Toxic Effect of Certain Marine Blue-Green Algae to Penaeid Shrimp

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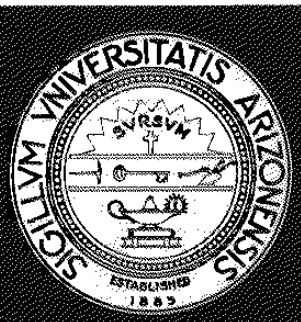

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SUMMARY

Hemocytic enteritis (HE) was found to be a disease of cultured marine penaeid shrimp and of the freshwater prawn, Macrobrachium rosenbergii. The principal lesion of HE disease is a necrosis and intense cellular inflammation of the mucosa of those portions of the shrimp's gastrointestinal tract that lack a chitinous lining (the midgut, and the anterior and posterior midgut caecum). HE has been observed in high density controlled environment culture tanks and raceways and in lower density pond culture, and has been induced experimentally in the laboratory. The Oscillatoriacean blue-green algae Schizothrix calcicola (Agardh) Gomont has been shown in numerous laboratory trials to cause HE when fed as live or freeze-dried unialgal cultures to juvenile penaeid shrimp. Another algae in this group, Spirulina subsalsa Oersted has also been circumstantially and experimentally linked to HE, but not with the same statistical validity as was the case for Sc. calcicola.

The use of chemotherapeutics and algicides to control or prevent HE provide either variable results or no effect. The most effective method used to control or manage HE in cultured shrimp and prawns is to prevent it by preventing the growth of toxin containing benthic blue-green algae, such as Sc. calcicola. This is usually accomplished by manipulation of water depth and/or water turbidity in such a way as to either "shade" out the benthic blue-green species or to favor beneficial algae species.

INTRODUCTION

History of Project

The University of Arizona's Environmental Research Laboratory (ERL) has been active since 1973 in the development of an intensive culture method for marine penaeid shrimp. ERL calls its method Controlled Environment Aquaculture, CEAq (Salser et al. 1978), and it developed this new technology initially near the fishing village of Puerto Peñasco, Mexico with the cooperation of the University of Sonora. Political difficulties in Mexico caused the University of Arizona portion of the Peñasco program to be terminated in Mexico in July of 1980. Consequently, the shrimp culture research and development activities of the ERL were moved to a new site on Oahu, Hawaii.

The research covered by this report was partially funded by a series of three 1 year-duration grants to the ERL from Sea Grant. Funding began October 1, 1978 and concluded December 31, 1981. The research time lost in 1980-1981, while the project was moved from Mexico to Hawaii was made up at no additional cost to Sea Grant from January 1, 1982 to the project's termination on June 30, 1982.

In CEAq of shrimp, as developed in Mexico and Hawaii, shrimp are reared in shallow, rectangular tanks or raceways (Figure 1), which are enclosed in air-inflated plastic structures, called "aquacells" (Mahler et al., 1974).

These units combined with the use of seawater wells, permit the control of most environmental variables that would otherwise adversely affect normal shrimp growth and health. Water temperature, flow rates, exchange rates, salinity, and light intensity are among the environmental variables that can be controlled in aquacells, regardless of season. In addition, the shrimp's diet is controlled, and all predators and many shrimp disease organisms are excluded or managed. The use of these systems has resulted in high levels of shrimp production in a relatively small area (Salser et al., 1978).

The very nature of high density culture provides the physical and environmental conditions that can enhance the occurrence, spread, and severity of certain types of diseases in cultured populations. On the positive side, however, this enhancement of disease processes permits the recognition of those diseases that may cause significant losses in other types of culture systems (i.e. ponds) without ever being detected. Hemocytic enteritis (HE) is an example of a disease of cultured penaeid shrimp that was first recognized as the cause of significant losses in high density CEAq, but has since been found to be a potentially important disease in pond-cultured (marine) penaeid shrimp and in (freshwater) pond-cultured Macrobrachium rosenbergii.

The purpose of the Sea Grant supported research reported here was to investigate the role of blue-green algae in the hemocytic enteritis syndrome as observed in cultured penaeid shrimp. The specific objectives are listed in the following section.

PROJECT OBJECTIVES

The objectives of each of the segments of this project are listed below by time periods:

A. October 1, 1978 - December 31, 1980

1. To determine if HE may be induced in juvenile penaeid shrimp by feeding unialgal cultures of blue-green algae species that have been circumstantially linked to the disease.

2. To determine the identity and optimum growth conditions of the blue-green algae associated with hemocytic enteritis.

3. To evaluate certain algicides for selective toxicity to the marine blue-green algae associated with HE.

4. To determine the nature of the toxins produced by the suspect blue-green algae.

B. January 1, 1981 - June 30, 1982

1. To compare endotoxin from Sc. calcicola to the living and freeze-dried alga for ability to produce hemocytic enteritis (HE)

disease in juvenile penaeid shrimp.

2. To determine the approximate composition and physical characteristics of the lipopolysaccharide (LPS=endotoxin) from Sc. calcicola.

3. To determine if juvenile penaeid shrimp may be immunized against HE disease by feeding them subtoxic amounts of the endotoxin from Sc. calcicola.

4. To describe, using light and electron microscopy, the development of the lesions of hemocytic enteritis in juvenile penaeid shrimp.

5. To determine if HE disease occurring in juvenile penaeid shrimp in culture facilities in Hawaii is caused by the same or different species of the Oscillatoriaceae as was found to cause the disease in Mexico.

RESULTS AND DISCUSSION

Hemocytic Enteritis

Pathology and Epizootiology:

Ingestion of certain species or stains of blue-green algae by penaeid shrimp have been shown to result in a particular disease syndrome called hemocytic enteritis (HE) (Lightner, 1978; Lightner et al., 1978; Lightner et al., 1980; and Lightner, 1983). Losses due to the disease in raceway-cultured populations of the blue shrimp, Penaeus stylirostris, reached 85% (Table 2) at the former University of Arizona-University of Sonora experimental shrimp culture facility at Puerto Peñasco, Sonora, Mexico (Salser et al., 1978; Lightner et al., 1978). More recently, the disease has been observed in raceway-reared juvenile P. stylirostris, P. vannamei, P. japonicus at the experimental shrimp culture facility near Laie, Hawaii (Lightner et al., 1980), and has been observed in pond-reared P. japonicus, P. monodon, and Macrobrachium rosenbergii (Tables 1 and 3). Because these later examples occurred in Brazil, the Philippines, Israel, and Hawaii, HE seems to be a ubiquitous disease of cultured (and possibly wild) shrimps and prawns.

The histopathology and pathogenesis of HE is identical in all species in which the disease has been recognized (Table 1). HE results from the effect of algal toxins (specifically algal endotoxin which is composed of lipopolysaccharides) released in the gut from the digestive breakdown of ingested algae (Lightner et al., 1978; Lightner, 1983). While other organisms, such as gram-negative bacteria possess similar endotoxins, HE has so far only been circumstantially or experimentally linked to certain endotoxin-containing species of the Oscillatoriceae of the blue-green algae phylum Cyanophyata.

Table 1. Geographic locations and the species of shrimps and prawns in which hemocytic enteritis has been observed.

Species	Geographic Location	Reference
Marine Penaeids:		
Penaeus duorarum	Florida	Nimmo et al., 1977
P. stylirostris	Mexico	Lightner, 1978 and Lightner et al., 1978
P. vannamei	"	
P. californiensis	Mexico	Lightner and McKee, unpublished
P. vannamei	Hawaii	Lightner, unpublished
P. stylirostris	"	
P. japonicus	"	
P. monodon	Philippines	Lightner, unpublished
P. stylirostris	Israel	Lightner, unpublished
Freshwater Prawn:		
Macrobrachium rosenbergii	Hawaii, The Philippines, Brazil	Brock, 1983 Lightner, unpublished

The principal lesion observed in HE is a necrosis and hemocytic inflammation of the mucosa of those portions of the shrimp's gastrointestinal tract that lack a chitinous lining. This includes the midgut proper, and the anterior midgut (or epigastric) and posterior midgut (or hindgut) ceca (Figure 2).

Electron microscopy of the midgut and anterior midgut caecum mucosal epithelial of animals with developing HE show the epithelium to be atrophying. Specific changes include a reduction in height of the mucosal epithelium cells from columnar to low cuboidal, and increase in cytoplasmic vacuoles and autophagosomes, a decrease in membranous granular endoplasmic reticulum, and a decrease in the height of surface microvilli. Eventually, cells so affected detach from the basement membrane and/or undergo cytolysis, and are sloughed into the gut lumen. Hemocytes, of both granular and hyaline types during the process of atrophy of the mucosal epithelium, infiltrate and accumulate basally to the epithelium, and are in extreme abundance by the time the mucosa has been sloughed.

Cellular lesions, not readily observed by light microscopy, were present in the hepatopancreas of juvenile P. stylirostris with acute HE. Polyhedral crystalline intranucleolar inclusion bodies (PNB) were observed in the nuclei of hepatopancreatocytes (Lightner and Redman, in press), and were relatively common in the hepatopancreas of shrimp with severe acute HE in the midgut.

The PNB's were not easily observed by light microscopy, but following their discovery by TEM, the larger ones were readily apparent in o-toulidine blue-stained plastic sections (Figure 3a), and they were occasionally detectable in H and E-stained paraffin sections. With H and E the PNB's stained red with eosin, indicating their composition to be mostly protein rather than nuclei acid (Lightner and Redman, in press).

PNB's were always present in the para amorpha portion of the nucleolus and were typically cubic in morphology (Figure 3b). Dimensions of PNB's ranged from 0.37 to 1.42 um, and were composed of "linked" subunits that averaged about 9 nm in diameter (Figure 3c). The same cells which contained PNB's also showed adverse cellular changes in the cytoplasm. These changes included a marked reduction in ribosomes, both free in the cytoplasm and attached to ER membranes (Figure 3b). An increase in the number of autolysosomes vacuoles in the cells (Figure 3b), and occasional necrotic cells undergoing cytolysis were also observed (Lightner and Redman, in press).

Intranucleolar crystals of this type are unusual if not unique. There is, at best, a paucity of information on PNB's of the sort described here in animals, and even less data on their occurrence in disease. The presence of proteinaceous crystals in the nucleoli of hepatopancreatic epithelial cells of shrimp with the HE syndrome may result from the toxic effect of the same algae-derived endotoxin that causes the midgut enteritis. This hypothesis seems

plausible as the specific action of many toxins, including bacterial endotoxin, is damage to the nucleolus (Cheville, 1976).

The cause of death in shrimp with HE may be due to osmotic imbalances or to poor absorption of nutrients from the midgut due to the destruction of the midgut mucosa. However, in most instances, death appears to be due to secondary bacterial septicemias. Vibrio sp., principally V. alginolyticus, is the organism most commonly isolated from the hemolymph of shrimp with septic HE (Lightner et al., 1978; Lightner, 1983). Mortality rates in populations of P. stylirostris with HE have reached 85% but, typically, were less than 50% of the affected population (Tables 2 and 3).

Besides causing mortalities in affected populations, HE also causes runting of those individuals that survive the acute phase of the disease. The histogram (Figure 4) demonstrates the chronic effect of HE in a random sample of a population in which HE incidence was about 5%. HE was diagnosed in 12 (by the presence of grossly apparent melanized hindgut ceca in animals with chronic HE) of 297 shrimp examined. These shrimp were the smallest shrimp in the sample, and represented a runted subpopulation in size distribution from the main population.

Table 2. Summary of hemocytic enteritis epizootics in blue shrimp (Penaeus stylirostris) which occurred during blooms or times of dominance of certain blue-green algae in raceways at Puerto Peñasco.

Approximate Dates of Epizootic		Size Range Wt. (g)	Initial Population	Approximate Accumulative Percent Mortality
July-Sept	1975	0.05 - 4.0	17,700	85
July-Aug	1976	0.62 - 2.4	21,000	81
Oct-Dec	1976	0.54 - 2.6	21,700	60
October	1976	0.24 - 1.0	19,600	46
November	1976	2.9 - 5.0	11,200	38
Jan-Mar	1977	12.3 -19.8	12,700	13
Oct-Nov	1977	0.01 - 0.5	26,000	61
April	1978	0.5	105,000	3
Mar-Apr	1979	0.01 - 0.5	68,000	12
May	1979	0.01 - 0.1	120,000	10-20
July-Aug	1979	0.01 - 0.1	506,000	10
Sept-Nov	1979	0.01 - 0.1	849,000	40
Sept-Nov	1979	0.01 - 0.6	563,000	15
November	1979	0.1 - 0.5	450,000	20
February	1980	0.1 - 2.1	208,000	5
February	1980	0.5 - 1.0	123,000	2
April	1980	0.01 - 0.5	310,000	20
April	1980	1.0 - 1.5	275,000	5
June-July	1980	1.0 - 1.5	73,000	10
June-July	1980	0.5 - 1.5	128,000	50

Table 3. Summary of naturally occurring cases of hemocytic enteritis (HE) observed since July 1, 1980, in Machrobrachium rosenbergii and several penaeid species.

Species	Approx. Avg. Wt.	Dates Observed	Type of Culture System	Location	HE-Caused Accumulative Percent Mortality
P. stylirostris	1-3 g	July 80	CEAq Post-nursery raceway	Peñasco, Mex.	~50
"	0.1 g	Aug-Sept 80	CEAq Post-nursery raceway	Hawaii	~20
"	3-9 g	Oct 80	CEAq Growout raceway	Hawaii	<10
"	1.5 g	Jan 81	CEAq Post-nursery raceway	Hawaii	<10
"	0.5-7 g	Apr-May 81	CEAq Nursery tank and Growout raceway	Hawaii	<10
"	0.3-0.5 g	July 81	CEAq Post-nursery raceway	Hawaii	<10
"	0.1 g	Aug 81	CEAq Post-nursery raceway	Hawaii	<10
"	0.6 g	Aug 81	CEAq Raceway	Guaymas, Mex.	~20
"	1 g	Apr 82	CEAq Post-nursery raceway	Hawaii	<10
"	15-18 g	June-July 82	CEAq Growout nursery	Hawaii	<10
"	~20 g	Nov 82	Earthen Pond	Israel	ND
"	60 g	Dec 82	Earthen Pond	Hawaii	ND
P. vannamei	10-60 mg	Dec 80	CEAq Nursery tank	Hawaii	<10
P. japonicus	0.2 g	Oct 80	CEAq Post-nursery raceway	Hawaii	<10
"	8 g	Apr 81	Earthen Ponds	Hawaii	<10
P. monodon	10 g	Sept 82	Earthen Ponds	Philippines	ND
Macrobrachium rosenbergii	1-4 g	Nov 81	Earthen Ponds	Brazil	ND
"	Juvenile	—	Earthen Ponds	Hawaii	ND[1]

[1]Brock, 1982

Causative Agent of HE:

At Puerto Peñasco, Mexico, three species of the Oscillatoriaceae (Spirulina subsalsa Oersted, Schizothrix calcicola [Agardh] Gomont, and Microcoleus lyngbyaceus (= Lyngbya majuscula) [Kutzing] Crouan), because of their relative abundance in shrimp rearing tanks and their presence in shrimp stomach contents during HE epizootics, were initially circumstantially suspect as the probable causative algae. One of the suspect algae, Schizothrix calcicola type B, had been originally identified as an Oscillatoria sp. by use of Desikachary's (1959) key (Lightner et al., 1978). However, isolates of this presumed Oscillatoria sp. were later identified as Schizothrix calcicola by Drouet (personal communications, Philadelphia Acad. Sci., PA). Two of these three species have since been found to be associated with HE epizootics in penaeids reared under similar conditions in Hawaii. The two species of algae associated with the Hawaiian epizootics were strains of Sc. calcicola and S. subsalsa, while M. lyngbyaceus (and related forms) were absent. Adding to this circumstantial evidence have been the results of controlled studies in which a strain of Sc. calcicola type B was shown to induce the disease in experiments in which it was fed as a unialgal culture to susceptible juvenile P. stylirostris or P. californiensis (Tables 4-7). The incidence of confirmed HE disease resulting from these experimental trials have ranged from lows of approximately 14% (Table 6) to highs of 78% in one trial (Table 5). Of the other two suspect algal species, S. subsalsa and M. lyngbyaceus, only S. subsalsa has been demonstrated experimentally to cause HE disease in juvenile P. stylirostris fed unialgal cultures of this alga (McKey, 1981). However, experimental results with S. subsalsa were

highly variable and confirmed incidence of HE in shrimp fed this species was much lower than was typical for S. calcicola, with incidence rates of HE in experiments usually ranging from 0 to 22% (McKee, 1981). M. lyngbyaceus was highly suspect as being also capable of causing HE disease because of its abundance in shrimp culture tanks (in Mexico) in which epizootics of HE were occurring and because shrimp in such culture tanks readily grazed upon that alga. It was not unusual to find shrimp with greater than 50% of their stomach contents being Microcoleus. Closer study, however, revealed that Sc. calcicola filaments were typically associated with the larger Microcoleus filaments. It seems likely, in hindsight, that it was this unnoticed association of Sc. calcicola with M. lyngbyaceus that was actually responsible for the HE epizootics once thought to be possibly due to M. lyngbyaceus. The same sort of association of Sc. calcicola with S. subsalsa blooms and the greater toxicity of the former alga in experimental studies, suggests that Sc. calcicola may be the alga actually responsible for the HE epizootics observed, and not S. subsalsa as had once been hypothesized (Lightner, 1978).

Table 4. Summary of results of a study in which Penaeus stylirostris[1] and P. californiensis[2] were cultured with live Schizothrix calcicola (type B) in 40 l glass aquaria.[5]

Treatment	Species	HE Incidence[6]	Percent Incidence	Accumulative Mortality
Schizothrix calcicola	P. stylirostris	4 of 15	27%	30%
Sc. calcicola	P. californiensis	0 of 16	0	20
Nutrient Control[3]	P. californiensis	0 of 15	0	15
Negative Control[4]	P. californiensis	0 of 20	0	0
Negative Control	P. stylirostris	0 of 18	0	10
Nutrient Control	P. stylirostris	0 of 17	0	15

[1,2] 0.2 g average weight
[3] Nutrient control: aquaria fertilized with PESW (Appendix) as were tanks containing algae.
[4] Negative control: Inoculated with Amphora sp. (a diatom).
[5] Experimental conditions: Temperature 25-27°C salinity 20 p.p.t.; 20 day duration; lighting by daylight fluorescent 100 ft-c continuously, algae introduced on day 1; all fed daily a shrimp pellet; initial population of 20 per tank.
[6] All survivors to day 20 taken for histologic study for HE.

Table 5. Summary of results of an experiment in which Penaeus stylirostris[1] and P. californiensis[1] were monitored for HE during continuous exposure to Schizothrix calcicola type B from Mexico.[2]

Treatment	Species	HE Incidence	Percent	Severity Avg. Degree	Accumulative Range	Accumulative Mortality
Schizothrix calcicola	P. californiensis	5 of 19	26	0.8	0-3	68%
Sc. calcicola	P. stylirostris	4 of 22	18	1.8	0-3	58
Sc. calcicola	P. californiensis	7 of 9	78	2.7	0-3	82
Sc. calcicola	P. stylirostris	7 of 17	41	3.8	0-3	66
Control	P. californiensis	0 of 13	0	0	0	73[3]
Control	P. stylirostris	0 of 18	0	0	0	64
Combined Data:						
Sc. calcicola	P. californiensis	12 of 28	43			
Sc. calcicola	P. stylirostris	11 of 39	28			

[1] Average size of both shrimp species was 0.4 g.
[2] Experimental conditions: Temperature 25-27°C; salinity 20 p.p.t.; 20 l fiberglass tanks; initial population of 50 per tank; experiment duration 30 days; fed artificial pellet daily; lighting by daylight fluorescent 100 ft-c continuously.
[3] Control tank due to ammonia/nitrite toxicity due to absence of algae.

Table 6. Summary of results of an experiment in which juvenile P. stylirostris were cultured with Schizothrix calcicola type B from Hawaii.[1]

Treatment	Species	HE Incidence	% Incidence
Schizothrix calcicola	P. stylirostris	1 of 7	14
Control	P. stylirostris	0 of 6	0

[1] Experimental conditions: Average weight shrimp 0.1 g; 25 shrimp each tank; lighting by daylight fluorescent 200 ft-c continuously; duration of experiment 15 days; water temperature 27-29°C, salinity 28-30 p.p.t., pH 8.1-8.4, undergravel filters in all tanks.

Table 7. Summary of an experiment in which live and freeze-dried *Schizothrix calcicola* type B and live *Spirulina subsalsa* were fed to juvenile *Penaeus stylirostris*.[1]

Treatment	HE Incidence	Severity % Incidence	Severity Average	Severity Range
Live *Sc. calcicola* + control diet	2 of 9	22	1.1	0-3
Live *S. subsalsa* + control diet	0 of 7	0	0	0
Freeze-dried *Sc. calcicola* (25%)	2 of 7	29	0.4	0-3
Freeze-dried *Sc. calcicola* (5%)	8 of 16	50	2.7	0-3
Control diet	5 of 11	45	2.8	0-3
Zero-day sample	0 of 10	0	0	0

[1] Experimental conditions
Shrimp : 25 mg average weight
Tanks : 40 l glass, recirculated with filtration and UV light in-line disinfection
Duration: 28 days
Lighting: 200 ft-c daylight fluorescent continuously

The discovery of blue-green algal-caused hemocytic enteritis in the controlled-environment culture of penaeids, raises the question of the importance of these blue-green algae in other forms of crustacean aquaculture, or perhaps, more importantly, as to whether marine members of the Family Oscillatoriaceae could affect the abundance and distribution of natural populations of certain crustaceans. On the aquaculture side of the question, cultured juvenile populations of the freshwater prawn Macrobrachium rosenbergii have, on occasion, experienced high mortalities in outdoor culture ponds in Hawaii by an, as yet, unidentified disease. The size and age of animals involved and the algae present in the ponds suggest that blue-green algae-caused HE disease may be involved, and occasional juvenile M. rosenbergii with HE have been found in Hawaii (Brock, 1983) and in Brazil (Lightner, unpublished data).

Other reports of toxigenic species of blue-green algae belonging to the Oscillatoriaceae are known from Japan, Hawaii, the Marshall and Gilbert Islands and Eniwetok Island in the Central Pacific (Banner, 1959; Moikeha et al., 1971; Moikeha and Chu, 1971; Hashimoto et al., 1976; Mynderse et al., 1977; Mynderse and Moore, 1978). Members of the Family Oscillatoriaceae shown to produce, or suspected of producing, toxins that can cause a contact dermatitis or possibly other toxic syndromes in man include M. lyngbyaceus (= Lyngbya majuscula), Oscillatoria nigroviridis, and Sc. calcicola (Moore, 1977). Because these organisms have been shown to be abundant in near-shore areas in the tropical Pacific and because they are similar to and, in two cases, thought by Drouet (1968 and personal communications) to be identical species to

those causing disease in penaeid shrimp, HE disease may not be confined to cultured penaeid shrimp and M. rosenbergii. In fact an analagous gastrointestinal disease caused by a freshwater strain of Sc. calcicola has been reported in man (Lippy and Erb, 1976; Keleti et al., 1979). A water-borne outbreak of gastroenteritis affected approximately 5,000 persons in Sewickley, Pennsylvania, during August of 1975. Extensive microbiological and chemical analyses of specimens obtained from patients and of water samples failed to identify a causative agent. However, investigation of the Sewickley water system revealed an accumulation of Sc. calcicola in the open finished-water reservoirs. Even one month after the outbreak, the water in the reservoir with the longest detention time was still contaminated by the alga (400,000 cells/ml).

It should be noted that not every animal species may be sensitive to the toxins present in these oscillatoriacean algae, or that only a few toxic forms are present in this group. The sea hare (Stylocheilus longicauda) is known to graze on L. majuscula without showing any harmful effect (Mynderse et al., 1977), and man and animals have safely consumed freshwater-cultured S. platensis and S. maxima without harmful effects (Hudson and Karis, 1974; Boudene et al., 1976; Stanley and Jones, 1976).

Isolation and Identification of Suspect Algae

Five strains of blue-green algae were isolated from the shrimp raceways in Puerto Peñasco, Mexico, and identified by Drouet (1980, personal communication) as: Microcoleus lyngbyaceus (Kutzing) Crouan, Spirulina subsalsa (Oersted), and three types of Schizothrix calcicola (Agardh) Gomont (designated as types A, B, and C to distinguish these morphologically distinct types of the same "species"). Identical strains of Sc. calcicola type B, and Spirulina subsalsa were also isolated from the ERL facility in Hawaii.

M. lyngbyaceus was isolated in Erdschreiber's Medium by the method using hormongia (Stein, 1973). An algal contaminant, later identified as Sc. calcicola A, was frequently observed as an epiphyte on M. lyngbyaceus. Single filaments of M. lyngbyaceus were reisolated in McLachlan's Medium plus vitamin B_{12} and stored in the culture chamber. The average width of M. lyngbyaceus was determined to be 16.6 µm (12-20 µm) (Table 8). This alga possessed a sheath that was readily observed by light microscopy. The ends of the filaments were rounded and hormogonia were frequently seen (Figure 5).

Table 8. Average widths of filaments for five strains of blue-green algae.

Type	Number of Filaments Measured	Average Width (μm)	Range (μm)
Microcoleus lyngbyaceus	40	16.6	12-20
Schizothrix calcicola A	20	1.3	0.9-1.5
Schizothrix calcicola B	23	2.6	2.1-3.0
Schizothrix calcicola C	20	2.6	2.0-3.0
Spirulina subsalsa	23	3.1	2.9-3.5

Hawaiian and Mexican isolates of S. subsalsa were obtained employing the spot plate method, and transferring isolated filaments to tubes of McLachlan's Medium (Stein, 1973; and Appendix). Four tubes were examined microscopically for contaminants and stored in a culture chamber. Filament widths averaged 3.1 µm (2.9-3.5 µm). S. subsalsa lacked a visible sheath and cell walls were not visible by light microscopy. Filaments were readily recognized by their tightly spiraled appearance (Figure 6).

Cultures of Sc. calcicola B from Hawaii and Mexico (Table 9) were isolated on 1% SSA (Appendix A) from a sample scraped from the raceway sides or off netting located above the raceway water line and transferred to tubes of McLachlan's Medium. Clonal cultures were established by dragging single algae strands over agar as previously described and placing them in tubes of McLachlan's Medium. The average filament width was 2.6 µm (2.1-3.0 µm). Sc. calcicola B is a straight filament with cross walls barely discernible by light microscopy, and it possessed a thin sheath that was occasionally observed (Table 9; Figure 7). Frequently one or two vacuoles per cell was evident by light microscopy.

Sc. calcicola A was isolated by scraping it off a filament of M. lyngbyaceus with a sterile glass loop and transferring it onto an agar plate. Filaments were incubated overnight at 100 ft-c continuous light, 32 ± 1°C, and isolated as described for Sc. calcicola B. The average filament width was found to be 1.3 µm (0.9-1.5 µm). Sc. calcicola A is a smooth filament (Figure 8) with some cross walls evident and with a sheath that was infrequently

observed (Table 9). After 3 wk in culture, certain strands within the algal mat tended to arrange themselves concentrically.

Sc. calcicola C was initially observed as a contaminant in control jars during a bioassay. It appeared as a brown mat on the bottom and sides of jars. A sample was placed on 1% PESW agar, incubated overnight, and then isolated as described for Sc. calcicola B. Filament widths averaged 2.6 µm (2.0-3.0 µm). Color in cultures ranged from brown to light green. Sc. calcicola C has pronounced constrictions at the cross walls and has a sheath that was occasionally observed (Table 8; Figure 9).

Ultrastructure

The fine structure of S. subsalsa, Sc. calcicola types A and B, and Microcoleus lyngbyaceus was investigated using scanning and transmission electron microscopy. The cellular morphology and structure of these algae are shown in Figures 5-8. These algae, while individually distinct in their structure and morphology, show numerous similarities to related forms (Fogg et al., 1973 and Humm and Wicks, 1980).

Table 9. Summary of the main features of strains of Schizothrix calcicola isolated from penaeid shrimp culture tanks.

Strain	Diameter	Colony Color(s)	Morphology Other Features	Source of Isolates
A	1.3 (0.9 to 1.5 µm)	blue green, brown yellow, reddish brown	Concentric whorls in algal mats in mature cultures; filaments smooth, with cross walls evident, seldom with sheath.	Mexico
B	2.6 (2.1 to 3.0 µm)	blue green to yellow green	Filaments smooth, with cross walls, with a thin sheath, frequently with one or two vacuoles per cell.	Mexico, Hawaii
C	2.6 (2.0-3.0 µm)	brown, red-brown, pale green	Filaments with constrictions at cross walls; occasionally with sheath.	Mexico

Optimum Growth Conditions

Culture Media. M. lyngbyaceus was grown in eight variations of five media (Table 10; Appendix). Two trials were run for periods of 24 and 15 days. For all media, except ASM, growth was noted by day 7 when filaments started to spread across the bottom of each flask. Good growth of this alga occurred in McL plus vitamin B_{12}, McL nutrients plus seawater, Erds, Erds plus vitamin B_{12}, and PESW. In Trial 1, the most dense growth was observed in the flask containing PESW; Erds promoted the best growth in Trial 2. This discrepancy can be partly explained by the method used in determining the concentration of the original inoculum which was only approximate and may have varied. Poor growth was observed in the flask containing soil-seawater medium (SS). No growth was observed in the flask containing ASM, and by day 9 the original inoculum had turned yellow.

Table 10. Relative measures of growth of four strains of blue-green algae with different media.[1]

Algal Species	ASM	PESW	McL	SS	ERDS	McL +B_{12}	McL+ Sea-water	McL+ B_{12}+ PO_4+ NO_3	McL+ PO_4	ERDS B_{12}	ERDS+ B_{12}+ Micro-nutrient
Microcoleus lyngbyaceus	0	++++	—	+	++	+++	—	—	—	+++	+
	—	++	—	—	+++	—	++	—	—	—	—
Schizothrix calcicola A	+	++++	+++	—	+	+++	+++	—	—	—	—
	—	++++	—	—	+	++	+++	—	—	—	—
Schizothrix calcicola B	0	++++	—	+	—	+++	—	—	—	—	—
	—	++++	—	—	+++	—	+++	—	—	—	—
	—	++++	—	—	—	+++	—	—	—	—	—
Spirulina subsalsa	0	++++	+++	+	+	—	—	+	—	—	—
	—	—	+++	—	—	—	—	+	++	—	—
	—	++++	+++	—	++	—	++	—	—	—	—

0 = no growth.
\+ = sparse growth (algae slightly spread out on bottom or faint green color in water column).
++ = moderate growth (bulk of algae spread out on bottom and faint green color in water column).
+++ = abundant growth (heavy on bottom, some on sides and water's surface).
++++ = luxuriant growth (heavy growth on bottom and sides of flask and on water's surface).
1 See Appendix for definition of media names used in this Table.

Sc. calcicola A was grown in six types of media during two trials lasting for periods of 21 and 15 days. Filaments grown in PESW produced the best results in both trials. McLachlan's nutrients plus seawater and vitamin B_{12} also promoted good growth of filaments (Table 10).

Sc. calcicola B was grown in six different media in trials conducted for periods of 7, 15, and 6 days. In all three trials, PESW was found to be the best growth medium. McL nutrients plus seawater also produced good growth (Table 10).

S. subsalsa was grown in nine media. Three trials were conducted having durations of 15, 33, and 15 days. The first and third trials indicated PESW as the best growth medium. McL and McL nutrients plus seawater also produced good growth. Trial 2 involved variations on McL. The addition of phosphate and phosphate with nitrate did not promote growth. The addition of vitamin B_{12} appeared to increase growth (Table 10).

Growth in PESW was consistently good for all algae (Table 10), but it was noted that filaments turned yellow in color sooner than those in McL. Since McL contained a greater amount of nitrate, a trial was conducted to determine if an increase of nitrate in PESW would alleviate that situation. Sc. calcicola A, Sc. calcicola B, and S. subsalsa were tested with two and three times the original amount of nitrate as sodium nitrate (Table 11). In all three cases, the addition of nitrate appeared to prolong the green color of the algae.

Light Intensity. M. lyngbyaceus was grown in McL plus vitamin B_{12} with a light intensity range of 100 to 400 ft-c for a period of 26 days. Growth was best at 200 to 400 ft-c, decreasing as light was decreased below 200 ft-c. A dark blue-green color was observed at both 100 and 200 ft-c. At 300 and 400 ft-c, a lighter green color was observed at three-quarters of the way through the trial (Table 12).

Table 11. Differences in color of three strains of blue-green algae with time and addition of extra nitrate as sodium nitrate to Provasoli's Enriched Seawater.

Algal Species	Number of Days	PESW	PESW + 2 x NO_3	PESW + 3 x NO_3
Sc. calciola B	5	YG	G	G
	14	Y	YG	G
	21	Y	Y	G
	29	Y	Y	G
Sc. calciola A	8	YG	G	G
	12	Y	YG	G
	14	Y	Y	YG
	19	Y	Y	Y
S. subsalsa	12	Y	YG	G
	14	Y	Y	YG
	21	Y	Y	YG

Y = Yellow
G = Green
YG = Yellow green

Sc. calcicola A was grown in PESW with a range of 50 to 300 ft-c for a 10-day period. A dark green color was observed at both 50 and 100 ft-c. Growth was most dense at 300 ft-c, but color ranged from light green to light yellow (Table 12).

Sc. calcicola B was tested in PESW with a range of 50 to 300 ft-c in three separate trials. Growth was best at 200 to 300 ft-c, but filaments were light green during most of the trial to almost colorless toward the end. A blue-green color was observed at lower light intensities of 50 to 200 ft-c (Table 12).

Three trials were conducted with S. subsalsa. The medium used for two trials was McL plus vitamin B_{12} and lasted for periods of 11 and 15 days. The third trial was conducted in PESW for 9 days. Best growth was observed at 100 ft-c. A dark blue-green color was observed at light intensities ranging from 25 to 100 ft-c. Above 100 ft-c, a light yellow color was present (Table 12).

Temperature. M. lyngbyaceus grew best at 32°C (Table 13). Three days after the trial began, the algae at 32°C were observed to have a darker blue-green color than the algae incubated at the other temperatures. Growth occurred at 24°, 32°, and 38°C by day 9. Growth at 32°C was dense by day 20 and the algae had started to turn yellow. This may have resulted from a depletion of nutrients. Growth in the other flasks was much less and the algae retained their blue-green color.

Table 12. Variations in color and growth of four algal strains under seven light intensities ranging from 25 to 400 ft-c.

Algal Strain	Duration of Trial (days)	Trial Number	Light Intensity (ft-c) 25	50	75	100	200	300	400
Microcoleus lyngbyaceus	26								
color			—	—	—	BG	G	G	LG
growth			—	—	—	++	+++	+++	+++
Schizothrix calcicola A	10								
color			—	BG	—	BG	LY	LY	—
growth			—	+	—	++	+	+++	—
Schizothrix calcicola B	12	1							
color			—	BG	—	G	LY	LY	—
growth			—	+	—	++	+++	+++	—
	7	2							
color			—	BG	—	G	G	LY	—
growth				+	—	++	++	+++	—
	7	3							
color			—	BG	—	BG	G	LY	—
growth				+	—	++	++	+++	—
Spirulina subsalsa	11	1							
color			—	—	—	—	—	LY	LY
growth			—	—	—	+++	++	++	++
	15	2							
color			BG	BG	BG	LG	—	—	—
growth			+	++	++	+++	—	—	—
	9	3							
color			BG	BG	BG	BG	—	—	—
growth			+	++	++	+++	—	—	—

BG = Blue-green
G = Green
LG = Light green
LY = Light yellow

\+ = Fair growth
++ = Moderate growth
+++ = Luxuriant growth

pH. S. subsalsa tested in PESW at 100 ft-c in Trial 1 and 200 ft-c in Trial 2 showed no differences in growth with varying pH (Table 14). S. subsalsa failed to grow in ASM plus B_{12} in Trial 3 at each pH tested.

Table 13. Growth of Microcoleus lyngbyaceus at four different temperatures and under 100 ft-c and continuous light.

Trial 1	
Temperature	Growth Rate
21°C	Fair
24°C	Moderate
32°C	Luxuriant
38°C	Fair

Table 14. Differences in growth of Spirulina subsalsa at four different pH levels incubated under 100 and 50 ft-c under continuous light at 32 ± 1°C.

Trial Number	Light Intensity (ft-c)	pH				
		7.1	7.7	8.0	8.5	9.0
1	100	—	++	++	++	++
2	50	—	++	++	++	++
3	100	0	—	0	0	0

\+ = fair growth
++ = moderate growth

Effect of Algicides

Algicides were tested to determine if HE could be prevented or managed in shrimp culture systems by identifying algicides with selective toxicity to the blue-green algae (Fitzgerald, 1971). Tested with potential algicides were the strains of blue-green algae (S. subsalsa and Sc. calcicola B) suspected or known to cause HE, representative beneficial algae (Amphora sp. and Enteromorpha sp., representing the diatoms and green algae, respectively), and Leucothrix mucor (a ubiquitous filamentous bacterium that possesses an LPS endotoxin [Charlton, 1978], is always present with cultured shrimp, and is similar in appearance and structure to certain oscillatoriacean blue-green algae [McKee and Lightner, 1982], but lacks chlorophyll). The results of those trials, summarized in Table 15, show that Sc. calcicola and S. subsalsa were generally as sensitive to the algicides tested as were Enteramorpha sp., Amphora sp. and L. mucor.

Aniline was included in the test because of its reported selective toxicity to blue-green algae (Batterton et al., 1978). Aniline showed no algicidal activity at the concentrations tested.

Of those products that showed algicide activity at the levels tested, Black Algaetrine, Roccal II, Cutrine-Plus, Algicide Inhibitor, and Hopkin's CCG showed potential use as algicides in managing blue-green algae in shrimp culture (Table 15). However, the concentrations of these algicides required to effectively manage (cidal effect) an alga like Sc. calcicola (Figure 11) approach or exceed the shrimp LC50 for each of the algicides (Lightner, unpublished data).

Hence, the use of algicides alone in controlling toxic blue-green algae in shrimp culture systems was found to be unrealistic.

Table 15. Summary of laboratory bioassays in which certain algicides were tested for selective toxicity to blue-green algae.

Algicide	*S. calcicola* B[a,b]		*S. subsalsa*[a]		*Amphora* sp.[a]		*Enteromorpha* sp.[a]		*Leucothrix mucor*[a,c]	
	MIC	MAC	MIC	MAC	MIC	MAC	MIC	MAC	MIC	MAC
Black Algaetrine	1	10	<10	10	1	10	<10	<10		
Formalin	>100	>100	100	100	100	100	100	100	100	100
Roccal II	2.5	5	<10	10	<10	10	<10	<10	1.25	10
AT-16	>100	>100	>100	>100	100	100	100	>100	—	—
Cutrine-Plus	1	2.5	100	100	100	100	<10	<10	1.25	100
Algigon	>100	>100	>100	<100	>100	>100	>100	>100	—	—
Algicide Inhibitor	0.5	10	<10	10	<10	10	<10	<10	1.25	10
Aniline	100	100	100	100	>100	>100	>100	>100	—	—
Hopkin's CCG	2.5	10	—	—	100	100	<10	100	25	100
Spotkill	100	100	<10	100	100	100	100	100	—	—
Super Spotkill	100	100	<10	100	100	100	100	100	—	—

[a,b,c] Range of concentrations tested were a) 0, 0.01, 0.1, 1.0, 10 and 100 mg; b) 0, 0.1, 0.2, 0.5, 1, 2.5 and 5.0 mg; and c) 0.625, 1.25, 2.5, 5, 10 and 20 mg principal active ingredient per liter of algae culture medium.

Nature of the Toxin of *Schizothrix calcicola*

The nature and composition of the endotoxin of *S. calcicola* has been described elsewhere (Keleti et al., 1979). *S. calcicola* endotoxin is a lipopolysaccharide (LPS). LPS molecules are constituents of the outer cell wall of some microorganisms, predominantly gram-negative bacteria and blue-green algae.

Crude *Sc. calcicola* toxin test:

The effect of semipurified and crude endotoxin from *Sc. calcicola* strain B was tested in a series of experiments with penaeid shrimp. In the first experiment, the alga was mass cultured in four 160 liter tanks of PESW (Appendix). The resultant algae produced was collected by filtration, freeze-dried, and then added at 0 (control), 5, and 25% (in place of wheat in a diet with a formulation similar to that described in Magarelli et al., 1979) of the diet.

Twenty 25 mg juvenile *P. stylirostris* were stocked into each of ten 40 l glass aquaria, connected to a recirculating seawater system. Effluent water from the aquaria was collected by gravity flow, passed again by gravity through a 40 l sand (biological filter, collected in a sump, pumped at 10 l/min through an UV-light water sterilizer (Model SL-1, Aquafine Corp., Burbank, CA), and returned to supply the test aquaria. Supplemental aeration was supplied to each aquarium with compressed air and airstones.

Two aquaria were fed (twice daily) the 0 (control), 5, and 25% freeze-dried Sc. calcicola diets; two other paired groups were fed the control diet once daily, but were reared in lighted aquaria inoculated daily with small amounts of live Sc. calcicola or S. subsalsa, respectively, until significant growth of each algae was present in the aquaria. At each feeding time, the experimental shrimp were observed for feeding behavior (i.e., acceptance or rejection of the added feed and whether or not the shrimp were or had been grazing on the added cultures of live algae).

The observations made during this study, and its final results answered a number of questions, and are summarized in Table 7. First, animals fed either live or freeze-dried Sc. calcicola developed HE within the 28 day experimental period. Those fed S. subsalsa did not develop HE. The pre-experiment control samples showed a zero percent incidence of HE in the shrimp population entering the experiment. The two control aquaria developed a luxuriant growth Sc. calcicola by day 28, and as expected, the shrimp in those tanks also developed HE. The proximity of these "non-algae" control aquaria to the "live" algae (Sc. calcicola) aquaria, and the absence of growth of Sc. calcicola throughout the remainder of the experimental aquaria, suggest that cross contamination or errant inoculation occurred in those tanks. However, it is significant that in every aquarium where Sc. calcicola was present, either live or freeze-dried, HE developed to significant levels.

Purified Sc. calcicola toxin tests:

Endotoxin was extracted from lyopholysed, unialgal, mass cultures of Sc. calcicola, using the phenolic extraction method used by Keleti et al. (1979). Two experiments were run with purified Sc. calcicola LPS endotoxin.

In the first experiment, purified LPS endotoxin in suspension in sterile saline was injected into the abdominal muscle of juvenile P. vannamei to provide doses to the shrimp of approximately 0, 0.1, 1.0, 10, 100, and 1000 mg of toxin per kg of body weight. Neither adverse behavior effects, nor gross or histopathological changes were noted by 72 hr postexposure. This finding is consistent with those of Keleti et al. (1979) who found Sc. calcicola LPS endotoxin to be non-toxic to mice when administered by intraperitoneal injection.

In a second experiment, 0.1 g average weight P. japonicus were fed diets contaminated with purified LPS endotoxin from Sc. calcicola, at rates that provided approximate doses of 10, 100 and 1000 mg/kg of diet to the experimental shrimp. No adverse effects directly attributable to the purified endotoxin were observed, but the endotoxin may have enhanced the development, and, hence recognition of a newly discovered disease syndrome of P. japonicus. This syndrome, named GNS (gut and nerve syndrome) is characterized by proliferative changes in the midgut and ventral nerve cord (Lightner, 1983; Lightner et al., submitted).

The absence of other types of toxins found occasionally in oscillatoriacean blue-green algae was confirmed by Dr. William Fennical (Scripps Institution of Oceanography, LaJolla, CA) and by Dr. Richard Moore (University of HI, Honolulu, HI). Unialgal cultures of Sc. calcicola type B, S. subsalsa, and M. lyngbyaceus that were known or suspected to cause HE in cultured shrimp were sent to Fennical, where they were mass cultured, concentrated, and extracted. The resultant extracts of these algae were then analyzed for content of several of the toxins reported by Moore and co-workers (1977) from these and related oscillatoriacean species from the Central Pacific and Hawaii. None were found.

We also sent to Moore a crude sample of algae (the dominant blue-green algae present were M. lyngbyaceus and Sc. calcicola) collected from a raceway in which an epizootic of HE disease was ongoing in a population of juvenile P. stylirostris. He found the algae and the extracts he prepared from the algae to demonstrate no toxicity to mice. According to Moore, similar extracts of the same or similar species of algae from Hawaii or the Central Pacific do show toxicity to mice, but no toxicity was noted in the sample from the shrimp culture raceway.

From the results of Fennical in California and Moore in Hawaii with shrimp farm-derived blue-green algae, it was concluded that highly toxic substances like debromaplysiatoxin are not present in the shrimp farm isolates.

The possibility that bacterial endotoxin might be responsible for HE was investigated. Blue-green algae often have bacteria closely associated with the

sheath material, and, in fact, obtaining bacteria-free axenic isolates of these organisms is extremely difficult (Stein, 1973). Hence, bacterial isolates were prepared on Difco Marine Agar 2216 (Difco Laboratories, Detroit, Mich.) from crude blue-green algae mats in tanks in which an HE epizootic was ongoing, and from a unialgal culture of Sc. calcicola. The results (summarized in Tables 16 and 17) revealed two significant points:

1) That the bacterial biomass present was small in relation to algal biomass and 2) that bacteria known to be pathogenic to shrimp were present on the algae only in trace amounts. The highest count of Vibrio spp. encountered was only 0.021% of the total viable count. The three dominant bacterial organisms present on total viable count (TVC) plates made from collected blue-green algae from the shrimp rearing tank with HE disease present were Enterobacter cloacae, Pseudomonas putrefaciens, and P. fluorescens (Table 17). None of these bacterial species are known shrimp pathogens and none have been isolated from the hemolymph of shrimp with septic HE disease. Vibrio spp., which are part of the normal gut flora of penaeid shrimp (Vanderzant et al., 1970; Lewis, 1973; and Yasuda and Kitao, 1980), are usually present in septic HE disease, indicating that these organisms are opportunistic, secondary invaders.

These data indicate that it is highly improbable that the bacteria associated with Sc. calcicola contribute significantly to the amount of endotoxin available to shrimp in shrimp culture ponds, tanks, and raceways, and that algal-derived endotoxin is responsible for the HE syndrome described here.

Table 16. Summary of bacterial counts per gram wet weight (total viable count, gram positive count, Enterobacter count and Vibrio count) made from a crude collection of mostly Microcoleus lyngbyaceus and Schizothrix calcicola from shrimp culture tanks or from unialgal culture of Sc. calcicola type B.

Count Category	Plating Medium*	Crude Collection from Shrimp Tank	Cultured Schizothrix calcicola B Run 1	Run 2
Total Viable Count (TVC)	Marine Agar 2216	1.6×10^7/g	5.2×10^7/g	1.1×10^9/g
Vibrio Count	TCBS Agar	2.1×10^3/g (0.013% of TVC)	$< 10^2$/g	2.4×10^5/g (0.021% of TVC)
Gram Positive Count	Phenyl Et. Agar	6.5×10^3/g	5.5×10^4/g	4.3×10^4/g
Enterobacter Count	Bismith Sulfite Agar	2.1×10^1/g (0.0001% of TVC)	$< 10^2$/g	9.9×10^4/g (0.009% of TVC)

*All media from Difco

Table 17. List of the identifications* of the three dominant colony forms picked from the TVC plates in Table 16.

Isolate Name	API Number
Entobacter cloacae	7305773 & 7315573
Klebsiella pneumoniae	7315733
Pseudomonas putrefaciens	0512004

*Identifications made according to the API analytical Profile Index 20E, Ayerst Laboratories, Inc., Plainview, New York.

Prevention and Control of HE

Penaeids apparently can develop an immunity or tolerance to blue-green algae endotoxin, provided that they are continuously exposed to toxin containing forms. Observations from the pond or tank systems in which animals with HE were obtained support that hypothesis. That is, HE seems to occur most often in tanks or shallow ponds with low turbidity and, hence, contain mats of benthic blue-green algae on the sides and/or bottom. A shallow pond, or one with less than an adequate plankton bloom, permits sufficient light penetration to the pond bottom for benthic blue-green algae to thrive and to produce "mats" of algae.

HE has been prevented in P. stylirostris by rearing the animals in covered tanks with insufficient light to support blue-green algae growth. Unfortunately, shrimp so grown become highly sensitive to the toxin of Sc. calcicola once exposed. Alternatively, shrimp tolerant to Sc. calcicola quickly lose that tolerance once the algae is removed from their access. Most algae in shrimp culture tanks, raceways, and ponds are actually beneficial (Lightner et al., 1979). In actual practice, certain types of algae are encouraged to grow in shrimp culture systems (in ponds, tanks, and raceways) because of their contribution to the shrimp nutrition and because of their ability to enhance water quality by their producing photosynthetic oxygen and their removal of carbon dioxide, nitrogenous wastes, and other potentially toxic micronutrients.

The most effective method used to control or manage HE is to prevent it by preventing the growth of toxin containing benthic blue-green algae such as Sc. calcicola. In pond and raceway culture of penaeids and Macrobrachium this is best accomplished by careful manipulation of water depth and/or water turbidity by maintenance of desirable plankton blooms. Water exchange rates, aeration, and the rate and frequency of feeding and/or fertilization may be manipulated to provide adequate stable blooms. The plankton in these blooms function in several ways to reduce and/or prevent HE. They compete with benthic forms for nutrients and sunlight energy, and if abundant enough (or if the water is deep enough), will "shade" out entirely those algae which require a substrate to grow well. By denying grazing shrimp or prawns access to toxic blue-green algae, or by reducing their relative biomass in relation to desirable algae species, HE may be controlled.

APPENDIX

Culture Media Used for Isolation and Culture of Blue-Green Algae

ASM Medium (McKey, 1981)

	um/l	mg/l
$NaNO_3$	8000	680
$MgSO_4$	1600	193
$MgCl_2$	1600	203
$CaCl_2$	800	118
K_2HPO_4	800	139
$FeCl_3$	16	4.3
H_3BO_3	80	5.0
$MnSO_4.H_2O$	56	9.5
$ZnCl_2$	1.6	.3
$CoCl_2$	.16	.038
$CuCl_2$	.0016	2.7×10^{-4}
Na_2EDTA	160	59.5

Weigh chemicals into a 1-liter flask. Dilute to 1 liter with distilled water.

Erdschreiber's Medium (Erds) (Stein, 1973)

1000 ml filtered seawater
50 ml soil-water supernatant
.2 g $NaNO_3$
.03 g $Na_2HPO_4.H_2O$

First day: Filter seawater through No. 1 filter paper and heat to 73°C.

Second day: (1) Reheat seawater to 73°C.
(2) Autoclave salt solutions (made up in distilled water so that 1 ml of each solution gives required amount for 1 liter of culture medium).

Third day: Add cold salt solutions to cold soil-water supernatant; then add these to cold seawater.

Variations:

Addition	Compound	Amount
$+B_{12}$	Cyanocobalamin	1 µg/l
+Micronutrients	McLachlan's Medium Stock No. 5	

McLachlan's Medium (McL) 0.41M (Stein, 1973)

Stock No.	Compound	g/l	M
1	$CaCl_2.2H_2O$	14.7	.01
	$MgCl_2.6H_2O$	44.7	.022
	NaCl	239.6	.41
2	$MgSO_4.7H_2O$	59.16	.024
3	K_2HPO_4	17.42	.0001
4	$Na_2SiO_3.9H_2O$	28.4	.0001
5	KNO_3	101.11	.001
	H_3BO_3	11.44	1.85×10^{-4}
	$ZnCl_2$	.109	8.0×10^{-7}
	$MnCl_2.4H_2O$	1.385	7.0×10^{-6}
	FeEDTA	.8	

$CoCl_2.6H_2O$	.0048	2.0×10^{-8}
$CuCl_2.2H_2O$	3.4×10^{-5}	2.0×10^{-10}

Dilute each stock to 1 liter with distilled water. Add 100 ml of stocks 1 and 2, and 1 ml of stocks 3, 4, and 5 to a 1-liter flask. Dilute to 1 liter with distilled water.

Variations:

Addition	Compound	Amount
$+B_{12}$	Cyanocobalamin	1 ug/l
$+PO_4$	$Na_2HPO_4.12H_2O$	.009 g/l
$+NO_3$	$Na\ NO_3$	.06 g/l
+ seawater	McLachlan Stocks 3, 4, and 5	Add stocks as described above to 1 liter filtered seawater.

Soil Seawater Medium (SS) (Stein, 1973)

Soil 1/4 to 1/2 inch over bottom of culture vessel.

Place soil in the bottom of a test tube (or bottle); cover with filtered seawater until container is 3/4 full; cover container. Steam for 3 h on 2 consecutive days.

Provasoli's Enriched Seawater (PESW) (Stein, 1973)

glass distilled water	100 ml
$NaNO_3$	350 mg
Na_2 glycerophosphate	50 mg
Fe (as 1:1 molar)*	2.5 mg
PII metals**	25 ml
vitamin B_{12}	10 µg
thiamine	.5 mg
biotin	5 µg
Tris buffer	500 mg

Adjust pH to 7.8. Add 2 ml of ES enrichment to 100 ml of filtered steamed seawater.

*Dissolve 351 mg of $Fe(NH_4)_2.6H_2O$ and 300 mg Na_2EDTA in 500 ml H_2O. 1 ml of this solution = 1 mg Fe.

**P II metal mix: to 100 ml distilled water add:

H_3BO_3	114 mg
$FeCl_3.6H_2O$	4.9 mg
$MnSO_4.H_2O$	12.4 mg
$ZnSO_4.7H_2O$	2.2 mg
$CoSO_4.7H_2O$	.48 mg
NaEDTA	100 mg

FIGURE LEGENDS

Figure 1a. Air inflated translucent plastic covered structures or "Aquacells" at E.R.L. Marine Culture Facility in Hawaii.

1b. Inside view of one of the two raceways contained in an aquacell.

Figure 2. Sections of gut of juvenile blue shrimp, Penaeus stylirostris. All hematoxylin and eosin staining.

2a. Anterior midgut with developing hemocytic enteritis. Remnants of the mucosal epithelium (E) are shown being sloughed into the gut lumen. Masses of hemocytes (H) are present on the luminal surface and under the mucosa. X400.

2b. Normal anterior midgut with an intact columnar mucosal epithelium (E). X800.

2c. Section of the anterior midgut caecum (C) with severe hemocytic enteritis. The adjacent anterior midgut (MG) is also severely affected, while the chitin-lined posterior stomach (S) is unaffected. X132.

2d. Section of the posterior midgut (MG) and posterior midgut caecum (PC) with severe HE. The adjacent chitin-lined hindgut (HG) is unaffected. X132.

Figure 3a. Light micrograph of hepatopancreatic tubule epithelial cells. Polyhedral intranucleolar bodies (PNB) are present in several of the hepatopancreatocyte nucleoli. O-toulidine blue. X1,970.

3b. A hepatopancreatic epithelial cell with cluster of PNB's that nearly fill the nucleolus. Also note the abundance of smooth endoplasmic reticulum (SER) in this cell and the adjacent cells, and the presence of a large autolysosome (L). X13,520.

3c. Higher magnification of the PNB's from 3b showing the cubic symmetry of the lattice structures and their subunit morphology. X23,260.

Figure 4. A histogram of the weight distribution of tails from 297 blue shrimp, Penaeus stylirostris. The distribution of sizes is bimodal, with the smaller size tails all being from shrimp with HE and the larger size being almost entirely from shrimp that were without HE.

Figure 5. Light micrograph and transmission (TEM) and scanning (SEM) electronmicrographs of Microcoleus lyngbyaceus filaments from shrimp culture tanks or from laboratory cultures.

5a. LM of a M. lyngbyaceus filament or trichrome (T) that is covered by a thick hyaline sheath (S), which extends well past the end of the trichrome. Single cells (C) are shown cleaving from the trichrome's free end. No stain. X560.

5b. SEM of a filament within the algae trichrome (T) shown protruding from its sheath (S). Gold sputter coated. X1,100.

5c. TEM of a trichrome (T), enclosed within its protective sheath, which has both inner fibrous (F) and outer mucilaginous (M) component. Lead citrate and uranyl acetate staining. X5,400.

5d. A higher magnification TEM of the filament shown in 5c. Photosynthetic membranes or thylakoids (M), cyanophycin granules (G), and dividing cells (D) are present in this portion of the trichrome. The fibrous nature of the inner layer of the sheath (S) is clearly evident. X19,700.

Figure 6. Light micrograph (LM) and transmission (TEM) and scanning (SEM) electronmicrographs of Spirulina subsalsa from laboratory cultures.

6a. LM of filaments of S. subsalsa. No stain. X660.

6b. SEM of S. subsalsa filaments or trichromes. Gold sputter coated. X4,000.

6c. TEM of S. subsalsa filaments. Note the absence of a sheath and the presence of cross walls (W), areas of nucleoplasm (N), and the photosynthetic membranes or thylakoids (M) within the alga's trichromes. Lead citrate and uranyl acetate. X19,700.

Figure 7. Light micrograph (LM) and trarsmission (TEM) and scanning (SEM) electronmicrographs of Schizothrix calcicola type B from laboratory cultures.

7a. LM of filaments of Sc. calcicola B from culture. Cross walls (arrows) are just visible in some filaments. A hormogonium (H) is also present. Interference contrast; no stain. X1,670.

7b. SEM of Sc. calcicola B filaments. Note the uniformity of the diameter of the filaments. Gold sputter coated. X1,500.

7c. TEM of trichromes (T) of Sc. calcicola with a fragment of sheath (S) material which was detached from its trichrome during processing. Bacterial (B) contaminants of the culture are also present. Lead citrate and uranyl acetate. X10,000.

7d. A higher magnification TEM of a Sc. calcicola trichrome less its sheath. Apparent within the trichrome is a cross wall (W), areas of nucleoplasm (N), photosynthetic thylakoid membranes (M), and numerous dense granules (G). Lead citrate and uranyl acetate. X35,400.

Figure 8. Light micrograph (LM) and transmission (TEM) and scanning (SEM) electronmicrographs of Schizothrix calcicola type A from laboratory culture.

8a. LM of a wet mount of Sc. calcicola type A from culture. Type A differs from type B primarily by its smaller filament diameter, the prominent cross walls (arrows), and the usual absence of sheaths. No stain. X1,670.

8b. SEM of filaments of Sc. calcicola type A. Note that individual cells comprising the trichromes are readily apparent due to indentations at the location of cross walls (arrows). Gold sputter coated. X2,000.

8c. TEM of Sc. calcicola type A trichromes. Type A differs from Type B not only by its smaller diameter, but by the usual absence of a sheath and by the presence of prominent identations at the site of the cross walls. Cell morphology in terms of the

organelles present and their positions with the algal filament are otherwise nearly identical in Types A and B. Lead citrate and uranyl acetate. X10,800.

Figure 9. Light photomicrograph of a wet mount of Schizothrix calcicola type C from culture. Cross walls in these trichromes are so prominent as to give this alga a pronounced "beaded" appearance. The diameter of its filaments are similar to that of Type B. No stain. X660.

Figure 10. A display of 0.45 um filter discs with collected algae (Schizothrix calcicola type B) from an algicide trial. The minimum algaestatic concentration (where the growth of the alga was inhibited) and minimum algicidal concentration (where the alga was killed) for the algicides listed was determined from this type of test.

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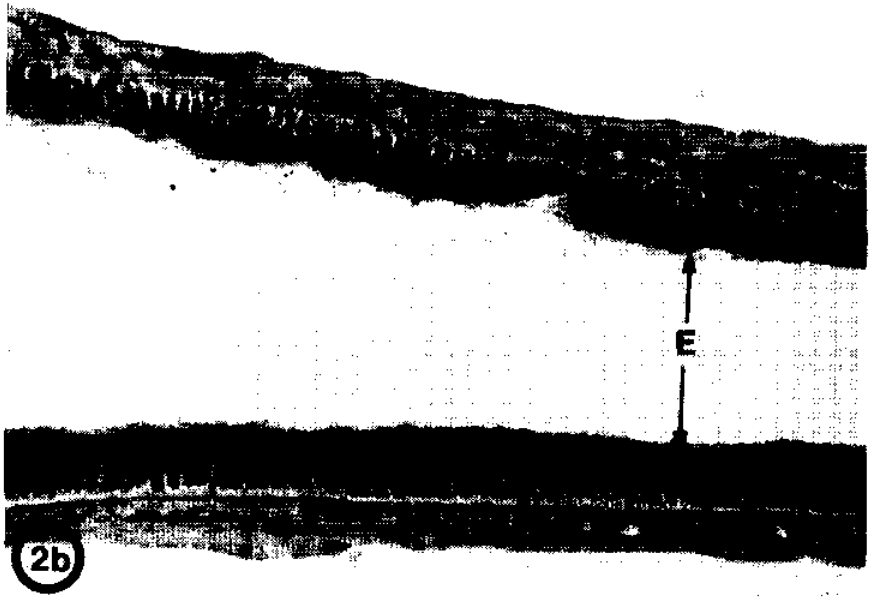
1a

1b


E
H
B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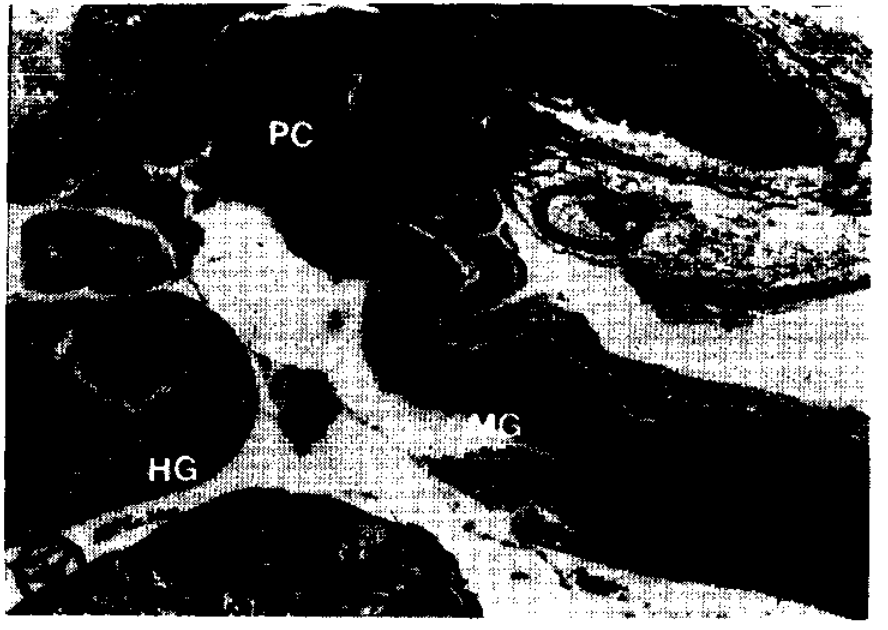
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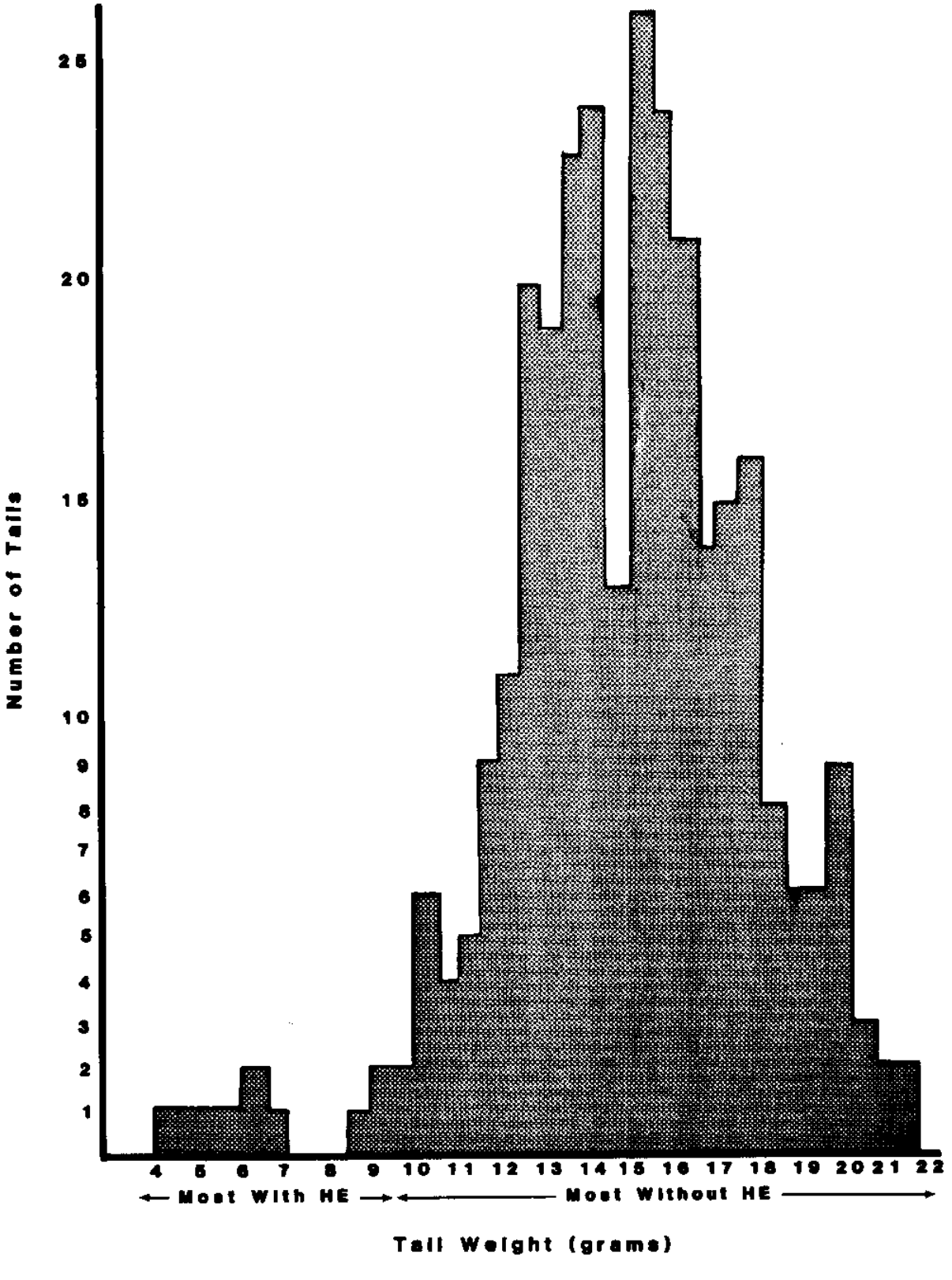
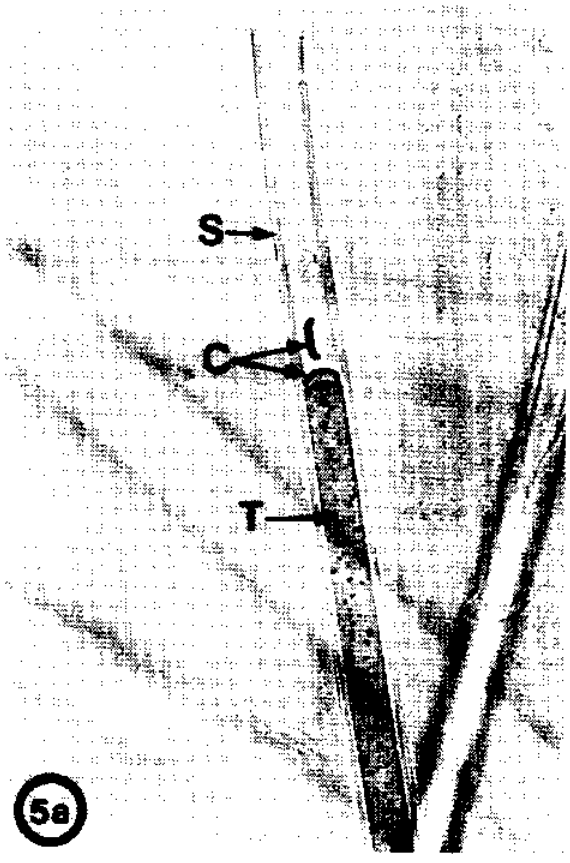
Histogram of Tail Weights (n is 297) of
Blue Shrimp, Penaeus stylirostris





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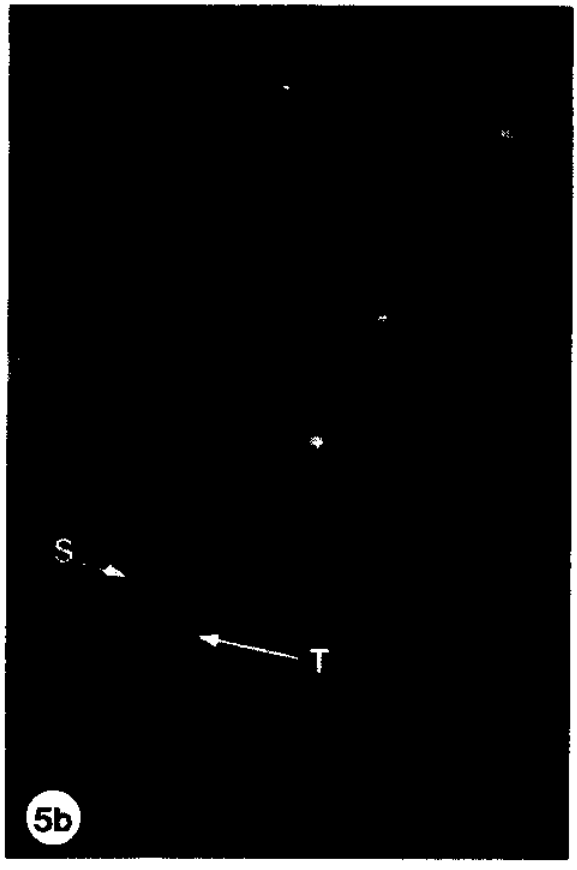

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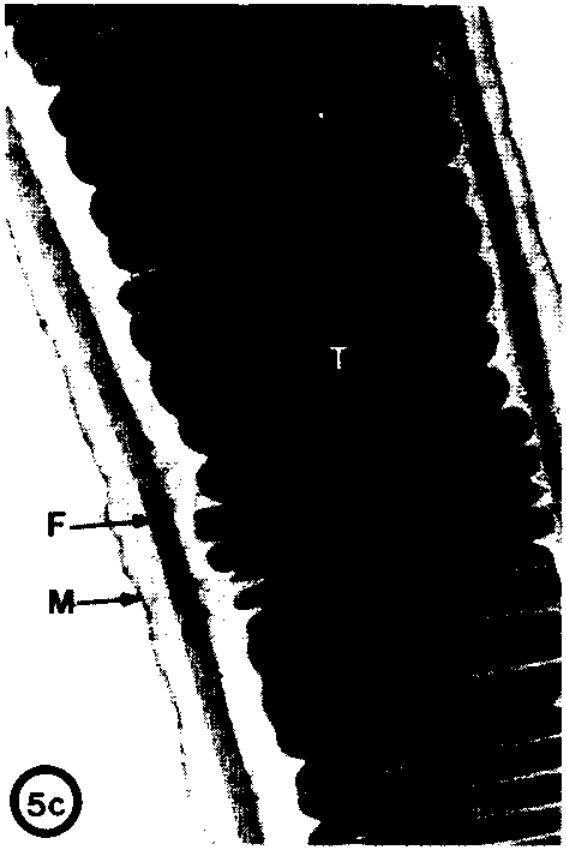

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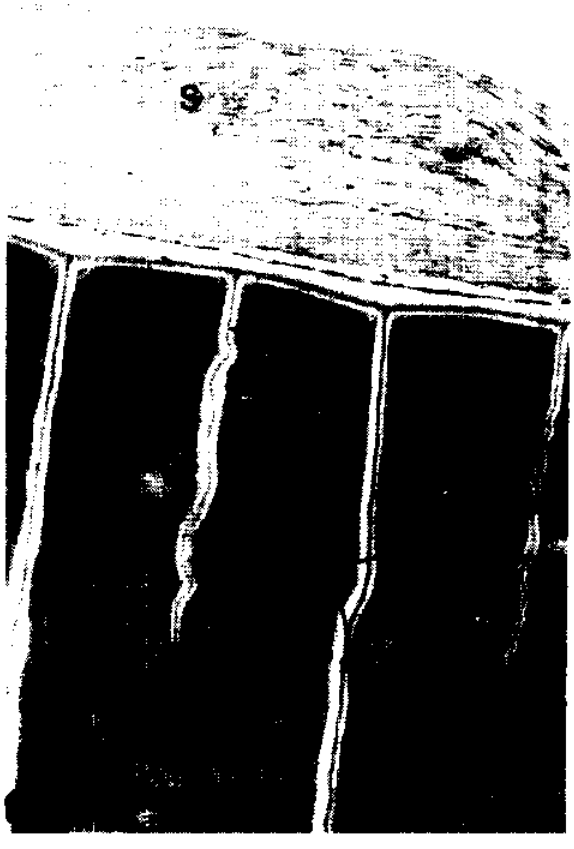

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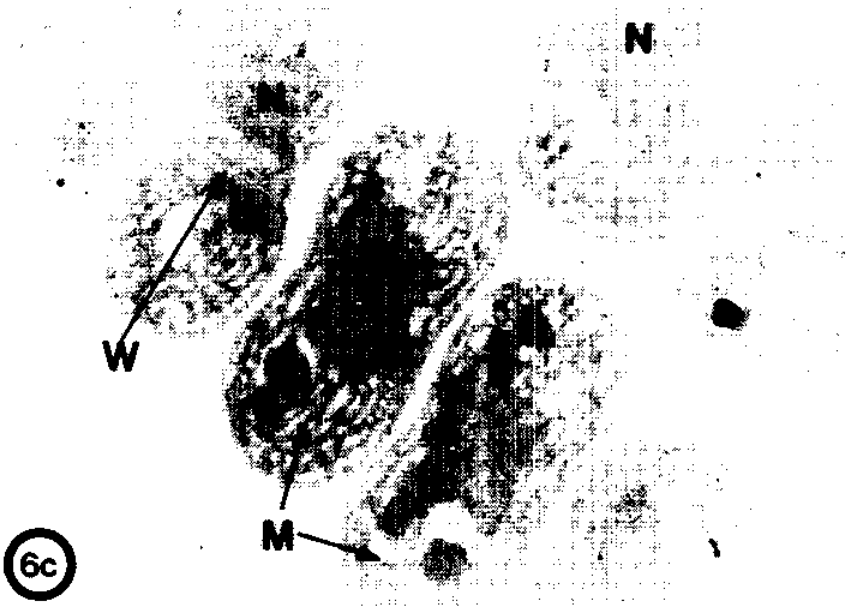

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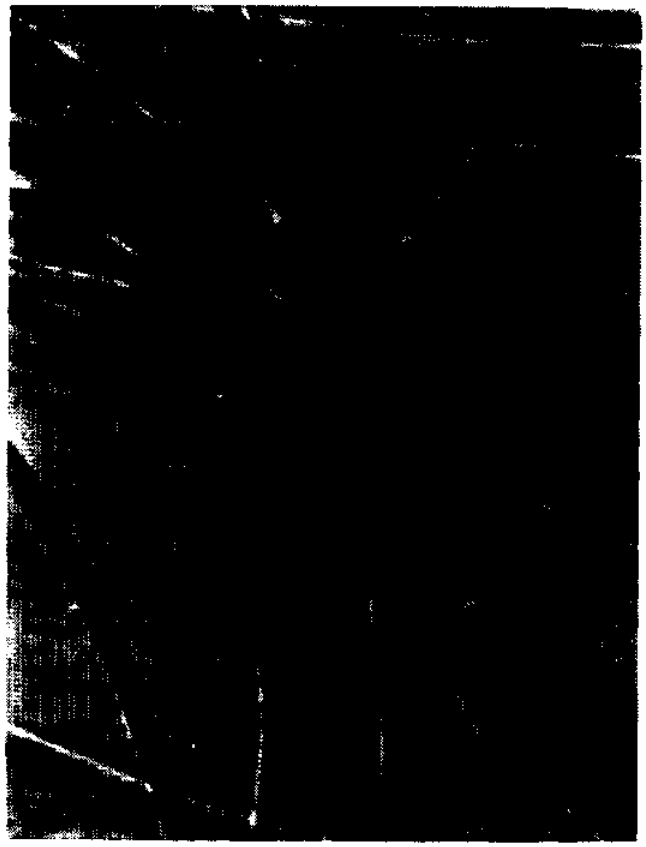

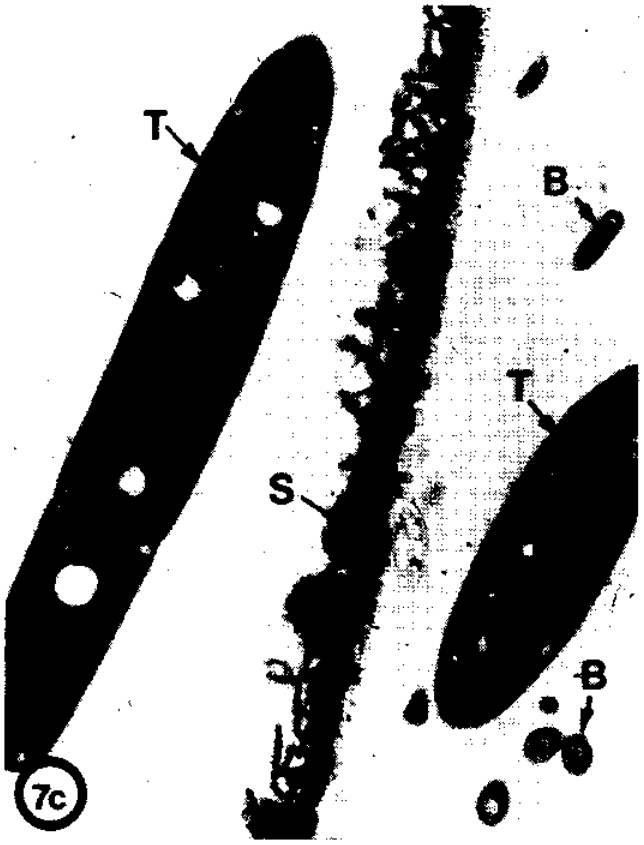

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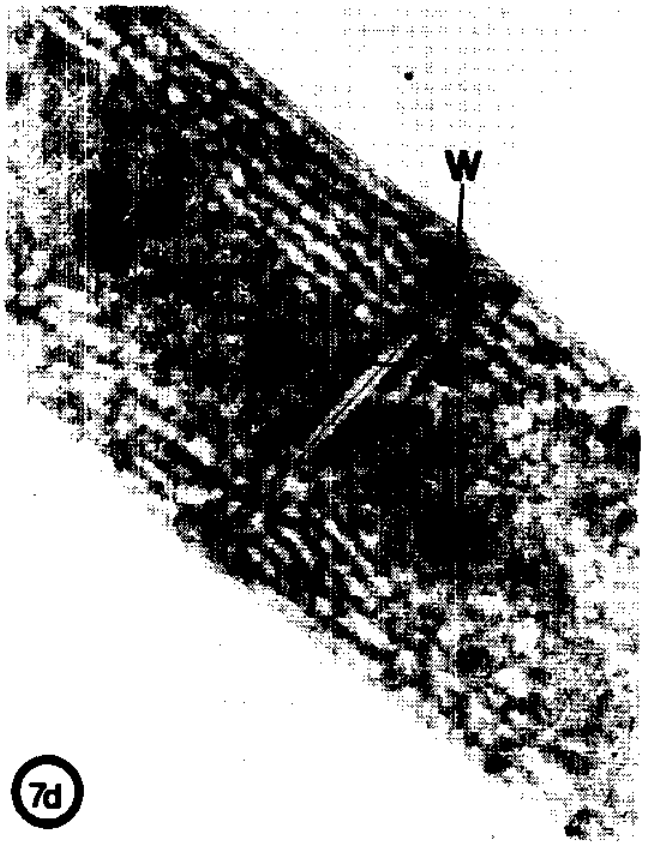

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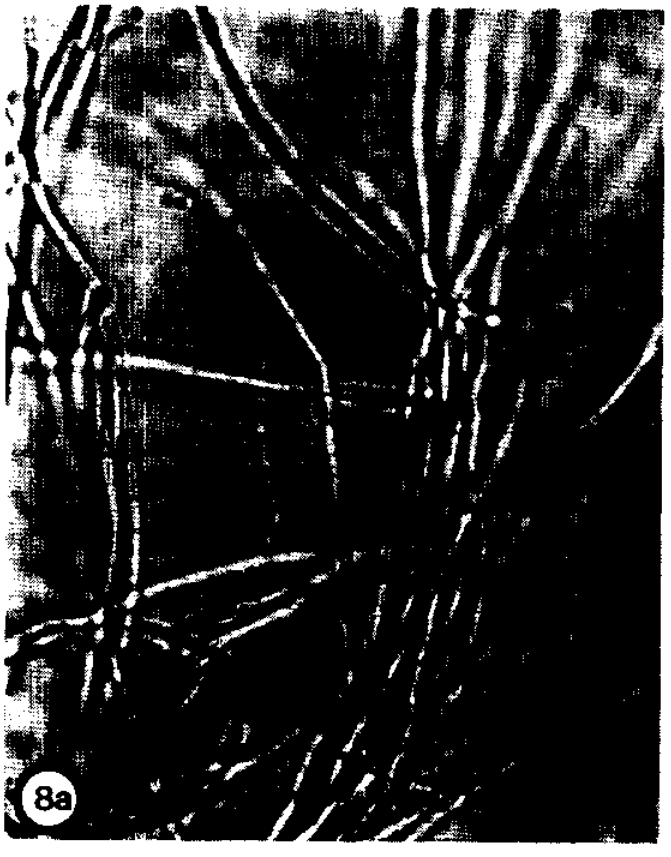
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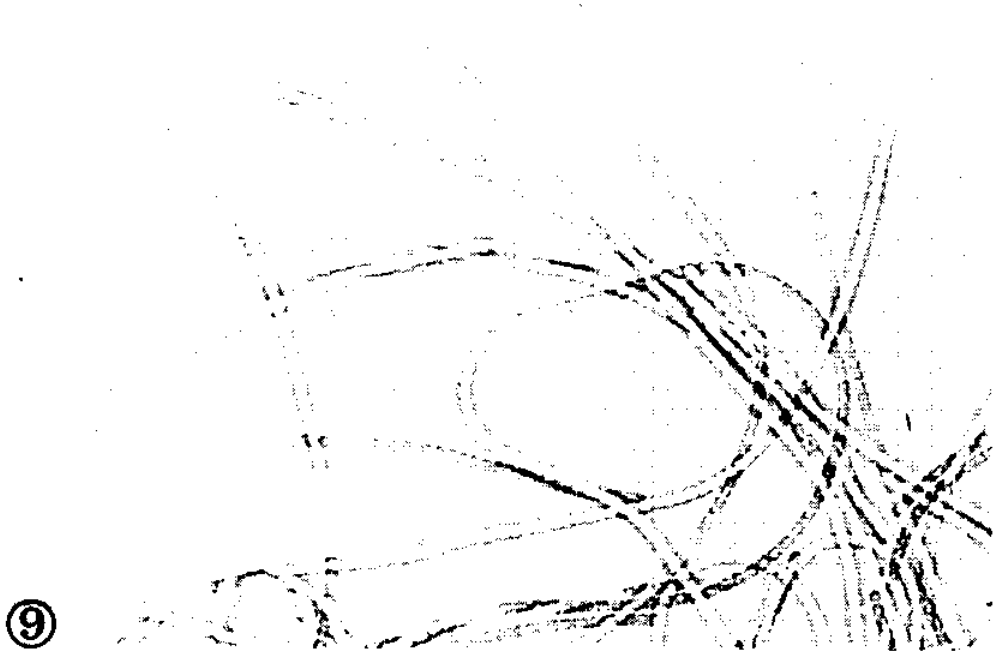

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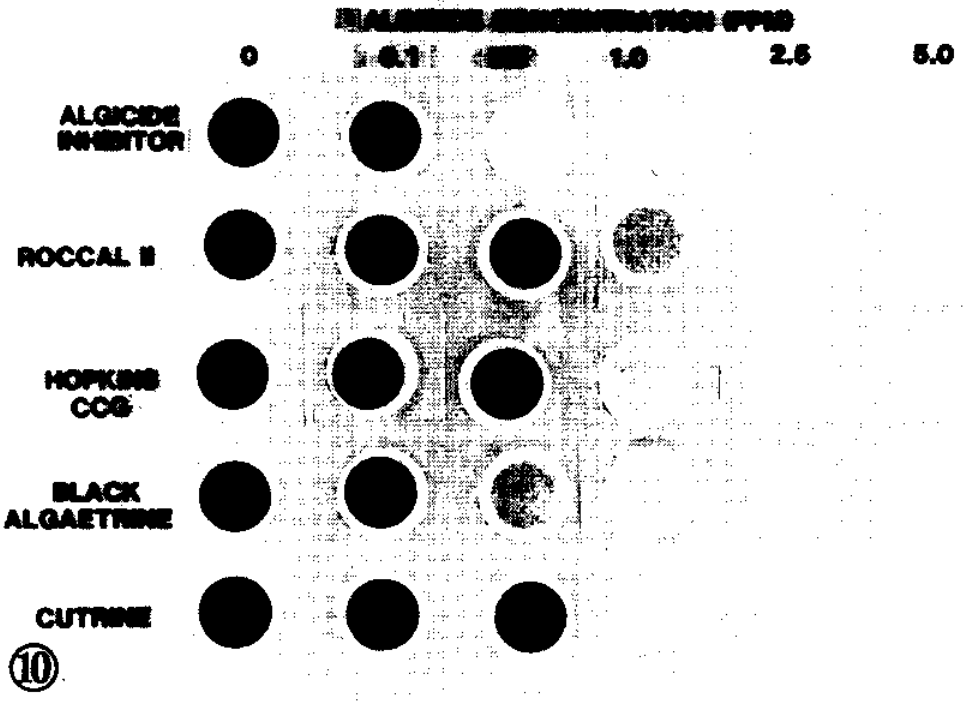
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