THE TOXIC EFFECT OF FIVE STRAINS OF BLUE-GREEN ALGAE ON *PENAEUS STYLIROSTRIS* STIMPSON

by

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ABSTRACT

Hemocytic enteritis, a disease syndrome affecting shrimp cultured in mariculture facilities in Puerto Peñasco, Mexico, and Oahu, Hawaii, has been epizootic in shrimp culture raceways containing an abundance of certain blue-green algae. The present study investigates the relationship between the presence of five strains of blue-green algae and incidence of hemocytic enteritis in the blue shrimp *Penaeus stylirostris* Stimpson. Growth conditions for four of the algal strains were also established.

Spirulina subsalsa Oersted, *Microcoleus lyngbyaceus* (Kutzing) Crouan, and three strains of *Schizothrix calcicola* (Agardh) Gomont (strains A, B, and C) isolated from shrimp culture raceways were established in unialgal cultures. Growth of filaments was most dense in Provasoli's Enriched Seawater and McLachlan's Medium at light intensities between 100 to 400 ft-c. Growth of filaments of *M. lyngbyaceus* was greatest at 32 °C when subjected to the temperature range of 21° to 38 °C. Differences of pH in the range of 7.1 to 9.0 did not have any effect on the growth of *S. subsalsa*.

In six bioassays, *P. stylirostris* was introduced into 3-liter jars containing unialgal cultures of the five strains of blue-green algae. The incidence of hemocytic enteritis increased in trials involving *S. subsalsa* and *S. calcicola* B. Only *S. calcicola* B in one out of the two caused a statistically significant increase in hemocytic enteritis.

INTRODUCTION

Blue-green algae have been associated with the occurrence of a disease syndrome, hemocytic enteritis (HE), in four species of penaeid shrimp, *Penaeus stylirostris* (Stimpson) (Lightner 1978), *P. californiensis* (Holmes), *P. japonicus* (Bate), and *P. vannamei* (Boone), reared in shrimp culturing facilities located in Puerto Peñasco, Sonora, Mexico, and Oahu, Hawaii (Lightner 1980, personal communication). The present study was undertaken to determine the relationship between this disease and five strains of blue-green algae.

Blue-green Algae

The procaryotic blue-green algae, being ubiquitous in nature, are common inhabitants of the raceways in which shrimp are raised. Their high resistance to adverse conditions is demonstrated by their occurrence in habitats ranging from hot springs with temperatures up to 80 °C (Odum 1971) to the low temperatures of the Antarctic. Fogg et al. (1973) stated that blue-green algae are usually limited to lighted habitats and prefer neutral or alkaline conditions, both of which are present in shrimp raceways.

Unialgal cultures of blue-green algae were isolated and subsequently identified employing numerous techniques due to their various forms and properties. Blue-green algae occur in coccoidal as well as filamentous forms that grow singularly, in mats, or as gelatinous masses. Both forms secrete a sheath which surrounds the cells. The

row of cells in filamentous forms is termed a trichome. The trichome and sheath collectively make up the filament. The trichomes of certain species fragment to form short segments termed hormogonia which frequently glide out of the sheath and form new filaments (Humm and Wicks 1980). Species of blue-green algae vary in color, size, shape, and texture of sheaths and trichomes according to different environmental conditions (Drouet 1968).

Toxigenic Algae

Algae have been found to produce toxins in many instances and may be producing a toxin that causes HE in cultured shrimp. Dinoflagellates such as *Gymnodinium brevis* (Davis) (Ray and Wilson 1957) comprise a large percentage of toxigenic algae, but blue-greens have also exhibited toxic effects in numerous cases (Steyn 1944; Gorham 1962; Shilo 1972; Carmichael, Briggs, and Gorham 1975).

Shilo (1972) stated that toxic algae may show mediated or direct toxicity. Algae of mediated toxicity are ingested by organisms that are not affected by the toxin but accumulate it in their tissues. The toxin is harmful when the accumulating organism is consumed by susceptible animals. Directly toxic algae such as blue-green algae produce a toxic effect upon ingestion or release toxin into the surrounding water, often upon death or decomposition of the algae.

Penaeid Shrimp

Penaeid shrimp, important in commercial fisheries, are also reared in culturing facilities. *Penaeus stylirostris* (Stimpson), the primary culture species, was used in this study for the bioassays with

suspect algae. *P. stylirostris*, of the family Penaeidae in the sub-order Natantia, is characterized by laterally compressed bodies with well-developed abdomens that have been adapted for swimming (Barnes 1974). Penaeid shrimp, in the wild, are found on sandy bottoms in 5 to 30 m of water (Barnes 1974) and typically feed on small crustaceans, polychaetes, and microfauna associated with detritus (Edwards 1978). Spawning occurs in coastal waters with eggs being shed directly into the surrounding water. Edwards (1978) stated that the larval stage includes five nauplius, three protozoea, and three mysis stages with shrimp then settling to the bottom during the post-larval stage leading to the benthic juvenile stage. Development from juvenile to adult is completed in 1 year.

Shrimp Culture

The Environmental Research Laboratory of The University of Arizona has operated shrimp culturing facilities in Puerto Peñasco, Sonora, Mexico, during the period 1972 to 1980, and in Oahu, Hawaii, starting in 1980. The method of shrimp culture used is controlled and experimental aquaculture that consists of an enclosed system in which many environmental factors such as light, water quality, and feed are controlled. Shrimp are reared in raceways covered by opaque plastic bubbles through which there is a supply of running seawater. Various species of algae grow on the sides and bottoms of raceways, enhancing growth of shrimp by providing a steady supply of needed nutrients in addition to artificial feed.

Shrimp are cultured at high densities and must be constantly monitored to prevent disease and stress-related problems from reaching epizootic proportions. At times when blue-green algae were predominant in the raceways, shrimp mortality rates often increased between 40 and 85% (Lightner et al. 1978). Affected shrimp turned blue-green in color, indicative of stress, whereas the body musculature became opaque instead of its usual clear color, and the hindgut caecum became thickened or showed signs of melanization. Upon histologic examination, lesions of the midgut and the hindgut caecum were evident, characterized by necrosis of epithelium lining the gut and caecum and a massing of inflammatory cells in its place. A normal cross section and longitudinal section are shown in Figures 1 and 3. Examples of diseased guts are demonstrated in Figures 2 and 4.

Statement of Problem

The purpose of the present study was to determine if one or more species of blue-green algae are the cause of hemocytic enteritis in cultured penaeid shrimp. Specific objectives were to:

1. identify the blue-green algae found in the shrimp culture raceways;
2. establish them in unialgal cultures;
3. determine optimum growth conditions for individual algae under unialgal culture conditions;
4. determine the toxicity of individual algae to penaeid shrimp under static bioassay conditions; and

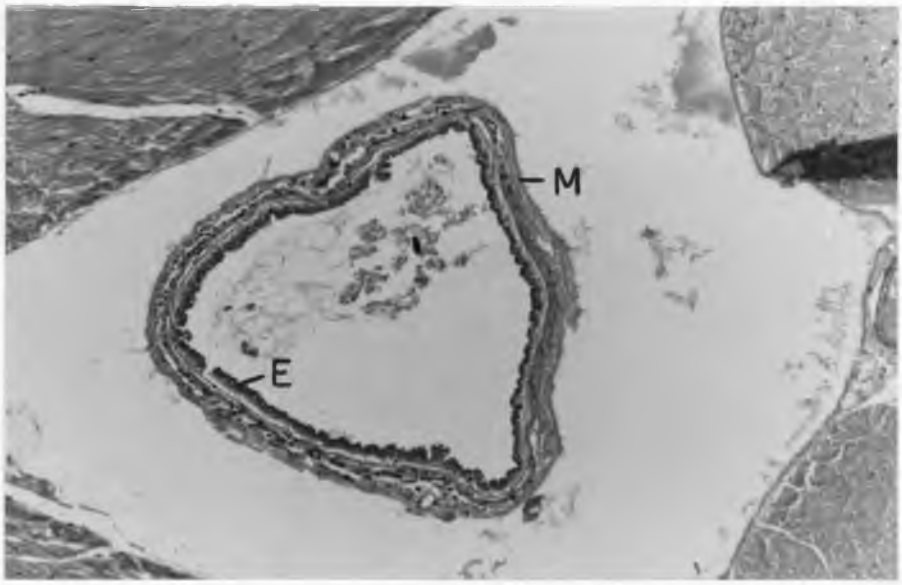

Figure 1. Cross section of a normal midgut *Penaeus stylirostris* showing longitudinal muscle (M) and columnar cells of the epithelium lining (E). -- Hematoxlyn and eosin staining. X132.

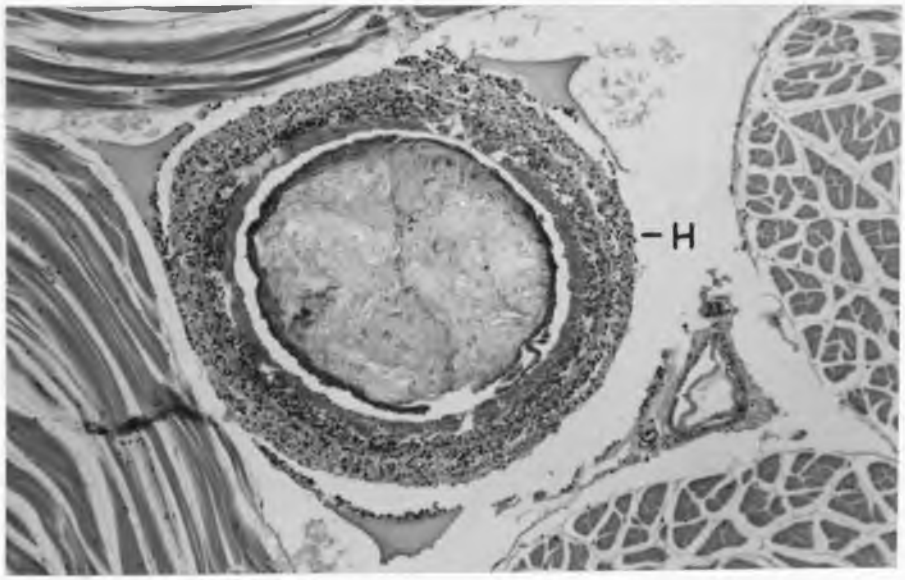

Figure 2. Cross section of a midgut of *Penaeus stylirostris* inflicted with hemocytic enteritis showing masses of hemocytes (H). The epithelium lining is absent. -- Hematoxylin and eosin staining. X132.

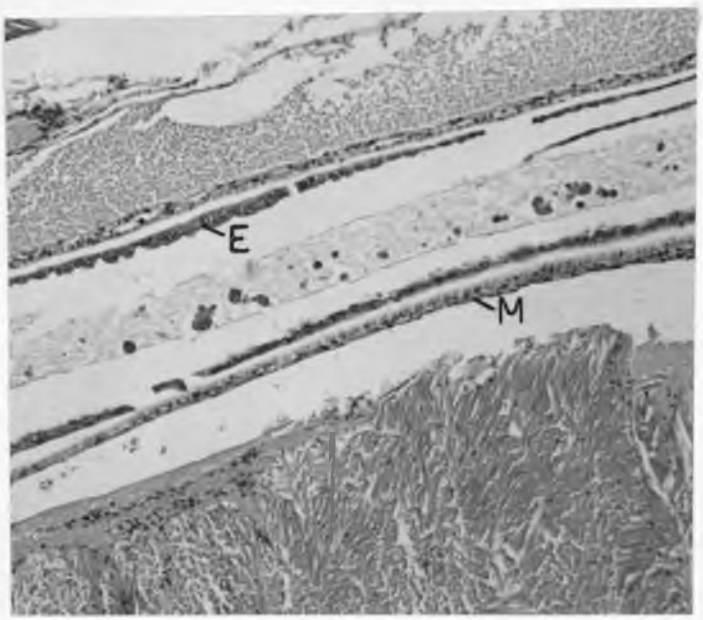

Figure 3. Longitudinal section of a normal hindgut of *Penaeus stylirostris* showing longitudinal muscle (M) and epithelium (E). -- Hematoxylin and eosin staining. X132.

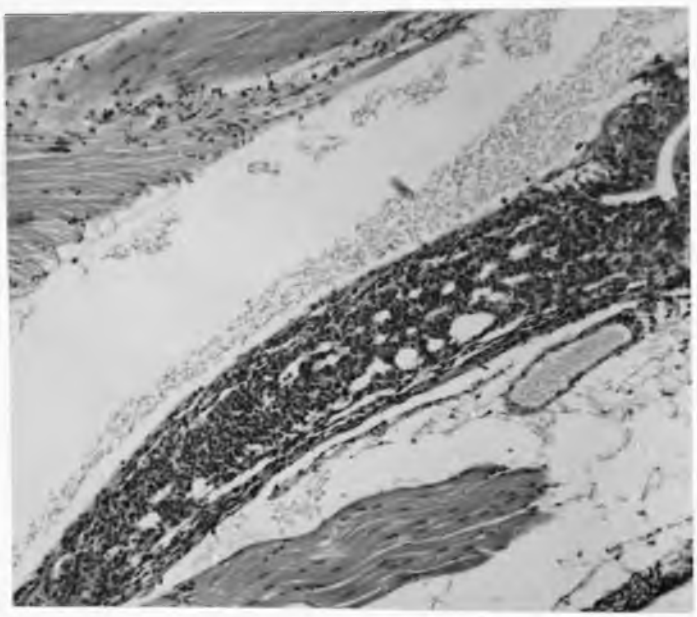

Figure 4. Longitudinal section of hindgut of *Penaeus stylirostris* affected by hemocytic enteritis. -- Hemocytes have infiltrated the gut and epithelium lining is absent. Hematoxylin and eosin staining. X132.

5. evaluate histologically the guts of moribund, dead, and surviving shrimp from the bioassays for evidence of HE.

LITERATURE REVIEW

There have been many unsuccessful attempts to culture blue-green algae. Blue-green algae were thought to have very slow growth and not to be amenable to culturing (Provasoli, McLaughlin, and Droop 1957). It was not until the 1950's that various media were developed that afforded good growth of these algae (Gerloff et al. 1950; Kratz and Meyers 1955) usually consisting of seawater with a few nutrients added to promote growth. Erdschreiber Solution (Starr 1964) containing soil extract was an important advance in the culturing of marine algae, allowing better growth through a wider range of species (Provasoli et al. 1957). Various artificial seawater media have been formulated containing many nutrients needed for growth including vitamins and trace elements. These media reduce the chemical variability of seawater. Species of blue-green algae grow better in different media, but vitamin B_{12} (Holm-Hansen 1968) seems to be a requirement of most marine species. As with eucaryotic algae, blue-greens also require the addition of nitrates and phosphates as well as various trace elements to the enrichment medium (Allen 1973). Their growth is better under alkaline conditions (Wolk 1973), and they tend to have a higher temperature range in general with some species growing well at 35 °C (Allen 1973). Optimum light intensities vary, but blue-green algae seem to prefer lower light intensities on the order of 100 ft-c (James 1974).

The Cyanophyta are often thought of as "problem algae." They can impart offensive odors or tastes in water and bloom in areas where eutrophic conditions are prevalent (Palmer 1959). Keating (1978) suggested that they may be able to produce a substance capable of inhibiting the growth of other types of algae.

There have been many reports of toxigenic blue-green algae. Gorham (1962) found the genera *Microcystis*, *Anabaena*, and *Aphanizomenon* to be prevalent in poisonings. Carmichael et al. (1975) noted that livestock and waterfowl died after drinking water containing a bloom of *Anabaena flos-aquae* (Lyngb.) de Breb. When the cells were lypholized, they were shown to contain a substance having some characteristics of a depolarizing neuromuscular blocking agent. Shilo (1972) found that a very fast death factor (VFDF) was produced and secreted into the surrounding water by *Anabaena*. Steyn (1944) noted that a toxin discharged into the water upon death of *Microcystis* was the cause of animal poisonings.

Keleti et al. (1979) have isolated an endotoxin consisting of lipopolysaccharides (LPS) from *Schizothrix calcicola* (Agardh) Gomont. LPS is found in the outer cell wall of certain bacteria and blue-green algae. *S. calcicola* was implicated in an outbreak of human gastroenteritis. Although definitive evidence was not found, it was speculated that LPS from this alga, common in the source reservoir, was related to the outbreak.

Numerous factors have been found to influence algal toxicity. Ray and Wilson (1957) studied the effects of a dinoflagellate, *G. brevis*, on fish. They found that its toxicity was influenced by

the concentration of the organism, the growth phase, pH, temperature, salinity of the test culture, the size and number of test fish, and bacterial growth. Shilo and Aschner (1953), in a study involving *Prymnesium parvum* (N. Carter) found that lowering the pH below 6.0 rendered the culture nontoxic, and when the pH was increased again to 7.0 the alga regained its toxicity. They found that the type of illumination affected toxicity of their cultures with daylight enhancing toxicity. Aeration and the presence of certain bacteria decreased *P. parvum's* toxicity. The addition of adsorbents such as kaolin and $CaSO_4 \cdot H_2O$ also decreased the alga's toxicity. The toxic material was found to be taken up by soil. Their toxic cultures of *P. parvum* remained nontoxic up to 2 weeks when mixed with pond bottom soil, showing the possible influence of environmental factors upon toxicity.

In studies on toxigenic *Anabaena* and *P. parvum*, it was shown by Shilo (1972) that conditions needed for maximum toxicity were not necessarily the same as conditions for optimum growth. Gorham (1962) determined that maximum growth of *Microcystis aeruginosa* (Kutz emend. Elenkin) occurred at 32.5 °C, whereas the optimum temperature for toxin production was 25 °C. Gentile (1971) found no correlation between maximum biomass and maximum toxicity. Gentile and Maloney (1969) demonstrated that toxin production of *Aphanizomenon flos-aquae* (L.) Ralfs varied with light intensity and temperature. Both the rate of growth and toxin production decreased at light intensities greater than 5000 lux. Toxin production was high (LD_{100} = 4 to 5 mg/kg) in

cultures incubated at 26 °C, but almost no toxin production was evident at 30 °C.

More than one type of toxin has been known to occur in a particular blue-green alga. Hughes, Gorham, and Zehnder (1958) found that *M. aeruginosa* produced two toxic factors, a slow death factor (SDF) and a fast death factor (FDF). When fresh cells of the algae were injected into mice, death occurred between 4 to 48 h. When "leaky" or disintegrating cells were injected, death occurred within 2 to 3 h. Freezing and incubation of the cells also caused the FDF to act. The SDF seemed to be due to bacteria associated with the alga (Gentile 1971), while the FDF was thought to be an endotoxin that had to be liberated before it could act (Hughes et al. 1958). Two toxins are known from the blue-green alga *Lyngbya majuscula* (Harvey) (syn. *Microcoleus lyngbyaceus* (Kutzing) Crouan). Cardellina, Marner, and Moore (1979) isolated a toxin, lyngbyatoxin A, from the shallow water populations of *L. majuscula*. The deep-water variety of this species had previously been shown to produce a different toxin, debromoaplysiatoxin.

There is some question as to the role of bacteria in the toxicity of certain algae. Shilo (1967) noted that bacteria may cause a synergistic effect in relation to the toxic alga, but cultures are difficult to obtain and maintain under axenic conditions (Shilo 1972). Burke et al. (1960) determined that axenic cultures of *Gonyaulax catenella* (Whedon et Kofoid) were more controllable as to toxin production than cultures contaminated with bacteria. When Gorham (1962) separately cultured bacteria associated with *M. aeruginosa* and tested

for possible toxicity, it was found that some of the bacteria produced a slow death toxin that was thought to play a secondary role in the qualities of *M. aeruginosa*.

Algal toxins have had various effects on invertebrates. Shellfish such as mussels, store and concentrate the toxin of *G. catenella* which causes paralytic shellfish poisoning (PSP) when such mussels are consumed by humans (Burke et al. 1960). Roberts, Henderson, and Medlyn (1979) tested the toxic effect of *Gymnodinium breve* (Davis) on three mollusc species and two species of crabs. The crabs appeared to be unaffected; mollusc mortalities ranged between 55 and 69% during the 48-h test period. Sievers (1969) found the shrimp *Palaemonetes pugio* (Holthuis) to be sensitive to the toxins of *Gonyaulax monilata* (Howell) and *G. breve* only during molting. In the same study, the annelid *Polydora websteri* (Hartman) and molluscs *Brachidontes recurvus* (Rafinesque) and *Crassostrea virginica* (Gmelin) were affected solely by *G. monilata*.

There have been few investigations of blue-green algal toxins and their affect on invertebrates. Lightner (1978) studied the possible toxicity of the blue-green alga *Spirulina subsalsa* (Oersted) to penaeid shrimp. Blooms of this alga were related to the occurrence of hemocytic enteritis and subsequent bacterial infections in the shrimp. *S. subsalsa* was commonly found in the stomachs of diseased shrimp.

ALGAL CULTURING

Materials and Methods

Algal Isolations

Samples of algae were collected from various sites in the shrimp raceways, including scrapings from sides and bottoms, areas just above the water line, and areas around pipes supplying water. These were either streaked on 1% Soil Seawater Agar (SSA) plates with a sterile inoculating loop according to procedures outlined by Hoshaw and Rosowski (1973) and incubated 8 to 16 d under 20-W cool-white fluorescent lamps at 500 ft-c on 16:8 light:dark (LD) at 22 ± 1 °C, or were immediately isolated into clonal cultures as described below.

Colonies of algae growing on SSA were observed after 1 week following the inoculation. Those determined to be blue-green algae were transferred by means of a sterile loop to tubes of McLachlan's Medium and incubated at least 3 weeks under the above conditions until good growth was obtained. Those cultures confirmed as being unialgal were reisolated to assure clonal cultures. This was accomplished by placing a few drops of the culture, using a capillary pipette, on 1% SSA plates and incubating overnight at 500 ft-c on 24:0 LD at 32 ± 1 °C in a Percival Model I-36L reach-in incubator. The plates were then inspected under dissecting and compound microscopes for evidence of hormogonial migration. A block of agar approximately 2 mm in diameter was cut around each hormogonium that appeared to be in an area uncontaminated by other algae or bacteria. These blocks were aseptically

transferred to tubes of McLachlan's Medium, Erdschreiber's Medium, or Provasoli's Enriched Seawater Medium and incubated at 500 ft-c, continuous light cycle at 32 ± 1 °C.

Algae that did not exhibit the property of hormogonial migration were isolated by other methods. One of these involved cutting a small piece of an algal filament (approximately three to five cells), washing and transferring the piece three times with a capillary pipette to sterile media as was described by Hoshaw and Rosowski (1973). A second method consisted of streaking algal filaments on 1% SSA. Single filaments were isolated and dragged over the agar with a sterile glass loop. A block of agar was cut around each filament as previously mentioned and transferred to tubes containing media. Cultures were incubated for at least 3 weeks at 50 to 100 ft-c under continuous light at 32 ± 1 °C. All cultures were stored in a Lab-Line Environeers V.I.P. "fifty-six" controlled environmental room at 50 ft-c on 16:8 LD at 22 ± 1 °C.

Samples of morphological types of blue-green algae isolated were identified with reference keys in the Cyanophyta by Desikachary (1959), Drouet (1968), and Humm and Wicks (1980). The identification of the five isolates were confirmed by Drouet (1980, personal communication).

Optimum Growth Conditions

Five different media and various light intensities (25 to 400 ft-c), temperatures (21° to 38 °C), and pH (7.1 to 9.0) were tested to determine the conditions that promoted good growth and healthy

looking algae. Blue-green algae vary in color from reddish brown to different shades of green ranging from light green to dark blue-green depending on environmental conditions. Algae that were dark green to blue-green in color were considered to be healthy; those light green to almost colorless were considered to be in poor condition.

Media. The five media tested were Provasoli's Enriched Seawater (PESW), McLachlan's Medium (McL), Erdschreiber's Medium (Erds), ASM, and Soil Seawater (SS) (Appendix A). Attempts were made during some of the experiments to improve the basic recipes of McL, Erds, and PESW by supplementing them with additional nutrients such as vitamin B_{12}, phosphate, and nitrate. These changes are listed in Appendix A with the original recipe for that specific medium. All media unless otherwise stated were steam pasteurized for 3 h on 2 consecutive days.

At the onset of each media trial, samples of algae were equally divided into clumps approximately 5 mm in diameter in an attempt to obtain a standard size of original inoculum, put into Erlenmeyer flasks containing 100 to 200 ml of the various media, and incubated at 100 ft-c, continuous light cycle at 32 ± 1 °C, for periods of 6 to 33 d. Growth rates were visually determined and recorded relative to each other.

Light Intensity. Samples of algae were divided into equally sized clumps as described in the previous paragraph and placed in Erlenmeyer flasks containing 100- or 200-ml McL, and incubated for 7 to 26 d under continuous light at 32 ± 1 °C until differences in

growth and color were observed. Light intensities were measured using a GE light meter, type 213, and ranged from 25 to 400 ft-c. Observations were recorded according to two properties of algal filaments, growth, and color. Color of filaments ranged from dark green to light yellow. Growth was recorded in the manner described in the preceding section.

Temperature. Some species of blue-green algae taken from shrimp raceways had been previously shown by Maluf (1980, personal communication) to grow well at 32 °C. One species, *Microcoleus lyngbyaceus*, was tested during the temperature experiments. Equal amounts of algae, approximately 5 mm in diameter, were placed in four 250-ml Erlenmeyer flasks containing 200 ml PESW and incubated 20 d at 100 ft-c, continuous light, at the following temperatures: 21°, 24°, 32°, and 38 °C. Differences in growth were visually observed and recorded.

pH. The growth of *Spirulina subsalsa* (Oersted) was tested under different pH conditions. Equal samples of algae were placed in five 125-ml Erlenmeyer flasks containing 100 ml of McLachlan's or ASM media. The original pH before addition of algae was 7.7 in McLachlan's Medium and 7.1 in ASM. The pH was adjusted to 8.0, 8.5, and 9.0 at the start of the trial by adding 0.1 NaOH and measured using a Coleman Metrion III pH meter. The pH was not measured during or after the trial. Flasks were inclubated for 16 d at both 100 and 50 ft-c under continous light at 32 ± 1 °C.

Results

Algal Isolations

Five strains of blue-green algae were isolated from the shrimp raceways and identified by Drouet (1980, personal communication) as: *Microcoleus lyngbyaceus* (Kutzing) Crouan, *Spirulina subsalsa* (Oersted), and three strains of *Schizothrix calcicola* (Agardh) Gomont (designated as strains A, B, and C to distinguish these morphologically distinct strains of the same species).

M. lyngbyaceus was isolated in Erdschreiber's Medium by the method using hormongia. An algal contaminant, later identified as *S. 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 1). This alga possessed a sheath that was readily

Table 1. Average widths of filaments for five strains of blue-green algae

Type	Number of Filaments Measured	Average Width (µm)	Range (µm)
Microcaleus lyngbyaceus	40	16.6	12-20
Schizothrix calcicola A	20	1.3	.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

observed. Ends of the filaments were rounded and hormogonia were frequently seen (Figure 5).

S. subsalsa was isolated by Maluf (1980, personal communication), employing the spot plate method, and transferred to tubes of McLachlan's Medium. Four tubes were examined microscopically for contaminants and stored in the 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 with a compound microscope. Filaments were readily recognized by their tightly spiraled appearance (Figure 6).

S. calcicola B was isolated on 1% SSA from a sample scraped off netting located above the raceway water line and transferred to tubes of McLachlan's Medium. Clonal cultures were established by dragging single 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). *S. calcicola* B is a straight filament with cross walls barely discernible under a compound scope and possessed a thin sheath that was occasionally observed (Figure 7). Frequently one or two vacuoles per cell was evident.

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

Figure 5. Filament of *Microcoleus lyngbyaceus* and hormogonium (H). -- Note absence of sheath on hormogonium. No stain. X264.

Figure 6. Spiraled filaments of *Spirulina subsalsa* in Provasoli's Enriched Seawater. -- No stain. X900.

Figure 7. Straight filaments of *Schizothrix calcicola* B possessing thin sheath. -- No stain. X528.

Figure 8. Filaments of *Schizothrix calcicola* A in Provasoli's Enriched Seawater. -- No stain. X528.

possessed a sheath that was infrequently observed. After 3 wk in culture, certain strands within the algal mat tended to arrange themselves concentrically (Figure 9).

S. calcicola C was initially observed as a contaminant in control jars during bioassay Trial 2. 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 *S. calcicola* B. Filament widths averaged 2.6 µm (2.0-3.0 µm). Color in cultures ranged from brown to light green. *S. calcicola* C is constricted at the cross walls and has a sheath that was occasionally observed (Figure 10).

Optimum Growth Conditions

Media. *M. lyngbyaceus* was grown in eight variations of five media (Table 2). Two trials were run for periods of 24 and 15 d. 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 SS. No growth was observed in the flask containing ASM, and by Day 9 the original inoculum had turned yellow.

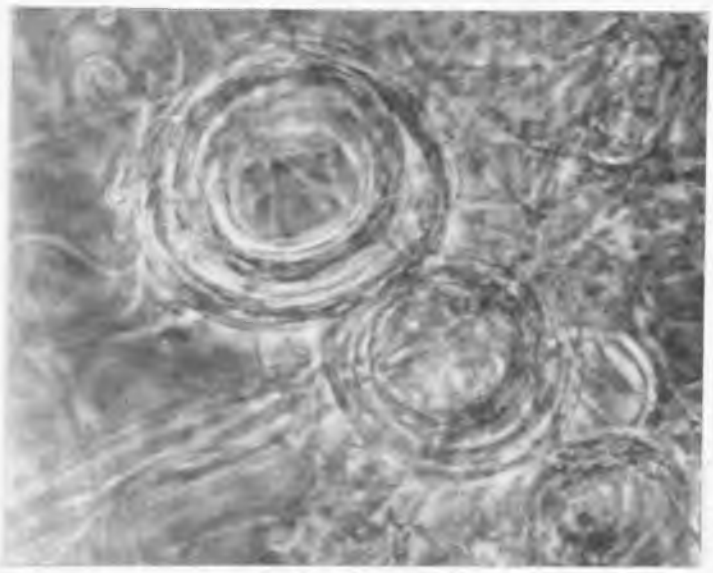

Figure 9. Mat of *Schizothrix calcicola* A containing strands of concentrically arranged filaments. -- No stain. X900.

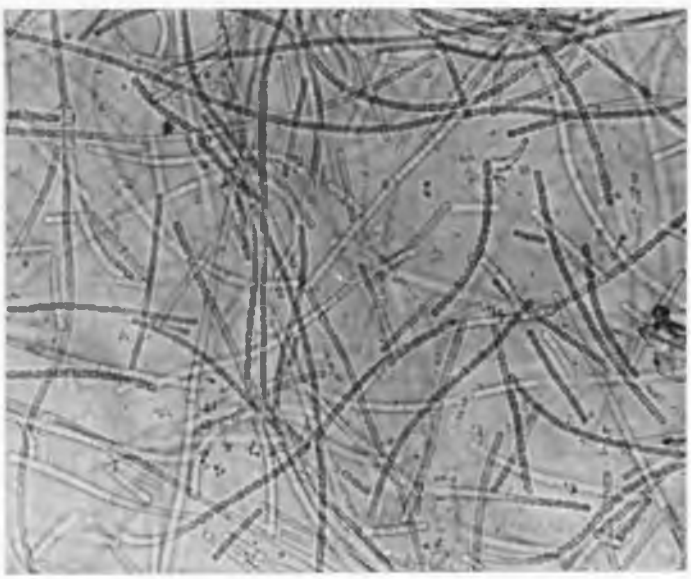

Figure 10. Filaments of *Schizothrix calcicola* C with constricted crosswalls. No stain. X528.

Table 2. Relative measures of growth of four strains of blue-green algae with different media

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).

S. calcicola A was grown in six types of media during two trials lasting for periods of 21 and 15 d. 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.

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

S. subsalsa was grown in nine media. Three trials were conducted having durations of 15, 33, and 15 d. 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.

Growth in PESW was consistently good for all algae, 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. *S. calcicola* A, *S. calcicola* B, and *S. subsalsa* were tested with two and three times the original amount of nitrate as $NaNO_3$ (Table 3). 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

Table 3. Differences in color of three strains of blue-green algae with time and addition of extra nitrate as $NaNO_3$ to Provasoli's Enriched Seawater

Algal Species	Number of Days	PESW	PESW + 2 x NO_3	PESW + 3 x NO_3
S. calciola B	5	YG	G	G
	14	Y	YG	G
	21	Y	Y	G
	29	Y	Y	G
S. 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

26 d. 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 4).

S. calcicola A was grown in PESW with a range of 50 to 300 ft-c for a 10-d 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.

S. 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.

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 d. The third trial was conducted in PESW for 9 d. 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.

Temperature. *M. lyngbyaceus* grew best at 32 °C (Table 5). 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 lack of nutrients. Growth in

Table 4. 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	LG	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

Table 5. 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

in the other flasks was much less and the algae retained their blue-green color.

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 6). *S. subsalsa* failed to grow in ASM plus vitamin B_{12} in Trial 3 at each pH tested.

Table 6. 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

Discussion

The five types of media tested afforded a wide range of growth of the four strains of blue-green algae. *M. lyngbyaceus*, *S. calcicola* A, *S. calcicola* B, and *S. subsalsa* were found to grow well in PESW and in variations of McL. Growth of filaments in Erds was moderate to good, while SS afforded sparse growth. Growth of all four algal strains was inhibited in ASM and only *S. calcicola* A remained viable in that medium. PESW appeared to consistently be the best growth medium and was utilized in subsequent experiments.

Light intensity also had an affect on growth of the algal strains. As was suggested by James (1974), growth was most dense at light intensities between 100 to 400 ft-c. At light intensities between 300 to 400 ft-c algae became light yellow in color and growth of *S. subsalsa* was inhibited. Later trials were conducted with light intensities ranging from 100 to 200 ft-c which afforded good growth of filaments still retaining their blue-green color.

All four blue-green algal strains grew well at 32 °C. Filaments appeared healthy but grew slower at 21° and 24 °C. When incubated at 38 °C, *M. lyngbyaceus* did not grow well. Although the filaments grew longer, they did not spread out on the bottom of the flask as with those incubated at lower temperatures.

The range of pH tested, 7.1 to 9.0, did not appear to affect the growth of *S. subsalsa*. A wider range of pH should be tested in further investigations.

S. calcicola B exhibited rapid growth under conditions of 100 ft-c, continuous light at 32 ± 1 °C. Appreciable amounts of this alga were usually evident within a few days of incubation. *S. calcicola* A and *S. subsalsa* had somewhat slower growth, but dense cultures could be established within 1 to 2 wk of incubation. *M. lyngbyaceus* turned yellow sooner in regards to culture density in PESW than the other three algal strains tested indicating a lack of nutrient(s). Although there was no change in growth rate, denser cultures of *M. lyngbyaceus* were obtained after supplementing PESW with additional nitrate.

BIOASSAY OF ALGAL TOXICITY ON SHRIMP

Materials and Methods

Six trials were conducted during which five strains of blue-green algae were fed to *P. stylirostris*. Sixteen 60-L tanks, each containing 35 L of germicidal ultra-violet light-treated seawater with Provasoli's enrichment medium, were used for the first trial. One-gallon jars containing 3 L of autoclaved or steamed Provasoli's Enriched Seawater were used for the remaining trials. Seawater was either autoclaved at 121 °C, 15 psi for 15 min in a Market Forge Sterilmatic autoclave Model 120, or steamed for 3 h on 2 consecutive days in a copper steamer (Wahmann Manufacturing Co., Baltimore, Maryland). Air was supplied to the jars and tanks through air stones or pasteur pipettes. The 60-L tanks were sterilized by wiping with 95% isopropyl alcohol prior to Trial 1. The 1-gal jars were sterilized by baking in a 170 °C oven for 2 h or by steaming.

Air filters, when used, were constructed from tygon tubing, 6 cm in length and 2 cm in diameter, stuffed with cotton and plugged with cork plugs containing glass tubing. These filters were autoclaved prior to use.

Round, plastic undergravel filters (Aquatrol, Inc., Anaheim, California), 10 cm in diameter, were employed in all trials after Trial 3. These had been previously baked in an oven at 80 °C for 3 h. Autoclaved, crushed oyster shell was used as the substrate. Air filters were not utilized in these trials since they impeded air flow.

Algae were grown in a 20-L tank for Trial 1 under 200 ft-c, continuous light at 32 ± 1 °C. Six liters of this culture were transferred to a 60-L tank and grown for 9 d under 200 ft-c, continuous light at 24 ± 1 °C. The algae from this tank were then transferred to the remaining 11 tanks and grown under the previous conditions for 6 d. In most other cases, algae were grown in 2 L of PESW in 2800-ml Fernbach flasks for 1 to 3 wk under 200 ft-c, continuous light at 32 ± 1 °C. When good growth was evident, the contents of each flask was transferred to 4 1-gal jars containing 2.5 L PESW. These were incubated under the above conditions until algal mats were observed in each jar. Algae in the final trial were grown separately in Fernbach flasks and fed to shrimp twice each day.

In all trials, the temperature was reduced from 32° to 25 °C 1 d before shrimp were added. Light intensity varied from 30 to 625 ft-c. The light cycle was either 12:12 LD or continuous light (Appendix B).

Prior to each trial, shrimp were removed from a raceway and placed in a 1000-L round fiberglass holding tank with a constant flow of UV-light treated seawater at approximately 25 °C. Shrimp were acclimated for at least 3 d. Ten shrimp were selected and weighed to determine their average body weight (Appendix B). In an effort to reduce the amount of contaminating algae adhering to the shrimp, an algicidal treatment was administered for 3 consecutive days. The water flow was turned off and 0.25 ppm $KMnO_4$, 0.5 ppm Cu as Cutrine-plus, 55 ppm formalin, and 0.1 ppm malachite green were added to the tank. Water flow was resumed after a 6-h period. In preparation for

Trial 1, shrimp were placed in experimental tanks 1 d after the last chemical treatment. In the remaining trials they were removed 1 h after the last treatment, placed in sterile seawater for 5 to 10 min, and then transferred to experimental jars.

At the start of each trial, 5 to 10 shrimp were placed in each jar or tank containing PESW. The algal strain to be tested was present in the experimental jars while control jars were devoid of algae.

Shrimp were fed a dry pelletized shrimp food twice a day. One-third of the feed was administered at 8 a.m., and two-thirds at 5 p.m. The amount of feed given daily (full feed) was 7% of shrimp weight. Shrimp in control jars were fed normal feed (full feed), one-half the normal feed (1/2 feed), or no feed (0 feed). Shrimp in experimental jars were fed 1/2 feed or 0 feed.

Algal and water samples were taken from each jar approximately every other day and microscopically examined to determine the presence of contaminating organisms (Appendix B). Many small colorless flagellates were observed in the size range of 1 to 6 μm. Due to their small size and, hence, the difficulty of classifying them, they were collectively grouped as "small" and "large" flagellates. Macroscopic observations were also recorded for each jar.

Shrimp were counted daily. Those that died within the first 2 d of the trial were assumed to have died from stress or mishandling and were replaced. Dead and moribund shrimp were removed and fixed by injecting approximately 0.2 cc of Davidson's solution (Appendix A) into their hepatopancreas. Shrimp were then placed in Davidson's solution for 72 h and transferred to vials containing 75% ethanol.

Any remaining shrimp at the termination of the trial were preserved in a similar manner. In preparation for histological evaluations, shrimp were cut longitudinally, dehydrated in an American Optical tissue processor (Appendix A), immersed in xylene for clearing, infiltrated with paraffin, and finally embedded in paraffin utilizing a Tissue-Tek II tissue embedding center. Thin sections of embedded shrimp were cut on a microtome, mounted on slides, and stained (Appendix A).

The finished slides were observed under a microscope for hemocytic lesions, characterized by massing of hemocytes. The hepatopancreas; anterior, central and posterior midguts; and epigastric and hindgut caeca were observed for each shrimp. Observations were recorded as follows: NS = not sectioned, 0 = normal, + = slight buildup of hemocytes or one hemocytic lesion, ++ = moderate buildup of hemocytes or two hemocytic lesions, +++ = heavy buildup of hemocytes or three or more lesions. If more than two areas were not sectioned in a shrimp that appeared normal, the shrimp was not utilized in later analyses. Shrimp that were positive in any section, with the exception of the hepatopancreas, were considered to have HE. Autolyzed shrimp were not diagnosed because it was too difficult to discern necrotic or inflamed tissue from normal tissue and hemocytes were not readily recognized.

Development of Technique

Numerous problems were encountered during the shrimp trials. At the termination of each trial, adjustments were made in an effort

to improve results of the following trials. This section summarizes those improvements.

The culture used in Trial 1 was contaminated with various ciliates and flagellates prior to the addition of shrimp. Various contaminating organisms were sighted throughout the trial (Appendix B). In an effort to reduce contamination, 1-gal jars were employed in the remaining trials instead of 60-L tanks. These jars were tightly closed with screw-on tops, reducing contact with the air in comparison to the tanks originally used that had large openings covered by plastic sheeting. In addition, air filters were placed on incoming lines. To assure that the air filters were adequate, one control jar in Trial 2 contained no shrimp, but had air supplied through an air filter.

The UV-light treated water was also a suspected source of contamination in the first trial, since pipes supplying water to the tanks had not been cleaned after untreated water had flowed through them. Autoclaved or steamed seawater was utilized in the remaining trials.

High mortality was observed in Trial 2 due to exceptionally heavy growth of *S. subsalsa* in which shrimp became entangled. This was remedied in Trial 3 by scraping algal mats off the bottom and sides of jars which spread the algae throughout the water column.

High mortalities were also observed in control jars which were devoid of algae. The water became heavily fouled as the trial progressed from decaying food and waste products. Undergravel filters were implemented in Trial 4, which appeared to improve water quality, but a high 80% mortality rate was still observed. It was thought that

air was being released from the system too close to the water's surface. In later trials, the air outlets were located near the middle of the water column, which decreased mortalities by 20%.

The shrimp were found to be the main source of algal contamination during the trials, and it was thought that shrimp were becoming recontaminated with various organisms in the period between treatments and transfer to the experimental jars. In an effort to reduce this, shrimp were transferred to sterile seawater 1 h after the last chemical treatment and then transferred to experimental jars.

Occasionally control shrimp were found to have HE. It was thought that these shrimp had acquired the disease before the experiment began since they had been taken from raceways in which the disease was enzootic. Shrimp were collected and held in the dark between Cutrine treatments for a period of 2 wk prior to the start of the final trial. It was hoped that the absence of algae for this period would reduce the incidence of HE.

In Trial 4, 2 control jars contained plain seawater instead of PESW to determine if low nutrient levels would reduce fouling of the water and if PESW was inducing HE in control shrimp.

Algal growth was usually dense in jars at the onset of the trials. This occasionally interfered with shrimp causing a number of mortalities as was exemplified in Trial 2 with growth of *S. subsalsa* on shrimp gills. In Trial 6 algae were added 1 d before the start of the trial. Additional algae were added each day until there was appreciable amounts of algal growth. This decreased the amount of

algae in contact with the shrimp while allowing them ample algae to consume.

Results

Trial 1. *Schizothrix calcicola* B

There was dense growth of *S. calcicola* B in all tanks when shrimp were added. Algal mats had grown on the bottoms and sides of tanks and on the water surface. The amount of *S. calcicola* B in each tank declined throughout the trial and was comparatively sparse by its termination, although in all cases there were algae in sufficient amounts for shrimp to consume. Algae were dark blue-green in color at the start of the trial, turning lighter green as the trial progressed, until they were light green within 2 wk of the 16-d period.

The culture was contaminated with ciliates, flagellates, and protozoa prior to the addition of shrimp. Various contaminants (Appendix B) including a diatom, *Amphora* sp., appeared during the first week; however, profuse growth of these diatoms was observed only in control tanks. *S. calcicola* B was clearly dominant in all experimental tanks throughout the duration of the trial.

Shrimp were counted daily, but there were more frequent checks for moribund shrimp. Shrimp tissue autolyzed rapidly and had to be fixed within an hour of death in order to make a good diagnosis. Shrimp are also cannibalistic and frequently dead shrimp were partially consumed before being discovered. These two factors reduced the number of shrimp that were available for analysis.

There were no mortalities of shrimp in the control, indicating that the various contaminants did not have a noticeable effect on shrimp survival (Table 7). There were high mortalities in tanks containing *S. calcicola* B. The majority of these deaths occurred after Day 10 of the trial. The cause of these deaths was not determined in most cases since the shrimp were frequently too autolyzed to be analyzed. Only 25% of the shrimp that died during the trial were diagnosed as having HE. Other factors could have contributed to these mortalities. Shrimp were occasionally found tangled up in algae, and through rapid algal growth were often completely surrounded. Mortalities were greatest in tanks not fed commercial shrimp food. It is possible that the algae did not supply enough nutrients or was not eaten in sufficient quantities to prevent weakening of the shrimp. Microscopic examinations revealed the presence of *S. calcicola* in shrimp stomachs in only a few cases. A trial conducted at a later date indicated that shrimp not fed at all had a 50% mortality rate over the same span of time.

The majority of shrimp used in the final analysis were those that survived the trial and were capable of being well fixed. Many of these shrimp, although they appeared healthy, were found to have mild to advanced cases of HE. Of the 35 shrimp examined, 34% possessed symptoms of HE (Appendix B).

Table 7. Number of shrimp diagnosed for hemocytic enteritis during the 16-d *Schizothrix calcicola* B bioassay (Trial 1)

Tank Number	Treatment	Feed	Original Number of Shrimp	Remaining Number of Shrimp	Number Negative for HE	Number Positive for HE
1	Control	Full	5	5	5	0
2		Full	5	5	1	0
3		Full	10	10	1	0
4		Full	10	10	1	0
7	*S. calcicola* B	Full	10	9	6	2
8		Full	10	8	3	1
9		Full	10	3	0	3
10		1/2	5	4	2	0
11		1/2	5	5	3	2
12		1/2	10	6	0	3
13		0	10	2	0	0
14		0	5	3	2	1
15		0	5	1	2	0
16		0	10	3	0	0
17		0	10	2	2	0
18		0	5	1	3	0

Trial 2. *Spirulina subsalsa* and *Microcoleus lyngbyaceus*

S. subsalsa grew in thin mats on the bottoms and sides of jars. When shrimp were added, their movements caused the algae to rise to the surface in large clumps. A new thin mat was observed on the bottoms of the jars on Day 15 of the 18-d trial. There was always an excess of algae for shrimp to consume. *S. subsalsa* was dark blue-green in color for the entire 18-d period.

S. subsalsa was unialgal prior to the addition of shrimp. Protozoans, flagellates, and amoebas were present in all jars by the end of the first week. A few diatoms of the genus *Amphora* were sighted during the last 2 d of the trial (Appendix B).

There was an exceedingly high mortality rate of shrimp in the jars containing *S. subsalsa* during Day 1 of the trial. The shrimp died when they became entangled in the algae. It was noticed that once the shrimp had scraped the algae off the bottom surfaces of the jar and the algae rose to the surface, the shrimp no longer became entangled. Five replacement shrimp were added to 1 jar that had 100% mortality by Day 2. This initial mortality drastically decreased the amount of *S. subsalsa*-treated shrimp in this trial. Approximately halfway through the trial, *S. subsalsa* was observed growing on shrimp gills. This could have impaired water circulation through the gills, possibly contributing to later mortalities.

Of the 18 shrimp analyzed, 22% were found to have HE. Four of the five shrimp with HE lived throughout the trial.

M. lyngbyaceus, when inoculated into 5 jars, floated at the water's surface. The alga did not appear to grow during the trial and, although blue-green in color at the onset of the trial, a brown color appeared after the first week, indicating poor condition of the alga. It remained approximately half brown and half blue-green for the remainder of the trial. The culture was contaminated with flagellates, *Amphora* sp. and protozoans before the addition of shrimp. During the trial, 1 jar was observed to contain spherical green unicells and 2 other jars contained small patches of *S. calcicola* C. There was a 72% mortality rate in jars containing *M. lyngbyaceus*. A piece of cloth was added to 1 jar in an attempt to give the algae a substrate to grow on. It was inferred that the cloth restricted the flow of oxygen, causing 100% mortality in that jar on Day 2 of the trial. Shrimp occasionally became entangled in the algae, which increased the mortality rate. Shrimp were frequently observed hanging upside down on the algal mats while grazing on them.

Only 1 shrimp of the 11 analyzed from the jars containing *M. lyngbyaceus* was found to have HE (Table 8). This shrimp was in the jar containing the cloth and died under the conditions described above. The shrimp had an early case of HE with a slight build-up of hemocytes in the hindgut caecum.

Jars with controls were observed to contain contaminants after the addition of shrimp. *S. calcicola* C was noticed growing in 2 of these jars on the last day of the trial.

There was an 80% mortality of control shrimp which was attributed to poor water quality. The accumulation of waste products and

Table 8. Number of shrimp diagnosed for hemocytic enteritis during the 18-d *Spirulina subsalsa* and *Microcoleus lyngbyaceus* bioassays (Trial 2)

Jar Number	Treatment	Feed	Remaining Number of Shrimp[a]	Number Negative for HE	Number Positive for HE
1	*S. subsalsa*	1/2	0	1	0
2		1/2	1	1	0
3		1/2	3	0	2
4		1/2	1	1	0
5		1/2	3	2	0
14		0	4	4	1
15		0	0	3	0
16		0	1	1	0
17		0	2	1	1
18		0	0	0	0
6	*M. lyngbyaceus*	1/2	0	2	0
7		1/2	3	2	0
19		0	4	4	0
20		0	0	0	0
21		0	0	2	1
8	Control	1/2	5	4	1
9		Full	0	0	0
10		Full	0	2	0
11		0	0	1	0

[a]All jars originally contained five shrimp.

decomposing feed was noticed by Day 5 and became increasingly worse as the trial progressed. The water was considerably fouled by the trial's termination. One early case of HE was identified in a control shrimp that had survived the trial.

Trial 3. *Schizothrix calcicola* A

The thin mat of *S. calcicola* A present on the bottom and sides of each jar was scraped into the water column prior to the addition of shrimp. The algae in various sized clumps either floated near the water's surface, sank to the bottom, or accumulated on the capillary pipette that was providing air. Algal growth was seen only in Jar 15 where a thin layer was observed growing on the sides and bottom of the jar by Day 8. In most jars algae were dark green, although algae in Jar 15 exhibited a light-green color.

The culture of *S. calcicola* A was unialgal prior to the addition of shrimp. Various contaminants (Appendix B) were observed after shrimp were introduced, but *S. calcicola* A was always abundant in all jars throughout the 19-d trial.

This strain of algae was readily eaten by shrimp, but seemed to be excreted intact, possibly indicating undigestibility of this alga. Examination of such algae under a microscope revealed that filaments were still whole and were slowly oscillating. No other tests were conducted to confirm their viability.

There was a comparatively low number of mortalities in the jars containing *S. calcicola* A (Table 9). This was thought to be

Table 9. Number of shrimp diagnosed for hemocytic enteritis during the 19-d *Schizothrix calcicola* A and raceway sample bioassays (Trial 3)

Jar Number	Treatment	Feed	Remaining Number of Shrimp[a]	Number Negative for HE	Number Positive for HE
1	*S. calciola* A	1/2	3	1	1
2		1/2	4	3	0
3		1/2	5	4	0
4		1/2	4	4	0
5		1/2	3	1	1
15		0	0	0	0
16		0	4	4	0
17		0	0	1	0
18		0	3	3	0
19		0	3	3	0
6	Raceway scraping	1/2	4	3	0
7		1/2	5	5	0
20		0	3	3	0
8	Control	1/2	5	2	1
9		1/2	4	4	0
10		Full	0	0	1
11		Full	0	1	0
12		0	3	1	0

[a]All jars originally contained 5 shrimp.

due, in part, to the lack of algal growth which could have lessened the amount of entanglement and clogging of gills.

Only 2 of the 26 shrimp used in the final analysis exhibited symptoms of HE. Both of these shrimp were alive at the trial's termination. One shrimp had an early case of HE, evident only in the hindgut caecum; the other possessed an advanced case extending through the entire gut.

Three jars in this trial contained scrapings from a raceway that suffered a recent outbreak of HE. The scrapings, which grew into mats after 1 wk of incubation, contained mostly *M. lyngbyaceus*, a coccoid blue-green, *Entophysalis* sp., and smaller amounts of *S. calcicola* A and *S. calcicola* C. Growth of all algae continued at a slow pace throughout the experiment. At its termination, none of the 11 shrimp examined had evidence of HE.

Mortalities ranged from 0 to 100% in jars containing controls. Losses were greatest in jars given full feed, and these losses were inferred to be caused by poor water quality due to accumulations of waste products and decomposing feed.

The jars containing control shrimp became contaminated with algae and organisms (Appendix B) after the addition of shrimp. The most noted algal contaminant, *S. calcicola* C, formed clumps on the sides of jars. Two of the 10 shrimp examined were found to have HE. It was thought that the presence of *S. calcicola* C might have induced the disease and this was consequently tested for in a later trial.

Trial 4. *Schizothrix calcicola* C

Five jars were inoculated with 1 L of *S. calcicola* C and incubated for 5 d under 200 ft-c, continuous light at 32 ± 1 °C. There was little growth during the 5-d period and the majority of algae were in mats floating at the water's surface or in smaller pieces on the bottom. The shrimp readily ate the algae and by Day 4 very little algae was left in each jar. *S. calcicola* C started to recolonize in the jars mid-way through the trial and mats had grown on bottoms and sides by Day 16 of the 20-d period.

S. calcicola C was always present in each jar, and although there were a few contaminants it was always clearly predominant. The mats of algae were brown, sometimes exhibiting a pinkish tinge. Under microscopic examination, individual strands appeared to be green.

There were no mortalities in the 2 jars receiving half feed, and 100% mortalities in those jars receiving no feed (Table 10). None of the 17 shrimp used in the final analysis exhibited symptoms of HE.

Three of the 5 control jars contained undergravel filters. Mortalities in these jars did not decrease, but this was thought to be due to poor aeration and was corrected in the remaining trials. Two jars contained plain seawater instead of Provasoli's Enriched Seawater. Although this did not appear to reduce fouling of the water, the amount of mortalities decreased. One shrimp in the seawater medium was found to have HE, suggesting that PESW was not the cause of HE in control shrimp.

Table 10. Number of shrimp diagnosed for hemocytic enteritis during the 20-d *Schizothrix calcicola* C bioassay (Trial 4)

Jar Number	Treatment	Feed	Remaining Number of Shrimp[a]	Number Negative for HE	Number Positive for HE
1	Control	Full	0	1	0
2	Control	1/2	0	1	2
3	Control-seawater	1/2	3	2	1
4	Control-seawater	1/2	4	4	0
5	Control	0	0	0	0
6	*S. calcicola* C	1/2	5	5	0
7		1/2	5	5	0
8		0	0	4	0
9		0	0	1	0
10		0	0	2	0

[a]All jars originally contained five shrimp.

Contamination in jars containing control shrimp was fairly low. *Amphora* sp. was first sighted on Day 10, increasing slowly in number through the remainder of the trial.

Of the 11 control shrimp examined, 3 had HE. One died on Day 11, while the others lived through the trial. All three had early cases of HE.

Trial 5. *Microcoleus lyngbyaceus*

An additional trial was performed with *M. lyngbyaceus* because results in Trial 2 were inconclusive. In an attempt to obtain a more actively growing culture, two new isolates were established and inoculated into 2 jars. A third jar contained the original isolate of *M. lyngbyaceus*. All jars were inoculated 5 d prior to the addition of shrimp.

During Day 22 of the trial, the new isolates grew at a slow pace, but the older isolate in Jar 3 grew at a more rapid rate than in previous cultures. Tufts of filaments approximately 50-mm long developed along the air line by the termination of the trial. *M. lyngbyaceus* was dark blue-green in color in all jars for the entire trial. Contaminating organisms such as protozoans were occasionally observed (Appendix B).

Although shrimp were observed grazing on algae, they seemed to prefer artificial shrimp food when available. To increase grazing on *M. lyngbyaceus* the daily amount of feed was reduced to 0.25 the full amount in jars containing algae on Day 12. No feed was administered

to Jars 1 and 3, and Jar 2 received only the morning feed beginning with Day 18.

Of the 10 shrimp analyzed, 20% were confirmed as having HE. These two shrimp were killed on Days 19 and 20 of the trial. They both had slightly enlarged hindgut caeca. An additional shrimp exhibiting similar symptoms on Day 17 was consumed by other shrimp before he could be removed.

Mortalities of control shrimp decreased in 2 out of 3 jars (Table 11). It is not known why there was 100% mortality in Jar 4 since conditions were similar in all 3 jars. A few contaminants were observed but were generally scarce (Appendix B) with the exception of *S. calcicola* C. There was profuse growth of this alga in Jar 6 by Day 18.

One of the 10 control shrimp had an early case of HE in the central midgut. This shrimp lived through the entire trial.

Table 11. Number of shrimp diagnosed for hemocytic enteritis during the 22-d *Microcoleus lyngbyaceus* bioassay (Trial 5)

Jar Number	Treatment	Feed	Remaining Number of of Shrimp	Number Negative for HE	Number Positive for HE
1	*M. lyngbyaceus*	1/2	4	3	0
2		1/2	4	4	1
3		1/2	1	1	1
4	Control	1/2	0	1	0
5		1/2	5	5	0
6		1/2	4	3	1

Trial 6. *Schizothrix calcicola* B

In this final trial, *S. calcicola* B was grown in separate flasks and added after the addition of shrimp. Algae were pipetted into the jars in various sized clumps ranging from 1 to 10 mm in diameter twice a day at the same time shrimp were normally fed. Algae were drawn into the substrate by the undergravel filter within a half hour of feeding. Shrimp appeared to have ample time to consume the algae, and larger pieces were readily available at all times.

Algal growth was evident by Day 9 of the 19-d trial, and became so dense by Day 13 that the addition of algae was discontinued. *S. calcicola* B was dark blue-green throughout the trial. By the trial's termination, algal mats had grown on sides and bottoms of jars and on the air lines.

Contamination in this trial was considerably lower than observed in previous trials. On the last day of the trial, a few green unicells (approximately 4 µm in diameter) of unidentified species were observed.

There were very few shrimp deaths until Day 13 of the trial, which corresponded to the increase in algal growth. Most of the shrimp were observed to have empty guts during the first week of the trial. After that period of time, shrimp were occasionally seen with small amounts of what appeared to be *S. calcicola* B in their guts. Shrimp were frequently observed swimming toward a clump of algae that had just been added, bringing it up toward their mouths, and then rejecting it, indicating dislike of this alga as a food source.

Table 12. Number of shrimp diagnosed for hemocytic enteritis during the 19-d *Schizothrix calcicola* B bioassay (Trial 6)

Jar Number	Treatment	Feed	Remaining Number of Shrimp	Number Negative for HE	Number Positive for HE
1	Control	1/2	3	1	2
2		1/2	3	3	1
5	*S. calcicola* B	1/2	4	2	3
6		1/2	2	2	1
7		0	1	3	0
8		0	1	1	0

One-third of the 12 shrimp examined had advanced cases of HE starting at the central midgut and extending through the hindgut caecum. (Table 12). One of these shrimp was killed on Day 13 of the trial to present a well-fixed specimen for histological processing. It exhibited the same symptoms of a slightly enlarged hindgut caecum as a shrimp that had died on the previous day. The other 11 shrimp lived through the trial. Both jars containing control shrimp remained clear throughout the trial. Contaminants were few in number and type (Appendix B).

Three of the seven control shrimp had advanced cases of HE ranging from the central midgut into the hindgut caecum. One of these shrimp died on Day 12, while the other two lived through the entire trial.

Discussion

Two of the six strains of algae tested, *Schizothrix calcicola* B and *Spirulina subsalsa*, appeared to promote the occurrence of hemocytic enteritis in *Penaeus stylirostris*. When statistical analyses were performed on incidence of HE using contingency tables and chi square, significant values were observed only in Trial 1 for *S. calcicola* B (Appendix B). There were no significant differences between shrimp treated with *S. calcicola* B and control shrimp in Trial 6. Two additional trials showed a 27% and 28% incidence of HE in 15 and 19 shrimp, respectively (Lightner 1980, personal communication). Later testing of another species of shrimp, *Penaeus californiensis*, with *S. calcicola* B also showed significant increases in the occurrence of HE with 43% (Lightner 1980, personal communication) and 21% incidence in 28 and 14 shrimp, respectively. None of the control shrimp were found to have HE in these later trials.

The main problems encountered throughout the trials were the occurrence of HE in control shrimp, mortalities due to poor water quality, dense algal populations, and low numbers of shrimp suitable for histological analysis because of autolysis. Despite efforts to keep shrimp separated from algae for various periods before each trial, HE was present in control shrimp in most trials. Three of 10 shrimp tested for Trial 6 were found to have HE. These shrimp were held in a 20-gal tank during the 19-d period of that trial. This indicated that some shrimp may have been inflicted with HE before the trial began. In future experiments, shrimp should be kept in the dark just before entering the post-larval stage, providing several weeks of

algae-free growth before being introduced into jars for testing, hopefully reducing the occurrence of HE. Water quality was improved with the use of undergravel filters, but for future trials, a continuous flow system or an increase in quantity of seawater per gram of shrimp per day in a closed system should be employed. In most trials, algal growth was very dense, causing mortalities due to entanglement and clogging of gills. This was alleviated by feeding algae to shrimp in smaller quantities that kept algae in lower densities throughout most of the trial, reducing early mortalities. Larger numbers of shrimp should be used to increase the amount of shrimp available for the final analysis. Very few shrimp that died during the trials were well fixed due to rapid autolysis of shrimp tissue. This was unfortunate since shrimp that may have died of HE were not detected. Mortality rate was not a valid parameter for measurement of HE due to the previously mentioned problems of water quality and physical contact with algae.

Shrimp numbers were originally reduced to allow the use of smaller jars in which algal contamination could be readily controlled. Although this reduced contamination, the major source was the shrimp themselves. Later testing indicated that the amount of formalin and Cutrine-plus used to treat the shrimp may not have been in sufficient concentrations to kill the associated algae. In future trials, increases in these concentrations and/or the covering of shrimp from light for a lengthy period prior to testing should help decrease contaminant algal densities. Experimental tanks should also be equipped with tight-fitting covers to reduce contamination from the air.

Although *S. calcicola* B appeared to promote the occurrence of HE during various trials, certain discrepancies remain unanswered. Microscopic evaluations of samples taken from raceways exhibiting outbreaks of HE failed to show appreciable amounts of *S. calcicola* B. The predominant alga was usually *M. lyngbyaceus*. Trial 3 included 3 jars of algae taken from a raceway that suffered a recent outbreak of HE. No shrimp were found to have HE in the 11 individuals analyzed. It was also observed in Trial 6 that shrimp did not eat *S. calcicola* B unless no other food source was available.

The study of toxigenic algae has shown the variability of toxin production. Environmental and physiological factors play an important role in the toxicity of algae. A given algal species may be toxic under certain conditions and harmless under others. *M. lyngbyaceus*, found in the waters off of Hawaii, has been found to be occasionally toxic, causing acute dermatitis in humans (Hashimoto et al. 1976). It has been speculated that its toxicity is lost with exposure to strong sunlight. Frequently, there is lack of correlation between algal densities and toxin concentrations (Shilo and Aschner 1953). It is possible that conditions during the present trials interfered with toxin production while conditions in the raceways promoted high toxicity even under low algal densities.

The presence of bacteria was not taken into consideration during the trials. It is possible that bacteria associated with the algae produces or contributes to the toxigenic agent.

Due to the appearance of HE in control shrimp additional tests should be performed to confirm *S. calcicola* B as the toxigenic agent.

Although the increase in the number of shrimp inflicted with HE when fed *S. subsalsa* was not statistically significant, additional trials should be undertaken with a larger number of shrimp to confirm these results.

SUMMARY

Five strains of blue-green algae, *Spirulina subsalsa*, *Microcoleus lyngbyaceus*, and *Schizothrix calcicola* (strains A, B, and C) were isolated from shrimp culture raceways in Puerto Peñasco, Mexico, and established in unialgal cultures.

Growth of *S. subsalsa*, *M. lyngbyaceus*, *S. calcicola* A, and *S. calcicola* B was tested in Provasoli's Enriched Seawater, Erdschreiber's Medium, Soil Seawater, ASM, and McLachlan's Medium. Provasoli's Enriched Seawater and McLachlan's Medium afforded the best growth of filaments.

Differences in growth and color of the above four strains were observed with varying light intensities from 25 to 400 ft-c. Growth of filaments appeared to be greatest at light intensities between 100 to 400 ft-c. Color of filaments turned from blue-green to light green at light intensities greater than 300 ft-c.

All five strains of algae tested grew well at 32 °C. *M. lyngbyaceus* was found to have the most dense growth at 32 °C when tested in temperatures ranging from 21° to 38 °C.

S. subsalsa was grown at pH values ranging from 7.1 to 9.0. No differences in growth rate at any of the pH values was detected.

Unialgal cultures of the five strains of blue-green algae were utilized in six bioassays in an effort to induce hemocytic enteritis in *P. stylirostris*, *S. subsalsa*, and *S. calcicola* B appeared to promote the occurrence of hemocytic enteritis in the shrimp, but only the

values in one out of two trials for *S. calcicola* B were found to be statistically significant while the value for the trial involving *S. subsalsa* was not significant.

APPENDIX A

MEDIA, FIXATIVES, STAINING PROCEDURES, AND TISSUE PROCESSING

Media

ASM

	µm/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
$MnSO_4 \cdot H_2O$	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 (Bold and Wynne 1978)

1000 ml filtered seawater

50 ml soil-water supernatant

.2 g $NaNO_3$

.03 g $Na_2HPO_4 \cdot H_2O$

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	1 ml/l

McLachlan's Medium .41M (McLachlan 1960)

Stock No.	Compound	g/l	M
1	$CaCl_2 \cdot 2H_2O$	14.7	.01
	$MgCl_2 \cdot 6H_2O$	44.7	.022
	NaCl	239.6	.41
2	$MgSO_4 \cdot 7H_2O$	59.16	.024

Stock No.	Compound	g/l	M
3	K_2HPO_4	17.42	.0001
4	$Na_2SiO_3 \cdot 9H_2O$	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}
	$MnCl_2 \cdot 4H_2O$	1.385	7.0×10^{-6}
	FeEDTA	.8	
	$CoCl_2 \cdot 6H_2O$	.0048	2.0×10^{-8}
	$CuCl_2 \cdot 2H_2O$	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 µg/l
$+PO_4$	$Na_2HPO_4 \cdot 12H_2O$	.009 g/l
$+NO_3$	$NaNO_3$	.06 g/l
+ seawater	McLachlan Stocks 3, 4, and 5	Add stocks as described above to 1 liter filtered seawater

Soil Seawater Medium (Starr 1964)

Soil 1/4 to 1/2 inch

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 (Starr 1964)

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 $Fe(NH_4)_2 \cdot 6H_2O$ 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
$FeCl_3 \cdot 6H_2O$	4.9 mg
$MnSO_4 \cdot H_2O$	12.4 mg
$ZnSO_4 \cdot 7H_2O$	2.2 mg
$CoSO_4 \cdot 7H_2O$	.48 mg
NaEDTA	100 mg

Fixatives

Davidson's Fixative (Humason 1967)

Formalin	20 ml
Glacial Acetic Acid	10 ml
95% Ethyl alcohol	30 ml
Distilled water	30 ml

Staining Procedure

Eosin-Hematoxylin stains (Preece 1965)

Immerse slides in the following manner:

Xylene -- 10 dips (2x)
Absolute alcohol -- 10 dips (2x)
95% alcohol -- 10 dips (2x)
80% alcohol -- 10 dips (2x)
50% alcohol -- 10 dips
Distilled water -- 10 dips
Hematoxylin stain -- 5 minutes
Running water -- 5 minutes
Eosin stain -- 5 minutes
95% alcohol -- 4 dips (3x)
Absolute alcohol -- 4 dips (3x)
Xylene -- 10 dips (4x)

Tissue Processing

Immerse shrimp in contains of ethyl alcohol in the following manner:

70% alcohol -- 1 h
80% alcohol -- 1 h
95% alcohol -- 1 h
95% alcohol -- 1 h
100% alcohol -- 1 h
100% alcohol -- 1 h
100% alcohol -- 1 h
100% alcohol -- 1 h

APPENDIX B

BIOASSAY AND CONTAMINANT TABLES

Table B-1. Experimental conditions of unialgal bioassays

Trial Number	Algae Tested	Filter	Number Days Trial	Average Weight of Shrimp (g)	Light Cycle lt:dk	Number Control Jars	Number Experimental Jars
1	*S. calciola* B	N	16	.2	24:0	4	12
2	*S. subsalsa*	A	18	.42	24:0	4	11
	M. lyngbyaceus	A	18	.42	24:0	4	5
3	*S. calciola* A	A	19	.45	12:12	5	9
4	*S. calciola* C	U	20	1.0	12:12	5	5
5	*M. lyngbyaceus*	U	22	.6	12:12	3	3
6	*S. calciola* B	U	19	.26	12:12	4	8

N = none

A = airfilter

U = undergravel filter

Table B-2 Percent of shrimp with hemocytic enteritis during unialgal bioassays

Trial No.	Algae Tested	Number Control	Number Positive Control	% Positive Control	Number Experimental	Number Positive Experimental	% Positive Experimental	x_2 Control vs. Experimental	Significance
1	*S. calciola* B	8	0	0	35	12	34	12.00	.999
2	*S. subsalsa*	10	1	10	18	4	22	.9602	NS
	M. lyngbyaceus	--	--	--	11	1	9	.0031	NS
3	*S. calicola* A	10	2	20	26	2	8	1.5311	NS
	Raceway	--	--	--	11	0	0	N/A	N/A
4	*S. calciola* C	11	3	27	17	0	0	N/S	N/A
5	*M. lyngbyaceus*	10	1	10	10	2	20	.4174	NS
6	*S. calcicola* B	7	3	43	12	4	33	.1711	NS

NS = not significant

N/A = not applicable

Table B-3. Contaminants observed during unialgal bioassays with *P. stylirostris*.

Organism	Trial 1		Trial 2			Trial 3			Trial 4		Trial 5		Trial 6	
	Control	*S. calciola* B	Control	*M. lyngbyaceus*	*S. subsalsa*	Control	*S. calciola* A	Raceway Sample	Control	*S. calciola* C	Control	*M. lyngbyaceus*	Control	*S. calciola* B
Small flagellates (1-5μm)	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Large flagellates (5-8μm)	x	x					x	x					x	x
Cilista														
Hymenstomatida	x	x	x	x	x		x	x	x	x	x	x	x	x
Actinopoda											x		x	
Spirotricha	x	x	x	x	x		x	x		x		x	x	x
Vorticella							x				x		x	x
Mastigophora														
Amoebida	x				x	x				x	x			
Bacillariophyta														
Rhizosoleniaceae				x				x	x					
Amphora sp.	x	x	x		x	x	x	x	x					
Cyanophyta														
S. calciola A						x		x						
S. calciola C			x	x		x		x			x			
Entophysalis sp.								x			x			
M. lyngbyaceus								x						
Chrysophyta														
Platychrysis neustophila						x				x				
Pyrrhophyta														
Ceratium	x													

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