THE EFFECTS OF THE TOXIC PHYTOPLANKTER (*HETEROSIGMA CARTERAE*) ON THE COON-STRIPE SHRIMP (*PANDALUS DANAE*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

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The Effects of the Toxic Phytoplankter (Heterosigma carterae) on the Coon-Stripe Shrimp (Pandalus danae) and Rainbow Trout (Oncorhynchus mykiss).

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Abstract

The raphydophycean phytoplankter, *Heterosigma carterae,* produces oxyradicals that can kill finfish. However, effects of toxic *H. carterae* on shrimp are unknown and formed the objective of this study. In this investigation, *Pandalus danae* was studied as a representative shrimp, while rainbow trout was used as a toxicity bioindicator.

Toxic *H. carterae* cells did not kill *Pandalus danae* within 24 h of exposure in spite of the injury to the shrimp gills that occurred between 4 and 24 h, i.e. hypertrophy and fusion of lamellae, rupture of the lamellar membranes, and atrophy of the filaments. These lesions impaired the aerobic respiratory system of this shrimp. The shrimp responded to the injury of the gills by hemocytic swarming at the lesion sites on the lamellae.

Blood hypoxia occurred as the O_2 concentration in the hemolymph decreased significantly (Tukey's test; $P<0.05$) with time during 24-h exposure to a toxic culture of *H. carterae*, whilst lactate concentrations increased ca. 5 fold within a 12-h exposure before decreasing to the pre-exposure value. Lactate concentrations in the gills dropped significantly with time during a 24-h exposure to the toxic *H. carterae* culture. The reduction of lactate concentrations in the hemolymph and in the gills upon exposure to the toxic *H. carterae* culture suggest that the animal might have utilized lactate for energy under toxic-*H. carterae*-induced hypoxia.

The gills of shrimp exposed to toxic *H. carterae* cells showed significantly reduced catalase activities after 18 h of exposure, a significant increase in peroxidase activity at 6 h of exposure, followed by a steady decline to values not significantly different from the pre-exposure value. These data suggest that oxyradicals secreted by *H. carterae* might destroy catalase activity, whereas peroxidase remained active during the exposure to the toxic algal cells.

After re-introduction to seawater for 6 weeks, the injured lamellae of the shrimp gills remained hyperthropied. However, healing processes had continued as indicated by hemocytic swarming at the site of the lesions of the lamellae. Molting process may have fostered the healing process by more evenly distributing the hemocytes along the lamellar membranes.

Dedication

To:

My lovely wife Liena for your prayers, love and patience,

My beloved children Patrick and Gloria who give joy for your dad,

and

My mother, father, brothers, sisters for your everlasting prayers and encouragement.

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INTRODUCTION

Among the approximate 5,000 species of extant marine phytoplankters, some 300 species are potentially toxic, while 40 or so species secrete sufficiently potent toxins to affect humans as well as wild and cultured marine finfish (Hallegraeff, 1993). Species such as *Heterosigma carterae*, *Chaetoceros concavicorus*, *Chattonella marina*, *Chrysochromulina polylepsis* and *Gymnodinium breve* are particularly toxic or harmful to finfish (Black *et al.*, 1991; Chang *et al.*, 1990; Gaines and Taylor, 1986; Oda *et al.*, 1992a; Rosenberg *et al.*, 1988; Yang *et al.*, 1995). Indeed, in many cases, natural blooms of these algae have caused extensive mortalities of aquacultured finfish (Boustead *et al.* 1989; Chang *et al.*, 1990; Gowen, 1987; Smayda, 1990; Taylor, 1993).

H. carterae (formerly *H. akashiwo*) is one of the most toxic (to finfish) marine phytoplankters (Black *et al.*, 1991; Taylor *et al.*, 1994). It is found in coastal waters with 2 to 50 ppt salinity, temperatures of 3 to 30 ^{o}C, and low to high light intensities (Gaines and Tayor, 1986). According to Chretiennot-Dinet *et al.* (1993), *H.carterae* (Hada 1968) belongs to the family Vacuolariaceae (Luther 1899), order Raphidomonadales (Chadefaud 1960), and class Raphidophyceae (Chadefaud ex Silva 1980).

H. carterae is a bean-shaped single cell, usually 12 to 22 um long, with a surface that varies from smooth to bumpy [when preserved in Lugol's Iodine (Taylor *et al.*, 1994)] with a potato-like outline (Gaines and Taylor, 1986). The cell surface of this alga has a relatively thick glycocalyx (Yokote *et al.*, 1985) and small peripheral mucocysts (Taylor *et al.*, 1994). Each cell contains between 20 and 50 greenish-brown chloroplasts (Gaines and Taylor, 1986).

H. carterae possesses two flagella (Gaines and Taylor, 1986) and can migrate vertically with a velocity of 1.0 to 1.3 m h^{-1} (Yamochi and Abe, 1984). Under bloom conditions, *H. carterae* cells can be found to depths of 30 m (Taylor *et al.*, 1994).

Blooms of *H. carterae* in coastal seawaters can cause fish mortalities (Black et al., 1991; Chang *et al.*, 1990; Gaines and Taylor, 1986; Yang *et al.*, 1995). Table 1 lists several finfish that have been reported to be killed by toxic *H. carterae* cells.

Table 1. List of finfish reported to be killed by toxic *H. carterae.*

Fish	Location	Reference(s)
1. Yellowtail (*Seriola quinqueradiata*) and flat-fish	Japanese waters	Gaines and Taylor (1986)
2. Chinook salmon (*Oncorhynchus tshawytscha*)	Big Glory Bay, New Zealand	Chang *et al.* (1990)
3. Chinook salmon (*O. tshawytscha*)	San Mateo Bay, B.C., Canada	Black *et al.* (1991)
4. Rainbow trout (*Oncorhynchus mykiss*)	Laboratory study in Canada	Yang et al. (1995)
5. Sockeye salmon (*Oncorhynchus nerka*)	Laboratory studies in Canada	Whyte (1993); Yang *et al.* (1995)

Since the late 1960's, in B.C. waters, particularly within the Strait of Georgia and in Barkley Sound on the west coast of Vancouver Island, *H. carterae* blooms have been found to occur annually between May and July and can last for four months or more (Taylor and Haigh, 1993). For example, a massive bloom of *H. carterae* in 1989 covered ca. 7000 km^2 along 750 km of B.C. and Washington coastline (Taylor *et al.*, 1994) and lasted between June to October (Taylor and Haigh, 1993).

Chang *et al.* (1990) suggested that chinook salmon (*Oncorhynchus tshawytscha*) die in the presence of *H. carterae* due to loss of gill function, while Yang *et al.* (1995) provided evidence that *H. carterae* is toxic to rainbow trout, largely due to the production

of oxygen radicals (oxyradicals). The latter authors suggested that these oxyradicals may cause loss of gill function in rainbow trout.

Yang *et al.* (1995) demonstrated that superoxide dismutase (destroys superoxide radicals) and/or catalase (catalyzes hydrogen peroxide degradation to water and oxygen) additions to a toxic *H. carterae* culture markedly reduced its toxicity to juvenile rainbow trout. These authors showed that under laboratory conditions, ca. 10^4 *H. carterae* cells released sufficient superoxide radicals to damage the secondary lamellae of fish gills.

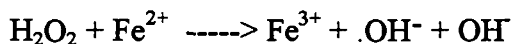
Oda *et al.* (1992b) previously reported that *C. marina*, a Raphydophycean closely related to *H. carterae*, produces superoxide and hydroxyl radicals as well as hydrogen peroxide. Additions of SOD and/or catalase to a *C. marina* culture in which bacteria were suspended could protect these bacteria against the toxic action of this phytoplankter (Oda *et al.*, 1992a).

Another Raphydophycean, *C. antiqua*, also reportedly generates oxyradicals that can kill finfish (Tanaka *et al.*, 1994). By use of a laser scanning microscope, these authors observed the release of many small particles, which they called mucocyts, by a *C. antiqua* cell, concomitant with the discharge of O_2^- for a few tenths of a second, following the exposure of the cell to the mucus from gill lamellae of a yellowtail (*Seriola quinqueradiata*).

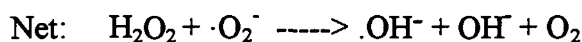
These findings strongly indicate that free oxygen radicals (oxyradicals) are the main, if not the sole, mechanism by which certain Raphydophyceans kill finfish.

In general terms, a free radical is any chemical species that has one or more unpaired electrons (Halliwell & Gutteridge, 1984). According to Abele-Oeschger *et al.* (1994) oxidative damage by oxyradicals is initiated by a radical chain reaction, often called the Fenton reaction. The reaction occurs as hydrogen peroxide diffuses through cell membranes, and inside the cells, simultaneously liberates other reactive oxygen species, mainly $.OH^-$ (hydroxyl radical). Iron is intimately involved in this reaction:

$$Fe^{3+} + .O_2^- \text{ -----> } O_2 + Fe^{2+}$$



$$H_2O_2 + Fe^{2+} \text{ -----> } Fe^{3+} + .OH^- + OH^-$$



$$\text{Net: } H_2O_2 + \cdot O_2^- \text{ -----> } .OH^- + OH^- + O_2$$

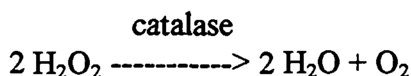
Thus a simple mixture of an iron salt (e.g. $FeSO_4$) and H_2O_2 can provoke a series of radical reactions (Halliwell & Gutteridge, 1984). In addition, other divalent cations, such as Cu^{2+} and Mn^{2+}, may also enhance the radical reaction.

These oxyradicals cause the formation of membrane lipid peroxides, and the oxidation of membrane-bound proteins, including enzymes as well as and other macromolecules such as DNA (Ahern, 1991). The destruction of many of the key structures in a cell leads to membrane leakage and the loss of cellular metabolism with resultant cell death and tissue damage.

Fundamentally, all aerobic organisms possesses a complex antioxidant defense against oxyradicals (Winston & Di Giulio, 1991). There are two types of biological antioxidants in organisms: 1) low molecular weight scavengers, e.g. ascorbate, glutathione, vitamins A and E, and carotenoids, and 2) enzymes, e.g. catalase, peroxidase, glutathione reductase and superoxide dismutase (SOD).

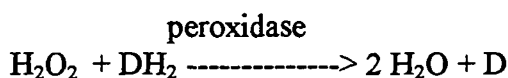
The catalytic activities of these antioxidant enzymes facilitate the breakdown of oxyradicals (Jakoby, 1980). Examples are the degradation of H_2O_2 by catalase and peroxidase through the following reactions:

a) Catalase (hydrogen peroxide oxireductase E.C.1.11.1.6.), according to Aebi (1983):



$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

b) Peroxidase (hydrogen peroxide oxireductase E.C.1.11.1.7), according to Putter and Becker (1983):



$$H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2\,H_2O + D$$

where, D = hydrogen donor e.g. 2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulphonic acid] (ABTS).

Beside removing hydrogen peroxide as the precursor of lipid peroxidation, the actions of both enzymes can prevent the formation of $.OH^-$ (Halliwell and Gutteridge, 1984).

In aquatic environments, the gill of an animal is of special interest as it is continously exposed to the external environment. Consequently, the gill might be very susceptible to the hazardous effects of toxins, such as the free oxyradicals secreted by *H. carterae*. Accordingly the gill might have a suppression of its critical physiological functions, including gas exchange, ionoregulation, maintenance of acid-base balance, and excretion of nitrogenous wastes.

Within the coastal seawaters in which *H. carterae* blooms, finfish, which are susceptible to the toxic effects of this phytoplankter, likely swim away from a water when it becomes toxic. However, there are other gilled animals that cannot readily leave the immediate vicinity of toxic blooms of *H. carterae*. Examples of such animals are invertebrates such as crustaceans.

If these gilled animals are indeed affected by toxic *H. carterae* blooms, the dynamics of the marine ecosystem in the immediate vicinity of a toxic *H. carterae* bloom may be affected. Accordingly, I chose to investigate the effects of a toxic *H. carterae* culture on a representative crustacean, the coon-stripe shrimp *Pandalus danae* Stimpson [order Decapoda, suborder Pleocyemata, infraorder Caridea (Bowman and Abele, 1982)].

P. danae is a shrimp that is native to coastal waters of North America from Alaska to northern California (Holthuis, 1980; Meinkoth, 1981; Neilson, 1981). *P. danae* is found in bays, estuaries, and eelgrass beds, occasionally in tidepools. It usually occurs in waters from 18 to 200 m in depth (Holthuis, 1980; Meinkoth, 1981).

In British Columbia, *P. danae* is one of eight pandalid shrimp which are economically important for shrimp fishery (Neilson, 1981). Along the continental shelf of B.C. coastal

waters, pandalid shrimps usually inhabit waters with mud or soft sand bottom (Neilson, 1981).

Male *P. danae* can reach maximum total and carapace length of 88 and 22 mm respectively; the female shows values of 105 and 29 mm respectively (Holthuis, 1980).

The Scope of this Study

The first objective of this study was to determine the relative toxicity of *H. carterae* to the coon-stripe shrimp, *P. danae*, as compared to rainbow trout. I used rainbow trout as a bioindicator of *H. carterae* toxicity because this species is relatively sensitive to the algal toxins as reported by Yang *et al.* (1995). In addition, rainbow trout is used as one of several standard animals for acute lethality tests (EPS, 1990). Gill histology and, assays of catalase and peroxidase activities and lactate concentrations in the gills, as well as measurement of lactate and oxygen concentrations in the hemolymph were used to evaluate the extent of respiratory epithelium damage.

The second objective of this study was to investigate the ability of the gills to heal following injury by a toxic *H. carterae* culture. This was assessed by observing the inflammation process in injured gills. In addition, the effects of exposures to *H. carterae* on subsequent molting were assessed.

MATERIALS AND METHODS

General procedure

The procedure of the research is outlined in Fig. 1.

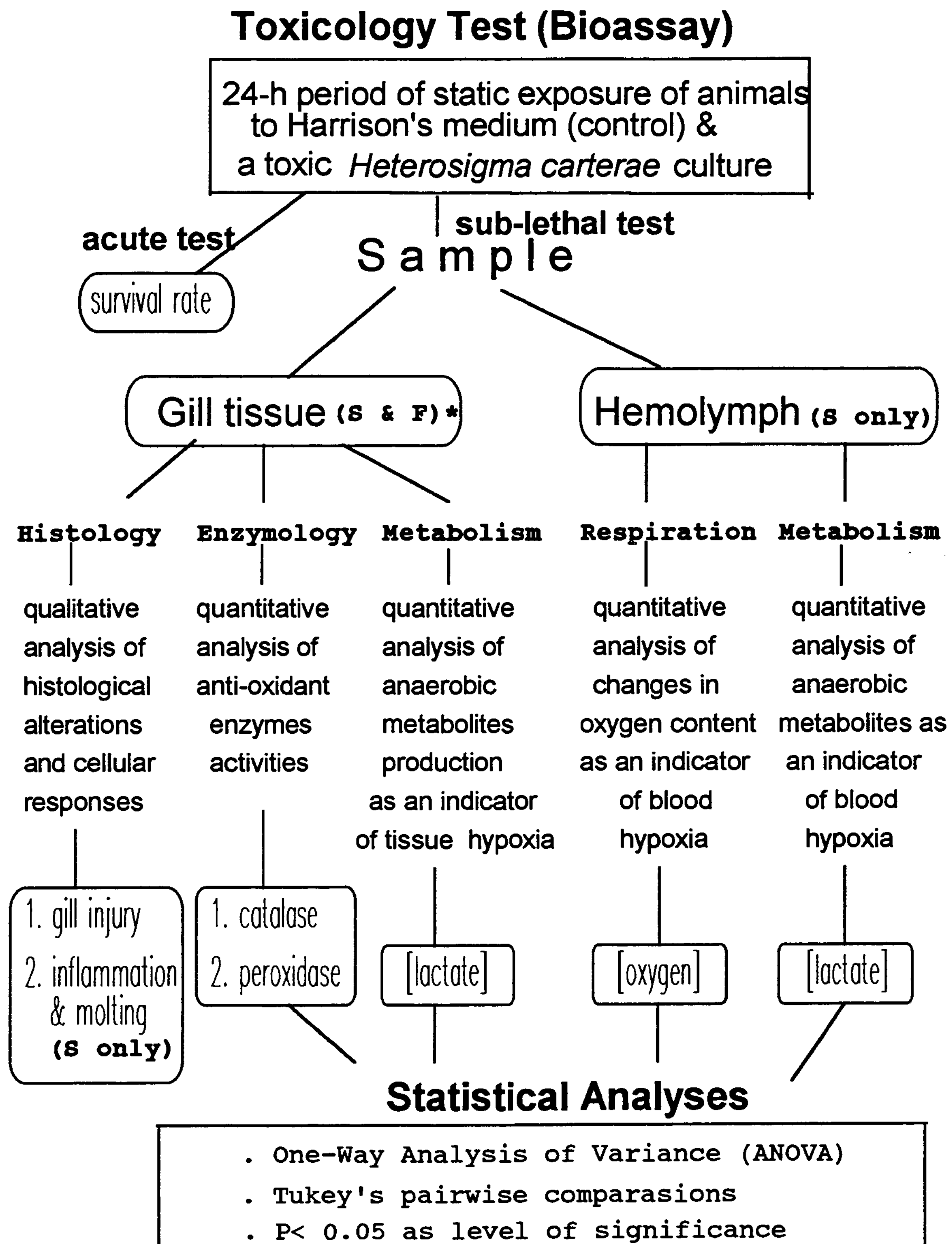
Heterosigma carterae culture

The unialgal, but not axenic, culture of *H. carterae* strain was obtained from Dr. I. Whyte (Pacific Biological Station, Canada Dept. of Fisheries and Oceans, Nanaimo, British Columbia). Cultures of this isolate were grown in 5 L of Harrison's medium (Harrison *et al.*, 1980) at an initial pH of 8.2. Each culture was contained in a 6-L boiling flask with aeration under 1000 lx of continuous illumination at 22 ^{o}C. The culture was subcultured every ten days by adding 2.5 L of culture to 2.5 L of fresh medium.

The cell concentrations in each culture were determined using the Utermohl technique (Utermohl, 1958). Two mL of each *H. carterae* sample were put in a Utermohl chamber and mixed with 10 drops of 1% (v/v) Lugol's preservative. Filtered seawater was then added to completely fill the chambers. The samples were then stored in the dark for 15 to 24 h to allow the cells to settle on the bottom of the chamber. Twenty fields were viewed at 100X for each settled sample using an inverted Olympus microscope. The mean salinity and pH of the culture were 28 ppt and 8.8, respectively.

In this study, a toxic culture of *H. carterae* is defined as the ability of the culture to cause mortality to at least 50% of a population of rainbow trout (mean weight = 12 - 18 g) within 3 h. A moribund fish (did not move when touched with a metal pincher) was counted as a dead fish.

Fig.1. Flow Chart of Research Procedure



*) F = fish; S = shrimp

Rainbow trout

All experiments were carried out using 12 - 18 g rainbow trout which were obtained from West Creek Trout Farm, Aldergrove, B.C. All fish were transported to Simon Fraser University, where they were cultured for at least four weeks in aerated fresh water before being used for experimental purposes. The fish were fed daily using a commercial salmon food. Two days prior to exposure to a toxic culture of *H. carterae*, fish were transferred to a 77-L circular tank containing 70 L of aerated seawater of approximately 25 ppt salinity and 8.3 pH at 16 °C. While in this acclimating tank, the fish were not fed.

Coon-stripe shrimp

P. danae were supplied by Dr. J. Marliave (Vancouver Aquarium, Vancouver, B.C.) and Seacology (North Vancouver, B.C), a commercial wild aquatic animal supplier. All shrimp were maintained in aerated seawater until used for experimental purposes. They were fed daily with minced canned shrimp meat, but starved for 2 days prior to use. At that time, a group of shrimp were randomly selected and placed in a holding tank containing seawater at 16 °C, pH 8.3 and 25 ppt salinity.

Toxicity assays

Six aquaria (each with dimensions of 18 x 36 cm, and a depth of 23 cm) were used. Aquaria 1 and 3 each contained 4.5 L of Harrison's medium, while 4.5 L of a toxic culture of *H. carterae* was placed in each of aquaria 2 and 4. Aquaria 5 and 6 contained 3.0 L of Harrison's medium and 3.0 L of a toxic *H. carterae* culture, respectively. All media and culture were continuously aerated and acclimated to approximately 16 °C for 2 h, at which time 16 trout (mean weight of 17.5 g each) were then added to each of aquaria

1 to 4. At the same time, 20 shrimp (mean weight 2.2 g each) were placed in each of aquaria 5 and 6.

Fish in aquaria 1 and 2 functioned as bioindicators of toxicity of the Harrison's medium and the *H. carterae* culture, respectively. Animals in aquaria 3 to 6 were used to obtain gill samples for histology.

Shrimp were classified as dead if no movement could be detected after gentle prodding with a glass rod. Any animal classified as dead was immediately removed from the aquarium. Trials were carried out in duplicate to provide adequate data for statistical analyses.

Gill histology

Gill samples of two fish and two shrimp from their respective holding tanks were collected immediately at the time the experiment commenced. These samples provided the normal histology of the gills.

Two fish were randomly removed from each of aquaria 3 and 4 at 4 and 12 h after the experiment started. Two shrimp were randomly selected and removed from each of aquaria 5 and 6 at 4 and 24 h after the test was started. The gills of the animals were excised immediately and treated as described by Humason (1979) for fish, and Bell & Lightner (1988) for shrimp.

The gills were fixed in Davidson's fixative for 24 h, routinely processed with ethanol, embedded in paraffin wax, sectioned at 6 (fish) or 5 um (shrimp), stained with hematoxylin and eosin (H&E) and examined under light microscopy for their histopathological status.

Observations of histopathological status in the gills included:

1) Types of injuries on the gill lamellae i.e. edema, hypertrophy, atrophy and necrosis,
2) The presence or absence hemocyte swarming and/or melanized nodules as well as intralamellar septae in the shrimp gill lamellae.

Assays of antioxidant enzymes - Experimental design

The following design applied for all experiments used to obtain samples of trout or shrimp gills for catalase and peroxidase assays.

Four aquaria were used for each assay set. Two aquaria contained 4.5 L each of Harrison's medium and the other two each contained 4.5 L of a toxic *H. carterae* culture. All media and culture were continuously aerated and acclimated to approximately 16 ^{o}C for 2 h, at which time 12 trout (mean weight of 12 g) were then added to each aquarium. At 2 and 4 h after the experiment was commenced, 3 fish were sampled from each aquarium.

Immediately after the commencement of each experiment, 6 fish were removed from the acclimating tank containing seawater and their gills were excised. Enzyme activities in the gills of these animals were considered as activities at time zero.

The same experimental design and procedure were also employed in the experiments using shrimp (mean weight of 3 g each), except 16 shrimp were added to each aquarium, each test lasted for 24 h, and 4 shrimp were collected every 6 h. To obtain the enzyme activities in the gills in time zero, eight shrimp were randomly sampled from the shrimp acclimating tank immediately following initiation of each experiment.

Collection and treatment of samples

All fish were killed by a quick blow on the head, whilst shrimp were sacrificed by incision of the animal transversely at the junction of the cephalothorax and abdomen. The gills of all animals were immediately aseptically incised, placed in clean plastic bags and stored at -20°C. Further analyses were carried out within 24 h.

Preparation of tissue extract

Preparations of the gill tissue extract were as per Lemaire *et al.* (1993), Aebi (1983), and Aksnes & Njaa (1981).

Frozen samples of fish gills were thawed to 0 °C in an ice bath. The gills were then washed with a cold isotonic saline (0.15 M NaCl) to remove erythrocytes and other cell debris. The gill filaments were then incised from the gill arches on an ice-cold surface. The gill filaments were then blotted dry on a tissue paper before being weighed to provide 0.1-g samples for further treatment. Gills of an individual fish constitued one sample, whilst gills from two or more shrimp were pooled to yield a 0.1-g tissue sample.

Assay of catalase activity

One-tenth g of fish or shrimp gills was made to 10 % solution with 50 mM phosphate buffered solution (PBS) (w/v) at 7.0 pH. The mixture was then homogenized with 20 strokes using a Potter-Elvehjem homogenizer placed in an ice bath, followed by centrifugation of the sample at 10,000 x g for 20 min in 4 °C. The resulted supernatant was mixed with detergent (1% Triton X-100) at a ratio of 10:1 (v/v). The mixture was diluted further with PBS, pH 7.0 (1:100). Prior to the measurement of catalase activity, ethanol was added to the diluted aliquot (0.01 mL EtOH/mL aliquot), and incubated in ice for 15 min.

Measurement of the catalase activity was as per Cohen *et al.* (1970) and Aebi (1983), using a Spectronic®-3000 Array spectrophotometer connected to an IBM PS/2 computer loaded with a Rate Analysis Program for enzyme kinetics and an output printer (Epson LX-810). The decrease of substrate (H_2O_2) concentration was followed by reading the changes of absorbances at 240 nm at room temperature.

The catalase activity (s^{-1}) in a cuvette was calculated according to the following formulae:

$$k = (2.3/\Delta t).(\log A1/A2)$$

where,

Δt = time interval for absorbance decrease from A1 (initial absorbance) to A2 (absorbance at time t); Δt = 15 s.

The specific catalase activity (in $\mu mol.min^{-1}g^{-1}$) was derived as follows:

$$z = k.\text{dilution factor}.60/\text{wet weight}$$

where: the wet weight = 0.1 g.

Assay of peroxidases activity

One-tenth g of fish or shrimp gill materials were diluted to 10 % with 0.067 M aqueous phosphate buffer (w/v) at 6.0 pH. The mixtures were then homogenized and centrifuged as described earlier. The supernatant fluids were diluted to 5% with the phosphate buffer (pH 6.0) and used for peroxidase analyses.

Measurement of the peroxidases activity was as per Putter & Becker (1983), using the same computerized spectrophotometer as for the catalase assay. Peroxidases activity was determined by the formation of an oxidized compound ($ABTS^{+}$) from ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulphonic acid]. The increase of absorbances was followed at wave-length 405 nm in room temperature.

The peroxidases specific activity in the gills (in $\mu mol.min^{-1}g^{-1}$) was calculated as follows:

$$Z = \frac{dA.V.1000}{e.d.W.\Delta t}$$

where:
dA = absorbance difference of 1 min and 6 min after addition of the sample to the cuvette.
V = assay volume ($2.4x10^{-3}$ l)
e = absorption coefficient ($1.86\ lxmmol^{-1}xmm^{-1}$)
W = wet weight of the tissue (0.1 g)
d = light path (10 mm)
Δt = reaction time (5 min)

Assay of lactate - Experimental design

Four aquaria were used. Three L of Harrison's medium were added to each of two aquaria and 3 L of a toxic *H. carterae* culture were added to each of the other two

aquaria. All media were aerated continuously. After the media had been acclimated to 16 °C for 2 h, a group of 10 fish or 8 shrimp (mean weight of 3.0 each) were added to each aquarium.

Following 5 h of this treatment, three fish were randomly sampled from each aquarium to obtain gill samples for lactate assays. Gills from one fish represent one sample. Two shrimp were randomly selected from each aquarium at 12 and 24 h after the experiment was started. Samples of gills and hemolymph of shrimp from replicates of each treatment were pooled to provide adequate samples for lactate analyses.

To obtain lactate content at time zero, gills of 3 fish and gills and hemolymph of 6 shrimp were randomly sampled from their respective acclimating tank immediately following the initiation of the experiment.

Treatment of samples for lactate assay

The lactate assay was based on the methods of Low *et al.* (1993) and Henry *et al.* (1994).

Immediately following its removal from an aquarium, the dorsal region of each shrimp was blotted dry and hemolymph was withdrawn from the hemocoel using a 1-mL syringe with 27 gauge needle. Approximately 50 uL of hemolymph was deproteinized in 100 uL cold 8% perchloric acid (PCA) and stored in ice. The mixture was assayed for lactate using the Sigma (St. Louis, MO) diagnostic kit (a spectrophotometric enzymatic analysis).

Following removal of the hemolymph, the cephalothorax of a shrimp was incised from the abdomen and immediately frozen in liquid nitrogen for approximately 1 min. While still frozen, the gills were separated from the cephalothorax and stored at -20 °C before further treatment.

Immediately following its removal from an aquarium, each fish was sacrificed with a blow to the head. The gill was immediately excised and frozen in liquid N for

approximately 1 min. Gill filaments were excised from the arch on an ice-cold surface while the tissue was still frozen, and stored at -20 °C before further treatment within 24 h.

Each sample of fish and shrimp gill was thawed in ice and then homogenized in 8% PCA (approximately 0.25 g tissue in 1 mL PCA). The crude homogenate was centrifuged at 13,000 xg for 20 min at 5 °C. The resulted supernatant was assayed for lactate as described above.

Oxygen content of hemolymph

Two aquaria were used; one aquarium was filled with 3.0 L of Harrison's medium and the other with 3.0 L of a toxic *H. carterae* culture. Both media were aerated continuously and incubated at 16 °C for 2 h, when 30 shrimp (mean weight of 1.0 g each) were placed in each aquarium. Using a 1-mL syringe with 27 gauge needle, hemolymph of 10 shrimp from each aquarium were sampled and pooled at 6, 18 and 24 h after the experiment was started. Each hemolymph sample was treated further to determine its oxygen content. To provide the value for time zero, immediately after the test was commenced, hemolymph of 10 shrimp in the holding tank were taken, pooled and their oxygen contents were measured.

An Oxycon® Blood Oxygen Content Analyzer (Cameron Instrument Co., Port Aransas, Texas) was used to measure the oxygen content of hemolypmph. The results were expressed in % vol (mL O_2/100 mL hemolymph).

Molting and healing process of damaged lamellae

This experiment used sixteen 250-mL flasks, twelve of which contained 150 mL of aerated toxic *H. carterae* culture, two with aerated Harrison's medium and two with aerated seawater. One shrimp was then placed in each flask. Molting of shrimp in each flask was observed for 24 h.

After 24 h, the gills of the shrimp from 2 flasks containing toxic *H. carterae* culture were incised for histological examination. At the same time, algal culture and Harrison's medium in the remaining flasks were removed and replaced with 150 mL of aerated seawater. All shrimp were fed daily and their molting cycles were observed for 6 weeks.

The gills sampled from shrimp in flasks previously contained *H. carterae* were taken for histological examination at: 24 h, 3 d, 1 w, 3 w, and 6 w after their re-introduction to seawater. These samples were examined histologically to observe the presence and development of inflammation i.e. hemocytes swarming and/or melanized nodules, and of intralamellar septae.

Statistical Analyses.

Results were presented as means $\pm$ standard error measurements (SEMs). Data were subjected to evaluation by One-Way Analysis of Variance (ANOVA) followed by Tukey's pairwise comparison. Differences with $P<0.05$ were regarded as statistically significant. All analysis were executed by a computerized statistical program, Minitab Release 10.2 for PC Windows.

RESULTS

Concentration of *H. carterae* cells

The cell concentrations of the *H. carterae* cultures used in this study ranged from 6.5 - 8.5 x 10^4 cells mL^{-1}.

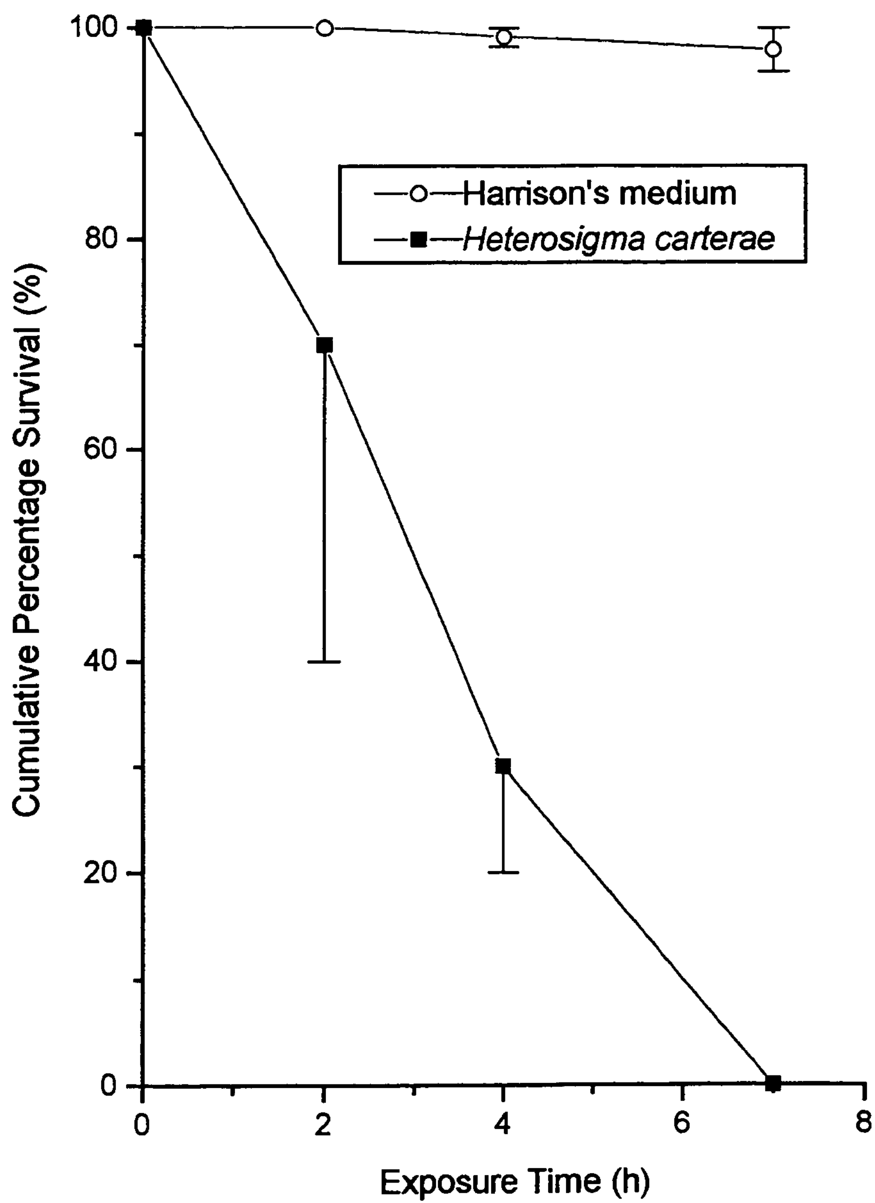
Toxicology

When exposed to a toxic culture of *H. carterae* both the rainbow trout and coon-stripe shrimp initially reacted by jumping and/or swimming rapidly. However, after approximately 15 to 20 min, the shrimp usually became calm and evenly distributed themselves on the bottom of the aquaria. Unlike shrimp, some fish displayed loss of balance when initially exposed to the toxic *H. carterae* culture. With increased exposure time to the toxic *H. carterae* culture, some fish became sluggish. These sluggish fish swam very slowly in an upside-down position along the bottoms or sides of the aquaria. Moribund fish rested on their sides on the aquaria bottoms.

The mean survival time of the rainbow trout (2 toxicity trials) is presented in Fig. 2. There were marked differences in the survival of the trout and shrimp exposed to *H. carterae*. Approximately 30% of the fish died 2 h after initiation of exposure to a toxic *H. carterae* culture. Within 7 h, 100% and approximately 3% of the fish had died in the toxic *H. carterae* culture and Harrison's medium respectively. No shrimp died in the toxic *H. carterae* culture and Harrison's medium within 24 h. Indeed, during the same period, 3 and 4 shrimp molted in *H. carterae* and Harrison's medium, respectively.

The exoskeleton and hemolymph of the shrimp exposed to the toxic *H. carterae* appeared darker (yellowish brown) than those in Harrison's medium (colorless).

Figure 2. Cumulative percentage survival of seawater-acclimated rainbow trout in Harrison's medium and *H. carterae* culture. Values are the means of 2 trials. Bars = SEMs.


100
80
60
40
20
0
Cumulative Percentage Survival (%)
0
2
4
6
8
Exposure Time (h)
Harrison's medium
Heterosigma carterae

Histological observations

The control and treated gill histology of trout are shown in Fig. 3. Gills of rainbow trout exposed to *H. carterae* exhibited extensive hypertrophy of epithelial cells, and epithelial lifting and edema of the lamellae (Figs.3B and 3C). No fusion of lamellae and filaments were noticed in the trout gills exposed to the algae.

Normal histology of a shrimp whole-gill and lamellae is shown in Figs. 4A and 5A, respectively. No pathological changes were observed in the gills of shrimp exposed to Harrison's medium. Shrimp exposed to the toxic *H. carterae* culture for 4 h displayed gill injury, including hypertrophy of the lamellae (Fig. 4B) and rupture of the intralamellar septae (i.e. walls dividing the pillar cells processes) and lamellar membrane (Fig.5B). Fusion of the lamellae and atrophy of the filament were detected in the shrimp exposed to the toxic algae for 24 h (Fig. 4C), while swarming of hemocytes was apparent on the gill central axis and injured lamellae.

Lactate in trout gills

After exposure to Harrison's medium and *H. carterae* for 5 h, lactate concentrations in the fish gills greatly increased (Fig. 6) and were significantly different from the concentration at time zero. Moreover, after 5 h of exposure to the toxic *H. carterae* culture, the mean lactate content in the gills of trout in the algal culture was higher, but not significantly so, than that in the gills of trout in Harrison's medium.

Activities of catalase and peroxidase enzymes in trout gills

The activities of catalase and peroxidase in the gills of the trout exposed to Harrison's medium and the toxic *H. carterae* culture are shown in Table 2.

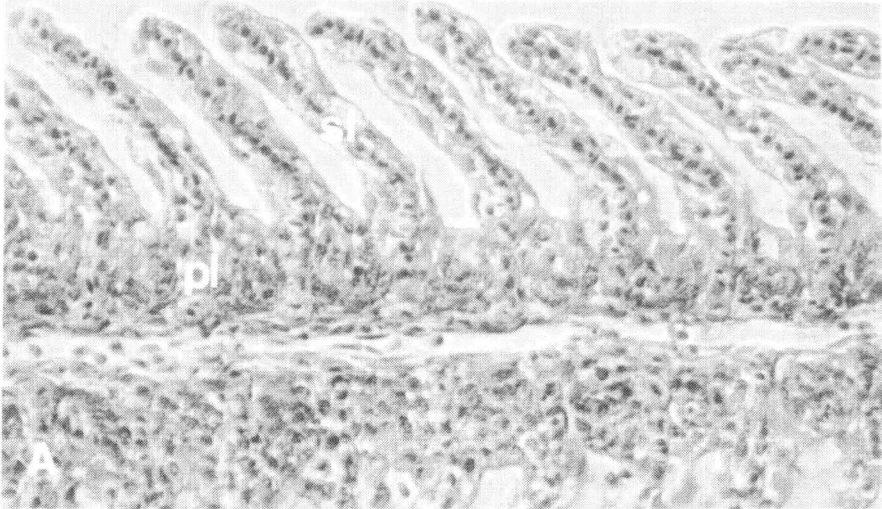
Catalase activities of the gills of fish in Harrison's medium did not significantly decrease with time, while those of the gills of the fish in the toxic *H. carterae* culture did show a significant decrease after 1 h of exposure.

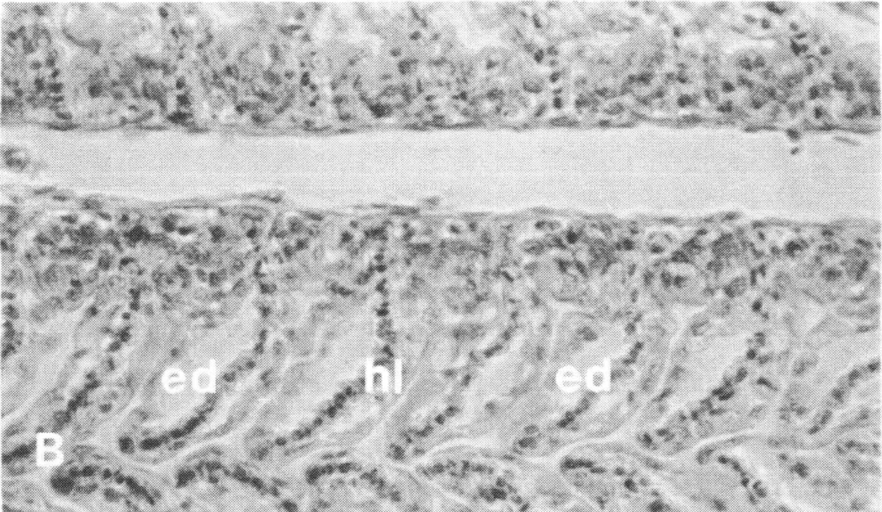
Figure 3. Gill lamellae of rainbow trout:

A) Acclimated in seawater for 48 h, not showing any recognizable changes. **pl** = primary lamellae; **sl** = secondary lamellae. H&E staining. 466X.

B) Exposed to toxic *H. carterae* for 4 h, showing hypertrophy of epithelial cells **(hl)** and edema **(ed)**. H&E staining. 466X.

C) Exposed to toxic *H. carterae* for 12 h, showing edematous and hypertrophied lamellae, and lifting of the epithelial membrane. H&E staining. 466X.


sl
pl
A


ed
hl
ed
B

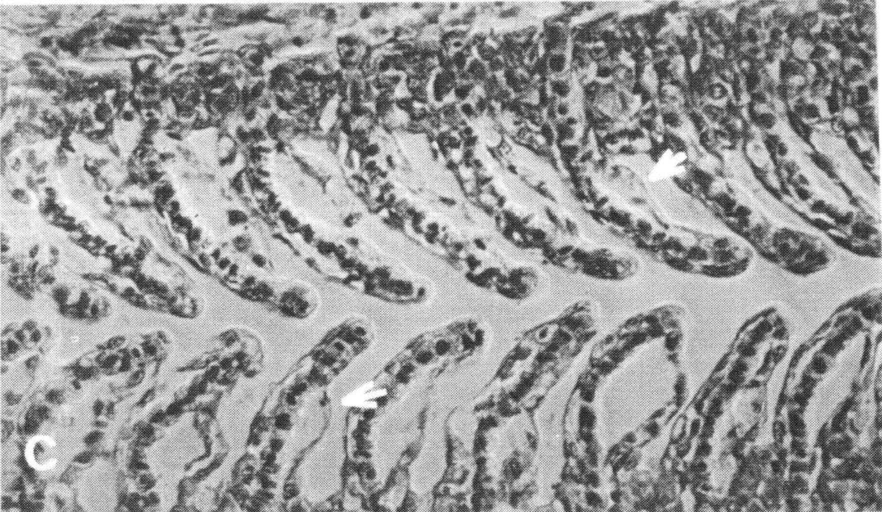
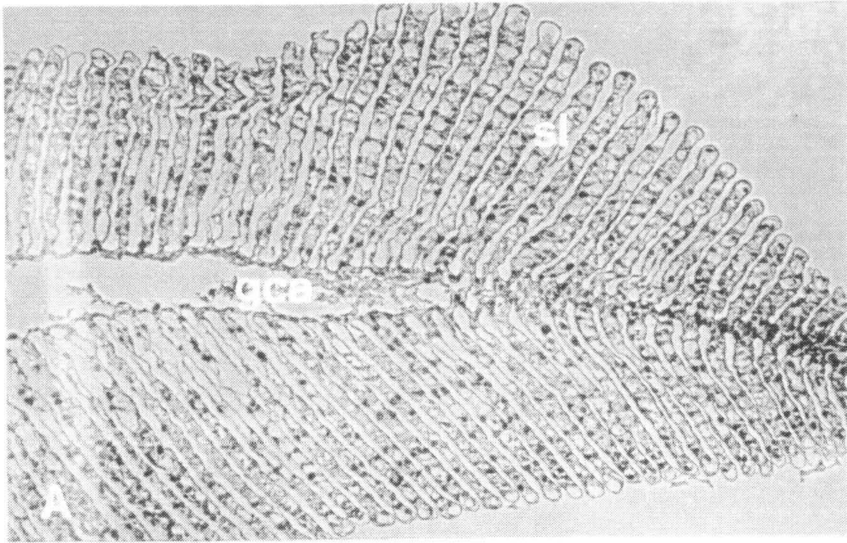

C

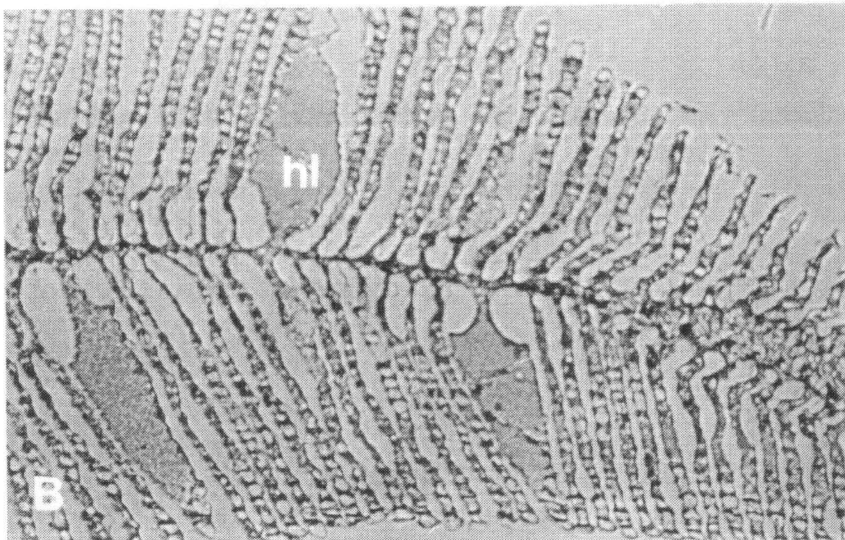
Figure 4. Whole gills of *P. danae*:

A) Normal gill. ***gca*** = gill central axis; ***sl*** = secondary lamellum (gill lamellum). H&E staining. 365X.

B) Exposed to toxic *H. carterae* for 4 h showing hypertrophy and necrosis of the lamellae (***hl***). H&E staining. 365X.

C) Exposed to toxic *H. carterae* for 24 h showing hypertrophy, necrosis and fusion of the lamellae (**lf**), rupture of the lamellar membrane and edematous filament, and hemocytic swarming (***hs***). H&E staining. 1460X.


sl
gca
A


hl
B

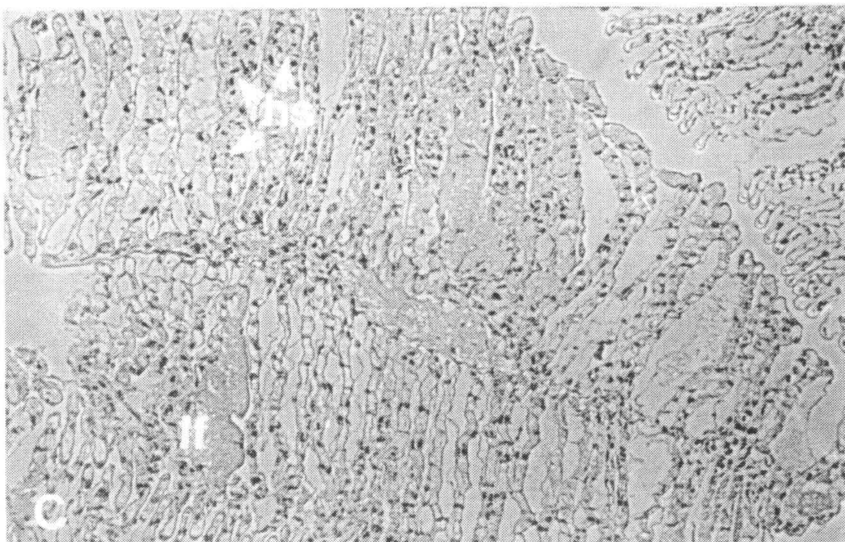
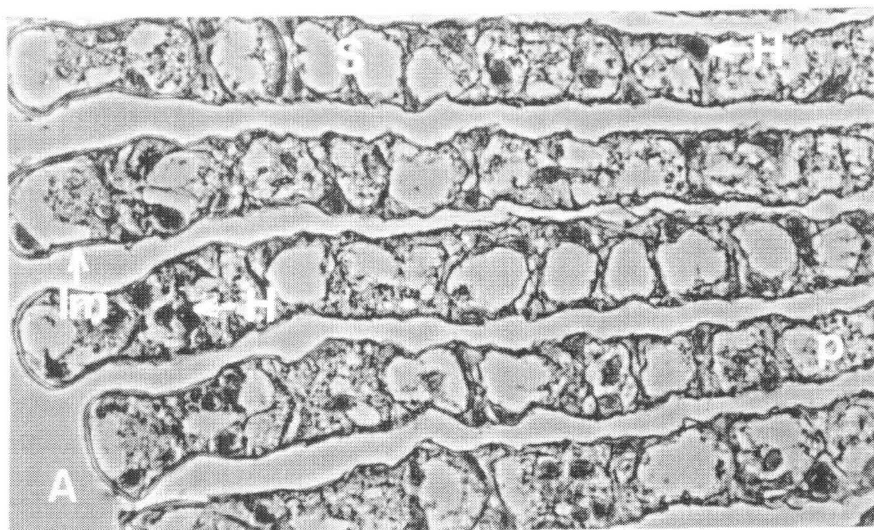

hs
lf
C

Figure 5. Gill lamellae of *P. danae*:

A) Normal gill lamellae (1460X). H&E staining. **H** = hemocyte; ***lm*** = lamellar membrane (cuticular wall); ***p*** = pillar cell processes; **S** = intralamellar septae.

B) Exposed to toxic *H. carterae* for 4 h showing hypertrophy of lamellae and rupture of lamellar membrane (***rlm***). H&E staining. 1460X. **H** = hemocyte.


S
H
lm
H
p
A

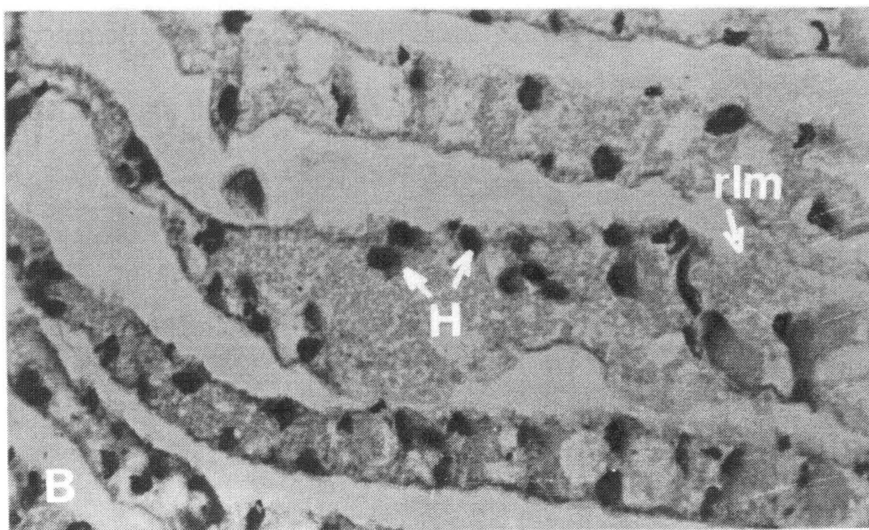
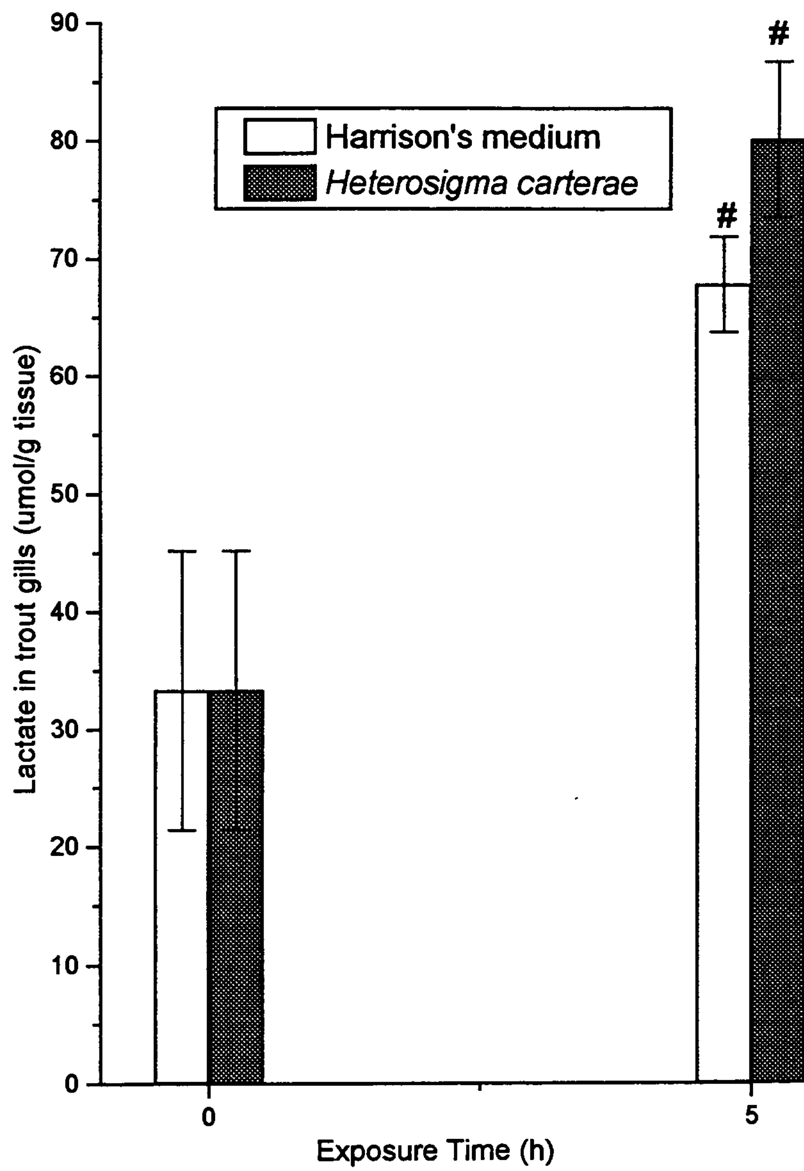

rlm
H
B

Figure 6. Lactate concentrations in the gills of rainbow trout exposed to Harrison's medium and *H. carterae*. Values are the means of 4-6 determinations. Bars = SEMs. The bars with common symbols are not significantly different at $P<0.05$ (Tukey's), but significantly different from the initial value.



90
80
70
60
50
40
30
20
10
0
Lactate in trout gills (umol/g tissue)
Harrison's medium
Heterosigma carterae
#
#
0
5
Exposure Time (h)

Table 2. Activities[a)] of catalase[b)] and peroxidase[c)] in the gills of seawater-acclimated rainbow trout exposed to Harrison's medium as control and toxic *H. carterae*.

Enzyme	Time (h)	Treatment	
		Harrison's medium	*Heterosigma carterae*
Catalase	0	976.0(60.3)	976.0(60.3)
	1	848.0(80.3)	488.7(33.3)[1)]
	4	763.3(59.2)	323.2(16.1)[1)]
Peroxidase	0	15,071(1910)	15,071(1910)
	2	11,209(4587)	11,515(3284)
	4	9,759(976)	11,206(1936)

Notes:

a) Catalase activity in µmol H_2O_2 oxidized/min/g tissue, while peroxidase activity in umol $ABTS^+$ formed/min/g tissue. b) Each value represents the mean $\pm$ SEM for N of 4-10 c) Each value represents the mean $\pm$ SEM for N of 6-12. For each enzyme, values with common superscripts are not significantly different at $P<0.05$ (Tukey's test). [1)] is significantly different to the value at time zero.

Peroxidase activities of the gills of fish in Harrison's medium and the toxic *H. carterae* culture were not significantly affected during 4 h of exposure to either medium.

Oxygen concentration in hemolymph

As shown in Fig. 7, oxygen concentrations of shrimp exposed to either Harrison's medium or toxic *H. carterae* cells decreased with time. Except for the value at 6 h for shrimp in Harrison's medium, all values are significantly lower than the initial concentration at time zero.

There is a significant difference between oxygen concentrations in hemolymph of shrimp exposed to toxic *H. carterae* culture for 18 and 24 h. Oxygen concentration in hemolymph of shrimp exposed to Harrison's medium for 6 h was significantly higher than that of shrimp exposed to the medium for 24 h. No significant differences between oxygen concentrations in hemolymph of shrimp exposed to either medium occurred at time 24.

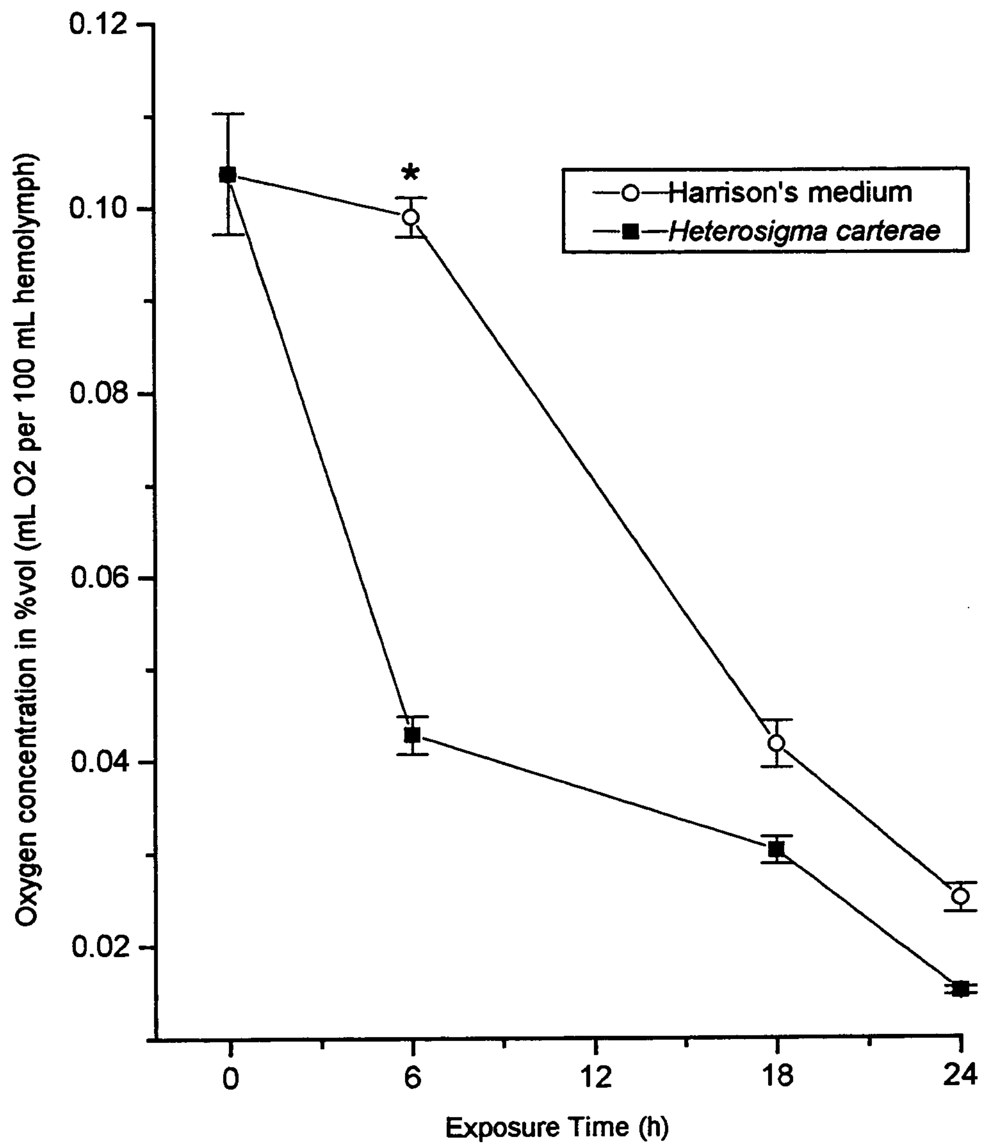
Lactate in shrimp gills and hemolymph

Lactate concentration of gills and hemolymph of shrimp treated with Harrison's medium or *H. carterae* are presented in Figs. 8 and 9, respectively.

The lactate concentrations in the gills of shrimp immersed in either medium decreased significantly approximately 12 h after the test was started (Fig. 8).

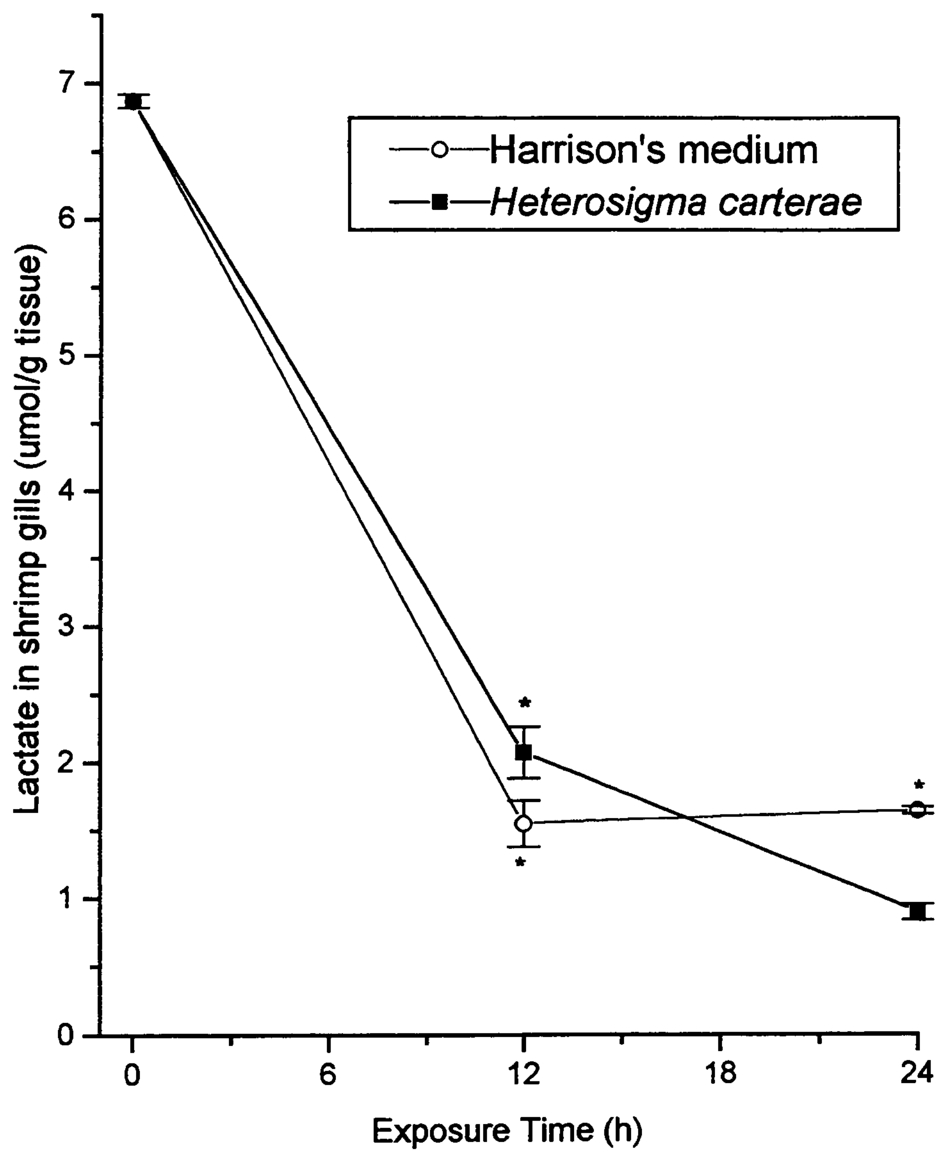
Lactate concentration in the hemolymph of shrimp exposed to Harrison's medium during the experiment did not significantly different from the initial value, even though the lactate concentration slightly increased at 12-h exposure time (Fig. 9). The lactate content in the hemolymph of shrimp exposed to *H. carterae* for 12 h was approximately 5 times higher than that at time zero, and significantly different from other values.

Figure 7. Oxygen concentrations in the hemolymph of *P. danae* exposed to Harrison's medium and a toxic *H. carterae* culture. Values represent the means of 4-5 determinations. Bars = SEMs. The bar with an asterisk is not significantly different at $P<0.05$ (Tukey's test) from the initial value.



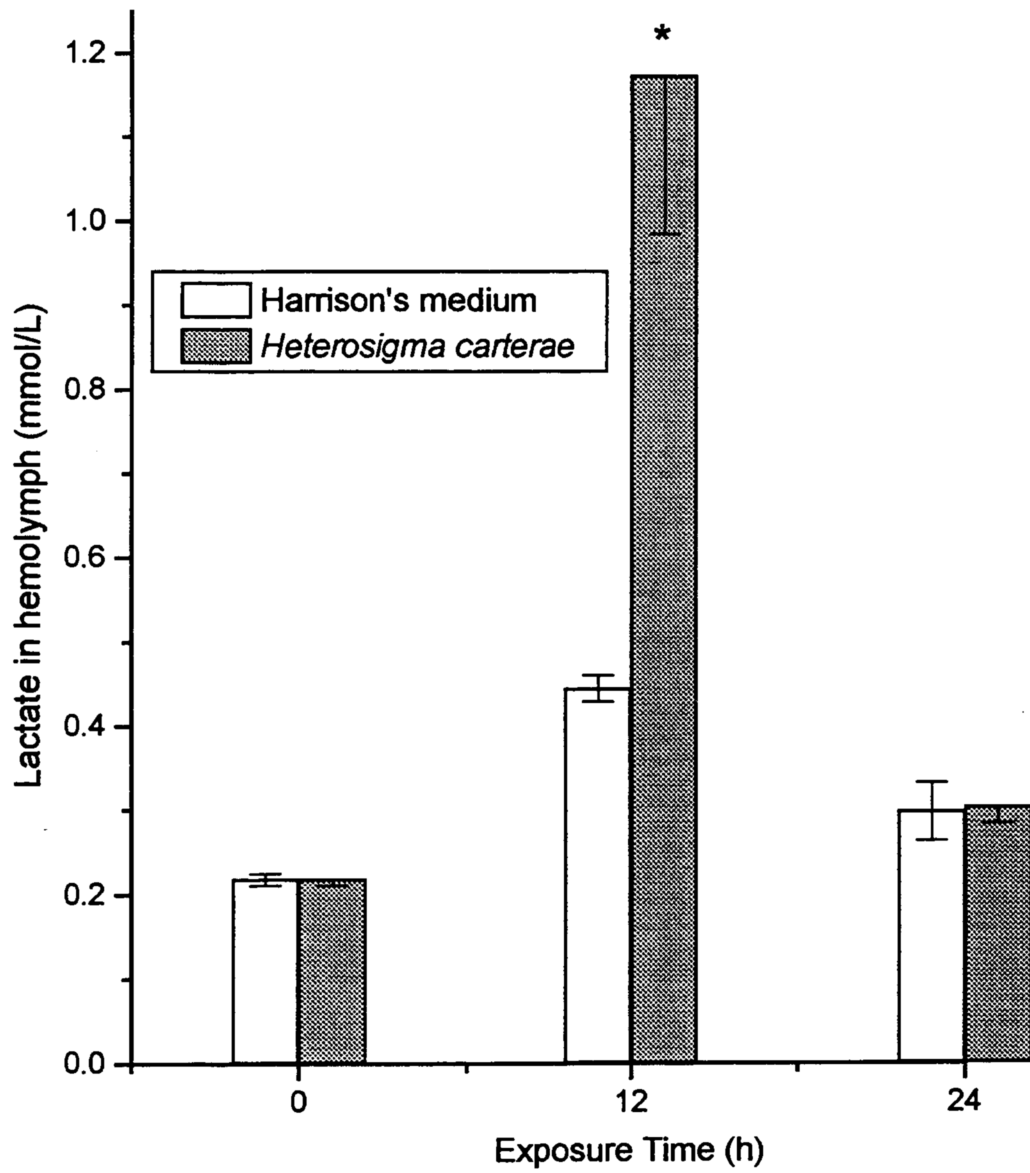
0.12
0.10
0.08
0.06
0.04
0.02
Oxygen concentration in %vol (mL O2 per 100 mL hemolymph)
*
Harrison's medium
Heterosigma carterae
0
6
12
18
24
Exposure Time (h)

Figure 8. Lactate concentrations in the gills of *P. danae* exposed to Harrison's medium and a toxic *H. carterae* culture. Values represent the means of 3-4 determinations. Bars = SEMs. The bars with asterisks are not significantly different at $P<0.05$ (Tukey's test).



Harrison's medium
Heterosigma carterae
Lactate in shrimp gills (umol/g tissue)
Exposure Time (h)
0
1
2
3
4
5
6
7
0
6
12
18
24

Figure 9. Lactate concentrations in the hemolymph of *P. danae* exposed to Harrison's medium and a toxic *H. carterae* culture. Values are the means of 3-4 determinations. Bars = SEMs. The bar with an asterisk is significantly different at $P<0.05$ (Tukey's test).


Lactate in hemolymph (mmol/L)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
Harrison's medium
Heterosigma carterae
0
12
24
Exposure Time (h)

Catalase activity in shrimp gills

Changes of catalase activities in the gills of shrimp exposed to Harrison' medium and *H. carterae* are shown in Table 3.

The means of catalase activities in the gills of shrimp exposed to Harrison's medium decreased significantly by 4-h time, and remained low thereafter.

The activity of catalase in the gills of shrimp immersed in *H. carterae* remained approximately the same for the first 12 h of exposure (Table 3). However, after 18 h of exposure, the activity was significantly decreased.

Overall, there were no differences between the means of catalase activities in shrimp gills exposed to Harrison's medium at and after 6-h exposure time with those in shrimp gills exposed to *H. carterae* at and after 18-h exposure. In each case catalase activity was minimal.

Peroxidase activity in shrimp gills

The activities of peroxidase in the shrimp gills are shown in Fig. 10. The enzyme activities in the gills of shrimp immersed in Harrison's medium changed in a biphasic fashion within 18 h; the values increased significantly up to 12-h exposure time, followed by a decrease at 18-h time. Nevertheless all values remained higher than the activity at time zero.

The gills of shrimp subjected to a toxic *H. carterae* culture showed a significant increase of peroxidase activity at 6-h exposure time, followed by a steady decrease up to 18 h.

Healing process of injured lamellae

No shrimp molted in the flasks during the first 24-h of treatment. Of the 12 shrimp reintroduced to seawater after 24-h exposure to a toxic *H. carterae* culture, one molted approximately 1 week after the reintroduction.

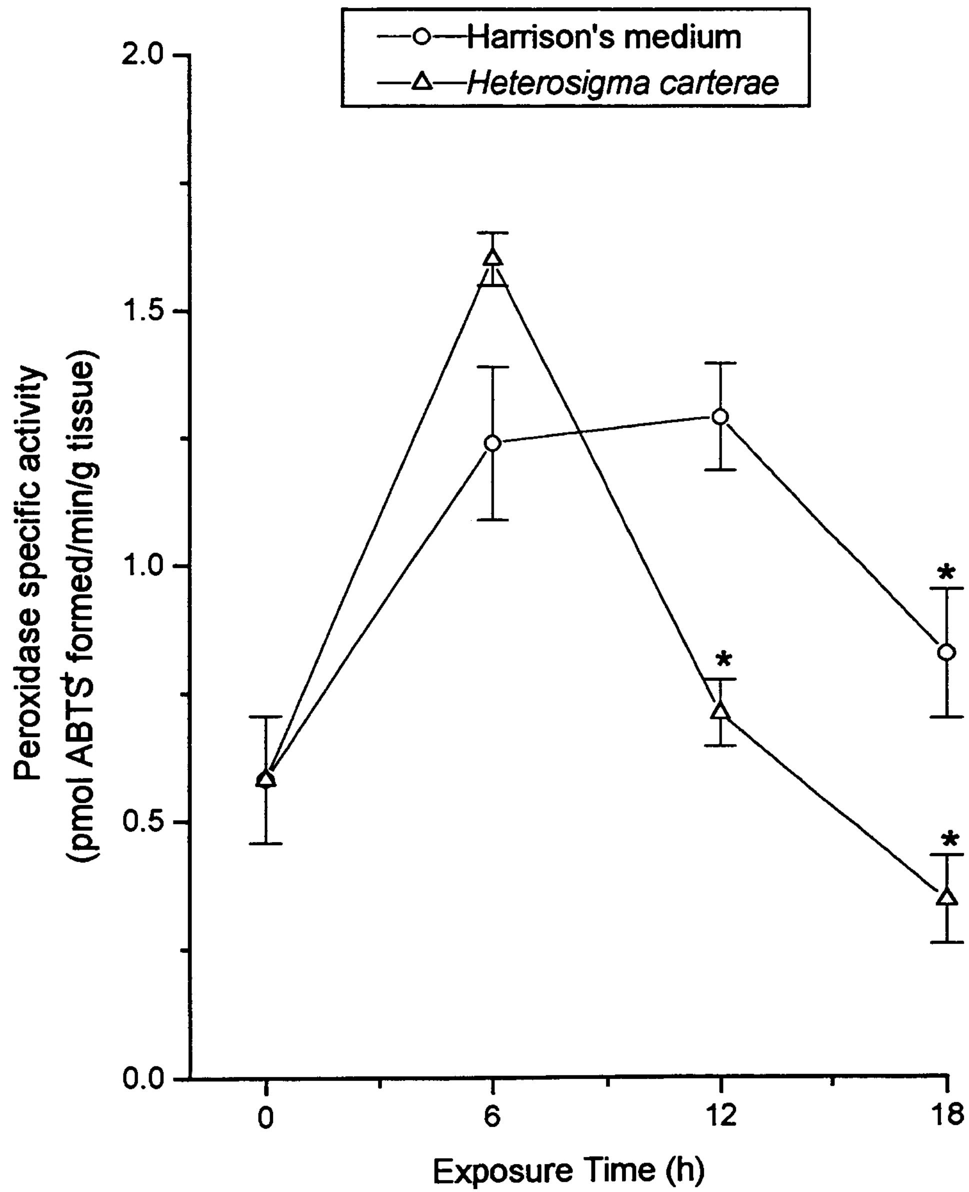
Table 3. Activities[a)] of catalase[b)] in the gills of *P. danae* exposed to Harrison's medium as control and toxic *H. carterae*.

Time (h)	Treatment	
	Harrison's medium	*H. carterae* culture
0	5321.00(1412)[1)]	5321.0(1412)[1)]
4	56.20(7.26)	4551.9(72.1)[1)]
12	119.02(0.98)	5863.0(535)[1)]
18	n.d.	79.3(9)
24	124.98(4.80)	102.1(7.6)

Notes:

a) Catalase activity in μmol H_2O_2 oxidized/min/g tissue. b) Each value represents the mean(±SEM) for N of 6-10. n.d. = not detected. Values with common superscripts are not significantly different at $P<0.05$ (Tukey's test).

Figure 10. Peroxidase activity in the gills of *P. danae* exposed to Harrison's medium and a toxic *H. carterae* culture. Values are the means of 4-5 determinations. Bars = SEMs. The bars with common symbols are not significantly different from the initial value ($P<0.05$; Tukey's test).


Harrison's medium
Heterosigma carterae
Peroxidase specific activity
(pmol ABTS+ formed/min/g tissue)
Exposure Time (h)
2.0
1.5
1.0
0.5
0.0
0
6
12
18
*
*
*

Histological observations showed that the gills of *P danae* were injured upon 24-h exposure to a toxic *H. carterae* culture, while hyperplasia due to swarming of hemocytes on the sites of injured lamellae was also apparent (Fig. 4C).

Approximately 3 d after *P. danae*, previously exposed to toxic *H. carterae* culture for 24 h, were reintroduced to sea water, large numbers of hemocytes were present in the gill central axis and the basal areas of normal lamellae. Some hypertrophied lamellae contained very few hemocytes and had no intralamellar septae (i.e. walls dividing the pillar cells processes). Few hypertrophied lamellae embodied elongated intralamellar septae filled with 1-2 hemocytes.

In shrimp which did not molt during and 1 week after the exposure to *H. carterae*, the hemocytes still packed the gill central axis and were present in larger numbers in the distal than in the basal areas of normal lamellae. Hypertrophied lamellae had very few hemocytes (mostly along the inside of lamellar membrane of the injured lamellae). The shrimp which molted 1 week after re-introduction to seawater had less numbers of hemocytes in the gill central axis, and the hemocytes evenly distributed along the normal lamellae. The hypertrophied lamellae contained hemocytes along the inside of the lamellar membrane, while less than 3 intralamellar septae were present.

After 3 weeks in seawater, the gill central axis and distal areas of normal lamellae were packed with hemocytes. Hypertrophied lamellae contained more hemocytes along the inside of lamellar membrane, in the pillar cells, and more intralamellar septae than those in shrimp reintroduced to sea water for less than 3 weeks.

Approximately 6 weeks after being reintroduced to sea water, hemocytes still packed the gill central axis and along the length of normal lamellae of *P. danae* gills. Hypertrophied lamellae had thick, but discontinued, intralamellar septae, and hemocytes along the inside of lamellar membrane.

No melanized nodules were observed in the injured gills of *P. danae* after 24-h exposure to a toxic *H. carterae* culture and subsequent reintroduction to sea water.

DISCUSSION

A toxic effect can be defined either as acute or chronic (e.g. Aldridge, 1988; EPS, 1990; Sprague, 1990). The acute term is usually used for effects induced in the test organisms within a short period of exposure to, or administration of, a test material, usually ≤ 4 days for fish (Aldridge, 1988; EPS, 1990). The acute effect can be lethal (causing death or coming to an end point quickly) or sublethal (not causing death) (EPS, 1990). The chronic term refers to long-lasting effects during a portion of the full life-cycle of a test organism (Sprague, 1990). In this investigation, acute effects of toxic *H. carterae* cells on the shrimp *P. danae* were studied.

The use of dose-response relationship was not possible, in this investigation, since the concentrations of biotoxins secreted by *H. carterae* depend on external factors (e.g. nutrients) that affect the physiology of this alga (Perkins, 1996). Taroncher *et al.* (1993) observed that influences of nutrients and/or bacteria-phytoplankter consortia may either promote or inhibit toxin biosynthesis by a toxic phytoplankter. For example, an oxyradicals-producing phytoplankter, *Ostreopsis lenticularis,* reportedly increased its specific toxicity during the stationary phase of growth caused by factors such as nutrient limitation under non-axenic culture conditions (Gonzales *et al.*, 1993). In addition, the concentration of the most toxic of the oxyradicals ($.OH^-$) produced by *H. carterae* cannot be measured. Therefore, time to death was used to measure the toxicity of *H. carterae* on the chosen aquatic animals. The test duration under the static test conditions used in this investigation had to be kept relatively short (≤ 24 h) such that the toxicity of *H. carterae* cultures remained relatively constant.

The toxic isolate of *H. carterae* used in this study induced acute, but sublethal, toxic effects on the shrimp *P. danae*; and, acute and lethal effects on juvenile rainbow trout. There are no reports in the literature on toxic effects of this phytoplankter on marine invertebrates, including shrimp. However, acute and lethal effects have been

shown by other individuals using freshwater- or seawater-acclimated salmonids (e.g. by Black *et al.*, 1991; Chang *et al.*, 1990; and Yang *et al.*, 1995).

In the experiments described here, the rainbow trout were partially acclimated in seawater 48 h prior to use in each test. The partial acclimation might enhance the sensitivity of the animal to the algal toxins since the fish might experience salinity-related stress. Bath and Eddy (1979) reported that upon entering seawater, freshwater rainbow trout experience ionic and osmotic imbalances which lead to increases in internal salt levels and dehydration of the body (including shrinkage of the branchial epithelium followed by a drop in arterial oxygen tension). These factors can suppress the seawater survival of freshwater rainbow trout and other salmonids. However, in this study, the salinity effect was not significant since the survival rate of rainbow trout exposed to toxic *H. carterae* cells was negligible relative to that of the fish placed in Harrison's medium which contained seawater salts (Fig. 2). The same conclusion was also reported by Black *et al.* (1991) who found no differences in cumulative mortality between freshwater- and seawater-acclimated sockeye salmon on their exposure to a natural, toxic *H. carterae* bloom at San Mateo Bay in Alberni Canal, Vancouver Island, British Columbia. The mortalities of salmonids observed in this study were therefore predominantly caused by *H. carterae* toxins, rather than by salinity-related stress; and can serve as an indicator of relative toxicity of the algal cultures.

Free radicals such as superoxide and hydroxyl radicals, are highly reactive in biological systems (Fridovic, 1983). Yang *et al.* (1995) reported that under laboratory conditions, approximately 10^4 *H. carterae* cells released superoxide radicals or hydrogen peroxide with an amount equivalent to that released by 10^6 mouse peritoneal macrophage cells, which can damage the plasma membranes of microorganisms and mammalian cells. They also observed an increasing superoxide radical and hydrogen peroxide production by *H. carterae* with an increase of algal concentrations greater than 10^4 cells mL^{-1}. Thus, the authors suggested that the amounts of superoxide radicals (and subsequent formation

of hydroxyl radical) and hydrogen peroxide released by the *H. carterae* culture were sufficient to damage the secondary lamellae of fish gills they studied. I found the shrimp gills were also damaged by the toxic *H. carterae* cultures (Figs. 4B, 4C and 5B).

The types of injury observed in *P. danae* gills exposed to toxic *H. carterae* cells were similar to those observed in the rainbow trout used in this study (Figs. 3B and 3C) as well as in other finfish (Chang *et al.*, 1990; Shimada *et al.*, 1983). Chang *et al.*(1990) noted severe acute exudative and degenerative changes to the gill tissue of moribund chinook salmon in the presence of toxic *H. carterae* cells. Similar results have been reported by Shimada *et al.* (1983) who observed the effects of *C. antiqua* on gills of yellowtail. They found a significant loss of goblet cells on the afferent ridges of the respiratory epithelium occurred 1 h after exposure of the fish to the toxic algal culture. And, mucous cells of the gill primary lamellae were also markedly decreased in number. The cell layer on both ridges exposed to the alga appeared to be thinner than that of control. The cell bodies of the internal multilayered mass shrank and intercellular spaces markedly expanded. Therefore, the authors suggested that the edematous gill lamellae might be caused by the disappearance of the mucous coat, leading to locally impaired osmoregulation. As a result, gas exchange on the gill lamellae would be disturbed. Chang *et al.*(1990) similarly proposed that local impairment of gill osmoregulation might induce death of salmonids exposed to *H. carterae*. However, *P. danae* which suffered similar gill injuries did not die, even though a significant drop of oxygen concentration occurred in the hemolymph of shrimp exposed to *H. carterae* during the experiment (Fig. 7). This indicates that blood hypoxia may have occurred because of the disruption of the anaerobic respiratory system.

Under hypo- and anoxic conditions, crustaceans may survive by employing metabolic adaptation i.e. utilization of anaerobic metabolism (Lockwood, 1967; Low *et al.*, 1993; Anderson *et al.*, 1994; Henry *et al.*, 1994). This anaerobic energy production is marked by an accumulation of tissue lactate, as the primary metabolic acid produced in

both muscle tissue and hemolymph of decapod crustaceans (McDonald *et al*., 1979). An investigation by Henry *et al.* (1994) on the fate of the lactate in three species of aquatic and terrestrial decapod crustaceans suggested that it is metabolized to glucose, amino acids and CO_2. However, the metabolic potential appears to be geared towards the metabolism to amino acids rather than carbohydrates. The authors argued that it is possible that gluconeogenesis is important in maintaining a constant circulatory supply of glucose to the central nervous system (CNS) in higher vertebrates after exercise (a situation resembling hypoxia in muscle tissue). However, in crustaceans, which lack a well-developed CNS, there was no selective advantage in the efficient recycling of lactate to circulating glucose. Hervant *et al.* (1995) stated that under hypoxic conditions crustaceans normally excrete lactate. However, the authors noted that the hypogean amphipod, *Niphargus rhenorhodanensis,* under hypoxia was able to remetabolize lactate, and removed lactate quickly when recovering from hypoxia.

The causes of hypoxia in the shrimp's hemolymph after 6-h (Fig. 7), and lactic acidosis in the gills of the fish and the shrimp (Fig. 6), upon exposure to Harrison's medium are unknown. In each case, Harrison's medium induced these metabolic responses by both animals without structural injuries to the animal gills. I suggest that the presence of trace metals in Harrison's medium might contribute to hypoxia and subsequent lactate accumulation in the gills of both animals (see below).

Harrison's medium contains trace metals that are essential for algal growth, such as Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, and Co^{2+} (Harrison *et al*., 1980). These trace metals are mostly toxic when present in the divalent cationic states in aqueous environments (Pagenkopft, 1986). However, their toxicities can be diminished or neutralized when they form complexes with ligands (Eichenberger, 1986), such as mucus which covers the surface of fish gills (Van Oosten, 1957). The metal-mucus complexes reduce diffusion rates of the metals across the epithelium cells (Eichenberger, 1986). Hence mucus may function as a chemical barrier against metal toxicity. However, high accumulation of

mucus-metal complexes in a gill surface may also pose as a physical barrier that is less permeable to oxygen. This condition, which is commonly found in fish exposed to polluted waters containing metal salts (Van Oosten, 1957), leads to hypoxia in fish.

A sharp decline of lactate concentrations in the shrimp gills during, and in the hemolymph after, approximately 12-h exposure to the toxic *H. carterae* culture suggests the existence of a time-dependent utilization of lactate under hypoxia. Presumably, lactate in the hemolymph is used after lactate in a tissue is depleted.

The activation of anaerobic metabolism in the gills of rainbow trout exposed to either a toxic *H. carterae* culture or placed in Harrison's medium (Fig. 6) was likely the results of hypoxia (Van den Thillart *et al.*, 1994; Wood *et al.*, 1983). In freshwater fish, high intracellular lactate loading causes water along with Cl^- and K^+ to move into cells leading to reduced blood volume (hemoconcentration) (Graham *et al.*, 1982). The reverse response of fish in a seawater environment (hypertonic medium), as used in the recent study, should be anticipated. However lactic acidosis may not be the ultimate cause of death of fish under stress conditions (Wood *et al.*, 1983). This is shown by the survival of rainbow trout in Harrison's medium under lactic acidosis. Thus, even though lactic acidosis might be a contributing factor, the mortalities of the fish in the *H. carterae* culture were more likely due to severe disruption of osmotic and ionic balance across the gills as a result of gill destruction by the toxins of *H. carterae* (Figs. 3B and 3C). These cause edema and lethal inhibition of oxygen uptake by the damaged lamellae (Chang *et al.*, 1990; Shimada *et al.*, 1983).

Under hypoxia, rainbow trout increase the ventilatory volume of their gills (Smith and Jones, 1982), and tend to lower their metabolic rate below the standard metabolic rate (Hochachka and Guppy, 1987). The former increases the metabolic energy requirement, while the latter decreases it. If the former exceeds the latter, hypoxia can therefore produce an energy debt in the gills. Fish compensate for the energy debt by the means of anaerobic metabolism indicated by lactate accumulation in the tissues and blood (Van den

Thillart *et al.*, 1994). In this respect, the accumulation of lactate reflects the energy debt in the fish (Wedemeyer *et al.*, 1990). The energy debt in rainbow trout exposed to Harrison's medium, in this study, may also be partially caused by energy needed to neutralize the toxic effects of trace metals on the fish.

Although the data of Figs. 7, 8 and 9 show that Harrison's medium and *H. carterae* cells (in spent Harrison's medium) are both toxic, the latter is clearly more toxic. This is likely due to the production of oxyradicals by the toxic *H. carterae* cells. It is likely that when the gills of *P. danae* were injured in this fashion, the shrimp activated its anaerobic metabolism by means of glycolysis (i.e. formation of lactate from glucose) and subsequently used the lactate in the hemolymph and gills as a source of energy. This metabolic mechanism enhances the survival of planktonic, benthic, freshwater and marine crustaceans in hypoxic environments (Wolvekamp and Waterman, 1960).

Beside disruption of gill structure, the toxins secreted by *H. carterae,* also affected the activities of the anti-oxidant enzymes of catalase and peroxidase (see below).

The catalase activity in the gills of *P. danae* exposed to toxic *H. carterae* cells decreased after approximately 12-h exposure to the toxic algal culture (Table 3). Thus, initially catalase in the shrimp gills retained activity and perhaps helped to protect the gills against oxyradicals produced by *H. carterae*. However, oxyradicals can oxidize subcellular macromolecules such as enzymes and/or enzyme-containing organelles e.g. peroxisomes, leading to loss of enzymatic activities (Fridovic, 1983). Therefore, the reduced catalase activity in the shrimp gills after 12-h exposure to toxic *H. carterae* cells might be caused by the degradation of the peroxisomes and/or degradation of catalase by oxyradicals. Another possible reason for this is inhibition of the catalase activity by quinones which are products of melanogenesis in melanocytes (see below).

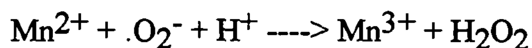
Crustaceans, when exposed to waters containing oxyradicals such as singlet oxygen and hydrogen peroxide, can utilize their pigments, e.g. melanins and carotenoids, as protective agents (Ghidalia, 1985). Melanin can detoxify oxidizing agents, such as

hydrogen peroxide, by acting as electron acceptors or donors (Ghidalia, 1985). Moreover, melanin production in crustacea involves the production of quinones, e.g. dopaquinone, eucodopa-quinone, and 5,6-indolequinone (Ghidalia, 1985). According to Hoffmann-Ostenhof (1963), quinones inhibit catalase activity by attacking the sulfhydryl groups of the enzyme, as the catalytic functional groups of oxidative enzymes. I observed that the shrimp had orange to brown hemolymph following exposure to *H. carterae*, while those in Harrison's medium remained colorless. If the coloration of the former was due to melanins, then the quinones-affected inhibition of catalase activity might occur.

As shown in Table 2, the catalase activity in the gills of fish exposed to Harrison's medium did not decline significantly with time compared to that in fish exposed to *H. carterae*, even though fish in both media experienced similar gill acidosis. Halliwell and Gutteridge (1985) stated that the activity of catalase can be influenced by the pH of a solution, i.e. catalase can dissociate easily into its subunits on exposure to acid or alkali. However, the reduced catalase activities of the fish gills in my study were not caused by lactic acidosis in the gill tissues. Perhaps these decreases may be related to the presence of trace metals in Harrison's medium, and their effects on the production of H_2O_2 *in vivo* (see below).

As shown in Table 3, catalase activities in the gills of shrimp exposed to Harrison's medium decreased significantly after 4-h exposure. It is believed that many trace divalent metallic cations play important roles in a redox reaction resulting in a radical production *in vivo* (Pryor, 1976). Of particular interest is that an iron salt, in the presence of $.O_2$, can readily produce oxyradicals as well as H_2O_2 through the Haber-Weiss cycle or auto-oxidation of cell tissue of subcellular organelles (Halliwell and Gutteridge, 1985). This mechanism of radical production has been frequently observed using isolated mitochondria *in vitro*; the rates of H_2O_2 production primarily depend on the oxygen concentration and mitochondrial metabolic state (e.g. reviews by Forman & Boveris, 1982; Halliwell & Gutteridge, 1985; Pryor, 1976). For example, in pigeon and rat heart mitochondria, the

production of H_2O_2 can equal approximately 1-2% of the oxygen takeup (Forman and Boveris, 1982). The formation of H_2O_2, mainly by the generation of $.O_2^-$ as the precursor of H_2O_2 (Forman & Boveris, 1982; Halliwell & Gutteridge, 1985), can arise from the auto-oxidation of ubiquinone or enzyme Q (a protein in the cell membrane of oxidative organism) as the primary source of $.O_2^-$ (Forman & Boveris, 1982). The H_2O_2 and $.O_2^-$ could be eventually involved in the Fenton reaction upon coming into contact with iron, unless inhibitory mechanisms, such as enzymatic termination by SOD, catalase and peroxidase, are present. Beside iron, Mn^{2+} can also elicit H_2O_2 production (Halliwell & Gutteridge, 1985), via the following reactions:



$$Mn^{2+} + .O_2^- + H^+ \text{---->} Mn^{3+} + H_2O_2$$

Harrison's medium contains both iron and manganese with concentrations of 564 $mg.L^{-1}$ Fe^{2+}, 38 $mg.L^{-1}$ Fe^{3+} and 134 $mg.L^{-1}$ Mn^{2+} (Harrison *et al.*, 1980). If certain metallic cations do indeed exacerbate the toxicity of *H. carterae*, then blooms of *H. carterae* that occur along with high concentrations of trace metals, such as Fe^{2+} and Mn^{2+}, in seawaters may enhance the toxicity of the algal blooms to shrimp or fish.

Peroxidase activity in the gills of shrimp exposed to the toxic *H. carterae* culture initially increased before decreasing to values not significantly different from the pre-exposure value (Fig. 10), while that in fish gills did not decrease significantly with time (Table 2). Although the underlying mechanisms are not known, these data indicate that peroxidase remained active during the exposure of both the shrimp *P. danae* and rainbow trout to toxic *H. carterae* cultures. However the activities of this enzyme were not sufficient to protect the shrimp and the fish gills against the toxins of this phytoplankter since tissue destruction did occur (Figs. 3, 4 and 5).

Algal cells utilize trace metals as micronutrients for their growth (Darley, 1982). Thus, the concentrations of trace metals in *H. carterae* cultures might have been reduced by the algal growth prior to use for each bioassay. If this is so, then trace metals might possess little or even no effects on the activities of catalase and peroxidase in the gills of

shrimp or fish exposed to a toxic *H. carterae* culture. However, if metalic cations in Harrison's medium do indeed exacerbate the toxicity of *H. carterae*, then the presence of excessive amount of trace metals (e.g. Fe^{2+} and Mn^{2+}) in natural seawater where a toxic *H. carterae* bloom occurs, would enhance the toxicity of the algal cells to marine animals.

The inflammatory response is a major system for repair of cellular and tissue injuries in crustaceans (Metchnikoff cited by Sparks, 1985; Sindermann, 1990). Metchnikoff concluded that inflammation occurs by accumulation of masses of hemocytes beneath surface wounds of *Daphnia* and other primitive crustaceans, until the wound is completely healed. In penaeid shrimps, injured areas are infiltrated by a dense network of fibrocytes followed by formation of melanin from hemocytes (Fontaine and Lightner cited by Sindermann, 1990). In Decapods, congestion of gill lamellae by melanized nodules occurs when the gill tissue is necrotized by hazardous agents (Sparks, 1985).

I found that inflammation occurred on the injured lamellae at least 24 h after *P. danae* was initially exposed to a toxic *H. carterae* culture (Fig. 4C). The inflammation, shown by swarming of hemocytes, was similar to the observations of Metchnikoff (cited by Sparks, 1985) on *Daphnia* and other primitive crustaceans. However, I observed no melanized nodules in the injured lamellae.

The gill healing process seemed to intensify after the shrimp were re-immersed in sea water for more than one week. The presence of hemocytes along the lamellar membrane of injured lamellae after the shrimp were re-introduced to seawater indicates that hemocyte swarming was initiated in the lamellar membrane before moving to the intralamellar septae.

Six weeks after the re-introduction of the injured shrimp to seawater, the injured lamellae remained hypertrophied. This indicates: 1) the healing process may need more than 6 weeks after a cessation of a natural bloom of toxic *H. carterae* cells, or 2) the injured lamellae cannot heal. That the healing processes can be delayed, or cannot occur, may be due to damage to DNA, which is responsible for wound repair, by the oxyradicals

(Ahern, 1991). Thus, although the toxic *H. carterae* do not cause lethal effects on *P. danae*, the alga caused gill damage that lasted in excess of six weeks.

During their life cycle, crustaceans molt several times as they grow (Skinner, 1985). During a molt, several events take place, including the degradation of old exoskeleton (ecdysis), synthesis of several layers of a new exoskeleton, atrophy of somatic muscle in the chelae that is replaced following ecdysis. Molting process can therefore affect the healing process in shrimp gills through sloughing of the damaged lamellar membrane at ecdysis (Fontaine and Lightner cited by Sindermann, 1990; Sparks, 1985).

My results showed that the gills of shrimp which molted one week after being re-introduced to sea water had less swarming hemocytes, than those in shrimp which did not molt. This may be caused by the elimination of the hemocytes beneath the cuticular walls of the lamellae at ecdysis (Sparks, 1985). In addition, in both cases the injured lamellae remained hypertrophied. However, hemocytes were distributed evenly along the inside of lamellar membrane in the injured lamellae of the molting shrimp. This may indicate that the molting process might aid the distribution of hemocytes to injured lamellae to fasten their repair.

Overall, this study showed that the shrimp *P. danae* survived, while rainbow trout died, when exposed to toxic *H. carterae* cultures. Although the gills of both the shrimp and the rainbow trout were severely damaged by the algal toxins, the shrimp utilized anaerobic metabolism to remain viable whereas the fish could not do so. The roles of anti-oxidant enzymes, pigments and wound-healing ability in the survival of *P. danae* upon exposure to toxic *H. carterae* cells were inconclusive. Moreover, Harrison's medium appeared to be toxic to both shrimp and fish. However, *H. carterae* cells appeared to be more toxic than Harrison's medium as the alga caused histopathological changes in the shrimp and fish gills.

As reported by Black *et al.* (1991), Chang *et al.* (1990) and Gaines and Taylor (1986), blooms of toxic *H. carterae* in coastal seawaters caused mass mortalities to caged

finfish, e.g. salmonids, yellowtail and flatfish. These caged fish cannot swim away from the bloom waters whereas wild fish can do so. Therefore, the toxic *H. carterae* cells can damage the fish gills, and in some cases kill the fish. To a certain extent, the spatial migration of shrimp, *P. danae,* may be as limited as that of the caged finfish where this shrimp inhabits bays, estuaries and tidepools along the continental shelf of North America (Holthuis, 1980; Neilson, 1981). Under bloom conditions, *H. carterae* cells can be found to depths of 30 m (Taylor *et al.*, 1994), while the shrimp usually occurs in waters starting from 18 m in depth (Holthuis, 1980; Meinkoth, 1981). Thus, the shrimp may not be able to avoid toxic blooms of *H. carterae* at many locations.

The *H. carterae* cultures used in my study, which had a concentration range of 6.5-8.5 x 10^4 cells mL^{-1}, damaged fish and shrimp gills. By way of comparison, in the Strait of Georgia, a body of coastal seawater between Vancouver Island and the B.C. mainland coast, blooms of *H. carterae* can reach concentrations of 2 to 8 x 10^8 cells mL^{-1} at certain times of the year (Taylor *et al.*, 1994; Whyte, 1991). Thus, blooms of this toxic alga in coastal seawater would potentially cause injury to the shrimp gills. Perhaps high concentrations of trace metals in coastal seawater during late spring to summer periods in subtropical waters (Ishio, 1990) may exacerbate the *H. carterae* toxicity.

However, in the natural environment the shrimp, *P. danae*, have the ability to survive the toxic *H. carterae* blooms because they can utilize anaerobic metabolism pathways when the aerobic metabolic pathway has been damaged by *H. carterae* toxins. In addition, the shrimp may repair the gill damage after the *H. carterae* bloom wanes. Thus, metabolic adaptation and wound-healing ability of *P. danae* provide ways for this shrimp species to survive *H. carterae* blooms in coastal seawaters that are toxic to the finfish. Since many crustacean species possess the ability to survive anoxic conditions using anaerobic metabolism (Lockwood, 1967; Wolvekamp & Waterman, 1960), it is likely that they also possess the ability to survive exposure to toxic *H. carterae* blooms under natural conditions.

CONCLUSION

This study showed that a toxic *H. carterae* culture caused acute, lethal effects on rainbow trout (*O. mykiss*), and acute, sublethal effects on the coon-stripe shrimp, *P. danae*.

The toxic *H. carterae* cells damaged the gill lamellae. This caused hypoxia and lactic acidosis, in the blood and gill tissue of both species. As a result, the fish died while the shrimp did not because the latter species had the ability to utilize anaerobic metabolism, while the fish could not do so at rates commensurate with metabolic needs.

The shrimp demonstrated their ability to at least partially heal injured lamellae through hemocyte accumulation at the site of injury. This cellular defense mechanism may enhance the ability of the shrimp to survive exposure to the algal toxins.

Peroxidase activities of the gills of both animals remained active in toxic *H. carterae* cultures, whereas catalase activities were depressed by the toxic alga.

Harrison's medium caused hypoxia, lactic acidosis, depression of catalase activity, but did not suppress peroxidase activity, in the shrimp. Trace metals in the medium may have contributed to the phenomena.

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