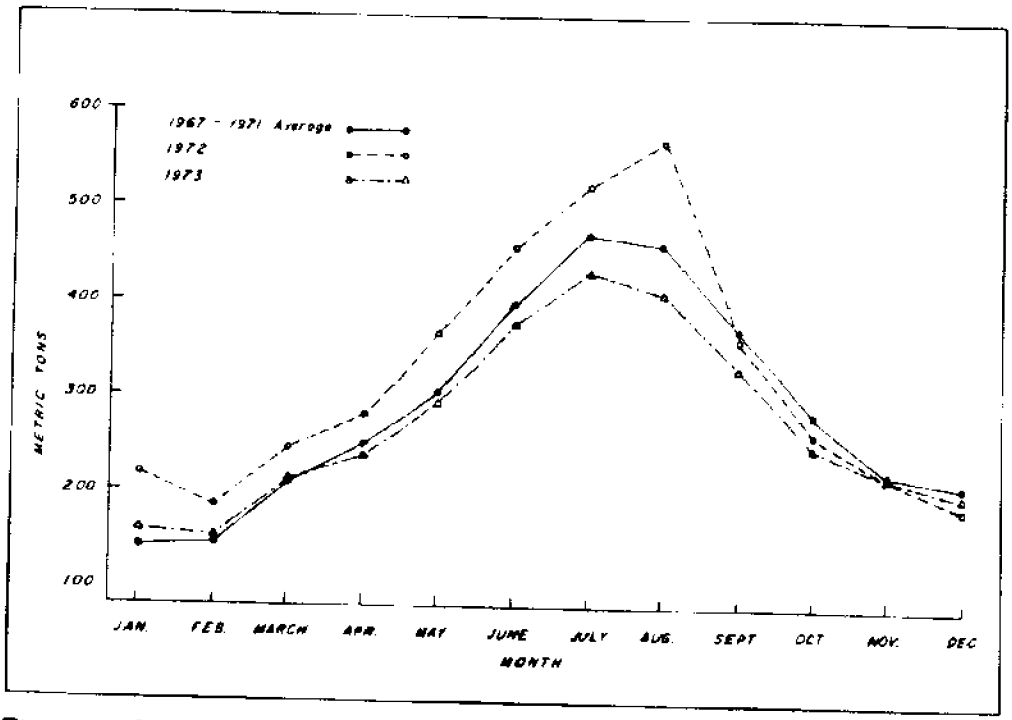

Figure 1. Monthly landings of hard clam meats from New York waters for 1967-1973 and the average for 1967-1971.

Table 1

NEW YORK LANDINGS OF HARD CLAMS, 1967-1973

(Metric Tons)

Year	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total	Value ($ Million)
1967	187	145	210	220	258	341	426	437	316	254	212	204	3210	7.1
1968	116	114	218	242	279	345	428	443	342	269	199	179	3174	7.0
1969	151	169	222	264	298	371	419	460	364	285	215	198	3416	7.3
1970	129	156	203	259	329	402	531	468	371	289	239	215	3591	9.0
1971	122	125	191	268	352	530	543	495	452	308	247	251	3884	10.7
1972	218	185	244	279	364	458	523	568	360	260	220	184	3863	13.2
1973	161	154	213	237	294	379	437	411	336	251	220	198	3291	10.9
Average (1967-1971)	141	142	209	251	303	398	469	461	369	281	222	209	3455	8.2

waters, usually in the embayments of Long Island Sound. I have investigated several of them which had achieved the characteristic color often described as "diluted tomato soup". One bloom, sampled on August 4, 1972, had a well-mixed and diversified planktonic flora that included the following genera:

Diatoms	*Dinoflagellates*
Thalassiosira	*Gonyaulax*
Chaetoceros	*Peridinium*
Navicula	*Ceratium*

None of the organisms was found in concentrations greater than 1,000 cells per liter and no *G. tamarensis* was found. Juvenile silversides (*Menidia menidia*) swam about in the bloom with no apparent ill effects and the technician collecting the water samples immersed his bare legs in the bloom with no discomfort. Occasionally, large numbers of menhaden (*Brevoortia tyrranus*) die off coincidentally with the blooms but analysis of the water usually shows very low levels of dissolved oxygen (ca. 0.5 to 2.0 ppm) which probably is the cause of the die-off.

IMPROVING THE INFORMATIONAL SYSTEM

The rumors and misinformation that were generated by the 1972 New England red tide and PSP outbreak clearly demonstrated that an informational system was needed to provide governmental agencies and the public with factual, accurate, and up-to-date information. The regulatory agencies and the shellfish industry cooperated closely in the information efforts.

Industry Efforts

The seafood industry moved quickly to provide the best available information to its members and to the new media. Special issues of informative newsletters were mailed by SINA to its members. In addition, officers of the organization met with Federal officials to exchange accurate information. SINA also issued news releases but these were not always used. Full page advertisements were taken out by industry in local newspapers to bring the message directly to the public (Figure 2).

Agency Efforts

The N. Y. State Department of Environmental Conservation has the responsibility for protecting the consumer from unwholesome seafood products and for assisting the seafood industry to produce the best products in sufficient volume consistent with sound fishery conservation principles. After

An Old-Fashioned Yankee Cusses:

The red tide has given us a black eye and we're darned mad!

We can tell you, unequivocally, that the algae invasion of coastal New England waters, called the Red Tide, has nothing to do with Snow's Clam Products.

Absolutely nothing.

But we've suffered because of it. The sale of our wholesome and nutritious products has been hurt. They have even been removed from some grocery shelves by mistake.

We are not interested here in examining who is responsible or how the scare led to confusion between fresh, frozen and canned products.

We are, however, justifiably irate.

None of our products come from coastal waters. They are taken from deep sea fishing grounds, an area untouched by and not expected to be affected by this problem.

All Snow's Clam Products belong back on the grocer's shelf, and on yours too.

If you have any doubts in this matter, please feel free to ask your grocer.

He'll be the first to reassure you.

Thank you.

Snow's Clam Products

There's a Streak of Yankee Cussedness In Every Can (But No Red Tide)

Figure 2. An industry-sponsored newspaper advertisement from the *Long Island Press* (N.Y.) issued to counter unfavorable publicity in the 1972 PSP outbreak.

determining that the 1972 PSP outbreak was not affecting local shellfish, the author prepared a news release that was issued by the Department under the name of the Commissioner. It was widely used by local newspapers and radio stations. The release explained the current situation and explained the safety of locally-produced shellfish and the efforts of the Department to safeguard the consumer. I also spoke directly with The Long Island Caterers and Restaurant Association, providing them with factual information to use in their own publicity releases. In addition, the Regional Office of the Department at Stony Brook distributed a list of Department officials to be notified in emergency situations affecting public health and safety in relation to shellfish. These persons also serve as sources of information for the news media, the public, and regulatory agencies in other states and in the Federal government.

Communications Alert and Liaison System

In 1974, NMFS established the Communications Alert and Liaison System (CALL) to provide a way for prompt sorting and dissemination of facts during times when fishery products may be linked to a public health problem. CALL features a Central Coordinator in Washington, 16 Relay Points that serve as information focal points, and Field Contact Points. The Relay Points include NMFS Regional Directors, representatives of FDA and the Environmental Protection Agency, and executive officers of fishery trade and regional associations.

The effectiveness of CALL was demonstrated in early June, and again in late August, 1974, when PSP was detected in shellfish from New England coastal waters. Reporting on the outbreaks, the Executive Director of the Atlantic States Marine Fisheries Commission wrote to its members, "A new outbreak of...paralytic shellfish poisoning along the New England coast is related in a memorandum from the CALL Central Coordinator. The incident has been reported to the news media, and in a commendable fashion...so that no adverse publicity has resulted. There was no need to counter any rising tide of bad, misinterpreted or misleading publicity".

CONCLUSION

It is doubtful that for the foreseeable future we shall be able to prevent, alleviate, or control blooms of toxic dinoflagellates and outbreaks of PSP. The best we can do is to learn to live with and work around these natural phenomena. By using the best available systems for keeping informed about the occurrence and distribution of the blooms and PSP, fishery managers can greatly reduce the public health hazard. They can also avoid the "halo effect" — the damage to those portions of the fishing industry not directly involved in the PSP outbreak — that results from uninformed and sometimes erroneous unfavorable publicity about a red tide and PSP.

The 1972, New England red tide never was a direct threat in N.Y. waters. The State acted promptly and effectively to prevent the importation of shellfish from the affected areas. Thus, consumers can rest assured that the CALL and shellfish sanitation system works.

ACKNOWLEDGMENTS

The *Long Island Press* and the Snow Food Products Division of Borden Foods generously gave permission to reprint the newspaper advertisement. Dr. J. L. McHugh reviewed the manuscript and made helpful suggestions.

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THE 1972 RED TIDE IN NEW HAMPSHIRE

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ABSTRACT

An extensive paralytic shellfish monitoring program was conducted on Common Island, Hampton Harbor, New Hampshire during the 1972 New England red tide. In addition, many shellfish samples from other locations and various water depths were analyzed, cell counts from surface waters made, and other ecological observations noted. Field and laboratory depuration studies showed a simultaneous decline in toxicity values. This information represents baseline data for possible future red tide outbreaks.

Since New Hampshire does not have a commercial shellfishery and has not had an ongoing PSP monitoring program, the 1972 red tide presented a public health emergency and problems unique to this state.

INTRODUCTION

More than 20 species of marine dinoflagellates have been implicated in poisonings during or after red tide blooms (3). There are, apparently, two major ways in which these toxic cells affect other organisms higher in the food chain. First, the release of toxins into the water during cell lysis after peak red tide conditions and/or by cell secretion, produce massive fish mortality. Two species from the Gulf of Mexico (*Gymnodinium breve* and *Gonyaulax monilata*) are well known examples (1, 6). These also affect many invertebrate species that occupy intermediate positions in the food chain (9). Second, toxic dinoflagellates may exert effects through primary consumers called "transvectors" that are not permanently affected themselves. If these intermediary consumers (bivalve molluscs) and then ingested by animals still higher in the food chain (birds, mammals), paralytic shellfish poisoning (PSP) may result. Two dinoflagellates (*Gonyaulax tamarensis* and *G. catenella*) from temperate waters are in this category (3, 7) and are the source of a potent material called saxitoxin (2). In addition, *G. tamarensis* contains another potent material that is currently being purified and tested in our laboratory (see Buckley *et al.*, 1974 conference proceedings) at the University of New Hampshire. Interest in this species and its toxins has been recently stimulated by the 1972 red tide bloom that occurred in the coastal waters in the southern Gulf of Maine. While the presence of this species in this area is not rare, it had not previously been reported as present in red tide concentrations. In fact, there are no reports to our knowledge of toxic shellfish in New Hampshire prior to the late summer of 1972.

FIELD AND LABORATORY OBSERVATIONS

During mid-September 1972, a red tide bloom occurred in coastal New Hampshire waters. The causative organism was identified at the University of New Hampshire as *Gonyaulax tamarensis*. The peak of the bloom conditions occurred on 14 and 15 September, when colored water masses were flushed in

and out of Hampton Harbor during the tidal cycles. Red water patches several miles long were observed from aircraft and boats within 2 miles of the New Hampshire and northern Massachusetts coastline. Smaller patches were noted some 6 miles offshore at the Isle of Shoals. Interviews with local fishermen revealed the presence of patches of colored water offshore as much as 10 days prior to the onshore bloom. These reports, the presence of dead and dying birds, and the nonfatal poisoning via shellfish of 2 New Hampshire clam diggers were all subsequently related to the *Gonyaulax tamarensis* red tide. Surface water samples during the bloom had cell counts between 7×10^5 and 2.6×10^6 cells per liter. Counts decreased in about 2 days and the cells disappeared from the surface waters sampled within 5-7 days. Microscopic examination revealed an essentially unialgal population of *G. tamarensis*. Concentrated, then homogenized cell samples injected intraperitoneally into laboratory mice were extremely potent. Although relatively few bivalves perished during or after the red tide, many soft-shelled clams (*Mya*) were observed to be in a lethargic condition, i.e. insensitive to physical stimuli. Bivalves in this condition that were placed in a laboratory running seawater system recovered in 24 hours.

A total of 620 waterfowl, gulls and shorebirds representing 13 different species were found dead at the Parker River National Wildlife Refuge at Plum Island, Massachusetts. Representative samples of gut contents from these birds showed the presence of small filter-feeding bivalves such as *Mytilus edulis* (blue mussel), *Siliqua costata* and *Ensis directus* (razor clams) in all cases examined. Many other birds apparently perished after feeding on toxic shellfish, but were not recovered. Personnel of the Parker River Refuge estimated that more than 1,600 Black Ducks died in this way. However, the large bird kills were not reflected in the winter count conducted by the New Hampshire Audubon Society (Arthur C. Borror, Pres. NHAS, personal communication).

BIOASSAY FOR PSP

The potency of dinoflagellate toxins is usually measured by challenging mice, fish or other organisms with active extracts from molluscs or cell cultures and determining survival time. The means of expressing and comparing potency has relied on mouse units (MU) (i.e. the survival time of a specified mouse strain in a given weight range) with doses given intraperitoneally. At least 2 different interpretations of the mouse unit are currently being used. One is used for the slow acting ciguatera-like toxin from *Gymnodinium breve* (4, 11) and the other to evaluate the fast acting PSP from *Gonyaulax catenella* and *G. tamarensis* (10, 3). The latter was employed in our studies of bivalve toxicity along the New Hampshire and northern Massachusetts coastline during recent red tides. The MU, in this case, is defined as the amount of PSP that will kill a 20 gram mouse in 15 minutes. The strain of mouse used was the $B6DF_1/J$ obtained from Jackson Laboratories, Bar Harbor, Maine. The

method is based on comparing partially extracted field samples of bivalves against a reference standard of pure saxitoxin (PSP) obtained from the U.S. Public Health Service (3,8). The MU's measured are converted to micrograms of poison per 100 grams of bivalve tissue. The safety level has been set at 80µg/100 gms and scores above this value indicate that shellfish are potentially hazardous for human consumption.

RED TIDE TOXICITY SCORES

The sites selected for our monitoring program for the state of New Hampshire were Common Island, Hampton, N.H. and for comparative

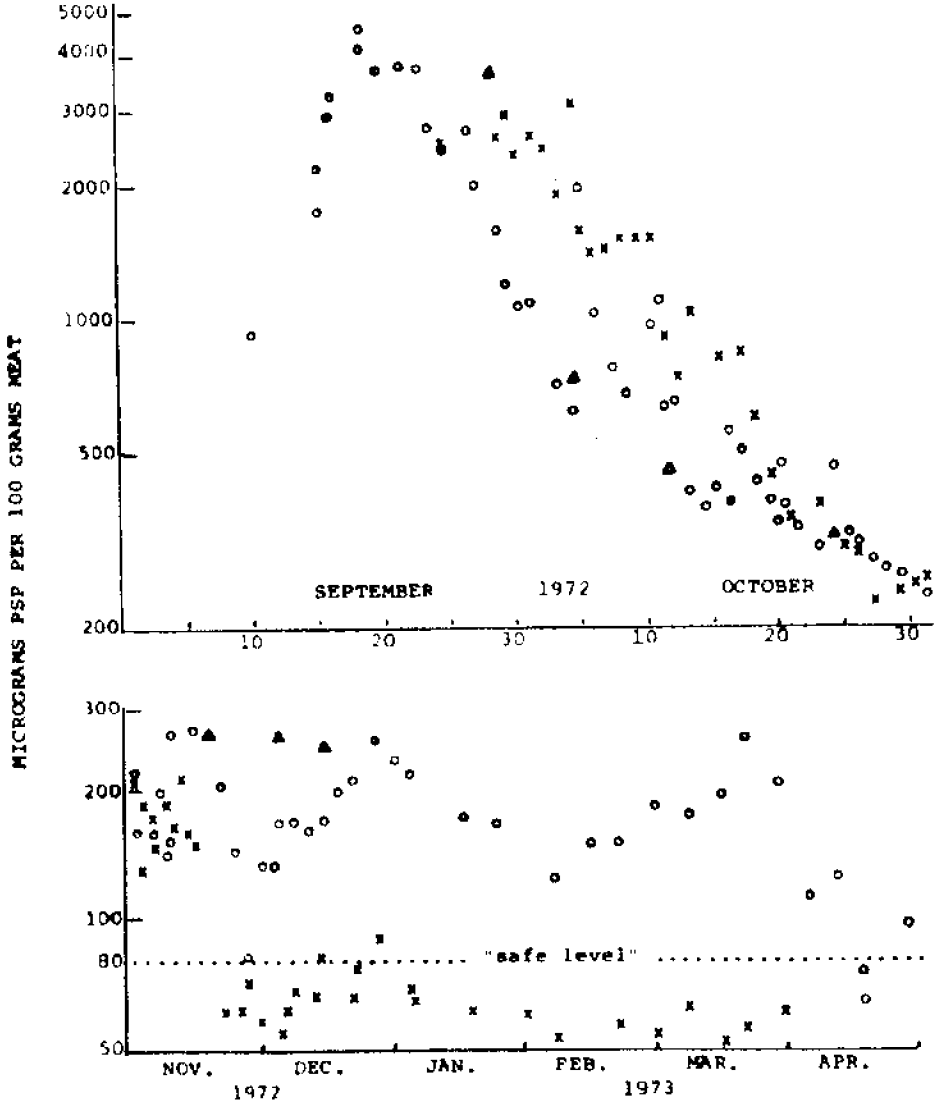

Figure 1. Toxicity Scores for *Mya* (o), and *Mytilus* (x) collected at Common Island, Hampton, N. H. 1972-73. (Δ) indicates *Mya* clearance under laboratory conditions.

purposes, the Parker River National Wildlife Refuge at Plum Island, Massachusetts. The former was chosen because of its proximity and abundance of shellfish that are not harvested by commercial or recreational clam diggers. We chose to bioassay the Hampton bivalves more often than would be necessary in a general monitoring program because we wanted a significant amount of baseline information for this area, - since the 1972 Red Tide caused the first recorded outbreak of PSP in this state. Figure I shows the scores obtained in our monitoring program conducted at the University of New Hampshire. The peak values in mid-September approach some of the highest recorded in northern Maine and the Canadian Maritime Provinces, but represent only approximately one half of the values obtained in some Massachusetts samples where PSP scores of up to 9500 were recorded. Scores from *Mytilus edulis* fell below the safe level toward the end of November 1972, but *Mya arenaria* values oscillated and were not "safe" until the end of April or the first part of May 1973.

Bivalves from Parker River showed the same clearance rates as those from Common Island. *Mytilus* scores fell below 80μg/100 gm tissue in late November 1972, while *Mya* values remained at or above 150μg until mid-December 1972, when we stopped sampling at that site because of heavy ice conditions. *Mya* specimens were collected at the peak of the red tide in September 1972, and placed in clean-running seawater at the University of New Hampshire's Jackson Estuarine Laboratory. This laboratory population was checked periodically and was found to clear at a rate similar to *Mya* from both sampling sites. In mid-December 1972, *Mya* scores from Common Island, Parker River, and the laboratory were 2 to 3 times above the safe level although all *Mytilus* samples were below 80μg.

Table I shows representative scores from deep water (to 30M) bivalves taken over several months. These values are similar to those obtained in Canada (5)

Table I
Shellfish Samples From Hampton[1], NH, Great Bay[2], NH., And Plum Island[3], Mass.

Date	Species	Common Name	Score μg/100 grams tissue
9/21/72	[1]*Siliqua costata*	razor clam	1727
9/21/72	[1]*Ensis directus*	razor clam	735
9/21/72	[1]*Modiolus modiolus*	horse mussel	359
9/21/72	[1]*Spissula solidissima*	surf clam	2939
9/21/72	[1]*Arctica islandica*	black clam	1010
9/21/72	[2]*Crassostrea virginica*	oyster	43
10/20/72	[2]*Crassostrea virginica*	oyster	<40
2/13/73	[3]*Placopectin magellanicus*	scallop(edible muscle only)	48
2/13/73	[3]*Placopectin magellanicus*	scallop (viscera only)	1914
2/13/73	[3]*Spissula solidissima*	surf clam	682
2/13/73	[3]*Arctica islandica*	black clam	213
2/13/73	[3]*Lunatia (Polinices) heros*	moon snail	1329
2/22/73	[1]*Modiolus modiolus*	horse mussel	40
3/14/73	[1]*Placopectin magellanicus*	scallop (edible muscle only)	<40
3/14/73	[1]*Placopectin magellanicus*	scallop (viscera only)	1550

following outbreaks of paralytic shellfish and shows that deep water forms retain high toxin levels for prolonged periods of time. No samples of these species were available from pre-red tide times for comparative purposes. However, *Mya* and *Mytilus* samples collected prior to mid-September 1972 showed no toxicity.

DISCUSSION

When compared to other coastal New England states affected by red tides, New Hampshire is in the unique position of not permitting the commercial digging of clams. The state maintains only a recreational shellfishery whereby some 12,000 licensees pay $4.50/yr. to dig soft-shelled clams (*Mya arenaria*) on weekends and holidays. The standing crop of *Mya* has been estimated at 16,000 - 20,000 bushels in Hampton Harbor alone (other smaller areas not included). If one assumes a minimal value of $4.00/peck for *Mya* then the standing crop has an in-ground value in excess of $300,000. The N.H. Fish and Game Department realizes a $50,000 to $60,000/year income from clam licenses. If the average recreational clam digger harvested no more than 1 bushel/year, the dollar value would be $180,000 to $200,000 to the citizens of New Hampshire.

The type of PSP associated with the dinoflagellate *Gonyaulax tamarensis* has been routinely assayed in other coastal states because of the potential hazard to human health. In September 1972, the first red tide in the southern Gulf of Maine closed the clam flats in New Hampshire for many months. In June and again in September 1974, closure of clam flats was necessary because of danger to humans. After 3 periods of unsafe bivalves over a 2 year span, the Governor's Council appropriated $10,000 in September 1974 so that the N.H. State Dept. of Public Health can establish a PSP monitoring program.

ACKNOWLEDGEMENT

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HISTORY OF PARALYTIC SHELLFISH POISONING ON THE MAINE COAST 1958-1974

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ABSTRACT

Following an epidemic of shellfish poisoning in New Brunswick, Canada in 1957, Maine was encouraged by the U. S. Public Health Service to investigate the possibility of the presence of paralytic shellfish poisoning in Maine. The initial study in the summer of 1958 indicated that Maine indeed had a problem in portions of eastern Washington County adjacent to New Brunswick, Canada. Maine developed a shellfish monitoring program for this area which has worked quite well. Measurable amounts of poison have been detected each year, with shellfish closures necessary nearly every year. Coastwide monitoring of shellfish after high scores have been found in this area has demonstrated potential trouble areas. Two coastal islands are permanently closed because of PSP.

The sudden rise of PSP in western Maine and Massachusetts in 1972 was not predicted by this monitoring program and has necessitated an expanded monitoring program.

INTRODUCTION

Following the epidemic of shellfish poisoning in New Brunswick, Canada in 1957, Maine was requested by the U.S. Public Health Service to investigate the probable presence of PSP in Maine's shellfish growing areas adjacent to Canada. The initial study in 1958 indicated the presence of poisonous shellfish in eastern Washington County. Following the study in 1958, Maine developed a monitoring plan for eastern Washington County which is as follows: At six sampling stations shellfish are collected and assayed every other week from May 1 until the first rise in poison (June 15 +), at which time sampling is conducted weekly until the quarantine level is reached which requires the closing of the area. The weekly sampling continues until October 1 or such time as the area may be re-opened. From October 1 to May 1 four stations are sampled at monthly intervals. The winter samples may be regarded as providing background information as scores have never exceeded 80*.

PSP in 1972

Until 1972 this sampling scheme appeared to give early warning of any possible rise of poison. We sampled the entire coast only when scores in Washington County were high (600+ in *Mya*). For example, in 1961, a year of high toxicity in eastern Maine, we detected poison along the entire Maine coast. In all sampling stations except Monhegan and Matinicus Islands the scores were well below quarantine level. Monhegan and Matinicus Islands are permanently closed to shellfishing because of PSP. These two islands are not consistently toxic but their remoteness and low shellfish resources make it

* scores = microgram of poison per 100 grams shellfish meat.

difficult to adequately determine the periods of toxicity. In 1972, at the offshore Quoddy Bar-Lubec "key" station, the highest score was 284 for *Mya* on August 9. At Monhegan Island, which we regard as an early warning station for western Maine, a score of 291 in *Modiolus* was obtained on the same day. Our previous experience had indicated that the relatively low score at Monhegan precluded the possibility of high scores in any other areas in western Maine, so no other samples were collected. Our previous experience had also indicated that Quoddy Bar would give an indication of what to expect at Monhegan Island. Inasmuch as the scores at Quoddy Bar decreased after August 9 (77 on August 13, 81 on August 22, 74 on September 1, 70 on September 8, 64 on September 17), no additional samples were collected at Monhegan. On September 15, 1972, with the sudden rise in PSP in western Maine, New Hampshire, and Massachusetts, it became apparent that we could no longer depend on Quoddy Bar to predict Monhegan's scores, and thus western Maine's scores.

On September 16, 1972, a sample of *Mytilus* collected at Monhegan had a score of 5,846. It is regrettable that we did not sample Monhegan between August 9 and September 16 as in all probability we could have predicted the rise of poison in western Maine which occurred at that time.

On September 15 the *Mya* samples collected between Portland and Kittery indicated a high level of poison (1,000-3,000 in *Mya*) and these shellfish areas were closed.

On September 16 the remaining portion of the Maine coast was sampled with sporadic areas testing above quarantine. Due to the undependability of this quick sampling plan, the whole Maine coast was closed on September 17. Extensive sampling following this closure enabled us to re-open the coast on September 30, 1972, with the exception of areas west of Cape Elizabeth to the New Hampshire line. Much of this area remained closed until September, 1973.

PSP in 1973

PSP scores in 1973 were low, and any scores above 80 μg/100 g could be easily attributed to the remaining residual poison from 1972.

PSP in 1974

In 1974 the first rise of poison was noted on May 30 between Cape Elizabeth and the New Hampshire line. Extensive sampling in Casco Bay north of Portland found areas in excess of quarantine necessitating the closing of a portion of this area. These two closures were rescinded in early July.

The Washington County area showed the expected annual rise of poison early in August necessitating the closure of all shellfish growing areas east and north of Moose Cove-Trescott and the Canadian border. The scores in this area were not high, never exceeding 300 in *Mya*. This closure was repealed on October 11, 1974.

The second rise of poison in 1974 in western Maine was noted on August 24. An apparent early warning station for western Maine, and in all probability eastern Massachusetts as well, is Hampton, New Hampshire (Maine runs New Hampshire samples). The first rise of poison in *Mytilus* was noted on the August 21st sample with a score of 268, up from less than 58 on August 11. By August 25 the *Mytilus* scores were 1,489 and with a high of 8,398 on September 4 the rise at Hampton, New Hampshire preceded any rise of poison in Maine. This warning enabled Maine to adequately sample all of its growing areas. The poison levels apparently progressed from west to east, with the most easterly line at Schoodic Point-Winter Harbor on September 17. Poison levels after this date decreased dramatically, permitting the re-opening of all shellfish areas east of Pine Point-Scarborough on October 12, 1974. Quarantine scores in *Mytilus* only are noted in the remaining closed area.

Maine is anticipating investigating an expanded sampling program in late April or early May of 1975, based on the 1974 experience. The sampling plan of eastern Maine will be extended to the entire coast.

SUBJECT INDEX