

Aquaculture: Public Health, Regulatory and Management Aspects
Proceedings of the 6th U.S. Food and Drug Administration
Science Symposium on Aquaculture

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July 1982

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AND MANAGEMENT ASPECTS

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TABLE OF CONTENTS

	Page
IMMUNOLOGICAL APPROACHES TO UNDERSTANDING MARINE TOXINS -- Y. Hokama, L.H. Kimura and J. Miyahara.....	1
PARALYTIC SHELLFISH POISONING IN SHELLFISH AQUACULTURE: THE STATE REGULATORY PROBLEM -- J.W. Hurst, Jr.....	21
PRELIMINARY OBSERVATIONS ON PARALYTIC SHELLFISH POISONING IN CENTRAL PUGET SOUND -- L. Nishitani and K. Chew.....	33
SHELLFISH AQUACULTURE AND PARALYTIC SHELLFISH POISONING -- Y. Shimizu.....	38
PROGRESS OF SHELLFISH TOXIN RESEARCH: IMPLICATIONS OF TOXIC RESTING CYSTS FOR AQUACULTURE -- C.M. Yentsch and L.C. Incze.....	49
THE OCEAN HAS ITS BEZOAR, TOO --A.A. Benson.....	63
CHEMOTHERAPY AND CHEMOPROPHYLAXIS FOR CONTROL OF BACTERIAL DISEASES OF FISH -- G.L. Bullock.....	71
THE SECRETORY IMMUNE SYSTEM IN FISH: AN OVERVIEW OF RECENT ADVANCES -- C.J. Lobb and L.W. Clem.....	83
PRINCIPLES OF CONTROL OF DISEASES OF FISH AND SHELLFISH -- S.F. Snieszko.....	100
FATE AND TRANSPORT OF VIRUSES IN MARINE WATERS -- S.M. Goyal, R.L. LaBelle and C.P. Gerba.....	112
ACCUMULATION AND PERSISTENCE OF ENTEROVIRUSES IN BLUE CRABS -- T.W. Hejkal and C.P. Gerba.....	126
POTENTIAL MICROBIOLOGICAL PROBLEMS ASSOCIATED WITH THE USE OF HUMAN AND ANIMAL WASTES AS A FOOD SUPPLEMENT IN AQUACULTURE -- E.P. Larkin.....	134
DISEASES ACQUIRED BY EATING FISH AND SHELLFISH IN THE UNITED STATES, 1975-1979 -- J.G. Morris.....	148

	Page
1,3,5-TRICHLORO-2-(4-NITROPHENOXY)BENZENE (CNP) IN FISH, SHELLFISH, AND SEAWATER IN TOKYO BAY, 1977-1978 -- T. Yamagishi and K. Akiyama.....	156
SELECTIVE BREEDING PROGRAMS AND GENETICS AS LIKELY IMPACTS ON FUTURE AQUACULTURE PRODUCTION SYSTEMS AND REGULATION OF AQUA- CULTURE -- A.C. Longwell.....	167
THE ROLE OF THE FOOD AND DRUG ADMINISTRATION IN THE APPROVAL OF NEW ANIMAL DRUGS -- B.F. Corey.....	180
A BALANCED GOVERNMENTAL ROLE IN AQUACULTURE -- R.D. Wildman and W.N. Shaw.....	190
INVESTIBLE AQUACULTURE IN THE UNITED STATES -- H.H. Webber.....	198

IMMUNOLOGICAL APPROACHES TO UNDERSTANDING MARINE TOXINS

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INTRODUCTION

The application of immunological technics for recognition of low molecular weight carbohydrates, peptides, fats, drugs and many other compounds has increased markedly in the past decade. The ability to conjugate these small molecules covalently to appropriate antigenic carriers via their functional groups has led to the production of specific antibodies to the haptenic molecules following administration into appropriate animals. Primary functional groups or moieties amenable to conjugation, provided they are accessible to chemical coupling, include amino, hydroxyl and carboxyl groups. Some common methods for coupling include carbodiimide, nucleophilic substitution, diazo and azide coupling. In some cases, for example with lipid antigens, noncovalent complexes have been utilized using methylated bovine serum albumin as the carrier (8).

The availability of a specific antibody to the low molecular weight compounds can be utilized for the development of sensitive and specific immunological procedures such as radioimmunoassay (RIA) and enzyme-linked immunoabsorbent assay (ELISA) for detection of minute amounts of antigens or antibodies. In addition, the antibodies are useful in examining other areas of study such as tissue distribution, synthesis and in some instances structure and function of the haptenic molecule.

The application of the sensitive immunological methods for the detection of marine toxins, such as saxitoxin, ciguatoxin and tetrodotoxin, merits strong consideration in light of the minute amounts of these toxins in the natural environment. These minute amounts in tissues have constituted serious hazards to the consumer and create anxiety within the fishing and shellfish industries.

This study discusses the immunological approaches for the development of a radioimmunoassay procedure in the utilization for the detection of a marine toxin such as ciguatoxin (9). The methodology presented is applicable for development of the immunological procedures for the other low molecular weight marine toxins (saxitoxin, tetrodotoxin, and Gymnodinium breve toxins). In addition, for correlative analysis, the guinea pig atrium procedure (12) for quantitation of ciguatoxin and maitotoxin from crude extracts of fishes is also included in this study. Comparison of the RIA procedure with the cat, mouse MLD and M.U. tests is also presented ,

GENERAL PROCEDURES

Methods of Coupling Low Molecular Weight Compounds: The methods of coupling haptens are dependent on the functional groups on the haptens as

well as on the carrier protein. The functional groups on the carrier molecules to which haptens may be attached are summarized in Table 1.

TABLE 1. Functional groups of carrier molecules to which haptens may be conjugated.

<u>FUNCTIONAL GROUP</u>	<u>RESIDUES ON CARRIER</u>
Amino	N-terminal AA, Lysine
Carboxyl	C-terminal AA, Aspartic and Glutamic Acids
Guanidino	Arginine
Imidazo	Histidine
Indolyl	Tryptophan
Phenolytic	Tyrosine
Sulfhydryl	Cysteine

Some of the chemical methods employed include the following:

(a) diazotization; (b) nucleophilic; (c) carbodiimide; and (d) azide reactions. These are illustrated in Figures 1-A, B, C and D.

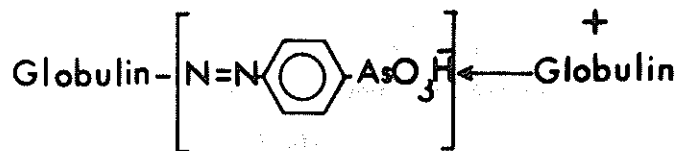
Diazotization: This is applicable to aromatic amines containing a NH_2 group which is diazotized with cold dilute nitrous acid. The diazonium salt formed is conjugated to the carrier protein in an alkaline solution. The most likely site of diazo bond formation to the carrier is the aromatic ring of tyrosine and histidine and the ϵ -group of lysine. A mono- or di-substitution may occur depending on the diazonium salt concentration used. Diazo bonds may possibly occur with tryptophan and arginine residues also. In the final analysis, however, the nature of diazo bond conjugation is dependent on the accessibility of the reactive groups. Preferentially at low diazo-salt concentrations tyrosine and histidine are generally the active sites of binding. Diazo coupling has been utilized in coupling glycosides to aromatic rings. For example, p-aminophenylglycoside following diazotization was coupled to protein carriers and antiserum to the oligo-saccharide protein conjugate was prepared. The diazotization procedure is applicable to a wide variety of small molecular weight compounds for which derivatives of aromatic amines could be prepared. Figure 1-A illustrates the diazo type of coupling.

Nucleophilic Substitution: One of the most widely used haptens for the examination of the chemical basis of immunogenicity, for the sites and energy of antibody binding, and receptor activation of B cell and T cell are the dinitrophenyl compounds. These include, 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP). These compounds have proven of great value because of their intense and easily distinguishable absorption spectra, stability of covalent bond formed and especially because of their immunodominant characteristics on carriers contributing to immunogenicity. DNP is attached to the protein carriers through the nucleophilic substitution reaction in which the active chlorine or fluorine residue is in

(A) DIAZOTIZATION

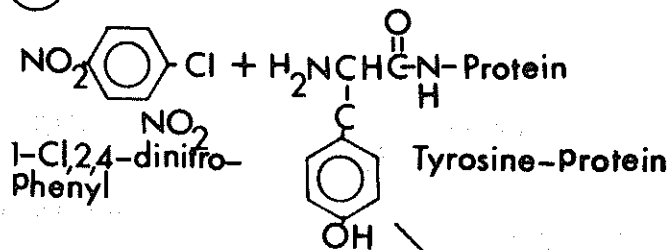


p-aminobenzene- nitrous p-benzenearsonate
 arso ate acid diazonium salt



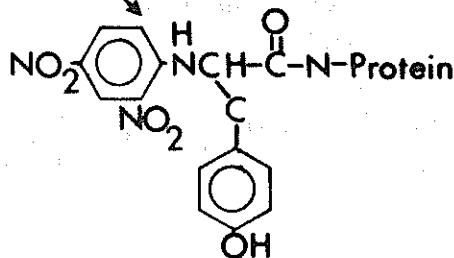
p-azobenzenearsonate Globulin

(B) NUCLEOPHILIC



1-Cl,2,4-dinitro-
 Phenyl

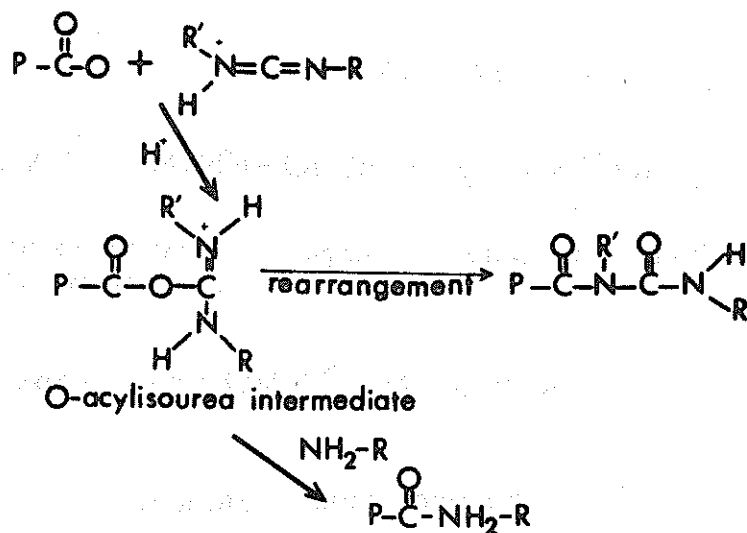
Tyrosine-Protein



N-2,4,-dinitrophenyl-L-tyrosine-protein

Figure 1 A-B. Various chemical reactions for covalent coupling of haptenic groups to carrier antigens. A. diazotization; B. nucleophilic reactions.

(C) CARBODIIMIDE



(D) AZIDE

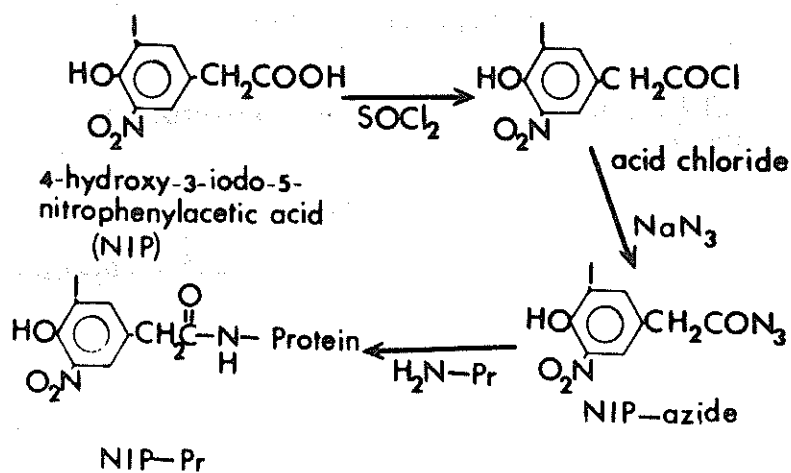


Figure 1 C-D. Various chemical reactions for covalent coupling of haptenic groups to carrier antigens. C, carbodiimide coupling; and D, azide coupling.

position 1 of the benzene molecule. The halogen is activated by the NO_2 groups in position 2, 4, and 6.

The halogen is readily displaced by electron donating groups such as $-\text{NH}_2$ of lysine, $-\text{OH}$ of tyrosine, and $-\text{SH}$ of cystine of the protein carrier. The chemical reaction of this mode of conjugation is shown in Figure 1-B. As with the diazo coupling, the DNP haptenic determinant is heterogenic; coupling is dependent on the position, number and accessibility of the electron-donating residues on the carrier protein. Homogenic haptenic determinants can be achieved in some cases with use of a less reactive derivative, DNP-sulphonic acid. This compound tends to react essentially with the $-\text{NH}_2$ residue of lysine in the carrier molecule, an advantage being that it is soluble in aqueous solutions. The DNP residue can be quantitated at 360 nm spectrophotometrically using the extinction coefficient of ϵ -DNP-lysine as standard.

Carbodiimide Coupling: Of particular value for coupling amines or carboxylic acid haptens to proteins is the use of carbodiimide reagents. These compounds with the general formula: $\text{R}-\text{N}=\text{C}=\text{N}-\text{R}^1$, where R and R^1 represent aryl and alkyl groups react with amines or carboxylic acids to form peptide bonds via condensation reactions as shown in Figure 1C, with the elimination of a substituted urea, $\text{RNHC}=\text{ONHR}^1$. Aqueous soluble carbodiimide solutions are readily available and have been used to couple proteins to haptens containing NH_2 or COOH residues. For example, the conjugation of bradykinin (a nonpeptide) to carrier proteins has been achieved and specific antibodies to bradykinin elicited in rabbits. Many other peptides have been coupled to protein or polypeptide carriers with carbodiimide. These include ACTH, calcitonin, gastrin, and vasopressin. The method of introducing insolubilized and water-soluble carbodiimides appears to be easily and simply applied, versatile, and has found wide ranging applications, especially where the formation of peptide, ester, thioester or phosphoester bonds is required.

Azide Coupling: Haptenic groups can be introduced into proteins, erythrocytes, viruses and other carriers via the azide derivatives. The chemical coupling is depicted in Figure 1-D. The most widely used family of compounds are the 4-hydroxy-3-iodo-5-nitrophenyl-acetic acid (NIP) and related derivatives. The free carboxyl group of the hapten is transformed to an acid chloride derivative with thionyl chloride (SOCl_2). The acid chloride then reacts with sodium azide (NaN_3) to yield a crystalline reactive haptenic azide. This compound is stable when kept frozen (-20°C) and stored in dimethyl formamide solution. The haptenic azide can then be coupled to the carrier. The internal spacer of the haptenic azide minimizes the carrier effects such as steric hinderance and contributes to specificity.

The coupling studies of non-immunogenic low molecular weight compounds have contributed toward the understanding of the chemical basis of immunogens and more importantly, opened doors to the advancement of clinical medicine. This is evidenced by the development of RIA procedures for assessment of polypeptide hormones in man, an enormous advancement of knowledge in this area. That these procedures can be utilized for the advancement of knowledge of marine toxins are evident.

Synthetic Antigens as Carriers: The advent of synthetic monopolymers

(a single amino acid polymer) and copolymers (two or more different amino acids) has contributed immensely to the understanding of the immunogenicity of molecules. These synthetic polymers are capable of inducing antibody synthesis in rabbits and also of reacting in vitro or in vivo by the various immunological procedures. The synthetic polymers have also been successfully emphasized as carriers and in some cases have proven to be better than protein carriers. Figure 2 presents a schematic diagram of a monopolymer and copolymer.

Carriers may contribute to the specificity of the molecule acting as a complete determinant which is attributable to the increase in energy of the interaction of the hapten with the B cell receptor site. On the other hand, carriers may initiate an allosteric affect in the receptor which could be transmitted as a signal throughout the immunocyte. The hapten would be serving as the immunodominant point towards which the antibody specificity is directed with the carrier providing the additional element of the complete determinant. The carrier may also contribute to the metabolizability of the complete hapten-carrier and thus provide appropriate hapten-carrier fragments (antigenic determinant) of enhanced immunogenic specificity.

Animals and Immunization: A variety of animal species has been used to prepare precipitating antibodies. These include rabbit, guinea pig, goat, sheep, horse, donkey and many other species. The rabbit and goat have been utilized most frequently for production of antibodies to many proteins and low molecular weight compounds (haptens). The choice of animal is generally dependent on availability of the amount of the antigen, its toxicity and immunogenicity.

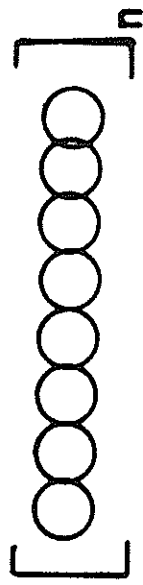
The mode of administration of the antigen may be orally, subcutaneously, intraperitoneally, intravenously and intramuscularly. For enhancement of the immune response, immunoadjuvants such as Freund's complete adjuvant (FCA) Corynebacterium parvum, or the recently available dipeptides of 6-O-mycolyl-N-acetylmuramic acid (6-O-mycolyl-N-acetylmuramyl-L-seryl-D Isoglutamine) (1,17) are generally given with the antigens either mixed together or separately.

The antigen and immunoadjuvant may be administered in three consecutive days, initially at low concentrations (this is important with highly toxic haptens such as ciguatoxin or saxitoxin) with a rest period of 4 days. These series of injections are repeated weekly. Alternatively, antigens may be given every other day three times a week. The administration would vary according to the animal and the route of the injection.

The animals are bled at weekly intervals (large animals) after the third week following the initial antigen injection to assess the production of antibody. When a satisfactory level of antibody is raised, the animal is bled, and the serum is removed from the clot and stored at -20°C until ready for examination.

Methods of Antibody Assay: The assessment of the antibody level can be made by a variety of methods (15). The procedures include immunodiffusion or immunoelectrophoresis in agar or agarose gels, complement-fixation, passive hemagglutination with sheep erythrocytes (SRBC), agglutination and radioimmunoassay or enzyme-linked immunoabsorbent assay for low concentrations of antibodies or antigens.

(A) Homopolymer: Poly-L-lysine



○ L-lysine

◇ L-tyrosine

(B) Copolymer: Poly-L-tyrosyl-L-lysine

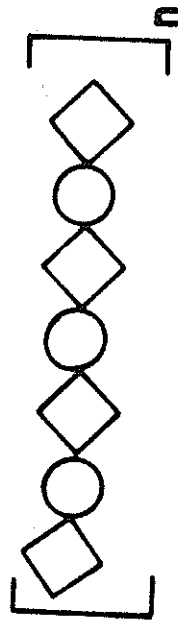


Figure 2. Schematic diagrams of examples of homopolymer and copolymer synthetic polypeptide antigens.

Methods for Ciguatoxin Analysis: The development of the RIA procedure for the direct detection of (CTX) from fish tissue has followed the procedures alluded to in the discussion under General Procedures.

Purified ciguatoxin (16) isolated from livers of toxic eels was conjugated by the carbodiimide procedure (14) to human serum albumin (HSA). The latter is the carrier for the lipid hapten-ciguatoxin. Precipitation of the ciguatoxin-HSA (CTX-HSA) with cold acetone indicated that CTX was in the precipitate (CTX-HSA) and not in the acetone phase. This suggested that most if not all CTX was bound to HSA.

The antibody to CTX-HSA was raised in sheep given 3 weekly subcutaneous injections of a CTX-HSA Freund's complete adjuvant mixture (1:1). A total of 500 µg of CTX and 50 mg of HSA (CTX-HSA) was administered to the sheep in a period of 7 weeks. The sheep was bled 10 days after the last injection and the serum removed and stored in 30 ml aliquots at -20°C.

The details of the determination for the presence of anti-CTX-HSA by immunoelectrophoresis and mouse studies and for the development of the RIA procedure have been described (9). In brief, the RIA method consists of the following steps: (1) sampling of tissues from the dorsal and ventral muscles, tails or gonads of the fish; (2) dehydrate samples in oven at 70°C for 1 hour; (3) after cooling, weigh 15 mg samples; (4) rehydration with buffered saline; and then removal of buffer; (5) add 0.2 ml of ¹²⁵I-labelled sheep anti-CTX-HSA (sp. act., 1.03 mCi/mg protein) containing 50,000 cpm/ml; (6) shake for 3 hours at room temperature; (7) add 3.0 ml buffered saline; (8) aspirate buffer thoroughly; and (9) count the sample in gamma-counter. After background correction, the cpm/tube is divided by 15 mg to give cpm/g tissue. Samples are determined in triplicates, or duplicates in some instances. Toxic and non-toxic fish tissue samples are examined with each set of unknown determinations.

The present study includes examination by the RIA procedure of fishes recovered from clinically diagnosed ciguatera poisoning cases and non-toxic fishes from commercial sources. The toxic fishes were obtained through the courtesy of the Hawaii State Health Department. The routine examination of a variety of fishes caught around Midway was also determined by the RIA procedure. The fish samples were prepared in vials marked with numbers only. These samples were obtained through the courtesy of R. Shomura, Director of the National Marine Fisheries Service Honolulu Laboratory.

A correlative study of the RIA procedure and the *in vitro* guinea pig atrium assay was carried out for several samples of toxic and non-toxic fish tissues. The details of the guinea pig atrium analysis have been reported recently (12). The crude extracts of the fish tissues were prepared according to a previously described procedure (19). A 20 g tissue is homogenized and extracted with 200 ml hot methanol. The residue is removed by filtration and the methanol phase concentrated to 10% of the original volume. The concentrated solution is diluted with distilled water (5 volumes) and the lipid solutes extracted with diethyl ether. The ether is evaporated and the crude lipid residue dissolved in minimal methanol and stored at -20°C until ready for study. Assay for the cat, and mouse toxicity procedures have been described previously (3,18).

The cat and the mouse MLD and MU results were carried out by Dr. Bagnis of Tahiti and the RIA determined on the same tissues. The RIA was determined prior to the information given for the cat and mouse results.

Based on the examination of fishes from human ciguatera poisoning and previous results obtained by RIA (9) the following arbitrary criterion has been established relative to the cpm/gm tissue values.

<u>CPM/gm Tissue</u>	<u>Toxicity Index</u>
< 350,000	Negative
351,000 - 399,999	Borderline
≥ 400,000	Toxic

The diagnosis of ciguatera poisoning of patients following consumption of suspected toxic fishes is based on the clinical symptoms described previously (13,2).

RESULTS

RIA Examination of Fish Samples: The results of the examination of individual cases of fish samples recovered from clinically diagnosed ciguatera poisoning are indicated in Table 1. A variety of species has been implicated, which include the following species: Scarus sp., parrot fish/uhu; Elagatis bipinnulatus, rainbow runner; Seriola dumerili, amber-jack/kahala; Caranx and Carangoides sp., jack/ulua; Mugil cephalus, mullet/ama'ama; Aprion virescens, grey snapper/uku; Monotoxis grandoculis, mu; Priacanthus sp., big red eye/aweoweo; Lutjanus gibbus, paddle-tail snapper and Epinephelus mario, sea bass. Two samples, a Seriola dumerili and a Caranx sp. gave RIA values in the non-toxic range. The mean \pm 1 S.D. (434,654 \pm 110,184) value for the 32 toxic samples is summarized in Table 3. This value is significantly ($p < 0.001$) different than the mean \pm 1 S.D. (264,202 \pm 44,291) value of the 91 non-toxic samples of essentially the same species shown in Table 2. In a similar analysis with a single species (Seriola dumerili), a highly significant difference ($p < 0.0001$) in the RIA values of non-toxic and toxic S. dumerili is demonstrated (Table 3). These differences have been the basis for the criterion established in assessing the RIA findings described in the preceding section under methods.

Preliminary Survey of Various Species of Fishes from Midway: Using this criterion of designating toxic, borderline and non-toxic based on cpm/gm tissue, an extensive exploratory examination of various important species of fishes from the Leeward Islands, Northwest of Hawaii, up to Midway has been initiated by the National Marine Fisheries Service (NMFS). The results presented are preliminary findings from fishes caught around Midway and these are summarized in Table 4. A detail study will be presented at a later date by NMFS. Of the 126 samples of flesh tissue examined, approximately 12.0% showed borderline to toxic RIA values. Those fishes showing borderline to positive values include many implicated in ciguatera poisoning (S. dumerili, Caranx sp., Lutjanus sp., etc.). The available gonad tissues from the same fishes were also examined and not surprisingly showed greater incidence of borderline to toxic values (37.0%). It is of interest in this regard that in an earlier report Banner (4) indicated that

Table 2. RIA examination of fish samples from clinically documented ciguatera poisoning.

NUMBER OF SAMPLES	FISH SPECIES	COMMON/ HAWAIIAN NAME	RIA RESULTS CPM/GM TISSUE	REMARKS
3	<u>Scarus</u> sp.	Parrot Fish/Uhu	421,064 463,039 524,106	Toxic
1	<u>Elagatis bipinnulatus</u>	Rainbow Runner/Kamanu	463,636	Toxic
5	<u>Seriola dumerili</u>	Amberjack/Kahala	412,600 415,870 436,778 447,045 549,534	Toxic
1	<u>Seriola dumerili</u>	Amberjack/Kahala	296,667	Toxicity not detected by RIA
8	<u>Caranx</u> or <u>carangoides</u> sp.	Jack/Ulua	400,867 414,489 420,667 448,936 492,634 505,779 497,406 711,346	Toxic
4	<u>Caranx</u> or <u>carangoides</u> sp.	Jack/Ulua	351,400 387,553 362,920 370,597	Borderline
1	<u>Caranx</u> or <u>carangoides</u> sp.	Jack/Ulua	300,300	Toxicity not detected by RIA
2	<u>Mugil cephalus</u>	Mullet/ama'ama	449,967	Toxic (Viscera Sample)
1	<u>Aprion virescens</u>	Grey-snapper/Uku	462,753	Toxic
1	<u>Monotoxis grandoculis</u>	Porgy/Mu	482,133	Toxic
1	<u>Priacanthus cruentatus</u>	Red big eye/Aweoweo	660,556 382,584	Toxic Borderline
1	<u>Lutjanus gibbus</u>	Paddle-tail Snapper	442,000	Toxic
2	<u>Epinephelus mario</u>	Sea bass	479,339 490,392	Toxic
1			375,189	Borderline

based on bioassays, gonads tended to have 3-5 times the ciguatoxin level of flesh in the same fish. However, the liver and viscera appeared to have the highest concentrations.

Table 3. Summation of RIA values for fishes from clinically defined "ciguatera poisoning" cases and non-toxic fishes.

FISH	CPM/GM TISSUE (MEAN \pm 1 S.D.)		p ⁺
	Non-Toxic	Toxic	
<u>Seriola</u> <u>dumerili</u> (Kahala or Amberjack)	249,253 \pm 46,452 (n = 150)	426,416 \pm 81,028 (n = 6)	< 0.0001
Several Species [*]	264,202 \pm 44,291 (n = 91)	434,654 \pm 110,184 (n = 32)	< 0.001

⁺ p value based on two-tailed analysis.

^{*} See Table 1 for the individual species listed for the toxic group; the non-toxic fishes in this category were sampled from the same species implicated in the "ciguatera poisoning" or the toxic group.

Comparison of the RIA with Cat Assay and the Mouse Toxicity, MLD and M.U.: Figures 3, 4 and 5 summarize a comparative study of the RIA with the cat assay and the mouse toxicity, MLD and M.U. of reef fish tissues obtained from Tahiti through the courtesy of Dr. R. Bagnis of the Institut de Recherches Medicales Louis Malarde. The incidence of ciguatera poisoning is high in French Polynesia in contrast to other areas of the Pacific. The coefficient of correlation, r, is fair between the RIA:Cat and moderate between RIA:MLD (inverse correlation and RIA:M.U.). These r values are summarized in Table 5. The RIA:Mongoose r value from the previous report (9) is also shown in Table 5.

Comparison of RIA with the Guinea Pig Atrium Assay: This correlative study between the RIA and inotropic effect of ciguatoxin on guinea pig atrium is presented in Figure 6 with the r value shown in Table 5 (r= 0.88). Though the sample number is small, a significantly good correlation is shown between the RIA and guinea pig atrium assay. The latter assay appears to be a valuable method for ciguatoxin studies in correlation with the RIA and other biological procedures.

Table 4. RIA results of fish tissue of various species from Midway.

NUMBER OF SAMPLES	TISSUE EXAMINED (FLESH DORSAL AND VENTRAL)	%	REMARKS
	<u>CPM/GM TISSUE*</u>		
126	162,566 - 349,089 245,800 + 45,342	88.1	Negative
15	351,533 - 392,689 363,813 + 13,495	10.5	Borderline
2	406,533 - 453,422 429,997 + 33,129	1.4	Toxic
	<u>TISSUE EXAMINED (GONADS)</u>		
34	183,911 - 348,067 267,192 + 56,683	63.0	Negative
5	350,900 - 392,445 373,756 + 18,834	9.2	Borderline
15	413,356 - 720,489 482,509 + 79,416	27.8	Toxic

* Values are ranges and mean \pm 1 S.D.

Table 5. Summation of the comparison of the RIA procedure with the other biological methods for the evaluation of ciguatoxin.

NUMBER OF SAMPLES	SYSTEM	COEFFICIENT of CORRELATION (r)	REFERENCE
79	RIA:Cat	0.39	--
66	RIA:M.U. (Mouse)	0.56	--
68	RIA:MLD (Mouse)	-0.55	--
66	RIA:Mongoose	0.56	Hokama, et al., 1977
13	RIA:Guinea Pig Atrium	0.88	--

M.U. = Total weight in gm of mice killed within 48 hours by a 1 ml extract from 20 gm of fish tissue (Yasumoto, et al., 1971)

MLD = Minimum lethal dose in μ g

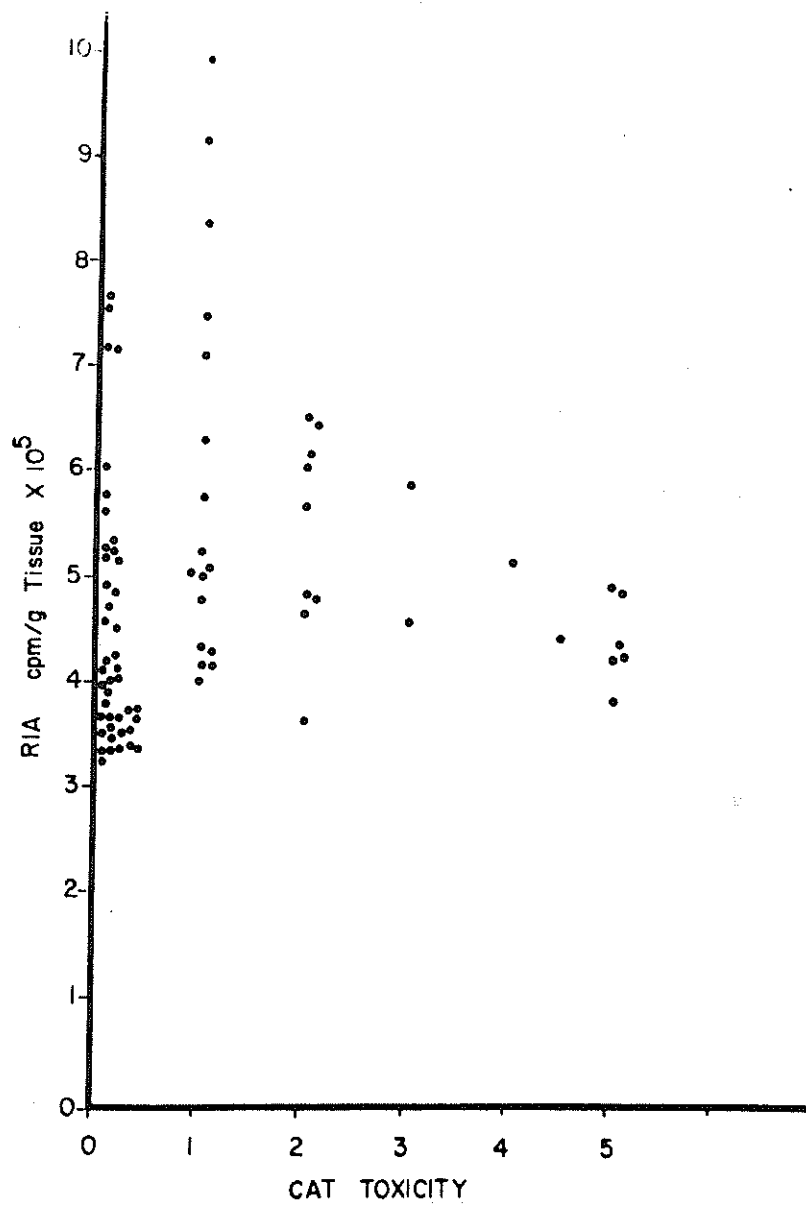


Figure 3. Comparison of the RIA procedure for ciguatoxin detection and the cat assay; each circle represents a sample (RIA values are average of triplicate samples).

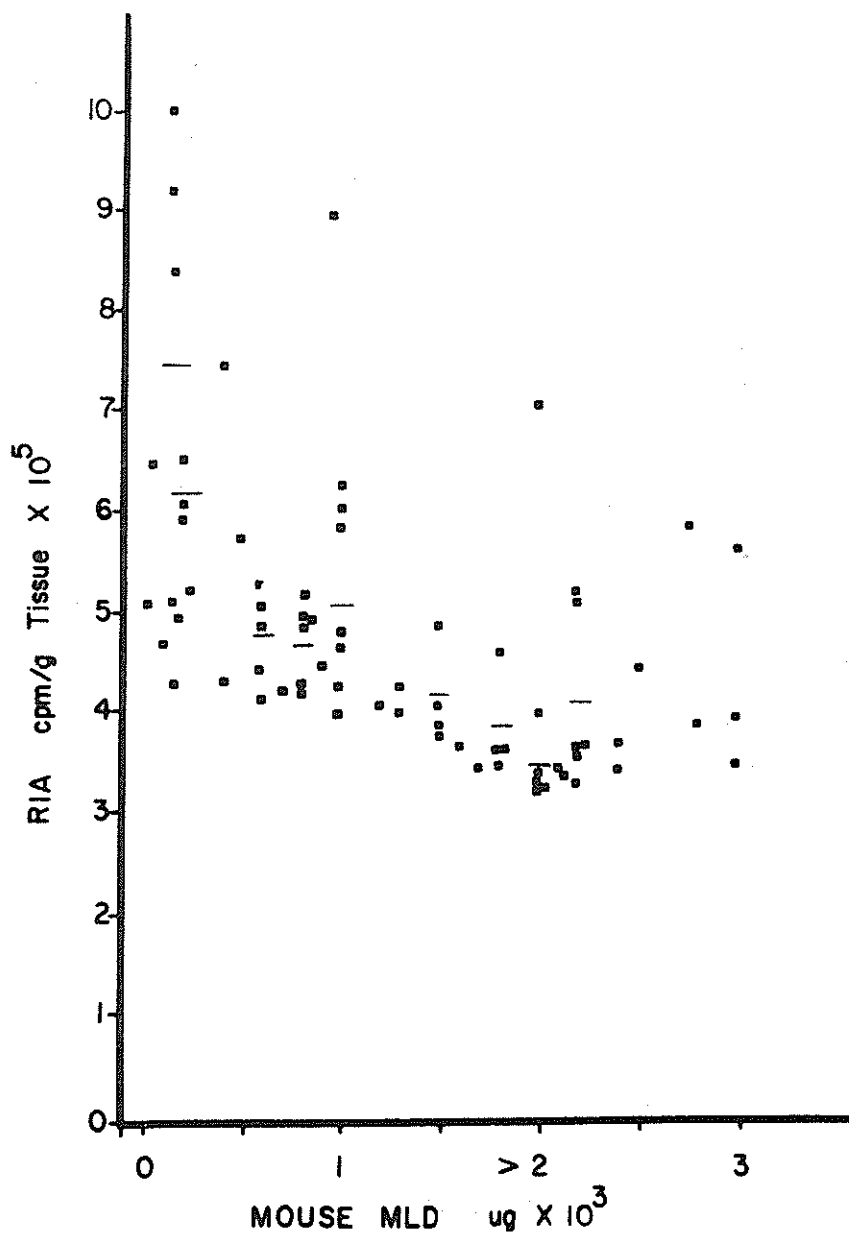


Figure 4. Comparison of the RIA procedure for ciguatoxin detection and the mouse toxicity based on MLD; each circle represents a sample (RIA values are average of triplicate samples).

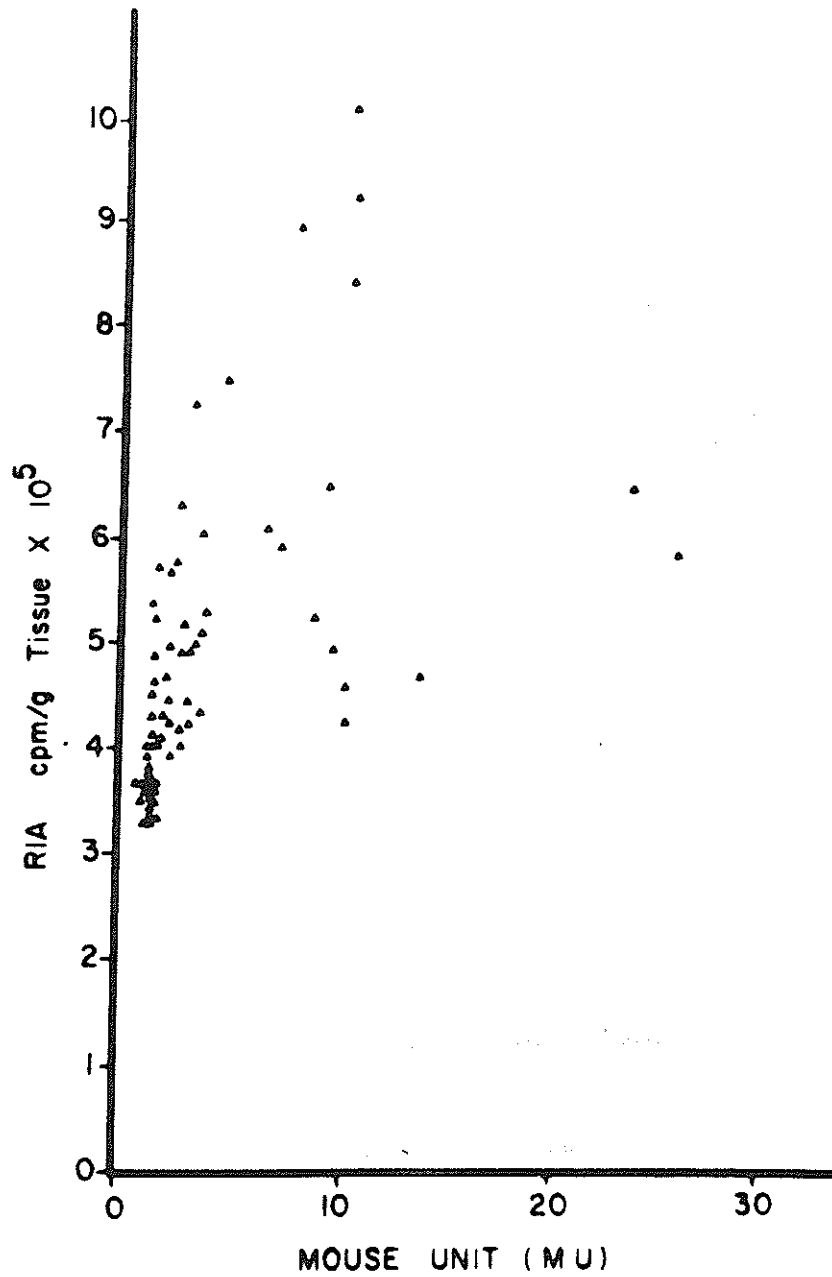


Figure 5. Comparison of the RIA procedure for ciguatoxin detection and the mouse toxicity based on M.U.; each circle represents a sample (RIA values are average of triplicate samples).

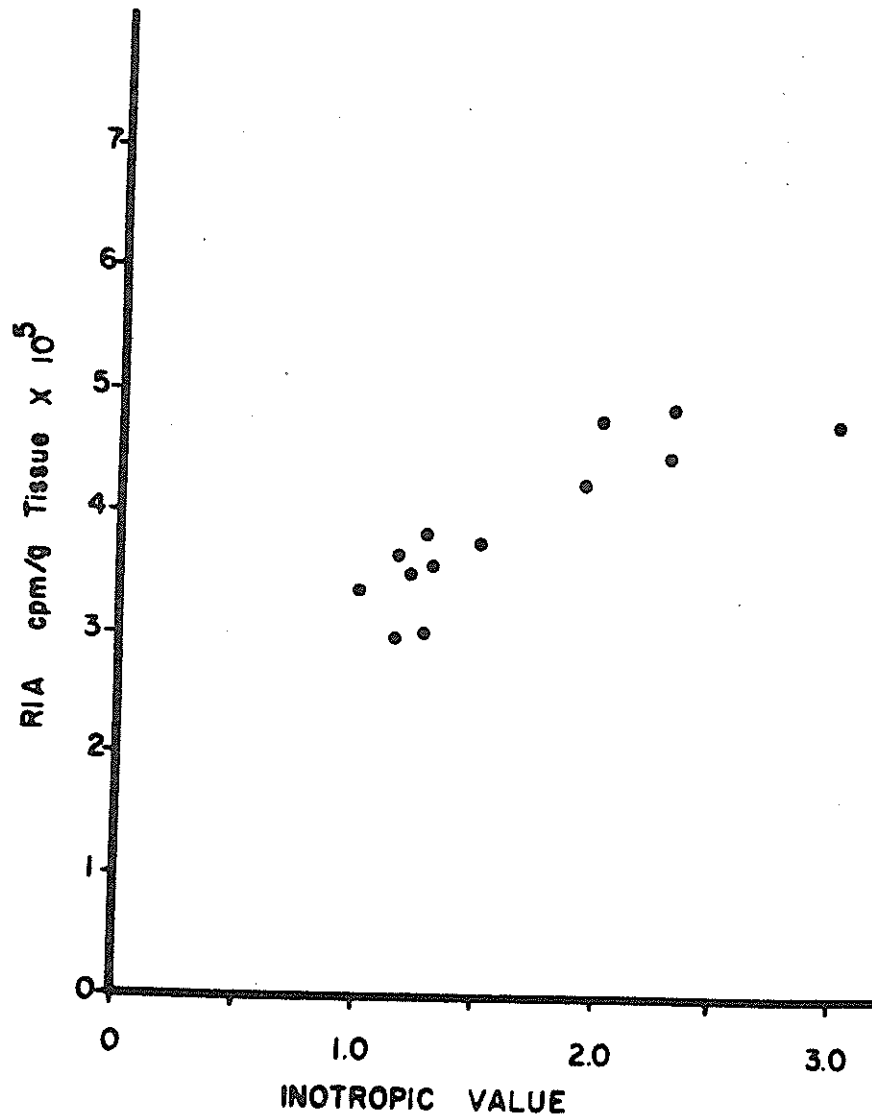


Figure 6. Comparison of the RIA procedure for ciguatoxin detection and the inotropic effect on guinea pig atrium. The inotropic values indicated are obtained by dividing the increase in amplitude (inotropic effect) by the base line contraction.

DISCUSSION

A general approach for the use of immunological procedures for application to small molecules has been presented. The significant factors discussed include: (1) the importance of coupling of the low molecular weight compounds to appropriate antigenic carriers, that is conjugation in positions of accessibility, without gross changes in the haptenic molecule; and (2) selection or choice of the antigenic carrier. Of equal importance is the choice of animal for the production of antibody and the mode of administration of the hapten-carrier conjugate. The analysis and development of the immunological methods for the antibody raised will depend on the levels of the antibody and the haptenic molecule to be examined (11, 15).

Essentially following the general procedures discussed, the development of an immunological procedure for the detection of ciguatoxin (a lipid hapten) has been initiated earlier (9). This report presented the further evaluations of this RIA procedure by examining various species of fishes including those specimens implicated in ciguatera poisoning.

The comparative Examination of fish samples from Tahiti with the RIA, cat and mouse (MLD and M.U.) procedures gave r values which appeared to be similar (Table 5) to that demonstrated earlier for RIA and mongoose. The lack of a better correlation may be due to the highly sensitive nature of the RIA test as discussed previously (9). The capability and limitations of the biological procedures have been discussed earlier by other investigators (5,6,10).

The present state of the RIA procedure is significantly sufficient to distinguish toxic from non-toxic fishes. Furthermore, the test appears to be helpful for the examination of various species of fishes from different geographical areas for the evaluation of the presences or absences of toxic fishes. For example, coral reef fishes from Tahiti tended to have a higher incidence of toxic levels and cpm/gm tissue than similar species caught in Hawaii. (See Figures 3, 4 and 5 for fishes from Tahiti and compare with Table 3 fishes from Hawaii and the Leeward Island Chain. Thus, in its present state the RIA procedure can be utilized for the exploration in different geographic areas of the levels of toxicity and distribution among various fish species, especially where ciguatera poisoning is endemic. This would aid in the development of the fishing industry.

Preliminary studies for screening Seriola dumerili prior to marketing has been in progress for 11 months with good results. Prior to initiating the RIA test, several outbreaks of ciguatera poisoning have occurred; and since the testing was begun, no ciguatera poisoning has occurred from the tested and marketed fishes. The high count fishes rejected by the test represented about 10% of the total catches. This study is continuing, and the details will be reported at a later date.

Nevertheless, the limitations of the RIA procedure for wide scale use at present include: (1) restrictions on the number of sampling per day by a technologist; and (2) a minimum of approximately 6 hours for the completion of the test. Other difficulties include: (1) sampling error, that is the uncertainty of toxin distribution or localization in various parts of the tissue; (2) a tendency for false positive reactions due to

nonspecific binding; and (3) reproducibility due to the fact that tissues are utilized and even within close proximity "hot spots" of ciguatoxin may vary.

Efforts are presently being directed towards decreasing the test time, devising an ELISA procedure, using homogenous crude lipid extracts from large tissue areas, minimizing the cost of the test and, in addition, increasing specificity by attempting to isolate and concentrate the specific antibody involved.

SUMMARY

The steps involved in the utilization of immunological procedures in the study of small molecules including marine toxins are as follows: (1) conjugation of the small molecule to appropriate antigenic carriers; (2) immunization of animals for the production of antibodies; and (3) development of an immunological method for analysis of the small molecule. The development of the radioimmunoassay for ciguatoxin, a lipid was carried out, accordingly. The RIA procedure for detection of ciguatoxin showed fair to moderate correlations with the cat feeding assay ($r = 0.39$), mouse MLD ($r = 0.55$) and mouse M.U. ($r = 0.56$) tests, respectively. These results were similar to that reported for the RIA and mongoose ($r = 0.56$). A good correlation of the RIA with the in vitro guinea pig atrium assay was demonstrated ($r = 0.88$). Examination of non-toxic fishes and fishes recovered from patients with clinically defined ciguatera poisoning with the RIA procedure demonstrated that the test clearly differentiated toxic from non-toxic samples. It is suggested that the RIA test in its present state is useful for the assessment of toxic from non-toxic fish tissue in a variety of fish species from various endemic areas. Currently, work is being carried out to develop a simpler enzyme-linked immunoabsorbent assay with increased specificity.

ACKNOWLEDGEMENTS

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Paralytic Shellfish Poisoning in Shellfish Aquaculture
The State Regulatory Problem
Presented at the Sixth FDA Science Symposium
Office of Health Affairs
U.S. Food and Drug Administration
February 14, 1980

by
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ABSTRACT

P.S.P. In Shellfish Aquaculture
The State Regulator Problem

Maine has a vigorous P.S.P. monitoring program designed to protect the public's health and to lessen the impact of P.S.P. on its valuable shellfish industry. The P.S.P. monitoring program is described as well as the ongoing research on the various parameters of the causative organism. The relationship of these studies to shellfish aquaculture is discussed as well as potential management in shellfish aquaculture around P.S.P.

Maine has a very valuable shellfish industry. For example, the landed value of the soft-shell clam in 1979 was approximately \$8,000,000 with a consumer value of about \$48,000,000. For the shellfish industry to remain viable, the public must have confidence in shellfish as to its safety as a food. The Department of Marine Resources is the state agency in Maine responsible for shellfish safety. Maine's Paralytic Shellfish Poisoning Monitoring Program has been described by Hurst and Gilfillan, "Paralytic Shellfish Poisoning In Maine"* (1977).

We began monitoring and closing for PSP in 1958 following a serious outbreak of PSP in nearby New Brunswick, Canada in 1957. Five monitoring stations were established in this eastern Maine area. Closures were made in portions of this area in 1958, 1959, 1961, 1964, 1969, 1970, and 1972 whenever scores exceeded 80 micrograms. This limited monitoring plan, coupled with up-to-date knowledge of the monitoring results in nearby Canada, has apparently given adequate public health protection in eastern Maine. Until 1972 no other areas were closed although our shellfish monitoring demonstrated occasional low PSP scores. Early in September 1972 it became evident that there were extremely toxic shellfish from Cape Ann, Massachusetts into western Maine. The aftermath of this discovery was the closure on September 15, 1972 from Cape Elizabeth to New Hampshire and the entire coast on September 17.

*John W. Hurst, Jr. and Edward S. Gilfillan, Paralytic Shellfish Poisoning in Maine. 10th. National Shellfish Sanitation Workshop, U.S. Dept. of Health, Education and Welfare, 1977.

Most of the coast, with the exception of Cape Elizabeth to New Hampshire, was reopened on September 30, 1972. Much of this area remained closed into 1973. 1973 did not require any closures other than the historical area in eastern Maine. 1974 was a year of high toxicity with a spring and summer peak. Inasmuch as we were without a precise sampling plan, 1974 was a year of multiple crises. Although hindsight demonstrated that while we were able to keep up with these crises, this method of PSP monitoring was not a responsible public health protection program. A lack of precise knowledge as to the areas which were toxic led to larger closed areas than were necessary in order to give adequate public health protection.

Late in 1974 we were able to obtain funds from the New England Regional Commission, NERCOM, Contract #10530699, to develop our monitoring plan. This permitted a greatly expanded PSP monitoring program to provide more precise information about distribution of PSP during an outbreak. One hundred nineteen monitoring stations were established. This monitoring program was initiated on April 1, 1975 and consisted of a series of 18 primary, 35 secondary and 63 tertiary sampling stations. These stations were established on the basis of previous historical data. Primary stations are sampled on a weekly basis throughout the danger period for PSP, that is, April through October. Primary sampling stations are those which in the past have been good indicators of the presence of PSP when it is present at a low level. Once PSP is identified at a primary sampling station, samples are taken at secondary and tertiary sampling locations. Secondary sampling locations are chosen on the basis of past results to be good indicators of what is expected to occur in given clam growing areas. Tertiary sampling stations are chosen to fill the gaps in these secondary sampling locations and further localize the distribution of PSP. In 1979, using the information derived from our sampling program, we were able for the first time, as a part of our management plan, to keep open a portion of the area, with the exception of mussels, which would have normally been closed for all shellfish for PSP. This entailed a heavy-handed sampling program. Although the growing area which we sampled intensively was relatively small, during the 55 day closure approximately 155 shellfish diggers harvested 17,050 bushels of soft-shelled clams with a landed value of \$426,250 and an estimated consumer value of \$2,770,625. Because of this obvious benefit to everyone concerned, we intend in the future to expand our ability to narrow down the areas which need to be closed for PSP. With this expanded monitoring program it is necessary to continually update the sampling stations and even sampling frequencies as conditions dictate. With experience and better knowledge, not only will it be possible to better define what areas need to be closed, but more importantly, what areas can safely remain open to harvesting.

Fig. 1
Fig. 2

Our PSP sampling program has documented how the different species of shellfish react to the poison (Fig. 3). This dramatic difference in accumulation of poison in the various species allows for at least some preplanning for our monitoring program in the spring. Mussels become toxic several weeks prior to the time that clams become poisonous, which allows for management of closures for species only. The mussel closure, in turn, forewarns the soft-shell clam industry of a potential closure, giving them

Fig. 3

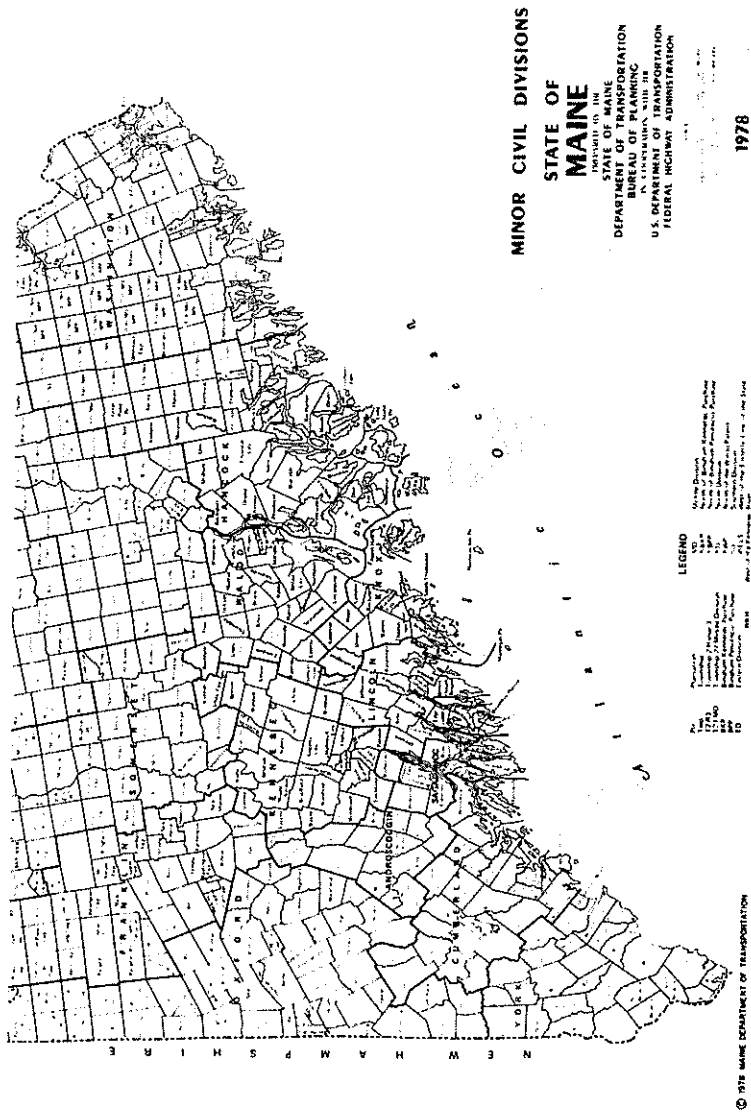


Figure 1. Coast of Maine.

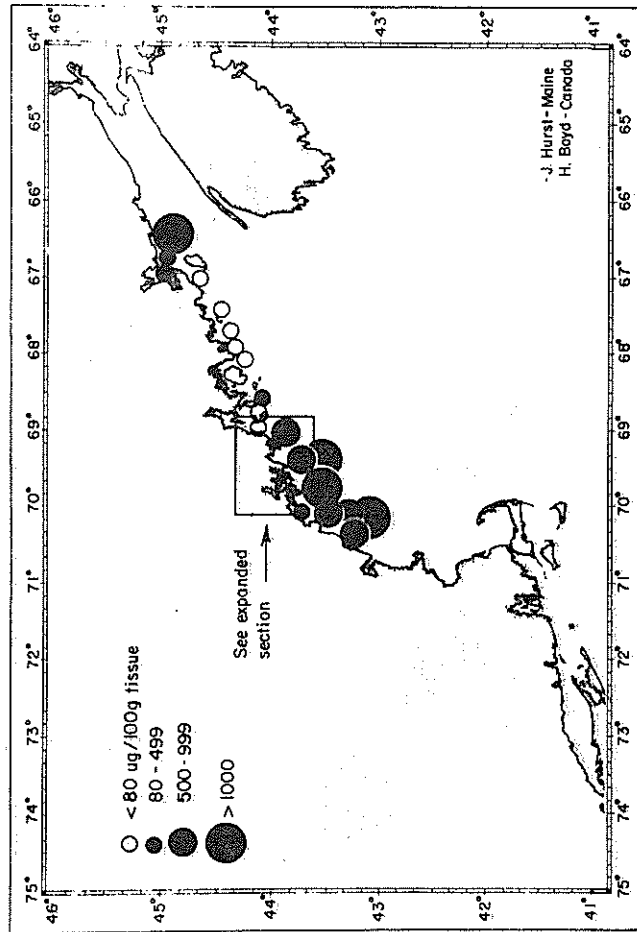


Figure 2. Location of PSP sampling areas showing degree of poison.

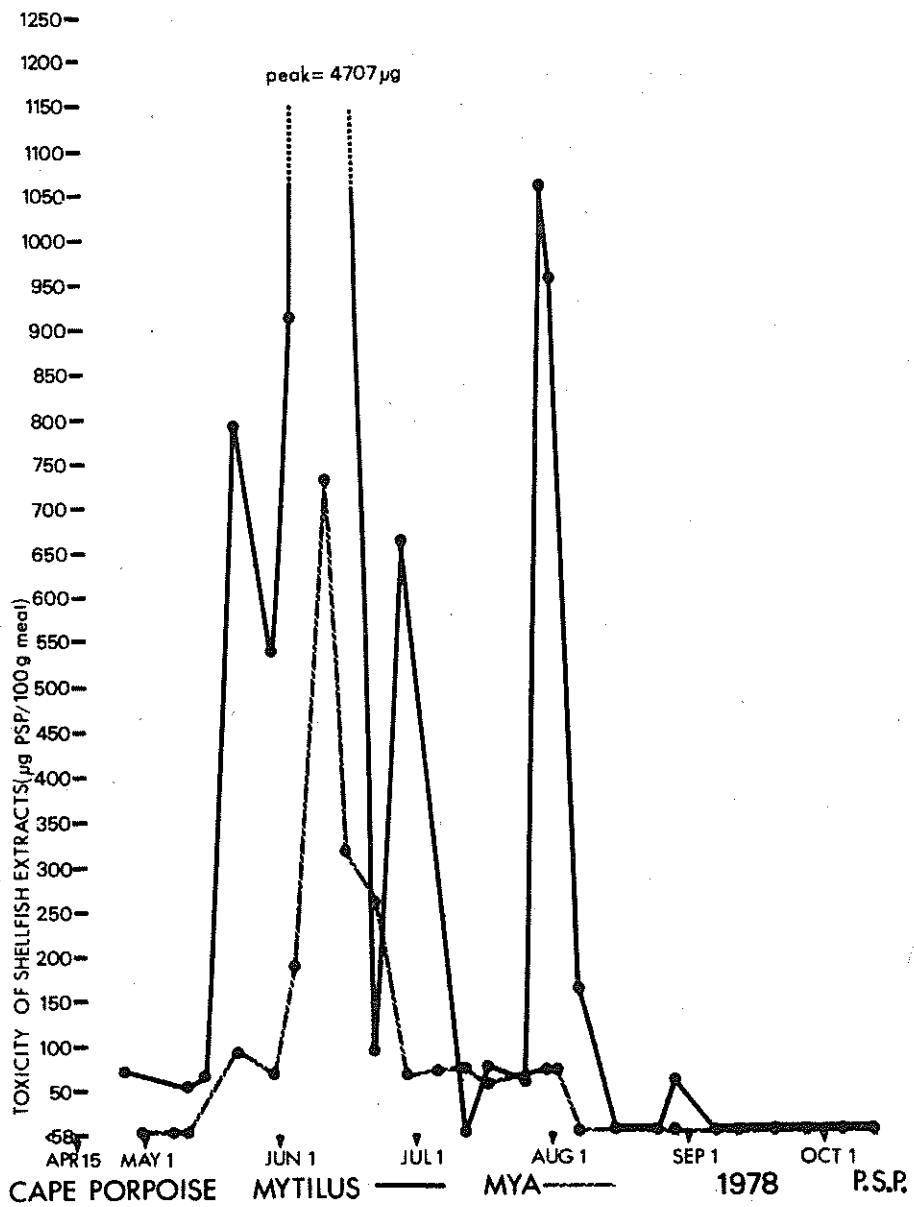


Figure 3. Variation in poison in species at Cape Porpoise.

time to locate alternate supplies of clams. In some years this has meant that we have only had to close for mussels because we have been able to demonstrate that clams were not becoming toxic. This has proved to be beneficial to the clam harvester and dealer because they are not put out of business because of a rise of poison only demonstrated in mussels. Prior to this expanded program, a rise of poison in one species has resulted in a closure for all species. I must point out, however, that when mussels become toxic, one must expand their sampling program to demonstrate that the other shellfish are not becoming toxic.

The monitoring plan also demonstrates that when there is a geographically widespread incidence of poison in mussels just below 80µg/100g, it is reasonable to question the advisability of having any of this area open to the harvesting of mussels. In mussels, 80µg/100g appears to be the threshold of a rapid rise of poison. We have noted a rise in mussels from 80µg/100g to 500µg/100g in a single day! Fortunately, this rapid rise is in reality not an instant thing; however, an up-to-date sampling program will give sufficient warning of what is going to take place (i.e., a widespread low level of poison). In the final analysis, mussel closures must be based upon the best available PSP monitoring data, on the State's ability to adequately monitor the harvest area, and on the overall value of the mussel resource in the area under consideration.

Maine has two distinct and separate areas in which PSP may be expected to occur. The first area in eastern Maine extends from the international border with Canada to the Machias River. The second area extends from Schoodic Point to New Hampshire. Between these two areas is an apparent PSP-free area. In addition to our shellfish monitoring program, we have demonstrated by our Gonyaulax cyst sampling program that this area is free of cysts. On either side of this area only low incidence of poison has been demonstrated. To date we have not explained satisfactorily the reasons for this area being toxin free, but undoubtedly it has to do with hydrographic and chemical conditions which are unfavorable for the growth of Gonyaulax. I conduct limited shellfish monitoring in this area to demonstrate that the shellfish continue to be safe. I am not going to become over confident in this area always being toxin free as I remember that until 1972, with the exception of Monhegan and Matinicus, there were no significant amounts of PSP south of our traditional eastern Maine area!

Fig. 4

Fig. 5

Additional information useful in decision making in evaluating of PSP in Maine has been derived from a F.D.A. contract (233-77-2314) to Bigelow Laboratory for Ocean Sciences entitled "Toward an Environmental Predictive Index for Toxic Dinoflagellate Blooms"* (1980). Although we have not yet been able to develop this index, we have made several important valuable advances in our understanding of the PSP phenomenon under this contract. This study has explored and given information on the following parameters:

- I. Patterns of intoxicification of shellfish in the Gulf of Maine coastal waters
- II. Taxonomy and life history aspects

Clarice M. Yentsch and John W. Hurst, Jr., Report of Investigations Toward an Environmental Predictive Index For Toxic Dinoflagellate Blooms. To Food and Drug Administration 1979.

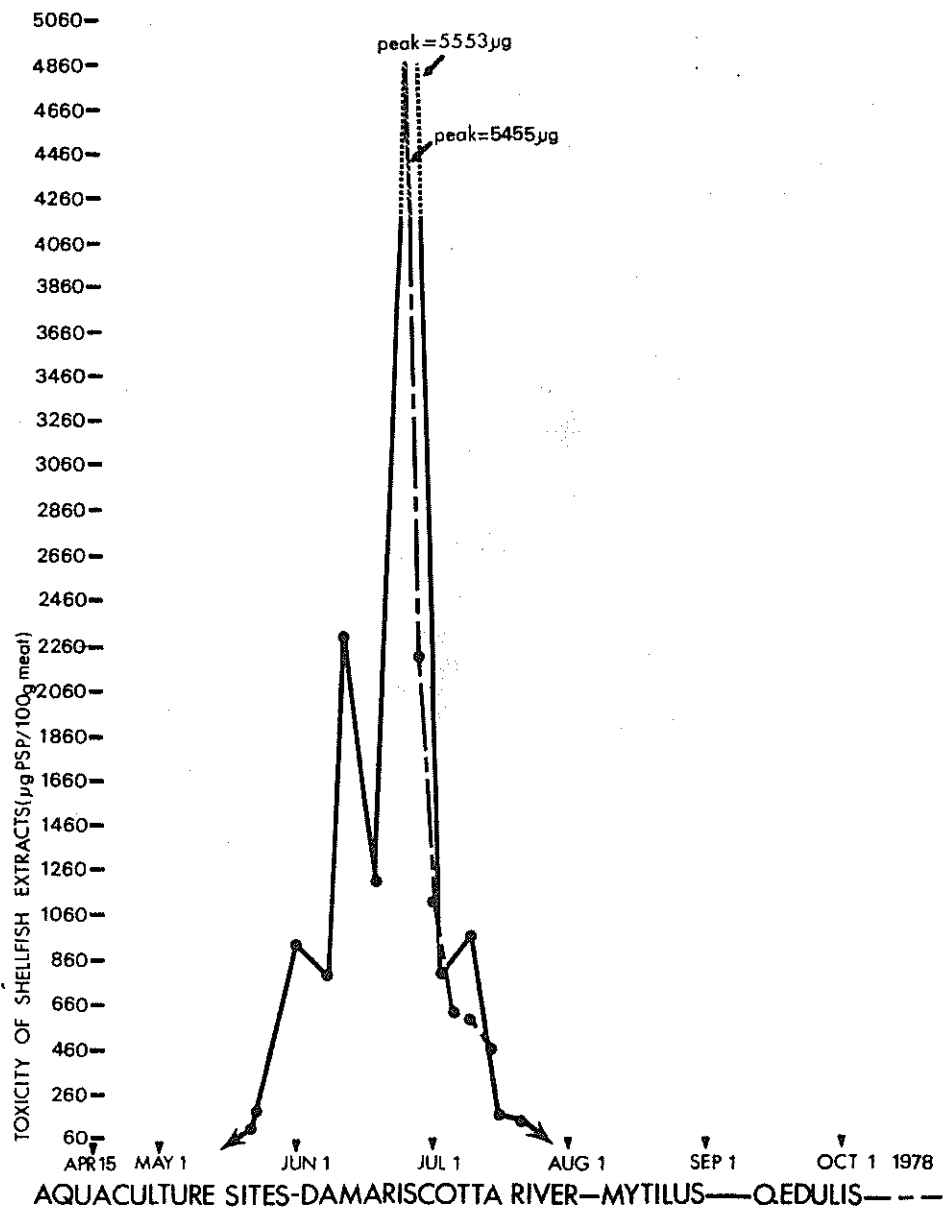


Figure 4. Degree of poison at aquaculture sites on Damariscotta River.

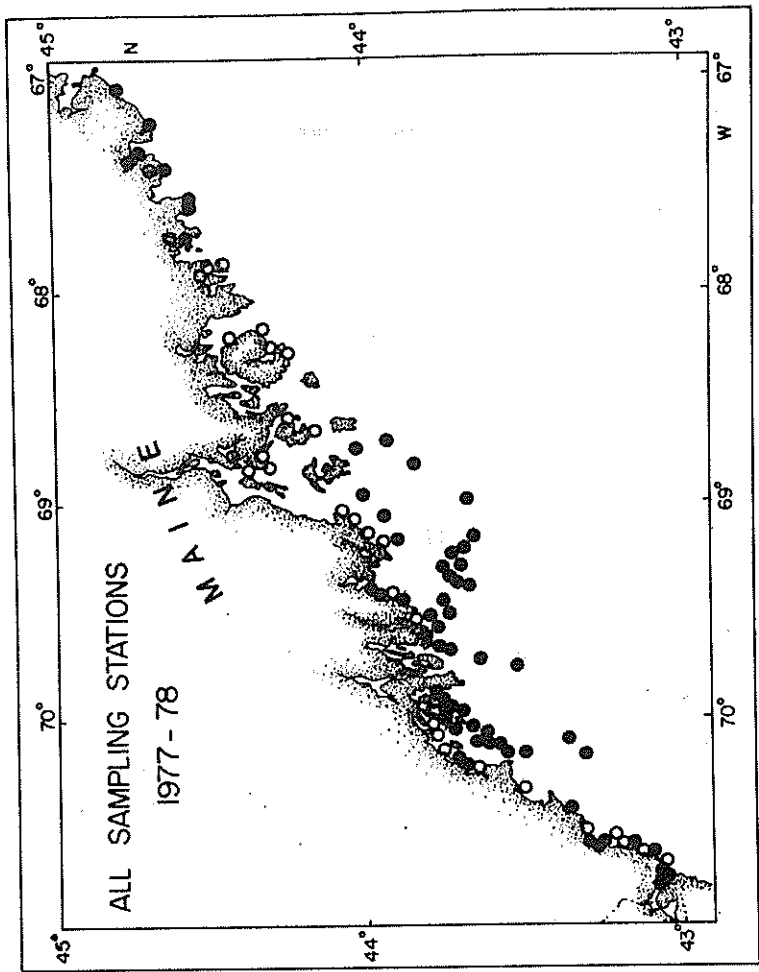


Figure 5. Cyst sampling stations - 1977-1978.

- A. Probing the occurrence of biological resting in the dinoflagellate, Gonyaulax excavata
 - B. Coexistence of toxic and nontoxic dinoflagellates resembling Gonyaulax tamerensis in New England coastal waters
- III. Patterns of distribution of Gonyaulax excavata
- A. Patterns of phytoplankton distribution: An apparent lunar tidal cycle of phytoplankton blooms and phytoplankton community succession in the Gulf of Maine
 - B. Distribution of Gonyaulax excavata resting cysts in the sediments of the Gulf of Maine
 - C. Patterns of resting cyst distribution
- IV. Mechanisms and conditions of intoxicification and dinoflagellate blooms
- A. Motile cells and cysts: two probable mechanisms of intoxicification of shellfish in New England waters
 - B. Dinoflagellate bloom initiation and perpetuation in the Damariscotta estuary
 - C. Current measurements and flow dynamics around Monhegan Island, Maine
- V. Physiology of nitrogen and toxins
- A. Light and nutrient limitation in Gonyaulax excavata: Nitrogen and carbon trace results
 - B. Toxicity and nucleic acid content of Gonyaulax excavata
 - C. Toxicity in resting cysts of the red-tide dinoflagellate Gonyaulax excavata from deeper water coastal sediments
 - D. Changes in toxicity of benthic resting cysts
- VI. Recent work with trace metals
- A. Iron in Maine coastal waters seasonal variation and its apparent correlation with a dinoflagellate bloom
 - B. Trace metal analysis by differential pulse ASV in water samples from the Gulf of Maine.

Shellfish aquaculture is expected to be an increasingly valuable addition to Maine's shellfish industry. As the aquaculturist evaluates potential locations for a new aquaculture enterprise, the occurrence of PSP is only one of the considerations that must be made in the selection of a growing area. The aquaculturist must investigate such parameters as site availability, distance from market, anticipated marketing practices and the overall environmental conditions of the growing area. It is entirely possible that the presence of PSP in an area may be an indication of a good growing area. The two species most favored by Maine aquaculturists are the blue mussel, Mytilus edulis, and the European flat oyster, Ostrea edulis. These two species will always become toxic during a Gonyaulax bloom! I have been fortunate in that early on I discovered that O. edulis becomes toxic. This oyster presents a very serious public health concern as it is consumed raw, thus a lower overall PSP level in this oyster may be expected to cause illness. I have no intention of finding out what this level is, however.

Shellfish aquaculture in Maine is centered in the Damariscotta River. Fig. 6
 This is due to the presence of the University of Maine's Darling Research Fig. 7

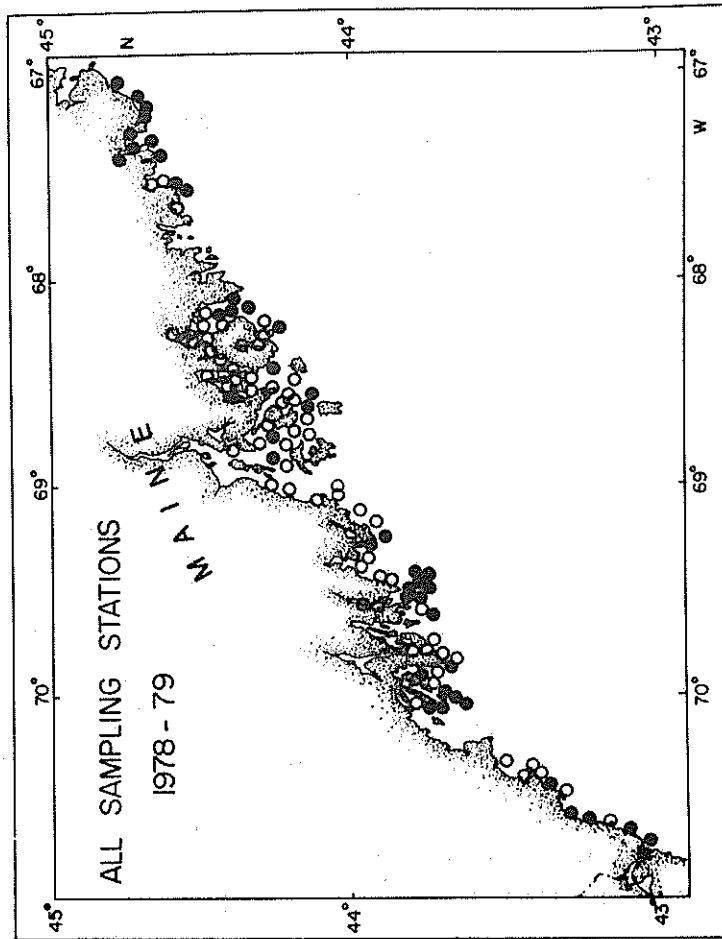


Figure 6. Cyst sampling stations - 1978-1979.

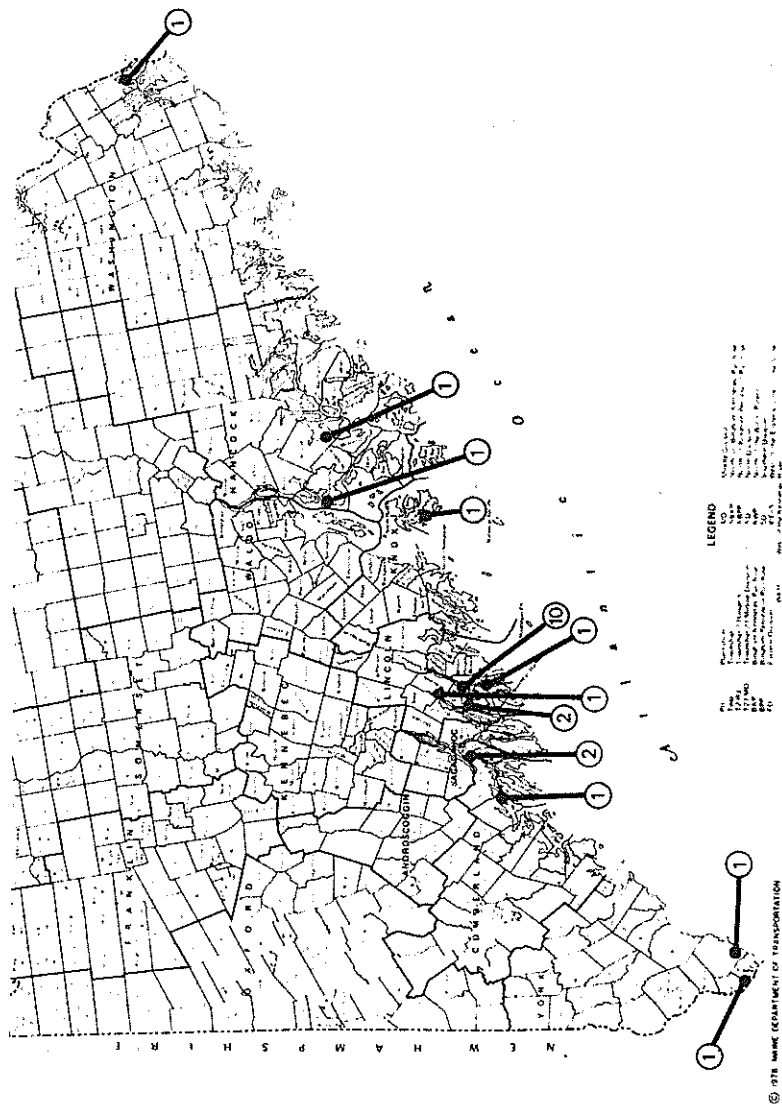


Figure 7. Locations of aquaculture sites.

Center on the river, which has promoted shellfish aquaculture. In respect to PSP, this centralized location of aquaculture is probably an unfortunate occurrence. This is because PSP can reasonably be expected to reoccur in the Damariscotta River every year. However, as aquaculture enterprises begin to expand into other areas it is reasonable to evaluate those areas for these enterprises to see how they shape up, paralytic shellfish poison-wise. As we evaluate the information available on PSP, it becomes increasingly evident that it is possible to utilize this information in the planning of new developments in shellfish aquaculture.

In the long run, the occurrence of PSP may work to the overall advantage of the aquaculture industry because it forces them to market their shellfish during periods when the overall quality is best. This is particularly true of mussels and European oysters; the quality of both species is poor during the months (May - October) when PSP may be expected to occur.

In conclusion, I anticipate an increasing awareness of PSP by the shellfish aquaculturists. Without a doubt, our increasing knowledge of the conditions which aggravate dinoflagellate blooms may be utilized by the shellfish aquaculturists and the Department of Marine Resources is developing a meaningful management plan for this potentially valuable addition to our shellfish resources.

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PRELIMINARY OBSERVATIONS ON
PARALYTIC SHELLFISH POISONING IN CENTRAL PUGET SOUND*

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ABSTRACT

The first outbreak of paralytic shellfish poisoning (PSP) in central Puget Sound occurred in 1978, with toxicity levels as high as 30,000 ug toxin/100g mussel tissue. Research initiated at that time has shown that Gonyaulax catenella, the species believed to have been the causative organism, forms cysts and that a maturation period may be required before excystment can occur. A shellfish monitoring program throughout central Puget Sound delineated the geographical limits of toxicity in 1978 and demonstrated a southward spread in 1979. Differences noted in patterns of uptake and loss of toxin in butter clams (Saxidomus giganteus) and mussels (Mytilus edulis) may be partially explained by the presence of cysts. A study of environmental parameters has been initiated to determine whether any of these factors can be used in prediction of occurrences of PSP.

BACKGROUND AND PURPOSE

In September, 1978, paralytic shellfish poisoning occurred for the first time in Puget Sound, a fiord extending deep into the more populous portions of Western Washington (Fig. 1). Prior to that time, toxic shellfish had been found almost annually since the 1930's along the northern coast of the Olympic Peninsula, and since 1975 in the Bellingham area as well. In the 1978 outbreak red water and extremely high levels of toxicity (22,000 to 30,000 ug toxin/100g mussel meat) were reported in three different areas of the Whidbey Basin of Puget Sound. Lower levels of toxicity and discolored water, reported several days later in the northern part of the Main Basin of Puget Sound, suggested that a southward spread of the causative organism was occurring.

A research program was initiated by the College of Fisheries at the University of Washington to study several facets of the PSP problem: the extent of the geographical spread, the identity of the causative organism and questions regarding cyst formation and distribution, rates of uptake and loss of toxicity in shellfish in Puget Sound, and water quality conditions which influence growth of the toxic alga.

* Emergency funding for this study was received from the U.S. Food and Drug Administration and the Washington Sea Grant Program. Mouse bioassays for shellfish toxicity were conducted by the Washington Department of Social and Health Services.

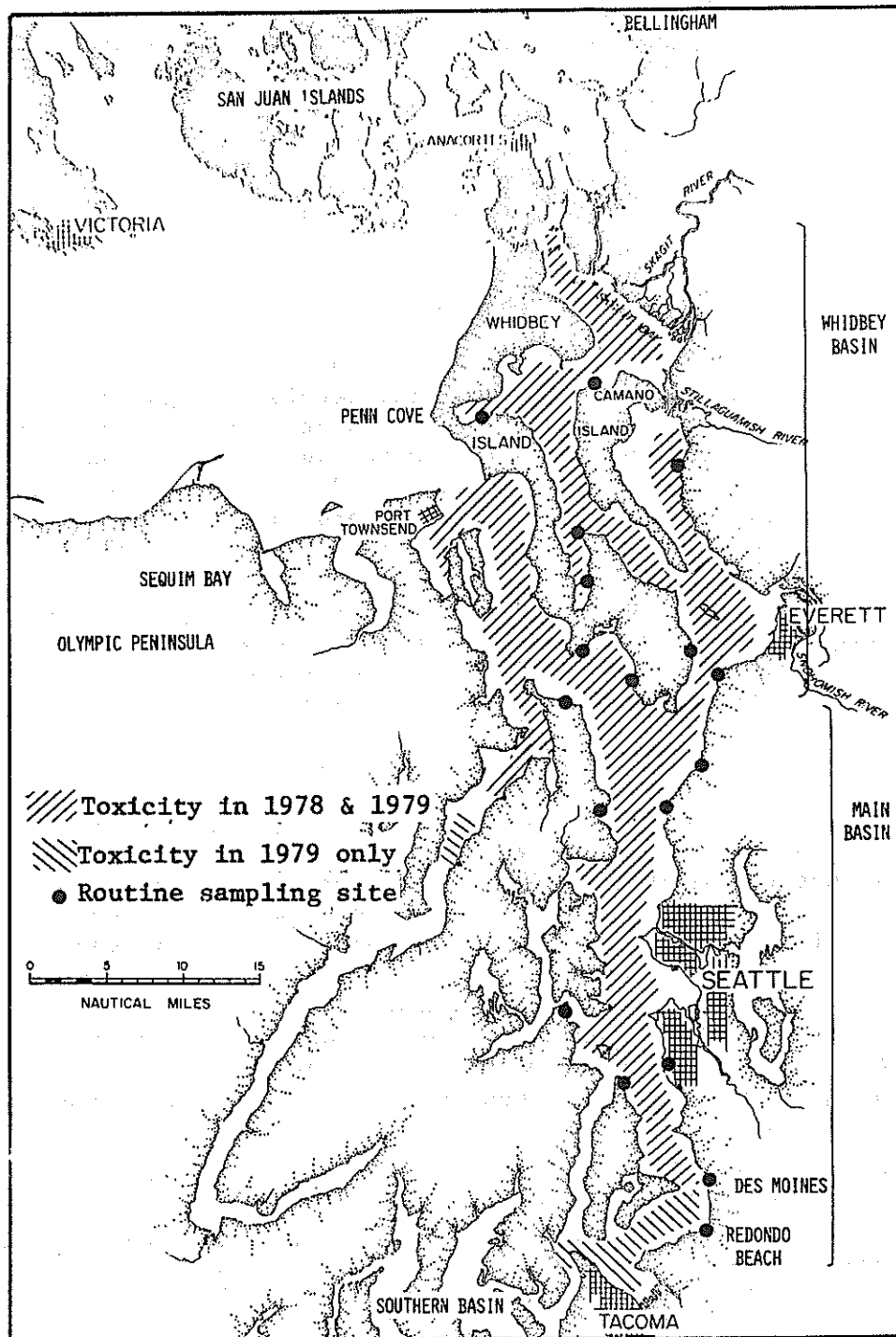


Figure 1. Distribution of PSP in Central Puget Sound, 1978-1979.

RESULTS AND DISCUSSION

A shellfish monitoring program with routine sampling at 16 sites and occasional sampling elsewhere on both sides of the Whidbey and Main Basins of Puget Sound showed that in 1978 shellfish were toxic throughout the Whidbey Basin and on both sides of the Main Basin as far south as Des Moines but not a Redondo Beach (Fig. 1). In 1979 shellfish toxicity occurred throughout the southern end of the Main Basin in areas which had not been toxic in 1978 with the highest levels of toxicity at Redondo Beach. This leads to the speculation that significant numbers of resting cysts may have been carried southward in the sediments by the bottom current, which has a net southward flow in this region (2), and deposited in the gyre off Redondo Beach. Such extension of geographical distribution by resting cysts has been proposed for Gonyaulax tamarensis and G. excavata (1). If, in the future, the southward extension of toxicity continues past Tacoma, it could jeopardize the Southern Basin oyster and clam industries with landing values of approximately \$5 million per year and pose very significant additional public health problems.

Plankton samples taken in the Whidbey Basin just after the bloom in 1978 contained a few motile cells of Gonyaulax catenella, a dinoflagellate known to cause shellfish toxicity on the Olympic Peninsula. It was considered probable this species had caused the toxicity observed. To determine whether this species forms resting cysts, as had been demonstrated for G. excavata (4), sediment samples were collected in Penn Cove, a bay on Whidbey Island in which toxin levels reached 22,000 ug toxin/100g mussel meat, and incubated in a suitable growth medium and under a temperature-light regime previously used for culturing G. catenella. In a series of such cultures set up at intervals beginning in October, motile cells first appeared in March. These results demonstrated the formation of cysts by G. catenella and suggested that a maturation period may be required before excystment can occur, as had been observed for G. excavata (5). Rough estimations of the density of cysts in Penn Cove were obtained by the technique of serial dilutions in microtiter plates suggested by Hall (6). Motile cells grew from 31 of the 34 sediment samples collected in three areas of Penn Cove (the head, middle, and mouth of the bay), with estimated densities ranging from 40 to 2,800 cysts/ml of the top centimeter of sediment. Thus, cysts were demonstrated to be patchily distributed throughout the bay and very dense in some samples.

The fact that benthic cysts are produced by this species contributes to our understanding of the patterns of uptake and loss of toxin, found to be similar in this study and in earlier work in Sequim Bay (9) and Alaska (7). Mussels became toxic when motile cells of G. catenella were present and, at most study sites, lost toxicity rapidly to undetectable levels within five to seven weeks after maximum toxicity was attained. An exception to that pattern was noted in those areas of the Whidbey Basin which had had very high toxicity in 1978. There, following the loss of 80% of the toxin in a few weeks, low levels of toxicity were maintained for three to twelve months. Butter clams became toxic several weeks after mussels and, in areas outside the Whidbey Basin, remained toxic longer than mussels. The different times of toxin increase in these two species observed by Neal in Alaska (7) led him to suggest that they may have different food

sources providing the toxin, namely motile cells of Gonyaulax for mussels and toxic detritus or possibly an encysted form of Gonyaulax for butter clams, as Bourne had suggested for sea scallops in eastern Canada (3). Our demonstration of resting cysts of G. catenella supports Neal's suggestion and appears to provide at least a partial explanation for the differences in timing of increase in toxicity in these two species. The longer maintenance of toxin in butter clams has been attributed to the binding of toxin to melanin and storage of the bound toxin in the siphon (8). It now seems probable that, in addition to such retention, continued feeding on benthic cysts may also contribute to the longer maintenance of toxin in butter clams. The exceptional pattern of prolonged toxicity in mussels, as well as fluctuations in toxin levels throughout the winter in areas which had had very high toxicity, raise the question of whether cysts may have been stirred up in the water column and ingested by mussels, thus providing a continued source of new toxin which compensated for metabolic loss.

During this study seven intertidal and three subtidal species of bivalves were found to be toxic. In addition, a large gastropod, Fusitriton oregonensis, from -55m dredges was found to be as toxic as the scallops (Chlamys hericium) in the same samples.

A study of water quality parameters, plankton composition, and toxin levels in shellfish was initiated in Penn Cove in 1979 to determine whether any measureable environmental conditions could be used in prediction of PSP. This study will be continued as funding permits. Because hazardous levels of PSP in shellfish in Washington occur much more frequently with low densities of G. catenella (20×10^3 cells/l) than with denser, visible blooms, continued studies of both population changes at very low densities and the role of cysts in intoxication of shellfish are essential to our understanding of the factors which may be of value in prediction of PSP.

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SHELLFISH AQUACULTURE AND PARALYTIC SHELLFISH POISONING

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ABSTRACT

The history and current status of paralytic shellfish poisoning problems in shellfish aquaculture are presented. The characteristics of the newly discovered toxins from economically important shellfish were introduced. It was confirmed that scallops have the ability to convert gonyautoxins to saxitoxin in the sequence: gonyautoxin-I → gonyautoxin-II → neosaxitoxin → saxitoxin. The scallop adductor muscle has the ability to detoxify; the PSP Alaska butter clam has also the ability to convert gonyautoxins to saxitoxin and store it in the siphon. Precautions considered to be necessary for shellfish aquaculture in connection with PSP are discussed.

Accumulation of noxious substances, both natural and synthetic, in fish and shellfish, transported through the food chain have been a subject of great public concern. Such substances are normally so minute in quantity that their existence can be recognized only due to the recent development of analytical methods and increased understanding of molecular toxicology. In a few cases, however, such metabolite transfer from organism to organism have been known to us because of the drastic biological activity of the involved compounds. One noted example is the shellfish toxins.

Paralytic shellfish poisoning is one of the most drastic forms of food poisonings which has been known from the ancient time. People empirically knew that shellfish in certain areas can become toxic in certain times of the year. Sommer and Meyer (1) for the first time showed the relationship between the toxicity of shellfish and a dinoflagellate in the Californian waters. The organism was named Gonyaulax catenella. Some years later, the similar observation was made with the PSP on the Canadian Atlantic coast. Their causative organism was identified as Gonyaulax tamarensis (2). The toxic principle responsible for the poisoning was determined to be a small molecular nitrogenous compound, saxitoxin. The name came from Alaskan butter clam, Saxidomus giganteus (3) from which the toxin was originally isolated. In certain Alaskan areas this normally non-toxic clam contains a considerable amount of toxins in its siphon. Schantz and his co-workers identified the same toxin in the Californian mussel, Mytilus californica, and the dinoflagellate, G. catenella (4). Since then, it was generally regarded that the PSP was caused by saxitoxin.

However, the recent studies mostly done by our group at the University of Rhode Island have revealed that in many cases toxic shellfish contain multiple toxins, and that saxitoxin represents only a small portion of the

of the total toxicity (5). This is true with all the toxic shellfish obtained from various parts of the world except Alaska butter clam in the Southeast Alaska (Table 1). The toxin components in the causative organisms also follow the same trend, and thus several toxins were discovered in the intact cells (6).

The chemical structures of these newly discovered toxins are all closely related to saxitoxin. Gonyautoxin-II, which is the major toxin in many samples, was assigned the structure of 11α -hydroxysaxitoxin by our group and gonyautoxin-III as its 11β -isomer (7). Later it was found out that both compounds actually exist in their sulfate forms (8). Neosaxitoxin, which was first discovered in Alaska butter clam, was given the structure of 1-hydroxysaxitoxin based upon chemical and spectroscopic evidence (9). Gonyautoxin-I has recently been assigned the structure of 11α -dihydroxynesosaxitoxin sulfate (10). Gonyautoxin-I and gonyautoxin-IV form an equilibrium mixture, in which the former is the predominant component indicating gonyautoxin-IV is the more sterically hindered 11β -isomer.

The pharmacological action of all the isolated toxins seems to be similar to that of saxitoxin; the blocking of sodium ion influx through excitable membranes. The manifestation of the poisoning is usually very fast and the direct cause of deaths is respiratory paralysis. The toxin is heat-stable at a lower pH and cannot be destroyed by heating. The canning process destroys only a part of the toxicity. The toxin is easily destroyed by oxidation at a high pH range, which is not applicable in the ordinary food processing.

In the past, the occurrence of PSP was considered to be limited to specific areas, e.g., Alaska, Northern California, etc. However, increasing numbers of incidents have recently been reported around the world. The socio-economical impact of PSP is now becoming a serious problem.

In September, 1972, a large scale red tide took place in New England coast north of Cape Ann, where PSP was not a serious problem before. The toxicity of shellfish in the area reached a very high level. Fortunately, the incident was discovered at a very early stage, but over thirty people were poisoned and some seriously. The most serious impact of this red tide was an economic one. The collection and transportation of shellfish was banned immediately. The bad publicity created by television, radio and newspapers implanted unjustifiable fear of seafoods in general among the public. People started to shun even fish and lobsters which are absolutely safe. One time the price of fish dropped to one-third of the ordinary prices at the Boston fish market. Sea food restaurants lost customers and tourism dwindled. It is difficult to estimate the total monetary loss in this incident, but it certainly reached the order of million dollars. The President declared the area as a federal disaster area.

In 1976 there were 268 PSP incidents widely scattered in western Europe. The poisonings were traced to mussels originated from Spain. Analysis of a sample confiscated by Swiss Federal Government showed the presence of gonyautoxins, neosaxitoxin and saxitoxin, a typical profile found in other PSP samples (11). The toxic mussel was cultured on the

TABLE 1. Toxin Contents in Various Shellfish Samples.

Toxic Shellfish	Causative Organism*	Collection Site	STX	GTX ₁	GTX ₄	Purified Toxins [†]												
						GTX ₂	GTX ₃	GTX ₅	neoSTX	GTX ₆	GTX ₇							
<u>Mya arenaria</u>	<u>G. tamarensis</u>	Essex, MA	++	++	+	+++	+	+++	+	+								
<u>Mya arenaria</u>	<u>G. tamarensis</u>	Hampton, MA	++	±		+++	+	+++	+									
<u>Mytilus edulis</u>	<u>G. catenella</u>	Oase Bay, Mie, Japan	+	+++	+	++	+	++	+	++								
<u>Tapes japonica</u>	<u>G. catenella</u>	Oase Bay, Mie, Japan	+	+++	+	++	+	++	+	++								
<u>Mytilus edulis</u>	<u>Gonyaulax</u> sp.	Vigo, Spain	+++	+	+	++	+	++	+	++								
<u>Mytilus edulis</u>	<u>G. catenella</u> ?	Haines, AK		+++	++	++	++	++	+	++								
<u>Mytilus edulis</u>	<u>G. catenella</u> ?	Elfin Cove, AK	+	+++	+	+	+	++	+	++								
<u>Saxidomus giganteus</u>	?	Porpoise Island, AK	+++															
<u>Placopecten magellanicus</u>	<u>G. tamarensis</u>	Bay of Fundy, N.S., Canada	+	++	+	++	+	++	+	++								
<u>Mytilus</u> spp.	<u>G. catenella</u>	Inland Sea, Japan	+			+++	++	+++	++	++								

* Tentative assignment: all Gonyaulax spp.

[†] See Figure 3: STX = saxitoxin; GTX, etc. = Gonyautoxins I etc.; neoSTX = neosaxitoxin

Northwest coast of Spain and was shipped to various parts of Europe in large quantities. Due to the insufficient international communication, the identification of the causative organism was not accomplished, but the toxin profile clearly indicated that a *Gonyaulax* species was involved.

In the northern part of Japan, the aquaculture of the scallop, *Patinopecten yezoensis*, is a very profitable enterprise. It started just some ten years ago and quickly spread to many parts of Hokkaido, Aomori Prefecture, and Iwate Prefecture. A tremendous amount of capital was invested for the hanging type culture gears. Usually several baskets are hung by a line attached to a raft or a line spun between buoys. The manipulation of the hanging lines are often done by a crane placed in a boat.

Since a few years ago, the large scale cultures at such places as Ofunato Bay or Funkawan Bay started to be plagued by PSP. The problem is most serious at Funkawan Bay in Hokkaido, where the loss due to the PSP amounted to \$10 million in 1978.

The toxicity level of scallop at Funkawan Bay reached 300 mouse units/1 g hepatopancreas in June, 1978, and did not go down until October (12). This year the matter is worse; they had to halt the shipment completely, which amounts to about 70% of the total Japanese scallop production. Japanese government set a tentative guideline of 400 mouse units per 100 g meat for the legal limit. The U.S. and Canadian governments set the limit, 400 mouse units or 80 µg saxitoxin dihydrochloride equivalent per one hundred gram meat. The World Health Organization recommendation also follows the U.S. guideline. The problem with the Japanese case is rather unique because Japanese people consume the whole scallop rather than only the adductor muscle. In a Canadian study it was reported that the adductor muscle part of sea scallop, *Placopecten magellanicus*, is devoid of toxicity. The toxicity is mostly localized in the hepatopancreas, and partly at the mantle, the foot, etc. The discarded part of *P. magellanicus* at Bay of Fundy indeed showed a very high toxicity, while the adductor muscle part was safely shipped for consumption (13). In this respect, our recent study revealed a very interesting phenomenon.

When the scallop toxin mixture was incubated with a homogenate of the adductor muscle in a buffer solution at pH 5.8, a substantial loss of toxicity was observed. This ability to inactivate the toxins is apparently limited to the adductor muscle. When the toxin mixture was incubated with the remaining parts of the scallop, there was no change of the total toxicity, but there were significant changes in the toxin composition (Figure 1). The changes indicate that there is the biotransformation of toxins in the shellfish body, which is summarized in Figure 2. All the toxins are eventually reduced to saxitoxin which involves the reductive removal of a sulfate group and a hydroxyl moiety. The similar transformation was also confirmed in Alaska butter clam, which may explain the accumulation of saxitoxin in the siphon (Table 2).

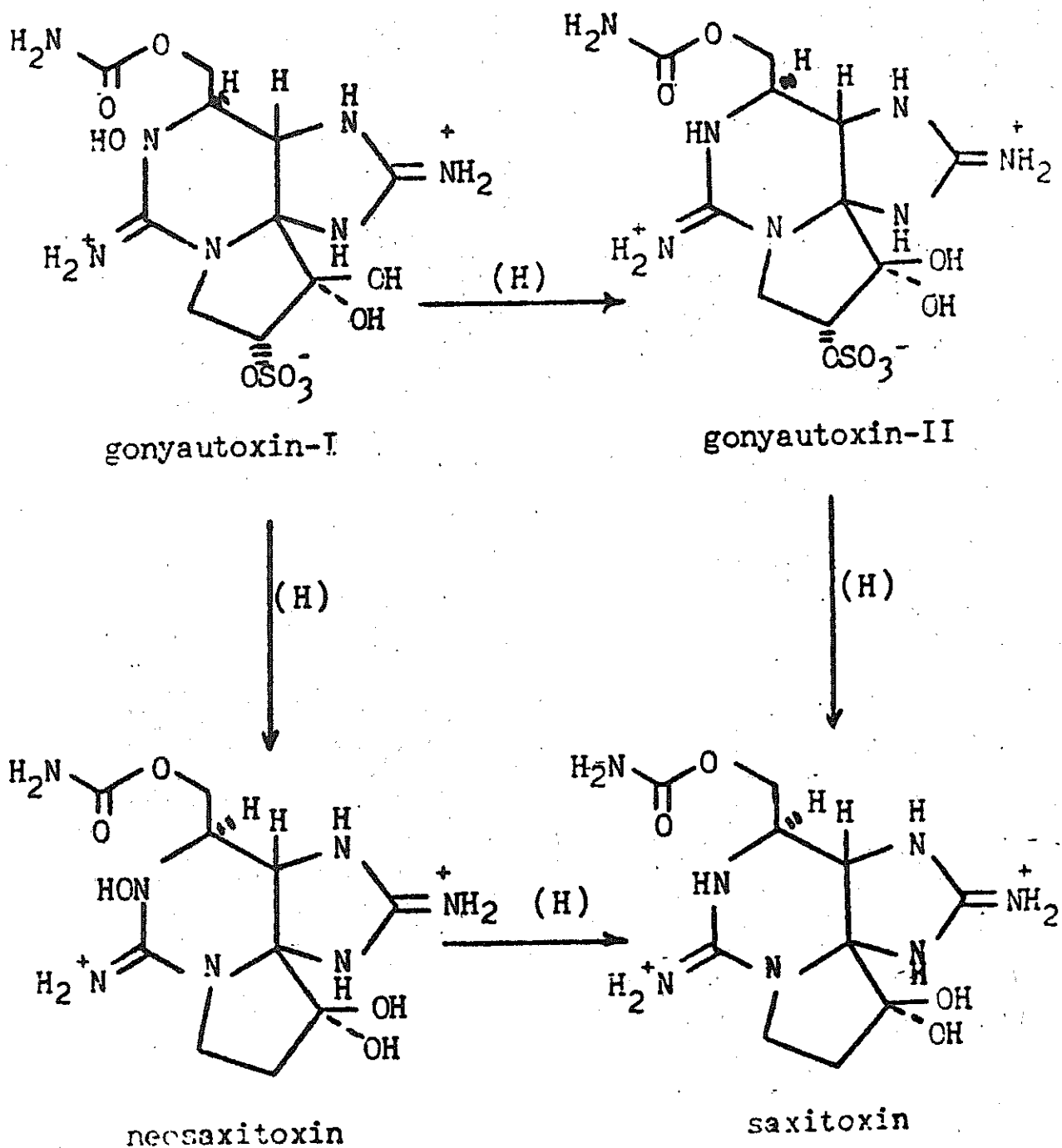


Figure 1. Toxin profiles in the toxin extracts incubated with scallop *Placopecten magellanicus* (a) gill, (b) foot, (c) visceral mass and (d) adductor muscle in acetate buffer (pH 5.9) for 3 days. STX: saxitoxin; GTX: gonyautoxin; neoSTX: neosaxitoxin. Chromatographic pattern on a Bio-Rex 70 column.

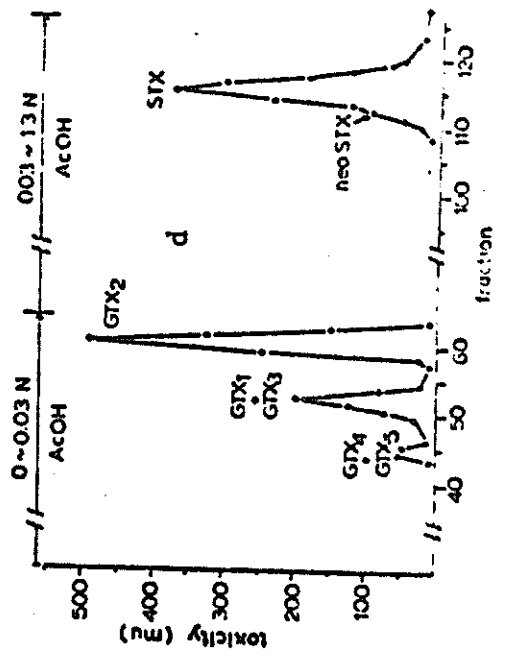
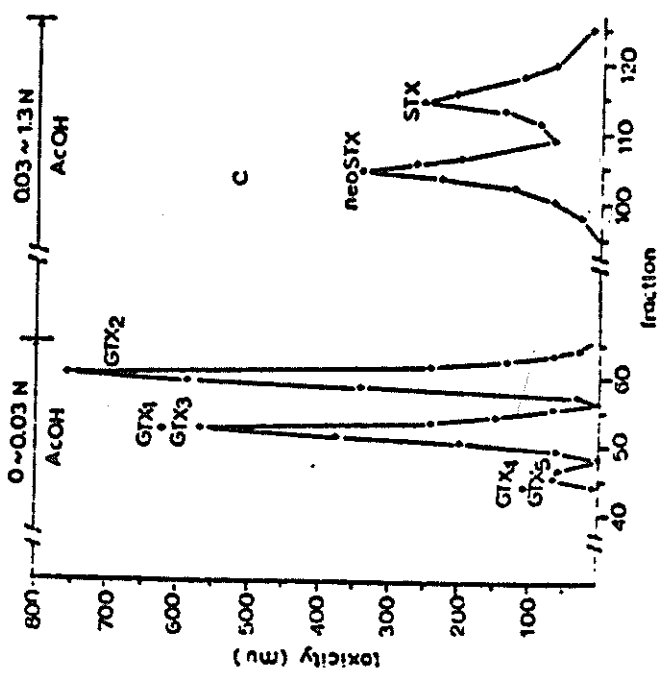
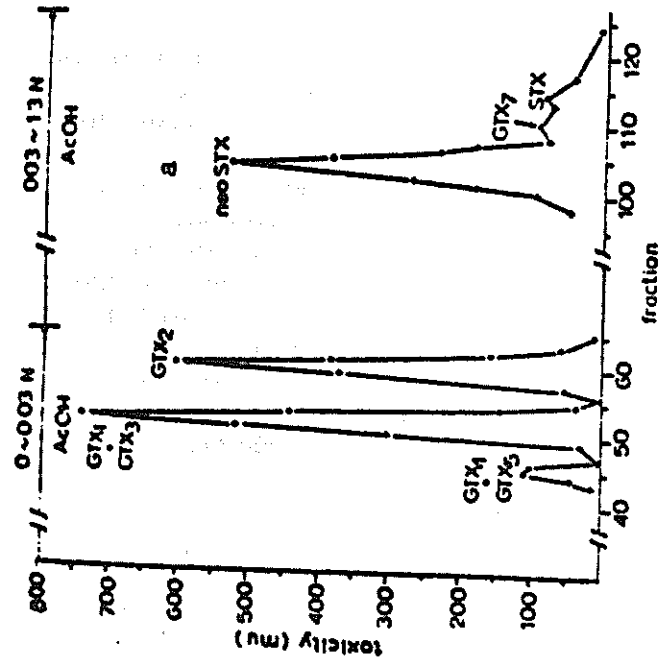
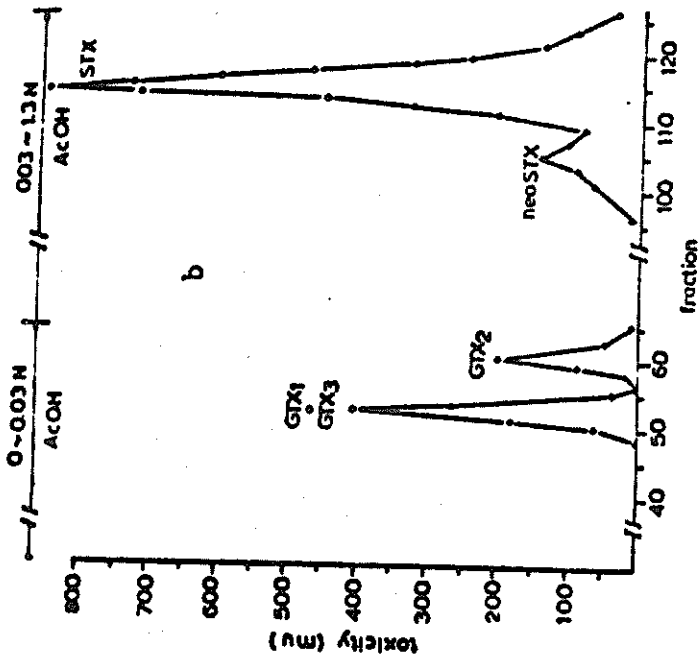


Figure 2. Transformation of paralytic shellfish poisons in the body of the sea scallop Placopecten magellanicus.

TABLE 2. Toxin Contents in Toxic Mussels and Butter Clams from Puget Sound, Washington.

Toxins	<u>Mytilus</u> sp. (whole meat%)	<u>Saxidomus giganteus</u> (whole meat %)	<u>S. giganteus</u> (siphon %)
GTX ₁	54.0	31.7	0
GTX ₂	11.3	21.4	0
GTX ₃	3.4	6.4	0
GTX ₄₊₅	25.2	12.2	0
neoSTX	6.2	6.7	0
STX	0	20.9	>95

The ability of the scallop siphon to detoxify the PSP toxins puts scallops at a uniquely advantageous position in aquaculture. The adductor muscle, which is only commercially valuable part in this country, is free of the toxins regardless of the toxicity of the remaining parts. Other bivalves such as mussels, oysters and clams, whose whole bodies are to be consumed, always face unpredictable risks. One incident of PSP could finish a well-established operation and wipe out the total investment overnight. Predictability of PSP occurrence--if one can predict the potentiality of an area for PSP occurrence or forecast the bloom of toxic dinoflagellates--will tremendously diminish the risk shellfish growers now are facing. In order to discuss this subject, it is necessary to explain the mechanism of the toxin accumulation in shellfish and the life cycle of the causative organisms.

Basically, filter-feeders like bivalves pick up the toxic dinoflagellates and the toxins in the cells accumulate in the hepatopancreas. It was generally assumed that visible blooms (red tides) are necessary to make the shellfish toxic. The accumulated toxins will gradually dissipate if there is no more new intake of the toxins. In reality, however, there are cases shellfish maintain the toxicity in the absence of any visible blooms. The extremely toxic deep-sea scallop in the Bay of Fundy is found in the depth of more than 100 m, where there is no possibility of existence of the photosynthetic motile dinoflagellate. A plausible explanation is that the shellfish collects the toxins from the cysts form of the dinoflagellate which is proved to be highly toxic (14). The cyst is considered to play a key role in the reoccurrence of blooms. Once the motile organisms exhaust nutrients or other conditions become inadequate, they become dormant cysts and stay in the sediments until the condition becomes appropriate for the next bloom. Theoretically, the examination of sediment for such cysts can determine the potentiality of particular area for toxic dinoflagellate blooms. We have recently analyzed three different strains of Gonyaulax spp. hatched from cysts obtained from proximate but different locations in Massachusetts. Although they are morphologically very close, their toxin productivity is quite different (Table 3) (15).

TABLE 3. Toxin Profile in Gonyaulax Organisms of Different Origins.

Organisms	STX	Purified Toxins					neoSTX	Toxicity
		GTX ₁	GTX ₂	GTX ₃	GTX ₄	GTX ₅		
<u>G. tamarensis</u> ¹	++	++	+++	+	+	+++	+++	1 mu/10 ⁴ cells
<u>G. tamarensis</u> ²	+	++	++	++	+	+	+	1 mu/5x10 ⁴ cells
<u>G. tamarensis</u> ³	±	++	++	++	+	+	+	1 mu/10 ⁵ cells
<u>G. catenella</u> ⁴	+	+++	++	+	+	++		1 mu/4x10 ⁴ cells
<u>G. catenella</u> ⁵	++	++	++	++	+	+	++	1 mu/10 ⁵ cells
<u>G. catenella</u> ⁶			(±)					1 mu/10 ⁶ cells

¹Ipswich, MA; ²Mill Pond, Cape Cod, MA; ³Perch Pond, Cape Cod, MA;

⁴Oase Bay, Japan; ⁵Sequin Bay, WA; ⁶LaJolla, CA.

This finding led us to assume that each confined area has a dominant indigenous strain of Gonyaulax organisms whose toxicity varies from non-toxic to strongly toxic. They recycle by the dormant cyst-motile form mechanism. In a closed area, the bloom will be confined, and the toxicity of the shellfish there will be determined by the toxicity of the native strain and its population. In areas whose indigenous Gonyaulax spp. are nontoxic, shellfish remain nontoxic despite high counts of organisms in the water. It is advisable to avoid locations where toxic Gonyaulax cysts are found in the sediment for shellfish aquaculture.

Constant monitoring of toxicity should be a required practice in shellfish aquaculture. The U.S. legal limit of 80 µg saxitoxin equivalent/100 g shellfish meat was set after a number of safety considerations. One of them is that once the toxicity reaches that level, it increases very rapidly. Another consideration is that even in close proximity, the toxicity varies tremendously.

The toxicity levels vary also according to depth. Despite the generally accepted concept that the organisms in bloom (red tide) exist near the surface of water, it was pointed out that the largest population was found at a certain temperature layer or at the interface of different water layers. Nishihama (16), working at Uchiura Bay in Hokkaido, found that the toxic Gonyaulax population slowly migrates from surface to depth as the water layer interface moves deeper. Accordingly, scallops in the upper (-10M) baskets reached the maximum toxicity in late May, but those in the lower (-25M) baskets on the same line reached the maximum toxicity in the middle of July. Since most shellfish aquacultures are done by hanging methods, this remarkable vertical variation in toxicity is very significant. Enough precaution should be taken in sampling toxicity test specimens.

Toxicity variation among different kinds of shellfish--not all shellfish accumulate the PSP toxins in the same manner even if they are placed at a same location and feed on an identical plankton mixture. It has been said for a long time that oysters have always lower levels of toxicity compared to other shellfish. This makes oysters a rather advantageous aquaculture product as far as PSP is concerned. The reason is not clear, but probably oysters have the ability to degrade the toxins. As mentioned earlier, sea scallops (probably other scallops too) have ability to transform some of the toxins. Especially the adductor muscle, with its enzyme system to detoxify PSP, remains nontoxic unless the muscles are contaminated in the shucking process. Mussels, Mytilus sp., are used as the test specimen in the PSP monitoring program. They are very fast in accumulating toxins. On the other hand, they also depurate very rapidly. So it is quite possible that even after the toxicity in mussels go below the danger level, still other shellfish in the area maintain a high level of toxicity. As a rule, if the causative organism disappears, the toxicity in mussels will dissipate in a few weeks. Alaska butter clam, however, converts the toxins to saxitoxin and accumulate in the siphon tissues where the toxin stays for a length of time. For that reason, Alaska butter clam may not be suitable for aquaculture in areas where PSP danger is eminent. It is still under investigation if this is true with other types of clams.

CONCLUSION

With increasing numbers of people who want to venture in shellfish aquaculture, the paralytic shellfish poisoning problems should be given the most serious consideration. In a culture of high density, one small scale PSP incident will suffice to wipe out the total investment. It is highly desirable to investigate the potentiality of a designated area for the presence or absence of toxic dinoflagellate cysts before any decision is made. It is also highly desirable to choose an area where no previous incident of PSP was observed. Cysts can be easily transported with shellfish, therefore, the seed should be brought in only from safe areas. Once the organisms are brought in, they very likely stay there permanently by the cyst-motile life cycle. Constant toxicity monitoring is important after the shellfish start to go into the market. Since it is not easy to run the mouse assay individually, the establishment of one assay center in the country will be a good solution.

Selection of appropriate shellfish will also bring more security to the venture. The scallop aquaculture, for example, is free of PSP threat as long as only the adductor muscle is the commercial product.

There is no means to prevent selectively the growth of the toxic dinoflagellates. Actually dinoflagellates along with diatoms are important food of shellfish. Complete elimination of such organisms from the water also means the starvation of shellfish. Despite the general belief, a visible bloom is not a requirement to have toxic shellfish; several hundred cells per liter are enough to make shellfish toxic.

ACKNOWLEDGMENT

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PROGRESS OF SHELLFISH TOXIN RESEARCH:
IMPLICATIONS OF TOXIC RESTING CYSTS FOR AQUACULTURE

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ABSTRACT

Resting cysts, of toxic dinoflagellates responsible for PSP, behave as fine silt particles within the sedimentary regime; some areas act as "sinks" for collecting them, while other areas remain relatively cyst-free. We are discovering large concentrations of toxic resting cysts in bottom sediments along the Maine coast, and they have been reported as far south as Long Island Sound. This calls for a new approach to the shellfish toxicity problem. Resting cysts probably account for shellfish toxicity where there is no obvious link with motile dinoflagellates (for example, in deeper waters, or in winter), and they probably contribute significantly to toxicity following blooms. If so, then the monitoring of plankton for shellfish toxicity will have to be broadened to include benthic resting cysts and the sedimentary process, and the practice of transplanting shellfish from one geographic area to another needs re-evaluation. Aquaculturists must be concerned about the implications: (1) that shellfish can become toxic by ingestion of cysts directly; and (2) that the distribution of toxic dinoflagellates can be spread to areas currently toxin-free by the transplanting of shellfish which contain mud with cysts of toxic species.

INTRODUCTION

Several coastal waters along North America are subject to sporadic increases in the density of toxin-producing algae of the genus Gonyaulax (see Figure 1). The various toxins elaborated by some of these algae may be accumulated by bivalve molluscs of commercial interest. Although most shellfish species are themselves relatively unaffected by the accumulated toxins, they present a threat to public health because human intoxication can result from consumption of these shellfish while they contain the toxins. The resulting intoxicated condition in humans is known as Paralytic Shellfish Poisoning (PSP). There are medical records of over 1,650 cases of this food poisoning worldwide which have resulted in at least 300 fatalities.⁵ Tingling lips and fingertips, dizziness, respiratory difficulties, and loss of equilibrium are symptomatic of PSP.¹⁰ In extreme cases, intoxication can lead to respiratory arrest and death.

Shellfish from most of the major growing areas of North America, however, contain high levels of these toxins for only short periods of the year; and during some years, toxin levels remain low enough so that they are not detected in many areas. Programs to monitor for the increase of toxins in shellfish are currently in effect to permit selective closures which allow resource harvesting to proceed with the least possible interruption.

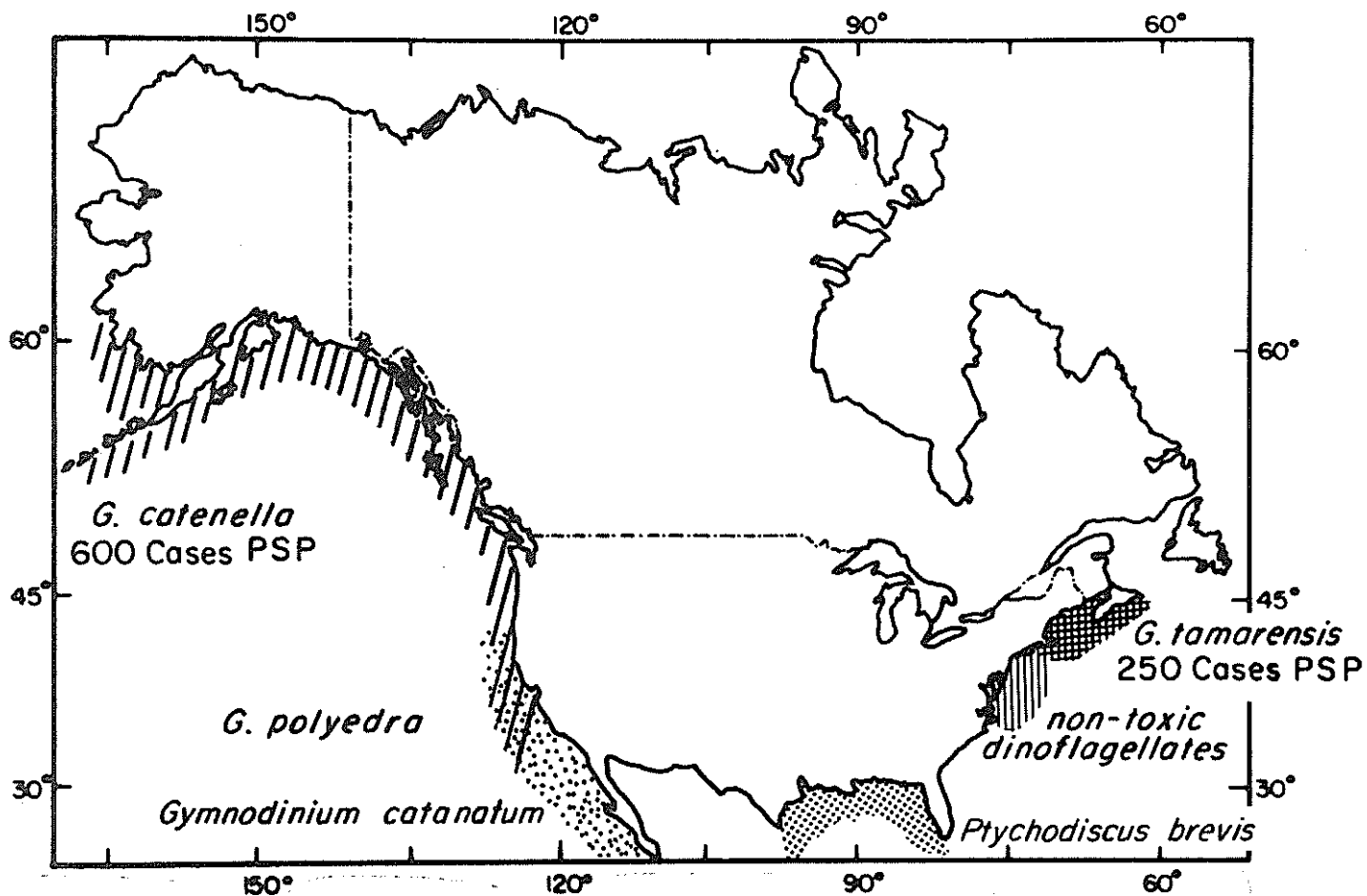


Figure 1 Coastal waters of North America and common distribution of species of Gonyaulax and other red tide dinoflagellates.

With expanded research, the timing and location of significant toxin levels in shellfish are becoming increasingly predictable, providing benefits to the future management of natural beds and to the development of aquaculture practices and the selection of aquaculture sites.

With the onset of a toxic dinoflagellate outbreak, the closing of a shellfishery sets off a barrage of problems which include not only individuals involved with the fishery itself, but the public as a whole.^{11,14} The outcry often vented against these closures is whether or not the established standards are too conservative. Of course, the shellfish industry by way of its own public relations is at stake; a severe outbreak of PSP caused by consumption of marketed toxin-bearing shellfish could lead to unrecoverable economic disaster for the shellfisheries in general.

At the moment, public health protection from PSP is under control, but there are other problems which must be addressed. There can result collapse of local fisheries and related industries. Socio-economic impacts are more frequent, widespread and longer lasting. These may be direct, e.g., the closure of the Alaskan clam fishery in 1947 (with a very limited reopening in recent years), or more recently the closure in 1972 and subsequent years in New England and 1978-79 in Puget Sound, or indirect, e.g., adverse consumer reaction to fishery products or tourist sites following a red tide. Both can span several years and affect communities some distance from the bloom site. Furthermore, pressures to develop alternatives or replacement fisheries at new locations may seriously affect the long-term economic stability of areas experiencing red tides.

The crisis nature of the problem, and the long standing concerns over the consequence of periodic blooms of toxic dinoflagellates, including their recent occurrence in new geographic areas, underscores the need for an organized collaborative effort to place these events in their proper scientific perspective. With the toxin reported from new locations, it is uncertain whether or not the apparent spreading of shellfish toxicity is a function of our increased awareness and monitoring zeal or a real phenomenon, since many countries and regions have not had long, ongoing programs studying the problem. It is equally uncertain whether or not the increased appearance of red tide problems is due to man's activities (pollution or inadvertent "seeding" via shellfish transplants) or whether these are merely natural events with long-term periods which have not been measured before.

Recently workers have begun to look more closely at the organisms responsible for the production of the toxins. The identification is complex and dependent on strict attention to minute details. As of this writing, there is no consensus as to whether the observed differences are diagnostic of apparent species, strains, forms or varieties. In addition to the taxonomic considerations, it is ecologically important to discern whether the different "types" have different environmental preferences and whether differences in cellular toxin levels result from differences in environment, or differences in genetic "type." Toxin levels in Gonyaulax spp. have been observed to vary dramatically from nontoxic²⁶ through highly toxic.^{1,7,22}

Recent developments also have caused a resurgence of interest in the life history of the organisms involved. It is known that, in addition to

the motile form present in New England waters from mid-April to mid-October, G. tamarensis produces a naturally occurring resting cyst. These cysts are the result of sexual reproduction.²⁴ They lack flagella and sink rapidly and accumulate in the flocculent layer at the sediment-water interface where they overwinter. The nearly neutral density of these cysts renders burial in the sediments unlikely. When wind-mixing disturbs the surface sediment, resettling occurs with the heaviest particles first, and the cysts last. These cysts appear to act as other sedimented particles, sorting out by particle size and density into "sinks" and have been used by some investigators to identify water movement patterns.³³ A resting period of 4 months appears to be mandatory,⁴ yet in some cases duration varies with temperature of storage, and can be as short as one month.²

Measurement of toxin levels in resting cysts indicates levels at least ten times higher than those of motile cells.⁶ Cysts have been found in the water column during periods of strong vertical mixing.²⁸ Large concentrations of highly toxic resting cysts have been discovered in bottom sediments along the Maine coast¹³ and have been reported in the literature as far south as Woods Hole, MA.³ Distribution and occurrence along the Pacific Coast is not as well known, but cysts have been reported from the Puget Sound area²⁹ and from Alaska.³⁰

The presence of these highly toxic cysts calls for a new approach to understanding shellfish toxicity problems.^{6,21} Evidence suggests that resting cysts may account for shellfish toxicity even when no motile dinoflagellates occur in the water column (Figure 2). Such cysts may also contribute to high toxicity in shellfish when both motile cells and cysts are in the water column. Examples of organisms exhibiting winter toxicity, which also suggest ingestion of cysts, include deep sea scallops (Placopecten magellanicus) at a depth of 100 m in the Bay of Fundy^{20,31} and butter clams (Saxidomus giganteus) and mussels (Mytilus edulis) from Haines, AK.³²

Recent findings about cysts serve to emphasize that red tides, that is, discolored water, are by no means diagnostic of toxic conditions because: (1) shellfish can accumulate harmful amounts of toxin even at Gonyaulax concentrations below those necessary to discolor water; (2) shellfish can intoxify when no motile cells are detected in the water column, but when cysts of the causative organisms are present;²⁸ and (3) red tides can result from concentrations of other, non-toxic dinoflagellates²⁶ or ciliates.^{16,19}

While dense concentrations of toxic dinoflagellates may result directly from seed beds of cysts, followed by rapid growth of the algal populations, physical (hydrographic) mechanisms also appear to be important. These concentrating mechanisms are unique in that they require no rapid reproduction. Instead, they provide a means for delivery of existing populations to a specific area where biological behavior, such as positive phototaxis, can result in dense concentrations.^{15,17,23,25} These mechanisms may be triggered by meteorological events, such as rainfall and wind.^{8,17,27}

Recent physical data suggest that frontal zones, or discontinuities between water masses, are the locations most likely to generate red tides.⁹ These fronts may result from tide- or wind-generated convergences and/or density discontinuities; they are frequently marked by pronounced differences in the vertical stability of the two water masses.^{12,18,23,25}

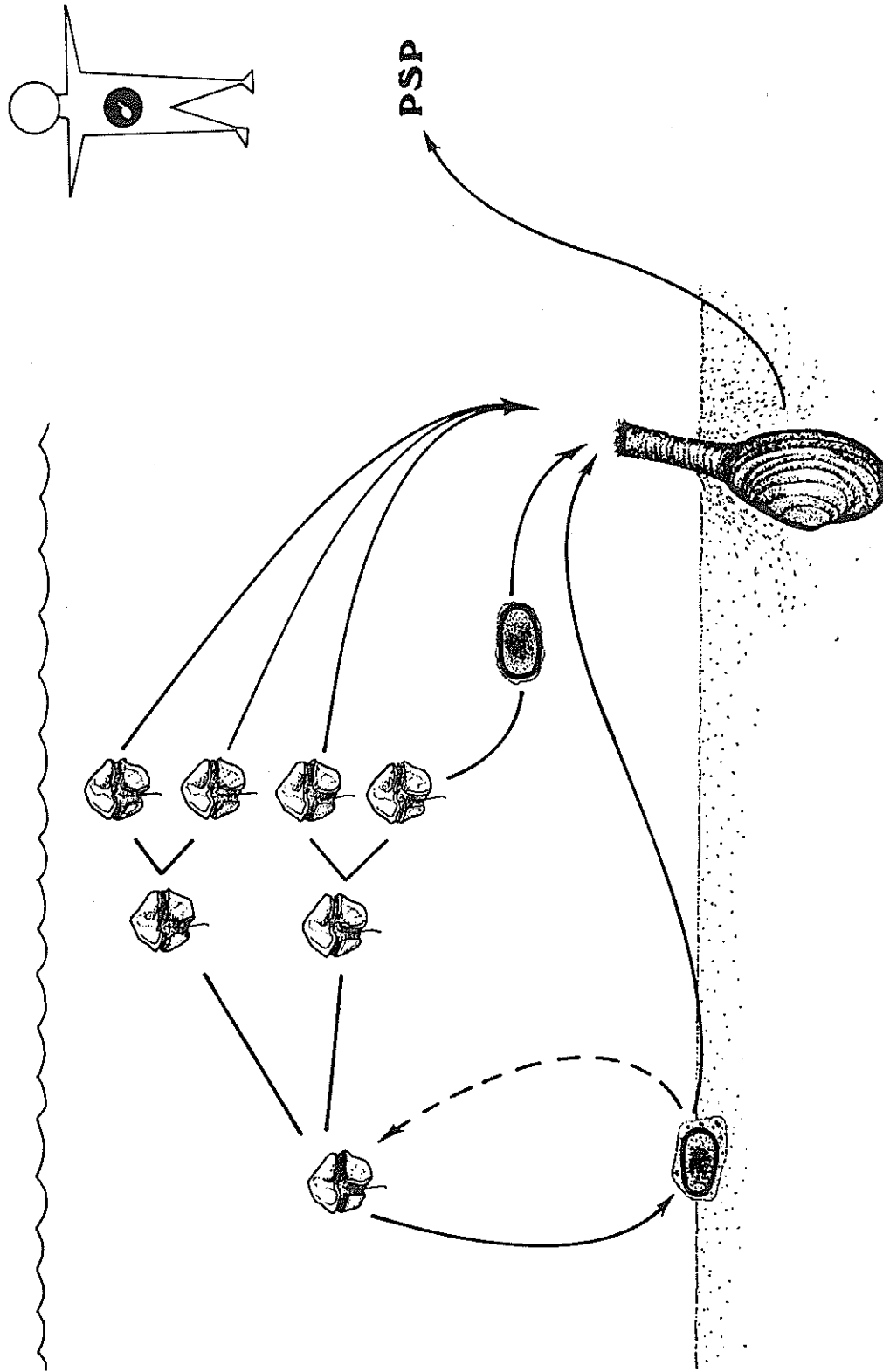


Figure 2 Motile cells developing into cysts, and their role in perpetuating shellfish toxin, leading to PSP in humans (from reference 4).

Fronts can be detected in surface temperature differences which can be sensed by infra-red carried aboard small aircraft or via TIROS, GOES and other satellites. These fronts may also produce surface and/or water column chlorophyll maxima which can be detected by the CZCS (Coastal Zone Color Scanner) system.

The Monhegan Island area off the coast of Maine has been studied intensively by our research group over the past five years. The progress is given diagrammatically in Figure 3 a-e.

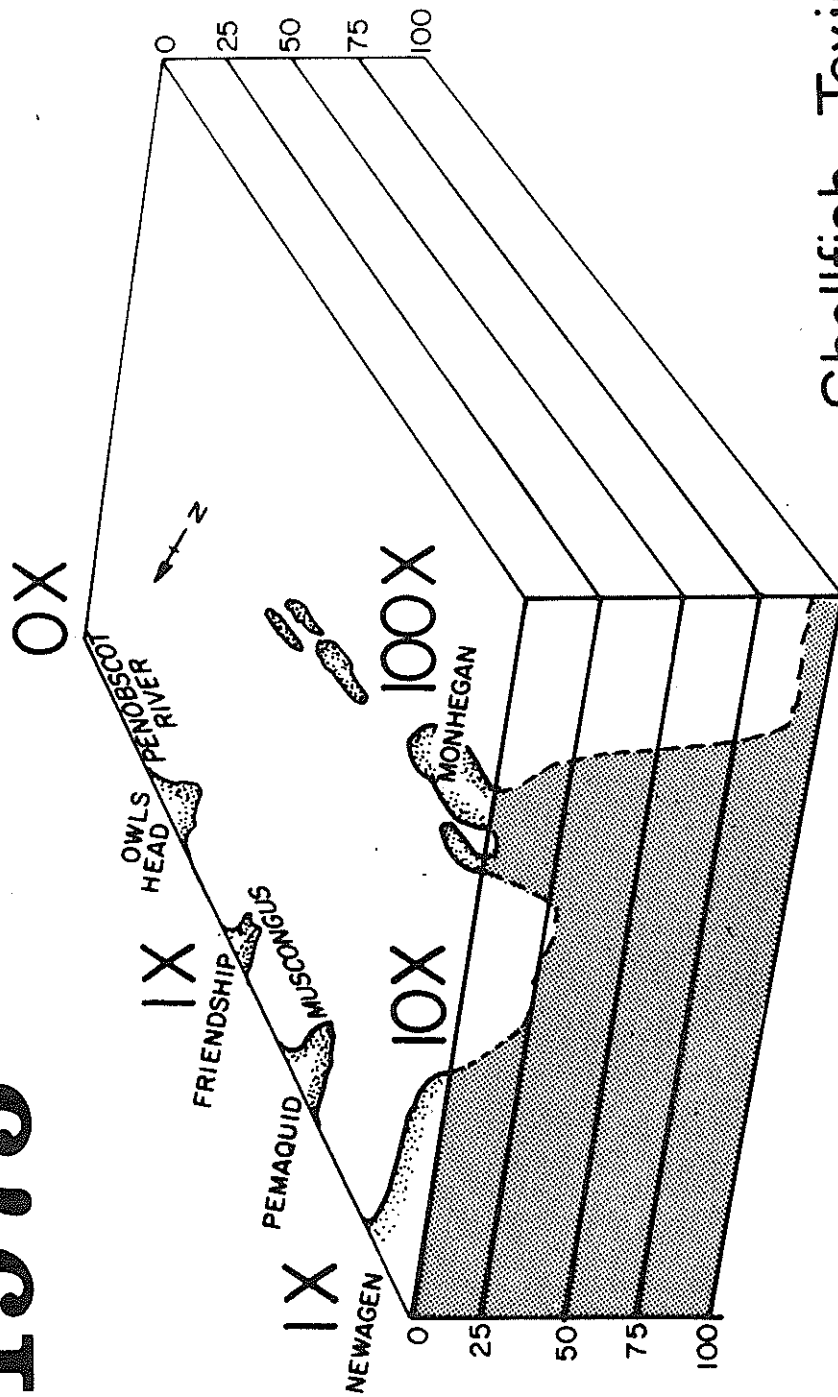
Despite our increasing knowledge of dinoflagellate ecology, it is still not possible to predict specific toxin occurrences. In recent years, toxic shellfish have been found in areas where toxins had been previously unknown; it thus appears that no guarantees can be made for toxin-free locales. In areas that to date have witnessed no shellfish toxin, dinoflagellates routinely bloom, and there is fear that a toxic species might replace a non-toxic species. It is, therefore, unrealistic for commercial management to focus attention only on areas historically free from shellfish toxins.

The current thought on developing a predictive index for shellfish toxin is changing from the original concept of monitoring dinoflagellates in the plankton to the general concern for long-term studies on climatological and satellite-sensed hydrographic factors which may favor "blooms" of dinoflagellates. The fact that cysts are now being implicated as a cause of shellfish toxicity complicates the predictive index concepts, but increases our understanding of toxin occurrences in nature. This, in turn, should lead to better approaches to managing shellfish resources.

ACKNOWLEDGEMENTS

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1975

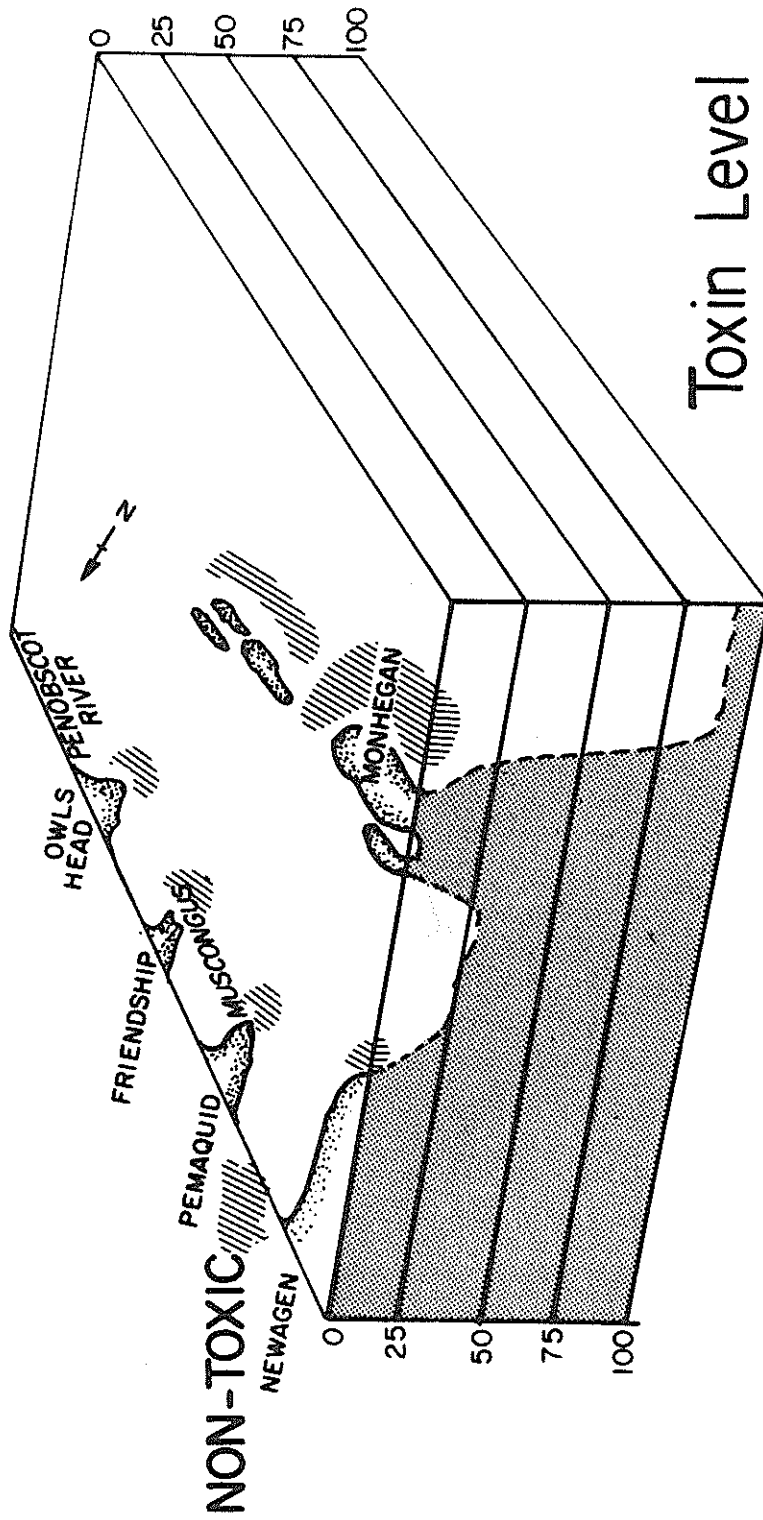


Shellfish Toxin Level Patterns

Figures 3a-3e Diagrammatic representation of findings 15 miles offshore near Monhegan Island, Maine. Depth is in meters.

Figure 3a 1975 -- Patterns of toxicity reflected that island systems are 100X the toxic levels of bays. The region east of the Penobscot River is virtually toxin-free.

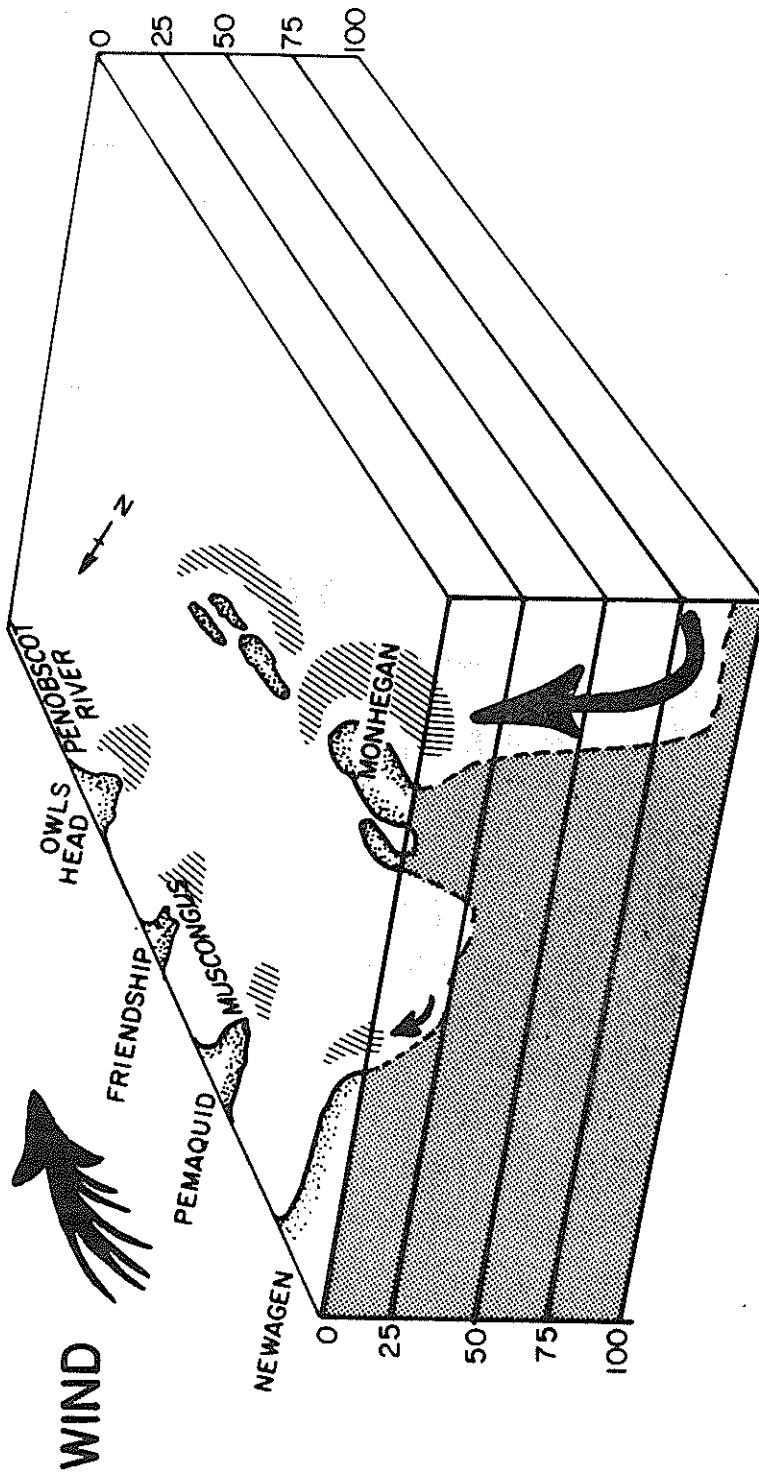
1976



Toxin Level Patterns Coincident With *Gonyaulax*

Figure 3b 1976 --- Patterns of toxin level were found to be coincident with motile Gonyaulax in the water column. The one exception was the Damariscotta Estuary, between Cape Newagen and Pemaquid. In that region, there was extensive blooming of a non-toxic Gonyaulax.

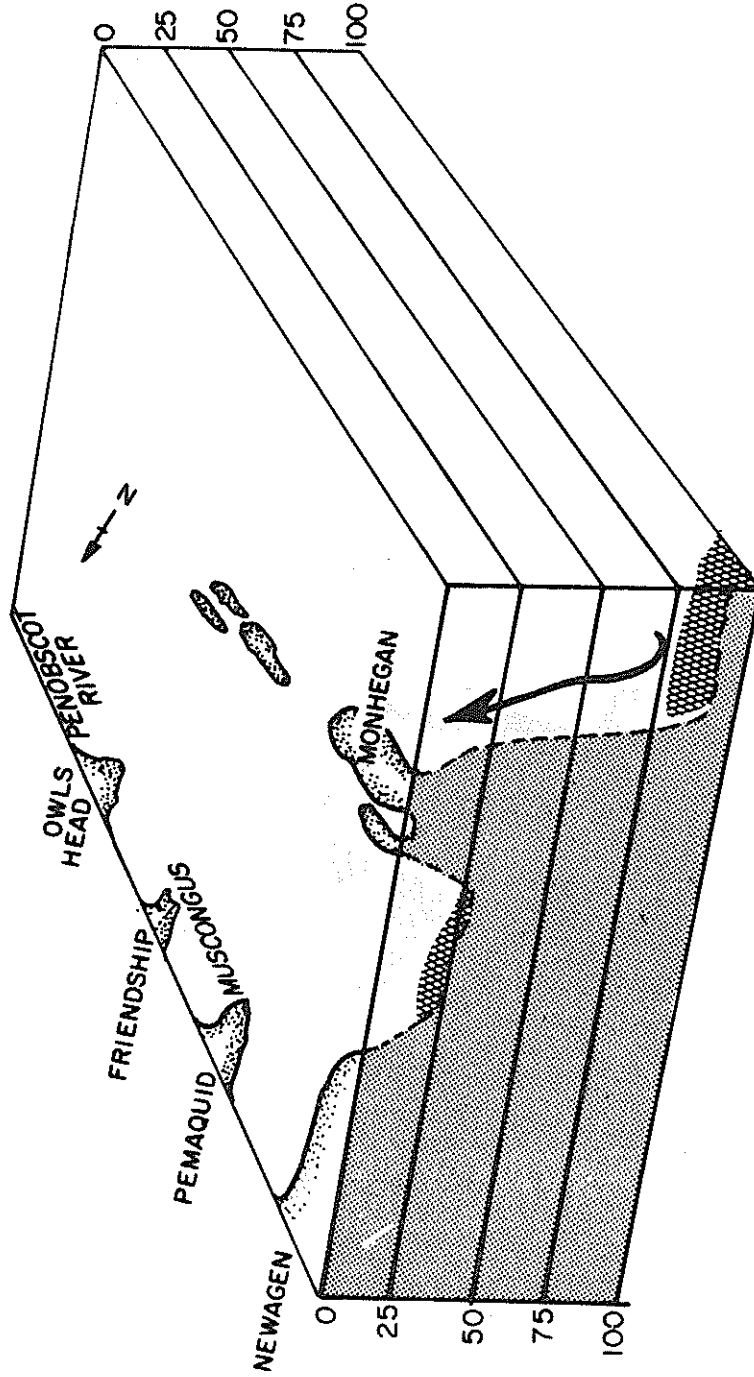
1977



Gonyaulax Coincident With
Coastal Upwelling (N,P);
Fronts and Offshore Winds

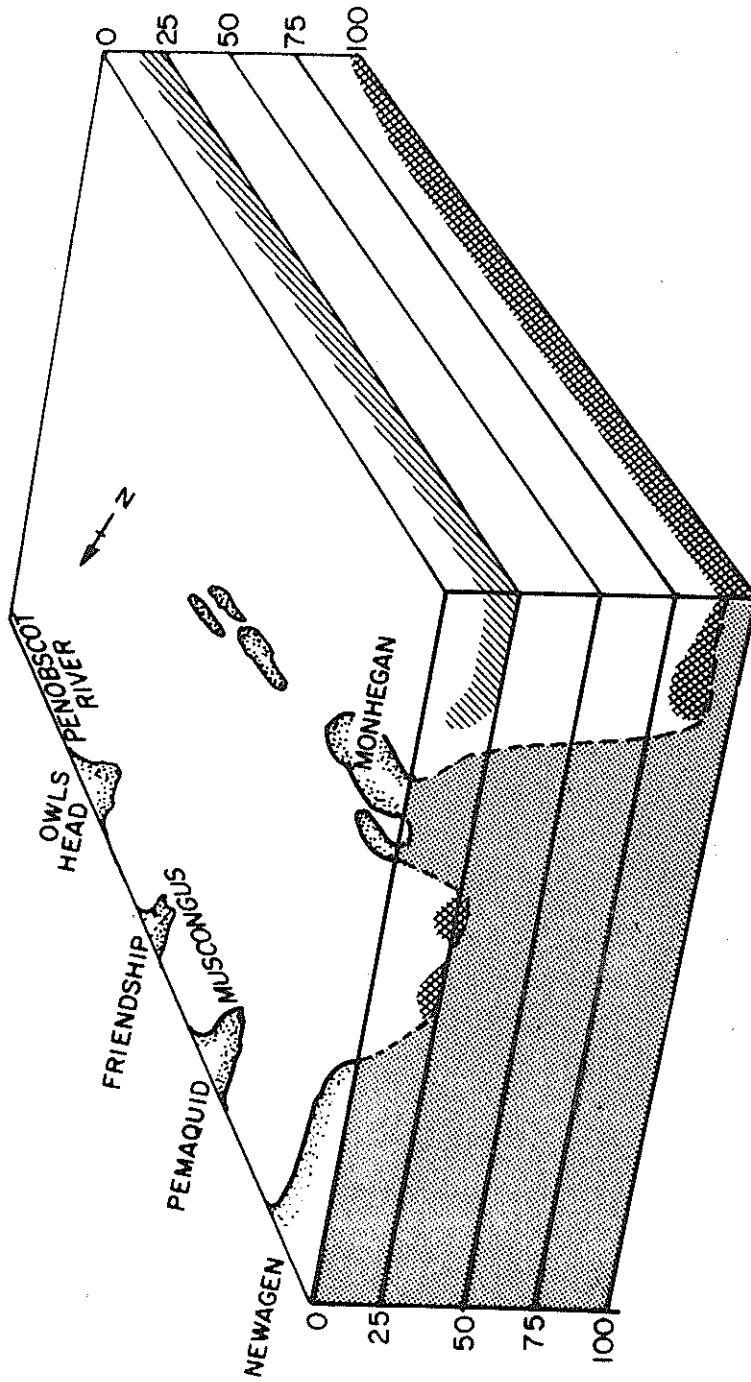
Figure 3c 1977 -- Coastal upwelling was documented which was driven by offshore winds. Surface Gonyaulax populations were most abundant in frontal regions.

1978



Cyst Accumulations Offshore; Highly Toxic

Figure 3d 1978 -- Winter cyst accumulations were found offshore and were demonstrated to be highly toxic.



Cyst Distribution General Offshore; Dinoflagellate Subsurface Maxima - Offshore Fronts

Figure 3e 1979 -- Cyst distribution was found to be general offshore in the sediments, both winter and summer; and motile dinoflagellates were found in extensive subsurface maxima. No surface patches were reported.

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THE OCEAN HAS ITS BEZOAR, TOO

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ABSTRACT

Phosphate concentrations of surface waters approach those of arsenate in much of the ocean. Consumed by phytoplankton, phosphate drops to the $2-6 \times 10^{-8}$ molar level of oceanic arsenate. Since uptake mechanisms for both ions appear to be identical, the algae of low phosphate waters are faced with the problem of detoxicating arsenate.

We have examined the products of ^{74}As -arsenate uptake by many diatom and dinoflagellate species, by corals, and by several tropical algae using radio-chromatographic methods. A major arsenic-containing product identified as 5-dimethylarsenosylphosphatidylglycerol, is derived from a stable anionic phospholipid. The detoxication process, then, involves successive methylation of arsenite and transfer of the product to ribose to yield 5-dimethylarsenoso-5-deoxyribose. The products of deacylation of the phospholipid accumulate in varying but comparable concentrations in algae. Bacterial degradation aids removal of arsenic from the algal cell. Nature's elegant mechanism for arsenate detoxication appears to be regulated by the availability of environmental phosphate. The Persians' Bezoar Stones, too, were used for arsenic detoxication for the past thousand years. We now know how they could have functioned in removing arsenate and arsenite from wines or in cases of poisoning. They were indeed no more magical than Nature's own process.

INTRODUCTION

The Bezoar Stone played a central role in pharmacy from its introduction into the West during the Middle Ages (Elgood, 1935; Monardus, 1580) until the ultimate differentiation of pharmacy, chemistry, and magic in the eighteenth century. These intestinal concretions (Milton and Axelrod, 1951) from goats in the mountains of western Persia had been claimed, as early as 822 AD to "protect against poison" from whence their name, "Bezoar" Stones. Although the stones were at first claimed to protect against arsenic and aconite poisoning, their powers grew to include plague, epilepsy and jaundice (Bauhini, 1613), perhaps the reason for the wane in public confidence after about 1750.

Now that we understand chemistry and a little about biology we realize that the Bezoar Stone could have had some therapeutic functions. A drink, poisoned with arsenate, could have been purified upon contact

with a Bezoar Stone. Its crystalline calcium hydrogen phosphate (brushite) could release phosphate ions to produce the exactly isomorphous calcium hydrogen arsenate (pharmacolite) (personal communication, Professor G. A. Arrhenius). The more commonly used poison, arsenite, could have been bound by the Bezoar's matrix of degraded hair with its copious disulfide and sulfhydryl groups. It seems possible that these stones could have performed some useful function in detoxication of arsenic.

The ocean, too, has an arsenic problem. This ubiquitous element is widely distributed in seas, lakes, and soils. It arises from vulcanism, hot springs, and rivers. It is ultimately deposited in marine sediments leaving a rather constant level of 10^{-8} molar arsenate in seawater. Its presence, recognized by Rakestraw and Lutz (1931), was determined by Johnson and Pilson (1972) who refined phosphate analyses for several low nutrient areas of the ocean. Both ions give the same characteristic phosphomolybdate blue color. The Sargasso Sea, the Caribbean Sea and tropic seas in general, where upwelling does not restore the phosphate consumed by surface phytoplankton, have very low phosphate concentrations. In fact, their phosphate levels approach those of natural arsenate, 10^{-8} molar. Under such conditions algae, being unable to discriminate between phosphate and arsenate, absorb both ions in comparable quantities. They would die of arsenic poisoning were it not for immediate function of their mechanism for biochemical detoxication of arsenate and arsenite. It serves as a built-in Bezoar Stone to make life possible in the tropic ocean.

RESULTS

Experimental approach. Since arsenic concentrations in algae and other organisms are generally only a few parts per million, one can hardly isolate and identify metabolic intermediates. Edmonds et al. (1977) succeeded in isolating from Western rock lobster (Panulirus longipes cygnus) muscle a crystalline compound identified as arsenobetaine (Fig. 1).

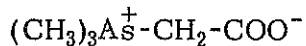


Fig. 1. Arsenobetaine

Its identity relieved concerns for arsenic toxicity of this important Australian marine product. Arsenobetaine, like arsenocholine, had been synthesized forty years earlier and fed to rats and chicks at a 1% of diet level with no apparent ill effects (Welch, 1936, 1938, 1942; Jukes and Welch, 1942). Subsequent studies with natural arsenic metabolites, too, suggested that the products were readily excreted by mammalian kidneys.

Radiochemical studies with ^{74}As -arsenate in our laboratory revealed that the nearly universal products of algal metabolism of arsenate are the water soluble compounds related to an amphipathic arsenolipid

(Cooney et al., 1978). The structure of these compounds were determined using nmr and IR by Edmonds and Francesconi, 1981. While not the same as arsenobetaine and arsenocholine, the 5-dimethylarsenoso-5-deoxyribosylglycerol derivatives they identified are similar enough in structure and, probably, in metabolic fate to allay concern over their possible toxicity.

Mechanism of arsenate detoxication. For some time it has been clear that arsenate detoxication involves reduction to arsenite, methylation to produce methanarsonate, further methylation to yield cacodylate (dimethylarsinate), and final transfer to a ribose derivative yielding dimethylarsenosoribosylglycerol (Fig. 2).

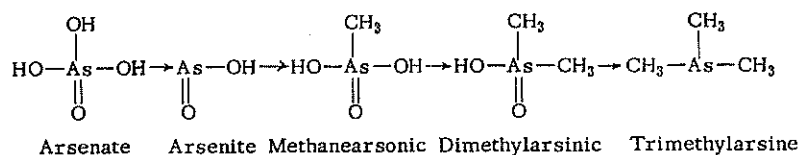


Fig. 2. Reduction and methylation of arsenic in biological systems.

The first intermediates are toxic. Yet each can be an end product of the detoxication process in certain groups of organisms. For example, we find that arsenite is excreted by the alga, *Dunaliella tertiolecta*, and presumably by a wide variety of organisms such as corals and bacteria. Methanearsonate accumulates in certain bacteria which fail to methylate it further. Yeasts often accumulate cacodylate and go no further. A group of notorious fungi synthesize and release volatile trimethylarsine, the "Gosio Gas" which caused numerous deaths when fungal metabolism produced it in wallpapers of European bedrooms. This garlic-smelling gas is released from soils, bogs, and decaying marine tissues from available arsenite (Woolson, 1977).

Fortunately, trimethylarsine reacts avidly with the ubiquitous metabolite, phosphoenolpyruvate, to produce trimethylarsoniumlactate (Fig. 3).

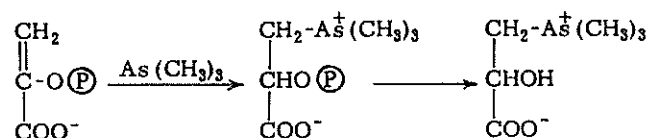


Fig. 3. Reaction of trimethylarsine with phosphoenolpyruvate producing trimethylarsoniumlactate.

Finally, the arsenic is in an innocuous form. This zwitterionic compound in which the arsenic mimics the nitrogen of isoserine betaine could be an arsenical which is readily excreted by the mammalian kidney. It could be the precursor of the arsenobetaine isolated and characterized in rock

lobster muscle. It must be pointed out, however, that although we have produced arsenobetaine chemically by oxidative degradation of trimethylarsoniumlactate all attempts to find it in biological systems allowed to metabolize arsenical intermediates have been unsuccessful. Even the American lobster Homarus americanus is apparently so different that it accumulates dimethylarsenosoribosylglycerol and not arsenobetaine when fed arsenic-labeled algae (Cooney and Benson, 1981).

Arsenic transfer in the marine food web. the classic feeding experiments of Coulson et al. (1935) were designed to demonstrate metabolism or excretion of the dietary arsenicals derived from shrimp. Based on elemental arsenic assay, the arsenic compound(s) were excreted through the kidneys of rats. The compounds so excreted probably included dimethylarsenosoribosylglycerol and/or arsenobetaine. Not all kidneys, however, are so effective in excretion of arsenic compounds derived from the diet. The wide range of arsenic contents of marine organisms appears to stem from variation in ability to excrete arsenic metabolites as well as the nature of the dietary arsenic source.

We have initiated a survey of arsenic accumulation in tissues of animals feeding on phytoplankton and algae growing under phosphate-deficient conditions (Benson and Summons, 1981). From a collection of animals obtained on the Great Barrier Reef we cite some dramatic examples in Table 1. It is seen that several symbiotic systems involving algae and animal hosts accumulate unusual levels of arsenic. The ascidians listed are hosts for their symbiotic prochloron (green eukaryotic algae) and the Hippopus and Tridacna are large molluscs which consume phytoplankton and harbor symbiotic zooxanthellae (dinoflagellate algae, Gymnodium microadriaticum) which appear to senesce and undergo degradation. Their large kidney (nephridium) appears to accumulate levels of arsenic higher than any recorded. Identification of the arsenical component(s) in these kidneys at a 0.2% level is in progress.

Bacteria play an important role in the removal of arsenic from marine algae. One would expect that phytoplankton and macroalgae phosphate-deficient waters might accumulate arsenic. There is little evidence that they do (Table 2). In preliminary experiments with ordinary and axenic algal cultures or with field collected and surface-sterilized macroalgae, R. V. Cooney and J. M. Herrera-Lasso (unpublished) observed dramatic effects of superficial bacteria upon the arsenicals of algae. It seems that the arsenophospholipid, incorporated in the surface membranes of algae, is attacked by bacteria or their extracellular enzymes to produce cacodylate and/or methanearsonate. One may envision the arsenoglycosylphosphatide (Fig. 4) located in the plasmalemma of an alga with its water-soluble 5-dimethylarsenosoribosyl group exposed in the external water phase.

Table 1. Arsenic and phosphorus in seawater (Johnson and Pilson, 1972).

	Arsenate μM	Phosphate μM
Sargasso Sea	0.03 0.05	0.01 0.02
Azores	0.01 0.02	0.01 0.01
Bahamas	0.02	0.00
Jamaica	0.01 0.02	0.01 0.01
Key West	0.06	2.01
North Atlantic (50°W)	0.04	0.27
Strait of Georgia, B.C.	≈ 0.03	2-3

Table 2. Arsenic content of selected marine organisms.

Name	Species	Source	Parts per million	Reference dry weight
Sargassum weed	<i>Sargassum filipendulum</i>	Fort Pierce, Fla.	32	3
Seaweed	<i>Laminaria hyperborea</i>	Trondheimfjord	139	2
Chlorophyceae	<i>Enteromorpha</i> sp.	Vancouver Is., B.C.	0.93*	3
Chlorophyceae	<i>Enteromorpha</i> sp.	Savannah, Georgia	23	3
	<i>Caulerpa</i> sp.	Great Barrier Reef, 14°35'South	4.5	1
	<i>Caulerpa</i> sp.	Florida Keys	2.5	
	<i>Halimeda</i> sp.	Florida Keys	1.5	3
	<i>Halimeda</i> sp.	Great Barrier Reef	21	
Shrimp	<i>Pandalus borealis</i>	Oslofjord	36	2
Herring	<i>Clupea harengus</i>	Western Norway	28	2
Foraminifera	<i>Marginopora vertebralis</i>	Lizard Island, Great Barrier Reef	3	1
Oyster	<i>Ostrea</i> sp.	Lizard Island	58	1
Rock oyster	<i>Ostrea</i> sp.	Jervis Bay, N.S.W., Great Barrier Reef	8	1
Mussel	<i>Mytilus edulis</i>	Western Norway	12	2
Hippopus clam kidney	<i>Hippopus hippopus</i>	Lizard Island	561	1
Hippopus clam gonad	<i>Hippopus hippopus</i>	Lizard Island	65	1
Tridacna clam kidney	<i>Tridacna maxima</i>	Lizard Island	1004	1
Ascidian	<i>Didemnum ternatanum</i>	Lizard Island	226	1

References: 1, Benson and Summons, 1980; 2, Lunde, 1973; 3, Sanders, 1979

* 43% organic, 57% inorganic As

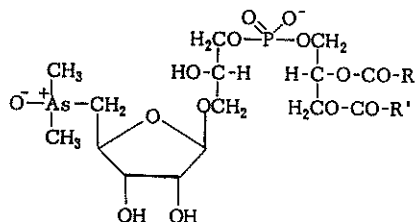


Fig. 4. Dimethylarsenosoribosylphosphatidylglycerol, the arsenophospholipid.

Oxidative attack at the dimethylarsenoso group yields cacodylate. This is also a product of in vitro thermal hydrolysis and air oxidation of these compounds in the laboratory.

Arsenic toxicity. Marine organisms, although facile in metabolism of natural levels of arsenate and arsenite, cannot tolerate exposures to very much higher levels of these ions. In experiments designed to estimate tolerance levels for corals (Acropora formosa) it was observed that arsenate or arsenite at levels only a few-fold greater than that of seawater caused ejection of zooxanthellae and death of the coral. Such sensitivity suggests that the productivity of marine organisms in phosphate-deficient waters may be seriously limited by the energy consumed and deleterious effects resulting from the requirement for arsenate detoxication. It is observed that the ratio of photosynthesis to respiration in algae grown under optimal nutrient conditions is 20 while the ratio for oceanic phytoplankton under limiting nutrient conditions is 3 to 5. The difference, a greatly enhanced respiratory requirement, may be the result of the energy consumed in reduction and methylation (Wood et al., 1978) of oceanic arsenate. The effect of increased arsenate levels upon respiratory ratios has been studied by Giddings and Eddlemon (1978).

Arsenic contents of marine products (Table 2) vary greatly. It is unfortunate that so much work has been reported without adequate documentation of the source of the sample and the phosphate content of the water in which it grew. The latter is extremely important in interpreting the bases for arsenic accumulation and estimating the effects of higher or lower phosphate levels upon arsenic content. One may note in Table 2 that samples of Enteromorpha from high phosphate coastal waters of British Columbia had very little arsenic which a similar species from Georgia coastal water had 23 times more arsenic. A similar relationship is seen in oyster samples from phosphate-rich waters of New South Wales (8 ppm) and low phosphate water of the Great Barrier Reef (58 ppm).

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CHEMOTHERAPY AND CHEMOPROPHYLAXIS FOR CONTROL OF
BACTERIAL DISEASES OF FISH

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ABSTRACT

The development of chemotherapeutic procedures for treating bacterial fish diseases and the drugs currently used in treatment of these diseases are briefly described. Efficacy studies with a potentiated sulfonamide for control of furunculosis and enteric redmouth disease, and in vitro studies on myxin, a potentially useful drug in fish husbandry, are also presented.

INTRODUCTION

Fishes, like other vertebrates, are subject to a wide range of infectious diseases. Wild and cultured fishes are infected by the same agents, but the frequency of epizootics is greater among propagated fishes because physical, chemical, and physiological stresses often occur among or are imposed upon intensively cultured fish. Nutritional imbalance, oxygen depletion, unfavorable temperature, and crowding are examples of the stresses that, singly or in combination, predispose fish to disease. As a result of stress, disease can be caused by opportunistic pathogens such as *Aeromonas hydrophila*, or latent carriers of obligate pathogens such as *A. salmonicida* may release the organism and precipitate explosive epizootics. The role of stress in fish disease was extensively reviewed by Snieszko (1974) and Wedemeyer et al. (1976).

Disease control consists primarily of the application of avoidance or preventive measures and secondarily of remedial measures such as therapy. Prevention may be achieved by sanitary procedures, appropriate nutrition, temperature control, oxygenation, and sound husbandry. In addition, specific diseases may be prevented by immunization or chemoprophylaxis. However, even in properly managed hatcheries, epizootics sometimes occur and chemotherapy is necessary. Unfortunately, the indiscriminate use of drugs and chemicals or the substitution of these compounds for sound husbandry during the last two decades has resulted in a sharp increase in the occurrence of drug-resistant bacterial pathogens. This increase has been due in part to selection of existing resistant forms and in part to the plasmid-mediated transfer of resistance factors (Aoki et al. 1977; Aoki et al. 1974; Aoki et al. 1971).

This report reviews current use of chemicals and drugs in controlling bacterial diseases of fishes, presents results of studies on a potentiated sulfonamide, and discusses briefly several new drugs that have a potential for effective control of bacterial diseases. The term drug as used here includes sulfonamides, antibiotics, nitrofurans, and other products that usually exert a bacteriostatic effect. The term chemical denotes compounds that are usually used as bath, or external, treatments and are most often bactericidal.

PRESENT STATUS OF CHEMOPROPHYLAXIS AND CHEMOTHERAPY OF BACTERIAL DISEASES

Bacterial diseases of fishes are caused by members of the genera *Aeromonas*, *Pseudomonas*, *Vibrio*, *Yersinia*, *Pasteurella*, *Edwardsiella*, *Corynebacterium*, *Streptococcus*, and *Flexibacter*. With the exception of *Flexibacter*, many of the fish pathogens are similar or identical to those producing disease in man and other warm-blooded animals. To date, fish are the only reported hosts for the flexibacters. The application of chemotherapy to fishes is usually effected on a population rather than on an individual basis. Systemic bacteremias are treated by adding the appropriate drug to the diet, and external infections are controlled by chemical bath or flush treatments (Herman 1972).

As the names imply, chemoprophylaxis is intended to prevent disease outbreaks, and chemotherapy is used to treat disease that has occurred. In both, the dosage rate is usually the same but the length or number of treatments is greater in chemotherapy. However, when oral medication is used for systemic bacteremias, the distinction between chemotherapy and chemoprophylaxis is not clearly defined because medication benefits only the fish healthy enough to eat. Even though the drug may have been intended for chemotherapy, it may act chemoprophylactically.

Although chemotherapy is beneficial in disease control, some antibacterials are detrimental in fish or fish culture systems. For example, Collins et al. (1976) found that a single application of 50 mg/L of erythromycin inhibited nitrifying bacteria that are essential in removal of ammonia in recirculating fish culture systems. Nitrifying bacteria were not affected by chloramphenicol, sulfamerazine, or Terramycin (oxytetracycline) when used at 50 mg/L, but Chun et al. (1978) reported that at 60 mg/L Terramycin did inhibit nitrifying bacteria. Rijkers et al. (1980) also reported that injection or oral application of Terramycin suppressed the humoral response in carp (*Cyprinus carpio*). Cellular immunity in carp was suppressed by injection but not by oral application.

Control of Systemic Infections

One of the first attempts at chemotherapy of a systemic bacterial disease was made when Tunison et al. (1937) attempted to control ulcer disease, caused by an atypical variant of *Aeromonas salmonicida* in brook trout (*Salvelinus fontinalis*) by feeding food containing sulfanilamide. Trout were fed at the rate of 2 g of drug per kilogram of body weight per day (g/kg/day) for several days. There was no beneficial effect, and some evidence of toxicity. Studies carried out in the late 1940s and early 1950s were directed toward control of furunculosis, a major septicemic disease of salmonids caused by *Aeromonas salmonicida*. Gutsell (1946, 1948) tested several sulfonamides and Furacin (nitrofurazone) for controlling a natural outbreak of furunculosis in brook trout, and found that sulfamerazine was the best drug, and that it controlled the disease when fed at the rate of 176 mg/kg/day for 25 days. Additional research was conducted to determine efficacy (Gutsell and Snieszko 1949a; Gutsell and Snieszko 1949b), toxicity (Wood et al. 1955), and tissue levels (Snieszko and Friddle 1951) for sulfonamides. From the resulting data, the recommended dosage of

200 mg/kg/day for 14 days was established. Work in the early 1950s showed that Terramycin or Chloromycetin (chloramphenicol) controlled furunculosis when fed at the rate of 50-75 mg/kg/day for 10 days (Snieszko et al. 1952). Post (1959), who tested several nitrofurans for their ability to control furunculosis, found furazolidone to be the best. Additional experiments established the effective dosage at 75 mg/kg/day for 14 days (Post and Keiss 1962).

Erythromycin was found to be the drug of choice in treating bacterial kidney disease (Wolf and Dunbar 1959) caused by *Renibacterium salmoninarum* (Sanders and Fryer 1980). The commonly observed tendency of *R. salmoninarum* to occur intracellularly, coupled with effects of water chemistry (Warren 1963), may be the reason for the frequent failure of chemotherapy to control the disease. Recently, fishery workers on the West Coast have injected adult Pacific salmon with erythromycin to control prespawning mortality attributed to *R. salmoninarum*. Other workers claimed that vertical transmission of *R. salmoninarum* was prevented when they added 1 to 2 ppm erythromycin phosphate to newly fertilized eggs during the time of water hardening.

Shiomitsu et al. (1980) found that feeding erythromycin at 25-50 mg/kg/day for 4 to 7 days effectively controlled natural outbreaks of streptococcal septicemia in cultured yellowtails (*Seriola quinqueradiata*). Erythromycin therapy was more effective in controlling streptococcal infections than either Terramycin or ampicillin.

The antibacterials discussed above have been and continue to be useful in controlling several infections caused by gram-negative bacteria. However, their effectiveness is limited by the requirement that fish eat the medicated food to derive benefit.

The introduction of Furanace (ni-furpirinol) opened a new dimension in treatment of systemic bacterial diseases. Even though the drug was administered as a bath at a 1- to 2-ppm concentration, it was rapidly absorbed and concentrated in tissues, where it attained therapeutic levels. Furanace was found to be effective in controlling columnaris disease caused by *Flexibacter columnaris* (Amend and Ross 1970), cold-water disease caused by *Cytophaga psychrophila* (Amend 1972), and bacterial gill disease purportedly caused by *Flavobacterium* (Kimura et al. 1978). However, skin and gill lesions were reported in channel catfish (*Ictalurus punctatus* Rafinesque) exposed to 0.5 ppm furanace for 4 or 14 days. Lesions were developed on day 3 of the 4-day treatment and on day 11 of the 14-day treatment. Lesions continued to develop after catfish were moved to fresh water (Mitchell et al. 1978). Nitrofurans have also been shown to have mutagenic properties, and there is little likelihood that furanace will ever be approved by the U.S. Food and Drug Administration for use in food fishes.

Fish can also absorb and concentrate kanamycin when it is administered as a bath treatment (Gilmartin et al. 1976). A single 25 mg/kg injection of kanamycin controlled a *Pseudomonas* infection in white catfish (*Ictalurus catus*) (Meyer and Collar 1964). However, when Kanamycin was injected, a dosage of 10-20 mg/kg was toxic to steelhead trout (*Salmo gairdneri*) and caused gross and microscopic pathological alterations (McBride et al. 1975).

Japanese investigators have tested several newer antibacterials for treatment of systemic diseases. When tested in vitro, piromidic acid was active against a wide range of bacteria, and a 3-day treatment of 6.1-7.8 mg/kg/day controlled *A. hydrophila* and *Vibrio anguillarum* infections in goldfish (*Carassius auratus*) and eels (*Anguilla japonica*) (Katae et al. 1979). Piromidic acid also inhibited the conjugal transfer of R-plasmids. Continuous 9-day exposure to a 2-ppm bath treatment of oxolinic acid or nalidixic acid was effective against *Pseudomonas anguilliseptica*, the cause of red spot disease in eels (Jo 1978). Oral administration of 5 mg/kg/day oxolinic acid for 3 days was also effective against *P. anguilliseptica* infections. Pseudotuberculosis of yellowtails (*S. quinquerediata* and *S. purpurascens*) caused by *Pasteurella piscicida* was controlled by feeding ampicillin at 100 mg/kg/day for 8 days (Kusuda and Inoue 1977).

Potentiated sulfonamides constitute a rather new group of drugs that have recently been shown to be effective in treating systemic diseases of fishes. The potentiated sulfonamides are combinations of the older sulfas with diaminopyrimidines. Individually, the sulfonamides and diaminopyrimidines such as trimetoprim are bacteriostatic, but in combination their effects are bacteriocidal. The combination works by blocking sequential steps in the synthesis of tetrahydrofolates. Seven sulfonamides, when tested in combination with trimetoprim, were found to be more effective in their inhibition of gram-negative fish pathogens than were the sulfonamides alone (McCarthy et al. 1974a). As an example, a combination of 15 mg trimetoprim and 100 mg sulfamethylphenazole controlled experimentally induced furunculosis in rainbow trout (McCarthy et al. 1974b). Medicated diet was fed for 14 days, and furunculosis was controlled when treatment was initiated either immediately after injection of *A. salmonicida* or when clinical signs were first observed. However, if medicated feed was withheld until trout began to die from furunculosis, control was not achieved. The potentiated sulfonamide Ro5-0037, a combination of five parts sulfadimethoxine and one part ormetoprim was tested at the National Fish Health Research Laboratory and found effective for control of several gram-negative pathogens of fish. Details of these studies are discussed in a later section.

Control of External Infections

Several chemicals are used to control external bacterial diseases in fishes. The organic iodines such as Betadine or Wescodyne are routinely used at concentrations of 100 ppm iodine for 10-15 min for disinfection of salmonid eggs immediately after water hardening (McFadden 1969). Efficacy and safety are greatest at a pH range of 6.5 to 7.5. Although a number of the bacterial pathogens have been shown to be sensitive to iodine compounds (Ross and Smith 1972), disinfection with organic bound iodine has not prevented vertical transmission of bacterial kidney disease (Bullock et al. 1978), nor of infectious pancreatic necrosis virus (Bullock et al. 1976). Bacterial gill disease, one of the most serious diseases of juvenile salmonids, may be prevented by a single 1-h prophylactic treatment with 2-4 ppm Diquat cation, or 1-2 ppm active ingredient of quarternary ammonium compounds such as Roccal or Hyamine (Wood 1974). However, when epizootics of bacterial gill disease occur, three consecutive treatments are required to achieve control. Diquat at 2-4 ppm of the cation has also been used for

treating the external phase of columnaris disease and salmonid cold-water disease (Wood 1974). Columnaris disease in pondfishes is controlled by the addition of 0.5 ppm copper sulfate (Davis 1953) or 2 ppm potassium permanganate (Rogers 1971). A 24-h bath of 1 ppm oxolinic acid was reported to be effective in controlling columnaris disease of several cultured fishes in Japan (Endo et al. 1973). A bath treatment of 22 ppm (active ingredient) Furacin is recommended for control of external motile aeromonad and pseudomonad infections of striped bass (*Morone saxatilis*) (Bonn et al. 1976).

The above drugs and chemicals used for systemic and external bacterial diseases are only a partial listing. A more detailed listing can be found in the works of Snieszko (1978) and Herwig (1979).

STUDIES ON THE POTENTIATED SULFONAMIDE Ro5-0037

Among the compounds discussed above, only Terramycin and sulfamerazine have been registered by the U.S. Food and Drug Administration, and thus are the only drugs that can be used legally for systemic or external bacterial diseases of food fishes. Inasmuch as it was considered desirable to have a third antibacterial registered for legal use, biologists at the National Fish Health Research Laboratory chose Ro5-0037 for study because several gram-negative fish pathogens have been found to be sensitive to this drug. The initial work at the Laboratory was with furunculosis because increasing numbers of strains of *A. salmonicida* were shown to be resistant to sulfamerazine or Terramycin, or both. In vitro studies with 23 strains of *A. salmonicida* showed that Ro5-0037 inhibited growth of both sulfonamide-resistant and sensitive strains. Sulfadimethoxine and ormetoprim inhibited sulfonamide sensitive strains but both were ineffective against resistant forms (Bullock et al. 1974). Also, a 14-day treatment with Ro5-0037 at 50 mg/kg/day--one-fourth the usual sulfonamide dosage--effectively reduced mortality among rainbow trout with experimentally induced furunculosis. As shown in Fig. 1, the combination of sulfadimethoxine and ormetoprim was more effective than either drug alone. Sulfadimethoxine provided a satisfactory measure of control of furunculosis, ormetoprim was practically ineffective. Also, the development of resistant strains of *A. salmonicida* was three times more rapid after treatment with sulfadimethoxine and ormetoprim than with Ro5-0037 (Fig. 2). Although Ro5-0037 proved efficacious for furunculosis, tissue studies indicated that ormetoprim residues remained in the skin and scales of rainbow trout for as long as 3 months. The long withdrawal time necessitated by the lingering residue diminishes the usefulness of the drug. To overcome the problem, investigators reduced the duration of treatment from 14 days to 5 days. With the 5-day treatment, ormetoprim residues were cleared from all tissues within 5 weeks.

Large-scale clinical trials were then carried out in fish hatcheries to test efficacy of the 5-day Ro5-0037 treatment on furunculosis or enteric redmouth (ERM), another septicemic disease of salmonids caused by *Yersinia ruckeri*. All trials were conducted under an Investigational New Animal Drug Permit, which required that medicated fish be held 6 weeks after completion of the trial. More than 900,000 salmon or trout were involved in trials conducted in representative geographic regions across the United States. Ro5-0037 effectively controlled all but one of the ERM epizootics,

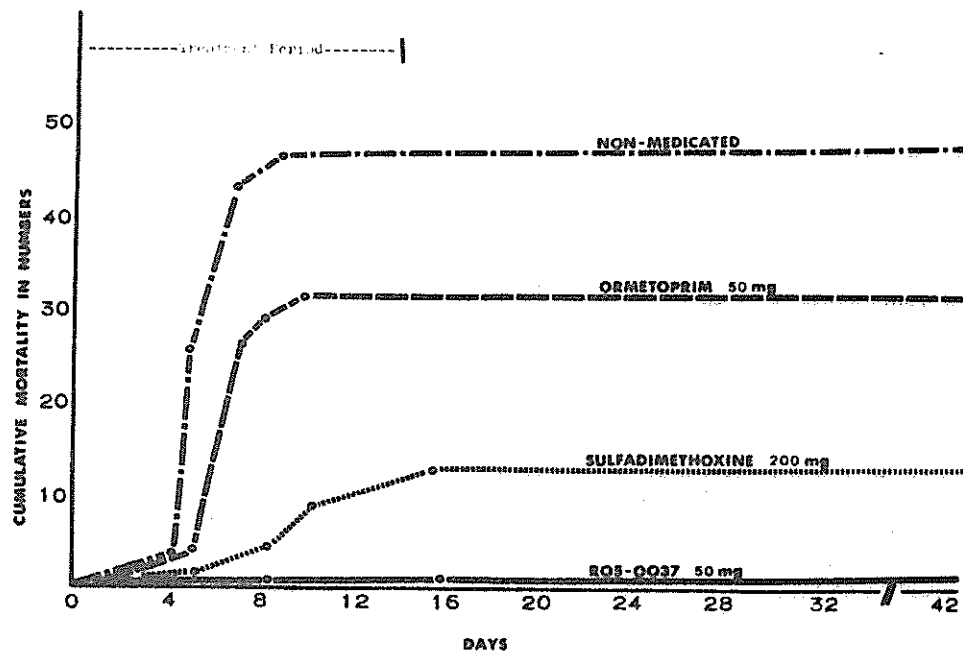


Fig. 1. Effect of 14-day treatment of furunculosis in rainbow trout (*Salmo gairdneri*) with Ro5-0037 and its components, sulfadimethoxine and ormetoprim.

not only during the 5-day period of treatment but also for the 3-week observation period that followed. However, Ro5-0037 was not effective against ERM when trout were under continuous stress from crowding and were receiving water from fish with active ERM disease.

Field trials also showed that the effectiveness of Ro5-0037 in controlling furunculosis epizootics varied. Generally, Ro5-0037 was effective in epizootics caused by strains of *A. salmonicida* that were resistant either to sulfamerazine or to Terramycin. However, the efficacy was less than complete against strains that were resistant to both drugs (Fig. 3). The effectiveness may have been reduced because the furunculosis outbreaks were well under way before treatment was started. I believe that if Ro5-0037 is fed when furunculosis or ERM is first recognized, and if proper cultural practices are followed, the drug will be effective. This is supported by results from a study that compared the effectiveness of Ro5-0037 and Terramycin in controlling a furunculosis outbreak in presmolt (23 g) coho salmon (*Oncorhynchus kisutch*) in Oregon. Salmon in two tanks (1200 each) were fed feed containing Ro5-0037 for 5 days at the rate of 50 mg/kg/day and non-medicated feed for 9 days. Salmon in two tanks were fed 50 mg/kg/day Terramycin for 10 days and nonmedicated feed for 4 days, while salmon in two tanks were fed nonmedicated feed for the 14 day test period. Total mortality in the three groups was 15% for the Ro5-0037 group, 40.6% for the Terramycin group, and 51.2% for the medicated group. These results clearly showed the 5-day Ro5-0037 treatment was superior to the 10-day Terramycin treatment. In addition, in vitro data indicate that Ro5-0037 will also control epizootics caused by *V. anguillarum* and *A. hydrophila*. Clinical trials on epizootics caused by these pathogens should be conducted.

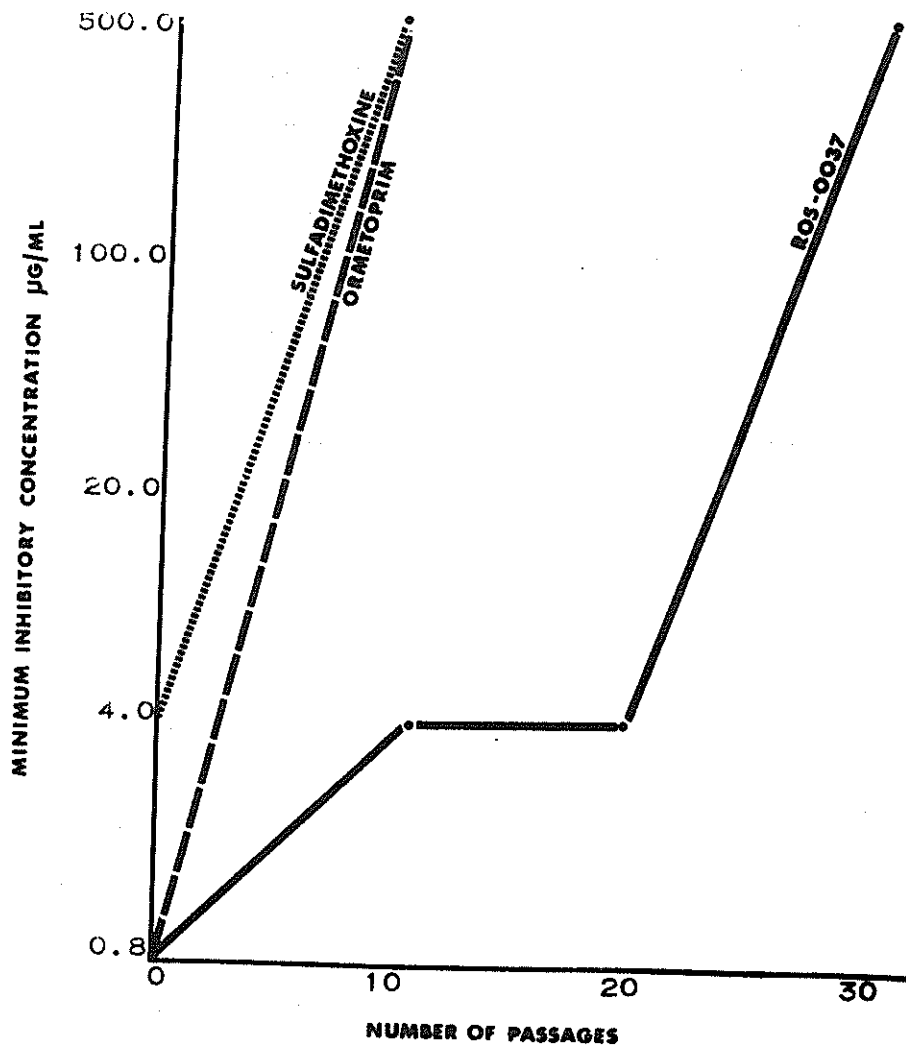


Fig. 2. Effect of repeated passaging of a sulfonamide sensitive strain of *Aeromonas salmonicida* in one-fourth minimal inhibitory concentrations of Ro5-0037, and its components.

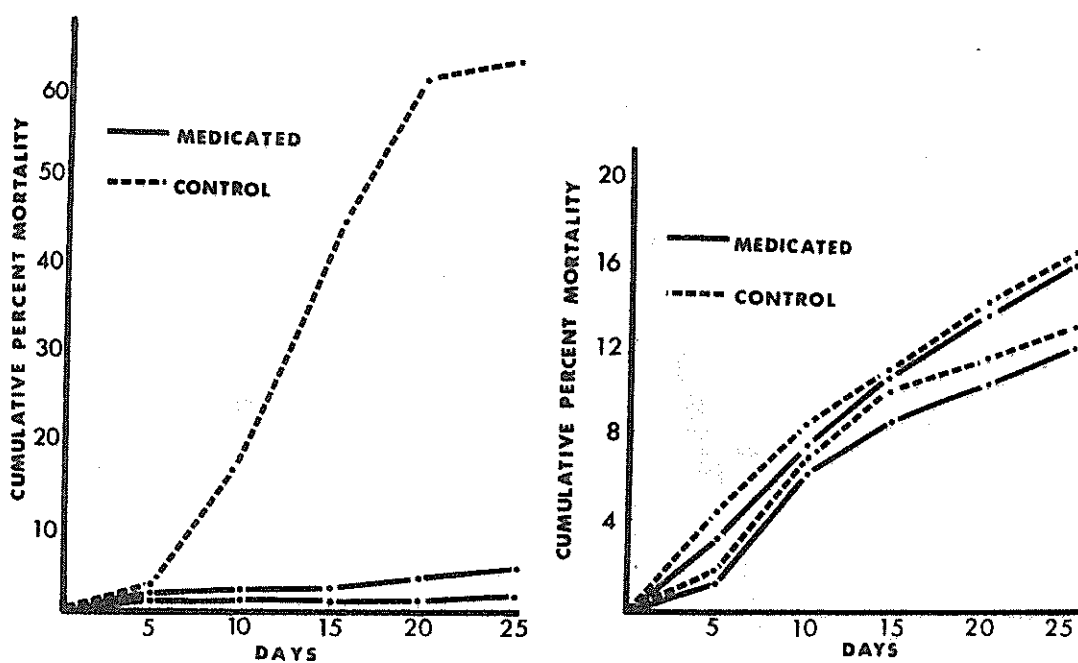


Fig. 3. Effectiveness of Ro5-0037 on furunculosis caused by sulfonamide or Terramycin sensitive (left) or resistant (right) *Aeromonas salmonicida*.

POTENTIALLY USEFUL ANTIBACTERIALS FOR USE IN FISH HUSBANDRY

Clearance of chemicals and drugs for use in fish husbandry is a continuing program. Candidate antimicrobials are screened in conventional disc sensitivity tests (Bauer et al. 1964). Sixteen drugs were recently tested for their effectiveness in inhibiting growth of 60 different cultures of gram-negative fish pathogens, which included representative strains of *A. salmonicida*, *A. hydrophila*, *P. fluorescens*, *V. anguillarum*, *Y. ruckeri*, *E. tarda*, and flexibacters.

Overall results of the disc sensitivity tests showed myxin (6-methoxy-1-phenazinol-5,10-dioxide) to be the best drug because all test cultures were inhibited by this compound. Nalidixic acid and oxolinic acid were also effective; however, these drugs only partially inhibited the *Pseudomonas* cultures. Additional in vitro tests were carried out with myxin, specifically the copper complex. In addition to having antibacterial properties, copper myxin is fungistatic and may be used in treating eggs or adult fish. Because it is not absorbed from the intestinal tract, its use would be limited to treatment of external infections. However, an effective treatment for external diseases such as columnaris, cold-water disease, bacterial gill disease, and fin erosion is badly needed. Additional tests showed the minimum inhibitory concentration of copper myxin for 17 flexibacterial cultures isolated from the above four diseases to be between 0.032 and 0.16 ppm. Because researchers at the La Crosse (Wisconsin) National Fishery Research Laboratory found the drug to be nontoxic to five species of fish at 10 ppm, there appears to be a wide margin of safety between therapeutic and toxic levels. Observations at the National Fish

Health Research Laboratory indicated that a level of 2 ppm copper myxin controlled external flexibacterial lesions in rainbow trout.

As mentioned earlier, proper application of chemotherapy and chemoprophylaxis is often a part of effective management of cultured fishes. The recently published text of Herwig (1979) lists hundreds of chemicals and drugs that are, or have been, used in fisheries. Little is known about efficacy or safety of many of the compounds, and for these and other reasons evaluation for potential registration is required. Registration is expensive; recent estimates are that \$8.8 million will be needed to register 33 chemicals now used or being considered for use (Schnick and Meyer 1978). Considering the cost and the time required to register even one product, it is not surprising that only two antibacterials may now be used legally on food fish. However, it is only by carrying out the definitive studies required to register compounds that intelligent selection of drugs and chemicals for use in fisheries can be made.

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THE SECRETORY IMMUNE SYSTEM IN FISH:
AN OVERVIEW OF RECENT ADVANCES¹.

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Fishes as a phylogenetic group, are able to undergo both humoral and cellular immune reactions akin to those observed in higher animals (reviewed 1). It therefore stands to reason that appropriate immunization should be an effective means of controlling certain infectious diseases of fish. This prediction has been verified numerous times in laboratory settings, but the difficulties in parenterally administering vaccines or other forms of antigens to large numbers of fish have precluded the large scale successful exploitation of the immune system as a means of controlling most diseases in fish. It would seem however that approaches at external vaccination by using hyperosmotic baths (2) or flush exposure (3) may solve this problem. In fact an alternative means, oral immunization, offers even greater potential for mass immunization. With this technique it would not be necessary to handle the fish and the antigen could be administered in pond situations where restricted water flow or crowding is not feasible. In man, the oral immunization approach has been particularly useful with the live oral polio vaccine. In fact the discovery of the so called local or secretory immune system involving a class of immunoglobulin designated IgA now provides a rational explanation for the success with this oral vaccine.

It is therefore particularly unfortunate that many efforts at oral immunization of various fish species with different vaccines has resulted in conflicting and/or nonreproducible results in terms of protection (reviewed 4). While the reason(s) for these discrepancies are not understood, there are several factors to be considered. The first would involve the question of whether or not locally synthesized secretory antibody exists in fish. Although serum immunoglobulins have been reasonably well characterized in a variety of fishes, the immunoglobulins in external secretions have not. Since many fish pathogens are suspected of having their portal of entry through mucosal surfaces, any antibodies present in secretions such as mucus and bile could be very important in protection against infection. In this context the studies on plaice by Fletcher and White (5) seem particularly noteworthy. Immunoglobulins were demonstrable in plaice secretions and, perhaps more importantly, it was found that animals immunized with vibrio vaccine exhibited differential antibody responses depending on the route of immunization. On one hand, orally immunized fish had higher antibody titers in intestinal mucus extracts than in serum whereas the converse was observed in parenterally immunized fish. These observations are strongly suggestive of local synthesis of antibody reminiscent of the situation seen with IgA in mammals. A second point of consideration in terms of oral

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immunization is the long standing, but until recently not understood, observation that the oral administration of certain antigens to higher animals (such as mice) not only fails to induce significant immune responses but in some cases induces tolerance or specific unresponsiveness to subsequent parenteral administration of normally immunogenic doses of antigen (6). Also certain parenteral immunization regimens, especially those involving low temperatures, have been shown to induce tolerance in fish (7). Therefore reports of negative findings in orally immunized fish must consider the possibility of the tolerant state.

It was with the above background that our laboratory undertook experimental approaches to determine whether or not fish have a secretory immune system distinct from the humoral system. The strategy employed involved initially purifying and characterizing the immunoglobulins in blood and the external secretions, cutaneous mucus and bile. Subsequently, experiments were aimed at determining if the immunoglobulins in external secretions were derived from blood. The fish chosen for this study was the sheepshead Archosargus probatocephalus, a marine teleost of ample size to provide suitable amounts of material for this study. The details of these experiments are published elsewhere (8, 9, 10, 11) but a summary of the pertinent findings is presented here to support the contention that the sheepshead does in fact have a secretory immune mechanism(s) that likely involves localized synthesis of immunoglobulins.

Serum immunoglobulins were purified from sheepshead by a combination of gel filtration and ion exchange chromatography and were found to exist in two major forms which appeared remarkably similar to the tetrameric and monomeric IgM-like molecules previously reported for the giant grouper, another marine teleost (12). One form was a 16S high molecular weight species ($\sim 700,000$ daltons) which appeared to be a tetramer composed of 2H-2L chain subunits. The presence of a peptide resembling J chain was also seen in this population of molecules. The second form of sheepshead serum immunoglobulin was a 6S molecule of lower molecular weight ($\sim 140,000$ daltons). It appeared to have a smaller H chain than that present in the 16S molecule (45,000 vs 70,000 daltons). This "missing" $\sim 25,000$ daltons on the H chain likely accounts for the antigenic deficiency of this 6S molecule when compared to the 16S form. In vivo and in vitro experiments using radioiodinated sheepshead 16S and 6S immunoglobulins indicated there was no interconversion between these two species. The sheepshead low molecular weight protein was not a degradation product of the 16S molecule as suggested by others for the goldfish (13).

Immunoglobulins were also identified in two external secretions, cutaneous mucus and bile, albeit in much lower concentrations than in serum. These low concentrations precluded large scale purification but sufficient material was obtained to allow certain generalizations. Bile contained only one demonstrable immunoglobulin which appeared to be a dimeric ($\sim 320,000$ daltons) molecule. Although the H chain of this protein appeared smaller than that of the 16S serum protein (55,000 vs 70,000 daltons), antigenic analysis indicated the two molecules to be identical. Cutaneous mucus contained both high and low molecular weight immunoglobulins with the predominant species being a dimeric form ($\sim 400,000$ daltons) with H chains indistinguishable from those of the serum

16S molecule. This protein also contained an additional ~95,000 dalton component that is likely the teleost equivalent of secretory component, a protein normally associated with immunoglobulins in secretions of higher animals (reviewed 10).

In order to directly approach the question of the origin(s) of the immunoglobulins in external secretions, radioiodinated sheepshead 16S or 6S serum immunoglobulins were injected intravenously into sheepshead. Determinations of specific activities (cpm/mg immunoglobulin) in serum, bile and mucus at various times indicated that little, if any, of the immunoglobulins in these secretions could have been derived from the predominant serum immunoglobulin. Hence this finding suggests very strongly that the sheepshead, and probably other fish, have at least one, and perhaps two, immunologic systems that are quite similar to the secretory immune system of higher vertebrates.

In conclusion, the studies discussed here serve to illustrate several important points regarding the immunoglobulins of the sheepshead. On one hand these findings substantiate previous reports of both high and low molecular weight humoral immunoglobulins in other fish species. Secondly, these studies have described in some detail the apparently different immunoglobulins present in two external secretions. Finally, the findings discussed emphasize unrealized (and perhaps unique) mechanisms for introducing immunoglobulins (and by inference protective antibodies) into external secretions. Future studies in this area must therefore consider ways of preferentially eliciting the production and secretion of these potentially important molecules.

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INFLUENCE OF REGULATIONS ON THE SPREAD OF FISH DISEASE

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ABSTRACT

Government regulation is only one of several approaches used to control diseases. Historically, control regulations for disease prevention have not been applied to aquatic animals as extensively as for other animals. However, during the past 15 years, prevention of the spread of fish diseases through regulations has become a significant management tool for protection of fish resources. Certain criteria must be met before regulatory control can be established for a specific disease: (1) Does the disease pose a major threat to the resource? (2) Is there a more economical method of control? (3) Are accurate and dependable detection methods available? (4) Is the infectious agent sufficiently confined to a specific geographical area? (5) Who will benefit? (6) Is it worth the expense? (7) Who will absorb the expense? and (8) Who will administer the program?

California was the first state to establish fish import regulations based on the health condition of fish (1956). In 1970, 14 states had some form of fish disease regulations and by 1979 the number had increased to 29 states. Lack of uniformity is the major problem with these state regulations. The regional concept of fish disease control (regulatory) appears workable. The Colorado River Drainage Fish Disease Control Policy being the first of these implemented in 1973. The Great Lakes Fishery Commission Fish Disease Control Committee adopted a regional policy in 1975. Although there is no national U.S. fish disease policy, the U.S. Fish and Wildlife Service has a functional program for the national fish hatchery system. Additionally, there is an international regulation (Title 50) that prevents the importation of trout into the U.S. unless certified free of whirling disease (Myxosoma cerebralis) and viral hemorrhagic septicemia. Canada has a National Fish Disease Control Policy that regulates interprovincial and international salmonid trade. The European community has a similar international program.

There are 17 diseases of fish that are included in the various state, regional or national policies. The most frequently listed disease agents are infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus, viral hemorrhagic septicemia virus, Myxosoma cerebralis, Ceratomyxa shasta, Renibacterium salmoninarum, and Yersinia ruckeri.

The effects of fish disease regulations on the spread of infectious agents are very difficult to objectively assess. There is no system whereby accurate reporting can be done as to how many, if any, diseases are effectively limited by such regulations. However, it is the feeling by most individuals involved in the regulatory process that regulations do aid in preventing the dissemination of some diseases of fish.

INTRODUCTION

Regulation is only one of several approaches to the control of diseases. Human and domestic animal diseases have been successfully controlled and their dissemination prevented through regulations for many years. Some diseases, including yellow fever and smallpox, have been virtually eliminated as a result of regulation and mandatory vaccination.¹ The U.S. Department of Agriculture administers an elaborate regulatory system to control the spread of many diseases of domestic animals. The best known domestic animal diseases that have been virtually eliminated from North America are black tongue, foot-and-mouth disease, and hog cholera.¹ However, until recently the Department of Agriculture has shown little interest in aquaculture. Regulatory control measures have not been applied to aquatic animal diseases as extensively as for other animals. One reason is that there are no serious diseases of fish that have zoonotic implication in public health. Consequently, they have escaped attention of the human and veterinary medical fields. Other reasons contributing to the lack of fish disease regulations are the disagreement on how it should be done, and the commercial industry as a whole is not convinced that disease regulations are economically beneficial. Nevertheless, as a result of the efforts of several organizations and groups, control of fish diseases through regulations is becoming a significant tool for aquatic resources.

CRITERIA FOR REGULATORY DISEASE CONTROL

If catastrophic diseases of other animals can be controlled through regulations why can those of fish, and other aquatic animals, not be similarly controlled? Most likely they can be, but some fundamental questions must be considered before this can be determined.

1. Does the disease pose a major threat to the resource? If unchecked, will the disease be highly detrimental to fish stocks?
2. Is there a more economical and efficacious method of control? Chemotherapy and immunotherapy must be considered.
3. Are accurate and dependable detection methods readily available? Of particular importance is detection of the non-diseased carrier state.
4. Is the infectious agent sufficiently confined so that a specific geographical distribution can be delineated? If a disease is ubiquitous, it would be difficult and impossible to regulate.
5. Who will benefit most as a result of the regulations--the industry or the organization charged with administering the program? Clearly the industry must benefit.
6. Is control of the disease through regulation monetarily worth the expense of the program? Control efforts must have a positive benefit-cost ratio.
7. Who will absorb the expense of the regulatory program?
8. Who will administer the program? Should it be the Department of Agriculture, which has the proper mechanism but not the expertise, or the Department of Interior, which has the expertise but not the mechanism? Or should this be a state responsibility?

The above series of questions are not intended to be all inclusive, but they are germane to establishing any regulatory disease program. Answers to these questions are seldom clear cut, but it is imperative that they are rationally addressed during the regulatory formulation period.

HISTORY OF FISH DISEASE REGULATION

Disease prevention is the most practical and economical method of controlling any type of disease, whether the method is voluntary or compulsory. Many fishery managers recognize this fact and through their management procedures help prevent diseases. Disease regulations are only a small part of fish disease prevention. In recent years, compulsory prevention of fish disease through regulations has received greater attention. California initiated such a program when the Department of Fish and Game issued their regulation on "Importation of Live Fish" in 1956.² Since that time, three reviews of the status of fish disease regulations in the United States were made. Herman³ surveyed the state fishery agencies and determined that 14 (28%) of the states had disease control regulation in 1978, 23 (46%) required transportation permits, and 15 (30%) had some form of disease inspection requirements before fish could be transported into their states. In later reviews of North American fish disease control regulations,^{4,5} 29 states (58%) had some degree of fish disease regulation with a similar number requiring disease inspection. A transportation permit was required by 39 (78%) states (Table 1). The increase in participating states indicates a greater interest in preventing the spread of serious infectious disease of fish. There has been a steady increase in the number of states participating in fish disease regulations from 1956 to 1979⁴ (Figure 1). Individual states are the logical regulators of aquatic resources which allows each state to adapt the regulations to fit its need. Unfortunately, it also allows variations in programs, a distinct disadvantage for live haulers and bait fish wholesalers supplying fish to different states.

During the period of 1972 to 1974, a strong move was made to establish a national regulatory policy concerning interstate shipments of diseased fish. These efforts failed to achieve congressional approval for several reasons. The major difficulty arose when the fish health community itself could not agree on what diseases should be controlled, or how the control of interstate movements of diseased fish should be implemented. Moreover, there was a lack of coordination and cooperation between those designing and supporting the regulations and the commercial fish farming industry, one aquacultural segment for which regulations were intended to assist. The industry was not aware of the benefits that would come to them as a result of the regulation and interpreted passage of the proposed law as a threat to their survival. In response to the proposed federal legislation, Clark White, then President of the U.S. Trout Farmers Association, published an article in the February, 1973, issue of the American Fish Farmer and World Aquaculture News,⁶ stating that: "Private and commercial fish farms will be threatened for their very existence if the present legislation sponsored by a group of federal and state biologist and disease experts is allowed to become a law." No doubt the majority of commercial farmers at that time had similar feelings; this attitude

Table 1. Summary of state fish disease control regulations in the U.S.

	Number of states (%)		
	1956	1970 ^{1/}	1979 ^{2/}
Fish disease regulations	1	14 (28%)	29 (58%)
Fish disease inspection	1	15 (30%)	29 (58%)
Transportation permit ^{3/}	1	23 (46%)	39 (78%)

^{1/} Herman³

^{2/} Busch⁴, Olsen⁵

^{3/} Includes permits designed to prevent importation of exotic species.

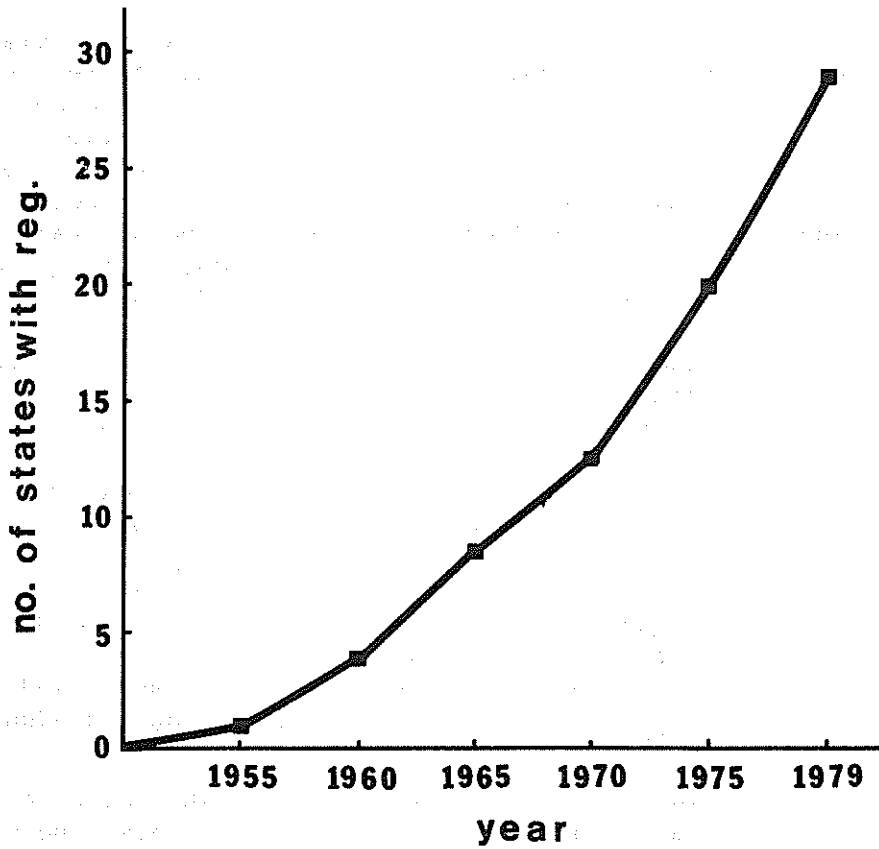


Figure 1. Increase of state participation in the establishment of fish disease regulations.

must change if a meaningful national policy is to be achieved. In my opinion, it is essential that future attempts to establish a national fish disease control policy must involve a "meeting of the minds" of the fish health and the aquaculture segments to arrive at common needs, benefits, and objectives. Certainly such programs should not be detrimental to an industry as a whole; however, it would inevitably affect some individuals more than others. Some distinct advantages would accompany a national fish disease control program, notably greater uniformity and less confusion. States or regions with unique or specific fish disease problems could supplement the national policy to suit their own needs.

Olsen⁵ pointed out that it may be somewhat easier and beneficial to develop fish disease control policies on a regional basis; there are two excellent examples of the regional approach currently in existence. The first, and possibly the strongest of these, is the Colorado River Fish Disease Program of the Colorado River Wildlife Council which was implemented in 1973. The Colorado River Program is a compulsory, cooperative arrangement between all states that have drainage into the Colorado River. This is a binding agreement between the states (Colorado, Wyoming, Nevada, Arizona, New Mexico, California, Utah, and the U.S. F&WS) that makes it unlawful to stock fish from any federal, state or private fish hatchery into the drainage unless the fish are certified free of certain diseases. The second regional fish disease regulatory program is that of the Great Lakes Fishery Commission Fish Disease Control Committee adopted in 1975.⁸ In contrast to the Colorado River policy, the Great Lakes program is voluntary between the states (Illinois, Indiana, Michigan, Minnesota, New York, Ohio, Pennsylvania, and Wisconsin), the Province of Ontario, the Canadian Government, the U.S. F&WS, and private industry. It was recommended by the Great Lakes Fish Disease Control Committee that all participating states develop legislative authority and regulations by 1980 to facilitate control and possible eradication of fish diseases. In each regional program, the diseases that are "certifiable" are specified and the programs are designed to certify facilities rather than individual lots or shipments of fish free of these diseases.

The U.S. Fish and Wildlife Service has a viable Fish Health Policy that applies to the national fish hatchery system.⁹ The policy not only categorizes hatcheries as to their health status, but also regulates the stocking of trout into waters based on the previous disease history of those waters and the fish to be stocked. The policy, based on strict periodic disease inspections, has been in force for 7 years, but inspections have been conducted at federal hatcheries for over 15 years.

The Canadian Fish Disease Control Policy (Fish Health Protection Regulations) came into being in 1977.¹⁰ This policy applies to interprovincial and international transport of salmonids and their products.

To prevent the spread of diseases from country to country, international regulations are necessary. The U.S. Fish and Wildlife Service took measures in 1968 to prevent the introduction of viral hemorrhagic septicaemia (VHS) into the U.S. by implementing Title 50 which prohibits the importation of trout or their products unless they are certified free of VHS and Myxosoma cerebralis (whirling disease).¹¹ M. cerebralis was already

in the U.S., but the law prohibits its continual introduction.

The European community has pursued an ambitious international plan for the control of the spread of fish diseases.¹² The Symposium on the Major Communicable Fish Diseases in Europe and their Control recommended that a Government Consultation or an International Convention for the Control of the Spread of Major Communicable Fish Diseases be convened. The first of the Government Consultations was convened in Scotland in 1974 and a second one was held in Paris in 1977 at the joint invitation of United Nations Food and Agriculture Organization (FAO) and the International Office of Epizootics (OIE). The second consultation was attended by representatives from 25 countries, the European Federation of Salmonid Breeders and the International Council for the Exploration of the Sea. This group was "Conscious of the economic and environmental damage caused by the spread of major communicable fish disease and the need to take concerted action to control and eventually eradicate such diseases."¹³ They were also concerned that the increasing international traffic in fish and fish eggs, without adequate precautionary measures, poses a serious danger to fish stocks when certain pathogens are introduced into countries previously free from such diseases. Therefore, the International Convention for the Control of the Spread of Major Communicable Fish Diseases recognized the need to adopt measures to facilitate international traffic in fish eggs, while simultaneously insuring adequate precautions against the spread of fish diseases.¹² Although not mandatory, it was recommended that all contracting parties of the European Economic Community adopt these programs through legislation.

DISEASES TO BE CONSIDERED

It is impossible to control the spread of all fish disease organisms; furthermore, it is not necessary to control all of these organisms. What disease to regulate has led to considerable disagreement among fish pathologists, farmers and legislators. Since the vast majority of the infectious organisms that cause diseases of fish are ubiquitous, only certain diseases must fall into a very narrow category and must conform to the basic questions outlined earlier concerning importance, seriousness, alternative control methods, detection methods, and geographical confinement.

There are 17 different diseases listed (Table 2) in various state or government regulations. Bear in mind that all regulations do not list all of these diseases. The various state regulations combined list all of these diseases but the USFWS lists only 7 diseases. The Colorado River Fish Disease Policy lists 9 while the Great Lakes Fishery Commission lists 7. Canada lists 8 while the European program lists only 3. VHS is the only disease that appears on every list. Range in the diseases listed illustrate the diverse needs of the various organizations involved.

Most of the diseases listed in Table 2 are legitimate candidates for control considerations; however, some of those listed should not be considered. It is doubtful if channel catfish virus (CCV) should be a "certifiable" disease due to the impossibility of detecting the carrier state. Motile aeromonas septicemia (Aeromonas spp.), pseudomonas septicemia

Table 2. Fish diseases that are specified as certifiable in regulatory.

Certifiable Disease	States ^{1/} (No.)	USFWS ^{2/}	Title ^{3/} 50	CRFDP ^{4/}	GLF ^{5/}	Canada	Europe
VIRAL							
1. Channel catfish virus	9			X			
2. Herpesvirus salmonis	3			X	X		
3. Infectious pancreatic necrosis	18	X		X	X	X	X
4. Infectious hematopoietic necrosis	19	X		X	X	X	X
5. Viral hemorrhagic septicemia	20	X	X	X	X	X	X
BACTERIAL							
6. Bacterial kidney disease	14	X		X	X	X	
7. Enteric redmouth disease	10	X		X	X	X	
8. Furunculosis	8	X				X	
9. Motile Aeromonas septicemia	2					X	
10. Myxobacteria infections	2					X	
11. Pseudomonas septicemia	2						
12. Vibriosis	3						
PARASITIC							
13. Salmonid ceratomyxosis	14			X	X	X	
14. Whirling disease	20	X	X	X	X	X	
15. Salmonid blood fluke	6			X			
OTHER							
16. Branchiomyxosis	7						
17. Ulcerative dermal necrosis	1						
TOTALS	17	7	2	9	7	8	3

^{1/} State regulations. The number is that number of states which include the specified disease in their regulations.

^{2/} United States Fish and Wildlife Service. National Fish Hatchery Fish Disease Policy.

^{3/} Title 50 is the national regulation controlling fish importation.

^{4/} Colorado River Wildlife Council Fish Disease Policy.

^{5/} Great Lakes Fish Commission Fish Disease Policy.

(Pseudomonas spp.), and myxobacteriosis should not be on any regulatory disease list. These saprophytic or facultative organisms are found in most waters and soil, and it is ludicrous for any facility or group of fish to be "certified" free of them.

Most specified disease affect salmonids and only three affect warm water species. One reason this is true is that disease of salmonids are more definable than those of warm-water fishes and are by and large geographically and environmentally more restrictive. Also more research has been done with salmonid diseases than warm-water disease, and the salmonid industry has been more willing to become involved in voluntary or compulsory regulation. That the severe effects of trout diseases are more readily apparent than of warm-water or pond-reared fish, also enhances salmonid disease control.

EFFECTS OF REGULATIONS

No one has attempted to document what effect regulations have had on the spread of communicable fish diseases. Unfortunately, there appears to be no systematic way to evaluate the regulatory programs; however, most do have some type of reporting system. Therefore, it is nearly impossible to objectively evaluate the effectiveness of regulatory programs in terms of actual curtailment of the spread of communicable fish diseases. In view of this, I will discuss some specific instances involving the regulatory processes. Myxosoma cerebralis (whirling disease) of salmonids serves as a model for the intercontinental dissemination of a fish disease, where no regulations applied, as it can be readily transmitted from one area (country) to another in infected fish. Hoffman¹³ traced the history of the spread of whirling disease from Europe to the U.S. and also within the U.S. after its introduction. At that time (1970), the disease was in nine states, most of which lacked fish disease import regulation. However, in 1966 the disease was reported from California¹⁴ that did have import regulations requiring inspections. Since 1970 the spread of M. cerebralis has stabilized somewhat¹⁵ and this may be due in part to the increase in regulation and in part due to increased surveillance and avoidance.

There is no doubt by U.S. Fish and Wildlife Service biologists and others that their Fish Health Policy is beneficial. Jim Warren, Hatchery Biologist in the Bureau of Sport Fisheries North Central Region, reports that after a series of hatchery inspections in that Region, all Fish and Wildlife Service trout hatcheries are free of diseases specified in the Fish Health Policy. Although inspections are an essential part of the Fish Health Program, proper hatchery management, restricted movement of diseased fish, and the use of certified specific disease free eggs are absolutely necessary for such a program to be successful.

Canadian fish health officials consider their regulations to be beneficial noting a decrease in international and interprovincial trade of salmonid fingerlings which they attribute to the implementation of regulations. This in turn reduces the opportunity for disease spread. More importantly, there have been no reports of "certifiable" disease outbreaks that were believed to originate from transport of salmonid eggs or juveniles since regulation implementation. Canadian regulations have report-

edly been accepted by government agencies and private industry inspite of the reduction of fish trade.

California has the oldest documented regulatory program in the U.S. and it was discussed by Wolf.¹⁶ During the early years of their inspection programs, approximately 6% of the fish shipments were rejected for various disease reasons; but the most historical reports involved a non-infectious etiology, "hepatoma", in 1960. When the industry became aware of the impact of this program, the percentage of rejects was reduced and in recent years no shipments were rejected. This probably reflects the producers recognizing California's determination, producers suspending fish shipments into California, and producers difficulty in complying with California's regulations. Wolf firmly believes that California's system is effective in preventing the establishment of certain diseases, but an objective evaluation of the program is difficult.

New York, a member of the Great Lakes Fishery Commission (GLFC), is one of the most recent states to establish a Fish Disease Control Program that is based on the procedures outlined by the GLFC Fish Disease Control Committee. Although the New York program, which is confined to state controlled facilities, was not adopted until 1979, disease control through inspections has been practiced for many years. The New York program includes criteria for hatchery classification, hatchery sanitation, epizootic control, and disposition of fish and eggs from hatchery stocks following disease epizootics. New York fish pathologists feel that definite benefits have resulted from their inspection and classification efforts and provided the following example. In 1977, prior to the Fish Disease Control Program, the spread of bacterial kidney disease (BKD) (Renibacterium salmoninarum) was disseminated to five different state hatcheries from a single hatchery where the disease was endemic. All fish were eliminated from the contaminated hatchery, the facilities were disinfected and restocked with fish from clear sources. There has been no reoccurrence of BKD, and its spread was contained. This is only one of many similar examples of the success of inspection programs. Eradication is not always possible or practical; this would be dictated by the water supply and the potential for reinfection.

New York has not expanded its fish health program to private industry due primarily to budgetary constraints, but lack of control on importation of fish to private farms poses some danger. Resulting from the lack of such controls, there was a serious outbreak of IHN at a private hatchery in 1978 where 95% of five million fish died. This may have been averted if importation regulations were functional.

The question still remains as to how effectively regulations control the spread of fish diseases. In discussing the effectiveness with a sampling of those involved, the concensus is that the spread of fish diseases is curtailed by regulations and many can cite specific examples when fish were rejected or destroyed because of their disease status. However, without a uniform monitoring system, which should be an essential part of any regulatory program, proper evaluation cannot be attained.

We must recognize that regulation governing the movement of diseased fish is not a fail safe approach. Diseases of fish will not be eliminated,

but their continued dissemination will be curtailed. It must be emphasized that cooperation of all organizations concerned is necessary for the beneficial effects of regulations to be maximized.

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PRINCIPLES OF CONTROL OF DISEASES OF FISH AND SHELLFISH

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ABSTRACT

Fish and shellfish are subject to diseases caused by physical and chemical changes in the environment, by malnutrition, and by infective agents. Diseases caused by infective agents are often triggered, or aggravated, by environmental stress.

Diseases caused by infective agents may be treated with therapeutic substances by adding the substances to water for external action; by systemic treatment via the intestinal tract; by parenteral administration; and by adding substances to the water that are absorbed and have systemic efficacy.

External treatments are complicated by the specific toxicity of chemicals, age of treated animals, water chemistry and temperature. External systemic treatments became popular and effective after the introduction of Furanace by a Japanese pharmaceutical company. This is compared with external fish immunization. Intestinal treatments are used only for treatment of parasites of the intestinal tract. Intestinal systemic treatments, the most common of all, are used with sulfonamides, antibiotics, and other drugs. Administration of drugs by injection has only limited application; however, this technique may be used in conjunction with immunization.

Aquatic invertebrates are susceptible to the same types of disease agents that attack fishes, and their treatment, in principle, is similar.

Aquaculture and use of drugs, like all human activities, have an impact on the environment. Therefore aquaculturists have the responsibility of using drugs and water wisely.

INTRODUCTION

Fish and shellfish are subject to diseases and kills caused by environmental conditions such as heat and cold, deficiency of oxygen, gas supersaturation, and pollution by waste materials and chemicals; by malnutrition; and by communicable diseases. Environmental diseases can be prevented by good management, and nutritional diseases by well-balanced natural or manufactured food; communicable diseases can be controlled by avoiding exposure to infection or infestation, and by disinfection and immunization. When infection or infestation does take place, treatment may be necessary. Since environmental stress facilitates outbreaks of communicable diseases and hastens their progress, the prerequisite for successful treatment is to remove, or at least reduce, the environmental stress.

Treatment or therapy may be preventive, when drugs are used to remove pathogens and parasites before overt signs of disease are apparent, or curative when disease is in progress. Use of drugs for treatment of

infections before, or after, the signs of disease appear is chemotherapy.

According to Poupard (1978), chemotherapy is applied to fishes in three ways:

1. External treatment.
2. Systemic treatment via the diet.
3. Parenteral treatment.

I add here a fourth mode of treatment, external systemic therapy. This is immersion of fishes in water containing a drug. The drug must penetrate the surface of the body easily to produce a therapeutic concentration in the tissues of the fish. This rather new method of treatment seems to be very effective with some drugs, and also with antigens that produce immunity. This is a very interesting and challenging subject that I discuss in greater detail later.

The literature lists many substances that are used for treatment of diseases in fish and shellfish (Herman 1970, 1972; Hoffman and Meyer 1974; Sindermann 1977; Snieszko 1954, 1978). A substance is considered effective if, during treatment, it harms the agent causing disease without endangering the life or health of the treated animal. This ideal is seldom, if ever, met because the efficacy and safety of drugs are relative quantities. Even such a common chemical as sodium chloride, which is physiologically necessary component may be harmful or even acutely toxic if used improperly. On the other hand its deficiency is deadly to marine fish and shellfish. Therefore, all drugs have a margin of safety, which depends on the type of pathogen or parasite, species and age of the treated animal, and numerous environmental factors. Some chemicals having a wide margin of safety may have a delayed harmful effect--for example, the ability to stimulate the appearance of malignancies.

Selection of drugs or chemicals that are relatively safe and effective is a laborious and expensive process. Certain governmental agencies have the power of licensing and controlling the use of drugs unless they are "generally regarded as safe" (GRAS). In the United States, drugs for control of diseases of fish and shell fish that are used as food are subject to licensing by the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), or by the Department of Agriculture. Most of the available lists of drugs give information on the chemical nature of drugs, their effectiveness, and dosage, without mentioning legal restrictions on use. These regulations are changing and are not internationally uniform.

The National Fishery Research Laboratory of the U.S. Fish and Wildlife Service, located at La Crosse, Wisconsin, and under the direction of Dr. Fred Meyer, is responsible for furnishing information necessary for drug licensing and registration. The most recent status reports were assembled by Meyer et al. (1976) and Schnick et al. (1979). I have prepared a list, to use with lectures, which was published in *Tropical Fish Hobbyist* (1975). The most recently published information is in the "Handbook of Drugs and Chemicals Used in the Treatment of Fish Diseases: by Herwig (1979). Along with other very useful information, it contains a chapter with an alphabetical list of drugs, their chemical nature, dosage, and efficacy.

Earlier I stated that fish and shell fish may be treated by drugs added to water, by oral administration (usually with food), and by injection. I also mentioned that drugs added to water may be absorbed by fish; thus external treatments may have both localized and general systemic effects. The external treatments are the oldest and most frequently applied.

EXTERNAL TREATMENTS

In principle, external treatments are simple: the desired chemical is merely added to water holding diseased fishes. In practice, however, they are more complicated. Even the simplest apparently harmless substance, like table salt, has a narrow margin of safety if used with freshwater fishes in concentrations higher than 1%. The safety margin varies for different species, and depends on the age of fish, temperature of water, and availability of dissolved oxygen. The toxicity and efficacy of other chemicals, such as copper sulfate, formalin, or benzalkonium chloride, vary in waters of different hardness and pH. Thus the success of external treatment depends on many factors that must be considered before treatment is begun. If external treatments are applied to fish in large bodies of water, like ponds, treatment may be very expensive because large quantities of chemicals needed and considerable manual labor is often required to ensure their even distribution.

When water is exchanged frequently, as in hatchery tanks, chemicals can be introduced in proper concentration with the inflowing water, or the flow of water may be stopped entirely. The flow can be stopped only for a short time, however--usually less than 1 h--unless water is aerated to prevent suffocation. Chemicals can be used in relatively high concentration for seconds or minutes if fish are dipped in the solution. This procedure causes considerable stress to fishes and its physiological after effects may last for a day or longer. Consequently most dip treatment of fishes is of questionable value. There is, however, one exception--treatment of fish eggs with disinfectants to reduce the chance of transmitting pathogens from the adult fish to the offspring, or from one hatchery to another. This procedure received a tremendous boost from the introduction of iodophors, which are in wide use as external disinfectants (Bogash 1956). A solution of 1 or 1.5% iodine in polyvinylpyrrolidone, a nontoxic substance that may be used as an expander of blood volume (Shelanski and Shelanski 1956), they were introduced to fish egg disinfection by McFadden (1969). Iodophors are widely used as a 10-minute dip in water containing 100 ppm iodine. This solution is nontoxic to eggs but highly toxic to hatched fish.

A large number of disinfectants, chemicals, dyes, pesticides, etc., were used for control of superficial infections or infestations, of fishes by bacteria, fungi, and animal parasites (protozoan and metazoan). Some of these treatments can be administered practically in large bodies of water without handling the fish. Only a few of these are listed here as examples: salt, formalin, copper sulfate, malachite green, potassium permanganate, Furanace, Masoten, and benzalkonium chloride. Concentrations vary greatly, for example from 3% for sodium chloride to as little as 0.05 ppm for Furanace or Masoten.

EXTERNAL SYSTEMIC

When any chemical is added to water containing fish the entire body of the fish is exposed to it. Chemicals in water may be absorbed by the skin. In fish, skin is in some respects similar to the mucous membrane of terrestrial vertebrates. Chemicals may also be absorbed by the gills, where only one or two layers of cells separate blood from water, or they may be absorbed through the lateral line. This symmetric sensory organ consists of small chambers in a linear arrangement along each side of the fish's body. The chambers open to the outside, and contain capillaries and nerve endings to receive various stimuli transmitted through the water. It is assumed that substances dissolved in water may enter the chambers in the lateral line and be absorbed faster there than through the skin. The osmotic equilibrium between the treated fish and water may be a deciding factor in the penetration of drugs from water. Freshwater fish, living in a hypo-osmotic environment, absorb water but do not drink it; saltwater fish, living in a hyperosmotic environment, lose water by osmosis and must drink water to compensate for this loss (Lagler et al. 1977). Therefore, it is more likely that marine fishes may absorb, through the intestinal tract, drugs dissolved in salt water.

Before studies were made on the absorption of drugs through the surface of fish, it was assumed that drugs dissolved in water would be effective in the control of organisms attached to the surface, and that drugs for control of systemic infections had to be introduced into the blood stream by way of intestinal absorption, or by parenteral injection. This approach changed about 10 years ago when a Japanese pharmaceutical company introduced Furanace (Dainippon Pharmaceutical Co. 1970). This nitrofurantoin drug is rapidly absorbed from water through the surface of fishes and quickly yields chemotherapeutically effective concentrations in the blood and other tissues.

When eels were immersed in water containing 1, 3, and 10 $\mu\text{g}/\text{mL}$ of Furanace for 30 minutes at 23°C the skin, muscles, and kidneys contained more of the drug than did the liver or blood. Also, quantities absorbed were directly proportional to the concentration of Furanace in water. When only heads, bodies but not heads, or the entire eels were immersed in water containing 10 $\mu\text{g}/\text{ml}$ of Furanace for 30 minutes at 26°C the highest concentrations were obtained when the entire eel was immersed. Concentrations were somewhat lower when only the head was immersed, and very low (except in the skin) when the body, but not the head, was immersed. This relation seems to indicate that the lateral line had little, if any, influence on the quantity of Furanace absorbed by eels.

In terrestrial vertebrates, the choice route of introducing nonliving antigen for immunization is by injection. This is also a very effective method in immunization of fish. However, it would be much more convenient, less expensive, and less stressful to fish if antigen could be introduced by another route and still produce sufficient immunity.

Oral vaccination of fish was first tried by Duff (1942). Research on this method continues, but the protection obtained is not yet consistently satisfactory.

Other interesting observations made on the penetration of various substances through the surface of fish were made in connection with research on immunization. To facilitate penetration of antigen through the surface of fishes, including gills and lateral line, investigators subjected fish to intermittently changing pressures, additions of hyperosmotic substances, and spraying. All these supplementary measures were found to be helpful. For example, Fender and Amend (1978) reported that increasing the osmotic pressure in water holding fish, by the addition of sodium chloride, facilitated the entry of bovine serum albumin to the lateral line in rainbow trout. Croy and Amend (1977) demonstrated that hyperosmotic infiltration and a simple immersion in suspension of *Vibrio anguillarum* resulted in the appearance of circulating antibodies in sockeye salmon (*Oncorhynchus nerka*). Anderson et al. (1980, 1979) reported that addition of antigen obtained from *Yersinia ruckeri* to water holding rainbow trout also resulted in immune response. This response was increased by increasing the concentration of the antigen or the ambient temperature.

These remarks on Furanace and immunization of fishes show that Furanace and antigens are absorbed by the surface of fish. Therefore, whenever a chemical, a drug, or antigen is added to water with fish, it may be assumed that the action of such a substance may not be limited only to the surface of fish, but may also become systemic. There are indications that flesh of the fish immersed in water with malachite green contains sufficient quantities of this substance to give it a transient greenish tinge (F. P. Meyer, personal communication). Quantitative data are not yet available. We do not know how many of the substances used for control of external infections also have a systemic action; however, we do know that Furanace and at least some of the antigens have both surface and systemic effects. This finding throws a new light on external treatment and opens the field to further interesting investigations.

INTESTINAL - LOCAL

Another well-established route for administration of drugs to fish is by way of the intestine. Drugs introduced into the intestine may be active only in the lumen, or they may be absorbed and reach blood and other tissues.

The earliest treatments of this type were for intestinal protozoans and helminths. Calomel, a mercurial, and carbarsone, an arsenical, were used successfully for the control of the intestinal flagellate *Hexamita salmonis*, and Kamala was used for tapeworms. These chemicals are no longer in use; they have been replaced by Enheptin and Metromidazole for *Hexamita* and di-n-butyl tin oxide for helminths. No attempts are being made to have these drugs registered for treatment of food fishes because control of these parasites is also possible by management or elimination of the intermediate host.

INTESTINAL - SYSTEMIC

Chemotherapy of fishes by oral administration of drugs for treatment of systemic diseases was introduced immediately after World War II, when sulfonamides and antibiotics became available for veterinary use. Gutsell

(1946) published the first results on selected drugs such as sulfamerazine, sulfathiazole, and Furacin. Of these, sulfamerazine gave the best results for treatment of fish furunculosis and was registered with the FDA in 1967. It is interesting to note that Gutsell used Furacin mixed with food and also dissolved in water. Furacin reduced mortalities when introduced by either route, although the reduction was less than that effected by sulfamerazine. Nevertheless it was the first test of a nitrofurane drug dissolved in water--some 25 years before the Japanese pharmaceutical company introduced Furanace (Dainippon 1970).

For several years nitrofurazone (Furacine, Furoxone) was used for control of furunculosis in trout hatcheries (Post 1962). However, because of its possible tumorigenic activity and the general use of oxytetracycline (Terramycin), it is no longer used in the United States. However, in countries where cultured fishes are important sources of protein for human food, nitrofurane drugs such as Furanace and nifurprazine (Carofur, Aivet) are widely used (Deufel 1970; Shiraki et al. 1970; Tanaka and Shiraki 1970).

Sulfadiazine was established as an effective drug for control of bacterial diseases of Pacific salmonids but is still not registered with the FDA.

Sulfisoxazole (Gantrisin) should be mentioned among other sulfonamides that were tested. The value of sulfisoxazole is particularly evident in the treatment of furunculosis and ulcer disease in brown trout (*Salmo trutta*); growth of the fish is stopped during therapy with sulfamerazine but is not affected by sulfisoxazole (Snieszko and Wood 1955).

Recently, a considerable amount of research has been carried out on the use of sulfonamides potentiated with ormetoprim for control of bacterial fish diseases. These very effective antibacterial drugs will be discussed in detail during the present symposium by Dr. G. L. Bullock.

Most of the research on antibiotics has been devoted to oxytetracycline, chlortetracycline, chloramphenicol, and erythromycin. Of these, only oxytetracycline is registered with FDA. Chloramphenicol is very effective but is not likely to be registered in the United States. Chlortetracycline was found to be bacteriostatic in vitro against some bacterial fish pathogens, but in vivo it had no effect on furunculosis, probably because it was stored in the liver of trout and therefore did not occur in the blood (Wolf and Snieszko 1963). Erythromycin is, thus far, the most effective antibiotic for the control of corynebacterial kidney disease (Wolf and Dunbar 1959).

Chemotherapy of diseases of fishes was reviewed by Snieszko (1954, 1978) and Herman (1970, 1972), who included detailed bibliographies.

INJECTION - PARENTERAL

Treatment of fish diseases by injection of drugs is used infrequently because handling is harmful to fish and should be avoided whenever possible. It is also time consuming and uneconomical. It is justified only when other ways of drug administration are not possible, or when the fish are very valuable--e.g. brood stock or rare ornamental species (Klontz 1978).

In countries where water is near 0°C during the winter, carp eat little if any food. In the spring many brood carp are debilitated because of starvation and production of gonads. Such fish are very susceptible to spring viremia of carp and bacterial infections caused by *Aeromonas hydrophila*. Schäperclaus (1956) investigated in Germany various means of protecting carp from disease and death during this most vulnerable period. Since carp usually do not feed when water is too cold, the only reliable method of treatment was by intraperitoneal injection of drugs. Among the many drugs tested by Schäperclaus, chloramphenicol gave the best results when injected intraperitoneally at a rate of 20 to 50 mg per kilogram of body weight.

When this injection was followed closely by an increase in water temperature, fish produced sufficient immunity during the protection period. However, if water temperature remained low, protection conferred by chloramphenicol was too short for production of immunity. To remedy this shortcoming, Schäperclaus (1970, 1979) injected the fish with a mixture of chloramphenicol and a polyvalent antigen from *A. hydrophila*. This procedure successfully combined long-range protection by immunity with short-range protection by the drug. Similar use of heat-killed suspension of *Vibrio anguillarum* and nitrofurazone for control of vibriosis in salt water culture of salmonids was mentioned by Fryer et al. (1977). Perhaps someone should try the immersion method for simultaneous application of a nitrofurazone and an antigen for short and long-range protection of fish from bacterial diseases.

Chemoprophylaxis and chemotherapy, though valuable tools in control of parasitic and bacterial diseases of fishes, are still not used in control of diseases caused by viruses. There are some indications, however, that disinfection of fish eggs with iodophors may have some effect on vertical transmission of viral diseases.

When fish are cultured in crowded conditions, or if biological filtration is not functioning properly, nitrites may accumulate in water and result in methemoglobinemia. This can be prevented by proper management. In acute cases, however, a rapidly acting (but temporary) remedy for methemoglobinemia may be achieved by intraperitoneal injection of methylene blue at a rate of 10 mg per kilograms of body weight of fish (Bortz 1976).

Anemia and lipid degeneration of the liver, with resulting respiratory insufficiency, may result from toxic diet (Snieszko 1972). This respiratory insufficiency may also be temporarily remedied by adding methylene blue to the diet. Methylene blue is effective for controlling some external parasites on ornamental fishes, and also may serve as an oxygen donor for alleviation of oxygen deficiency. Unfortunately methylene blue is toxic to nitrifying bacteria and should not be used where biological filters can be affected (Collins et al. 1975). Other drugs have similar effects (Collins et al. 1976).

DISEASES OF CULTURED INVERTEBRATES

Various invertebrates are of great significance in aquaculture. Most of these are marine crustaceans such as shrimps, crabs, and lobsters; and molluscs such as oysters and clams. All these animals are subject to

infectious diseases caused by viruses, bacteria, fungi, and protozoan and metazoan parasites (Sindermann 1970, 1974, 1977).

Shrimps and lobsters may be cultured throughout their life-span, or only during their larval stage. Since the disease agents of these invertebrates belong to the same groups as the pathogens of fishes, similar methods of control are applied. No chemicals or drugs are available for control of viral diseases. Bacterial diseases are controlled with the same drugs that are used in fishes. For example, mortalities of shrimps caused by vibrios are reduced by mixing Terramycin with food. Also, the addition of Furazolidone, sulfonamides, and erythromycin to tank water reduces mortalities. Gill filaments infected with *Leucotrix*, or blue-green algae, respond to treatment with salts of copper or potassium permanganate. Fungal infections may be controlled in larvae of shrimps with malachite green added to water at the rate of 1 to 6 parts per billion. For treatment of *Fusarium* in adult shrimps, concentration of malachite green may be increased to 0.05 to 0.1 parts per million.

Trapped lobsters are kept in marine enclosures. Their claws are immobilized with wooden pegs, or rubber bands, to prevent cannibalism. Crowding and handling injury often result in infection with *Aerococcus viridans* (*Gaffkya homari*) which may cause heavy mortalities. Sulfonamides and several antibiotics given with food are effective in reducing these losses; so is sanitation.

Bacterial shell disease of larval lobsters has been successfully treated by dipping the larvae in 20 ppm malachite green for 8 minutes every second day.

Among the molluscs, oysters in their larval stages are cultured on a large scale and fed cultured algae. They are therefore subject to infections with viruses, bacteria, and fungi. Due to the nature of oyster culture, administration of therapeutic drugs is not practical. For detailed information on control of diseases, readers are referred to textbooks (Sindermann 1974, 1977).

GENERAL REMARKS

At this conference, sponsored by the Food and Drug Administration, we are discussing the culture of fish and shellfish and control of their diseases. Both culture and disease control affect the environment. We now realize that human activities have a very significant influence on the balance of nature. Therefore we are also concerned about the effect of aquaculture--particularly the effect of chemicals used in aquaculture and disease control. To assess the significance of this influence, one should examine it in comparison with the effects of nonhuman activities on the balance of nature.

According to the present state of our knowledge, we believe the original atmosphere on earth was free from oxygen and was what we call "anaerobic." As a result of the photosynthetic activity of plants, oxygen was gradually added to the atmosphere and now composes 20% of it. This of course eliminated the living things that existed in the atmosphere without oxygen. To anaerobic life on earth, the introduction of oxygen was a deadly pollution.

Oceans originally contained much lower concentrations of dissolved mineral salts. However, most of the present marine animals cannot survive in fresh water.

We know, from paleontology, that long before humans appeared there were countless species of plants and animals that disappeared and were replaced by succeeding species. We had no hand in destroying them.

We are greatly modifying the aquatic environment by building dams and creating large artificial impoundments. We know what has happened during the past 50 years to the Columbia River and the accompanying profound modification of fish populations. We are desperately trying to save Pacific salmonids, for which conditions are becoming increasingly adverse. However, before humans appeared, beavers built dams everywhere and changed the aquatic environmental conditions to suit their needs. Termites and ants build big cities and even grow crops. We are doing this on a much larger scale, because with intelligence, characteristic for our species, we have learned to use other sources of energy to enormously implement our muscular strength.

We are worried, and justly so, about the disappearance of many species of plants and animals because of our activities. This is unavoidable because we are the most active species on earth. Our species has a tremendous appetite and therefore changes everything around it. This is the way of nature, and nature never stands still.

Fish and shellfish culture with all of its aspects is one of many human activities which affects the balance of nature. It is certainly our responsibility to be concerned with its effect on the environment and on the welfare of humans and of other animals and plants with which we are sharing what we believe to be the garden spot, the living paradise, of our solar system.

This presentation has concerned the control of diseases of fishes and shellfish and the effect of this activity on the rest of nature. This matter is highly complicated, and we are all frustrated by the slow progress that results from the multiple and controversial aspects of this activity.

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FATE AND TRANSPORT OF VIRUSES IN MARINE WATERS¹

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An understanding of the ecology of animal viruses in the marine environment is essential for controlling the spread of these viruses to man and commercially important marine organisms. We have conducted several field studies on the fate and transport of human enteric viruses in marine water along the Texas coast to determine the role of various environmental factors (particularly sediment) in controlling the occurrence and survival of viruses in marine water and shellfish and to determine whether indicator bacteria accurately reflect the presence of viruses in water, sediment and shellfish.

In general, we recovered viruses in greater numbers from sediment than from overlying seawater on a volume basis (Table 1). Several types of human pathogenic enteroviruses were isolated: coxsackieviruses B1, B5 and A16, echovirus 1, and poliovirus 2. The highest concentrations of viruses in water often occurred after periods of heavy rainfall, suggesting that sediment resuspension may play an important role in controlling the amount of virus in the overlying water.

Attempts were made to relate the occurrence of viruses to the biological and physicochemical characteristics of the local environment. Environmental variables studied were pH, salinity, turbidity, rainfall, total and fecal coliforms, viruses, *Clostridium* sp., and *C. perfringens* in water. The same data were also collected for sediments, with the exception of pH, salinity, and turbidity. Statistical analysis of these data yielded only one significant relationship: the number of viruses in sediment was found to be related to the number of fecal coliforms in sediment. No other physical, chemical or biological characteristic of seawater or sediment showed statistically significant association with virus numbers. Core studies analyzing the distribution of fecal coliforms suggested that viruses would probably occur in the upper 10 mm layer of sediment.

To study the effect of sediment on virus survival, seawater alone or a mixture of seawater and sediment was introduced in sterile dialysis tubing or in plexiglass chambers with polycarbonate membrane walls. Virus survival was also studied in sterile, sealed chambers at the field sites. For this purpose, sealed polypropylene centrifuge tubes (Corning Glass Works, Corning, NY) containing sterile-filtered artificial seawater with and without autoclaved sediment were suspended in estuarine water. The results of a typical experiment (Figure 1) show that poliovirus exposed to seawater alone was inactivated in both dialysis tubing and polycarbonate membrane chambers much faster than in the presence of sediment material. In this experiment, the addition of sediment under sterile conditions did not enhance virus survival, but survival in sterile artificial seawater was greater than when virus was suspended in natural (unsterile) seawater. In several other experiments, sediment prolonged virus survival under sterile conditions also. This indicates that there are other factors in

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TABLE 1. Isolation of Enteroviruses from Galveston Bay Water and Sediment

SITE	DATE	PFU/ 20 LITERS*	PFU/ 20 LITERS SEDIMENT†
H-86	11/15/77	6	200
	11/22/77	15	0
	04/12/78	374	0
	04/12/78	528	140
	02/27/78	0	350
	02/27/78	0	280
H-86a	11/15/77	6	200
	11/22/77	5	50
	12/01/77	30	480
T-50	11/15/77	0	170
H-86b	11/22/77	1	0
H-81	11/22/77	8	0
K-27	12/09/77	2	0
	12/21/77	1	0
H-86c	02/27/78	0	50
Oyster bed no. 1	03/03/78	7	0

*Number of viruses isolated per 20 liters of seawater.

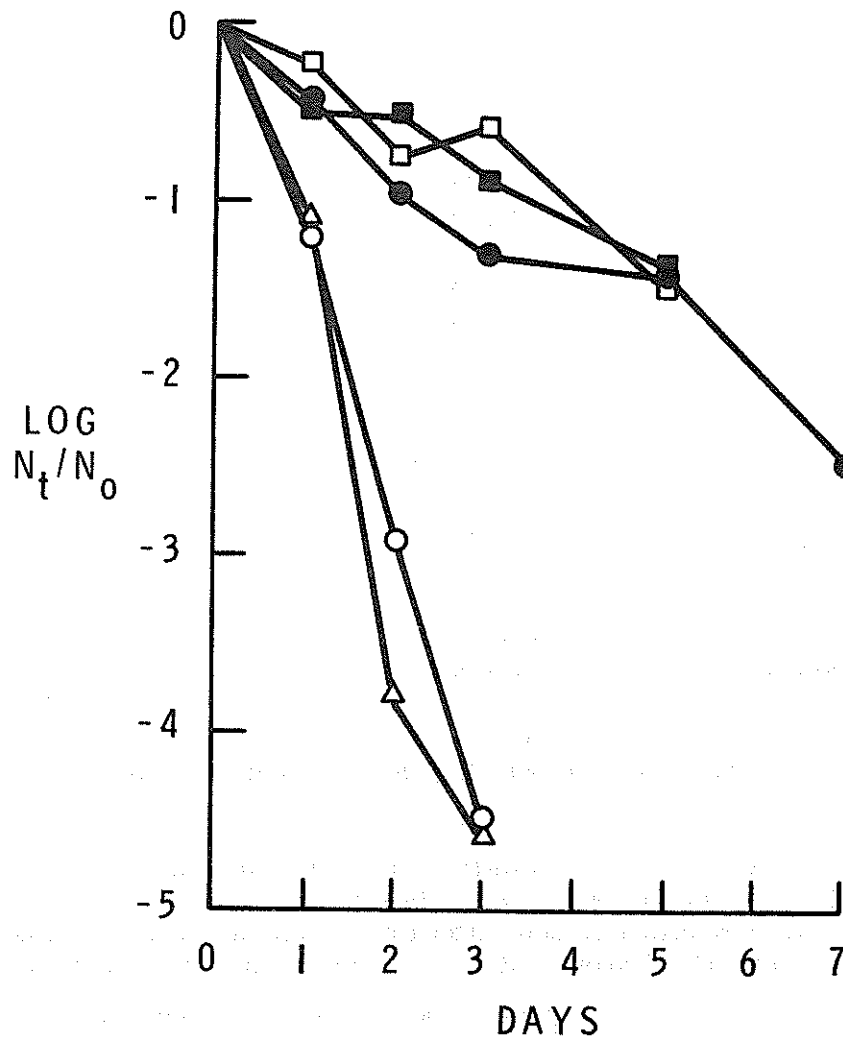
†Number of viruses isolated per 20 liters of sediment material.

addition to temperature and salinity that are involved in virus inactivation in seawater. Thus, sediment protected poliovirus 1 from the inactivating effects of microorganisms, temperature, and filterable materials such as salts and microbial enzymes. An anaerobic environment did not seem to affect virus survival.

To determine whether bacterial indicators in marine water and shellfish and environmental variables could be correlated with the occurrence of viruses, the results of field studies conducted by us in Galveston Bay were evaluated on a statistical basis. The variables were analyzed by multivariate regression. Although multivariate analysis indicated that the number of viruses detected in water was related to rainfall, salinity, and total coliforms in the water, the amount of variation in the number of viruses accounted for by these factors was not large enough to make them good predictors. Enteroviruses were detected 43% of the time in recreational water considered acceptable as judged by coliform standards and 44% of the time when judged by fecal coliform standards. Enteroviruses were detected 35% of the time in water that met acceptable standards for shellfish harvesting. Our failure to correlate the occurrence of enteroviruses in marine water with indicator bacteria, and the frequent occurrence of enteroviruses in water that met current bacteriological standards, indicate that these standards do not reflect the occurrence of enteroviruses, and perhaps other human pathogenic viruses, in marine water.

Laboratory studies with blue crabs have indicated that they also accumulate pathogenic human viruses when exposed to contaminated water. Preliminary results indicate that enteroviruses can survive at least 7 days in living crabs after the crabs are exposed to contaminated water.

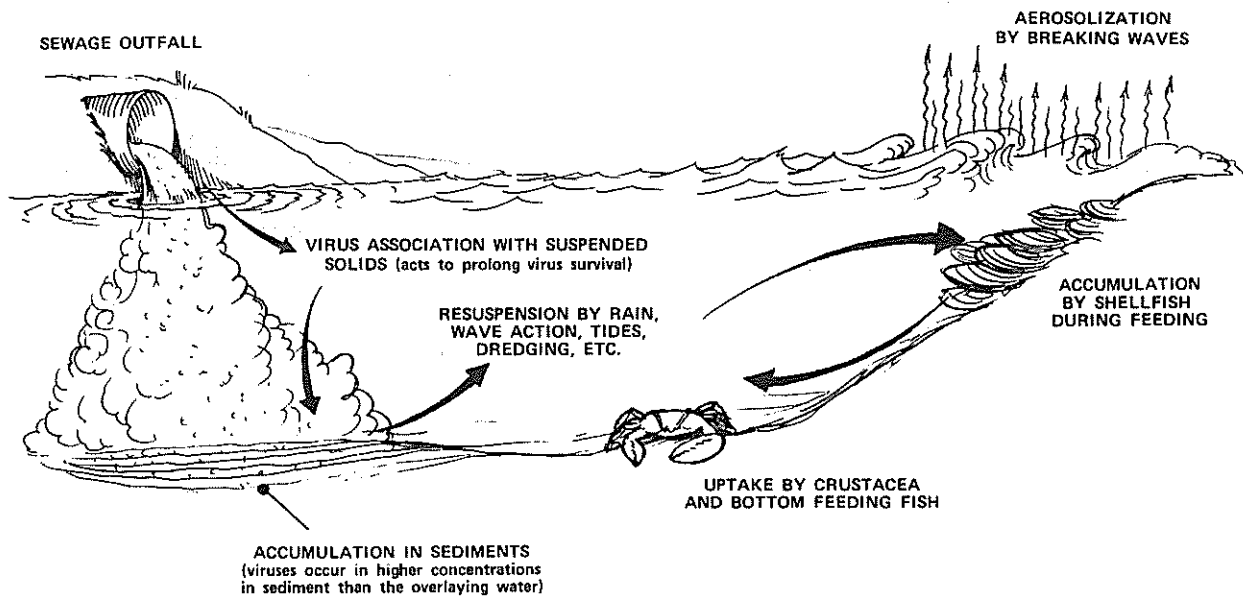
FIGURE 1. Poliovirus 1 (Strain LSc) Survival in Marine Water*



*Virus suspended in seawater in dialysis tubes (O); virus suspended in seawater and sediment in dialysis tubes (●); virus suspended in seawater in a plexiglass chamber with polycarbonate membrane (0.015 μm pore size) wall (Δ); sealed polypropylene centrifuge tube containing sterile artificial seawater (\square); sealed polypropylene centrifuge tube containing sterile artificial seawater and sterile sediment (\blacksquare). $\text{Log } N_t/N_0 = \text{log of (the virus concentration at the time of sampling/virus concentration at 0 hr)}$.

From our studies and those of others, some generalizations on the fate and transport of viruses in the marine environment can be made (Figure 2). Viruses entering marine waters through sewage outfalls or in contaminated river water readily associate with suspended matter, which acts to greatly prolong their survival. Such solid-associated viruses can accumulate in sediment material and not be detected in the overlying water. Rainfall, dredging, and tides can resuspend viruses in the sediment, allowing their uptake by filter-feeding shellfish and crustacea, where their survival can be further prolonged.

FIGURE 2. Fate and Transport of Enteric Viruses in the Marine Environment



PRELIMINARY STUDY ON EFFECTS OF UNREFINED VIBRIO
TOXINS ON SURVIVAL OF AND HEMOLYMPH COAGULATION IN
WHITE SHRIMP, Penaeus setiferus^{1/}

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ABSTRACT

Intramuscular injection of a 0.05-ml aliquot of crude endotoxin (CREND) or exotoxin (CREXT) extracts of Vibrio parahemolyticus or V. alginolyticus induced significant mortalities in juvenile white shrimp (Penaeus setiferus). In normal white shrimp, the hemolymph becomes viscous within 30 seconds and coagulated within one minute after withdrawal from the body. In CREND-treated shrimp, viscosity and coagulation may be delayed up to almost one hour and four hours, respectively, or may not occur at all. This anticoagulation phenomenon is contrary to the intravascular coagulation effect of endotoxin in mammals. It also differs from the in vitro enhancement of gelation of Limulus blood by endotoxin.

INTRODUCTION

Previous experiments indicated that a toxic factor or factors were involved in the death of penaeid shrimp infected with Vibrio bacteria (Leong and Fontaine, 1979). It will be of practical interest to determine the mode of toxigenesis in the shrimp. As a first step to probe into this question, preliminary experiments were conducted to test on juvenile white shrimp, Penaeus setiferus, crude toxic fractions prepared from Vibrio parahemolyticus and V. alginolyticus.

The procedure for obtaining crude toxic fractions was similar to that which was used to prepare crude bacterial endotoxin and exotoxin extracts. For immediate purposes, we did not further refine the extracts. Refinement work was projected to be done in a second phase

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of the research, which will be carried out at another time when circumstances permit. For convenience sake, we shall refer to the crude extracts as crude endotoxin, (CREND) and crude exotoxin (CREXT) in this paper.

MATERIALS AND METHODS

Juvenile white shrimp, 62-138 mm long, were maintained individually in glass aquaria converted from gallon-sized Mason jars (Leon and Fontaine, 1979). The animals were injected intramuscularly, according to a procedure adopted by Lightner and Lewis (1975), between the 4th and 5th abdominal segments with a 0.05-ml aliquot of a crude preparation of either CREND or CREXT obtained from V. parahemolyticus (strain GFC-76-36) isolated from postlarval white shrimp (Penaeus setiferus) in Galveston Laboratory, National Marine Fisheries Service, and identified by Dr. D. H. Lewis, Texas A&M University, College Station, Texas, and V. aliginolyticus (strain MS-670; NMFS culture code GFC-76-5) obtained from Dr. D. H. Lewis, Texas A&M University. These bacteria had been grown for 24 hr in brain-heart infusion medium (BHI).

CREXT was prepared by centrifugation (1,500 g, 10 min) of the bacteria from a BHI culture and passing the supernatant through a disposable Nalgene filter (0.2 micron) (Nalgene Co., New York²) seated in an ice-bath. The filtrate if not immediately used was frozen at -20°C and quickly thawed just before use.

To prepare CREND, the above bacterial pellet was resuspended in a sterile saline solution (2.5% NaCl) to the original volume and centrifuged. The washed cells were again resuspended in a similar saline solution to the original culture volume. The suspension was then steam-autoclaved (121°C, 15 psi, 15 min) to lyse the bacterial cells, to be followed by freezing and storage at -20°C.

Groups of 5 shrimp injected with different dilutions of either CREND or CREXT (Tables 1 and 2) were periodically monitored for mortality, while only shrimp injected with CREND were monitored for changes in hemolymph. Control groups consisted of shrimp receiving or not receiving injections of BHI or saline. To monitor hemolymph changes, a sterile hypodermic needle (gauge 27) fitted to a 1 c.c. tuberculine syringe was inserted dorsally into the pericardial sinus. The withdrawn hemolymph was expelled from the syringe (needle removed) into the cavity of a depression slide and left at room temperature (23°C). Periodic records were made of the viscosity and color of the hemolymph.

²Mention of trade names or commercial products does not constitute endorsement for use.

RESULTS AND DISCUSSION

Both CREND and CREXT from the two Vibrio species were highly toxic to P. setiferus via intramuscular injection (Figs. 1, 2). The undiluted toxin preparations yielded very steep mortality curve within 24 hr, indicating acute intoxication reaction in animals. These results confirmed the toxic effect of the crude extracts on the shrimp.

Vibrio bacteria are common in shrimp rearing tanks and can multiply rapidly in seawater containing a high level of organic nutrients. These bacteria release toxins during their life processes. How the presence of free bacterial toxins in the tank water affect the survival of the shrimp is unknown. There is evidence that shrimp food contaminated with Vibrio bacteria could cause death in penaeid shrimp, presumably due to the effect of bacterial toxins rather than direct invasion by the bacteria (Leong and Fontaine, 1979). In laboratory tests, a species of Pseudomonas (GFC-80-9), which was isolated from seawater used in culturing larvae of penaeid shrimp, Penaeus stylirostris, was observed to be lethal to naupliar and protozoal stages of shrimp larvae (J. K. Leong, unpublished data). Since there was no apparent tissue invasion by the bacteria, it was postulated that the lethal effect of GFC-80-9 was mediated through bacterium-associated toxins. This toxin-mediated-mortality concept was later shown to be valid by laboratory experimental findings (C.L. Huang, personal communication). In controlled experiments, Huang placed endotoxin, chemically extracted from Pseudomonas GFC-80-9, in seawater containing either naupliar or protozoal larvae of P. stylirostris or newly-hatched larvae of brine shrimp, Artemia salina, and found that the intoxicated culture water was lethal to the shrimp larvae. These observations and the fatal effects of CREND and CREXT in shrimp as demonstrated in the present study have implied the vulnerability of penaeid shrimp to bacterial toxins in culture water.

In normal white shrimp, the hemolymph became viscous within 30 seconds and coagulated within one minute after withdrawal from the body (Tables 1-3). In CREND-treated shrimp, viscosity and coagulation could be delayed up to 1 hr and 4 hr, respectively, or might not occur at all. This anticoagulation phenomenon is in contrast with the intravascular coagulation effect of endotoxin, which constitutes the Schwartzman reaction, in man and rabbits (Braude et al., 1973; Jawetz et al., 1972; Smith et al., 1964).

In experiments using live Vibrio bacteria instead of bacterial toxic extract as inoculum, Lightner and Lewis (1975) observed that hemolymph drawn from bacterium-infected moribund shrimp could not clot as rapidly as hemolymph taken from uninoculated shrimp, and that Giemsa-stained hemolymph smears from moribund shrimp contained hemocytes in greatly reduced numbers compared to normal shrimp. These

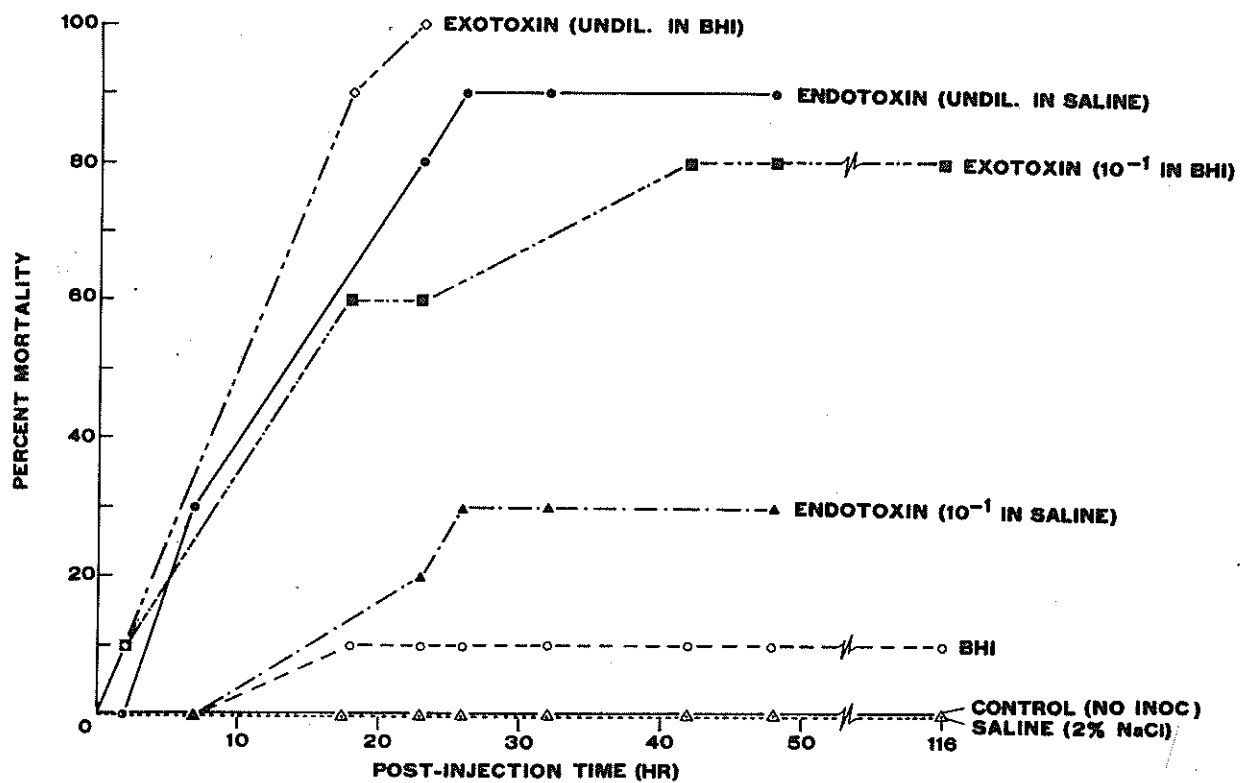


Figure 1. Mortality of Penaeus setiferus injected with crude endotoxin and exotoxin extracts of Vibrio parahemolyticus. Undil., undiluted; BHI, brain-heart infusion broth; Inoc., inoculum.

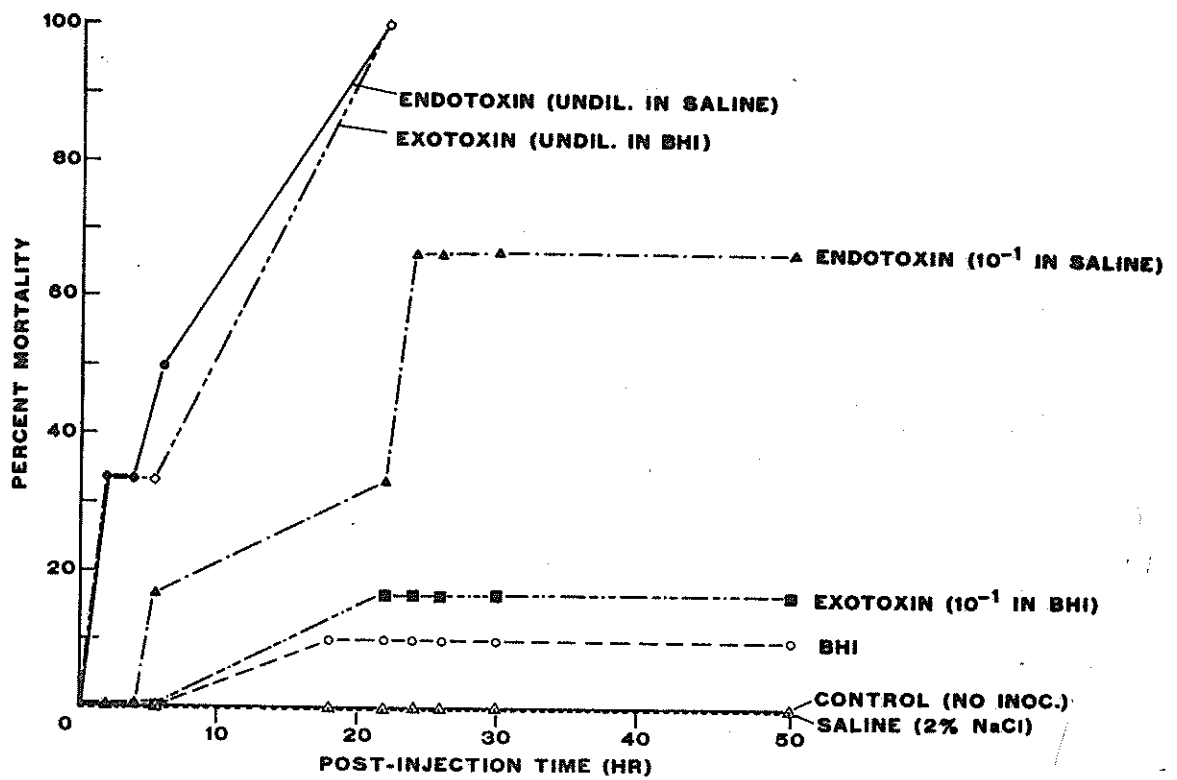


Figure 2. Mortality of Penaeus setiferus injected with crude endotoxin and exotoxin extracts of Vibrio alginolyticus. Undil., undiluted; BHI, brain-heart infusion broth; Inoc., inoculum.

Table 1. Changes in physical conditions of hemolymph withdrawn from white shrimp (*Penaeus setiferus*) one hour after injection of shrimp with unrefined endotoxin from Vibrio alginolyticus.

Inoculum	No. of Shrimp Tested	A		B		C		D	
		Time (min) To Viscous	Time (min) for Hemolymph To Gelatinous	Hemolymph Color	When Drawn	Condition of Shrimp	Shrimp Showing Conditions in Columns A - D	No.	%
Endotoxin (undil.)	5	7	15	LB ^a		Weak	2	40	
		14	17	LB		Weak	1	20	
		7	225	TBC ^b		Weak	1	20	
		NCC	NC(240) ^{d,e}	TBC		Weak	1	20	
Endotoxin (10 ⁻¹ dil.)	5	Inst (vv) ^f	1	LB		Normal	2	40	
		Inst (sv) ^g	8	LB		Normal	3	60	
Saline (2.5% NaCl)	5	Inst (vv)	1	LB		Normal	5	100	
None (Control)	5	Inst (vv)	1	LB		Normal	5	100	

^aLight blue

^bTrace of blue in a colorless background

^cNot changed

^dNot changed after 240 minutes

^eHemolymph remained fluid after further storage for 2 days at 4°C.

^fVery viscous instantly (within 30 seconds)

^gSlightly viscous instantly (within 30 seconds)

Table 2. Changes in physical conditions of hemolymph withdrawn from white shrimp (Penaeus setiferus) 5 hours after injection of shrimp with unrefined endotoxin from Vibrio alginolyticus.

Inoculum	A		B		C		D	
	No. of Shrimp Tested	Time (min) To Viscous	Time (min) To Gelatinous	Hemolymph Color Drawn	Condition of Shrimp	Shrimp Showing Conditions in Columns A - D	No.	%
Endotoxin (undil.)	5	Nca	60	TBC ^b	Very weak	1	20	
		SV (60) ^c	NC (240) ^d	TBC	Weak	1	20	
		NC	NC (240)	LBe	Very weak	2	40	
		(Shrimp died; hemol. not examined)		Dead	1	20		
Endotoxin (10 ⁻¹ dil.)	0	-	-	-	-	-	-	
Saline (2.5% NaCl)	5	Inst (vv) ^f	1	LB	Normal	5	100	
None (Control)	5	Inst (vv)	1	LB	Normal	5	100	

^aNot changed

^bTrace of blue in a colorless background

^cSlightly viscous within 60 minutes

^dNot changed after 240 minutes

^eLight blue

^fVery viscous instantly (within 30 seconds)

Table 3. Changes in physical conditions of hemolymph withdrawn from white shrimp (Penaeus setiferus) 24 hours after injection of shrimp with unrefined endotoxin from Vibrio alginolyticus.

Inoculum	A				B		C		D	
	No. of Shrimp Tested	Time (min) To Viscous	Time (min) for Hemolymph Changes To Gelatinous	Hemolymph Color When Drawn	Condition of Shrimp	Shrimp Showing Conditions in Columns A - D				
Endotoxin (undil.)	0	-	-	-	-	-	-	-	-	-
Endotoxin (10 ⁻¹ dil.)	5	Nca	3	TBCb	Normal	1	20			
		NC	NC (360) ^c	TBC	Normal	1	20			
		NC	NC (360)	TBC	Very weak	1	20			
		(Shrimp died; hemol. not examined)	-	-	-	2	40			
Saline (2.5% NaCl)	5	Inst (vv) ^d	1	LBe	Normal	5	100			
None (Control)	5	Inst (vv)	1	LB	Normal	5	100			

^aNot changed

^bTrace of blue in a colorless background

^cNot changed after 360 minutes

^dVery viscous instantly (within 30 seconds)

^eLight blue

observations coupled with the findings in our study have suggested that morbidity or death of shrimp in Vibrio infection might be due to non-coagulation of shrimp hemolymph, which could have been caused by bacterium-associated toxins such as endotoxin and exotoxin. The reduction in shrimp hemocytes as observed by Lightner and Lewis (1975) could have been caused by either invasion of bacteria or direct effects of bacterial toxins. Unfortunately, attempts to assess quantitatively the possible influence of CREND on destruction of shrimp hemocytes in our study were unsuccessful, primarily due to a great variation of hemocyte counts in different shrimp.

A different kind of response of crustacean blood to bacterial endotoxin had been described by Levin and Bang (1968) in Limulus, the horseshoe crab. Like shrimp blood, the whole blood of Limulus, after withdrawal, would quickly form a cellular clot. This clot contained aggregated amebocytes which later would degenerate and produce a liquid phase designated as pre-gel. The disruption of amebocytes would enhance the production of pre-gel. When pre-gel was exposed to bacterial endotoxin, coagulation occurred, and the rate of coagulation was directly related to the rate of concentration of endotoxin. Cell-free Limulus plasma was incoagulable. Disodium ethylenediamine teraacetate (EDTA) blocked the gelation effect of endotoxin. The sensitivity of the Limulus reaction has been well recognized and employed by health scientists for the detection of endotoxemia in Gram-negative sepsis (Levin et al., 1972; Wildfeuer et al., 1974).

Why CREND induced non-coagulation of shrimp blood instead of coagulation such as what endotoxin did to Limulus blood has not been understood. It could have been a matter of procedural differences in experimentation such as in vivo test in shrimp versus in vitro for Limulus blood. Nevertheless, the anticoagulation characteristic of Vibrio endotoxin in penaeid shrimp could be highly significant. It may facilitate studies on the mechanism of pathogenesis on vibriosis and the mechanism of hemolymph coagulation in shrimp. The underlying cause for hemolymph coagulation in shrimp is not well understood. It is difficult to study and analyze shrimp blood because it gels rapidly after leaving the body.

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ACCUMULATION AND PERSISTENCE OF ENTEROVIRUSES IN BLUE CRABS

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ABSTRACT

Laboratory experiments were performed to measure the uptake of human enteroviruses by blue crabs and thermoinactivation of viruses in crabs during cooking. The data provided information for assessing the health hazard due to viruses in blue crabs grown in contaminated water. The following results were obtained: (i) crabs accumulated virus within 2 h after being placed in contaminated artificial seawater; however, the concentration of virus in the crabs was generally less than in the surrounding water; (ii) the highest concentrations of virus were found in the digestive tract and hemolymph, with much lower concentrations in the meat; (iii) when contaminated crabs were placed in clean water, virus persisted for at least 6 days at 15°C; and (iv) when contaminated crabs were boiled, 99.9% of the virus was inactivated within 8 min.

INTRODUCTION

Human viruses accumulate in oysters, clams and mussels living in contaminated waters. This has been demonstrated by laboratory experiments and by isolation of viruses from shellfish collected from natural habitats. These studies have been reviewed by Gerba and Goyal (5). Since these types of shellfish are often eaten raw or partially cooked, even a very low level of viral contamination can create an important public health hazard. Therefore, viruses must be considered in any aquaculture system that might be contaminated with treated or untreated domestic wastewater,

DiGirolamo and coworkers reported survival of bacteriophage in oysters (2) and in West Coast shore crabs (4) after cooking. Since some virus remained after cooking, it was suggested that seafoods which are normally cooked before eating could still present a health hazard if harvested from contaminated waters. The present study was undertaken to determine the rate of accumulation of enteroviruses in crabs and the persistence of those viruses when contaminated crabs are placed in clean

water. Additionally, the effect of normal cooking procedures on viruses within blue crab muscle tissue was determined.

MATERIALS AND METHODS

Virus and virus assays. Plaque-purified stocks of poliovirus 1 (strain LSc), simian rotavirus SA11, and a natural isolate of echovirus (V239) were used. SA11 served as a model for human rotavirus, which can cause severe diarrhea in children. Echovirus 1 (V239) was tested because previous studies indicated it was relatively resistant to thermoinactivation. The enterovirus stocks were grown and assayed in BGM cells, a continuous monkey kidney cell line. Enterovirus assays were done by the plaque-forming unit (PFU) method as used in this laboratory (6). SA11 was grown and assayed in MA-104 cells, using a plaque assay technique developed in our laboratory (8).

Processing of samples. Samples of muscle and digestive tract were weighed and then homogenized in a 1:7 (wt/vol) amount of 0.05 M glycine containing 0.15 g NaCl per liter at pH 9.5. This homogenate was centrifuged at 1,500 x g for 30 min. The supernatant fluid was withdrawn, treated with antibiotics, and assayed for virus. Hemolymph and seawater were assayed directly.

Accumulation and persistence experiments. Virus was added to the aquarium containing artificial seawater and crabs. Water was constantly circulated, but not filtered, during the course of the accumulation experiment. After appropriate time intervals, individual crabs were removed, dipped in a dilute sodium hypochlorite solution to disinfect the carapace, rinsed in distilled water, and placed in plastic sample bags. Hemolymph was collected from one swimming leg into an equal volume of sodium citrate solution (10 g/l) to prevent clotting. The crabs were stored frozen at -20°C for 2 h. Muscle tissue was obtained from the claws and from the swimming muscles of each crab using care to avoid contamination from the digestive system.

In experiments to measure the persistence of viruses in the crabs, the crabs were contaminated by letting them accumulate viruses from artificial seawater. Water was recirculated and filtered through activated charcoal during these experiments.

RESULTS

The accumulation of poliovirus in the hemolymph, digestive system, and meat of blue crabs was measured in artificial seawater at a temperature of 15°C and a salinity of 10 g/kg. The results are shown in Figure 1. The initial concentration of virus in the water was 3×10^5 PFU/ml. A loss of 99% occurred in the seawater over the 70 h of this experiment.

Virus was found in the hemolymph and digestive tract within 2 min after the addition of virus to the water, and was detected in the meat after 2 h. Virus levels in the crabs rose rapidly in the first 8 h and then leveled off and decreased at a rate similar to that in the surrounding water. The concentration of virus in the crabs was consistently less than in the surrounding water.

ACCUMULATION OF POLIOVIRUS IN BLUE CRABS

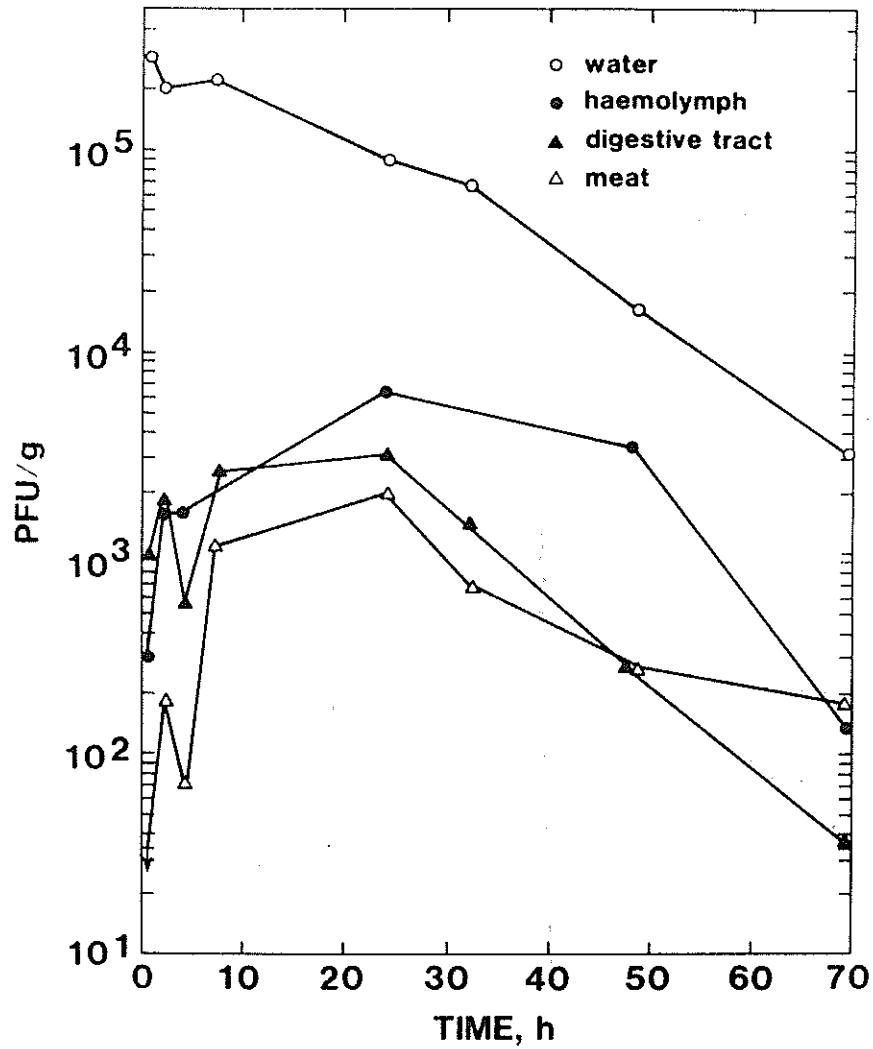


Figure 1. Accumulation of poliovirus in blue crabs. Crabs were held in contaminated artificial seawater at a temperature of 15°C and a salinity of 10 g/kg. Arrow indicates below detection limit.

The highest concentrations of virus were generally found in the hemolymph and digestive tract of the crab. However, after 24 h of accumulation, the concentration in the meat approached, and in some cases, exceeded, the concentrations in the hemolymph and digestive tract. After 72 h there were no substantial differences between the concentrations in the meat, hemolymph, and digestive tract. Also, the ratio of the concentration in the surrounding water to the concentration in the meat decreased from 320 after 2 h to 29 after 72 h of accumulation.

Figure 2 shows the persistence of poliovirus in blue crabs when contaminated crabs were placed in clean water at 15°C and at a salinity of 10 g/kg. Virus was still detectable up to 6 days in the hemolymph and digestive tract and up to 3 days in the meat. The loss of titer was 96% in the digestive tract, 98% in the hemolymph, and 98% in the meat in 6 days. This was slightly less than the loss of titer from seawater at 15°C (99% in 3 days; Figure 1).

Comparison of the ratios of the virus in hemolymph and digestive tract to the virus in the meat showed that there was little difference in the persistence of virus in these three portions of the crab. The ratios were variable, but again there was consistently less virus in the meat than in the hemolymph or digestive tract.

Finally, experiments were performed to determine the persistence of viruses in crabs when they are boiled. The swimming muscle of each crab was injected with 0.1 ml of virus suspension. A thermocouple was inserted into the opposite swimming muscle. Each crab was plunged into 3 l of boiling water and the container was covered loosely. At the appropriate time, the crab was removed and immediately placed on ice. The entire inoculated muscle was removed and assayed for virus as described above.

The three viruses tested all showed at least a 3.3 log₁₀ decrease, or 99.9% inactivation, after 8 min of boiling (Figure 3). Some virus was still detected after 16 min in two cases, but this represented a 4 log₁₀ decrease for echovirus 1 and a 4.3 log₁₀ decrease for the simian rotavirus SA11. The average internal temperature of the crabs was 70°C after 8 min boiling and 94°C after 16 min. No significant difference was detected between different viruses.

DISCUSSION

These data can be used to assess the health hazard due to viruses in crabs grown in contaminated waters.

First, blue crabs did not concentrate virus from the water as do filter-feeding bivalve mollusks. This is probably because of the different feeding mechanisms of crustaceans and mollusks. The amount of virus in crabs came to an equilibrium with the virus in the water at a level below that of the water. Therefore, unlike oysters and clams, crabs would not be expected to concentrate virus from water contaminated with very low levels of virus. They may, however, acquire virus by eating contaminated oysters or other organisms (3). It is also possible that viruses could be concentrated in the gills through which large volumes of water are filtered.

PERSISTENCE OF POLIOVIRUS IN BLUE CRABS

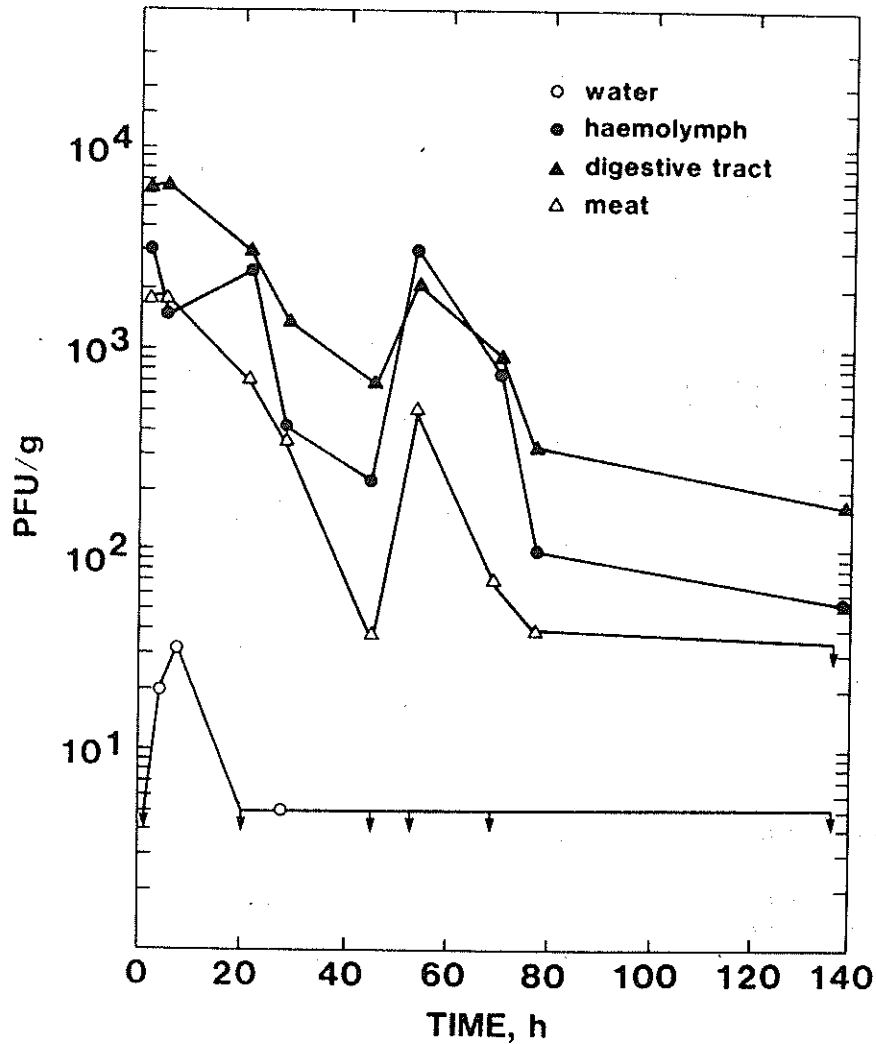


Figure 2. Persistence of poliovirus in blue crabs. Crabs were contaminated by exposure to artificial seawater containing 1.8×10^5 PFU of poliovirus per ml for 4 h. Contaminated crabs were then placed in clean artificial seawater at a temperature of 15°C and a salinity of 10 g/kg. Arrows indicate below detection limit.

THERMOINACTIVATION OF VIRUSES IN BLUE CRABS

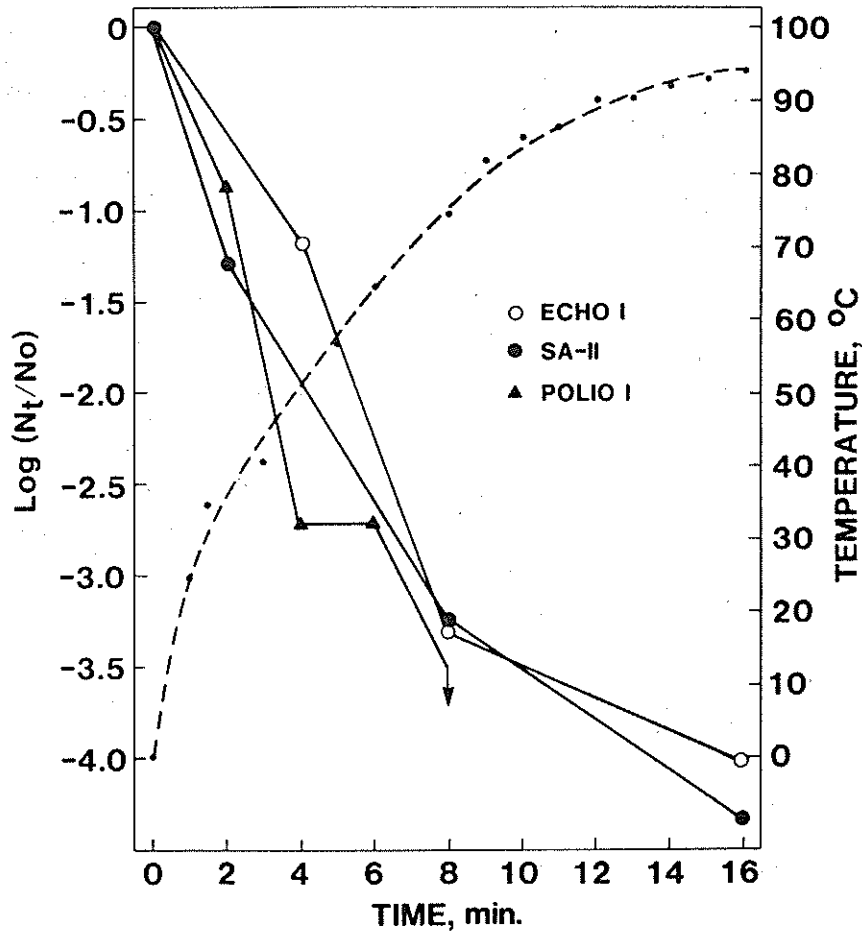


Figure 3. Thermoinactivation of viruses in blue crabs during boiling. One swimming muscle of each crab was inoculated with virus to give an initial concentration of about 1×10^5 PFU/g. The dashed line represents the average internal temperatures of the crabs. Arrow indicates below detection level. $\text{Log } N_t/N_0 = \text{log of the (virus concentration at the time of sampling/virus concentration at 0 hr)}$,

The highest concentrations of virus were found in the digestive tract and hemolymph. The location of the virus within the animal could be important for crabs since generally only the muscle tissue is eaten. Oysters also accumulate most of the virus in the digestive system (7), but since they are eaten whole the location is not as significant.

Virus was lost from crabs placed in clean water at a rate similar to the rates of inactivation reported for seawater at the same temperature (7), and a significant amount was retained for at least 6 days. The crabs did not appear to depurate the virus in the manner that oysters do, although oysters also appear to retain low numbers of virus for several days (1). Once virus was taken up by the crabs, it was not readily eliminated. This indicates that procedures for depurating shellfish taken from contaminated water would not be as useful for crabs.

Finally, the most important consideration of health hazards associated with viruses in crabs is the effect of normal cooking procedures on the viruses. Viruses in crab muscle are rapidly inactivated when the crabs are boiled (Figure 3). Boiling for 8 min inactivated more than 99.9% of the viruses tested. Commercially, crabs are cooked by steam under pressure or by boiling. Boiling for at least 12 min is used to maximize the yield of crab meat. If boiling times much less than this are used, a substantial proportion of the virus may survive. Also, if the water used for cooking is not fully boiling, the internal temperature of the crab will rise more slowly and more virus will survive. The most probable danger from viruses in blue crabs would come to individuals who cook live crabs for an insufficient time (e.g., until they turn red) or cook them in water that is not boiling. Since oysters are often eaten raw or not fully cooked, they have been implicated in the transmission of viral disease (5).

DiGirolamo and coworkers reported survival of up to 13% of the initial virus after frying contaminated oysters for 8 min (2). The calculated values for percent survival do not agree with the reduction in PFU which they reported. The percent survival based on the reported values of 1.2×10^4 PFU/g initially and 1.7×10^1 PFU/g after frying for 8 min is 0.14%. The calculated percentages of survival for stewing, baking, and steaming also differ from the reported reduction in PFU by one or two orders of magnitude. In another study, DiGirolamo *et al.* (4) found that bacteriophage T-4 in West Coast crabs survived boiling for up to 20 min. They reported 2.5% survival, but again this was miscalculated based on the reported PFU/g before and after cooking, which indicates a total survival of 1%. The present study shows that most of the virus in crab muscle can be inactivated by proper processing (i.e., boiling for 12 min or longer). However, it is possible that a small number of virions could survive this treatment if the crab were heavily contaminated.

The differences between crabs and oysters mentioned above suggest that crabs would present a lesser health hazard than oysters from a microbiological viewpoint. Similar evaluations which take into account accumulation, persistence, feeding habits, and the effect of normal processing can be made with other seafood organisms. The relative risks of growing these organisms in and harvesting them from potentially contaminated waters may then be estimated.

ACKNOWLEDGMENT

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POTENTIAL MICROBIOLOGICAL PROBLEMS ASSOCIATED WITH THE USE OF HUMAN AND ANIMAL WASTES AS A FOOD SUPPLEMENT IN AQUACULTURE

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ABSTRACT

Sewage and animal wastes have been shown to enhance the growth of phytoplankton and other marine biota that are food sources for fish and shellfish. Such wastes can contain viruses, bacteria, yeasts and molds and other fungi, mycoplasma, chlamydiae, rickettsiae and parasites. Most methods of sewage treatment reduce the concentration of contaminating organisms, but only a limited number of tertiary treatment processes are suitable for the elimination of microbial pathogens. Such processes are costly and require expensive monitoring systems and controls.

In addition to biological contaminants, numerous chemicals may also be found in the wastes that are detrimental to both marine and human life. Lead, cadmium, mercury, polychlorinated biphenyls and chlorinated organics are commonly found in sewage sludges, and medicinals, herbicides and insecticides are frequently detected in animal wastes. The limited treatment methodology applied to animal wastes does little to reduce the microbial and chemical contaminants, whereas many sewage treatment processes concentrate these contaminants in the sludge.

The use of sludges and animal wastes as a food supplement in aquaculture adds an additional microbial and chemical burden to a system that may already contain disease and pest organisms. The use of complete treatment and monitoring processes designed to eliminate biological and chemical contamination in the wastes is essential in order to safeguard the aquaculture system and the health of the consumer.

INTRODUCTION

Environmentalists have encouraged the recycling of wastes in an attempt to preserve natural resources and to reduce environmental contamination. Some recycling programs have been very effective, and in the future we will probably be required to recycle most wastes in order to preserve limited resources and to reduce energy requirements. For centuries man has disposed of many of his wastes in rivers and streams that quickly carried the diluted materials downstream. The increased population and the growth of large cities compounded waste disposal problems with the result that most of our streams and other bodies of water, as well as the air that we breathe, have been seriously polluted. One of the responses of the U.S. Congress to the pollution problem has been to regulate the disposal of sewage wastes into water-systems. These regulations required that municipalities retain a higher percentage of the solids in sewage wastes and, if possible, return these materials to the land.¹ Because of limited availability of land areas, many municipalities have searched for other sites in which to dispose of these solid wastes. One such disposal area of promise is the util-

ization of the sludge in aquaculture systems in anticipation that the food components present in sludge could be used directly by a number of aquatic animals as well as indirectly by providing essential elements for the growth of algae and detritus-feeding life that are food sources of fish and shellfish. Aquaculture in the United States is still in a preliminary state of development. However, sludges are being used experimentally as a supplemental food source for fish and shellfish aquaculture.²⁻⁴ Potential health problems exist when such wastes are used to produce foods that are directly consumed by humans.

Two agricultural by-products are being investigated as a potential food source for animals. Cattle feedlot and poultry wastes are produced in large quantities in localized geographic areas and have potential for use as an inexpensive animal food as well as for fertilizer on agricultural fields. The volume of such wastes from commercial establishments runs into thousands of tons per year. The limited geographical location of such establishments requires the expenditure of high costs when wastes are transported for use as fertilizers. It has been projected that such costs could be reduced considerably if animal wastes were disposed of in nearby aquaculture systems.

Sewage and agricultural wastes contain unknown quantities of microbial and chemical components that could be detrimental to the aquaculture systems as well as present a health hazard to the consumer. It is important that the use of human and animal wastes be carefully evaluated and, if a decision is made to use such wastes, that decontamination procedures be followed to ensure that the fish or shellfish grown in the aquaculture system will be acceptable to the consumer and regulating officials.

Biological Contaminants in Human and Animal Wastes

Human and animal wastes can contain at any time a wide variety of organisms including bacteria, viruses, parasites, fungi, rickettsia, chlamydiae and other forms of microscopic and macroscopic life. The organisms of importance in public health are those that are pathogenic for the human. In addition, the aquaculturist is interested in organisms that produce an adverse effect on the animals in culture that are detrimental to vegetative and animal life utilized as a food source by the fish or shellfish.

Bacteria

It is estimated that the daily fecal waste of a healthy human contains between 3 and 9 g of bacteria. If an average of 6 g is used in calculations, a city having a population of 100,000 will discharge 580,000 g (about 1276 lbs.) of bacteria per day in sewage. The bacterial titer per gram of feces has been estimated to be about 1×10^{12} . An indication of the variety of bacteria present in human feces is shown in Table 1.⁵ The numbers and types of bacteria present in feces will vary with the individual, his diet and his state of well-being. Fecal organisms of probable greater importance to human health are the salmonellae, shigellae and mycobacteria. These organisms vary in their disease-producing potential and in their ability to survive the sewage treatment process.⁶ Salmonellae have been detected in sludges and are probably the bacterial pathogens most commonly found as a contaminant in sewage wastes. Other organisms of varying pathogenicity are the enteropathogenic forms of Escherichia coli and the members

TABLE 1. Bacterial flora in human feces

Species or group	Occurrence %	Density range per gram
Aerobic bacteria		
Cocci (gram positive)		
<u>Staphylococcus</u>	0-93	< 1-10 ⁵
<u>Streptococcus</u>	0-100	< 1-10 ⁶
<u>Enterococcus</u>	74-76	10 ⁵ -10 ⁶
Bacilli (gram positive)		
<u>Corynebacterium</u>	6-21	-
<u>Mycobacterium</u>	43	5-60
Bacilli (gram negative)		
<u>Coliforms</u>	87-100	10 ⁷ -10 ⁹
<u>Pseudomonas</u>	3-15	10 ³ -10 ⁵
<u>Aeromonas</u>	0.2-0.7	-
<u>Alcaligenes</u>	0-2	-
<u>Proteus</u>	5-53	10 ⁶
<u>Enterobacter</u>	0-98	< 1-10 ⁹
Anaerobic bacteria		
Bacilli (gram positive)		
<u>Bifidobacterium</u>	-	10 ⁸ -10 ⁹
<u>Clostridium</u>	1-35	10 ⁶ -10 ⁷
<u>Lactobacillus</u>	66	10 ⁷ -10 ¹⁰
<u>Actinomyces</u>	50-90	10 ⁷ -10 ¹¹
Bacilli (gram negative)		
<u>Bacterioides</u>	100	10 ⁷ -10 ¹⁰
Spirillum		
<u>Borella</u> and <u>Treponema</u>	18-28	-

of the Pseudomonas and Proteus groups. The majority of the pathogenic bacteria are sensitive to chemical disinfection and to storage conditions usually employed in sewage treatment processes. However, chemical disinfection is usually not practiced in sludge treatment. Therefore, the potential is high that a number of these bacteria may be present in sewage wastes that might be utilized as a supplemental food source in aquaculture.

Viruses

A variety of viruses can be found in human and animal wastes. A list of the animal viruses and some of their characteristics are shown in Table 2. The families are listed according to their density, their nucleic acid content and their content of lipids or envelopes. The last five families

TABLE 2. Some characteristics of animal viruses

FAMILY	DENSITY	NUCLEIC ACID	SURFACE CHARACTERISTICS	
	(Cesium chloride)	(Type-stranded)	(Lipid)	(Envelope)
1. Arenaviridae	1.18 ^a	RNA, SS	+ ^b	+
2. Bunyaviridae	1.20-1.23	RNA, SS	- ^c	+
3. Coronaviridae	1.19-1.23	RNA, SS	-	+
4. Herpetoviridae	1.27-1.29	DNA, DS	+	+
5. Orthomyxoviridae	1.17-1.20	RNA, SS	-	+
6. Paramyxoviridae	1.21-1.24	RNA, SS	+	+
7. Poxviridae		DNA, DS	+	-
8. Retroviridae	1.16-1.18 ^d	RNA, SS	+	+
9. Rhabdoviridae	1.20	RNA, SS	-	+
10. Togaviridae	1.25	RNA, SS	+	+
11. Adenoviridae	1.33-1.35	DNA, DS	-	-
12. Papovaviridae	1.34	DNA, DS	-	-
13. Parvoviridae	1.38-1.46	DNA, SS	-	-
14. Picornaviridae	1.32-1.41	RNA, SS	-	-
15. Reoviridae	1.31-1.38	RNA, DS	-	-

a g/cm³; b + present; c - absent; d in sucrose gradient
Abbreviations: RNA - Ribonucleic acid; DNA - deoxyribonucleic acid;
SS - single stranded; DS - double stranded

contain no lipids or envelopes and are more dense than the other 10 families listed. A review of the literature showed that families 11-15 were the most resistant to physical and chemical agents and are more probably found in sewage wastes.⁷⁻⁹ In Table 3 a more detailed breakdown of the human viruses in these five families is shown. More information is available pertaining to the picornaviruses than other viruses because of the development of cell culture systems that are sensitive for the detection of many of these viruses. The polio, coxsackie and echo viruses are consistently found in human wastes. The polioviruses are found more frequently because of the continuous immunization programs in most areas of the United States. Surveys of viruses in sewage have shown that one type of enterovirus would be consistently found in the wastes for 2-3 weeks. The type of virus detected would then change and the second virus would be detected for several weeks. This variation was due to a change in the predominant infection present in the community. Monitoring sewage in this manner confirms the activity of viruses in infectious disease cycles in a community.

Preliminary data on municipal sewage indicate that rotaviruses may be discharged into the sewage more consistently than the enteroviruses. The human rotaviruses will not replicate in cell culture; however, viral anti-

TABLE 3. Human viruses with high resistance potential to inactivation during the sewage treatment process

1. Adenoviruses Human adenoviruses types 1-33	4. Picornaviruses Polioviruses 1-3 Coxsackievirus A 1-24 Coxsackievirus B 1-6 Echovirus 1-34 Enterovirus 68-71 Probably Hepatitis A
2. Papovaviruses Human BK and JC viruses	
3. Parvoviruses Human gastrointestinal viruses	5. Reoviruses Reovirus 1-3 Rotaviruses

gen produced in the culture system may be detected by immunofluorescent microscopy. Methods are available for detecting hepatitis A virus. However, the concentration of virus antigen present in sewage wastes is not great enough for consistent detection. Once this virus has been propagated in cell culture, it should be possible to determine whether infectious hepatitis A is a consistent contaminant in sewage wastes. Laboratory data are available, indicating that the adenoviruses, picornaviruses and reoviruses may be detected frequently in human wastes.

Animal wastes produced in large volumes that could be used as a food supplement in aquaculture systems are cattle feedlot and poultry wastes. An example of some viruses that could be present in cattle feces is shown in Table 4. Some of these animal viruses are infectious both for cattle and

TABLE 4. Viruses producing infections in cattle^a

Enteroviruses (+?)	BVD mucosal disease
Encephalomyocarditis virus +	Vesicular stomatitis virus +
Arboviruses +	Rabies virus +
Par influenza 3	Malignant catarrhal fever virus
Influenza virus +	3 adenovirus serotypes
Reovirus 1, 2 and 3 +	Bovine syncytial virus
Rotaviruses +	Bovine leukemia virus
Bovine diarrhea virus	

^a + Infectious for humans; +? Infectious for humans probable

humans. It is interesting that the percentage of cattle reported to be infected with rabies was higher than that for dogs in 1979. In some areas of

the world, antibodies to bovine diarrhea virus have been found consistently in the human population.

Similar viruses have also been found to infect poultry, as shown in Table 5. Virus diseases of poultry probably are not responsible for exten-

TABLE 5. Viruses producing infections in avian species^a

Influenza virus (Type A) +	Leukosis complex
Newcastle disease virus +	Infectious bronchitis virus
Enteroviruses	Marek's disease virus
Reovirus (5 species)	Herpes turkey virus
Rotaviruses +?	At least 10 adenovirus serotypes
Arboviruses +	Avian encephalomyelitis viral
Rous sarcoma +, Avian	arthritis agent

^a + Infectious for humans; +? Infectious for humans probable

sive infectious disease in humans. However, arbovirus infections in poultry probably result in the infection of insects which in turn may infect humans. The possible effect of avian viruses on aquatic life is unknown.

Parasites

Until recently little consideration had been given to the potential for parasite contamination in human and animal wastes. During the last 20 years, there has been an expanded foreign travel by U.S. citizens and the number of immigrants entering into the United States from Asian countries has increased. As a result the incidence of parasitic infection in residents of the U.S. has risen considerably. Limited sludge studies have shown that a variety of parasites or their infectious eggs or cysts may be found in sludges from sewage treatment plants. These data are shown in Table 6. In one study of sludges from 17 Army camps, 36% of the samples were found to be positive for at least one type of parasite. In other studies, the incidence of contamination ranged from 0 to 372 parasitic eggs or cysts per 100/g sludge.

Studies of parasitic persistence in various types of sludges showed that viable parasitic forms may survive for years. These data are comparable to studies of parasitic survival in soil where Ascaris eggs were detected in a viable state six years after wastes were disposed of on the soil.¹⁴ Because of the reported persistence of parasites in sludge and soil, the Food and Drug Administration has recommended that food crops that are consumed raw should not be grown in soils fertilized by sewage wastes until at least three years after the last sewage application. Such potentially resistant organisms could be a health problem if sludges contaminated with these organisms are used as supplemental food sources in aquaculture.⁶

TABLE 6. Parasite recovery from sewage sludge

SOURCE	TYPE OF PARASITE	NUMBER	REFER- ENCE
Municipal anaerobically digested sludge	* <u>Ascaris</u> sp.	11-120/100 g	10
	* <u>Toxocara</u> sp.	101-340/100 g	
	* <u>Toxascaris leonina</u>	0-16/100 g	
	* <u>Trichuris</u> sp.	0-23/100 g	
	⊖ <u>Taenia</u> and <u>Hymenlepis</u> sp.	0-33/100 g	
	ψ <u>Eimeria</u> and <u>Isospora</u> sp.	0-372/100 g	
Army Camp sludges (17)	⊖ <u>Hymenolepis</u> , * <u>Ascaris</u> ,	36%	11
	* <u>Trichuris</u> , ψ <u>Entamoeba</u> <u>histolytica</u> , *hookworm	positive	
Municipal, pri- mary treatment	<u>Ascaris</u> and ψ <u>Entamoeba</u> <u>coli</u>	5-27/L (in settled sewage)	12
Municipal wastes	Helminths	700/L	13
		(raw sludge) 200/L	

Helminths - *Nematode (egg) or (larva); ⊖ Cestode (egg). ψ Protozoa (cyst)

Other Biological Contaminants

Mycoplasma, chlamydiae, rickettsiae, fungi, and other forms of microscopic and macroscopic life are present in sludges, feedlot wastes and poultry litter. The extent of such contamination and the potential for human disease from such life forms is unknown. Such organisms may produce disease or may have an inhibiting effect on animals in the aquaculture systems.

Waste Treatment Processes

The usual treatment of animal wastes is some method of composting. Such systems are aerobic and anaerobic digestion in waste piles of varying size for varying time periods. During the process, much of the moisture is leached from the wastes and many of the complex organic compounds are metabolized by the microorganisms decomposing the waste materials. The time allotted for such processes varies with the type and size of farm operation. In some cases where large accumulations occur, removal from the treatment site might result in insufficient time for microbial decomposition to be completed, and wastes in various stages of decomposition would be transported to the aquaculture system.¹⁵

The extent of sewage waste treatment varies with the complexity of the treatment process. Some smaller installations have only limited treatment facilities, whereas larger municipalities have extensive complex systems such as activated sludge processes, chemical coagulation and a number of treatment processes including aerobic and anaerobic digestion, chemical

treatment and drying. Sludges that are used as aquacultural food supplements should be processed so as to eliminate pathogenic forms of life. Most systems in common use do not completely decontaminate sludges.¹⁶

All sludges and animal wastes intended for use in aquaculture systems should undergo treatment processes recommended by the Environmental Protection Agency and the Food and Drug Administration that completely inactivate pathogens in such wastes. Such treatment processes usually occur as a final stage in the tertiary treatment methodology of sewage wastes. When such procedures are utilized, the process must be monitored and be approved by regulatory officials.

Chemical Contaminants

A number of chemical contaminants could be present in sludges and animal wastes. Many sludges have been shown to contain heavy metals including cadmium and lead.¹⁷⁻¹⁹ In addition, the presence of organic contaminants is of concern chiefly because of the lack of information pertaining to the effect of these chemicals on humans. Such compounds could also have a carcinogenic, teratogenic or mutagenic effect on the animals in the aquaculture system. Many wastes have been shown to contain insecticides, herbicides and other persistent compounds such as PCBs and chlorinated hydrocarbons.²⁰⁻²² Many of these chemicals, their by-products, metabolites and oxidation products, and their effect on various forms of life are chiefly unknown. The potential for biological and public health problems requires that serious consideration be given before such recycled materials are added to the aquaculture system.²³

Pathogen Uptake

When wastes are added to an aquaculture unit, microorganisms present in sludges and animal wastes will be dispersed throughout the system.^{24,25} Studies have shown that the microorganisms are ingested by most forms of aquatic animals.²⁶ For example, viruses and bacteria are accumulated by shellfish during the feeding process, and after a short residence time in the animal, most of the organisms are expelled in the feces or pseudofeces. The wastes are consumed by detritis-feeding forms and the microorganisms enter their intestinal digestive tract. Many of these life forms are consumed by larger animal life in the aquaculture system, and the recycling process continues.

The intestinal contents of fish have been shown to be contaminated with varying types of microorganisms including bacterial pathogens and viruses. An interesting study was made on fish in the New York Bight and control fish a short distance from the polluted area. Determinations made from the blood of fish showed that increased antibody production against a variety of microbial contaminants in the surrounding water occurred in the fish in the polluted areas as compared to fish in the nonpolluted areas.²⁷ These data complement an earlier study reporting the detection of antibodies to a number of human bacterial pathogens in the serum of white perch. These fish were harvested from rivers that flow into the Chesapeake Bay.²⁸ Whether fish with high antibody titers had actually been infected by the microbial organisms is not known.

A number of studies have been made on the microbial content of shellfish harvested from approved and nonapproved waters, and pathogens have

been recovered. The bacteria associated with human diseases and shellfish consumption have chiefly been species of salmonellae and Vibrio parahae-
molyticus.^{29,30} Recently shellfish have been incriminated in a cholera
outbreak in the United States.³¹

A number of investigations have been made in an attempt to associate shellfish consumption with viral diseases. Epidemiologically, hepatitis A has been shown to be transmitted by shellfish that were consumed raw.^{32,33} A number of other viruses have been recovered from shellfish as seen in Table 7. Some of these isolations were made from market shellfish. The majority of such shellfish were harvested in countries other than the United States. In addition, viruses have been detected in oysters recovered from waters that were approved by the National Shellfish Sanitation Program. A number of studies have also been made on shellfish recovered from polluted waters and numerous viruses have been detected. It is probable that any viruses present in shellfish waters may be bioaccumulated, and for varying periods of time the shellfish will be contaminated.⁵⁰⁻⁵³ The viruses detected in shellfish have chiefly been those of the enterovirus group.

When shellfish feed, they filter large amounts of water, and contaminating particulates and organisms may be incorporated during the feeding process. However, when limited or no contamination is present in the water, the microbial pathogens will be flushed out during the feeding process and the shellfish will tend to cleanse itself. This system may be duplicated artificially by depuration of shellfish in large tanks of purified water.⁵⁴ Investigators have studied the uptake and depuration of viruses and bacteria and have determined that when low-level contamination occurred in the shellfish, the potential for decontamination by depuration was feasible. However, heavy contamination required much longer periods of time than that usually associated with the depuration process.⁵⁵⁻⁶⁰ The time involved for depuration of different types of shellfish was from 24 hours to more than three weeks. Usually, depuration plants operate on 48-hour cycles.

CONCLUSIONS

1. Sewage and animal wastes often contain microbial pathogens that are detrimental to the aquaculture system and to the consumers of the market product.
2. Chemicals are present in the wastes that may be concentrated by the fish or shellfish being grown in aquaculture. These chemicals may be toxic to the fish or shellfish and/or to the consumer.
3. Treatment processes commonly used to decontaminate sludges, feedlot wastes and poultry litter do not eliminate all pathogens.
4. Treatment methods to further reduce pathogens as recommended by EPA should be used to decontaminate the wastes. These processes must be monitored to ensure that all waste receives the recommended treatment.
5. Careful selection of the waste source and the use of recommended decontamination procedures could result in the production of a waste product that could be used effectively in aquaculture systems.

TABLE 7. Virus isolations from shellfish and fish

SOURCE	TYPE OF SHELLFISH OR FISH	PER CENT OF SAMPLES POSITIVE FOR VIRUS	VIRUS ISOLATES	REFERENCE	
1. Market samples	Mussels		Echovirus 3, 9, 13	34	
	Mussels	20	Coxsackievirus A, A16	35	
	Oysters	10	Coxsackievirus A, A16	35,36	
			Poliovirus 1, 2 and 3		
			Coxsackievirus B2, 3 and 4		
		Oysters	< 1	Echovirus 9	
		Fish fillets	9.8	Poliovirus 1	37
2. Samples from approved waters			Poliovirus 1 and 3 and Coxsackievirus B-4	38	
	Oysters	7	Poliovirus 1, 3	39,40	
	Oysters	20	Poliovirus 1	41	
	Oysters	20	Echovirus 1 and Poliovirus 1	42	
3. Samples from non-approved waters	Oysters	36	Poliovirus 1	41,43,44	
			Coxsackievirus B2, 3 and 4		
			Echovirus 9		
	Oysters		Echovirus 20, 23 and Poliovirus 1	45	
	Fish-bottom feeders		Poliovirus 1, 2 and 3	46	
	Mussels	14	Echovirus 5,6,8,12	47	
			Coxsackievirus A-18		
	Clams	33	Poliovirus 3	48	
			Enteroviruses	49	

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Diseases Acquired by Eating Fish and Shellfish
in the United States, 1975-1979

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ABSTRACT

Fish or shellfish were involved in 182 (9.3%) of the 1,963 foodborne disease outbreaks reported to the Centers for Disease Control between 1975 and 1979. Fifty percent of all fish-related foodborne disease outbreaks were caused by ciguatera fish poisoning; cases occurred most frequently in Hawaii and southeastern Florida, with grouper and amberjack the fish most commonly implicated in outbreaks. The other major fish-associated foodborne disease was scombroid fish poisoning, accounting for 24% of outbreaks. Bacterial pathogens were implicated in 19.6% of all shellfish-associated outbreaks, and paralytic shellfish poisoning in 15.7% of outbreaks; Vibrio species, including Vibrio cholerae and Vibrio parahaemolyticus, were responsible for half of the bacterial outbreaks. In more than half of the shellfish-associated foodborne disease outbreaks it was not possible to determine an exact etiology for the illness. At the present time we do not have a good understanding of many of the diseases associated with eating fish or shellfish, and further research into some of the unique problems which they present should be encouraged.

INTRODUCTION

Fish and shellfish form a small but significant portion of the American diet. There is great potential for growth in the industry, but to fully realize this potential, efforts need to be made to understand and to control those human diseases associated with eating fish and shellfish. Data are presented here on outbreaks of fish- and shellfish-associated foodborne disease reported to the Centers for Disease Control (CDC) in 1975-1979; although the data are limited, they provide some insight into current problem areas, and may help to establish priorities for further research.

MATERIALS AND METHODS

CDC has maintained a national foodborne disease surveillance system since 1967. This system is based primarily on reports from local and state health departments. Reports are submitted on a standard questionnaire. Each questionnaire covers a single outbreak: an outbreak involves one or more cases, with a case understood to mean illness in one person. Data requested for each outbreak includes: number of cases, persons hospitalized, and fatalities; clinical history of ill persons; incubation period and duration of illness; results of epidemiologic investigation, including vehicle

incriminated by epidemiologic evidence; place of preparation of contaminated item; place where eaten; manner in which incriminated food was marketed; factors, such as improper food handling, contributing to the outbreak; and pertinent laboratory data. All questionnaires received are reviewed by the CDC staff, and the preliminary diagnosis confirmed on the basis of established guidelines [1]. Data are presented here on the outbreaks reported to CDC for 1975-1979, as received through January 15, 1980.

RESULTS

In 1975-1979, CDC received reports of 1,963 foodborne disease outbreaks through the national foodborne disease surveillance system. In two-thirds of these outbreaks no specific cause for the reported illness could be confirmed. Twenty-one percent of outbreaks were attributed to bacterial pathogens, 8% to chemical food poisoning, 3% to parasitic organisms, and less than 1% to viral pathogens. *Salmonella* species were the most commonly implicated bacterial pathogens, with staphylococci the next most common.

One hundred thirty-one (6.7%) of the 1,963 foodborne disease outbreaks were associated with eating fish. These outbreaks involved 706 persons, of whom 58 were hospitalized and 2 died. Chemical food poisoning, including scombroid and ciguatera, accounted for 96 outbreaks and 431 cases. No etiology was determined for 22 outbreaks, 10 outbreaks were attributed to bacterial pathogens, and 3 were attributed to parasites (Table 1).

Table 1. Fish-Associated Foodborne Disease Outbreaks Reported to CDC, 1975-1979

Etiology	Out- breaks	Cases	# Hospi- talized	Deaths
Bacterial				
<u>C. botulinum</u>	7	19	19	2
<u>C. perfringens</u>	1	55	0	0
<u>Staphylococcus</u>	2	29	0	0
Viral	0	0	0	0
Parasitic	3	6	0	0
Chemical				
ciguatera	65	237	21	0
scombroid	31	194	7	0
Unknown	<u>22</u>	<u>166</u>	<u>11</u>	<u>0</u>
Totals	131	706	58	2

The single most common cause of outbreaks of fish-related foodborne disease was ciguatera fish poisoning, with 65 outbreaks and 237 cases reported. Twenty-one persons (9%) were hospitalized; there were no reported deaths associated with the outbreaks. The outbreaks were all reported from Hawaii (65%), California (3%), and Florida (32%); all of the latter occurred in the Dade County area. Cases from Hawaii were reported during all months of

the year, whereas in Florida the number of outbreaks peaked in May, with no outbreaks reported between July and December.

At least 11 different types of fish were involved in the ciguatera outbreaks reported. Grouper were implicated in 13 outbreaks (20%), amberjack in 12 outbreaks (18.5%), jack (ulua) in 9 outbreaks (13.8%), and snapper in 6 outbreaks (9.2%). Grouper were most commonly implicated in the Florida outbreaks, and amberjack and jack (ulua) were most often reported from Hawaii. It was not possible to determine from our reports whether the fish involved in the outbreaks were commercially or home caught, although it was reported that 47 of the 65 outbreaks (72%) involved fish prepared at home, and 6 (9%) involved fish prepared in commercial establishments. Place of preparation was not recorded for 12 outbreaks (19%).

The other common etiology for fish-associated foodborne disease was scombroid fish poisoning, with 31 outbreaks involving 194 persons reported between 1975 and 1979. Seven persons were hospitalized (3.6%); no deaths were reported. Outbreaks were reported from 11 different states, with Hawaii, Washington, and California accounting for 20 (65%). No seasonal incidence was apparent from the outbreak reports. Mahi-mahi or dolphin (a non-Scombroid fish) was the fish most commonly implicated in outbreaks. Tuna were implicated in 10 outbreaks (32.3%), while bluefish were involved in 3 outbreaks (9.7%).

When efforts were made to determine the factors contributing to the outbreak, it was found that the fish had been mishandled either immediately after being caught or during commercial processing or distribution in 18 outbreaks (58%). This includes 10 outbreaks in which the fish were known to have been imported into the United States: 1 outbreak involved jack (ulua) steaks from New Zealand; 3 were associated with mahi-mahi from Ecuador; and 6, all occurring in 1978, were related to mahi-mahi from Taiwan. In 7 outbreaks (22.6%) the fish had probably been mishandled during the retail process or while being prepared. No data on source of fish or possible errors in handling were available for the remaining 6 outbreaks.

Bacterial pathogens were identified in very few fish-associated outbreaks. The most frequently reported bacterial pathogen was Clostridium botulinum, accounting for 7 of the 10 outbreaks and all of the reported deaths from fish-associated foodborne diseases. Salmonella, the most commonly reported bacterial foodborne pathogen in the United States, was not identified as a pathogen in any fish-associated outbreak.

Fifty-one (2.6%) of the 1,963 foodborne disease outbreaks occurring in 1975-1979 were associated with eating shellfish. Twenty-nine (57%) of these outbreaks were of unknown etiology, while 10 (19.6%) involved bacterial pathogens, 10 (19.6%) chemical food poisoning, including paralytic shellfish poisoning, and 2 (3.9%) involved viruses (Table 2).

The 10 outbreaks associated with bacterial pathogens involved 2,026 cases. Vibrio parahaemolyticus was identified as the pathogen in 2 outbreaks associated with eating boiled shrimp. Both outbreaks were reported from Louisiana, one in 1975 involving 100 cases and the other in 1978 involving approximately 1,200 cases. Two outbreaks of non-O1 Vibrio cholerae

Table 2

Shellfish-Associated Foodborne Disease Outbreaks
Reported to CDC, 1975-1979

Etiology	Out- breaks	Cases	# Hospi- talized	Deaths
Bacterial				
<u>C. perfringens</u>	1	500	0	0
<u>Staphylococcus</u>	4	209	3	0
<u>Vibrio cholerae</u>				
01	1	11	8	0
non-01	2	6	4	0
<u>Vibrio parahaemolyticus</u>	2	1,300	20	0
Viral	2	27	8	0
Parasitic	0	0	0	0
Chemical				
Paralytic shellfish poisoning	8	21	11	0
Neurotrophic shellfish poison.	0	0	0	0
Monosodium glutamate	2	5	0	0
Unknown	<u>29</u>	<u>383</u>	<u>12</u>	<u>0</u>
Totals	51	2,462	66	0

involving 6 cases were reported in 1979; both were associated with eating raw oysters from Florida. One outbreak of Vibrio cholerae 01 associated with Louisiana crabs was reported in 1978.

Eight outbreaks of paralytic shellfish poisoning (PSP), involving 21 persons, were reported between 1975 and 1979. Eleven of the 21 persons required hospitalization. These outbreaks occurred in 2 clusters, 1 in Alaska in May, June, and July, 1976, and the other in Washington in September and October, 1978. The Alaska outbreaks involved clams harvested in the southeastern portion of the state; PSP toxin levels ranged from 1,400-6,000 micrograms/100g of meat. Mussels and pectins were implicated in the Washington outbreaks, and PSP toxin levels ranged from 1,400-30,000 micrograms/100g of meat. No neurotoxic shellfish poisoning outbreaks were reported to CDC during the 1975-79 period. Two outbreaks of monosodium glutamate toxicity were reported, both associated with overseasoning of scallop soup.

Two shellfish-associated hepatitis outbreaks were reported between 1975 and 1979. One was related to eating tiger shrimp, but the source of contamination was never determined. The other outbreak was associated with eating raw oysters in Georgia and Alabama, with the oysters implicated traced to commercial growing areas in Florida.

DISCUSSION

The number of outbreaks of fish- and shellfish-associated disease reported to CDC almost certainly represents only a small percentage of the

actual total. Mild cases of foodborne disease may not be reported to a physician, or even when reported may be attributed to "a virus." Even when illness is identified as due to foodborne pathogens, reports may not reach CDC; reporting for most diseases is voluntary, and thus depends to a large extent on physician and public health department time and interest. Because of these factors, the system tends to be skewed toward more severe or exotic diseases, such as botulism, while there is almost certainly substantial under-reporting of diseases characterized by mild, rather nonspecific gastrointestinal symptoms, such as seen with some of the bacterial pathogens. Despite these limitations, the system is valuable in identifying major problem areas and in helping to establish priorities for further investigations.

Ciguatera was the most common of the fish- and shellfish-associated foodborne diseases reported to CDC between 1975 and 1979. Described as early as 1555 and well-recognized by Spanish explorers in the West Indies [2], it is a major problem in areas where reef fish form a substantial portion of the diet. It is reported to be "common" in the Caribbean [3], although no valid estimates of incidence exist. Bagnis recently reported on 3,009 cases in the South Pacific, and commented that the number of cases appeared to be increasing [4]; no incidence figures were given in the study. In the United States valid incidence figures are again lacking; Lawrence has estimated the incidence in the Miami area at 5 cases per 10,000 population per year [5].

Research in the South Pacific has identified the dinoflagellate Gambierdiscus toxicus as the probable source of ciguatoxin [6]. G. toxicus is said to be present in most reef ecosystems, proliferating massively after such occurrences as large storms, human intervention on the reef, or pollution. Studies have shown larger fish to be more toxic [7], probably due to a concentration of toxin as it is passed up the food chain. The CDC data are not complete enough to allow correlation of cases with known natural disasters in reef areas or with fish size.

Geographic distribution of cases in the United States probably reflects the pattern of marketing of tropical reef fish, with most outbreaks reported from southeastern Florida or Hawaii. Studies in the Pacific do not show seasonal fluctuations in the number of cases of ciguatera fish poisoning, in agreement with the data presented here showing little or no seasonal variation of cases in the Hawaiian Islands. In contrast, ciguatera fish poisoning incidence appears to peak in Florida in the late spring and early summer. Because fish retain toxin for years [2], this observed variation probably cannot be attributed to seasonal change in the dinoflagellate population, and may simply reflect local fishing practices.

Public health regulations designed to prevent ciguatera fish poisoning have usually been based on prohibitions against marketing species that are frequently toxic. However, there are no national guidelines for ciguatera regulation, and local laws vary widely. The recently developed radioimmunoassay (RIA) technique gives promise of allowing rapid detection of toxin in individual fish [8], and has been used in Hawaii to screen amberjack sold in commercial fish markets in Oahu. Ideally, however, there should be adequate understanding of the ecology and epidemiology of the disease to

allow accurate predictions of which fish and which areas are toxic, with a regulatory system based on this knowledge; such an understanding will require expansion of current research efforts, both in epidemiology and in marine biology.

In contrast to ciguatera fish poisoning, which is caused by a toxin acquired while the fish is alive, scombroid fish poisoning results from toxic products produced by bacterial action on fish after they are caught [2,9]. Scombroid fish poisoning can be prevented simply by proper handling of fish; the high percentage of cases attributed to mishandling of fish immediately after they are caught or during commercial processing and distribution emphasize the importance of good handling techniques within the industry itself.

Bacterial pathogens, particularly Vibrio species, were implicated in a high proportion of shellfish-associated foodborne disease outbreaks. Vibrio parahaemolyticus, which was associated with more cases than any other single pathogen, is commonly isolated from seafood and seawater. Human gastrointestinal disease, however, is only associated with Kanagawa-positive strains, which constitute approximately 1% of marine isolates [10]. Adequate cooking should kill the bacteria, and the major 1978 Louisiana outbreak probably occurred because of mishandling of precooked shrimp; investigators did demonstrate, however, that V. parahaemolyticus could be cultured from shrimp that had been boiled for as long as 6 minutes [11].

Non-O1 V. cholerae have also been reported to be a relatively common environmental isolate, particularly in brackish waters of bays and estuaries [12]. Unfortunately, we do not yet have a method to identify strains potentially pathogenic for humans. Oysters implicated in the 1979 foodborne disease outbreaks involving non-O1 V. cholerae came from areas with high fecal coliform counts [13], suggesting that pathogenic strains may be sewage-associated. This contrasts with studies of environmental isolates, which show no relationship between occurrence of the organism and fecal coliform levels [12]. The 1978 cholera outbreak in Louisiana also suggested the presence of an endemic focus of V. cholerae O1 along the Louisiana coast. In that outbreak the organism was isolated from crabs, and from shrimp caught in the area [14]. Again, adequate cooking of seafood should kill V. cholerae, although the organism was isolated from crabs steamed as long as 25 minutes. Given the relatively common practice of eating raw or undercooked seafood, Vibrio species represent a potentially serious problem for the seafood industry.

Only 1 outbreak of viral hepatitis in the 1975-1979 period could be directly attributed to contaminated shellfish. Oysters implicated in the outbreak were from commercial areas and had been harvested when fecal coliform counts in the water were high [15,16]. In this instance the increase in fecal coliforms appeared to correlate with an increased risk of hepatitis, and the outbreak might have been prevented had current shellfish sanitation regulations for closing areas at such times been strictly observed. It again raised the question, however, of the accuracy of fecal coliform counts in predicting the relative risk of hepatitis. In 1973, oysters thought to have caused the major Houston outbreak came from areas with acceptable fecal coliform counts [17].

The major fish- and shellfish-associated foodborne diseases such as ciguatera fish poisoning, Vibrio-associated gastroenteritis, or hepatitis present a rather unique picture. Unlike outbreaks caused by staphylococci or Salmonella, which can usually be attributed to improper handling of food at the time of preparation, these diseases are primarily amenable to control prior to or at the time of harvesting. As such, their ultimate control will rest with the industry itself; there is a need for both industry and government to encourage further research so that appropriate control measures can be developed.

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1,3,5-Trichloro-2-(4-nitrophenoxy)benzene (CNP)
in Fish, Shellfish, and Seawater in Tokyo Bay, 1977-1978

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ABSTRACT

Concentrations of 1,3,5-trichloro-2-(4-nitrophenoxy)benzene (CNP) were measured in a total of 122 samples consisting of 36 fish (goby-fish and sea bass), 61 shellfish (short-necked clam) and 25 seawater samples collected at 5-12 sampling stations of coastal waters of the Tokyo Bay. The concentrations of CNP found in goby-fish (on the wet basis) were in the range of 2.6 to 91,600 ppb in liver, 0.1 to 360 ppb in muscle, trace to 2,900 ppb in short-necked clam (on the whole body of shucked samples), and not detectable to 0.8 ppb in seawater, respectively. Although the concentrations in these samples varied with the time and place of the sampling, the maximum levels were found on the samples obtained at all the sampling stations in May or June, both years 1977 and 1978. In this season in every year, CNP or other herbicides had been intensively applied in agricultural land of a rice paddy field in Japan.

INTRODUCTION

In the previous paper, we reported that residues of the herbicide, CNP (1,3,5-trichloro-2-(4-nitrophenoxy)benzene) had been found in certain fish and shellfish in the Tokyo Bay (14, 15). CNP is one of the herbicides used in a large quantity (annual production, 5,000 tons) for controlling various species of weeds in a rice paddy field in Japan (16). The acute toxicity of CNP to mammals (8) and fish (9) is lower than formerly used herbicides, such as 2,4-D (2,4-dichlorophenoxyacetic acid) and pentachlorophenol, which are now banned in Japan. However, it has been demonstrated that CNP is relatively stable (3), and highly bioaccumulated by freshwater fish (4) under the model ecosystem conditions. In field environment, although the residues had been detected in soil (11-13) and river water (11), no data had been reported on the concentration of environmental biota, and there were very few informations on the contaminations in the Tokyo Bay ecosystem.

An attempt was made to investigate fish and shellfish as an indicator of the marine environmental contamination by CNP. The purpose of the study is to find any differences in annual and regional bioaccumulation and correlations between the concentrations and organisms.

MATERIALS AND METHODS

A. Samples

A total of 122 samples containing of fish, shellfish and seawater were collected May and September, 1977-1978 from sampling stations along the coast of the Tokyo Bay (Figure 1). Thirty-three goby-fish samples (Acanthogobius flavimanus) were caught by fishing at 12 stations once every year. Three sea bass samples (Lateolabrax japonicus) were caught by net only in September, 1977 at the place of 5 km off A-6 station. Sixty-one short-necked clam samples (Tapes philippinarum) were collected at five stations once every month. Three each samples of freshwater fish (Zacco platypus) were collected and pooled at five each sampling sites along both of the Tone and Tama Rivers.

All fish and shellfish samples were immediately wrapped in hexane-washed aluminum foil and stored in a frozen state until analysis. Twenty-five seawater samples were collected only in 1978 at the same times and places at which the shellfish were taken. The seawater samples were stored at 4 °C.

B. CNP Determination

Individual goby-fish were dissected from the melt, and separated into liver and muscle. The tissue portions were pooled at the same sampling sites and times. The pooled samples were homogenized in a mixer. The shellfish samples were thawed and shucked. The whole meat was pooled and homogenized as in the case of fish samples. The homogenized samples were subjected to CNP determination according to the method described in the previous report (15) : sample extracts were cleaned up by the procedures of saponification and Florisil column chromatography for the separation of CNP from fat and p,p'- and o,p'-DDT (1,1-(2,2,2-trichloroethylidene)-bis[4-chlorobenzene] and 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)-ethyl]benzene) and PCB (polychlorinated biphenyls). The cleaned up solution was analyzed using gas chromatograph (Shimadzu 5AP₃FE) equipped with a ⁶³Ni electron capture detector. Columns used were 2% OV-1 (Gaschrom Q, 60-100 mesh) and 2% OV-17 (Chromosorb W, AW DMCS, 60-80 mesh).

RESULTS AND DISCUSSION

A. Annual Differences and Regional Differences

Results of the analyses for clarifying the annual differences and seasonal variations of CNP concentration were summarized in Table 1: a total of 99 samples consisting of 24 goby-fish, 50 short-necked clam and 25 seawater samples were collected from 5-12 sampling stations monthly from May to September in 1977-1978. The differences in the residual levels in both fish and shellfish samples were observed between 1977 and 1978 ($p < 0.01$): these levels in shellfish and fish (muscle and liver) in 1977 were much higher than those in 1978. The regional differences were found in both samples of fish and shellfish: the samples from B-1 station in 1977 were observed to have high levels during the period of May to September, compared with those of other stations. In 1978, the levels in fish and shellfish sampled at the same time

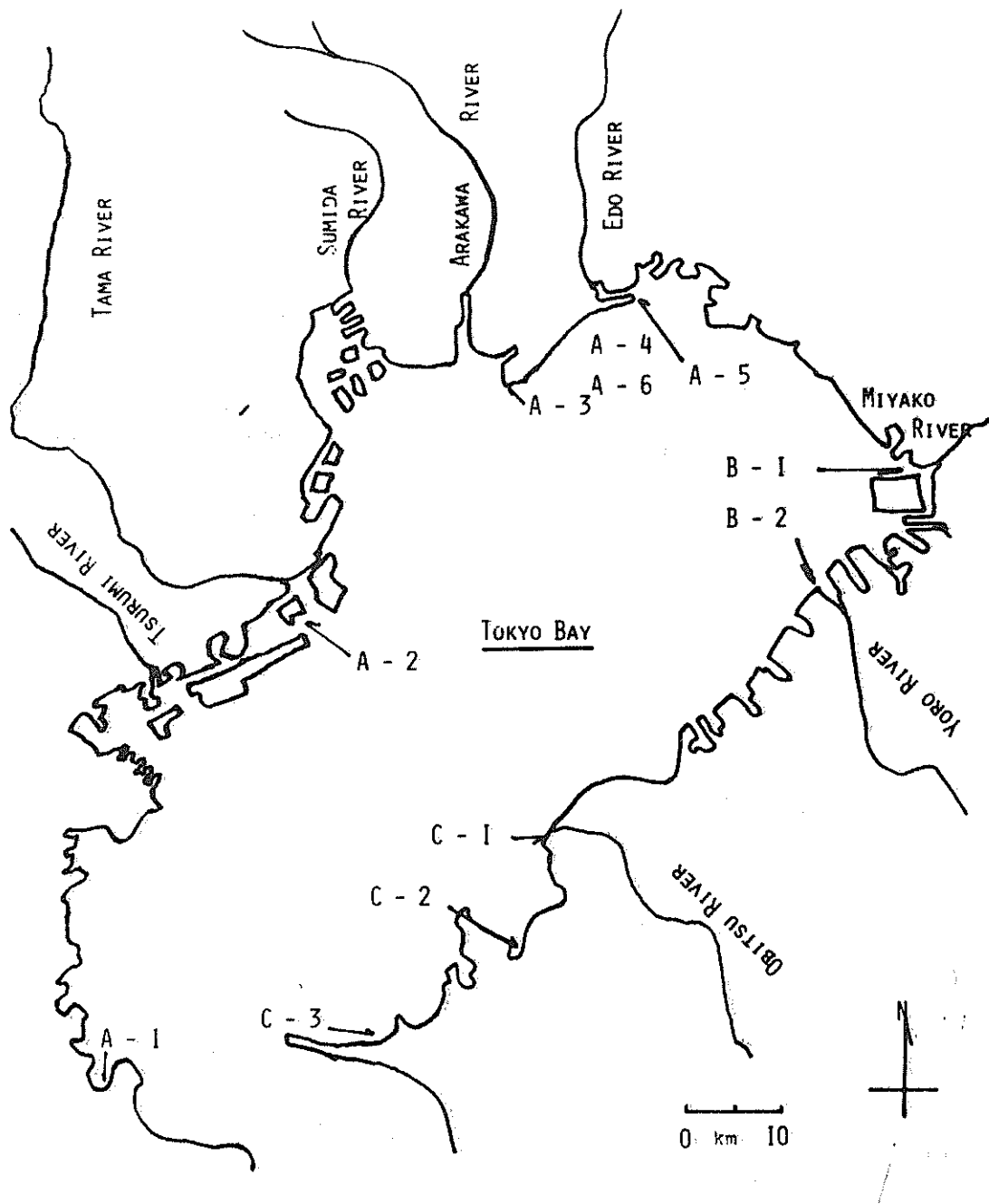


Fig. 1. Locations of sample collections

Table 1. CNP concentrations (ppb) in seawater, short-necked clam, and goby-fish from different sampling stations in Tokyo Bay

Sampling date	A-1	A-2	A-3	A-4	A-5	A-6	B-1	B-2	B-3	C-1	C-2	C-3
1978												
Seawater		0.020				1.90	0.040				0.080	0.040
		0.002				0.004	0.001				0.002	0.009
		0.002				0.001	0.002				0.001	0.001
		tr				tr	0.001				0.001	tr
		tr				tr	tr				nd	nd
1977												
Short-necked clam a		43				44	2,900				23	20
		91				31	2,100				18	4
		21				39	890				16	4
		3				9	240				4	5
		2				1	100				2	4
1978												
		4				24	18				15	4
		tr				2	tr				tr	tr
		2				tr	nd				1	tr
		1				tr	nd				1	tr
		1				1	nd				tr	tr
1977												
Goby-fish ^b												
Liver	670	950	6,880	2,920	408	59	91,400	80,000	4,280	1,080	814	809
Muscle	4	7	7	8	4	1	360	17	30	6	10	3
Liver	8	4	7	3	4	18	12	22	14	13	63	15
Muscle	0.2	0.1	0.1	0.3	0.2	0.3	0.1	0.2	0.1	1.3	0.3	0.2

nd: not detectable. tr: trace. a: Tapes philippinarum. b: Acanthogobius flavimanus.

and place were, however, approximately the same or less than those of other sampling sites. Data of the annual variation in the present study may indicate that there exist probably a quantitative relation between the residual levels in the marine biota and the consumed quantities of the herbicides, if the marine environmental pollution with CNP in the Bay is due to river water, which are discharged into the Bay. Since 1979, only small amounts of CNP were detectable in both biota, fish and shellfish, and the results are consistent with the treatment of some administrative warning and regulations which had been effectively taken by the local government on the use of CNP in a rice paddy field and for unsuited purposes. Although the cause of the regional differences is not clear, the distances from the spraying place of the herbicide may have potent influence on the residual levels in the marine biota.

B. Seasonal Change

The changes of the CNP levels in shellfish were similar in both years. In 1977, the levels gradually decreased from the maximum levels (in May or June) to 1/15 of them during the four months. As shown in Figure 2, periodical observations of the levels in shellfish at a fixed point demonstrated a linear relation between the logarithmic concentrations and the elapse of times after thirty days. The average half-life of the CNP residues in these shellfish was found to be 23.8 ± 8.3 days, and in each sampling station, the half-life was 10.0 days at A-2, 33.8 days at A-6, 23.5 days at B-1, and 27.1 days at C-1, respectively. In 1978, the variation of the CNP residues in shellfish hardly coincided with those in seawater.

It is well known that under the model experiment, this substance is one of the least persistent herbicides (6), such as atrazine (7), and these compounds generally persist for a few weeks. Present data indicate that the CNP accumulated by shellfish has a short existing period in body compared with that of DDT, and that CNP in seawater also is far less persistent. Such a rapid disappearance of CNP from marine system may involve microbial and chemical degradation as well as deposition and absorption. Although seawater and shellfish were thus polluted temporarily with CNP only a limited period, the CNP concentration in seawater influenced the levels in organism (Figure 3). Namely, CNP levels in shellfish were highest in May. This period of the high concentrations coincided with the herbicide spraying season (April to June) in agricultural areas.

Suzuki et al. (10) reported that the concentrations of CNP in river water, due to the runoff of the herbicide from rice paddy fields, had reached the maximum levels after one month from the start of the herbicide application, and that the levels had ranged from 0.009 to 16.7 ppb during June and September in North Kyushu, Japan. Ishikawa et al. (2) recently reported that the residual levels of CNP in zoobenthos (cord shell, Corbicula japonica)

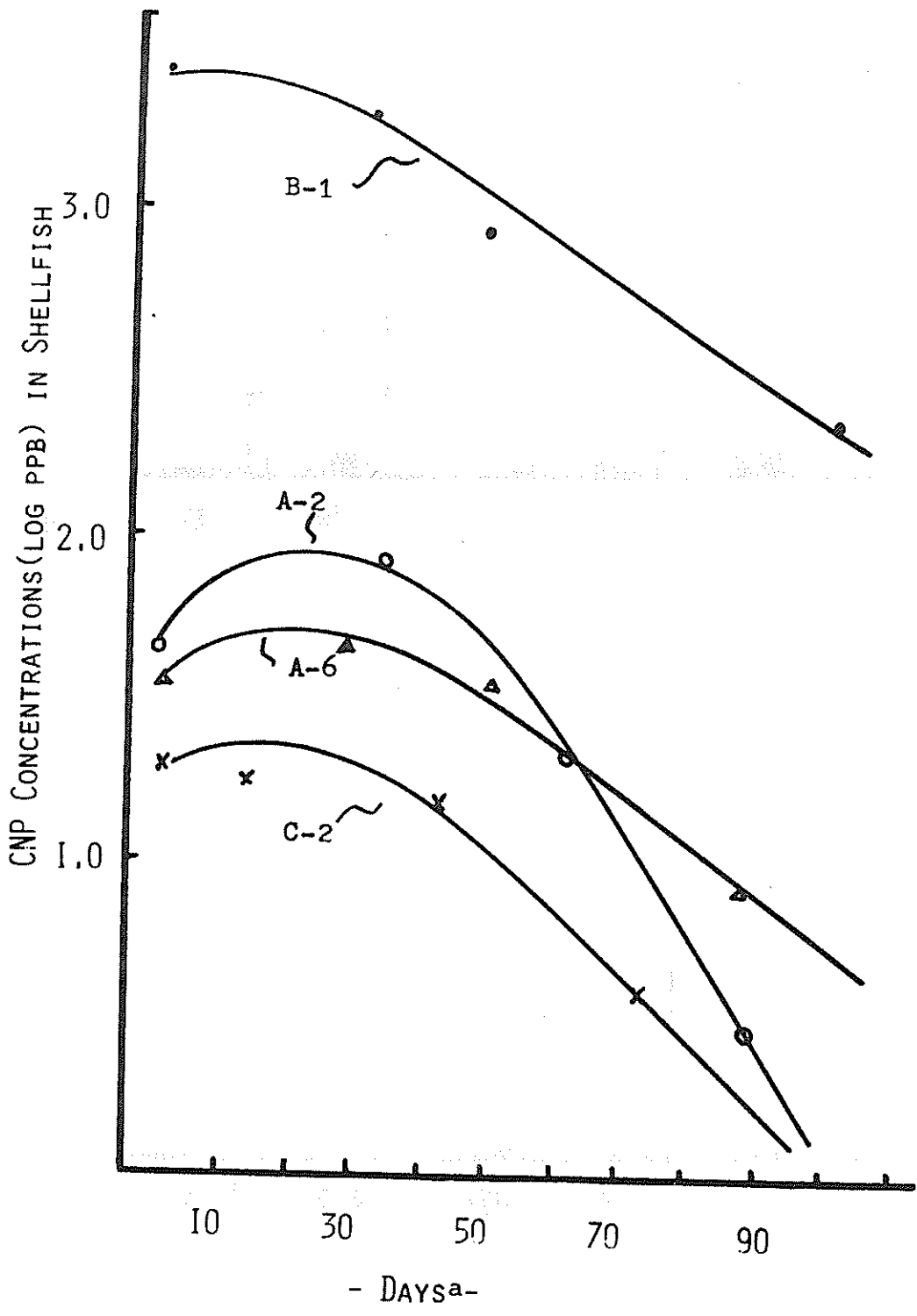


Fig. 2.
Change of
CNP levels
in short-
necked
clam which
were period-
ically sam-
pled at the
fixed points.

^a The first
sampling is
day 1.

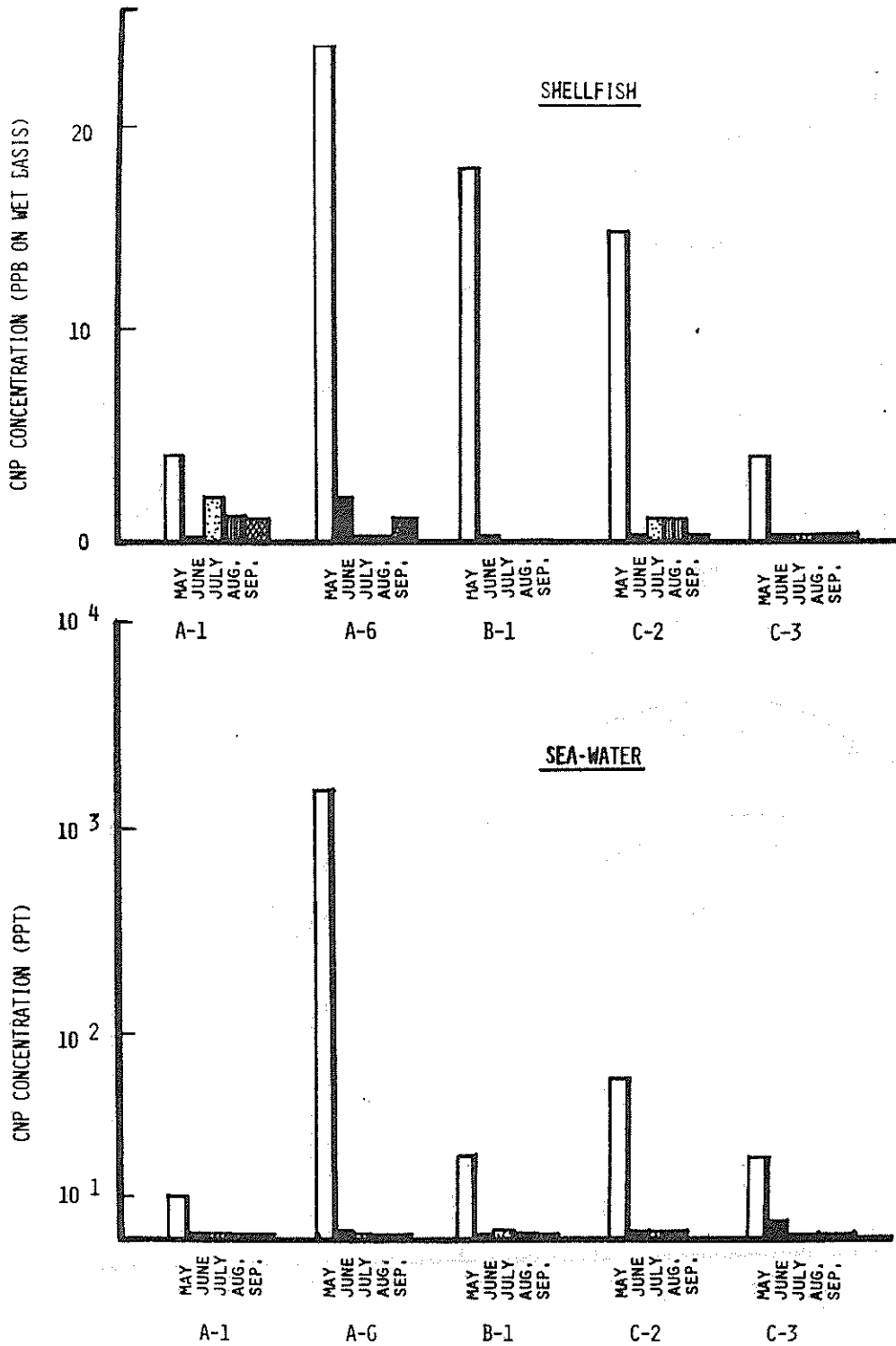


Fig. 3. CNP levels in shellfish and seawater collected at different sampling stations in 1978.

collected near the estuary were remarkably high in May, and the maximum level was 44.3 ppb. Thus, the season of the maximum levels reported in their studies are similar to that of the present study; however, the concentrations of the maximum level in seawater and shellfish are much lower than those of river water and the organisms.

In order to evaluate the fate of herbicides in the environment to which they may be applied, particularly the fate of CNP in river water, we have carried out a study. The residue levels of CNP in freshwater fish (Zacco platypus) were examined as an indicator of river water pollution.

Table 2. CNP concentrations in freshwater fish from the Tone and Tama River in June, 1979

Sampling sites ^a	Body weight (range, g)	CNP concentrations ^b (ppb, wet basis)
<u>Tone River</u>		
Oyama	16.1 - 18.6	76.3
Kano	15.4 - 18.7	32.0
Nogii	2.3 - 16.2	20.2
Noda	2.3 - 5.5	16.4
Nagareyama	23.5 - 23.7	19.7
<u>Tama River</u>		
Dam	15.6 - 9.4	nd
Haijima	11.0 - 18.5	nd
Koremasa	12.5 - 18.5	nd
Noborito	3.5 - 4.2	nd
Futago	3.5 - 4.5	nd

Freshwater fish: Zacco platypus

nd: not detectable

a

The sites are arranged from the upper to down stream.

b

Data represent mean of triplicated analyses of a pooled sample of three each fish.

In early June, 1979, samples of freshwater fish were collected at five each sampling stations in two rivers, the Tone River (upper stream of the Edo River) and the Tama River, which discharge into the Tokyo Bay. In the Tone River basin through agricultural areas, the CNP levels in freshwater fish were much higher than those in marine fish, as shown in Table 2.

While, in Tama River (industrial areas), CNP residues in freshwater fish

were not detectable. These results indicate that the marine environmental pollution with CNP in the Bay is mainly attributable to river water, which are discharged from agricultural watershed through areas of paddy fields after the herbicide application.

C. Tissue Distribution of CNP in Shellfish and Fish

Table 3 shows the distributions of CNP in the bodies of shellfish: the samples (3 kg) was collected at A-2 in May, 1977, and individually divided into two portions, viscera and muscle containing mantle and pseudopodium. The concentrations of CNP residues in viscera were about 5 times higher than those in muscle, and this difference may partly associated with relatively high content of lipid in viscera. As shown in Table 1, concentrations of CNP residues in goby-fish liver were about 120 times higher than those in muscle in all the samples examined. Furthermore, a linear correlation was observed in goby-fish samples when the residual levels in both liver and muscle were logarithmically converted.

Table 3. CNP concentrations in muscle and viscera of short-necked clam (Tapes philippinarum)

Tissues	CNP concentrations ^a (ppb, shucked, wet basis)	Ratio in each tissue (A/B)
A) Viscera	23.5 + 1.6	4.7
B) Muscle	5.0 ± 0.8	

Data represent mean ± S.D. of three pooled samples of thirty each shellfish

^aSignificant differences were not found between the three classes ($p < 0.01$)

Because the body sizes of the individual goby-fish were too small (10-30 g in whole weight), the tissue distributions of CNP were determined on another species, sea bass (3-5 kg in whole weight). The results are as follows; 1,660 ppb in gallbladder, 1,610 ppb in fat, 1,520 ppb in kidney, 1,350 ppb in liver, 210 ppb in heart, 44 ppb in gill lamella, and 43 ppb in muscle, respectively. The liver, kidney, and fat tissues thus, accumulate CNP selectively. Kanazawa et al. 4) revealed that under the model experiment, CNP bioaccumulation ratio for freshwater fish (topmouth gudgeon, Pseudorasbora parva) was relatively high, attaining to a magnitude of 1.1×10^3 , after the fish had been exposed to 20 ppb of CNP for 15 days, and that the levels in kidney, liver, and the other viscera were higher than those in muscle and head. Kobayashi 5) also reported similar results. However, it is not clear at present whether these residual levels in the organs would cause any func-

tional damage to internal organs, or have any biological effect on the sea bass and goby-fish.

D. Bioaccumulation and Body Weight

CNP concentrations in shellfish and fish were examined in relation to body size (Table 4 and 5). In both species, no significant differences in CNP concentrations existed between body sizes, medium, and large ($p < 0.05$).

The results indicate that CNP accumulation by these marine biota occurred quite recently in the Bay, and this may prove that the CNP residues in the marine biota were absorbed directly from seawater, and not from food chain.

Table 4. CNP concentrations in short-necked clam (*Tapes philippinarum*) by body weight

Sizes	Body weight (shucked, g)	CNP concentrations ^a (ppb, shucked, wet basis)
Small	0.9 ± 0.1	21.0 ± 1.4
Medium	1.9 ± 0.2	22.3 ± 1.5
Large	5.5 ± 0.3	19.6 ± 1.0

Data represent mean ± S.D. of three pooled samples of thirty each shellfish

^aSignificant differences were not found between the three classes ($p < 0.01$)

Table 5. CNP concentrations in goby-fish (*Acanthogobius flavimanus*) by body weight

Sizes	(n)	Body weight (g)	CNP concentrations ^a (ppb, wet basis)
Small	15	5.0 ± 0.6	0.90 ± 0.08
Medium	10	10.1 ± 0.2	1.13 ± 0.05
Large	5	13.9 ± 0.9	1.45 ± 0.13

Data represent mean ± S.D

^aSignificant differences were not found between the three classes ($p < 0.05$)

Biological indicators have been used by several investigators to monitor organochlorine insecticides or other pollutant in the marine environment. The use of such organisms have several advantages, especially the indicator species used in the present study have an advantage; to elucidate not only a route of marine environmental pollution due to river water but also the range and proportion of pollutants.

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Selective Breeding Programs and Genetics as Likely Impacts
on Future Aquaculture Production Systems and Regulation of Aquaculture

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I. Growing Interest in Genetic Applications to Aquaculture

The idea of applying genetic principles to aquaculture and very limited attempts to do so were still novel in the 1960s even though some knowledge of both would have led most to surmise that one might conceivably impact the other in a commercially significant sense.^{32,34} Because of the historically different development of genetics, animal and plant breeding, marine biology, and agriculture, papers on aquatic organisms, fresh water as well as marine, were truly a rarity at any genetics or scientific breeding meeting. To be a good subject for breeding studies the species must be one with a fully and easily controlled life cycle. The complicated life cycles of many marine species with their highly vulnerable, specialized juvenile stages still pose many problems to their fruitful research and economic culture.⁴⁶ This is not considering the even greater difficulties they present to a selective breeder or geneticist. Even so, genetics in culture systems is seen now less as a novelty and is viewed by an increasing number as a significant part of the future success of aquaculture. This could be particularly so for the more high-technology, less labor-intensive type of aquaculture envisioned as suitable to U.S. and West European economies. Most of the species plans prepared as an appendix to the U.S. National Aquaculture Plan, which the present Aquaculture Bill pending in Congress would require should it be passed, list selective breeding and genetics among highest priority areas for research and development.²⁷

As early as 1969, the USSR Academy of Sciences and fishing ministries sponsored a symposium on "Genetics, Selection and Hybridization of Fish" (ed. Cherfas), translated to English by NOAA, U.S. Dept. Commerce, Translation Services, 1972.⁴ Although it noted the significant scope of Soviet work on fish genetics and selection (largely fresh water), the conference concluded that such was yet not sufficient for the tasks facing fishery science. Many sound recommendations were made, but there is no evidence that these were followed up with much real support for the proposed breeding programs. Not unlike the belief in the U.S., it appeared to conference contributors that there was an underappreciation of the importance of genetics and special breeding in fish culture.

At the 1970 Second U.S. Food-Drugs from the Sea Conference, F. Schultz presented a paper outlining well, from the vantage point of one who has consulted extensively on poultry breeding, the potential value to aquaculture of population genetics, sex control mechanisms, artificial fertilization, chromosome manipulation, induced mutation, and biochemical genetics.⁴⁷ Later in 1978 the U.S. National Academy of Sciences' study on

"Aquaculture in the United States, Constraints and Opportunities" accepted the case for a role of genetics in aquaculture.^{39,33}

Also in 1978 the Japanese Society of Scientific Fisheries in conjunction with the Japanese Academy of Sciences sponsored a symposium on the "Present Status of Fish Genetics and Future Prospects of Breed Improvement" (ed. K. Fujino), soon to be available in English through NOAA Translation Services.¹² There now appears to be a real recognition in Japanese aquaculture of the importance of, and efforts to effect, breed improvement for increasing culture productivity.

Genetics has further been the object of serious consideration at international meetings. An FAO Ad Hoc Working Party on "Genetic Selection and Conservation of Genetic Resources of Fish" attributed the lag in applying genetic principles to aquaculture breeding to lack of complete control of reproductive phases, as well as to a generally inadequate awareness among aquaculturists of the importance of genetic work.⁹ At the FAO Technical Conference in Kyoto, Japan, in 1976, U.S., Norwegian, and Israeli geneticists described the theoretical basis for selective breeding and its application to aquaculture.¹⁰ They noted that, with few exceptions, aquaculturists still use undomesticated organisms, a situation they liken to using jungle fowl to operate a modern poultry farm. As to be pointed out, commercial hatchery operators do not yet have other options.

More recently, at the October 1979 Statutory Meeting of the International Council for the Exploration of the Sea, genetics in mariculture was one of two special topics addressed at sessions of the still-new Committee on Mariculture. Invited position papers and experience papers were presented by American, Canadian, and Norwegian researchers*. The Mariculture Committee elected to sponsor a study group on genetics to advise it on aquaculture breeding and, in regard to natural populations, still the primary source of breeding stocks.

Genetics is seen as having its most general, pervasive role in aquaculture as a basis for selection and other artificial, more experimental breeding programs (as chromosome manipulation to produce sterile triploid progeny) aimed at improving the organism for use in intensive, controlled production systems. In simple multiplication of stocks not intended for any selection purposes--the present status of most production systems--genetics can still advise as to how suitable levels of genetic variability can be maintained. Hopefully, it might direct the process of domestication in aquacultured organisms. Genetics is seen as aiding in the characterization of the diverse wild populations of commercial and related species, and in so doing assist in the selection of the best-adapted populations for particular purposes and environments. It can provide advice and be a stimulus for the conservation of native resources as irreplaceable gene pools for natural enhancement, and for future use in artificial breeding programs.

* For convenience, referred to here by their official ICES numbers: C.M.1979/F:21-22; C.M.1979/F:36-49; C.M.1979/F:56. Obtainable from International Council for the Exploration of the Sea, Charlottenlund Slot, DK-2920, Charlottenlund, Denmark.

An increase in more basic genetic information on cultured aquatics should contribute to the general data base still limited, particularly for marine species, relative to that which exists for terrestrial species of economic importance.

At the same time interest in applying genetics to aquaculture has been increasing, marine organisms have been found to be ideal subjects for basic population genetic studies employing biochemical genetic methods of gell electrophoresis. As stated at the 1978 NATO conference, "Marine Organisms, Genetics, Ecology and Evolution,"² marine organisms are playing a central role in what promises to be as major an impetus in the study of evolution as has occurred since Darwin's time. Some of the marine species subjected to these basic scientific studies are candidates for mariculture, and fish whose stocks must be managed. New advances in chromosome methodologies and even more widespread application of older chromosome methodologies to marine species spurred on by fresh interest may stimulate yet another burst of activity in evolutionary genetics with marine organisms and aquaculture species playing a key role. Heteromorphic sex chromosomes were recently identified in rainbow trout,⁴⁹ and populations observed with varying chromosome numbers, some possibly as high as those expected for cutthroat trout.¹³ Growing interest in the developmental biology and developmental genetics of fish is evidenced by papers presented at the 1978 Fourteenth International Genetics Congress, and by the recent symposium on "Development Biology of Fishes," held in conjunction with the annual meeting (1979) of the American Society of Zoologists. Certainly, there will accrue to aquaculture and fisheries some indirect benefits from this fundamental work.

II. Present Restrictions on Application of Genetic Principles to Aquaculture Breeding

A. Restrictions shared with aquaculture generally

Even though there are demonstrations of useful genetic variability in cultured species, this variability has not been exploited commercially to any great degree. Narrowing the gene pool through selection without a concomitant increase in control over the life cycle could confound rearing and maintenance problems. For economic gain, as opposed to scientific demonstration, selection programs should be integrated parts of pilot or full-scale production systems. Continuous generation-to-generation breeding of large numbers of organisms with relatively long-generation times and fragile juvenile stages--a necessity to benefit from the useful genetic variability demonstrated--suffers from all the non-scientific constraints on U.S. aquaculture programs as much and even more so than does aquaculture in general.³⁹ In the U.S. a vigorous industry would almost certainly play a significant role here in an undertaking, hardly the sole prerogative or responsibility of either universities or federal laboratories.

Probably the best example of gain to aquaculture from applied genetics is of seaweed, Laminaria japonica, in the People's Republic of China.^{45,50} In a breeding program started in 1951 in Tsingtao, China's main center for marine research, entailing use of X-irradiation to induce new mutations, inbreeding and selection, new varieties have been produced with higher temperature tolerance, faster growth, and higher alginate and iodine content.

Between 1950 and 1978, kelp production increased from 60 to 200,000 metric tons. A good portion of this increase is attributed to the improved strains.

B. Still wild genotypes of "uncultivated" aquaculture species

Geneticists would argue that to gain the measure of control over the life cycle required for production efficiency, the wild types aquaculturists currently use must undergo domestication or cultivation just as did domestic animals and crop plants long before the advent of genetics. Those high-producing successes of U.S. agriculture that lead aquaculturists to be interested in genetic technology were so changed by domestication before they underwent any deliberate improvements that they bear little genetic resemblance to wild ancestors. When the ancestors can be determined with any certainty, they are not even assigned the same species or genus rank. In this process of domestication, aquaculture species would, similarly to the agriculture species, accumulate those genes of benefit in high density commercial production with man's protection, and lose those genes that benefit the organism only in its survival in wild unprotected habitats, and are a detriment to economic aquaculture. It has been questioned, however, by geneticists themselves whether genetics can suitably direct the process of domestication, which is not clearly understood in terms of modern genetic theory.

It is known that in mammals, domestication results in great increases in the quality and range of genetic variability. A Soviet scientist, Lecturer of the American Genetic Association for 1978, by selecting foxes solely for non-aggressive behavior towards man, changed not only their behavior but their estrous, moulting, and morphological type which came to resemble that of some breeds of dogs.³ Aquaculturists, in selecting intensely for characters that natural selection would never favor in a stable environment, might similarly produce some unexpected associated responses. The process of domestication or cultivation is of considerable theoretical interest. Incipient domestication is presently being studied in the cultured freshwater prawn, Macrobrachium rosenbergii.³⁶

III. Application of Genetic Principles to Aquaculture Breeding, and Changing Requirements and Regulatory Needs of Aquaculture

A. In relation to need for genetic variability in founding stocks

Despite all the restrictions on application of genetic principles to aquaculture, should aquaculture move beyond its present stage of rearing essentially wild organisms, genetics would inevitably play an integral role. Breeding consecutive generations is an unavoidable basic component of such an advance. In the absence of directed selection by the breeder, natural selection will still operate in the hatchery and not necessarily to the benefit of the breeder. If not practiced properly, artificial selection can result in genetic deterioration of the stock. Properly practiced, there is potential for production gains.

Commercially successful application of genetic principles to aquaculture breeding would not result only in stocks with new genotypes. It would also bring about some change in views on new introductions, transport of stocks, disease control, and conservation. Current attitudes have, not inappropriately, been shaped out of consideration for protection of the

natural resource as opposed to the development and maintenance of artificial breeds. The 1971 FAO Ad Hoc Working Party on Genetic Selection and the Conservation of Genetic Resources of Fish⁹ recommended, as a high priority problem for research, the systematic exploration and testing of genetic resources which might offer better potential for aquaculture than strains already in use. Transfers and introductions of marine organisms for aquaculture are already on the increase, and epizootiological aspects of such have been reviewed.^{48,44,37,42}

For selective breeding--the cornerstone of most breeding programs--to succeed, there must be sufficient genetic variance for the traits to be selected for.⁸ Populations will differ in both the quality and quantity of these genes. Most traits of commercial interest are not inherited as simple dominant and recessive genes but rather as large numbers of genes, each with exceedingly small individual effects. The expression of these genes is greatly modified by the environment. Some estimates of genetic variance have been made for commercially important traits in several aquacultured groups. Estimates appear high enough to indicate some measure of success in properly conducted selection programs for the traits concerned as inferred above. For examples on oysters, lobsters, salmon, and trout see references 30, 21, 28, 43, 7, 35, 13, and 15. The normal state of most outbreeding organisms is one of considerable genetic variability. This appears to be true of marine species, as well as others, although fewer marine species have been studied.⁴¹

The outcome of selective breeding programs will depend on the number and composition of the founding stocks, as well as on the numbers of animals selected for hatchery breeding each generation.⁸ Too few founding individuals of too restricted a gene pool limit the outcome of such breeding programs even though peak selection responses would occur earlier. Reduced genetic variation also reduces the ability of the species to adapt environmentally. In extreme situations, having too few individuals in the founding stocks results in deleterious inbreeding depression in later generations after substantial funds and time have already been committed to the artificial breeding program. Use of new stock in the breeding schemes at this time causes loss of some gain already made. Carp and trout stocks are believed to have suffered from too much inbreeding in the past (Reference 9 and Ryman, personal communication regarding work in press).

When selection programs are to be founded with non-endemic or non-local stocks, restrictions on the numbers of animals to be brought in an area can, therefore, adversely affect the likely success of the undertaking, as well as guard against the likelihood of bringing in disease organisms and pests or establishing natural populations of a non-endemic species. The Revised Code of Practice of the Working Group on the Introduction of Non-indigenous Marine Organisms²⁵ recommends that brood stock be first established in approved quarantine, and only the first generation be transplanted to the natural environment. Particularly when such stock is to be used in hatchery breeding programs, care must be taken that large enough numbers of animals are used initially and that genetic variability is preserved in the breeding and rearing of such F₁ stock. This is no small undertaking considering current limitations of hatchery production. The ICES Working Group recognizes this, and recommendations specify that genetic requirements of sound

breeding programs be considered along with others when dealing with this matter (ICES Working Group on the Introduction of Non-indigenous Marine Organisms, 1979).

B. In relation to hybridization

Once breeders have tested and experienced the limitations of their own particular choice stocks, there should be an increased interest in any advantage hybrids might offer for artificial culture.³³ This, of course, further confounds the introduction problem. Hybrids might be made of stocks as closely related as strains, of population crosses of the same species or between species and, in the case of seaweeds,⁵⁰ possibly even between genera. Hybridization of a newly introduced form with an endemic stock or with an established hatchery line might be desirable as a means of increasing the genetic variability of the endemic stock in a selection program. Another reason for hybridization would be to combine in one stock desirable characteristics that exist separately in different forms. Another would be to produce especially vigorous or otherwise desirable hybrids for the market. Occasionally, a hybrid between two species will be sterile because of the lack of homology between chromosomes of the two parental species and would be made to obtain sterile progeny for commercial production. It was recently proposed by Israeli carp breeders and geneticists that wild fish populations of various types be improved for harvesting by hatchery rearing stocks which would hybridize in nature with natural populations to produce superior fish for traditional fishing;³⁸ a bold proposal for the fisheries, but hardly new to forage crop breeders. The potential use of such hybrids in restocking of public shellfish beds was remarked on earlier.³⁴

Wide hybridization is believed, furthermore, to have had a role in the cultivation process of crop plants by introgression of genes from one species into another, and some of the hardiness of crop plants may depend on occasional hybridization with associated weeds.^{16,11,31} It could similarly be important in the domestication of aquaculture species. It is also known that old, well-established subspecies usually have gene combinations so well-adapted to the environments they occupy that any new combinations created by hybridization will nearly always be less favorable. If, however, hybrids are to be made use of in an entirely new environment, some of the vast array of segregates appearing in later generations may be better adapted than any individuals of either parent groups.⁵¹ Certainly, hatcheries are a new environment for aquaculture species.

In plants, one of the major consequences of domestication associated with this wide hybridization was an enormous increase in the area of distribution of the species.²² (Most of our major crops came into cultivation in one or few regions, each with a restricted range and rather narrow climactic limits.) Major wheat cultivars of, for example, Canada, come from regions as widely separated and as climactically diverse as Poland, India, Russia, Australia, England, Egypt, and Portugal.^{29,17} Hardly any of the major agriculture species of the U.S. are native to this country. Too stringent restrictions on introductions for aquaculture at the current phase of development of the field may well impede its further development if agriculture is used as an example.

C. In relation to disease and prophylaxis

As the gene base of aquaculture species is narrowed and homozygosity increased in making for specific production efficiencies, any natural genetic resistance to disease risks becoming less or even lost entirely. Mortality or required destruction of stock because of disease after a breeding program is well underway is a particularly serious loss, and a not uncommon one. Dependence of the culturist on prophylaxis should then have to be increased on the loss of natural genetic resistance or general vigor. An alternative is to make deliberate use of disease resistance as a selection criterion from the beginning of any serious breeding program insofar as this is practical or possible. There would be the disadvantage here that the more characters selected for simultaneously, the slower the overall selection progress.

In spite of the spectacular advances in chemical protection, the use of resistant varieties still represents the only economical means of control against some of the most widespread and destructive plant pathogens.⁵¹ A rather common practice of plant breeders is the crossing of a resistant variety or species with a non-resistant local type or commercial form, followed by backcrossing to the more generally desirable but susceptible parental type, selecting each generation the disease-resistant offspring for the parents of the next generation. Such a breeding scheme might be adapted to some aquacultured species, or direct use even made of the F₁ progeny of a resistant type crossed with a non-resistant type.

While genetic resistance to disease has occupied a special place in plant breeding, by comparison to plants little has been done to improve the resistance of agricultural animals to infections or dietary deficiencies by breeding. Yet, this has not been due to lack of genetic variability to disease resistance,²⁶ but rather to reliance on prevention. It has not been possible to carry out comprehensive selection experiments with large farm animals, but a number of interesting observations have been made. In small animals, experiments have been performed in which disease resistance has been changed by selection.

For over 20 years Hutt and his co-workers at Cornell University carried out selection for high and low mortality to leukosis (a viral disease) by simply exposing poultry to natural infection.^{23,24} By selecting birds for breeding from families with low mortality, two highly resistant lines were produced also superior in other ways. In another line, selection was for high mortality. In the resistant lines mortality from leukosis was 2-3%, and in the susceptible, 40-60%.

Fish too appear to have genetic variation for disease resistance which might be exploited for aquaculture production systems. Higher resistance to disease in the eastern brook trout (Salvelinus fontinalis), along with increased growth rate and average number of eggs per female, was reported in 1930 by Hayford and Embury.²⁰ The development of disease-resistant strains of freshwater fish was briefly reviewed by Wolf in 1954.⁵² Donaldson⁶ reported higher resistance to disease in his selectively-bred chinook salmon (Oncorhynchus tshawytscha). Ayles¹ attributed the heritability of survival of alevins in splake hybrids to genetic differences in susceptibility to blue sac diseases. Gjedrem and Aulstad¹⁴ reported significant

differences in resistance to Vibrio disease between river strains of Atlantic salmon parr. Estimates of heritability within strains were 0.12 and 0.07, respectively, for the dam and sire components, low but significantly different statistically from 0. These researchers argue that resistance to Vibrio can be altered by selection. They are attempting to do so selecting for differences in Vibrio antibody titer in rainbow trout.

Wild oyster populations have developed resistance to the Malpeque Bay disease of still unknown etiology. About 1915 this disease wiped out nearly all the oysters in the hitherto productive waters of Malpeque Bay on the north shore of Prince Edward Island. By 1940, solely through the effectiveness of the pressure of natural selection for resistant oysters, these oyster beds were again productive.⁴⁰ Transfers of oysters from this area to others where the disease had not struck showed oysters from the former to be highly resistant. Until recently this has been the only reported case of a population of marine invertebrates acquiring genetic resistance to a disease.

The devastating mortalities of oysters in Delaware and Chesapeake Bays in the later 1950s were reminiscent of those occurring in Malpeque Bay. The disease-causing organism of the mid-Atlantic epizootic was identified as a haplosporidian parasite, Minchinia nelsoni.¹⁹ In a pattern similar to the Malpeque experience, evidence suggests that these native oysters are developing resistance to what has come to be referred to commonly as the MSX disease.¹⁸ Resistance seems to be correlated with lighter, more localized infections.¹⁸

Stocks of oysters with various histories of exposure to MSX have also been laboratory-reared, and their offspring tested for resistance.¹⁸ After nearly three years of intensive selection against MSX in each generation, first, second, and third generation resistant stocks have 4.6, 5.1, and 6.3 times as many survivors, respectively, as do unselected groups. Native resistant oysters have nearly three times as many survivors as do susceptible imports.

There is then reason to suppose that breeding for disease resistance in aquaculture could be successful, as well as important, in reducing somewhat dependence on prophylaxis.

D. In relation to needs for conservation of wild stocks

Even though those who would continuously breed and maintain aquacultured species generation to generation in the most artificial ways should eventually view stock introduction differently from those whose interests remain strictly with the natural resource, both groups have a continuing stake in the conservation of the natural resources of their particular organism. For the aquaculturist, this concern over conservation should include wide areas of the natural species distribution. Any highly successful aquaculture types of the future, grown under somewhat artificial, controlled conditions over widely varying environmental climates, may derive from only a few of the presently cultured types, those that best lended themselves to the little understood process of domestication or cultivation. There is no assurance that these will be endemic types. The world's major crops derive mainly from one or a few wild species with greatly expanded ranges.²²

Natural populations of wild stocks are, in addition, invaluable as the gene pool from which genetic variation for aquaculture breeding can be drawn in the future. Genes unwittingly discarded in intense hatchery selection can be regained later only by returning to wild stocks. Wild populations can never be maintained artificially and practically in a manner that would preserve their natural genetic integrity, making their conservation in nature all the more important. New introductions should accordingly be viewed in terms of the likelihood of any significant level of hybridization in nature with endemic populations, as well as in terms of ecological competition, and their usefulness in aquaculture selection programs.

While aquaculturists look forward to domesticated, selected lines, increasingly plant breeders must return to the primitive cultivars of the past created unconsciously by uncultured forbears for genes long discarded in artificial breeding programs.^{11,5} The growing concern of crop breeders and geneticists that their primitive germ-plasm resources may be lost should impress on aquaculturists the importance of preserving the wild gene pools of their own species for the selection programs that may be an integral part of the future of aquaculture. The proposal to categorize and inventory shellfish populations of Europe that Ireland presently has before the Committee on Science and Technology of the European Economic Commission may be a step in that direction (information from Prof. N. Wilkins, University College, and E. Sweeney, National Board of Science and Technology, Dublin). In 1977 funds were made available to the Department of Animal Genetics and Breeding, Agricultural University of Norway, to establish an egg bank with the preservation of salmon strains threatened with extinction as one of its three objectives.¹⁴ Proposals are also being prepared, in cooperation with the United Nations Environment Program, regarding the conservation of the genetic resources of fish (information from T. Pillay, Aquaculture Development and Coordination Programme, Fisheries Department).

IV. Summary

Aquaculture systems might be more efficient once they move beyond the present, general stage of rearing essentially wild organisms in hatcheries. Breeding consecutive generations is an inevitable basic component of such an advance. It is unavoidable then that application of genetic principles to aquaculture breeding would have to be an essential part of any such future development of aquaculture even though genetics can still play a lesser role in the present state of aquaculture. In the absence of directed selection by the breeder, natural selection will still operate in the hatchery and not necessarily to the benefit of the breeder. If not practiced properly, artificial selection can result in genetic deterioration of the stock. Properly practiced, there is potential for production gains.

Integration of selective breeding programs into production systems would effect a change in attitudes on introduction and transfers of non-endemic stocks, on conservation of wild resources as gene pools, and on disease control for reasons discussed in this paper. Current attitudes have, not inappropriately, been shaped largely out of consideration for protection of the natural resource as opposed to the development and maintenance of artificial breeds.

Beset as it is by all the current limitations of aquaculture and by a general lack of domesticated breeds, aquaculture breeding, as based on genetic principles, is still attracting increasing interest. At the same time, marine organisms are playing a central role in basic evolutionary studies in genetics. Domestication of marine organisms, viewed as essential to their maximum use in artificial production, could shed new light on genetic theories of evolution of domesticated and cultivated species, a process which can no longer be fully traced in agriculture species.

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THE ROLE OF THE FOOD AND DRUG ADMINISTRATION IN
THE APPROVAL OF NEW ANIMAL DRUGS

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Abstract

The Food and Drug Administration's responsibility in aquaculture, as with other methods of food production, is to assure the safety of food commodities produced in aquaculture systems that have utilized drugs for therapeutic purposes.

Animal feeds used in aquaculture operations which contain drugs to prevent or control disease must be in compliance with the same regulatory requirements regulating animal feeds in meat and poultry husbandry.

Introduction

The Food and Drug Administration (FDA) operates under the Federal Food, Drug, and Cosmetic Act (FFDCA) and regulations published in Title 21 of the Code of Federal Regulations (CFR).²

For products to be subject to FDA jurisdiction, they must be either foods, drugs, devices or cosmetics introduced into or delivered for introduction into interstate commerce.

Before a new animal drug can be legally marketed, it must be approved by the FDA on the basis of safety and effectiveness (table 1). The sponsor of the drug has the responsibility of providing this data. If the drug is for food-producing animals, not only must the safety of the animals be considered, but also the safety of food products derived from the treated animals that are intended for human consumption.

Therefore, all approved drugs used in man or other animals must be safe and effective in the proposed animal specie as well as safe to the consumer.

Table 1. The only FDA therapeutics currently approved for use in food fish are:

SULFAMERAZINE FISH GRADE--AMERICAN CYANAMID 21 CFR 558.582
FOR CONTROL OF FURUNCULOSIS IN RAINBOW TROUT, BROOK TROUT,
AND BROWN TROUT

WITHDRAW--21 DAYS PRIOR TO MARKETING OR STOCKING IN OPEN FISHING
STREAMS

DO NOT TREAT IN EXCESS OF 14 DAYS
(WET AND DRY DIETS)

DOSAGE--10 GRAMS PER 100 POUNDS OF FISH PER DAY

TERRAMYCIN ANTIBIOTIC PREMIX FOR FISH--C. PFIZER & CO.
21 CFR 558.450

(OXYTETRACYCLINE)

FOR *SALMONIDS TO CONTROL ULCER DISEASE, FURUNCULOSIS,
BACTERIAL HEMORRHAGIC SEPTICEMIA, AND PSEUDOMONAS
DISEASE

DO NOT ADMINISTER WHEN WATER TEMPERATURE IS BELOW 9°C (48.2°F)

FOR *CATFISH FOR CONTROL OF BACTERIAL HEMORRHAGIC
SEPTICEMIA AND PSEUDOMONAS DISEASE

DO NOT ADMINISTER WHEN WATER TEMPERATURE IS BELOW
16.7°C (62°F)

*TREAT FOR 10 DAYS 2.5-3.75 GRAMS PER 100 LBS. OF FISH.
WITHDRAW-21 DAYS PRIOR TO SLAUGHTER OR MARKETING.

FINQUEL

21 CFR 529.2503

(TRICAINA METHANESULFONATE)

FOR ANESTHESIA AND TRANQUILIZATION OF FISH AND OTHER
COLD-BLOODED ANIMALS

DOSAGE--ADD TO AMBIENT WATER AT A CONCENTRATION OF FROM
15-330 mg PER LITER DEPENDING UPON THE DEGREE OF
SEDATION DESIRED

DO NOT USE WITHIN 21 DAYS OF HARVESTING FISH FOR FOOD.
USE IN FISH INTENDED FOR FOOD SHOULD BE RESTRICTED TO
ICLATURIDAE, SALMONIDAE, PERCIDE AND ESOCIADAE, AND WATER
TEMPERATURE SHOULD EXCEED 10°C (50°F).

The FDA's responsibility in aquaculture, as with other methods of food production, is to assure the safety of food commodities produced in aquaculture systems that have utilized drugs for therapeutic purposes. According to the law (FD&C Act), a food is illegal if it contains a natural or added substance which may be unsafe or injurious to health.

Animal feeds used in aquaculture operations which contain drugs to prevent and/or control disease must be in compliance with the same regulatory requirements regulating animal feeds in meat and poultry husbandry.

Chemicals which are considered to be new animal drugs must be the subject of an approved new animal drug application (NADA). The legal requirements for approval of a new animal drug application, in brief, are as follows:

Contents of a new animal drug application (NADA) initial submissions should contain the information described in 21 CFR 514.1(B). It includes:

1. Full reports of investigations in the target specie to show that the drug is safe and effective for the use proposed.
2. A list of components of the drug (chemical characterization, active drug ingredient, its purity, all components of the formulation).
3. A full description of methods, facilities and controls used in manufacturing, processing and packing of the drug.
4. Copies of proposed labels and labeling.
5. A signed copy of Form FD 356.³
6. An environmental impact analysis report (EIAR), regulation 514.1(B)(14).
7. A freedom of information (FOI) summary of safety and effectiveness data.

Prior to the initiation of any studies, we recommend a complete literature review on the disease or syndrome for which control is desired.

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE FOOD AND DRUG ADMINISTRATION NEW ANIMAL DRUG APPLICATION (<i>Drugs for Animal Use</i>) <i>(Title 21, CFR § 514)</i>		FOR FDA USE ONLY NADA NO. _____ DATE RECEIVED: _____
NAME OF APPLICANT _____ ADDRESS (<i>Street Number, City, and Zip Code</i>) _____ FDA DRUG REGISTRATION NO. _____ DATE: _____		TYPE OF SUBMISSION (<i>Check one</i>) <input type="checkbox"/> ORIGINAL APPLICATION (<i>CFR 514.1(a)</i>) <input type="checkbox"/> AMENDMENT TO AN UNAPPROVED ORIGINAL APPLICATION (<i>CFR 514.6</i>) <input type="checkbox"/> AMENDMENT TO AN UNAPPROVED SUPPLEMENT TO AN APPROVED APPLICATION (<i>CFR 514.6</i>) <input type="checkbox"/> SUPPLEMENT TO AN APPROVED APPLICATION (<i>CFR 514.8 (a)</i>) <input type="checkbox"/> SPECIAL NEW ANIMAL DRUG APPLICATION SUPPLEMENT - CHANGES BEING EFFECTED (<i>CFR 514.8 (a)</i>)
CHEMICAL NAME (<i>In cases of multi-component drug mixtures a continuation sheet must be attached</i>) _____		GENERIC NAME: _____ PROPRIETARY NAME: _____

INSTRUCTIONS FOR PREPARING AND SUBMITTING THE NEW ANIMAL DRUG APPLICATION

- i. Assemble and bind three identical copies of the submission.
- ii. Identify each front cover with the name of the applicant, the proprietary name, if available, the name of the new animal drug and the dosage form.
- iii. Use separate pages for each numbered heading consistent with subparagraph (1) through (11) of this Application form. Number the pages of the new animal drug application. Each copy should bear the same page numbering.
- iv. Each copy of an original new animal drug application shall contain three complete sets of labeling.
- v. Submit separate applications for each different dosage form of the drug proposed. Repeating in each application basic information pertinent to all dosage forms is unnecessary if reference is made to the application containing such information. Such references should be made by volume and page. Include in each application information applicable to the specific dosage form, such as labeling, composition, stability data, efficacy data, method of manufacture and investigational new animal drug application number.
- vi. Forward amendments, supplements, reports and other correspondence submitted after the original application in the above format. Identify the submission with the assigned NADA number. If the submission is a supplemental application, full information shall be provided on each proposed change concerning any statement made in the approved application.
- vii. Submit to:
 - Food and Drug Administration
 - Bureau of Veterinary Medicine (*HFV-16*)
 - 5600 Fishers Lane
 - Rockville, MD 20857

The undersigned submits this application for a new animal drug pursuant to section 512(b) of the Federal Food, Drug, and Cosmetic Act. It is understood that the labeling and advertising for the new animal drug will prescribe, recommend, or suggest its use only under the conditions stated in the labeling which is part of this application and if the article is a prescription new animal drug, it is understood that any labeling which furnishes or purports to furnish information for use or which prescribes, recommends, or suggests a dosage for use of the new animal drug will also contain, in the same language and emphasis, information for its use including indications, effects, dosages, routes, methods, and frequency and duration of administration, any relevant hazards, contraindications, side effects, and precautions contained in the labeling which is part of this application in accordance with 21 CFR 201.105. It is understood that all representations in this application apply to the drug produced until changes are made in conformity with § 514.8. It is further understood that new animal drugs as defined in § 510.3, intended for use in the manufacture of animal feeds in any State will be shipped only to persons who may receive such drugs in accordance with § 510.7. The undersigned certifies, that the methods, facilities, and controls described under item 5 of this application conform to the current good manufacturing practice regulations in 21 CFR PART 200.

No new animal drug application may be processed unless a completed application form has been received (21 CFR 514.1).

SIGNATURE OF APPLICANT (<i>Responsible official or Authorized Agent</i>) _____	TITLE OF AUTHORITY _____	DATE SUBMITTED _____
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NOTE: This application must be signed by the applicant or by an authorized attorney, agent, or official, if the applicant or such authorized representative does not reside or have a place of business within the United States, the application must also furnish the name and post office address of and must be countersigned by an authorized attorney, agent, or official residing or maintaining a place of business within the United States.

(Warning: A willfully false statement is a criminal offense. U.S.C. Title 18, sec. 1001)

Enter the estimated time (manhours) to prepare this application. (<i>Include typing, photocopy and assembly only</i>)	Original Copy	
	Duplicate/Triplicate Copies	
	TOTAL (manhours)	

The following information, described in 21 CFR 514, shall constitute the requirements of this application:

1. **Identification.** Whether the submission is an original or supplemental application; the name and address of the applicant, the date of the application, the trade name(s) (if one has been proposed) and chemical name(s) of the new animal drug. Upon receipt, the application will be assigned an NADA number, which shall be used for all correspondence with respect to the application.
2. **Table of Contents and Summary.** The application shall be organized in a cohesive fashion, shall contain a table of contents which identifies the data and other material submitted, and shall contain a well organized summary and evaluation of the data in the following form:
 - i. **Chemistry**
 - a. Chemical structural formula or description for any new animal drug substance.
 - b. Relationship to other chemically or pharmacologically related drugs.
 - c. Description of dosage form and quantitative composition.
 - ii. **Scientific rationale and purpose the new animal drug is to serve:**
 - a. Clinical purpose.
 - b. Highlights of laboratory studies: The reasons why certain types of studies were done or omitted as related to the proposed conditions of use and to information already known about this class of compounds. Emphasize any unusual particularly significant pharmacological effects or toxicological findings.
 - c. Highlights of clinical studies: The rationale of the clinical study plan showing why types of studies were done, amended, or omitted as related to laboratory studies and prior clinical experience.
 - d. Conclusions: A short statement of conclusions combining the major points of effectiveness and safety as they relate to the use of the new animal drug.
 - e. List of references. References to pertinent information including information contained in files of the Food and Drug Administration as provided in 21 CFR 511.1.
3. **Labeling.** Three copies of each piece of all labeling to be used for the article (total of 9).
 - i. All labeling should be identified to show its position on, or the manner in which it is to accompany the market package.
 - ii. Labeling for nonprescription new animal drugs should include adequate directions for use by the layman under all conditions of use for which the new animal drug is intended, recommended, or suggested in any of the labeling or advertising sponsored by the applicant.
 - iii. Labeling for prescription veterinary drugs should bear adequate information for use under which veterinarians can use the new animal drug safely and for the purposes for which it is intended, including those purposes for which it is to be advertised or represented, in accord with 21 CFR 201.105
 - iv. All labeling for prescription or nonprescription new animal drugs shall be submitted with any necessary use restrictions prominently and conspicuously displayed.
 - v. Labeling for new animal drugs intended for use in the manufacture of medicated feeds shall include:
 - a. Specimens of labeling to be used for such new animal drug with adequate directions for the manufacture and use of finished feeds for all conditions for which the new animal drug is intended, recommended, or suggested in any of the labeling, including advertising, sponsored by the applicant.
 - b. Specimens of all labeling representative of those proposed to be used for finished feeds manufactured from the new animal drug.
 - vi. Draft labeling may be submitted for preliminary consideration of an application. Final printed labeling will ordinarily be required prior to approval of an application. Proposed advertising for veterinary prescription drugs may be submitted for comment or approval.
4. **Components and Composition.** A complete list of all articles used for production of the new animal drug including a full list of the composition of each article:
 - i. A full list of the articles used as components of the new animal drug. This list should include all substances used in the synthesis, extraction, or other method of preparation of any new animal drug and in the preparation of the finished dosage form, regardless of whether they undergo chemical change or are removed in the process. Each component should be identified by its established name, if any, or complete chemical name, using structural formulas when necessary for specific identification. If any proprietary name is used it should be followed by a complete quantitative statement of composition. Reasonable alternatives for any listed component may be specified.
 - ii. A full statement of the composition of the new animal drug. The statement shall set forth the name and amount of each ingredient whether active or not, contained in a stated quantity of the new animal drug in the form in which it is to be distributed (for example, amount per tablet or milliliter) and a batch formula representative of that to be employed for the manufacture of the finished dosage form. All components should be included in the batch formula regardless of whether they appear in the finished product. Any calculated excess of an ingredient over the label declaration should be designated as such and percent excess shown. Reasonable variation may be specified.
 - iii. If it is a new animal drug produced by fermentation:
 - a. Source and type of microorganism used to produce the new animal drug.
 - b. Composition of media used to produce the new animal drug.
 - c. Type of precursor used, if any, to guide or enhance production of the antibiotic during fermentation.
 - d. Name and composition of preservative, if any, used in the broth.
 - e. A complete description of the extraction and purification processes including the names and compositions of the solvents, precipitants, ion exchange resins, emulsifiers, and all other agents used.
 - f. If the new animal drug is produced by a catalytic hydrogenation process (such as tetracycline from chlortetracycline) a complete description of each chemical reaction with graphic formulas used to produce the new animal drug, including the names of the catalyst used, how it is removed, and how the new animal drug is extracted and purified.
 5. **Manufacturing Methods, Facilities, and Controls.** A full description of the methods used in, and the facilities and controls used for, the manufacture, processing, and packing of the new animal drug. This description should include full information with respect to any new animal drug in sufficient detail to permit evaluation of the adequacy of the described methods of manufacture, processing, and packing, and the described facilities and controls to determine and preserve the identity, strength, quality, and purity of the new animal drug, and the following:
 - i. If the applicant does not himself perform all the manufacturing, processing, packaging, labeling, and control operations for any new animal drug, he shall: Identify each person who will perform any part of such operations and designate the part; and provide a signed statement from each such person fully describing, directly or by reference, the methods, facilities, and controls he will use in his part of the operation. The statement shall include a commitment that no change will be made without prior approval by the Food and Drug Administration, unless permitted under 21 CFR 514.8.
 - ii. A description of the qualifications, including educational background and experience, of the technical and professional personnel who are responsible for assuring that the new animal drug has the identity, strength, quality, and purity it purports or is represented to possess, and a statement of their responsibilities.
 - iii. A description of the physical facilities, including building and equipment used in manufacturing, processing, packaging, labeling, storage, and control operations.
 - iv. The methods used in the synthesis, extraction, isolation, or purification of any new animal drug. When the specifications and controls applied to such new animal drugs are inadequate in themselves to determine its identity, strength, quality, and purity, the methods should be described in sufficient detail, including quantities used, times, temperature, pH, solvents, etc., to determine these characteristics. Alternative methods or variations in methods within reasonable limits that do not affect such characteristics of the new animal drug may be specified. A flow sheet and indicated equations should be submitted when needed to explain the process.
 - v. Precautions to insure proper identity, strength, quality, and purity of the raw materials, whether active or not, including:
 - a. The specifications for acceptance and methods of testing for each lot of raw material.
 - b. A statement as to whether or not each lot of raw material is given a serial number to identify it, and the use made of such numbers in subsequent plant operations.
 - vi. The instructions used in the manufacturing, processing, packaging, and labeling of each dosage form of the new animal drug, including:
 - a. The method of preparation of the master formula records and individual batch records and the manner in which these records are used.
 - b. The number of individuals checking weight or volume of each individual ingredient entering into each batch of the new animal drug.
 - c. A statement as to whether or not the total weight or volume of

- each batch is determined at any stage of the manufacturing process subsequent to making up a batch according to the formula card and, if so, at what stage and by whom it is done.
- d. The precautions used in checking the actual package yield produced from a batch of the new animal drug with the theoretical yield. This should include a description of the accounting for such items as discards, breakage, etc., and the criteria used in accepting or rejecting batches of drugs in the event of an unexplained discrepancy.
 - e. The precautions used to assure that each lot of the new animal drug is packaged with the proper label and labeling, including provisions for labeling storage and inventory control.
 - f. Any special precautions used in the operations.
- vii. The analytical controls used during the various stages of the manufacturing, processing, packaging, and labeling of the new animal drug, including a detailed description of the collection of samples and the analytical procedures to which they are subjected. The analytical procedures should be capable of determining the active components within a reasonable degree of accuracy and of assuring the identity of such components.
- a. A description of practicable methods of analysis of adequate sensitivity to determine the amount of the new animal drug in its final dosage form including finished feeds and in drinking water should also be included. Methods should be included for any premix or other intermediate mix for such drugs. Where two or more active ingredients are included, methods should be quantitative and specific for each active ingredient.
 - b. If the article is one that is represented to be sterile, the same information with regard to the manufacturing, processing, packaging, and the collection of samples of the drug should be given for sterility controls. Include the standards used for acceptance of each lot of the finished drug.
- viii. An explanation of the exact significance of any batch control numbers used in the manufacturing, processing, packaging, and labeling of the new animal drug, including such control numbers that may appear on the label of the finished article. State whether these numbers enable determination of the complete manufacturing history of the product. Describe any methods used to permit determination of the distribution of any batch if its recall is required.
- ix. Adequate information with respect to the characteristics of and the test methods employed for the container, closure, or other component parts of the drug package to assure their suitability for the intended use.
- x. A complete description of, and data derived from, studies of the stability of the new animal drug, including information showing the suitability of the analytical methods used. Describe any additional stability studies underway or planned. Stability data should be submitted for any new animal drug, for the finished dosage form of the new animal drug in the container in which it is to be marketed, including any proposed multiple-dose container, and, if it is to be put into solution at the time of dispensing, for the solution prepared as directed. If the data indicate that an expiration date is needed to preserve the identity, strength, quality, and purity of the new animal drug until it is used, the applicant shall propose such expiration date. If no expiration date is proposed the applicant must justify its absence.
- xi. Additional procedures employed which are designed to prevent contamination and otherwise assure proper control of the product. An application may be refused unless it includes adequate information showing that the methods used in, and the facilities and controls used for, the manufacturing, processing, and packaging of the new animal drug are adequate to preserve its identity, strength, quality, and purity in conformity with good manufacturing practice and identifies each establishment, showing the location of the plant conducting these operations.
- xii. The methods, facilities, and controls described under item 5 of this application conform to the current good manufacturing practice regulations in 21 CFR 200.
- xiii. An analysis of the environmental impact of the manufacturing process(es) of the article that is the subject of the requested action as specified under 21 CFR 25.1(g). Include:
 - (1) An identification of pollutants expected to be emitted;
 - (2) A citation of applicable Federal, state, and local emission requirements; and
 - (3) A certification that such emission complies with said requirements. Where there are no applicable Federal, state or local emission requirements, citation and certification shall be made to appropriate industry, advisory, or voluntary standards acceptable to the agency.
6. Samples. Samples of the new animal drug and articles used as components and information concerning them may be requested by the Bureau of Veterinary Medicine as follows:
- i. Each sample shall consist of four identical, separately packaged subdivisions, each containing at least three times the amount required to perform the laboratory test procedures described in the application to determine compliance with its control specifications for identity and assays. Each of the samples submitted shall be appropriately packaged and labeled to preserve its characteristics, to identify the material and the quantity in each subdivision of the sample, and to identify each subdivision with the name of the applicant and the new animal drug application to which it relates. Included are:
 - a. A sample or samples of any reference standard and blank used in the procedures described in the application for assaying each new animal drug and other assayed components of the finished new animal drug.
 - b. A representative sample or samples of each strength of the finished dosage form proposed in the application and employed in the clinical investigations and a representative sample or samples of each new animal drug from the batch(es) employed in the production of such dosage form.
 - c. A representative sample or samples of finished market packages of each strength of the dosage form of the new animal drug prepared for initial marketing and, if any such sample is not from a representative commercial-scale production batch, such a sample from a representative commercial-scale production batch, and a representative sample or samples of each new animal drug from the batch(es) employed in the production of such dosage form, provided that in the case of new animal drugs marketed in large packages the sample should contain only three times a sufficient quantity of the new animal drug to allow for performing the control tests for drug identity and assays.
 - ii. The following information shall be included for the samples when requested:
 - a. For each sample submitted, full information regarding its identity and the origin of any new animal drug contained therein (including a statement whether it was produced on a laboratory, pilot-plant, or full-production scale) and detailed results of all laboratory tests made to determine the identity, strength, quality, and purity of the batch represented by the sample, including assays.
 - b. For any reference standard submitted, a complete description of its preparation and the results of all laboratory tests on it, if the test methods used differed from those described in the application, full details of the methods employed in obtaining the reporting results.
7. Analytical Methods for Residues. Applications for new animal drugs shall include a description of practicable methods for determining the quantity, if any, of such drug in or on food, and any substance formed in or on food because of its use, and the proposed tolerance or withdrawal period or other use restrictions for such drug if any tolerance or withdrawal period or other use restrictions are required in order to assure that the proposed use of such drug will be safe. When data or other adequate information establish that it is not reasonable to expect the new animal drug to become a component of food, assay methodology is not required.
- i. The kind of information required by this subdivision may include: Complete experimental protocols for determining drug residue levels in the edible products, and the length of time required for residues to be eliminated from such products following the drug's use; residue studies conducted under appropriate (consistent with the proposed usage) conditions of dosage, time, and route of administration to show levels, if any, of the drug and/or its metabolites in test animals during and upon cessation of treatment and at intervals thereafter in order to establish a disappearance curve; if the drug is to be used in combination with other drugs, possible effects of interaction demonstrated by the appropriate disappearance curve or depletion patterns after drug withdrawal under appropriate (consistent with proposed usage) conditions of dosage time, and route of administration; if the drug is given in the feed or water, appropriate consumption records of the medicated feed or water and appropriate performance data in the treated animal; if the drug is to be used in more than one species, drug residue studies or appropriate metabolic studies conducted for each species that is food-producing. To provide these data, a sufficient number of birds or animals should be used at each sample interval. Appropriate use of labeled compounds (e.g. radioactive tracers) may be utilized to establish metabolism and depletion curves. Drug residue levels ordinarily should be determined in muscle, liver, kidney, and fat and where applicable, in skin, milk, and eggs (yolk and egg white).

As a part of the metabolic studies, levels of the drug or metabolite should be determined in blood where feasible. Samples may be combined where necessary. Where residues are suspected or known to be present in litter from treated animals, it may be necessary to include data with respect to such residues becoming components of other agricultural commodities because of use of litter from treated animals.

ii. If such new animal drug is one which has been shown to induce cancer when ingested by man or animal or after other tests which are appropriate for the evaluation of the safety of such drug and the Secretary is requested to find that, under the conditions of use specified in the proposed labeling and reasonably certain to be followed in practice, such drug will not adversely affect the animals for which it is intended and that no residue of such drug will be found in any edible portion of such animals, after slaughter or in any food yielded by or derived from the animal, methods of analysis shall be submitted in such form as to be suitable for publication in the Federal Register.

8. Evidence to establish safety and effectiveness.

i. An application may be refused unless it contains full reports of adequate tests by all methods reasonably applicable to show whether or not the new animal drug is safe and effective for use as suggested in the proposed labeling.

ii. An application may be refused unless it includes substantial evidence, consisting of adequate and well-controlled investigations, including field investigation, by experts qualified by scientific training and experience to evaluate the effectiveness of the new animal drug involved, on the basis of which it could fairly and reasonably be concluded by such experts that the new animal drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the proposed labeling.

iii. An application may be refused unless it contains detailed reports of the investigations, including studies made on laboratory animals, in which the purpose, methods and results obtained are clearly set forth of acute, subacute, and chronic toxicity, and unless it contains appropriate clinical laboratory results related to safety and efficacy. Such information should include identification of the person who conducted each investigation, a statement of where the investigations were conducted, and where the raw data are available in the application.

iv. All information pertinent to an evaluation of the safety and effectiveness of the new animal drug received or otherwise obtained by the applicant from any source, including information derived from other investigations or commercial marketing (for example, outside the United States), or reports in the scientific literature, both favorable and unfavorable, involving the new animal drug that is the subject of the application, and related new animal drugs shall be submitted. An adequate summary may be acceptable in lieu of a reprint of a published report that only supports other data submitted. Include any evaluation of the safety or effectiveness of the new animal drug that has been made by the applicants veterinary or medical department, expert committee, or consultants.

v. If the new animal drug is a combination of previously investigated or marketed new animal drugs, an adequate summary of preexisting information from preclinical and clinical investigation and experience with its components,

including all reports received or otherwise obtained by the applicant suggesting side effects, contraindications, and ineffectiveness in use of such components, shall be submitted. Such summary should include an adequate bibliography of publications about the components and may incorporate by reference information concerning such components previously submitted to the Food and Drug Administration by the applicant; with written authorization, information may also be incorporated from the material that another applicant has on file with the Food and Drug Administration. Each ingredient designated as active in any new animal drug combination must make a contribution to the effect in the manner claimed or suggested in the labeling, and, in the absence of express labeling claims of advantages for the combination such a product purports to be better than either component alone, it must be established that the new animal drug has that purported effectiveness.

vi. An application shall include a complete list of the names and post office addresses of all investigators who received the new animal drug. This may be incorporated in whole or in part by reference to information submitted under the provisions of 21 CFR 511.1.

vii. Explain any omission of reports from any investigator to whom the investigational new animal drug has been made available. The unexplained omission of any reports of investigations made with the new animal drug by the applicant or submitted to him by an investigator or the unexplained omission of any pertinent reports of investigations or clinical experience received or otherwise obtained by the applicant from published literature or other sources that would bias an evaluation of the safety of the new animal drug or its effectiveness in use, constitutes grounds for the refusal or withdrawal of the approval of an application.

9. **New animal drugs subject to section 512(n) of the act.** If the application is for a new animal drug subject to the certification provisions of section 512(n) of the act and the drug is included in regulations promulgated under section 507 of the act, the applicant may be exempted from the submission of some of the information required by subparagraph (8) of this paragraph if the application includes data adequate to prove that the new animal drug is comparable to the new animal drug for which certification has been previously provided.

10. **Environmental Impact Analysis Report.** The applicant is required to submit an environment impact analysis report analyzing the environmental impact of the ultimate use or consumption of the new animal drug pursuant to 21 CFR 25.1.

11. **Confidentiality of Data And Information In A New Animal Drug Application.** The availability for public disclosure of any record in the NADA file shall be handled in accordance with the provisions of 21 CFR 514.11.

i. After approval has been published in the Federal Register certain data and information (as indicated in 21 CFR 514.11(e)) in the NADA file are immediately available for public disclosure unless extraordinary circumstances are shown. This information shall be known as a Freedom Of Information (FOI) Summary.

ii. Preparation of the FOI Summary shall be by the applicant according to guidelines specified by the agency.

Literature reviews provide excellent input for the development of a protocol. They also provide an indication of the types of studies which have already been conducted and for which data exists. Literature reviews should include all information relevant to the safety and effectiveness of the drug including:

1. Publications and data on the specific disease organism;
2. Studies conducted in the proposed animal specie (target organism);
3. The incidence and impact of the disease;
4. Environmental or other factors causing the disease; and
5. Known techniques for controlling the disease in the target species or other animals.

Effectiveness Studies

Each disease claim must be supported by a minimum of two well controlled studies demonstrating the effectiveness of each label claim. Well controlled studies must include the following three groups of test animals: (a) non-infected, non-medicated control group; (b) infected, non-medicated control group; and (c) infected medicated control group.

Effectiveness studies must: (a) demonstrate the effective drug concentration to inhibit or kill the pathogen in vitro, including time and dose relationships; (b) establish the minimum effective dose for control of the pathogen in laboratory infected test organisms; (c) determine the acute LD₁ to host species to be treated and EC₉₉ to pathogen; (d) demonstrate effective control for the disease in laboratory infected host species; and (e) demonstrate efficacy under production conditions.

Regardless of the proposed use of a drug, an evaluation of toxicity must include, as a minimum, testing of the parent compound in both a rodent and a nonrodent mammalian species by daily, oral administration for at least 90 days.

Generally, the rat and dog are the preferred species because of the large base of reference data available for comparison.

Tissue residue studies should be conducted to show the levels of the drug or its metabolites in test animals upon completion of the treatment and at intervals thereafter in order to establish its rate of disappearance from the organism.

In order for government surveillance programs to detect excessive levels of drug residues, the sponsor must provide an analytical method for residue testing: (1) an acceptable written analytical method must be provided for the detection of residues in edible tissues; (2) an alternate residue method confirming the preferred method must be provided; and (3) the fate of the parent compound, metabolites and degradation products must be determined.

Safety Studies

1. Determine acute oral toxicity for rats.
2. Determine subacute (90-day) oral toxicity for rats and dogs.
3. Determine toxicity for target species under single and repeated dosages at recommended treatment levels (3x and 5x).
4. Determine residue dynamics (time to clear blood, bile, urine and edible tissues).
5. Identify metabolites and residues found in flesh.
6. Determine residue levels in the animal treated at recommended use rates under normal production conditions.
7. Identify residues and their persistence in soil, water and benthic organisms if the chemical is used under pond conditions.
8. If the compound does not degrade to non-toxic components, techniques for its removal or counter-action should be provided.

Labeling Requirements for NADA's

The label must indicate (1) the specie of animal to be treated; (2) dosage level; (3) indications for use; (4) adequate directions for use; and (5) cautions, warnings, and restrictions (including pre-slaughter withdrawal times).

References

1. Federal Food, Drug and Cosmetic Act (1979) U.S. Government Printing Office, Washington, DC 20402.
2. Title 21 Code of Federal Regulations (1980) Part 500 to 599, Subchapter E: Animal Drugs, Feeds, and Related Products. U.S. Government Printing Office, Washington, DC 20402.
3. New Animal Drug Application, Form FD 356 (1978), Department of Health and Human Services, Food and Drug Administration (HFV-140), 5600 Fishers Lane, Rockville, MD 20857.

A Balanced Governmental Role in Aquaculture

Robert D. Wildman and William N. Shaw*
National Oceanic and Atmospheric Administration
U.S. Department of Commerce

ABSTRACT

The Federal Government is very active in its attempt to develop aquaculture in the United States. This paper discusses two of these activities, i.e., drafting of a National Aquaculture Plan and the ongoing activities of the Joint Subcommittee on Aquaculture (JSA).

The first draft of the National Aquaculture plan is completed and is presently being reviewed. One of the main parts of the Plan includes 12 species plans -- baitfish, catfish, clam, crawfish, large-mouth bass, mussel, oyster, freshwater prawn, marine shrimp, salmon, striped bass and trout. These plans discuss approaches to resolve the problems confronting their commercial development.

JSA has been active since late 1975 and currently meets on a quarterly basis. The membership of this committee consists of representatives from all federal agencies involved in aquaculture. The committee contains a number of panels, e.g., legislation, translation, statistics, effluent utilization, regulation, extension services, financial assistance, and fish health. One of the major activities sponsored by the legislation panel of JSA is a study being conducted by Aspen Systems Corporation entitled, "State and Federal Regulatory Impact on Aquaculture Development".

INTRODUCTION

What is a balanced governmental role in aquaculture? Is the present role unbalanced? What is the present role the Federal Government is playing to enhance the development of aquaculture in this country? These are good questions and deserve straight-forward answers. But, when do you ever get a straightforward answer from a bureaucrat!

Many will claim that the Federal Government is playing a negative role. For example, no aquaculture bill has been passed and many believe it is because the "Feds" cannot decide among themselves who should be the lead agency. Many claim the permit system is the biggest factor that is inhibiting the growth of aquaculture; there is no low cost, speedy, one-stop system. Many believe the federal research effort is duplication, lacks goals, milestones, and coordination. Although there is some truth to the claims stated

above, there is presently an all-out effort by supporters of aquaculture in the Federal Government to enhance the development of aquaculture. In the next few minutes, I will relate to you the positive role being taken by the Federal Government.

Presently, several aquaculture bills are being considered by Congress. Of particular interest is Senator Inouye's bill S. 1650, "The National Aquaculture Act of 1979." This bill does not designate a single lead agency. For the first time, all three agencies (Commerce, Interior, and Agriculture) supported this bill at the October 16 hearings before the Committee on Agriculture, Nutrition, and Forestry and the Committee on Commerce, Science, and Transportation. There are two aspects of the bill I would like to highlight. First, the bill calls for the three Secretaries of Agriculture, Commerce, and Interior to be responsible for developing a National Aquaculture Development Plan in consultation with other appropriate federal agencies. Second, the bill calls for the creation of a Joint Subcommittee on Aquaculture. You should be aware that these two provisions of the bill are already being carried out and I would like to describe the actions of each at this time.

The National Aquaculture Development Plan

For the past six months, a Task Force containing members from each federal agency involved in aquaculture has been working on the first draft of a National Aquaculture Plan. The Plan will address such areas as: (1) the advantages the U.S. has in developing aquaculture; (2) potential for aquaculture in the U.S.; (3) current support of aquaculture research and development in the U.S. and internationally; (4) commercial investment in aquaculture; (5) current sources of financial programs for aquaculture industries; (6) barriers to success of aquaculture in the U.S.; (7) proposed advisory and coordinating activities; (8) proposed education and information programs; (9) required financial programs; (10) annual cost to implement plan and conduct programs for the first 5 years; (11) long term program needs; and (12) expected benefits of the national program. A major section of the Plan deals with proposed species development programs. In this section, there will be 12 species plans, i.e., baitfish, catfish, clam, crawfish, large-mouth bass, mussel, oyster, freshwater prawn, marine shrimp, salmon, striped bass, and trout. These plans include approaches to resolve the problems confronting their commercial development. Areas being identified include programs of research development, other activities required, and funding. The

National Plan is actually the first interaction of what will be a continuing process of updating and expanding the original plan document. The uniqueness of this Plan, over all other previously prepared for aquaculture, is that it is a joint federal plan, not one written by a single federal agency.

This past September, the preliminary draft was reviewed at a workshop held in Washington, D.C., by close to 200 members of the aquaculture community, including representatives from industry, academia, state and federal agencies, and Congressional staffers. As a result of this workshop, the Plan is being redrafted and will soon be reviewed by the aquaculture community. In essence, the Federal Government is a step ahead of the pending legislation which will call for a National Aquaculture Plan. If a bill is passed this year, the first draft of the Plan will have already been completed, thus giving us plenty of time to refine it.

Joint Subcommittee on Aquaculture

As I stated earlier, there are many that will claim that the government's role in aquaculture is fragmented and uncoordinated. Each agency is viewed as independently doing "their thing". Congress has criticized the federal role in aquaculture, therefore, in past and present aquaculture bills they call for an Interagency Committee on Aquaculture. Although chairmanship of this committee varies with the different bills, the functions of this committee are quite clear. They are: (1) to insure that there is a continuing exchange of information among the agencies represented on this Committee with respect to the nature and status of the programs or projects being carried out by such agencies which relate, or which may apply, to aquaculture; and (2) to review on a continuing basis the relevant programs and projects of all federal agencies.

As far back as 1975, the late David Wallace, then Associate Administrator for Marine Resources in NOAA, recognized the problem and on November 18, 1979, NOAA sponsored the first Interagency Aquaculture Workshop. As a result of this workshop, there was a unanimous agreement among the agencies that an interagency committee for aquaculture be formed for the following reasons:

- (1) there is no current existing mechanism for coordinating efforts and for informing others;
- (2) there are no lines of communication between those carrying out research and those involved in regulatory functions;

- (3) there are several agencies carrying out aquaculture research within overlapping areas;
- (4) Congress is distressed that there is a lack of coordination;
- (5) if we do not coordinate our own efforts, Congress will oblige us to do it; and
- (6) it would have appeal to OMB when we go before our examiners.

An interagency committee for aquaculture was formed and has met quarterly for the past three years. The committee is made up of a number of panels -- legislation, translation, statistics, effluent utilization, regulation, extension services, financial assistance, and fish health. Time does not permit me to describe the functions of each Panel, therefore, I will limit my remarks to the regulations panel since this is the theme for the Symposium we are attending today.

Dan Hunt, the moderator of the present session, is the chairman of this panel. It was recognized immediately by this group that regulations are one of the major reported restraints in the development of aquaculture in the U.S. Voices from all "necks of woods" were crying out that the permit system was impossible or too costly to deal with. For example, the certification of drugs was either not possible, too costly, or too time consuming; that aquaculture was not treated like agriculture; coastal zone planners did not recognize aquaculture as a priority use of the coastal environment, and so forth. Were all these cries for real? To date, they were independent voices and never documented into a single package by a single firm or individual. Therefore, the regulation panel, recognizing the problem, prepared a proposal and obtained the necessary federal support for a contract to study the "State and Federal Regulatory Impact on Aquaculture Development." See Appendix I for a description of the proposed study.

Briefly, the contractor is reviewing existing and pending federal and state regulations impacting on the development of commercial aquaculture activities. In addition, the contractor shall conduct case studies of ten commercial operations in the U.S., representing marine and freshwater species, to determine the practical effects of regulatory impacts.

In September, 1979, the contract was awarded to Aspen Systems Corporation. They are presently reviewing the State and Federal regulations which affect aquaculture. Initially, they have made a literature search of all

aquaculture publications that refer to regulations. A subcontract was given to Pan-Tek Corporation to look at the 10 commercial firms. These firms have been interviewed and a report is now being prepared. Although I am not at the liberty to give you the name of these firms, I can say they represent companies that are commercially rearing the following species: oysters, mussels, freshwater prawns, marine shrimp, catfish, baitfish, crawfish, trout, and salmon.

Some preliminary results of these interviews were made available to us by Pan-Tek and I will relay a few of them to you. The type of firms interviewed range from small operations to large corporations. Although the total costs to obtain the necessary permits for each aquaculture venture may be the same, these costs affect, as you can imagine, small companies far greater than large corporations. What I am really saying is that although big companies do not like the delays, etc., they can afford the costs.

What are the regulatory impacts on the 10 commercial firms that were interviewed by Pan-Tek? As one might expect they vary considerably based on the size of the operation, species being cultured, location, and method used to culture the species.

The number of regulatory impacts affecting these firms is quite large and it is not possible to give them all to you at this time. A complete analysis is being done by the contractor. Therefore, in the few minutes I have left, I will just mention a few examples. These are taken directly from Pan-Tek's trip reports.

1. FDA certification is expensive and slow. Only a few drugs and medicines have been certified to date. A number of others are being used in a quasi-legal manner in both commercial and governmental operations. A battery of options are important since the operator is always facing a high risk possibility of an unanticipated or currently untreatable epidemic. The aquaculture industry is too small to undertake this effort and, for the same reason, the commercial drug companies are uninterested. One firm stated that some drugs and medicines are often prohibited to commercial operators, but used in State and Federal operations. And, finally, a statement was made that the number one problem is to obtain FDA certification of an adequate inventory of drugs to treat diseases.

2. The most critical constraint on the potential aquaculture industry is the restriction on the importation of endangered and non-indigenous species and their fully commercialized technologies from overseas, i.e., Japan, Taiwan, China, etc.

3. OSHA has adopted deep water diving regulations which apply to shallow water aquaculture divers. A ratio of three stand-by divers to one operational diver was so prohibitive that one firm eliminated this function and the divers were discharged.

4. In the regulatory environment, one company stated that they dealt with 32 separate agencies. This company felt that the regulatory process was fraught with ignorance, inexperience, amateurism, confusion, delay and outright selfishness. Another firm stated it had to deal with 55 agencies at a cost of hundreds of thousands of dollars.

5. The following are statements from 4 firms related to expansion: (a) holding and expanding underwater land is a key regulatory constraint; (b) the time, money, and energy spent by the President and his staff on the permit process has clearly raised the problem to an emotional level; (c) the most important constraint concerned new land for expansion; and (d) local zoning applications have been erratic and destructive to the establishment of new facilities either in country or city areas.

The above are highlights of some of the regulatory constraints that are affecting commercial aquaculture firms. The picture would not be complete if I gave only one side. The following is a statement by one of the 10 firms that was interviewed: "The ... industry is not aware of much restrictive regulation or may not (be) complying with a lot of unnecessary verbage." I believe this statement speaks for itself.

In summary, I believe the Federal Government is making an attempt to enhance the development of aquaculture. The study being supported above is an example where regulatory constraints will be documented and, hopefully, this will lead to legislative or procedural changes that will alleviate some of the problems.

The development of a National Aquaculture Plan is a very positive step. For the first time, a document will be available which will state what needs to be done in what order, and how much it will cost. Finally, it is hopeful that an aquaculture bill will be passed this year. It is interesting to note that in the regulation study, several firms felt that this was number one priority and that many constraints on aquaculture stem from a lack of national recognition of this field, a declaration of aquaculture as a national priority, and a development plan. The minimum U.S. plan should ensure maximum interagency and interprogram support and a minimum of duplication, confusion and inhibiting dollar costs and delays. Simplification,

standardization and one-stop centers can remove some restrictions. Whether or not this will come to pass, I cannot say. Only time will tell!

APPENDIX I

Description of Proposed Study on the State and Federal Regulatory Impact on Aquaculture Development

Introduction

The purpose of the study is to collect information on regulatory restrictions facing commercial aquaculture operations in the U.S. The information is essential to the enactment of effective legislation for the development and support of aquaculture. It will also be helpful to State and Federal agencies with responsibilities in the field of aquaculture and will assist those in the private sector who are considering investing in fish culture projects.

A. Statement of Work

1. The contractor shall conduct a literature review and develop a cross-referenced descriptor list to identify the overall parameters of the issue. The resulting index terms with definitions shall be used to categorize the material gathered on the following work elements:
 - a. The contractor shall identify and index existing and pending federal regulations impacting on the development and operation of commercial aquaculture activities.
 - b. The contractor shall identify and index existing and pending State regulations impacting on the development of commercial aquaculture activities in eight States representing six separate geographic regions in the U.S. The states selected by the contractor must be approved by the Project Officer.
2. The contractor shall conduct case studies of ten commercial aquaculture operations in the U.S. representing marine and freshwater species to determine the practical effects of regulatory impacts. These commercial operations shall include but not be restricted to trout, catfish, salmon, shrimp (freshwater and marine species) and mollusks. Each case selected for study must be individually approved by the Project Officer.
3. The contractor shall develop a representative flow-chart time line utilizing information obtained in the items listed above (1.a. and b., 2) to identify

those regulations or requirements that must be dealt with during the development and operation of aquaculture activities in the U.S.

The objective of this contract is to develop a data base of legal restrictions impacting upon the development and operation of aquaculture in the U.S. for the purposes of improving or streamlining State and Federal regulations affecting aquaculture development and to provide guidance to the developing industry.

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INVESTIBLE AQUACULTURE IN THE UNITED STATES

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INTRODUCTION

Fortuitism has been the essential force shaping the development of aquaculture from its ancient origins in China and the Indo-Pacific region to recent times of modern research and development programming. Probably the first aquaculturist was Wen Wang¹, who in 1100 BC in the Honan Province of China, confined carps in ponds and studied their behavior and cultural requirements for the pleasure and artistic value of what is now known as koi culture. However, it was Fan Lee's Fish Culture Classic, published in China in 460 BC², describing the rearing of carp fishes as a food crop that may be considered the start of the practical aquaculture for sustenance and profit which interests us today. In the western centers of cultural development, particularly in the Mediterranean, fish and shellfish farming was practiced several thousand years ago, first by the Egyptians, then the Etruscans, and subsequently, the Romans with mollusc culture in Lake Lucrine³ and with fresh and brackish water carp, eel, and mullet culture.

Throughout the long history of the development of fishfarming, the rate of change has been extremely slow, and for the most part dependent solely on farmers' observations and empirically encountered influences on the growth and behavior of a few species of fish and shellfish in specific environments. Thus, observation and chance, rather than the hard evidence from designed, controlled experiments and carefully planned pilot farm trials had brought aquaculture as a food generating mechanism to a new point of departure following World War II.

Technology

Most of the recent technological advances have come from biological scientists whose essential contributions to an understanding of the life processes of the crop animals are crucial to the advances of the technology, but who have often neglected to consider the problems of systems engineering, scale, socio-economic factors, and business management. Furthermore, aquaculture, like most of the bioindustries, is extremely site-sensitive, and is highly dependent on the specific characteristics of the ecosystem and the society in which it is practiced. It has long been practiced as an art, tailored to the site and culture, and only recently, as we have gained better understanding of ecosystems management principles, bioengineering mechanisms, and institutional influences, have we begun to translate the art - by quantification, integration, and generalization - into a practical technology. This technology is now, in fact, transferable, within latitudinal limits around the globe, with appropriate adaptation to the environmental and social parameters of the recipient society.

Nevertheless, aquaculture, as a food production mechanism in the industrial nations, is actually in a very early stage of this kind of development, and is only now being considered as a vertically integratable industrial system, from sunlight to market. It can only now be subjected to a detailed business analysis in order to present an investible enterprise to the investment community.⁴ The most prolific aquaculture practiced in the world today, Chinese carp polyculture integrated with terrestrial animal husbandry, achieved a moderate level of effectiveness as a subsistence farming method probably three or four hundred years ago. Then the productivity probably did not exceed 200 to 300 kilograms of whole fish per hectare per year. It has only been within the last twenty years, however, that in an organized effort to respond to the pressure of the food needs of a massive population, China has established fish breeding centers and scientific laboratories to study the requirements for increased productivity.⁵ Controlled, induced spawning, selective breeding, larviculture, disease control, and nutrition are research areas which are receiving a good deal of support. The extensive use of polyculture systems has resulted in increases in productivity to more than 10,000 kilograms per hectare per year.

In Japan, Israel, Taiwan, and some few other countries, recently including the Philippines, similar intensification of culture technologies and careful coordination of the many components of the complex system have resulted in similar increases in productivity and economic justification. In these countries, the national planners have recognized the urgent need to produce high protein foods for dense human populations living on limited arable land. The national commitment to aquaculture is firm, and the technologies, as they have matured in recent years, follow closely the analogous methods of intensive husbandry practiced with terrestrial animals. There is a spectrum of techniques that ranges in intensity from the methods of wildlife management of low density populations surviving on the natural carrying capacity of unamended ecosystems⁶, yielding only a few hundred kilograms per hectare per year, to closed, high-technology, water recycling systems in which all of the known environmental parameters are fully controlled.^{7,8} There is a substantial energy subsidy provided to these latter high-technology production systems in the form of feed, water movement or exchange, temperature regulation, aeration, etc., resulting in the very high productivity of many thousand kilograms per hectare per year.

Intermediate between these extremes are the various systems of pond culture in which, for instance, animal manures or commercial chemical fertilizers are added to enhance primary productivity, not only for filter feeding crop animals or obligate herbivores, but for total biomass generation. When the farming system is freed from the dependence on a natural recruitment of juveniles from the wild population by having seed animals supplied from a hatchery facility, then stocking rates can generally be increased to densities far above the natural carrying capacity of the ecosystem. When this is accomplished, formulated rations must be supplied to supplement the natural feed. Under such conditions, the natural rate at which feces and other metabolic wastes can be assimilated by the pond ecosystem may well be exceeded, and a further energy subsidy in the form of aeration for increased oxidation or water exchange for increased dilution must be provided. When the latter mechanism is elected, the optimum

form of the culture confinement becomes anisometric, and a long, narrow raceway system proves most efficacious. Another means of accomplishing dilution of metabolites is by cage or pen culture which depends on operating in large water volumes, or in especially favorable hydrological conditions with favorable currents. In these cases of high intensity culture, the capital requirements may be quite high, but the commensurate increases in productivity may well justify the investment.

Valliculture, as it is practiced in the Mediterranean⁹, and tambak-culture in Indonesia and other parts of Southeast Asia, employ low technology, utilizing natural recruitment of juveniles for seed, no feed, and little control of predators and competitors.¹⁰ In this aquafarming system, capital investment is low, the operating budget is minimal; labor inputs are also low, and the required management skills are simple. Commensurate with the low risk, the productivity is low, but generally adequate to satisfy the goals of the subsistence farmer or the cooperative. When the purpose of the farming operation is subsistence of a single family unit or a village community, the operation is viewed as a way of life. The criteria for success or failure are those that relate essentially to the survival of the family, and economic profit or loss is rarely measured or judged.

Even with intensification of the production system employing small farm ponds and some degree of supplement to the natural carrying capacity in the form of green or animal manures, natural or formulated feeds, and some water quality management, the classic business rationale of these essentially artisanal culture systems remains the same.¹¹

An aquafarming venture resulting from further intensification of production methods utilizing numerous ponds in a coordinated system requiring more sophisticated water control measures and structures; higher density stocking, generally from a seed production facility such as a hatchery; and complex, formulated rations can no longer be considered subsistence farming. Such a system can only be operated successfully if it is centrally controlled and can generate a profit and a fair return on the considerable capital investment required to establish the farm. What may in fact be a tolerable set of operating conditions for subsistence aquafarming conducted as a way of life, may be a problem area of major consequence to corporate vertically integrated large-scale aquaculture (VIA).

It is not scale alone which differentiates between traditional aquaculture and the new aquatic agribusiness which can now be seen in its incipient forms in many areas of the world. This new VIA industry may be viewed by the investment community as being correlative with corporate terrestrial agribusiness. It is developing without necessarily recapitulating all the evolutionary stages of traditional agriculture, since the broad data base in the biological and engineering sciences, as well as the extensive experience with agribusiness management, provides a resource that was not available to terrestrial agriculture during similar stages of development.

By VIA enterprises, we mean those comprehensive aquafood businesses which are centrally managed, so that all the components of the system,

from the input of energy from the sun in the production phase, through processing, packaging, transport and the sale of the product in the marketplace, are coordinated functions, and are kept in balance.⁴ The management rationale receives its guidance from market parameters, from which, it must be realized, the enterprise gains its primary justification.

Species Selection and Market Criteria

Probably the first among the criteria that will measure the marketability of aquafoods are the traditional food habits and taboos of the variously differentiated human cultures. From among the thousands of aquatic plants and animals that inhabit the fresh and saline waters of the earth, only a certain few species are being selected as favored candidates for culture. The taste and food preferences reflect traditional availability, ease of capture or culture, preservation in storage, ease of preparation, wholesomeness and, of course, the degree to which the user's basic nutritional requirements can be satisfied. In addition, social identity and status-seeking play an important role in food preferences.¹² Flavor is probably used by most people as the first means of judging the acceptability of a food.

Other subjectively judged characteristics that serve as a critical early screen in evaluating acceptance are such appearance factors as color, luster, and form.

A flaky and firm-textured fish flesh appears to have wide acceptance, although there are many instances where aquatic foods of soft and 'mushy' texture are widely utilized. Raw seafoods, such as those eaten in the Orient, and to a lesser degree in Europe and North America, have textures that may turn away the uninitiated - even though the flavor may be highly rated. The aversion among some sectors of Western societies to eating raw oysters and other molluscs in the uncooked state may be an example of this behavior. On the other hand, among oyster-eating aficionados, the texture of 'fat' (high glycogen) raw oysters is an important determinant in acceptance.

Hedonic testing of aquafoods clearly reveals that consumer preference and acceptance is an integration of all the factors noted above.¹³ Such testing reflects the strong influence of traditional food habits and cultural experience. These important matters, along with the many biological parameters, must be factored into the decision regarding the choice of candidate species for the research and development programs that will lead to domestication and commercial culture of aquatic plants and animals.

Another group of market criteria which must be considered, relates to problems of manufacturing efficiency such as dress-out percentages. For example, the bone structure of a vertebrate animal will influence the dress-out weight in filleting, or the ratio of the weight of the exoskeleton to the weight of marketable product in a crustacean. The meat-to-shell weight ratio in a shucked oyster, or the recoverable and marketable components of the numerous other invertebrates, such as squid, that might be considered for commercial aquaculture are other examples.

Ultimately the essential market criteria that will influence the choice of candidates for culture must be the size and structure of the market and the elasticities of demand and price for the aquafood products that can be grown.

Locating The Aquafarm

Following the evaluation and selection of the candidate species appropriate to satisfy a given market, and the determination of the most appropriate culture technology that can be applied to produce the product, one must then perform a most critical judgment regarding site selection.¹⁴ In this regard, satisfying the ecological requirements such as water quality characteristics, soils, meteorological criteria etc. may not be sufficient in itself; for the economic and political climate as well as the cultural and sociological milieu can also be limiting influences on the successful application of a business plan that is otherwise well based on marketing and technological judgments.

Poor judgment in site selection has in many cases in the United States accounted for the collapse of the enterprise. Failures due to hurricanes, water quality and supply, regulatory constraints, or acceptance of the venture by the indigenous fishing community have occurred.

STATUS OF WORLD PRODUCTION

The last reported authoritative summary of the world status of aquaculture production was prepared by T.V.R. Pillay, of FAO, in 1975 in preparation for the FAO Technical Conference on Aquaculture, held in Kyoto, Japan. He reported some six million metric tonnes as the worldwide aquaculture production at that time. About four million tonnes were in finfish; one million tonnes in molluscs, including oysters, mussels, and clams; and about one million tonnes in seaweeds. Only 16 thousand tonnes were in shrimps and other crustaceans. According to Pillay, most of the finfish are produced in freshwater with over two million tonnes from mainland China alone, and most of the remainder produced in ten nations in Southeast Asia. The production is predominantly from the culture of several species of carps, in a vast number of small farm ponds, employing simple technologies and marketing methods. The entire capture fishery of the world has been producing in recent years about 65-70 million tonnes per annum. Thus, aquaculture, which had doubled its production in the five years prior to the 1975 survey, accounted for about 10% of worldwide aquafood production.¹⁵ All of the aquaculture production was for direct human consumption, whereas only about two-thirds of the capture fishery is consumed directly as human food, the remaining catch going into fishmeal and other industrial products.

There is a great variability among countries in their capability to produce cultured aquafoods. For instance, China, which consumes 9 kilograms, liveweight, of aquafoods per capita per annum, provides 40% of its total fish supply by culturing 2.2 million tonnes of finfish.^{15, 16} India, which consumes only 3 kilograms per capita, produces annually about 490,000 tonnes. Japan, which is the largest fishing nation in the world, capturing 10.6 million tonnes from the seas, supplies about 10%, or 500 thousand tonnes, of its total fish consumption by aquaculture. Japan depends heavily

on aquafoods, consuming 75 kilograms per capita per annum. Russia produces about 210 thousand tonnes through aquaculture and uses 28 kilograms per capita per annum. Most of the food seaweeds are produced in Japan and Korea and are consumed directly as human food. The macrophytic algae are also grown for the production of industrial phycocolloids; carrageenans, agars, and algin.

STATUS OF U.S. AQUAFOOD PRODUCTION

The development of aquaculture in the United States had its origin about 1850, but has not been motivated by the same economic forces which shaped aquacultural developments in Asia. Except for oyster farming, subsistence aquafarming found little justification in competition with other high protein food production systems in the United States. Terrestrial animal husbandry developments which resulted in substantial animal protein production, particularly in the geographical interior regions, satisfied the nutritional and gustatory need. The marine capture fisheries contributed an additional significant share of animal protein to the coastal communities. U.S. consumption of aquafoods remained static at about 4.5 kilograms per capita per annum for most of this century until the recent decade when a steady increase was realized. The per capita consumption in 1978 at 6.1 kilograms is a record.¹⁶

Although culture of warmwater fishes that subsist at the lower trophic levels for energy efficient protein production at least cost, holds interest for many U.S. research workers, there is little commercial interest yet expressed in the culture of such important food fishes as the carps and tilapias.

United States aquaculture has focused primarily on the culture of high-value species including penaeid and freshwater shrimps, the salmons and trouts, American channel catfish, oysters and other molluscs. The American lobster, Homarus americanus, and some high-value finfish such as pompano, Trachinotus carolinus, have received some considerable research attention. Several undercapitalized, abortive efforts have been based on the commercial culture of these luxury items. Generally, however, these ventures were poorly conceived and were not based on sufficiently sound understanding of the biology of the crop animal, systems engineering of the culture facility, or appropriate business management.

U.S. aquaculture in 1976 produced 65 thousand tonnes¹⁵, which based on food fish, was approximately 6% of U.S. landings and 3% of U.S. consumption. Although, in the aggregate, this cultured production is low compared to U.S. consumption, the influence of culture is now very important in some species. About 30% of our total landings of Pacific salmon, and over half of the Columbia River salmon caught by commercial and sports fishermen, are hatchery reared before being released into the wild for growout. Private aquaculture produces over 40% of our oysters, half our catfish and crawfish, and nearly all our rainbow trout. The U.S. imports 60% of the fishery products it consumes. The trade deficit in 1978 was about \$2.5 billion which represented about 30% of the total deficit from non-petroleum products. 16, 17

Other sectors of U.S. aquaculture include production of baitfish, marine baitworms and tropical aquarium fishes; stocking for enhancement of natural populations of sport fishes by federal and state hatcheries of striped bass, largemouth bass, and trouts; experimental studies with other food species such as pompano, anadromous Atlantic salmon, the northern lobster, eels, dolphin fish, rabbitfish, carps, abalone, bay scallops, clams, and certain other sportfishes, including walleye, sunfishes, pike; and low technology culture of some marine plants.

Salmonids

The earliest aquaculture in the United States, dating back to the mid-nineteenth century, entailed the management of salmonid populations. These may be considered in two groups - the non-anadromous species of Salmo and Salvelinus, e.g., rainbow trout, brook trout, cutthroat trout, lake trout, brown trout, and their hybrids; and the anadromous Atlantic salmon, Salmo salar, and the Pacific salmons, Oncorhynchus species, e.g., coho, chinook, chum, pink, and sockeye. With the development of hydroelectric dams on several of our northern rivers that interrupted salmon migration to the spawning grounds, research in hatching and culture to the smolt stage was undertaken to compensate for the loss of spawn and thus to sustain wild populations. The stimulus resulting from the basic biological research and hatchery engineering provided a greater awareness of the potential for commercial fishfarming management as a means of augmenting supplies from the catch of the wild fisheries.

Salmon

In the United States, the most valuable combined commercial, recreational, and subsistence fishery is based on the Pacific salmon resource. The retail value of U.S. commercial salmon products is estimated at \$1.2 billion annually, and the Pacific coast sport salmon fishery is valued at \$212 million.¹⁸ Over 700 thousand salmon are taken annually in the Indian tribal subsistence fisheries, a tradition of immeasurable importance to their culture and welfare.¹⁸ There are more than three thousand salmon producing streams in California, Oregon, Idaho, Washington, and Alaska, and many of these are subjected to intense use by competitive social and industrial functions as well as for agriculture and municipal waste disposal. More money has probably been spent on salmon biology and culture in the past than for all other West Coast fishery programs combined. Salmon culture in the United States is currently being practiced by both public hatcheries and by private ocean ranching and pen rearing commercial enterprises. The salmon and seagoing steelhead trout hatcheries of the Pacific Northwest are probably the most highly developed and technically sophisticated aquatic culture systems in the world. These hatcheries provide fish for a large segment of the U.S. salmon fishery, and the massive releases of juvenile salmon and steelhead trout are presently justified by favorable benefit-cost comparisons. Currently the contribution of public hatcheries to the commercial and sport fishery catch is estimated to be 40% for chinook salmon, 45% for coho, and 5% for chum salmon.

The investment in public salmon hatcheries is judged to be about \$500 million, and annual operating costs are estimated at \$40 million. The northwestern states and the federal government produced about 385 million smolts for release to public waters in 1977-1978.

Since 1950, the U.S. commercial salmon fishery has consistently ranked second or third in landed value among all U.S. fisheries and consistently ranked first in landed value among U.S. finfish fisheries.¹⁹

Food products from the traditional commercial salmon fishery are highly valued and in considerable demand throughout the world. The markets and distribution channels for salmon products are well established. The United States is the largest consumer of commercial salmon products, utilizing 28%; with Japan next, consuming 25%; followed by the USSR, 17%; Canada at 7%; and others, primarily European countries, consuming 23% of the world catch.²⁰

World demand for commercial salmon products was estimated by the U.S. National Marine Fisheries Service in a preliminary report, employing an assumption of an unlimited world supply condition, at 700 thousand tonnes. Under the same assumption, the United States demand could come to about 215 thousand tonnes. The implications are that if salmon supplies can be increased through efficient culture technology and habitat maintenance, world demand would increase by 51%, and the United States demand by 50% by the year 2000.¹⁸

The world supply of Pacific salmon, as inferred from commercial catch data, declined from its peak of 770 thousand tonnes annually during 1935-1939 to the 400 thousand tonne level of the seventies.²¹

There is a wide range in cost depending upon the type of hatchery technology employed in seed production, e.g., open versus closed systems, and the number and kind of ancillary facilities, e.g., rearing ponds, raceways, pens, etc. The capital cost of a hatchery in the U.S., geared to a production of approximately 22 million chinook salmon smolts, has been estimated at \$7.7 million. The annual operating cost for that scale of production is about \$475 thousand. The production cost is estimated at 0.6-1.5¢ per smolt released for pink and chum salmon, and 2.2-12.5¢ for chinook and coho salmon.¹⁸

Alaska, Oregon, and California now allow private ocean ranching. In these states, companies rear salmon in freshwater hatcheries up to migratory size, condition the smolts in seawater pens, and then release them into a river or estuary to complete their life cycle grazing on natural food in the ocean. After maturing in the ocean, they return as adults to the point of release where they are recaptured and marketed by the ocean rancher. The state of Washington does not yet allow ocean ranching. Although some considerable efforts have been made to legalize ocean ranching in Washington, opposition from the commercial salmon fishing industry has prevented passage of introduced legislation.²²

A form of ocean ranching which is somewhat different from public hatcheries and private ocean ranching is that operated by American Indian tribes. By treaty, the Indian tribes are allowed special fishing rights.²² These enterprises rear and release salmon which return as adults to designated tribal fishing areas that may be on or off the reservation. Tribal ocean ranching is now underway in Alaska and Washington and is proposed for Oregon.

Approximately \$35 million has been invested by private corporations and regional fishery associations in ocean ranching. Twenty-four private ocean ranching businesses are currently licensed in the three permitting states. Approximately 40 million salmon were released in 1977 from private hatcheries, compared to 385 million from public salmon hatcheries. Private ocean ranchers are expected to spend about \$1 million on research in 1979. Additional private efforts will be constrained by competition from existing ventures, for suitable locations, and egg supplies. Removal or relaxation of these constraints would allow an approximate doubling of the numbers of privately reared and released salmon within 5-10 years. However, recently, environmental concerns have been raised over the natural carrying capacities of estuaries and near-shore ocean environments. A mounting conflict exists between the traditional fishing interests and the new ocean ranchers. Public hatcheries operate to provide adult salmon to the commercial and sport fisheries, while private ocean ranchers want to minimize the numbers of fish caught by fishermen and maximize the numbers of fish returning to their recapture sites.

On an adult fish return basis, the cost is estimated at \$0.90 per kg of adult fish for pink salmon and \$1.28 to \$1.94 per kg for coho salmon.

The consequences of releasing very large numbers of smolts at the lower and upper ends of the species geographical ranges are not well known. Releases will be made at these extremes because that is where licenses are available. Potentially high risks for the investors exist in those situations. The fine points of the life history and behavior of the fish need to be better known in order to reduce the risk. For instance, reduction in genetic variability in an animal which is required to spend most of its life in the wild is thought to be undesirable, but little scientific evidence can yet be brought to bear on this question.

Among the institutional limitations on the growth of the industry are the permitting regulations. Approximately 31 federal, state, county, and local government permits are required in the state of Oregon by commercial ocean ranchers. The states and the federal government must decide whether private ocean ranching is to be encouraged. The chances of success are marginal at best if a public fishery continues to be allowed. Undue restriction and limitations - biological or political - will almost assuredly preclude success.

One of the social and political constraints on the development of ocean ranching has been the reluctance of large fisheries corporations to enter into the business. Consequently, by default, the commercialization is being developed by non-fisheries oriented corporations such as

Weyerhaeuser, Crown Zellerbach and by Union Carbide which recently sold its salmon cage rearing business to Campbell Soup.

Commercial net-pen rearing involves the culture of young salmon and trout in freshwater hatcheries and the transfer of the fish to floating saltwater pens for continued rearing. The fish are harvested after 9-12 months of saltwater rearing and marketed when they reach 170-400 grams in weight. Two Washington State based companies are currently producing pen-reared salmon.

Although net-pen rearing of salmon is now marginally profitable, certain problems limit its success. The major limitations are disease, availability of suitable feeds, saltwater farm sites, detailed knowledge of saltwater salmon husbandry, institutional and social barriers, and market constraints.

Approximately \$21 million has been invested by industry in pen-rearing operations. The two companies will produce approximately 800 tonnes of pan-sized salmon this year, plus a small amount of larger-sized fish.

Additional requirements in the technology include the development of more manageable confinements, better fish handling and sorting devices, improved feed delivery systems, and a means of transporting live fish from freshwater hatcheries to saltwater net-pens with little stress.

Pen-reared salmon represent a new food product - pan or single portion size salmon - which competes in part with trout and traditional fresh/frozen salmon products. Pan-size salmon has not fared particularly well in the market, and prices have been closer to trout prices than to prices of other salmon. Cost of production is estimated at \$2.20 per kilo, in the round, which is marginally competitive with raceway trout production costs. Unless pen-rearing of salmon can produce a very much larger fish as is done in Norway, the economic outlook for this form of salmon culture in the United States may not be favorable.

Trout

Trout farming in the United States is perhaps the most diverse of any segment of public and private aquaculture. The scope of commercial activities ranges from brood stock operations which sell only eyed-eggs, to those vertically integrated enterprises which produce and process a product for sale in restaurants and supermarkets.

Public hatcheries - both state and federal - produce fry, fingerlings, and legal sized trout for distribution to public fishing waters. According to available data, there are approximately 450 state and 100 federal hatcheries producing trout for distribution. The annual production is in the range of 400 million fish, which are stocked into an estimated 33 million acres of fishing waters.

In 1977, commercial cultured trout production in Idaho was 9 thousand tonnes, which had a gross value of \$24.7 million. Since Idaho produces

about 90% of the nation's commercial rainbow trout, the national commercial production of trout for human consumption during 1977 can be estimated at 10 thousand tonnes with a gross value of \$27.4 million.

The life histories and general biological nature of the species of Salmo and Salvelinus raised in public and private hatcheries have been well documented. Research and application of results in trout genetics have been centered in selective breeding and in hybridization.

The nutritional requirements for trouts have been extensively studied during the past two decades. An excellent summary of the results has been presented by Halver.²³ There are currently several commercial diets available for trout that are satisfactory. Most of the rations are of the dry, pelleted type; however, two moist feeds are also commercially available. Both types of feed are available in graded pellet sizes according to the size of the fish being fed.

In cases where water quality and quantity are not limiting, the production facility consists of flow-through raceways. Where water quantity is limiting, either multiple reuse water systems or recycled water systems requiring only small quantities of make-up water are employed. More efficient water recycling systems will have to be developed for the trout industry, since a limited water resource may be the chief constraint on significant increases in U.S. trout production.

It is the opinion of many that the cost of disease management is roughly twice what it should be when compared to such high density terrestrial animal husbandry as poultry.

In the early days of trout farming, culture confinements were most often earthen raceways, but in recent years, all new construction has been with reinforced concrete. Smaller, more intensive fish rearing units are often constructed with glass-fiber reinforced plastics or other synthetic materials.

The day-to-day operation of a modern trout farm - whether it is a public or a private facility - is fairly standardized and has changed little from the beginnings of American trout culture over 100 years ago. Eggs are incubated in various styles of incubators - some commercially available, and some uniquely fabricated. The yolk sac fry are placed into troughs and introduced to feed just prior to absorption of the yolk material. The swim-up fry are raised in troughs and tanks for a short period and then transplanted into rearing confinements - either raceways or aerated ponds.

The majority of large trout raising facilities are vertically integrated from egg production to processed fish. Several commercial facilities produce hundreds of millions of eyed trout eggs for sale to other private facilities, and to state and federal agencies. Egg production is now possible on a year-round basis, thus allowing for full utilization of the total farm facility.

In 1973, there were approximately 250 private trout raising facilities, 410 state trout hatcheries, and 40 national fish hatcheries raising trout. During that period, there was an estimated production of 16.4 thousand tonnes of trout. Facility costs have been estimated at \$3.30 to \$22 per kilogram of trout produced annually.

In 1965, the average cost of producing a non-anadromous salmonid in a public hatchery in the U.S. was \$1.54 per kilogram. During the last 15 years, the average cost of raising the same fish has nearly doubled to \$2.93 per kilogram.¹⁸

During the past decade, there has been no construction of new trout raising facilities by state and federal agencies. Higher production has been accomplished through renovating existing facilities and through more efficient rearing practices to increase productivity.

In 1977, the cost to the producer of raising rainbow trout in Idaho was \$1.06 per kilogram. The processors paid \$1.50 per kilogram round weight to the producer. The processor sold a variety of products ranging in price from \$2.62 to \$5.46 per kilogram. Of the sales price in 1977, 5.3% was labor, 43.7% was feed, 18% was processing cost, 10% was marketing cost, and 23% was fixed and administrative cost and pre-tax profit. The general consensus among the commercial trout growers is that a 5% return after taxes is an acceptable profit margin. However, the actual net profit is more likely to be 3-4%.

The annual increases in production costs and sales prices have kept pace with the national inflationary rate. In some instances, particularly with specialty products, the increased sales prices have exceeded the national inflationary rate which has been a good indicator of the financial soundness of the industry. In 1978, construction of new trout raising facilities in Idaho has been higher than the level during the preceding decade.

Although it is difficult to acquire data from commercial interests, it is estimated that it costs \$1.5 million to construct a 225-275 tonne annual production facility. This figure includes such costs as: purchase of land, site development, water source development, pond construction, dwelling construction, office and laboratory space and equipment, and the first year's operating expenses. The 250 tonne production scale is judged the break-even point.

Because of the high capital outlay for construction of a modern commercial trout farm, together with the high risk, and the comparatively low rate of return, the financial community is reluctant to assume large front-end financing of trout farming ventures.

The Idaho Department of Fish and Game has gathered statistics on the trout produced in Idaho for food in the years 1975, 1976, and 1977. They report a production of 8.9 thousand tonnes in 1977 and a selling price of \$1.50 per kilogram in the round. This represents a moderate increase in production over 1975 at 8.4 thousand tonnes, but a decrease in round weight

price from \$1.76 per kilogram in 1975. Prices of processed products increased by over 25% in this period, however, more than keeping pace with inflation.²⁴

Catfish

Catfish farming has been a valid commercial industry in the U.S. since the early 1960's. Biological information and effective techniques for spawning, hatching, and raising catfish are well-known and practiced. Catfish are grown in ponds, raceways, and cages and are marketed as fingerlings for stocking, as pan-size fish for food, as 'put and take' stocks for recreational fishing, and as large fish for brood stock.

Catfish farming currently is the largest aquaculture industry in the U.S. Over 3,800 operators use over 22,400 ha of water for catfish production. More than 36 thousand tonnes of catfish were produced in 1977, twice the amount produced in 1970. Catfish production and consumption has grown almost 20% annually in recent years and is expected to continue at that rate for at least another five years. Farm-raised catfish currently accounts for approximately 2% of all fish consumed in the U.S.²⁵

Catfish farmers sell directly to processing plants, especially in Alabama and Mississippi. Nationally about 27% of production is sold to processing plants. Farmers sell about 31% of their production live or dressed to local buyers. Catfish sold in recreational, fee fishing markets represent more than 40% of the annual production.

Total investment in the U.S. catfish industry is estimated to exceed \$2 billion, which makes commercial catfish farming one of the most capital-intensive forms of aquaculture.

The long-range potential is difficult to assess, but much of the acreage now devoted to catfish is suitable for other crops, such as rice, cotton, or soybeans. Depending upon the market for these crops, the industry may accelerate or decline by the year 2000.

Practical diets are now available for feeding fish destined for the food markets. However, continued research in areas such as nutrient requirements, ingredient substitutes, and improved feeding practices are still required to reduce feeding costs. This item is the largest variable in the operations budget, being approximately 60% of production costs. Further improvement in the diets of broodfish and fingerlings is also needed to achieve better growth rates, fecundity, and survival.

Aeration devices recently employed enable catfish farmers to increase productivity appreciably, but further research is needed on the development of more energy efficient methods of aeration.

Marketing is critical to the success of the industry. Variables that affect the quality of processed channel catfish include quality of culture water, quality of feed, slaughter methods, bacterial content of the initial and final product, and plant sanitation.

Because the product is new to most of the U.S., catfish does not enjoy long-term acceptance in an extensive marketplace. Little is known about regional markets that may have the highest demand, or about price and supply levels that achieve optimum profits. The industry has not yet measured the effect of consumer education to stimulate demand at various price and supply levels. Catfish farmers say that the most pressing of their immediate needs are in the marketing area. Market intelligence, including information on marketing alternatives, new product development, package design, and distribution practices would be of great value to existing enterprises.

Molluscs

Commercial molluscan culture in the U.S. is centered on oysters, clams, and mussels. Abalone has been grown in a few facilities, and is approaching commercial scale.

Oysters

U.S. commercial landings in 1978 of 23 thousand tonnes of oyster meats were valued at \$60.9 million.

In 1978, 20.3 thousand tonnes of American oyster, *Crassostrea virginica*, meat came from the East and Gulf Coast states, while 2.6 thousand tonnes of Pacific oysters, *C. gigas*, were harvested in West Coast states. An estimated 40% of U.S. oyster production comes from private aquaculture. Although increased by 2.25 thousand tonnes over 1977, these landings do not compare well to a record of 68 thousand tonnes in 1908, and a mean annual production of 31.5 thousand tonnes in the late 1940's. Imports of oysters, however, largely from Japan and Korea, increased from 50 tonnes of meat in 1947 to 10.6 thousand tonnes in 1978. Total U.S. consumption of oysters has thus remained between 31 and 36 thousand tonnes for the past 30 years, with Japanese and Korean imports making up for lost U.S. production.¹⁹

Reasons given for the decline in domestic production of oysters include overfishing, natural disasters, diseases, and pollution. Overfishing is said to have contributed to major declines after the turn of the century, but diseases and pollution probably caused the major declines since the late 1940's. The value of cultured oysters usually exceeds 40% of the total market value, because in most cases, they bring a higher unit price. Cultured Long Island oysters sell for nearly \$6.60/kg compared to approximately \$2.75/kg for oysters from public beds in Maryland.²⁶

All oysters harvested on the West Coast come from private beds, as do oysters from New England, New York, and Delaware Bay. The majority of oysters landed from Maryland, the Southeastern States, and along the Gulf Coast are taken from public beds. Laws governing private molluscan aquaculture vary considerably from state to state, and one must, therefore, examine the laws carefully before initiating an aquaculture venture.

Bottom culture, with intensive management, can produce 5,000 kg/ha per year; while public grounds, with little or no management, produce only 10-100 kg/ha per year. Studies of off-bottom culture estimate that 0.1 ha covered by rack cultures could yield 6.5 tonnes per year.¹⁸

If the market could be expanded, a large potential exists for expansion of oyster production by aquaculture. One estimate predicts an increase in consumption of oysters in the U.S. from 34 thousand tonnes in 1970 to 56 thousand tonnes by the year 2000. Although significant increases probably cannot be achieved by expanding the fishery of wild stocks, the technology of private oyster culture used in other parts of the world could be applied in the U.S. to significantly increase production.

By manipulation of environmental parameters, reproduction can be controlled so that oysters can be spawned on demand throughout the year. The growth and behavior of oysters are reasonably well understood, but the industry has not taken full advantage of this knowledge. Oysters grow much faster and have better survival in suspended culture, but the fragile shell that results from suspended culture due to the fast growth may affect its marketability as a half-shell oyster.²⁷

Considerable effort has gone into the design and operation of oyster hatcheries. Present systems for growing small, cultchless oysters in trays are labor-intensive and costly. Engineering research now in progress should result in the design of low-cost, off-bottom growing systems which can be managed with a minimum of labor. If such controlled, intensive systems prove technically feasible, then the oyster farming industry may be stimulated to new levels of productivity.

In many areas of the U.S., natural seed-setting has become sporadic or, in some years, a total failure. To augment natural sets, commercial hatcheries were constructed, especially on Long Island Sound and on the West Coast where seed production is a major problem. Because of lower cost, farmers prefer naturally-caught spat to hatchery seed. In the Maryland and Virginia portions of Chesapeake Bay, natural setting has been light to a total failure since 1970. Hatchery seed production is advocated to supplement natural sets.

Economic incentives depend heavily on the development of new markets. Growers recognize the necessity of developing and promoting other product forms besides fresh oysters, but little market development has been accomplished by the industry. Lack of consumer demand coupled with high spoilage losses and the problems of shucking make food store and restaurant proprietors hesitant to stock fresh oysters. Some retail outlets prefer the canned product. However, the potential exists for development of improved packaged products that will gain consumer acceptance.

Clams

The hard clam, *Mercenaria mercenaria*, is harvested commercially in 16 states, and in terms of employment, it comprises the largest clam fishery in the United States. In 1978, the catch was six thousand tonnes, and was worth \$29 million, or about \$18 per bushel (over 7¢ per clam). Hard clams are usually sold by the bushel or by individual count. The prices vary for different sizes with the smaller sizes or grades usually having a greater value than larger clams.¹⁶

There is a chronic shortage of the smaller size littleneck and cherry-stones. The demand for these smaller sizes is strong and continues to increase. Harvests of clams have declined in areas that have traditionally been major producers in the past (New York, New Jersey, and Virginia). The continued shortages and the increased prices paid for clams have helped create more interest in culturing clams, either as a private venture or as a public service project to replace over-harvested stocks in public areas.

Hard clam aquaculture remains in the early stages of large scale commercialization, although methods of spawning and growing hard clam larvae were described as early as 1927.

A number of companies tried various methods of growing clams, but were generally unsuccessful. The major problems were not in producing seed, but in avoiding high mortalities and bringing the seed to market size in large enough numbers, and at a reasonable cost. By 1970, some new technology and new materials contributed to the development of several promising methods for the aquaculture of hard clams.

At the present time, there are 15 companies that are culturing clams. Six are involved exclusively in clam aquaculture, and the others are involved in producing other species besides clams. There are six commercial seed hatcheries in operation. Despite their efforts, there is a chronic shortage of some larger seed sizes for the existing industry. This inadequate seed inventory forces the culturists to plant smaller sized seed, or grow the seed to larger size in troughs or on rafts. Besides seed production which is from a quarter to almost a half billion seed per year, the total production of marketable sized clams based solely on aquaculture is only about two million clams (about 8000 bu).

In 1970, a cost analysis made by the Virginia Institute of Marine Sciences (VIMS) indicated a favorable profit ratio could be expected if clams were grown by the method at VIMS that was available at that time. The price of clams has increased over 15% per year since 1976 (inflation rate was about 8% per year during that period), and costs have been reduced through optimization of growing methods.

The favorable financial picture plus the reduction in catch in some of the traditional clamming areas should encourage entrepreneurs to investigate clam aquaculture. Probably the greatest lack in clam culture development is finance. Low interest, guaranteed loans or tax incentives would, no doubt, encourage further development.

Market sized clams can be produced for about \$.015 each, or about \$3.75 per bushel, plus an additional \$1.75 to \$2.00 harvest costs, or a total cost of \$5.50 to \$5.75 per bushel. The current price of clams is about \$17 to \$19 per bushel to the harvester.

There is no culture of the soft clam, Mya arenaria, which was the basis of the fried clam market. The harvest of this clam has declined, and the unsatisfied demand is being met by surf and other clams, and to some extent recently by mussels. Some persistent research efforts in Maine promise a commercial aquaculture of soft clams in the Gulf of Maine.

There is no commercial culture of several other clams important to the capture fishery, including the butter, manila, geoduck, and surf clams, but some culture activities have been undertaken. However, large scale culture is not yet economic.

Mussels

Mussel, Mytilus edulis, aquaculture in the United States is in the early stages of development. University researchers and private companies or individuals have engaged in various aspects of mussel culture for several years. The annual combined harvest from six commercial operations approaches 5,000 bushels, or approximately 34 tonnes of wet meat.

Inadequate culture, harvest, and processing technology restrain development and expansion of the industry. Some aspects of culture and processing operations must be improved, such as the design and construction of an efficient rope-stripping device, and washing, grading, and debussing processes. Losses from predation by sea ducks and disease are perhaps the largest biological problems facing the mussel industry.

The quality of cultured mussels is consistently high, while marketing of wild mussels with low-quality meats and high pearl incidence produces an adverse influence on the industry.

Although several young companies plan for rapid expansion of their current small production, the mussel industry in the United States confronts the need to develop a substantial market in addition to solving its production problems. The cultured mussel is generally superior to most of the harvest from the wild, but this mollusc is not yet appreciated in the United States as it is in Europe, and although the market absorbs the current small production at prices favorable to the producer, a mass market demand is not yet available.

Crustaceans

Crustacean foods are widely accepted by most human societies, with a few ethnic exceptions. They generally command high prices in current markets in the industrial countries which are frequently supply-constrained. Most of the wild populations of the oceans have been identified, and the dynamics characterized; and the fishing pressure is probably at maximum sustainable yield, if not greater. This is certainly true of the wild crustacean populations in U.S. waters which include the penaeid and pandalid shrimps, homarid and palinurid lobsters, and the various crabs in the genera, Callinectes, Menippe, and Cancer. There is a widely held consensus that if increased supplies are to find their way into U.S. markets, aquacultural production must be the source. This may be the case, not only because of the population limits, but also because of the increasing cost of capture due to the high cost of fuel, labor, vessels, and gear. Furthermore, most marine crustaceans of commerce occur naturally in the littoral, or near off-shore regions of the sea, where pollution concentrations are greatest.

In the case of penaeid shrimps, the nursery grounds for juvenile

development are in the coastal wetlands and intertidal marshes. The effects of pollution, and the many competitive uses and abuses of the coastal zone are materially limiting the catches.

Penaeid Shrimps

The persistent high market demand in the U.S. for penaeid shrimps is the most compelling justification for public and private sponsored research efforts to develop a shrimp aquaculture technology. For at least the past couple of decades, shrimp has been the highest value U.S. fishery, and in 1978, the landed catch at dockside earned for the shrimp fishermen \$385 million. At the same time, the U.S. imported 90 thousand tonnes worth \$422 million. Such massive imports are a significant drain on our foreign exchange.¹⁹

Commensurate with the increased consumption of marine shrimps in the U.S. markets, the European and Japanese demand has also been very strong, and competition for the finite supply of wild shrimp has stimulated higher prices and further justification for investment in shrimp and aquafarming.

High quality shrimp, particularly live shrimp for the tempura market, has traditionally commanded high prices in Japan. High prices for shrimp in Japan, as well as the scientific motivations, encouraged M. Fujinaga to undertake a lifetime of research in elucidating the biology of the native "Kuruma" shrimp, Penaeus japonicus. This fundamental research is the basis on which U.S. and worldwide shrimp aquaculture technology has been constructed.²⁸

A major contribution to the establishment and management of high density shrimp populations came out of the efforts of Japanese and American scientists in the past two decades to capture gravid females from wild populations, induce spawning, manage larviculture by establishing appropriate environmental conditions and feeding methods, protect and nourish postlarval shrimp through nursery and growout phases, and harvest a marketable crop. This cultural technology has been greatly augmented by the recent successful induction of sexual maturity which frees the culturist from a dependence on the procurement of wild gravid females and provides an opportunity for the establishment of genetic improvement programs to develop more growth efficient breeds with improved marketability.²⁹

Whereas shrimp aquaculture was first investigated on the coasts of the Gulf of Mexico and Florida, it was soon found that without the insertion of considerable heat energy to maintain high rates of growth over most of the year, the economics of shrimp farming in those regions were not favorable. Consequently, as commercial, production-scale farming approached technical and economic feasibility, U.S. shrimp farming interests were shifted to the lower latitudes of Central and South America. Penaeid species indigenous to the Pacific coast waters of Middle America were found to be superior to the Gulf and Caribbean species which were the subjects of the earlier American studies. Penaeus vannamei and P. stylirostris, both white shrimp species, proved to be hardy, fast-growing, easier to bring to sexual maturity, and efficient in feed conversion and are now the species of choice in current Western Hemisphere shrimp farming.³⁰

Although an early pioneering shrimp farming effort in Honduras, sponsored by Armour Company and the United Fruit Company, was stopped by a management decision that was completely unrelated to the technical and economic validity of the development program, it served as an encouragement and attractant to several other U.S. investors to undertake similar developments in Central America. The Armour/United Fruit shrimp farming facilities were subsequently procured by General Mills and have been considerably augmented to support a commercial freshwater shrimp farming venture in Honduras.

Sea Farms of Honduras, which was initiated by venture capital supporting a U.S. shrimp capture and processing company, now has ConAgra participation and management.

Maricultura, S.A., located in Chomes, Puntarenas, Costa Rica, is a commercially successful joint venture between Costa Rican and U.S. investors with considerable financial support by International Finance Corporation of the World Bank.

Ralston Purina, which initiated its shrimp investigations in Crystal River, Florida, soon after established a commercial pilot farm facility in Aquadulce, Panama. This Panamanian facility is now being expanded to a large-scale commercial venture.

Several other shrimp farming efforts by smaller U.S. companies have been undertaken in Central America and Panama. Some of these have failed due to being undercapitalized or poorly managed, but some have favorable prospects for success. Intensive shrimp culture technology based on the use of highly aerated raceways and biological filtration to restore water quality allowing for water recycling, has been studied for several years at the National Marine Fisheries Service, Biological Laboratory at Galveston, Texas.³¹ This intensive culture system was further advanced by research and development at the environmental laboratories of the University of Arizona at Puerto Penasco on the Sea of Cortez in Sonora, Mexico. This work has been supported by Coca-Cola International and is being further expanded in Mexico.³²

Several major U.S. corporations and private investors have made significant commitments in Ecuador by the insertion of capital, high technology for intensive culture, and management to vertically integrated corporate farming, processing, and marketing. Ecuador has had for the past decade a rapidly expanding shrimp farming activity operated at the lowest level of technology and requiring extensive land but small additional capital investment. The ecological endowments of large areas of the coastal zone of Ecuador are very favorable and have allowed for an extensive farming system based on the confinement of a natural recruitment of juveniles in large ponds with no feeding. This system results in 200-300 kg/ha per year of large size shrimp and has been profitable for some of the farmers. However, there is the need for a reliable source of seed to allow for high density stocking which will, no doubt, exceed the natural carrying capacity of the ponds and thus, require an energy subsidy in the form of feed, and aeration and/or frequent water exchange. By the establishment of hatcheries for seed production and feed formulating plants, an intensive

shrimp farming system can be instituted in Ecuador which will increase the productivity of the farms to yields in the order of 4,000-5,000 kg/ha per year. This intensification is being accomplished by the insertion of U.S. technology, management, and capital through joint ventures with Ecuadorian farmers and processors.

The oldest and largest shrimp farming business in the U.S. is Marifarms, Incorporated, located at Panama City, Florida. This operation was initially predicated on the production of large numbers of penaeid juveniles produced through hatchery techniques to stock a net enclosed 1,000 hectare natural embayment. The culture environment is treated with Rotenone for removal of predators, and heavily stocked with juvenile shrimp. However, this embayment approach to culture has not proven successful due to storms, predation and competition, net fouling, and other facilities management problems. The company has persisted, however, and now operates its hatchery for stocking large pond systems.

It has shifted from the use of indigenous Gulf species to the faster growing Pacific whites, P. vannamei and P. stylirostris which it can procure earlier in the growing year by purchase from Central American companies. This enables management of a longer growing season.

Operations and expansion of the facility at Panama City are seriously constrained by the numerous U.S. regulatory agencies which make the conduct of aquafarming in the coastal zone of the U.S. very difficult and economically unattractive.

Freshwater Prawns

Despite recent commercial failures, freshwater prawn, Macrobrachium rosenbergii, aquaculture is steadily progressing through an early development phase. Some twenty individual growers currently produce prawns in Hawaii. More than 200 ha of ponds producing a crop worth in excess of \$5 million are projected for 1980. Large commercial farms on Oahu owned by Lowe Farms, and on Kauai, owned by C. Brewer, will contribute significantly to the Hawaiian production.

Aquaculturists in Hawaii report production levels of 2.8-3.9 tonnes of whole prawns per hectare per year from year round production ponds. Overall production levels are roughly estimated to be 79 tonnes in Hawaii in 1978 and 80 tonnes in the rest of the U.S. in the same year.

General Mills International has a major pilot-production facility in Honduras and markets its products through outlets in the U.S. including its own Red Lobster restaurant chain. Other corporations have already developed, or plan, prawn-production facilities in Central and South America.

According to one estimate, probably \$25 million in private and government funds have been invested over the past decade in Macrobrachium farming.

Land and warm water resources suitable for Macrobrachium aquaculture

exist not only in Hawaii, but also in Puerto Rico, the Southeastern and Gulf states, California, and perhaps other areas of the U.S. with heated waters, as well as in many foreign locations at the lower latitudes. Because of the lack of knowledge regarding nutrition and the fact that feed accounts for an estimated 20-40% of operating costs, lack of an inexpensive, appropriate diet represents a major constraint on freshwater prawn aquaculture. The high price of postlarvae, as high as \$50 per thousand at retail, is a further restraint on commercialization, if the venture is based on purchased juveniles.

Facilities and equipment for prawn aquaculture have evolved empirically through trial-and-error, and innovative engineering studies are now required to facilitate scale-up from research facility to pilot farm, to commercial culture system.

Available estimates indicate that the start-up cost for prawn farming is high compared to most other agricultural alternatives - \$18,900/ha for a hypothetical 4 ha prawn farm in Hawaii. Lack of reliable data on risks and potential returns associated with such investments makes it difficult for interested investors to justify the necessary capital. Because of the high initial costs, lack of financial assistance may preclude significant involvement of small farmers in Macrobrachium aquaculture. This probably is not a major deterrent to the involvement of large corporations.

As volume of production increases, so does the need for processing, especially on the mainland where nearly all shrimp are sold frozen or in some processed form. Currently, most prawns produced in Hawaii are sold fresh, on-ice, or alive. Because of the inferior image in the market of freshwater shrimp imported into the U.S. from Southeast Asia, market development will be required in addition to processing and product development.

Crawfish

Crawfish culture is practiced in Louisiana, Texas, Arkansas, Mississippi, North Carolina, and California. Three genera have been grown, but only Procambarus spp. have been produced for human food. Orconectes spp. and Pacifastacus spp. are raised for bait.

Crawfish culture for human food in Louisiana yields from 225 to 1,125 kg/ha annually, while intensively managed Texas ponds produce about 2,250 kg/ha. Crawfish culture is generally profitable for the farmer who invests little capital, uses little sophisticated technology, no feed, and does not purchase young. Current prices to the farmer are about \$1.14/kg live weight. Hectareage devoted to crawfish culture has increased from 2,400 in 1966 to almost 20,000 in 1979. Crawfish is often rotated with rice and/or soybeans.

Since crawfish are trapped over a period of months, harvesting is a major problem in the economics of culture. Many farmers lease trapping rights for a share of the profits. Most of the Louisiana catch is marketed locally in the live condition. The processing capability is very limited, and in periods of excess production, tails are dumped at very low prices.

Crawfish farming in the U.S. has not yet been developed into a technology that is likely to be transferred readily to other environments, since the culture practices have emerged as a consequence of very specific ecosystem parameters.

Lobsters

For the past half-century, U.S. consumption of the northern lobster, Homarus americanus has remained constant at about 23 thousand tonnes, with from one-third to one-half of the supply imported from Canada.¹⁹ The value of the U.S. catch in 1978 was a record \$64.6 million.¹⁶ The consistent, but limited supply, and the consequent high selling price in the face of a growing demand, have provided an incentive to entrepreneurs and publically supported research centers to seek ways to increase the supply. The release into coastal waters of juvenile lobsters reared in a hatchery as a means of enhancing the capture fishery, was first tried as far back as the turn of the century. There is very little confirming evidence that this practice does, in fact, increase the catch. However, the early effort to produce juveniles led to a better understanding of the life-cycle of this animal, and now reproduction in captivity is readily achieved.

This command over a supply of juveniles has encouraged several efforts, both in the U.S. and in Europe, to grow out the young to marketable sizes in controlled environments. This research entailed not only the ongoing quest for suitable diets which still requires a substantial portion of the current research budgets; and studies of disease and parasite control; but also the study of physiology and behavior of the rapidly growing animals. The outstanding behavioral characteristic affecting culture is expressed as aggressive territoriality. This latter characteristic has dictated engineering design for growout facilities in which individual lobsters are isolated from each other in compartments. Such a growout system demands high capital investment, special engineering for water quality maintenance and exchange, a feed distribution system, harvesting, and solution of other materials handling problems.

As a consequence of the specialized growout system, and the efforts to enhance growth rates by improved feeds, water temperatures, flow patterns, and so forth, the costs of producing lobsters by aquaculture have remained high, and to date have precluded a commercial venture.

However, there is expert opinion based on empirical evidence and observations of behavior that advocates mass rearing of lobsters. Optimizing feed quality and availability and the growth environment, cannibalism can be reduced sufficiently to enable high survival rates and commensurate reduction of production costs to make lobster farming a commercially valid enterprise. If this is true, lobster farming will probably be conducted in water temperatures between 20 to 24^o C, which are somewhat higher than in the lobster's native environment. The elevated temperatures increase growth and survival rates, and thus, enable a crop period of eighteen to twenty-four months.

The annual price to the lobster fisherman in 1978 was \$4.14/kg, but

prices are highly seasonal and may be almost twice that amount in late winter and early spring. A culture operation would, therefore, be designed to put its product into the marketplace at the most advantageous time to realize the best prices.

Baitfish

Among the most popular recreational activities in the U.S. is sports fishing. It has become a family activity demanding a considerable expenditure for travel, food and lodging, tackle, and a very sizeable expenditure for bait.

The current demand for fish bait far exceeds the natural resources, and today the major portion of the supply of freshwater baitfish is provided from commercial aquaculture. Whereas the culture of baitfish began as early as 1915, it has only been in the last two decades that the demand has justified a considerable increased investment in baitfish farms. Half of the baitfish produced in the U.S. is produced in Arkansas in the intensive aquaculture of about 8,800 hectares. In 1978, there were 1,544 baitfish farms in the U.S. using 17,600 hectares.

There are five fishes which dominate this production. In order of importance, they are the golden shiner, Notemigonus crysoleucus, the fathead minnow, Pimephales promelas; the goldfish, Carassius auratus; the white sucker, Catostomus commersoni, and the chub sucker, Erimyzon spp. These fishes command prices ranging from \$2.20 to 6.60/kg wholesale, and range over the same prices per dozen at retail. Baitfish farms often are located on marginal land not suited for row crops or pasture, but usually net to the farmer \$500 per hectare. This is a favorable return when compared with the returns in the U.S. of \$125 to \$188 per hectare for such crops as corn, soybeans and rice.

Modern baitfish farming is now capital-intensive, requiring on the order of \$4,000-\$5,000 of investment per hectare, but the return on equity is sufficient to warrant a continuing expansion of the industry. A conservative estimate of current investment in baitfish farms in the U.S. is over \$100,000,000.

There is a substantial opportunity to improve returns in the baitfish industry by increasing productivity. Yields per hectare currently range from 500-600 kg/ha. The application of improved technologies developed in the culture of other fishes should triple or quadruple the productivity while requiring little additional capital cost and only incremental operating funds for the procurement of seed and feed.

Potential Aquacultures

There is a group of candidate species in the U.S. which has been studied as potential culture animals. Any one of these species may soon emerge as a significant crop for a new aquatic animal husbandry business.

Striped Bass

The striped bass, Morone saxatilis, is an important food and game fish in the U.S. This is an anadromous fish which spawns in freshwater streams, but has wide salinity tolerance and is found in fresh, brackish, and full salinity environments. It has been successfully introduced into some reservoirs and lakes which now support large natural populations if they are served by appropriate fast-running streams which provide natural spawning grounds.

In the early 1960's, several reservoir introduction programs encouraged efforts to rear striped bass juveniles in controlled environments. A substantial research effort resulted in the development of techniques for hormone induced ovulation and spawning, larviculture, and adult rearing. There are now twelve striped bass hatcheries in the U.S., and over 15 million fingerlings are produced annually for stocking reservoirs and estuaries. Although a commercial aquaculture venture was attempted in the mid 1970's and failed, there are now several new private ventures being undertaken for food fish production.

Large-mouth Bass

The cultivation of large-mouth bass, Micropertus salmoides, was begun prior to the turn of the century, primarily for the introduction of the species into new environments to enhance sports fishing. This aggressive predator has established itself in many natural waters, and is prized by many sportsmen as a game fish. However, in recent years, bass has been employed as a part of a polyculture system with channel catfish in food-fish aquaculture programs. It is used to control catfish competitors (trashfish) in the ponds, enhancing the growth of the primary crop animal, while at the same time creating a secondary crop which commands high prices in the food market. Pond raised bass sell for \$6.60/kg in the round, and may soon become a primary aquaculture crop.

Pompano

The pompano, Trachinotus carolinus, is a high-value foodfish caught from the wild on the Atlantic and the Gulf coasts of the U.S. According to the latest statistics, only 400 tonnes per year of this fine foodfish were landed, which may account for the high value that it commands in the marketplace. It is appropriate to consider whether high market prices would be sustained if a significant quantity of cultured fish were introduced regularly.

In recent years, pompano fry have been captured in the surf and confined in raceways or ponds to be cultured to market size. Resistance from the fishermen to the capture of wild fry has encouraged efforts to induce spawning in captivity. Success has been variable, and several commercial efforts have failed, although there is a persistent resurgence of interest in the commercial aquaculture of this fish. There is now encouragement that recent advances in the technology of controlled reproduction and culture of other species may be applied to the culture of pompano with success.

FUTURE PROSPECTS

U.S. aquaculture will almost certainly follow the world trend toward the more effective utilization of aquatic resources for food and energy production. It may not suffer the same limitations by which other national aquaculture programs are currently constrained, i.e., a dearth of investment capital and competent management, but in U.S. territorial waters, aquaculture, nevertheless, is seriously constrained by overwhelming cultural-legal regulatory mechanisms. The numerous competitive uses of the environments in which aquaculture can be practiced, both in the coastal zone and in inland waters, make it difficult, if not impossible, for profitable businesses to be conducted.

Federal, state, and local regulatory bodies exercise such overwhelming controls over the uses of the resources, and the design and conduct of the business system, that the imposed risks may be greater than the rewards necessary for prudent investment. To counter these institutional constraints, there is a requirement for the society to provide commensurate incentives to attract investment. Considerable investment is needed to support further research and development programs and in pilot and full scale production systems. These incentives may take the forms that terrestrial agriculture required to enable it to build the essential technological base and some degree of assurance that the economic outcome of the effort would justify the investment.

The scientific and sociopolitical climate within which U.S. agriculture matured into the successful food and wealth generating system that it has become, could serve as a realistic model for a parallel development in aquaculture.

A mechanism must be created to provide incentives to the investors in aquaculture in the forms of favorable credit terms, realistic crop insurance programs, possibly some short-term tax abatement or tax credits, accommodation of some of the more rigorous regulatory requirements, and a greater role of the public sector in the research and development of baseline data to guide the applied technology into higher productivities and profitability.

In view of the rigorous demands being imposed on the natural aquatic resource in the U.S., American aquaculture is more likely to develop a greater dependence on high-technology and high-capital demanding, intensive systems of culture. Such production systems require the rigorous management of closed environments, in which all of the parameters influencing the biological and economic variables are monitored and controlled to yield the most efficient growth and survival at least cost. Thus, freeing production from the dependence on the vagaries of nature will enable a responsiveness, in kind and degree, to the demands of the market. It, perforce, dictates vertical integration to allow for central control and balance of the numerous components of production, processing, and marketing.

The present advancing state of the biological sciences and of the arts of bioengineering applicable to aquaculture, the demands for energy

efficiency, and the capability in the marketplace to continue to absorb more diverse, new, and improved foods, constitute an indomitable force shaping the next increment of aquaculture developments in the U.S.

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