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# THE INFLUENCE OF THE HARMFUL PHYTOPLANKTER, *CHAETOCEROS CONCAVICORNIS* ON THE SURVIVAL AND PHYSIOLOGY OF THE SALMONIDS *ONCORHYNCHUS MYKISS, O. TSHAWYTSCHA* AND *O. KISUTCH*.

by

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M.Sc., Xinjiang University, 1988

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THE REQUIREMENTS FOR THE DEGREE OF
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of
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*Our file    Notre référence*

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THE INFLUENCE OF THE HARMFUL PHYTOPLANKTER, CHAETOCEROS CONCAVICORNIS ON THE SURVIVAL AND PHYSIOLOGY OF THE SALMONIDS, ONCORHYNCHUS MYKISS, O. TSHAWYTSCHA AND O. KISUTCH.

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# ABSTRACT

Experiments were conducted to characterize the lethal effects of *Chaetoceros concavicornis*, a harmful diatom which possesses spines with barbs, upon salmonids (rainbow trout, chinook and coho salmon) and to determine effective ways of reducing finfish mortality caused by this phytoplankter. Under laboratory conditions, *C. concavicornis* at concentrations of 40-60 cells $mL^{-1}$ in seawater killed up to one-third of the treated fish within 10 days of exposure. Scanning electron microscopic examination of salmonid gill tissues showed that large numbers of *C. concavicornis* cells and their spines became lodged between the secondary lamellae with some of the spines penetrating the lamellar cells. These irritating barbed spines stimulated the production and accumulation of excess mucus on and between the secondary lamellae. Histopathological examination showed that the gills of *C. concavicornis* treated fish had severe edema, a collapsed pillar cell system, detachment of epithelial cells, localized hemorrhaging, hyperplasia, hypertrophy, and fusion of secondary lamellae. The excessive amounts of mucus which accumulated on the primary and secondary lamellae greatly inhibited dissolved oxygen uptake; as a result the blood became hypoxic. This condition induced a cascade of events which included decreases of

arterial blood pH and oxygen consumption and increases of
hematocrit, ventilation volume, ventilation frequency, and
anaerobic metabolism. Exposure of juvenile chinook to
harmful concentrations of *C. concavicornis* caused
lymphocyto-, neutrophilo- and thrombocytopenia and increases
in blood erythrocyte concentrations. An increase of blood
cortisol concentrations was also observed in rainbow trout
exposed to *C. concavicornis*. The interrenal and chromaffin
cells of rainbow trout exposed to harmful concentrations of
*C. concavicornis* became hypertrophied. These changes were
indicative of a suppressed immune system. The mortality
rates of coho salmon exposed concurrently to a harmful
concentration of *C. concavicornis* and to a sub-lethal
concentration of the bacterial pathogen, *Vibrio anguillarum*,
were greatly increased. By comparing the time course of
appearance of *V. anguillarum* in the kidneys, gills, and
spleens of coho salmon simultaneously treated with harmful
concentrations of *C. concavicornis* and this bacterial
pathogen, the data indicate that the damaged gills may be
the organ for the initial entry of this pathogen under these
conditions. The results indicate that salmonid mortality due
to *C. concavicornis* was caused by: (1) suffocation due to
excess mucus production and accumulation on the gills and
(2) greater susceptibility to bacterial pathogens because of
reduced immunological competence. Oral treatment of coho
with L-cysteine ethyl ester, a mucolytic agent, greatly
reduced mucus production and significantly lowered their

mortality rates when they were cultured in the presence of harmful concentrations of *C. concavicornis*.

# ACKNOWLEDGMENT

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# GENERAL INTRODUCTION

Phytoplankton is a collective name which describes drifting photoautotrophic microscopic plants which occur at any depth in marine or freshwater (Boney, 1989). The larger colonial forms possess individual cells that are usually of uniform structure and appearance. Some phytoplankton form chains of loosely associated cells and some are filamentous. However, many phytoplankters occur as single, free-floating cells. The main components of the phytoplankton assemblage which can be lethal or toxic to finfish in seawater are diatoms, dinoflagellates, coccolithophorids, and several flagellates, while in freshwater, blue-green algae, diatoms, and dinoflagellates are prominent.

Because most of food web of the oceans depends on them, phytoplankters are almost always beneficial. However, some species of phytoplankton are harmful or toxic to finfish. And, under certain conditions, they can threaten certain other organisms. In 1972, in the Seto Inland Sea of Japan, over US $35 million worth of caged fish were killed by toxic phytoplankton (Iwasaki, 1979). During several weeks in May of 1988, about 600 tons of caged rainbow trout on west coast of Sweden and on the south coast of Norway were lost due to toxic phytoplankton (Rosenberg, 1988). Similar events have occurred in Norway and Denmark (Gaines and Taylor, 1986). In

north and west Scotland and along the Norwegian coast, phytoplankton blooms have caused extensive mortalities in farmed Atlantic salmon costing 4.5 million pounds sterling (Bruno *et al.*, 1989). Along the west coast of Canada significant phytoplankton blooms occur annually, and kill farmed Pacific salmon (Margolis and Evelyn, 1987 cited in Bruno *et al.*, 1989). Since 1986, losses in the salmon farming industry in the northeast Pacific of the United States and coastal British Columbia have been in excess of Canadian $15 million (Rensel *et al.*, 1989; Black, 1990). In Japan, blooms caused by toxic phytoplankton have produced catastrophic kills of caged yellowtail (*Seriola quinqueradiata*) (Gaines and Taylor, 1986).

*Chaetoceros* spp. belong to the *Bacillariophyta*, *Bacillariophyceae* family. Although there are more than 50 species of *Chaetoceros* (Gaines and Taylor, 1986; Rines and Hargraves, 1988), to date only a few have been found to be harmful to finfish. The most important harmful diatom species in seawater along the British Columbia coast are *Chaetoceros concavicornis and Chaetoceros convolutus*. These have been reported to cause the deaths of finfish, including wild and penned salmonids (Evelyn, 1972; Bell *et al.*, 1974; Brett *et al.*, 1978). Since the first report that spines of *C. convolutus* can penetrate into the gill tissue of lingcod (*Ophiodon elongatus*) and kill it (Bell, 1961), sockeye (*Oncorhynchus nerka*), coho (*Oncorhynchus kisutch*), chinook (*Oncorhynchus tshawytscha*), chum (*Oncorhynchus keta*) and

pink (*Oncorhynchus gorbuscha*) salmon have also been reported to be killed by harmful *C. concavicornis* and *C convolutus* species (Evelyn, 1972; Brett *et al.*, 1978; Bruno, *et al.*, 1989; Albright *et al.*, 1992).

The genus *Chaetoceros* characteristically has seta (spines). The spine is an outgrowth of the valve surface forming a very elongate structure (Priddle and Fryxell, 1985). The most important characteristic of harmful *C. concavicornis* and *C. convolutus* is that their spines are studded with many smaller spinules (barbs) along their length. The spinules are directed toward the tip of the spine (Gaines and Taylor, 1986). All species of *Chaetoceros* possess spines and form chains, as do *C. concavicornis* and *C. convolutus*, but because most of them do not have spines with barbs, they are not harmful to fish (Gaines and Taylor, 1986).

The mechanisms by which *C. concavicornis* and *C. convolutus* cause finfish mortalities are believed to include (1) microbial infections of damaged gill tissue. (2) hemorrhage of gill capillaries and/or (3) suffocation from excess mucus production and accumulation at the sites of penetration of the gills by the spines (Bell, 1961).

*C. concavicornis* and *C. convolutus* can directly cause finfish mortalities (Bell, 1961). The numerous barbed spines of this unicellular marine phytoplankton break away from the diatom, penetrate and become imbedded butt-first in the lamellar cells of salmonid gills. There, they are held in

place by the barbs (Brett, et. al., 1978, Gaines and Taylor, 1986).

Concentrations as low as approximately 5 cells of harmful *Chaetoceros* spp. $mL^{-1}$ of seawater can cause damage to finfish (Bell, 1961; Bell *et al.*, 1974). In 1989, Bruno reported that in phytoplankton blooms which occurred at Loch Torridon and Shetland, the dominant species were harmful *Chaetoceros* spp. The histopathology strongly suggested that the finfish mortalities resulted from the direct clotting of excess mucus on the surface of gills and abrasion of the delicate gill structures by the silicified frustules from these species. The gill sections from affected fish showed extensive necrosis and sloughing, with separation of the secondary gill lamellae and moderate hyperplasia at the base of the filaments. Bruno suggested that other diatoms and silicoflagellates may also cause the same irritation of the fish gills and as a consequence, cause respiratory failure.

Of the salmonids, sockeye salmon appear to be the most vulnerable to harmful *Chaetoceros* damage (Brett *et al.*, 1978; Kennedy *et al.*, 1976).

L. J. Albright (person communication, Simon Fraser University) has observed that at many British Columbia seawater salmon farm sites, mortality rates of penned chinook salmon (many due to vibriosis epizootics) increase after apparently sub-lethal (less than 5 cells $mL^{-1}$) concentrations of harmful *C. concavicornis* and/or *C. convolutus* occur in the water column. He also reported that

anecdotal information of several farmers, particularly those at salmon farms sited within Jervis Inlet and the northern Strait of Georgia, indicates that mortality rates of penned chinook and coho salmon increase after exposure of the fish to apparently sub-lethal concentrations of harmful *Chaetoceros* spp. Some farmers have attributed these increased mortalities to *C. concavicornis*- and *C. convolutus*- induced diseases such as vibriosis and bacterial kidney disease.

Harmful phytoplankters are of great economic significance to production of farmed finfish, especially salmon culture along the British Columbia coast. Knowledge of the interaction of harmful phytoplankton with finfish might allow one to better control fish losses due to these harmful phytoplankters. At present, except for reports of finfish mortalities (Bell, 1961; Evelyn, 1972; Brett *et al.*, 1978; Bruno *et al.*, 1989 and Albright *et al.*, 1992) and pathological changes of gill tissues of salmon, there are few data on the effects of harmful *C. concavicornis* and *C. convolutus* on finfish. The purposes of this investigation were to determine the mechanism(s) of finfish mortality caused by the harmful diatom, *C. concavicornis*, and to develop an effective method to reduce finfish mortality caused by this phytoplankter. Several aspects have been systematically explored : (1) the harmful effects of *C. concavicornis* on the respiratory system of several salmonids; (2) the harmful effects of *C. concavicornis* on a

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portion of the immune system of several salmonids; (3) the harmful effects of *C. concavicornis* on a portion of the hypothalamic-pituitary-interrenal (HPI)-axis of several salmonids including changes of plasma cortisol concentrations and two hormone secreting tissues; (4) the susceptibility of salmonids to sublethal concentrations of the bacterial pathogen *V. anguillarum* when exposed to this phytoplankter, and (5) the identification of a therapeutic agent which can reduce mortalities of salmonids when they are exposed to harmful concentrations of *C. concavicornis*. These investigations are described in the following chapters.

# CHAPTER ONE

**The Effects of the Harmful Diatom** *Chaetoceros concavicornis*
**on Respiration of the Rainbow Trout** (*Oncorhynchus mykiss*).

# INTRODUCTION

Death of finfish by harmful *Chaetoceros* spp. has been suggested to be caused by: (1) microbial infections of damaged gill tissue, (2) hemorrhage of gill capillaries, or (3) suffocation from excess mucus production at the sites of penetration of the gills by the spines (Bell, 1961).

Within as little as 48 h of the onset of a harmful *Chaetoceros* spp. bloom, mortalities of penned salmonids can occur. Because of this very short period of time between the appearance of harmful concentrations of diatoms and the onset of mortalities, the latter two of the above listed possibilities appear to be the most likely causes of death. If this is so, the impairment of gill function in salmonids affected by the harmful *Chaetoceros* spp. should cause significant changes in the fish's oxygen transport system. This portion of the study examined the effects of harmful concentrations of *C. concavicornis* on the oxygen transport system, such as changes of gill lamellar structure and arterial blood oxygen tension of rainbow trout (*O. mykiss*).

## MATERIALS AND METHODS

*Culture.* *C. concavicornis* (Fig. 1) was obtained from the Northeast Pacific Culture Collection at the Department of Oceanography, University of British Columbia. This culture was originally isolated from a bloom at Jericho Beach, Vancouver, British Columbia. *C. concavicornis* was cultured using Harrison's medium (Harrison *et al.*, 1980). Namely, $NaNO_3$ (46.67 g), $Na_2$glycerophosphate (6.67 g), $NaSiO_3 \cdot 9H_2O$ (30.00 g), $Na_2EDTA \cdot 2H_2O$ (4.53 g), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (2.34 g), $FeCL_3 \cdot 6H_2O$ (0.16 g), $MnSO_4 \cdot 4H_2O$ (0.54 g), $ZnSO_4 \cdot 7H_2O$ (0.073 g), $CoSO_4 \cdot 7H_2O$ (0.016 g), $H_3BO_3$ (3.80 g), $Na_2SeO_3$ (0.00173 g), Thiamine (0.1 g), Vitamin $B_{12}$ (0.002 g), and Biotin (0.001 g) were dissolved in 1 L of seawater, and the pH was adjusted to 8.2. Each 2 L culture volume was cultured in a Fernbach flask. The temperature and ratio of light:dark were about 14 $^{\circ}C$ and 14:10 h, respectively. To determine concentrations of *C. concavicornis*, two- or 10-mL of *Chaetoceros*-containing culture was prestained with 4'6-diamidino-2-phenylindole (DAPI) and filtered onto 0.8 μm pore size, 12.5 mm diameter Nuclepore[r] filters. The filters were examined using an epifluorescence microscope fitted with barrier and excitation filters with a wavelength of 365 nm. Fifteen grids, each 1 $mm^2$, were counted per filter. The total number of *C. concavicornis* were calculated. Only the cells which possessed spines were counted. Cultures were used while in the early stationary phase.

Fig. 1. Scanning electron microscopic views of *C. concavicornis* cells.

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07-2 20.0 23 001 002
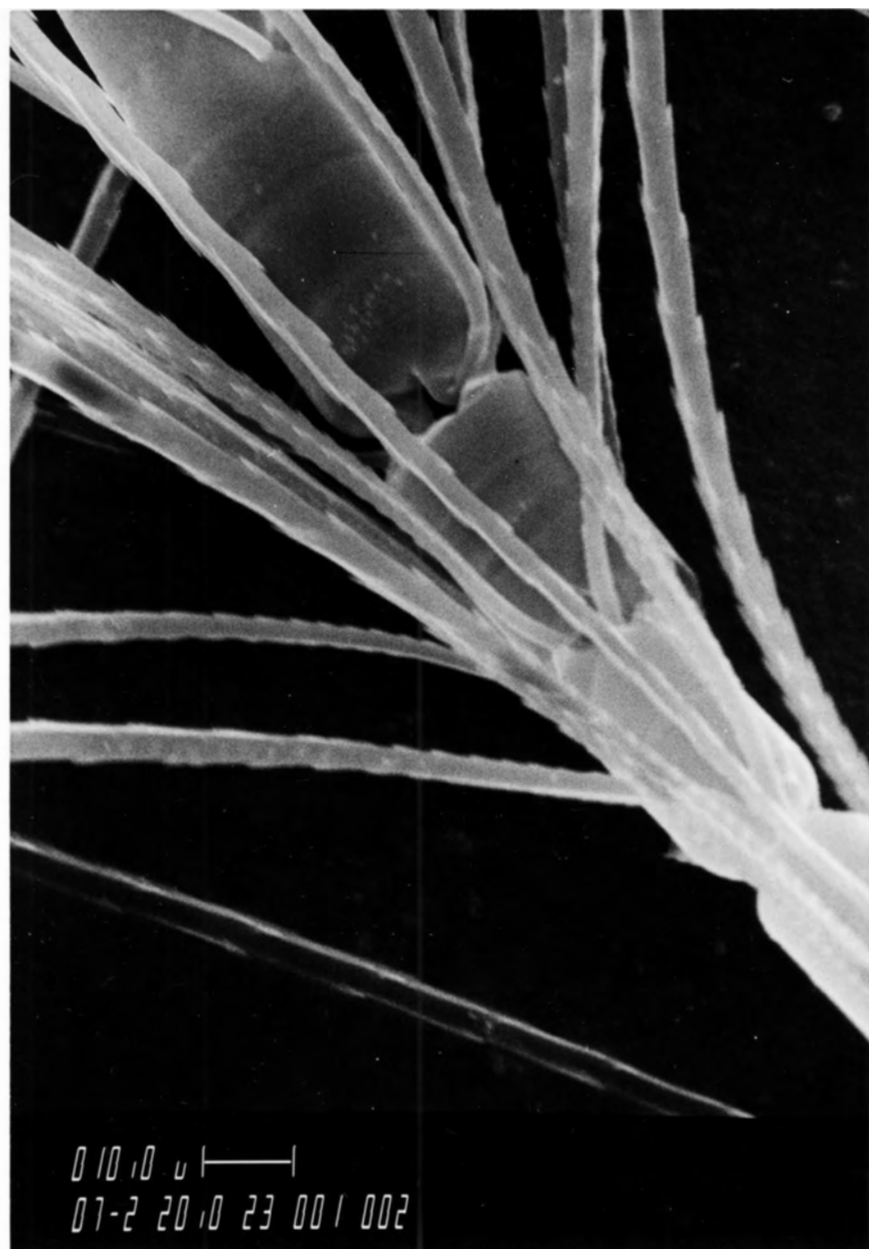
*Fish.* All experiments were carried out using 180 to 336 g rainbow trout which were obtained from a commercial trout farm. All fish were transported to Simon Fraser University and placed in 20 ‰ salinity water at least 15 days prior to cannulation. The oxygen concentrations, pH values, and temperatures of the dilute seawater in which the fish were placed varied from 8.3 to 9.7 mg mL$^{-1}$, 7.2 to 8.0 units, and 14 to 16 $^{\circ}$C, respectively. Feeding was discontinued 7 days prior to cannulation.

*Anesthesia and surgical procedures.* While immobilized in a net, each fish was anesthetized by immersion in neutralized tricaine methanesulfonate (MS 222, 0.1 g L$^{-1}$) for approximately 4 min. Following this treatment, the gills were perfused with oxygenated water containing a small amount of MS 222 (0.05 g L$^{-1}$) by means of a recirculating pump. Throughout the subsequent surgical procedures, cooled recirculating water at 5-6 $^{\circ}$C was used which greatly extended the period over which the fish could be maintained under anesthesia.

While under anesthesia the fish were cannulated at the following sites:

(1) Buccal cavity: One of the nasal openings was enlarged using a no. 16 needle (Saunders, 1962). Eighty cm of PE 90 polyethylene tubing was immediately passed through this hole. The end of the tubing was then heat-flared to anchor it on the inside of the mouth, and cotton thread was wrapped

around the tubing where it emerged from the nasal opening to prevent inward movement of the cannula.

(2) Opercular cavity : A hole was punched in the centre of one operculum with a no. 16 needle, and 80 cm of heat-flared, PE 90 polyethylene tubing was anchored in the hole as described above.

(3) Dorsal aorta: This vessel was cannulated using a modification of the method of Soivio *et al.* (1975). The cannulas were prepared from PE 50 polyethylene tubing. A steel wire (0.5 mm diameter) with the tip filed to a sharp point was inserted in the cannula. Before the operation the cannula was filled with Na-heparinate (Sigma), $200 \, \text{IU mL}^{-1}$ in isotonic (1 %) NaCl solution. The tip of the steel wire and cannula were pushed through one of the nares and then inserted into the blood vessel at the point between the first and second gill arches. Following insertion, the cannula was then pushed into the aorta until blood exited from the distal end of the cannula. The steel wire was then pulled out while the cannula was held in place in the aorta. The nearer end of the cannula was then pulled out through one of the nasal openings and fixed on the dorsal fin. The end was stoppered with a no. 23 1/2 gauge needle and a 1 mL disposable syringe.

*Experimental procedure*: Following cannulation, each fish (of fourteen) was placed in a metabolic chamber described by Maren *et al.* (1968). The chambers were then placed in four 75 x 60 x 22 cm black boxes: 8 chambers in

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the first two boxes containing treated fish, 6 in the other two boxes containing untreated control fish. Seawater of 20 ‰ salinity was recirculated through each chamber; the seawater was changed once each day. The pH and temperature of the recirculated waters varied between 7.3-8.0 units and 14-16 °C, respectively. The oxygen partial pressure was >130 mmHg during the course of the experiments. Following cannulation, the fish were allowed to recover for 48 h in the respiration chambers. During this recovery time the aortic cannulas were flushed once with the heparinized saline.

Forty-eight h after cannulation, *C. concavicornis* was added to the seawater of the first 2 boxes to yield a final concentration of approximately 50 cells $mL^{-1}$. The fish were sampled for all experimental parameters immediately prior to exposure to *C. concavicornis*.

*Analytic techniques*: Arterial blood pH was measured using a Radiometer G 2971 G2 electrode in a thermostated cuvette and recorded using a Radiometer PHM 73 meter. Arterial blood oxygen tension ($Pao_2$), inspired water oxygen tension ($Pio_2$), and expired water oxygen tension ($Peo_2$) were measured using a Radiometer E 5046/0 electrode in a thermostated cuvette and recorded using a PHM 73 meter. The meter was calibrated prior to each $Po_2$ measurement. The electrode was exposed to humidified $N_2$ prior to the determination of $Pao_2$, $Pio_2$ and $Peo_2$ to improve response

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time and decrease hysteresis, as recommended by Moran *et al.* (1966).

*Hematocrit values*: These were determined by the microcapillary method.

*Plasma lactate and glucose concentrations*: These were determined spectrophotometrically using Sigma (St. Louis, MO,) diagnostic kits.

*Light and scanning electron microscopy*: At the end of each experiment, branchial arches were removed from randomly selected, treated and control trout and washed in 1.1 % saline solution. Parts of these washed gills were then fixed in Davidson's solution, dehydrated in ethanol, impregnated with wax, sectioned, stained with hemotoxylin and eosin, and examined by light microscopy. Parts of the gills were fixed in glutaraldehyde, post-fixed in 1 % $OSO_4$, dehydrated in ethanol and amyl acetate, critical point dried, and then coated with gold. These samples were examined using an Autoscan scanning electron microscope.

*Determination of oxygen consumption*: After the respiratory chamber was sealed with rubber stoppers, water within the chamber was sampled immediately (time = t0) using a needle punched through each stopper. The oxygen tension ($Po_2$) at t0 was determined using a Radiometer E 5046/0 electrode. At intervals of 5 min thereafter, oxygen tensions were determined in a similar manner. These data were used to determine the rate of decrease of oxygen tension in the water. Following each experiment, the weight of each fish

was determined and its volume was measured. The volume of each respiratory chamber was also measured. These values were used to determine the specific rate of oxygen consumption (mg $O_2$ $kg^{-1}$ $h^{-1}$) of each trout.

*Determination of ventilation volume*: Ventilation volume was calculated by the Fick principle (Guyton, 1981) using the following equation:

$$Vg = Vo_2/ (Pio_2-Peo_2) ao_2$$

where:     Vg     = ventilation volume  (mL $min^{-1}$ $kg^{-1}$)

$Vo_2$     = $O_2$ uptake (mL $min^{-1}$ $kg^{-1}$)

$ao_2$     = solubility coefficient of oxygen in
           water (mL $mL^{-1}$ mmHg)

P          = partial pressure of gas (mmHg)

  subscripts

i refers to water in the buccal cavity (inspired)

e refers to water in opercular cavity (expired).

*Determination of intracellular pH*:  Red blood cells obtained by centrifuging 600 µL of blood at 20,000 x g for 5 min were frozen to - 20 $^{O}$C, thawed for 5 min, and refrozen to -20 $^{O}$C. To prevent the acid shift observed when lysed samples remain unfrozen, measurements of intracellular pH (pHi) were made immediately after the second thawing using a Radiometer G 2971 G2 electrode in a thermostated cuvette and recorded using a Radiometer PHM meter.

*Statistical analyses*: Values are expressed as the arithmetic mean +/- standard error (S.E.). All comparisons between treated and control fish were made by a two-way analysis of variance (ANOVA) with repeated measurements.

## RESULTS

The mean concentration of *C. concavicornis* cells remained at between 54 and 61 cells $mL^{-1}$ during the 72 h course of the experiments described below.

The inter-secondary lamellar spaces of the trout quickly accumulated a compact mass of *C. concavicornis* cells (Fig. 2). And, some of the spines of these diatoms appeared to penetrate the lamellae cells (Fig. 3). Coincident with this entrapment of diatoms in the inter-secondary lamellar spaces and the apparent penetration of the cells with the barbed spines, there was an accumulation of mucus-like material on the secondary lamellae surfaces (Fig. 4); indeed, in some cases, a mass of *C. concavicornis* and what appeared to be mucus filled the spaces between many adjacent secondary lamellar cells (Fig. 5).

Histological examination of gills of fish treated with *C. concavicornis* showed hyperplasia, hypertrophy, and partial or complete fusion of secondary lamellae (Fig. 6). The fish also showed severe edema, collapsed pillar cell systems, detachment of epithelial cells from the walls of the blood capillaries and localized hemorrhaging of some secondary lamellae (Fig 7) in comparison to the lamellar cells of untreated fish (Fig. 8). The mucous cells of the secondary lamellae of treated trout were more numerous and more prominent than those of this organ in untreated control trout (Fig. 9).

Fig. 2. Scanning electron microscopic views of the primary and secondary lamellae of rainbow trout exposed to *C. concavicornis* for 72 h.

100,0 μ ⊢──┤
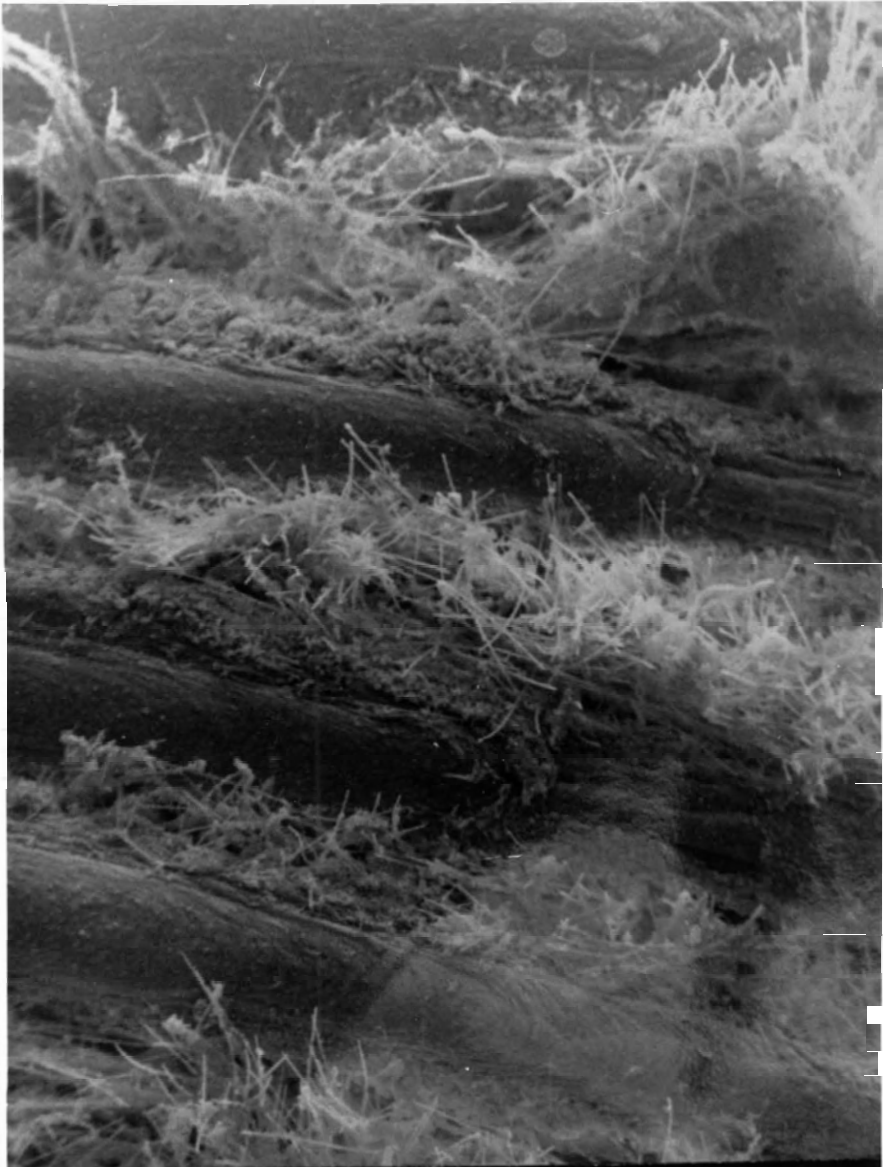
Fig. 3. Scanning electron microscopic views of the primary and secondary lamellae of rainbow trout treated with *C. concavicornis* for 72 h. The arrow indicates where the spines have penetrated the lamellae.

100,0 µ

Fig. 4. Scanning electron microscopic views of the primary and secondary lamellae of rainbow trout exposed to *C. concavicornis* for 72 h showing mucus accumulation.
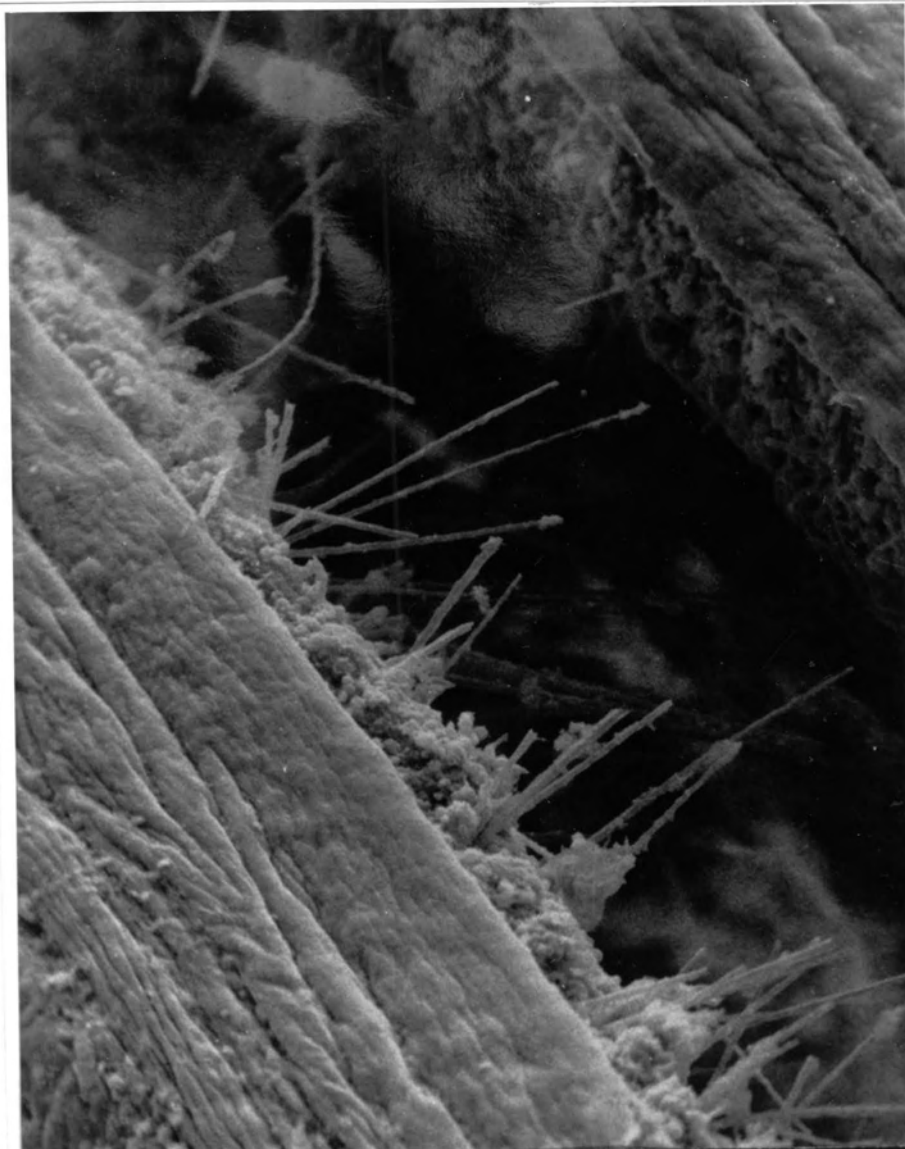
010,0 µ H

Fig. 5. Scanning electron microscopic views of the primary and secondary lamellae of rainbow trout exposed to *C. concavicornis* for 72 h showing mucus mixed with *C. concavicornis* forming a film filling spaces between adjacent primary lamellae.

100,0 µ ⊢————⊣

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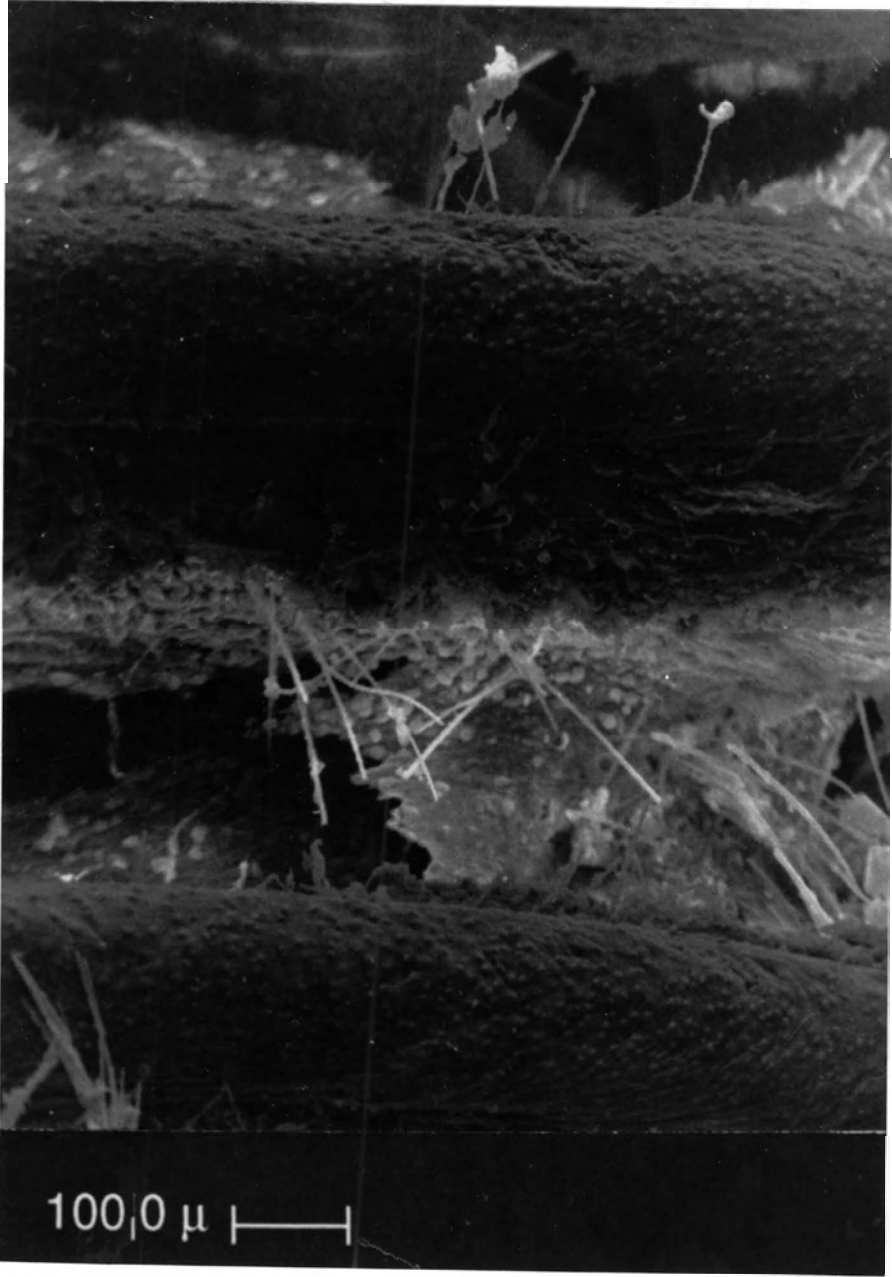
Fig. 6. Light microscopic views of the gills of rainbow trout  treated with *C. concavicornis* for 72 h; note hyperplasia, hypertrophy and partial and complete fusion of secondary lamellae. H & E staining. 150 x
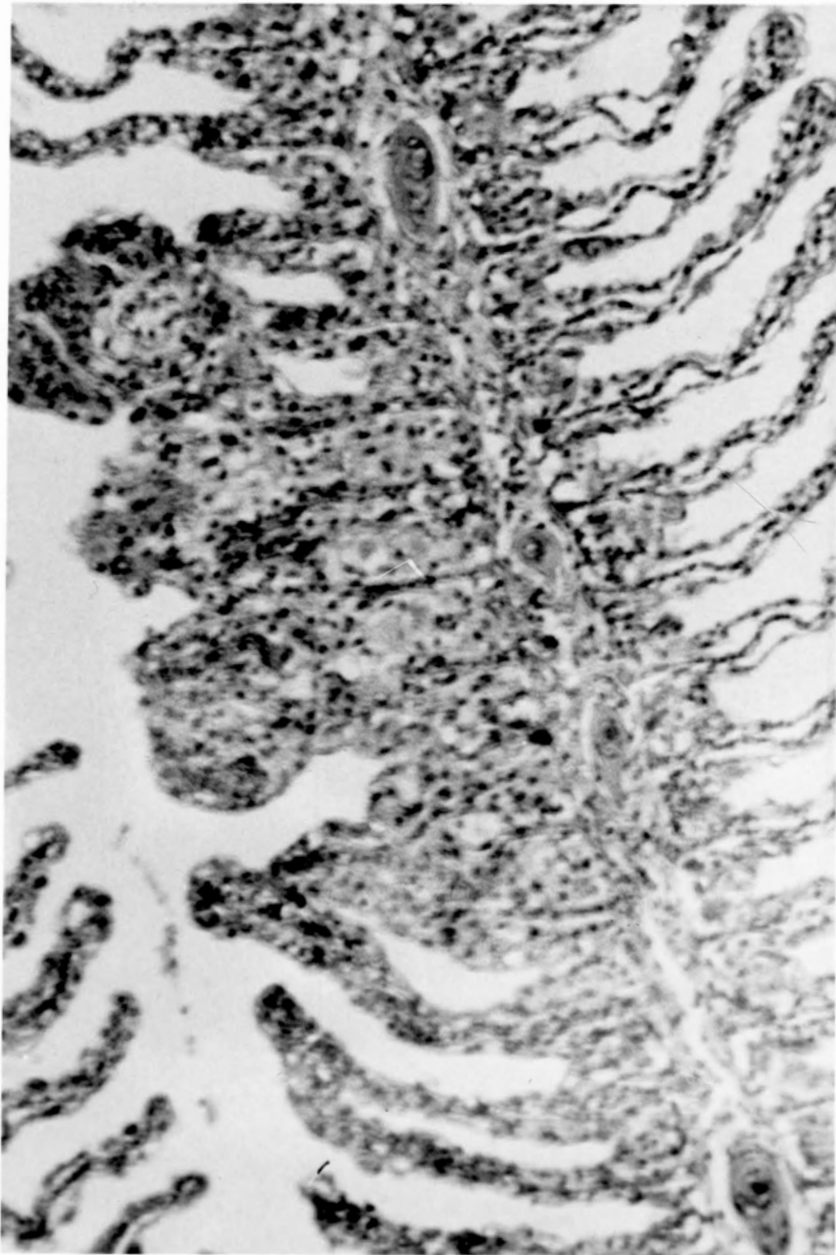
Fig. 7. Light microscopic views of gills of rainbow trout
treated with *C. concavicornis*; note severe edema, collapsed
pillar cell systems, detachment of epithelial cells from the
walls of the blood capillaries, and localized hemorrhaging
(arrowheads) of some secondary lamellae. H & E 240 x
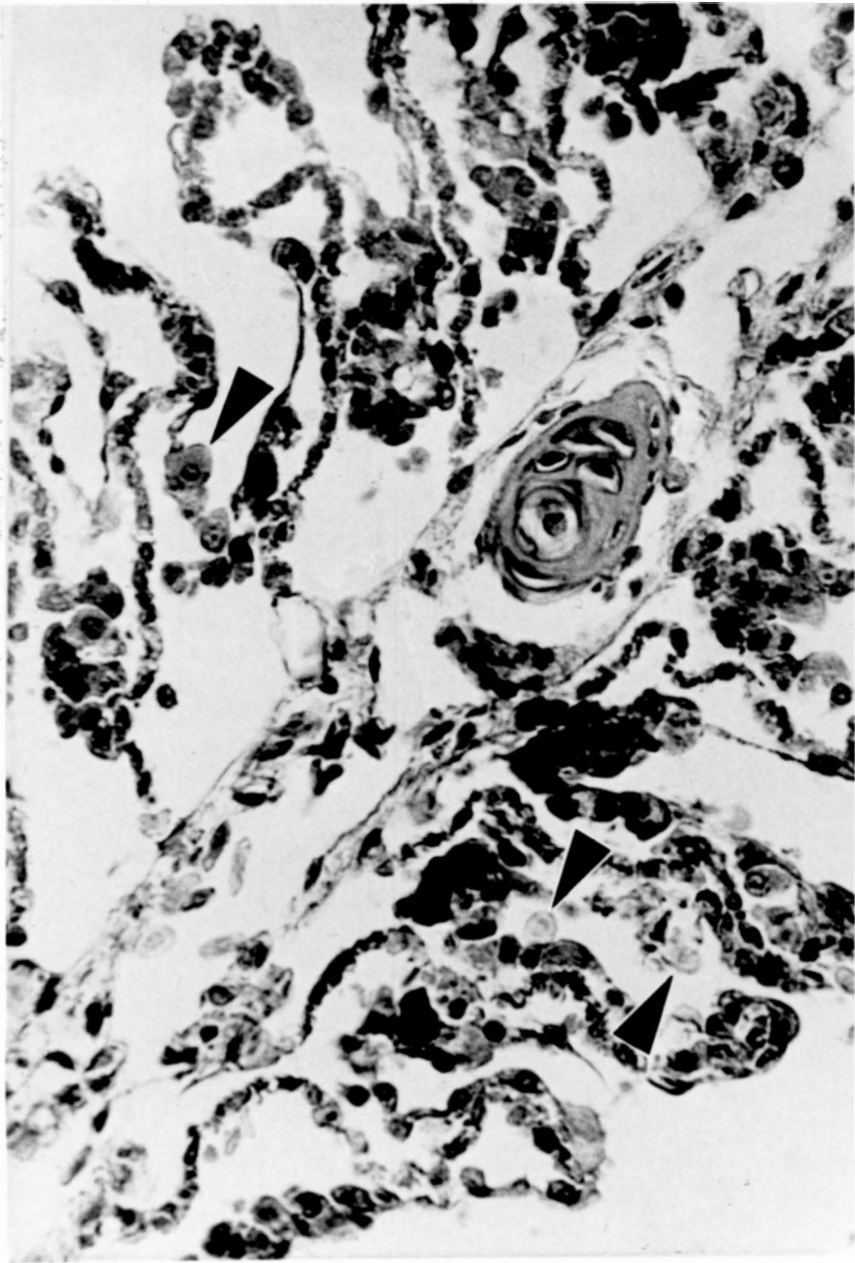
Fig. 8. Gill lamellae of untreated control rainbow trout. H
& E staining. 150 x
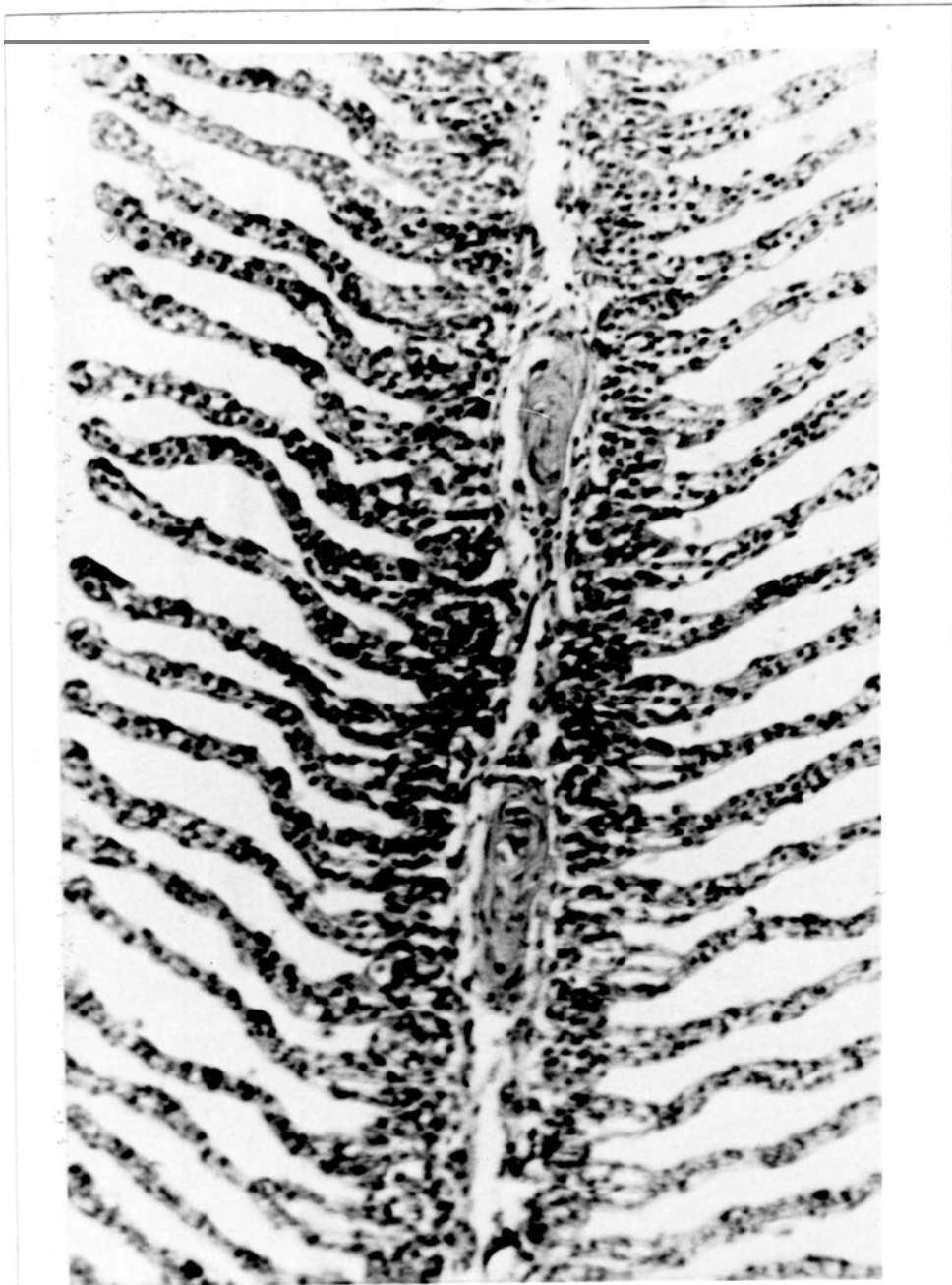
Fig. 9. Light microscopic views of gills of rainbow trout treated with *C. concavicornis* for 72 h; note the mucous cells (arrowheads) of the secondary lamellae are very numerous and very prominent.
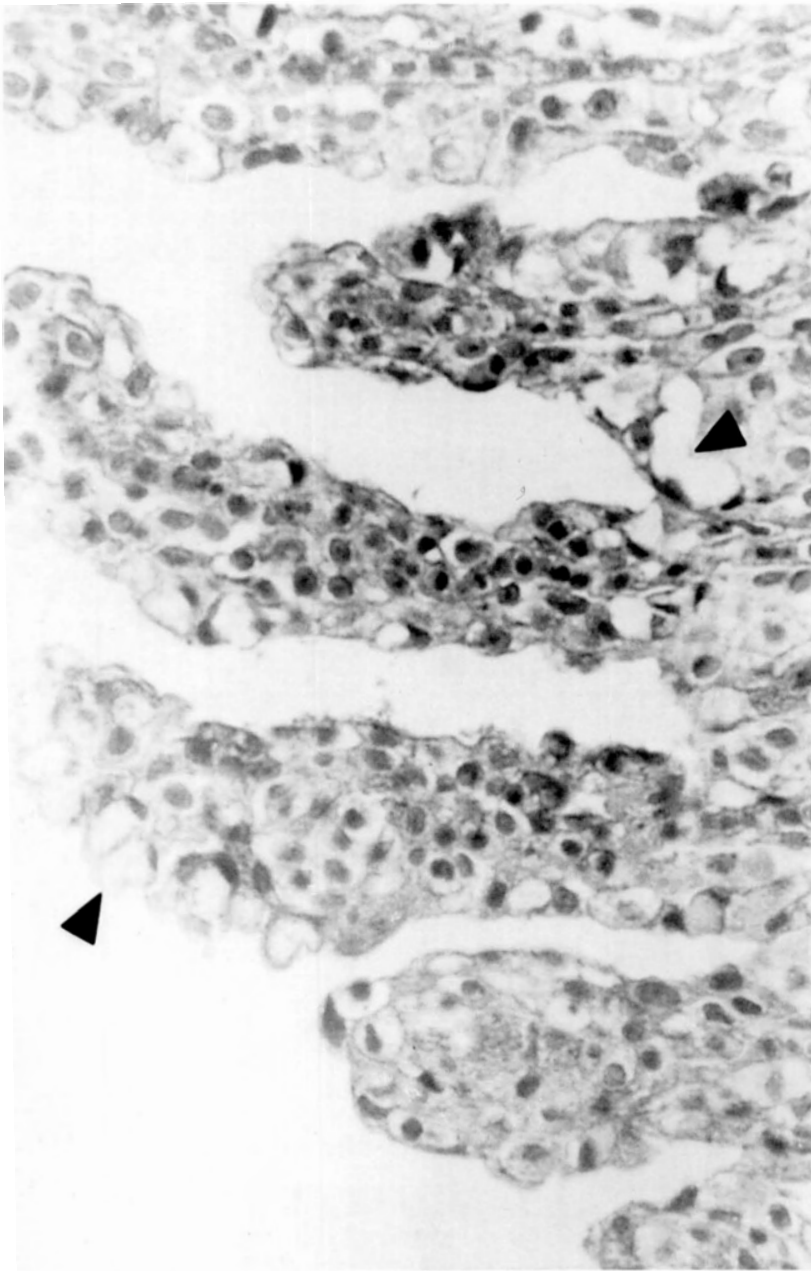
The mean $P_{O_2}$ of the blood sampled from the dorsal aorta of the treated fish decreased from approximately 100 mmHg to approximately 50 mmHg over a 72 h period when the fish were treated with water containing between 51-58 cells $mL^{-1}$ of *C. concavicornis* (Fig. 10). The mean $P_{O_2}$ of blood sampled from the dorsal aorta of the control fish untreated with *C. concavicornis* remained approximately the same over the 72 h period (Fig. 10). The $P_{O_2}$ values in the treated fish were significantly different from those of the untreated control fish at hours 12 ($P<0.01$), 24 ($P<0.01$), 48 ($P<0.01$) and 72 ($P<0.001$). The overall $P_{O_2}$ between treated and control fish groups was also significantly different ($P<0.05$).

The mean pH of the dorsal aorta blood of the fish decreased from 7.78 to 7.60 following *C. concavicornis* treatment for 72 h while the mean pH of the untreated control fish remained relatively constant at 7.77 - 7.73 (Fig. 11).

The mean ventilation volume of treated fish increased significantly to 3666 from 1258 mL $kg^{-1}$ $min^{-1}$ during the 72 h exposure to *C. concavicornis*, while the mean ventilation volume of the untreated control fish remained within the range of 1211 to 1298 mL $kg^{-1}$ $min^{-1}$ during this same period (Fig. 12). The ventilation volume in the treated fish were significantly different from those of the untreated control fish at hours 12 ($P<0.001$), 24 ($P<0.01$), 48 ($P<0.001$) and 72 ($P<0.001$). The overall ventilation volume between treated

Fig. 10. The partial pressure of oxygen in the dorsal aorta blood of rainbow trout treated with *C. concavicornis* in comparison to that of untreated trout. Error bar indicates +/- s.e.m. ** and *** indicate significant differences from the control values at the same sampling time (P<0.01) and (P<0.001), respectively.
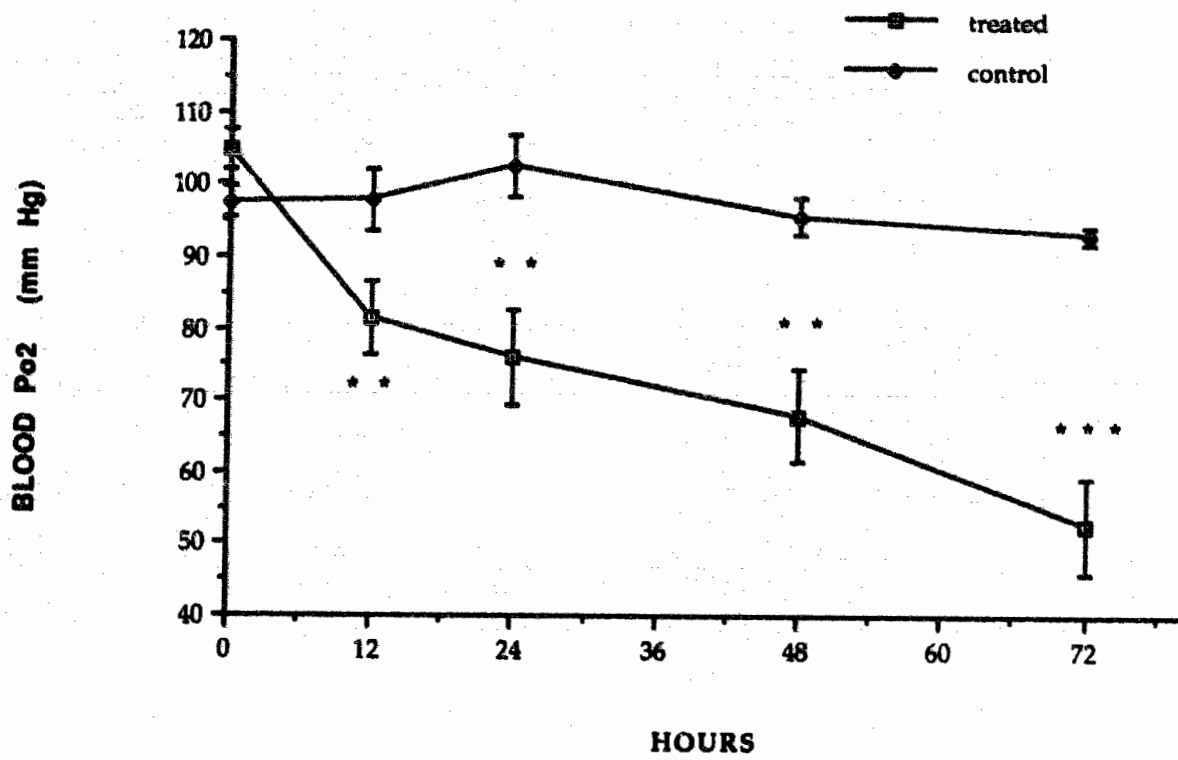
Fig. 11. The dorsal blood pH (pHa) of rainbow trout treated with *C. concavicornis* in comparison to that of untreated trout. Error bar indicates +/- s.e.m.
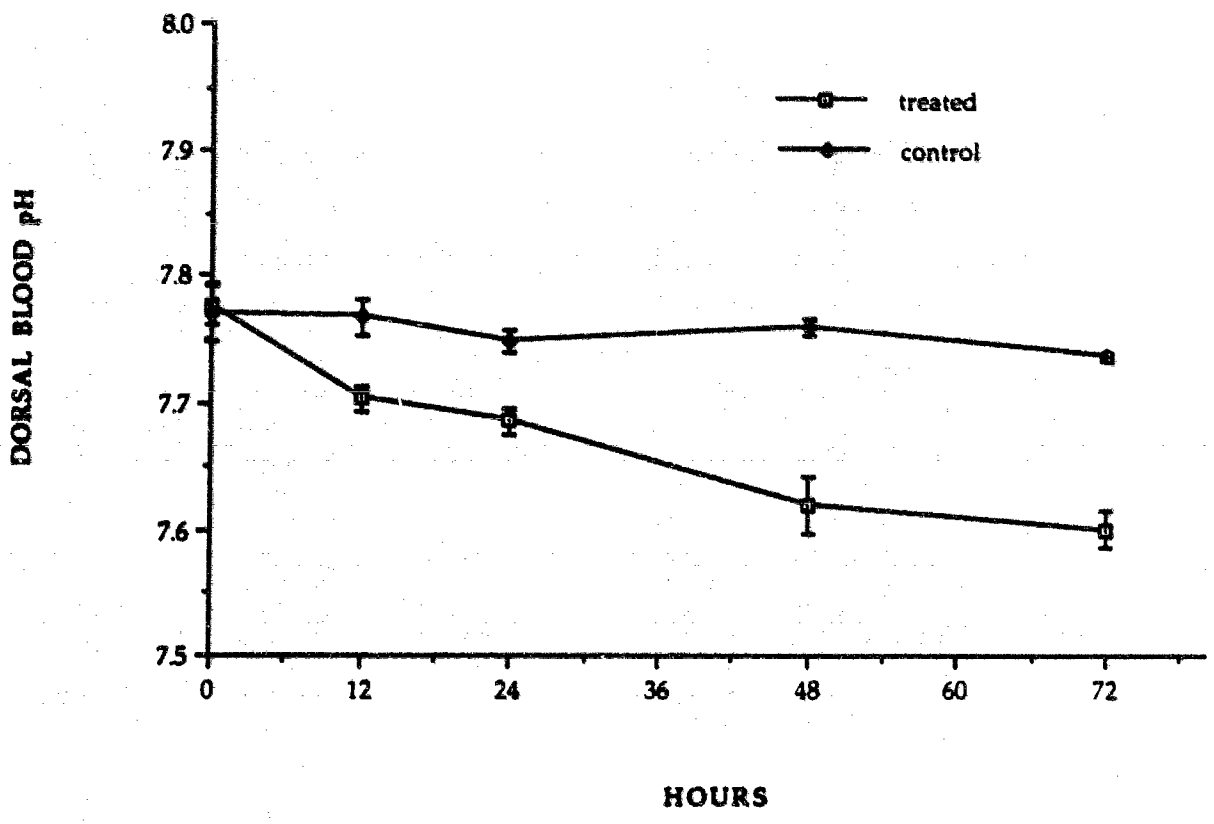
Fig. 12. The ventilation volume of rainbow trout treated with *C. concavicornis* in comparison to that of untreated trout. Error bar indicates +/- s.e.m. ** and *** indicate significant differences from the control values at the same sampling time (P<0.01) and (P<0.001), respectively.
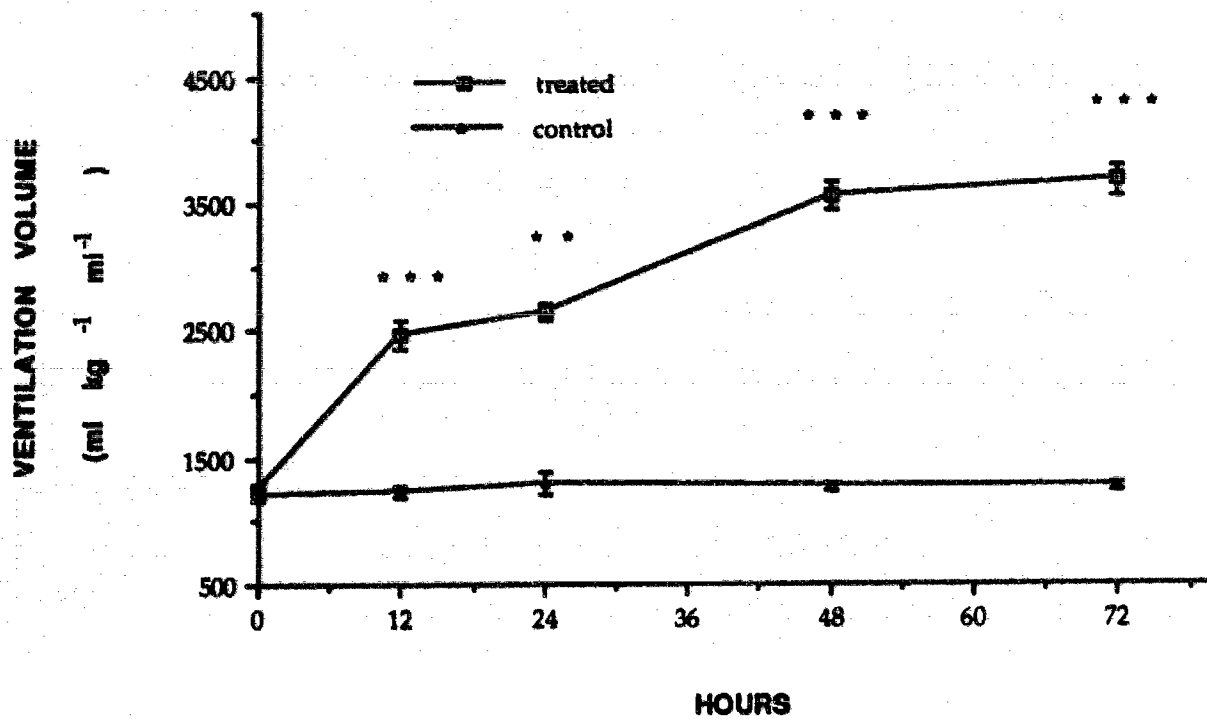
and control fish groups was also significantly different (P<0.001).

The mean ventilation frequency of treated fish increased to 131 from 79 times $min^{-1}$ following a 72 h exposure to *C. concavicornis* (Fig. 13). The mean ventilation frequency of the control fish remained within the range of 71 to 81 times $min^{-1}$. The ventilation frequency in the treated fish was significantly different from those of the untreated control fish at hours 12 (P<0.05), 24 (P<0.01), 48 (P<0.01) and 72 (P<0.05). The overall ventilation frequency between treated and control fish groups was also significantly different (P<0.001).

The mean $O_2$ consumption of treated fish decreased from 95 to 88 mg $kg^{-1}$ $h^{-1}$ following a 72 h exposure to *C. concavicornis* (Fig. 14). The mean $O_2$ consumption of the untreated control fish remained within the range of 93 to 97 mg $kg^{-1}$ $h^{-1}$ (Fig. 14). There was no significant difference between the $O_2$ consumption of the treated and untreated fish during the first 12, 24 and 48 h (Fig. 14). However, there was a significant difference between the $O_2$ consumption of the treated and untreated fish at 72 h (p<0.05).

Twelve h after exposure to *C. concavicornis*, the mean hematocrit value increased to approximately 22.1 % from approximately 18.9 % (The value of the control fish at 12 h was approximately 18.8%) (Fig. 15). The hematocrits in the treated fish were significantly different from those of the untreated control fish at hours 12 (P<0.05) and 48 (P<0.05),

Fig. 13. The ventilation frequency of trout treated with *C. concavicornis* in comparison to that of untreated trout. Error bar indicates +/- s.e.m.* and ** indicate significant differences from the control values at the same sampling time (P<0.05) and (P<0.01), respectively.
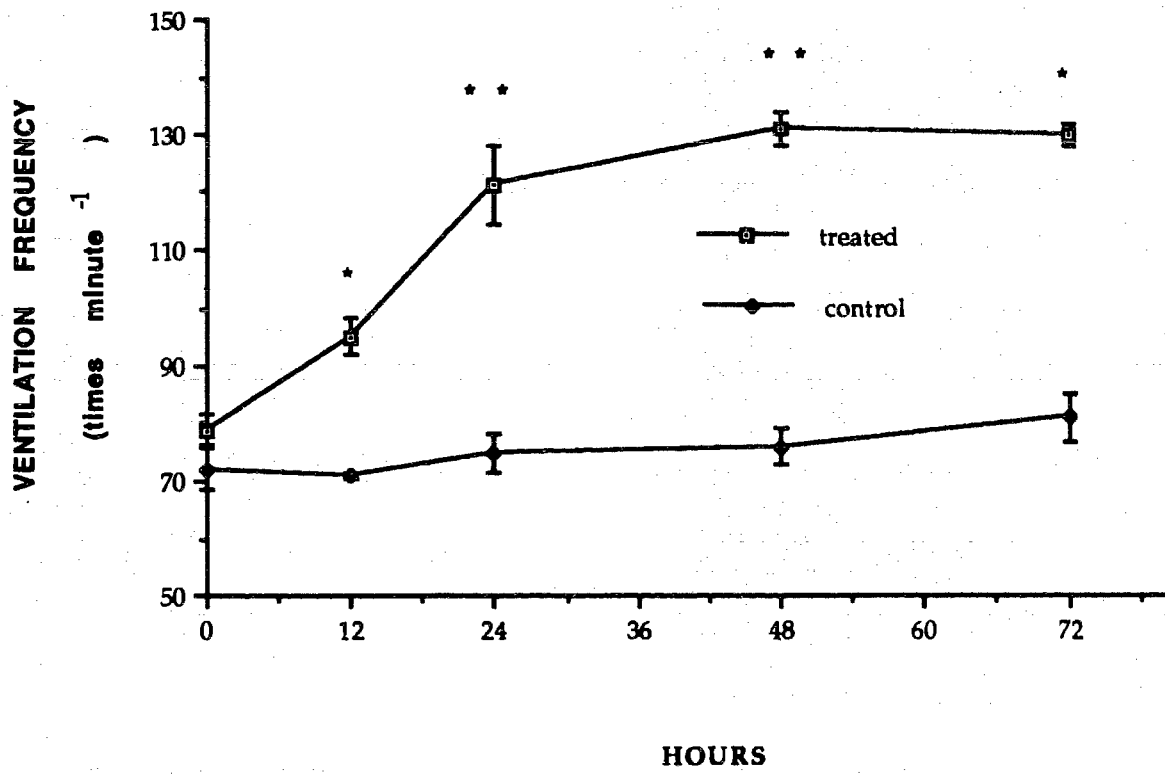
Fig. 14. The oxygen consumption of rainbow trout treated with *C. concavicornis* cells in comparison to that of untreated trout. Error bar indicates +/- s.e.m.* indicates significant difference from the control value at the same sampling time (P<0.05).
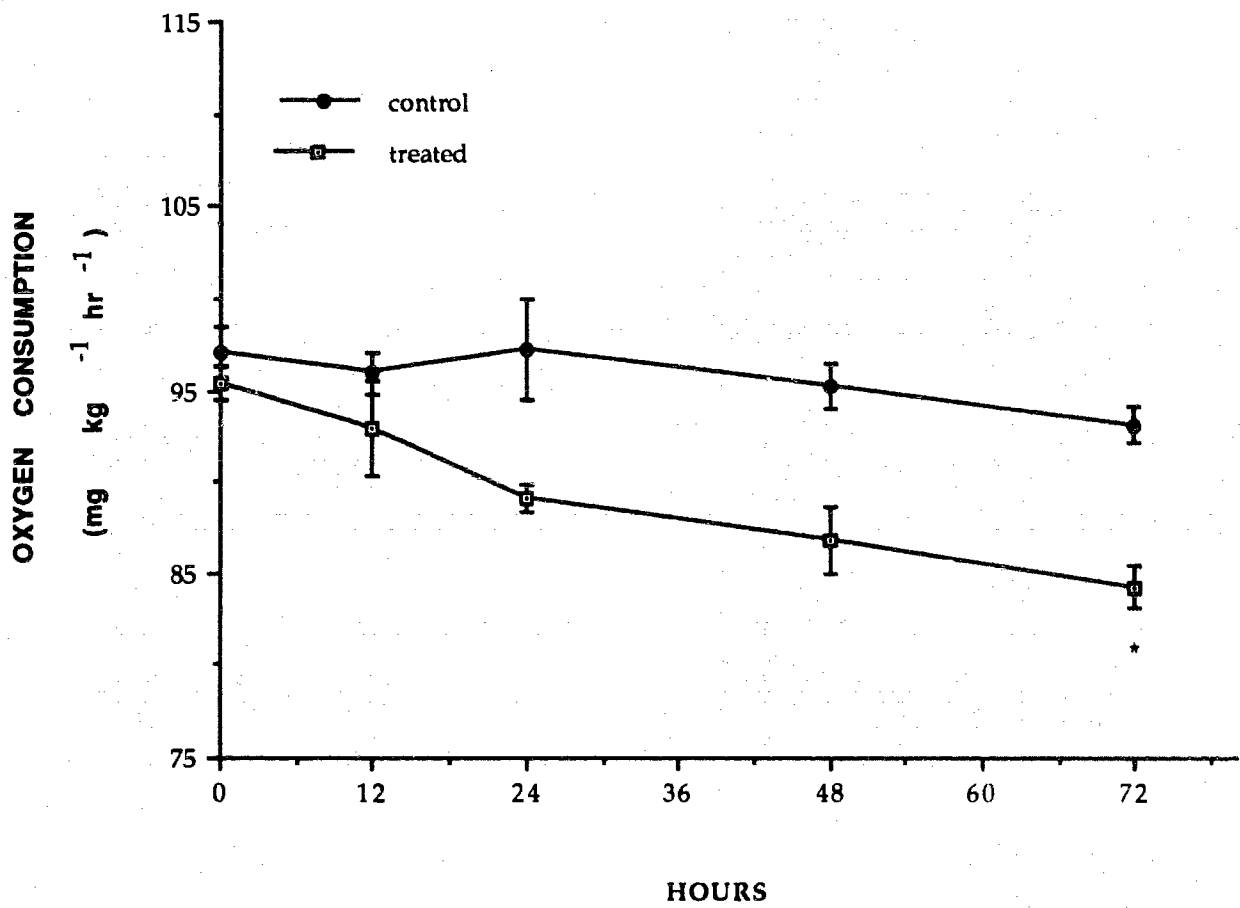
Fig. 15. The hematocrit of rainbow trout treated with *C. concavicornis* cells in comparison to that of untreated trout. Error bar indicates +/- s.e.m.* indicates significant difference from the control value at the same sampling time (P<0.05).
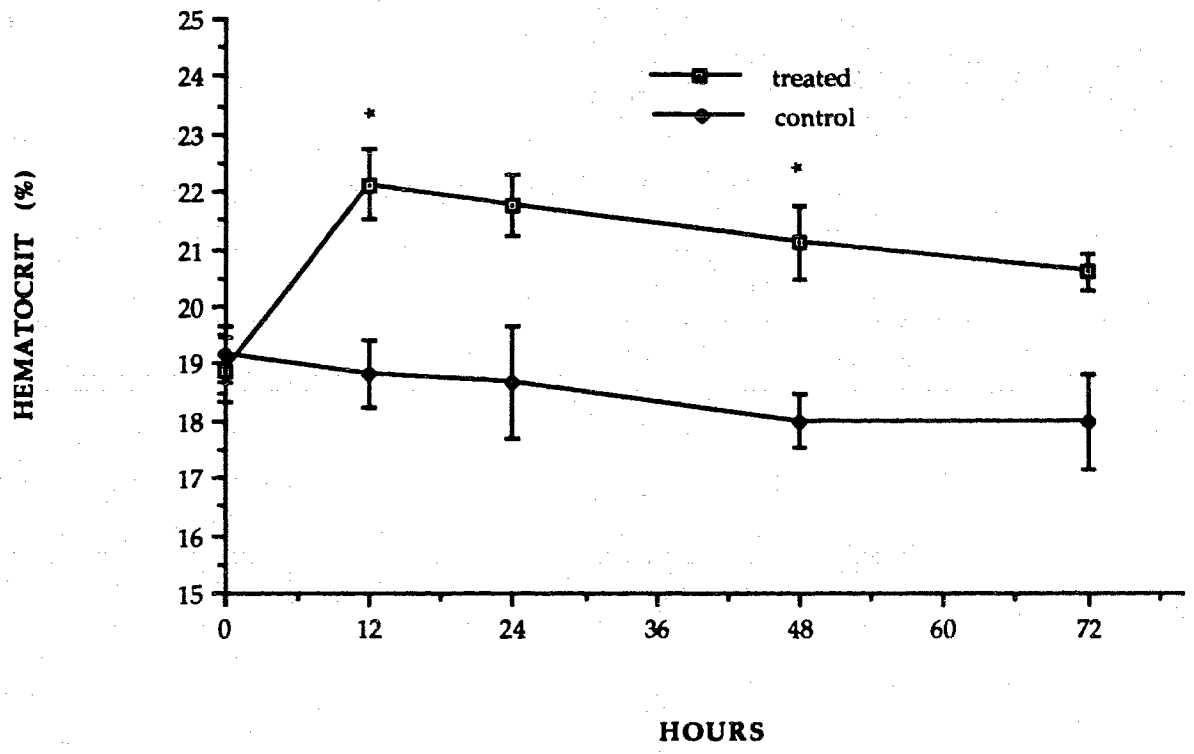
but not at hours 24 and 72. The overall hematocrit between treated and control fish groups was significantly different (P<0.05).

The mean plasma lactate concentration of the treated trout increased to 36.9 mg 100 mL$^{-1}$ following *C. concavicornis* treatment for 72 h in comparison to a value of 25.0 mg 100 mL$^{-1}$ prior to treatment (Fig. 16). The mean plasma lactate concentration of the untreated control trout remained approximately the same during the 72 h of the experiment (Fig. 16). The lactate concentrations in the treated fish were significantly different from those of the untreated control fish at hours 24 (P<0.05), 48 (P<0.05) and 72 (P<0.001). The overall lactate concentrations between treated and control fish groups was significantly different (P<0.05).

The mean glucose concentration of blood of the trout increased to 60.9 mg 100 mL$^{-1}$ following a 72 h treatment with *C. concavicornis* (Fig. 17); the mean glucose concentration of these trout prior to *C. concavicornis* exposure was 43.6 mg 100 mL$^{-1}$ (Fig. 17). The mean concentration of blood glucose of the untreated fish remained between 46.2 and 39.3 mg 100 mL$^{-1}$. The glucose concentrations in the treated fish were significantly different from those of the untreated control fish at hours 48 (P<0.05) and 72 (P<0.01). The overall glucose concentrations between treated and control fish groups was significantly different (P<0.05).

The mean erythrocyte pHi of the treated fish increased from 7.02 prior to treatment to 7.33 following *C. concavicornis* treatment for 72 h (Fig. 18). The mean pHi values of the control fish varied from 7.01 to 7.09 during the 72 h of the experiment (Fig. 18). There was a difference between these curves.

Fig. 16. The blood lactate concentrations of rainbow trout treated with *C. concavicornis* cells in comparison to those of untreated trout. Error bar indicates +/- s.e.m.* and ** indicate significant differences from the control values at the same sampling time (P<0.05) and (P<0.01), respectively.
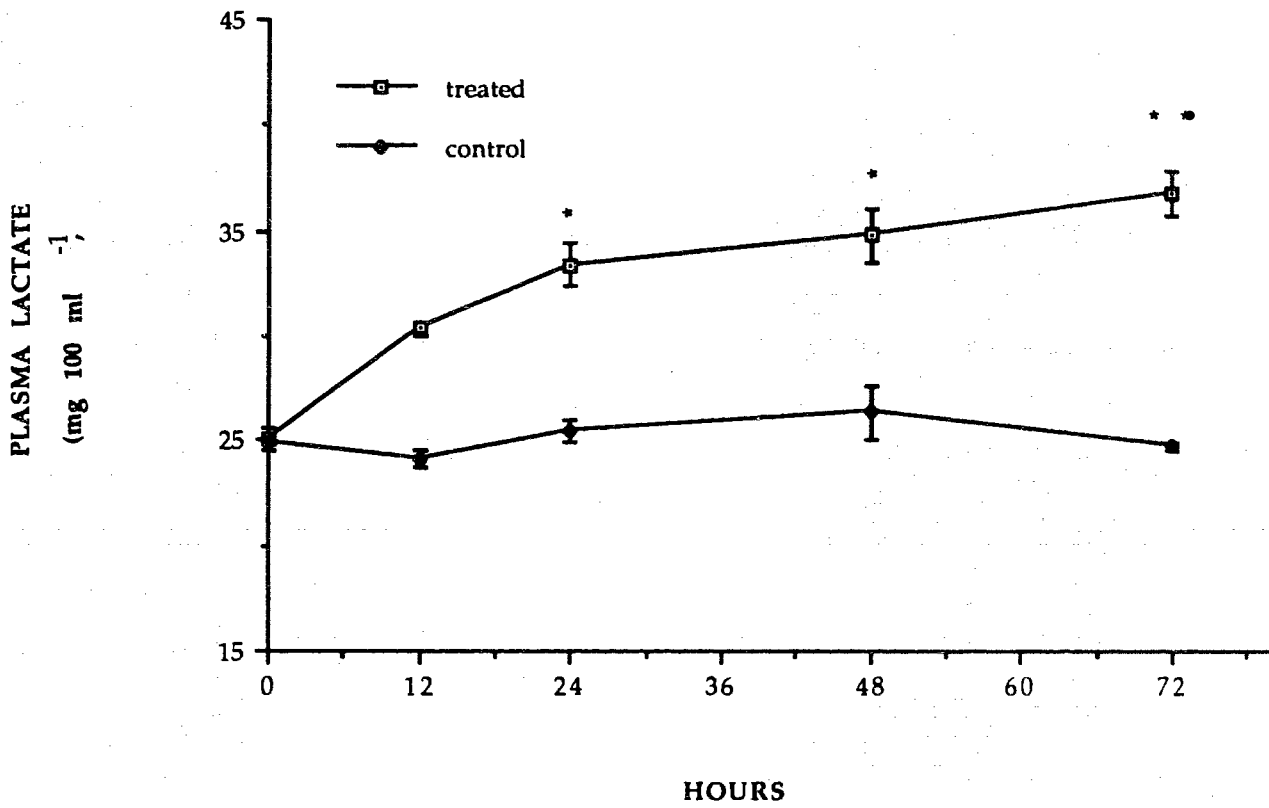
Fig. 17. The blood glucose concentrations of rainbow trout treated with *C. concavicornis* cells in comparison to that of untreated trout. Error bar indicates +/- s.e.m.* and ** indicate significant differences from the control values at the same sampling time (P<0.05) and (P<0.01), respectively.
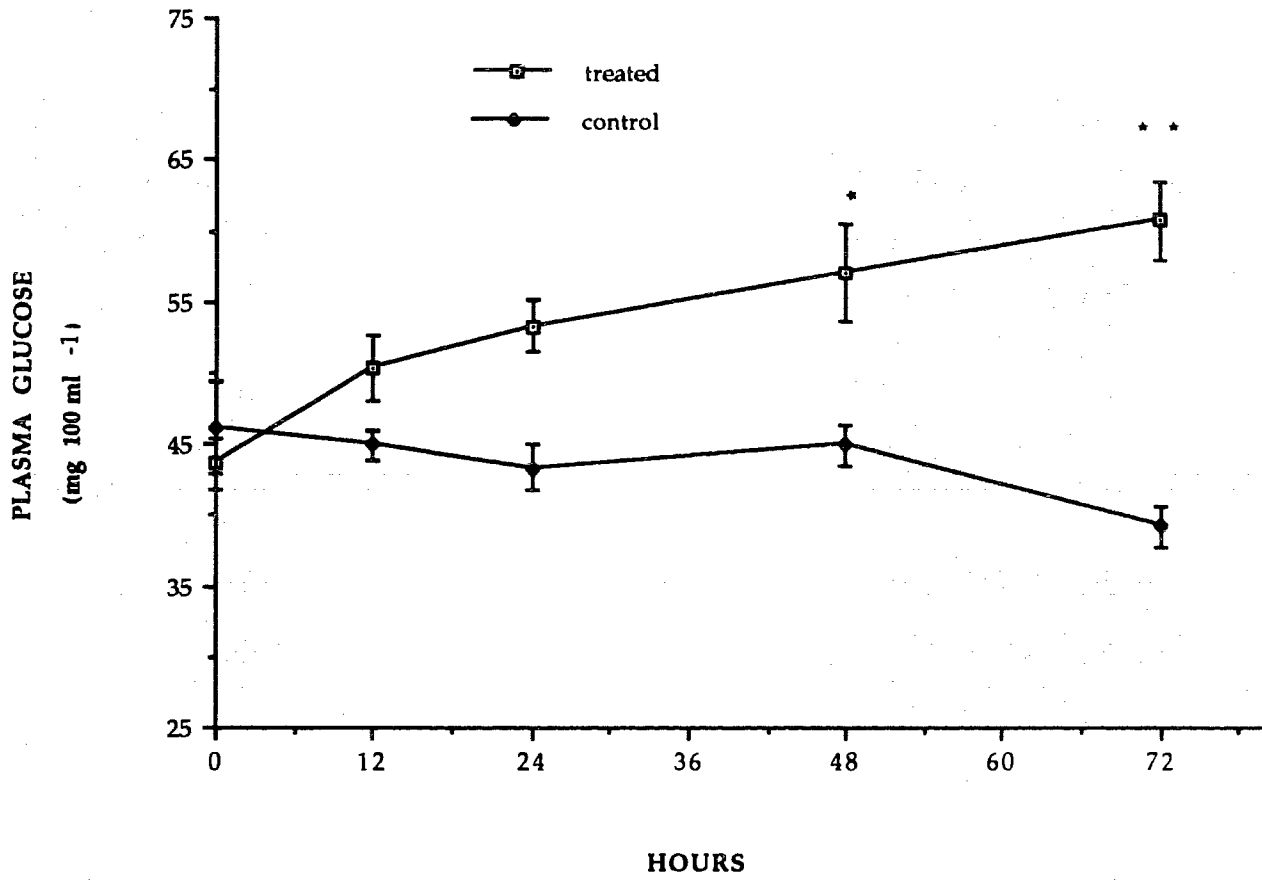
Fig. 18. The intracellular erythrocyte pH values (pHi) of rainbow trout treated with *C. concavicornis* cells in comparison to those of untreated trout. Error bar indicates +/- s.e.m.
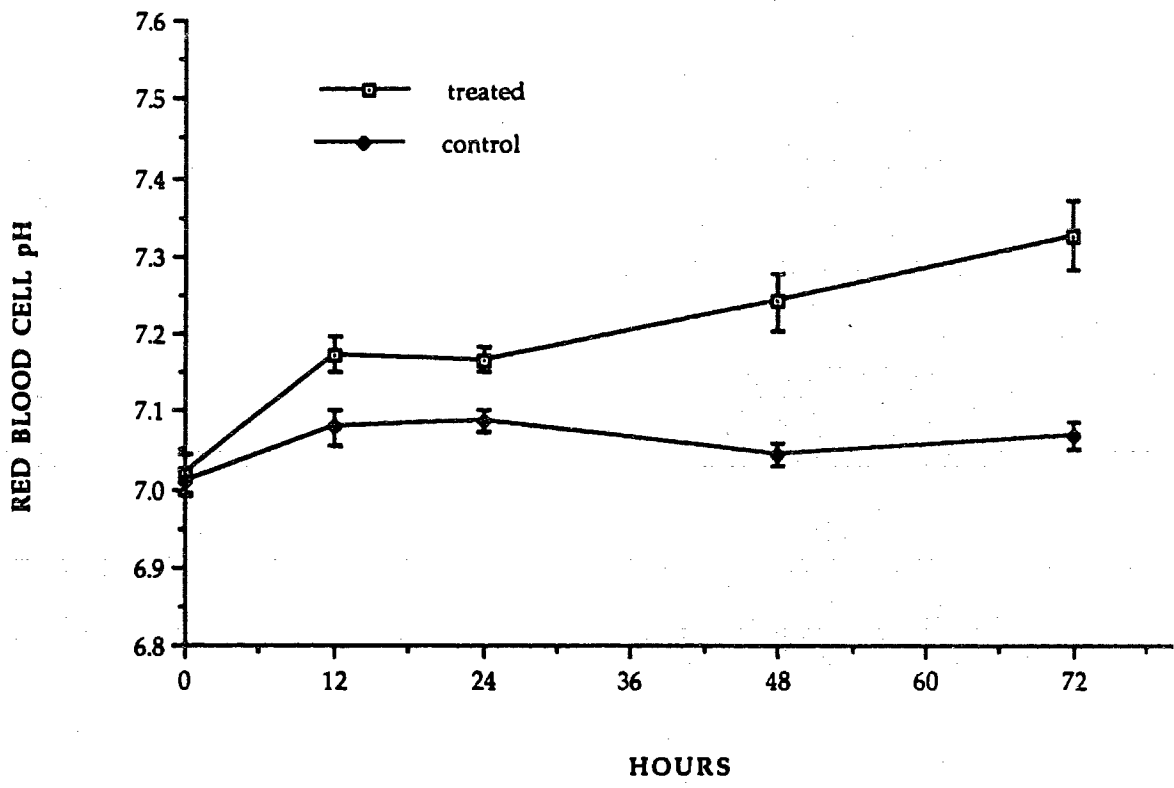
# DISCUSSION

Scanning electron micrographs clearly showed that *C.
concavicornis* cells and their barbed spines became wedged in
the inter-secondary lamellar spaces of the fish to form what
appear to be "windrow-like" masses of immobilized diatoms
and their broken spines (Fig. 2). Many of the spines have
broken off the diatom frustules and some appear to have
penetrated the lamellar cells (Fig. 3). A careful
examination of these micrographs (especially Figs. 4 and 5)
and those of Figs. 6 and 7 indicates that the cells of this
tissue appear to respond to this injury in several ways. One
of the responses was the formation of what appears to be
excessive amounts of mucus on and between the secondary
lamellae of the treated fish (Figs. 4 and 5). A histological
analysis of this tissue further showed that many of these
lamellar cells showed hypertrophy and hyperplasia 72 h after
*C. concavicornis* treatment (Fig. 6). These observations
cannot be used to determine the sequence of events which
occurred upon exposure of the trout lamellae to harmful
concentrations of *C. concavicornis*. Nevertheless, it would
appear likely that the production of mucus would be
initiated first, followed by cell hyperplasia. Cell
hypertrophy might occur coincident with or following the
initiation of mucus production.

A small amount of hemorrhaging of secondary lamellae was observed (Fig. 7) but there were no overt signs of secondary infections following exposure to *C. concavicornis* during the 72 h of this experiment. Both of these processes were suggested by Bell (1961) to be mechanisms by which *C. concavicornis* might cause death of affected finfish.

Based upon the above observations, it appears that the production of excess mucus, hypertrophy, and hyperplasia of the respiratory epithelium cells, caused by exposure to this harmful phytoplankter, physically limits gill functions, including gaseous exchange and waste products elimination (e.g. $CO_2$, $NH_4$, urea and trimethylamine oxide). The reduced oxygen diffusing capacity of the gills, in particular, would in turn initiate a cascade of events in the respiratory chain of the trout. One would therefore predict changes to functions such as aorta blood $O_2$ pressure and pH, ventilation volumes and frequencies, plasma lactate and glucose concentrations, hematocrit, and erythrocyte pH.

The effects of environmental hypoxia on respiratory gas transport in teleost fish have been studied extensively, and adaptational responses have been found in the respiratory chain. The responses include changes in arterial blood oxygen tension, blood pH, ventilation volume, ventilation frequency, and $O_2$ consumption (Randall, 1982, Holeton and Randall, 1967a). Specific responses of the various portions of the trout respiratory transport system as they relate to exposure to *C. concavicornis* are discussed below.

The continuous depletion of $O_2$ with time from arterial blood in *C. concavicornis*- treated fish (Fig. 10) is likely directly caused by diffusion limitation at the secondary lamellae due to excess mucus production. This reduction in arterial $O_2$ pressure then affects the trout's ventilation volumes and frequencies. Rensel (1993) also observed severe reduction in arterial $O_2$ pressure of Atlantic salmon when exposed to *C. concavicornis*. Smith and Jones (1982) have shown an effect similar to the one noted herein, where they found that a reduction of blood oxygen pressure by hypoxia in rainbow trout stimulated an increase in the animal's ventilation volume. Similar effects have been noted by Randall and Jones (1973) and Holeton and Randall (1967a).

Under hypoxic conditions the fish attempts to maintain its blood $O_2$ at required concentrations by increasing both its ventilation volumes and frequencies, the actual values varying with the species (Smith and Jones, 1982). Holeton and Randall (1967b) found that rainbow trout ventilation frequency increased from approximately 80 to approximately 120 $min^{-1}$ with advanced hypoxia. In our experiments, although the ventilation frequencies of the control trout varied between 70 and 80 $min^{-1}$, the *C. concavicornis*- treated trout had frequencies in excess of 130 $min^{-1}$ (Fig. 13), indicative of acute hypoxia.

The approximately 0.2 pH unit increase in acidity of the blood within approximately 24 h of exposure of the trout to *C. concavicornis* could involve an increase in plasma

metabolic acids and/or a change in plasma ionic composition such as increase of $H^+$ and decrease of $HCO_3^-$ and $CO_3^{2-}$. The excess mucus on secondary lamellae inhibits release of $CO_2$ in Atlantic salmon (*Salmo salar*) (Rensel, 1993). Consequently, in the blood there is a shift of the equilibria: $CO_2 \iff H_2CO_3 \iff HCO_3^- + H^+ \iff CO_3^{2-} + H^+$, in such a way as to produce a increase in $H^+$. $H^+$ is also extruded from erythrocytes under anaerobic conditions. The $HCO_3^-$ and $CO_3^{2-}$ concentrations probably decrease due to the intervention of a $Cl^-$ --- $HCO_3^-$ exchange process as suggested by Thomas and Hughes (1982). Following hypoxia, $H^+$ is believed to be extruded from erythrocytes, triggered by the release of catecholamines to the circulation which in turn stimulates $Na^+/H^+$ exchangers located in their membranes. This has been shown *in vitro* for adrenaline treated (Nikinmaa, 1982) and isoproterenol treated (Nikinmaa and Huestis, 1984) erythrocytes of rainbow trout. Nikinmaa (1982) and Cossins and Richardson (1985) have suggested that the $H^+$ extrusion could increase the hemoglobin oxygen affinity via the alkalization of the intracellular pH. The increase of lactate levels in blood reported herein may be associated with the fall of pH. Tetens and Lykkeboe (1985) reported a decrease in blood pH after fish were exposed to hypoxia. They suggested that the fall of pH in the blood was possibly caused by a release to the circulation of protons from muscle lactate.

Hypoxia of rainbow trout can cause metabolic acidification of the blood (Thomas and Hughes, 1982). The pHi increase (Fig. 18) and pHa decrease (Fig. 11) noted in our experiments are consistent with what has been observed in other experiments with teleosts undergoing hypoxia (Fievet *et al.*, 1988; Claireaux *et al.*, 1988; Thomas and Hughes, 1982).

The very rapid increase in hematocrit values of dorsal aorta blood within 12 h of *C. concavicornis* treatment may have been due to the division of erythrocytes (Murad *et al.*, 1990), plasma skimming and/or elevation of plasma catecholamine levels of the fish which could induce swelling of erythrocytes, and entry of erythrocytes into the circulatory system from storage organs, such as the spleen, under the influence of hypoxia ( Hughes, 1981; Milligan and wood, 1986; Johansen and Hanson, 1967; Ostroumova, 1964; Randall and Perry, 1992; Stevens, 1968; Yamamoto *et al.*, 1985). Whatever the reason, the increased hematocrit concentrations observed herein were typical of those observed when teleost fish undergo hypoxia.

Lactate and glucose concentrations have been shown by many investigators to be sensitive indicators of hypoxic stress in rainbow trout (e.g., Holeton and Randall, 1967a) as well as several other fresh and seawater fish species (e.g. Hattingh, 1967; Yu and Woo, 1987). The pattern of lactate and glucose concentration increases noted in the present work (Figs. 16 and 17) indicates that low blood $O_2$

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concentrations likely triggered anaerobic metabolism in the trout treated with *C. concavicornis*. Boutillier *et al.* (1988) noted that when environmental $O_2$ concentrations fell below 50 mmHg, plasma lactate levels in rainbow trout rose as increased hypoxia boosted the rate of anaerobic metabolism. In the experiments reported herein, the concentration of oxygen exceeded 50 mmHg in the water. However, this gas was unable to pass the secondary lamellar cells at rates commensurate with the maintenance of aerobic conditions in the arterial blood, allowing the $O_2$ concentrations to fall far below the concentrations which one would expect with this high concentration of $O_2$ in the water.

The data presented herein clearly indicate that the barbed spines of the harmful diatom *C. concavicornis* damage the physical integrity of the respiratory epithelium of rainbow trout, and by extension, that of other salmonids. The trout responds to this physical impairment of gill tissue by producing excessive amounts of mucus. The accumulation of this mucus on and between the secondary lamellae inhibits the gill functions of $O_2$ diffusion (and likely metabolic waste product release.) The hypoxic conditions which result within the fish then cause a cascade of events, including anaerobic metabolism, as the trout attempts to compensate for loss of arterial blood $O_2$. In the experiments described herein, impairment of respiration of the trout was not sufficiently great to cause mortality

of the animal by approximately 50 *C. concavicornis* cells ml$^{-1}$ seawater within the 72 h of the *C. concavicornis* treatment. However, most of the parameters assayed showed significant increases (or decreases) with time. Had the *C. concavicornis* treatment of the fish proceeded beyond 72 h, mortality of the fish would likely have occurred due to this harmful diatom.

# CHAPTER TWO


Leucocytopenia and High Mortality of Yearling Chinook Salmon

(*Oncorhynchus tshawytscha*) Caused by *Chaetoceros*

*concavicornis*

# INTRODUCTION

Previous research has shown that the spined (with barbs) diatoms *Chaetoceros concavicornis* and *C. convolutus* are overtly lethal to salmonids at concentrations in excess of approximately 5 cells mL$^{-1}$ (Bell *et al.* 1974). Albright *et al.* (in press) have subsequently shown that at several seawater sites along the coast of British Columbia, *C. concavicornis* and *C. convolutus*, at concentrations less than 5 cells mL$^{-1}$, may exacerbate penned salmonid mortality rates due to diseases such as vibriosis and bacterial kidney disease.

In the previous chapter, it has been shown that spined and barbed cells of *C. concavicornis* irritate the respiratory epithelium of fish such as the rainbow trout such that there is an overproduction and accumulation of mucus on the primary and secondary lamellae. This accumulation of mucus inhibited oxygen uptake by the respiratory epithelium, causing the arterial blood of the finfish to become anaerobic. If the blood becomes sufficiently anaerobic, mortality of the fish occurs.

Because harmful concentrations of *C. concavicornis* had such a marked influence on various arterial blood parameters of the rainbow trout, a more thorough investigation was warranted to (1) determine if another salmonid species (i.e. chinook salmon) would show the same response in the blood components and to (2) determine the influence of this

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phytoplankter on several leucocytic components of the immune system of the salmonid e.g., neutrophils, lymphocytes, and thrombocytes.

# MATERIALS AND METHODS

*Experiment 1*

The experiment was carried out using 9 to 18 g coho salmon, which were obtained from Capilano Fish Hatchery in West Vancouver, B.C. All fish were transported to Simon Fraser University and supplied with aerated, 20 ‰ salinity water for more than one month prior to use.

Three-hundred coho were randomly divided into six groups of 50 fish each. Each group was then placed in a separate 200-L tank. The salinity, pH, temperature, and dissolved oxygen of the water were 20‰, 7.2 to 7.8, 12.5 to 13.5° C, and 8.2 to 10.2 mg $L^{-1}$, respectively during the experiment.

The water in tanks 1, 2, 3, 4 and 5 was treated daily with *C. concavicornis.* The water of tank 6 was untreated with *C. concavicornis.*

During the experiment, dead and/or moribund fish were removed each morning and the mortality rates were calculated.

*Experiment 2*

Yearling chinook salmon (*O. tshawytscha*) of 4.8 g mean weight and 8.9 cm mean fork length were provided by the Pacific Biological Station, Canada Dept. of Fisheries and Oceans, Nanaimo, B.C. The fish were transferred to the

Aquatic Facility at Simon Fraser University where they were cultured for at least four weeks in freshwater of 9.5 to 10.5° C, dissolved oxygen content of 9.3 to 9.8 mg L$^{-1}$, and pH 5.8 to 6.5 until used for experimental purposes. The fish were fed daily using a commercial salmon food.

Two weeks prior to each experiment, a group of 50 fish was placed in each of two-200 L oval culture tanks. Each tank contained 100 L of aerated seawater of approximately 20‰ salinity. The temperature, dissolved oxygen, and pH ranges in the water contained in these tanks during the course of each experiment were 10.5 to 11° C, 8.1 to 10.1 mg L$^{-1}$, and 7.3 to 7.9, respectively. Constant 12L:12D photoperiod was used.

During the course of each experiment, the water in tank 1 was treated daily with *C. concavicornis*. The water of tank 2 was untreated with *C. concavicornis*.

Throughout each experiment, dead and/or moribund fish were removed each morning and the weights and lengths of each determined. Each fish was examined for gross pathological signs and the mortality rates were calculated. Where appropriate, the gills of selected moribund fish were examined by light and scanning microscopy as described below.

At days 1,3,5, and 10 following the initiation of each experiment, ten live fish were randomly selected and removed from each tank and immediately placed in 20 ‰ salinity seawater containing the anesthetic, 2-phenoxyethanol (0.4 mL

$L^{-1}$). When anesthetized, each fish was removed from the treated seawater and measured for weight and fork length. Blood samples were taken by severing the caudal peduncle and collecting this fluid in heparinized microcapillary hematocrit tubes.

Five-$\mu$L aliquots of each blood sample were diluted in 1% NaCl solution (final dilution 1:40000), fixed with Lugol's iodine, sedimented in polypropylene tubes with a known volume of pellet, and mounted on glass microscope slides. Total blood cell counts were made on the sedimented blood samples.

Differential counts were made from air-dried, methanol-fixed, stained (hematoxylin and eosin), duplicate blood smears. All cell counts were determined using a light microscope. Absolute concentrations of erythrocytes, lymphocytes, neutrophils, and thrombocytes were calculated from the total and differential blood cell counts.

The remaining blood samples were stored on ice and then centrifuged. The resultant plasma was stored at $-20^{\circ}$ C until assayed for glucose and lactate (Pickering & Pottinger, 1987a; Pickering et al., 1982).

For light microscopy, each gill was fixed in Bouin's solution, dehydrated in ethanol, sectioned, stained (hematoxylin and eosin), and examined by light microscope.

For scanning electron microscopy (S.E.M.) each gill was fixed in 3% glutaraldehyde, post-fixed in 1% $OSO_4$, dehydrated in ethanol and propylene oxide before being

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critical point-dried and then gold-coated. Material prepared in this way was examined using an Autoscan S.E.M.

Plasma glucose concentrations were determined using a Sigma Chemical Co. (St. Louis, MO) diagnostic kit. The assay is based upon enzymatic phosphorylation of glucose by hexokinase and oxidation by glucose-6-phosphate dehydrogenase. Plasma lactate levels were determined using a Sigma diagnostic kit: the assay is based upon a single enzymatic oxidation of lactate.

*Statistical analyses*: Values for hematocrits, blood cell counts, glucose, and lactate concentrations were analyzed for differences of each variable between control and treated fish using a two-way analysis of variance (ANOVA).

Each experiment was carried out in triplicate. The data from all triplicate experiments are presented as the means +/- S.E.M. for each parameter assayed.

*Experiment 1*

*Chaetoceros number*

The mean concentration of *C. concavicornis* cells in tanks 1, 2, 3, 4 and 5 remained at 4 to 6, 15 to 19, 24 to 29, 36 to 40, 46 to 52 cells $mL^{-1}$, respectively, during the experiment (Fig. 19).

*Cumulative mortality rates of C. concavicornis-treated fish*

No fish died in the untreated control group (tank 6) during the course of the experiment while a total of 1, 4, 8, 15 and 21 fish died in tanks 1, 2, 3, 4 and 5, respectively. The data were pooled and presented as cumulative mortalities. The mean cumulative mortality rates of the fish treated with *C. concavicornis* in tanks 1, 2, 3, 4 and 5 were 2, 8, 16, 30 and 42%, respectively, within 5 days of treatment (Fig. 20).

*Experiment 2*

*Chaetoceros number*

The mean concentration of *C. concavicornis* cells remained at between 47 and 57 cells $mL^{-1}$ during the experiment (Fig. 21).

Fig. 19. The mean concentration of *C. concavicornis* cells in treated tanks 1, 2, 3, 4, and 5. Error bar indicates +/- s.e.m.

The y-axis is labeled CHAETOCEROS CELLS ml$^{-1}$ with values 0, 10, 20, 30, 40, 50, 60. The x-axis is labeled DAYS with values 0, 1, 2, 3, 4, 5, 6. Legend: tank1, tank2, tank3, tank4, tank5.
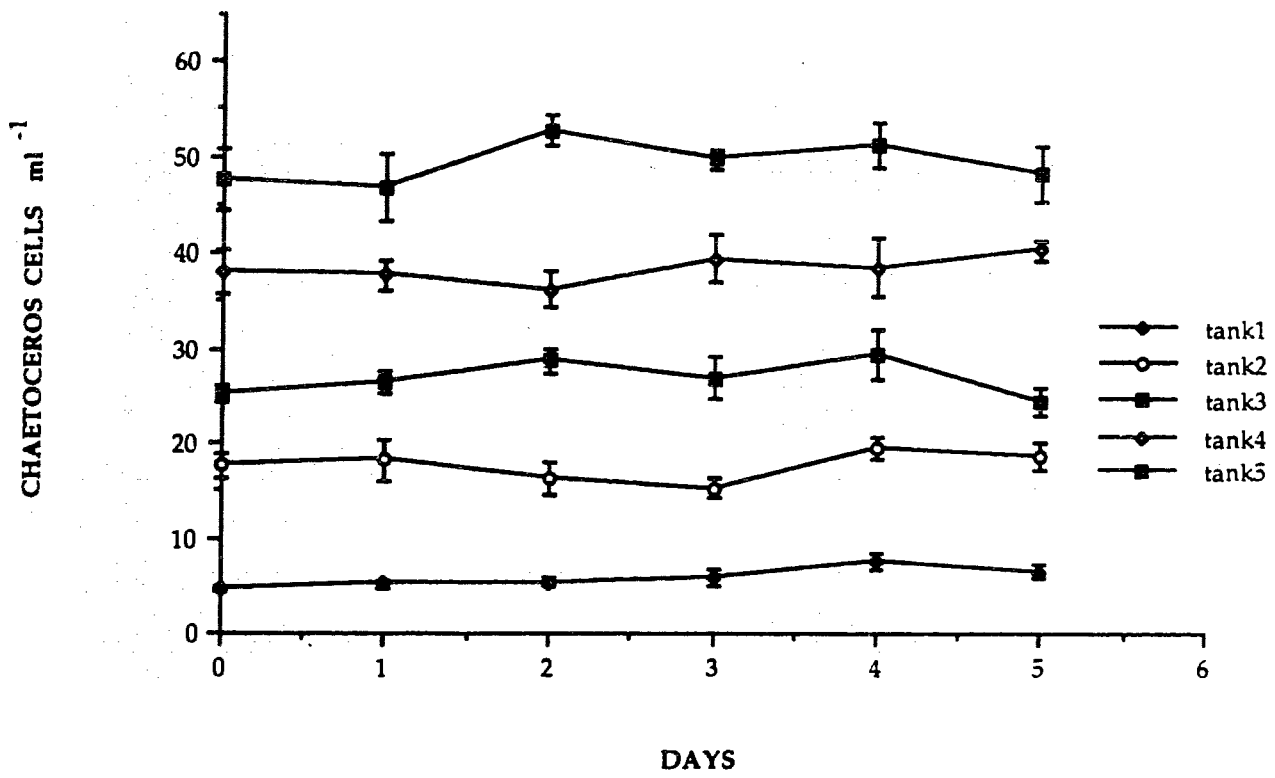
Fig. 20. The cumulative mortalities of *C. concavicornis* treated fish in tanks 1, 2, 3, 4, and 5 and untreated control fish in tank 6.
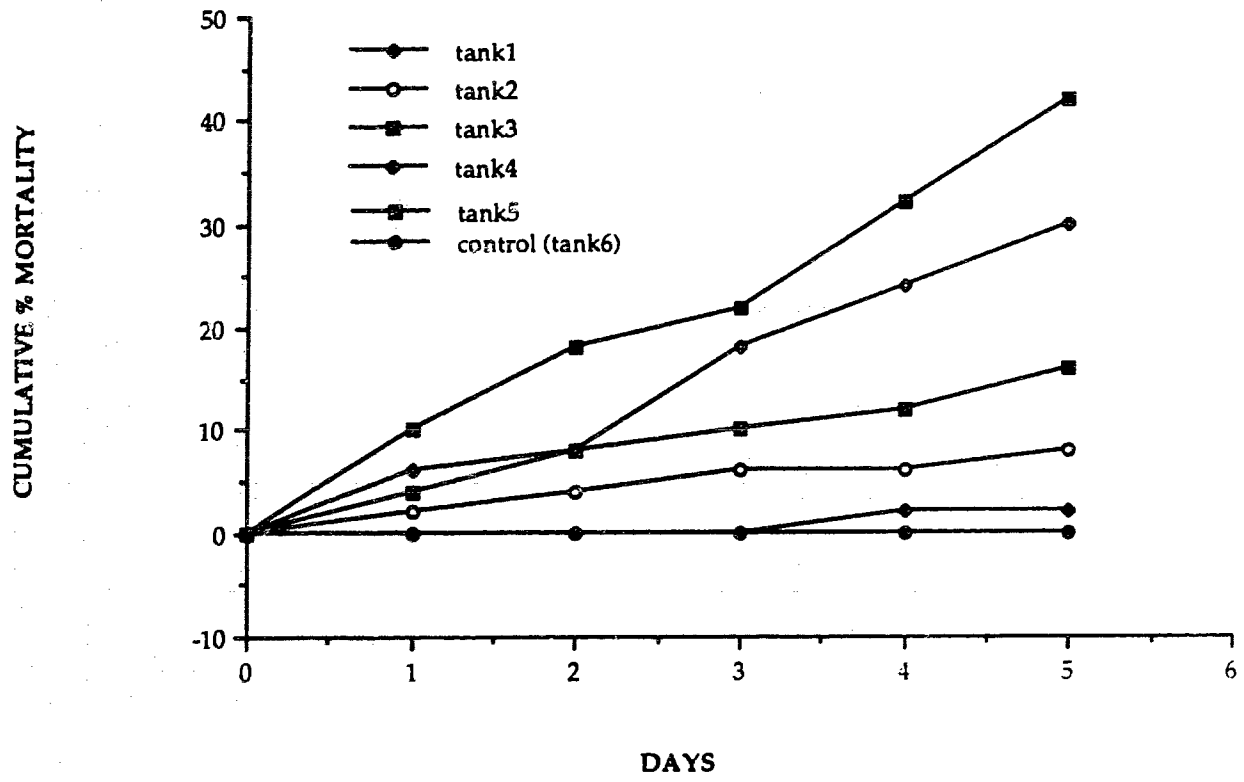
Fig. 21. The mean (of three trials) concentration of *C. concavicornis* cells in treated seawater. Error bar indicates +/- s.e.m.

CHAETOCEROS NUMBER

(cells ml$^{-1}$)

DAYS
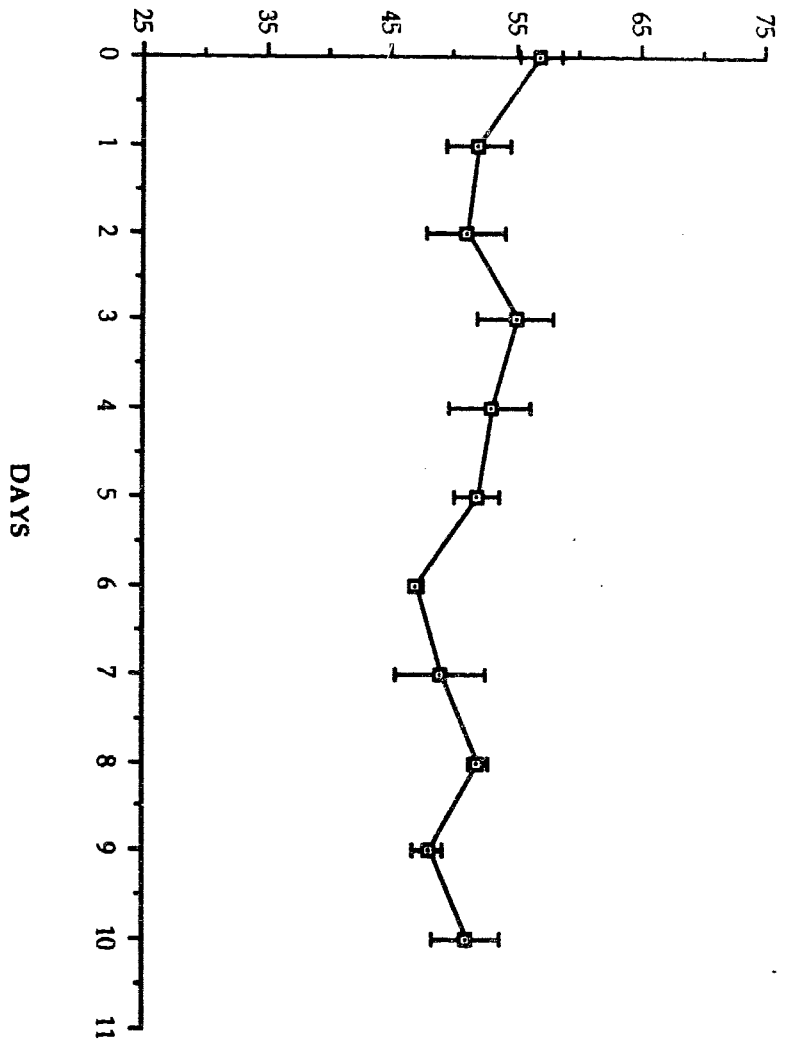
*Gross examination of chinook salmon treated with C. concavicornis*

C. *concavicornis*-treated fish moved slowly, sought the edge of the tank and the water surface, and collected around the aeration bar. Excessive amounts of yellow to green-colored mucus mixed with detritus (mainly algal remnants) collected on the gill surface of moribund fish. The mouths and opercula of dead fish remained open. The gills and pelvic fins of moribund fish were hyperemic.

*Histopathology*

Some of the spines of C. *concavicornis* became imbedded in the secondary lamellae (Fig. 22A). After 48 h of exposure to C. *concavicornis*, there were some degenerative changes, necrosis, and exfoliation of the secondary lamellae cells in treated fish (Fig. 22B).

Fig. 22. A. A spine (arrow) of *C. concavicornis* imbedded in a secondary lamella of a chinook salmon; the lamellae show local fusion, hypertrophy, and hyperplastic changes. B. Necrosis and exfoliation of secondary lamellae cells and congestion in the capillaries of gill in chinook salmon exposed to *C. concavicornis* for 48 h.
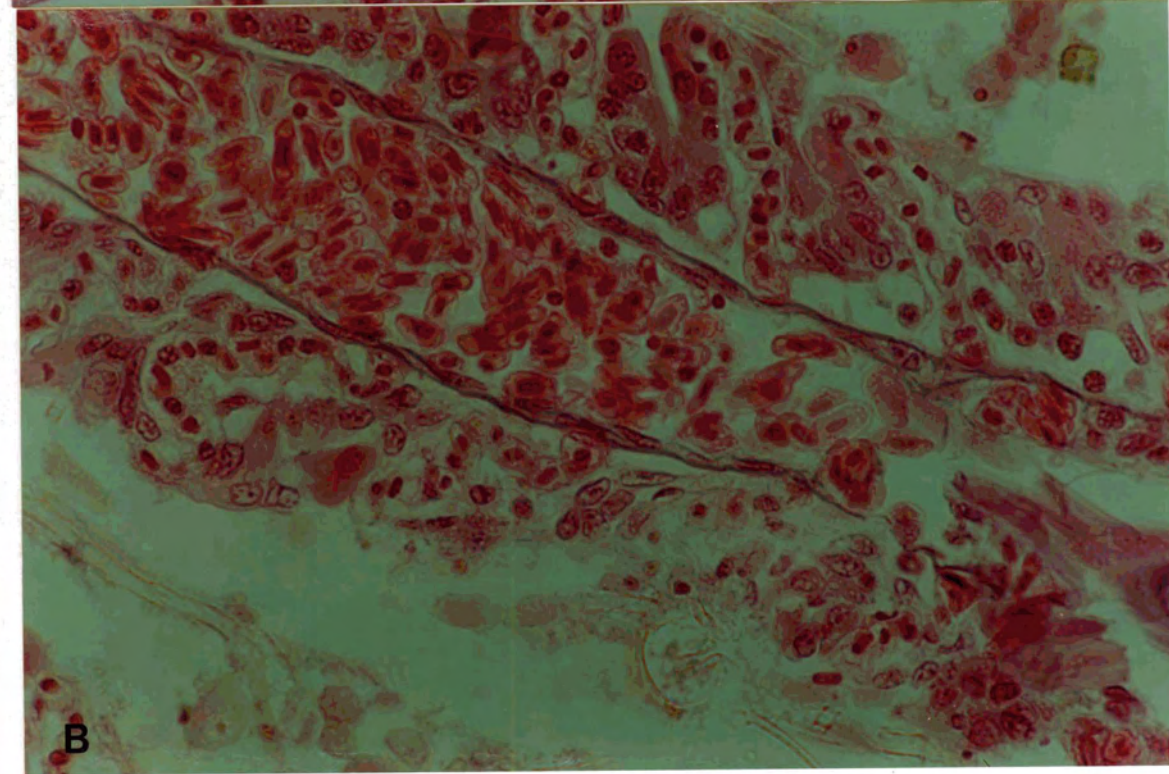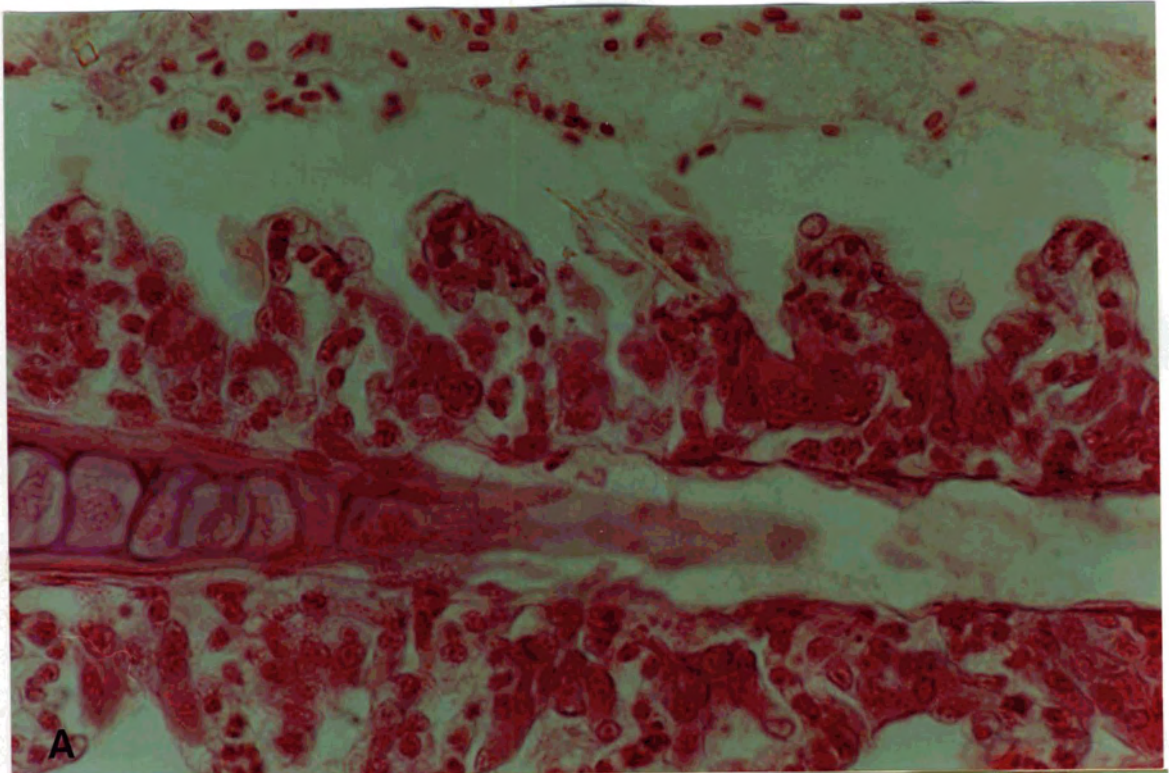
There were also intra-epithelial edema, small hemorrhages, and local congestion of the secondary lamellae in treated fish (Fig. 22 A and B).

Gill damage increased with increased exposure time to *C. concavicornis*. With time, increased necrosis, edema, and detachment of the epithelium of the secondary lamellae were observed. Local fusion, hypertrophy, and hyperplasia (Fig. 22A) appeared in the secondary lamellae.

*SEM examination*

Scanning electron microscopic (SEM) examination of the gill tissue of moribund fish which were treated with *C. concavicornis* showed that many barbed spines were trapped between the secondary lamellae and some were embedded in the lamellar tissue (Figs. 23 and 24). After 24 h of *C. concavicornis* treatment, microridge degeneration of filament cells was widespread in treated fish (Fig. 25). Goblet cells of the primary lamellae of treated fish (Fig. 25) were much more prominent in size and number than those of the untreated control fish following 144 h of exposure. There was excessive mucus on and between the secondary lamellae of treated fish.

Fig. 23. Scanning electron microscopic view of chinook salmon treated with *C. concavicornis* showing many barbed spines trapped in the inter-secondary and primary lamellae space.
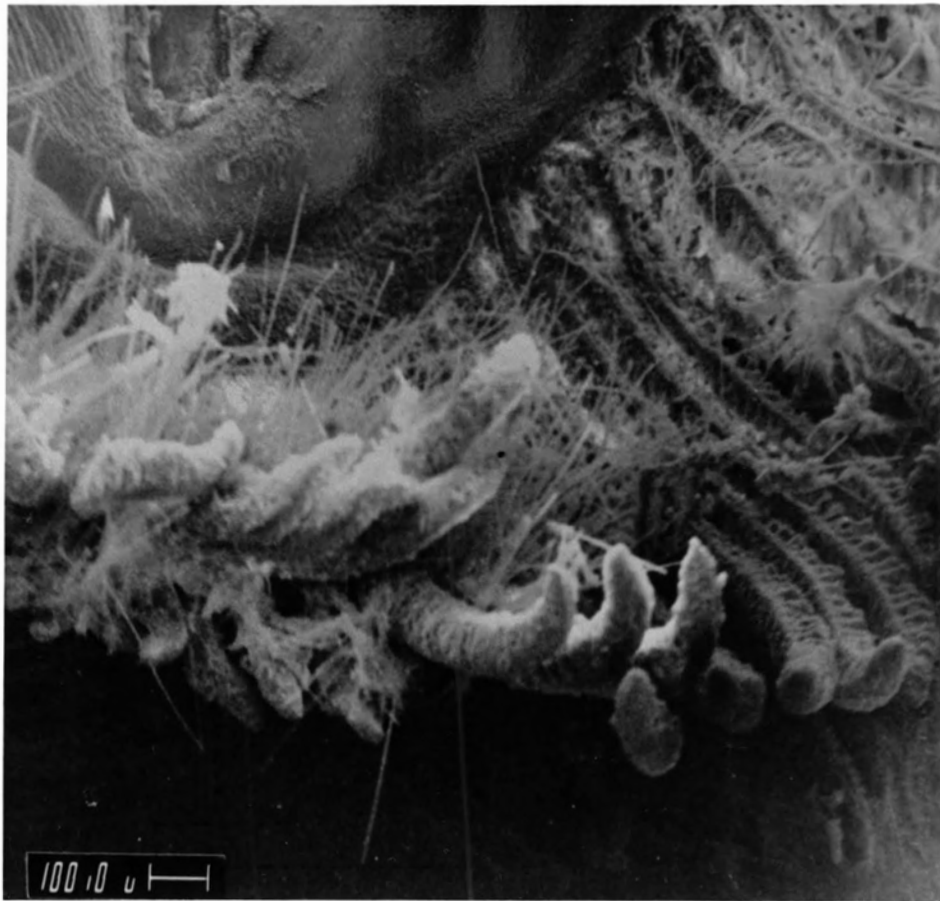
Fig. 24. Scanning electron microscopic view of chinook salmon treated with *C. concavicornis* for 48 h showing barbed spines embedded in the lamellar tissue and excessive mucus on the surface of the secondary lamellae.
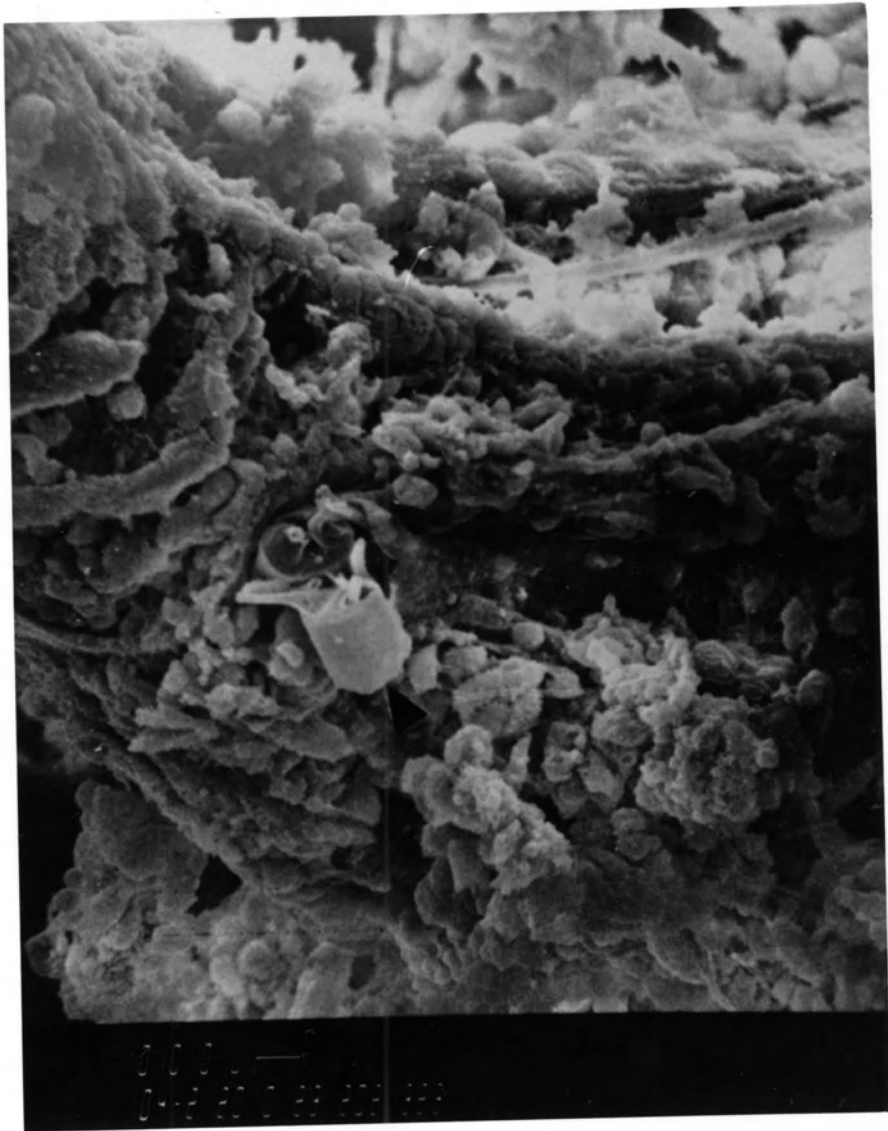
Fig. 25. Scanning electron microscopic view of the goblet cells and the microridges present on the primary lamellae of untreated (A) and treated (B) chinook salmon exposed to *C. concavicornis* for 144 h.
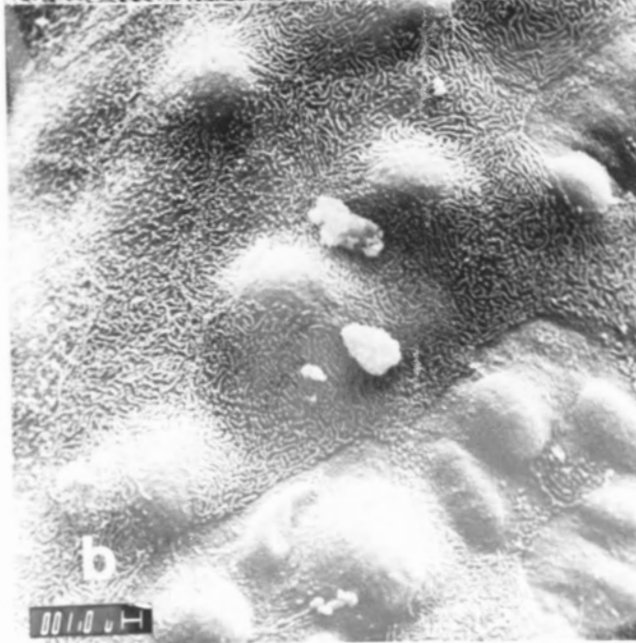
One mortality occurred in the untreated control group during the course of the experiment while a total of 46 fish died in the treated group. The data were pooled and presented as cumulative mortalities. These were calculated by the formula, $Zn = (lnN_T - lnN_t)/(t - T) + Z_{(n-1)}$, where $Zn$ is cumulative mortality of fish at n day(s). $N_T$ and $N_t$ are the numbers of fish at $T$ and $t$ days, respectively. T is the day on which fish were first sampled and t is the day on which fish were next sampled (Pickering and Pottinger, 1988). The mean cumulative mortality rate of the fish treated with *C. concavicornis* was approximately 35% within 10 days of treatment. The mortality rate of the untreated control fish was 0.60% (Fig. 26).

*Hematocrit values.*

Immediately prior to *C. concavicornis* treatment the mean hematocrit values of the treated and untreated fish were 37.0 and 37.8, respectively. Following treatment with the phytoplankter, the hematocrit value of the treated fish increased to a mean of 47.2 within 24 h (Fig. 27). The hematocrit value of the untreated fish showed a minor increase at 24 h followed by a slow decline to day 10 (Fig. 27).

Fig. 26. The mean (of three trials) cumulative mortalities of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m.
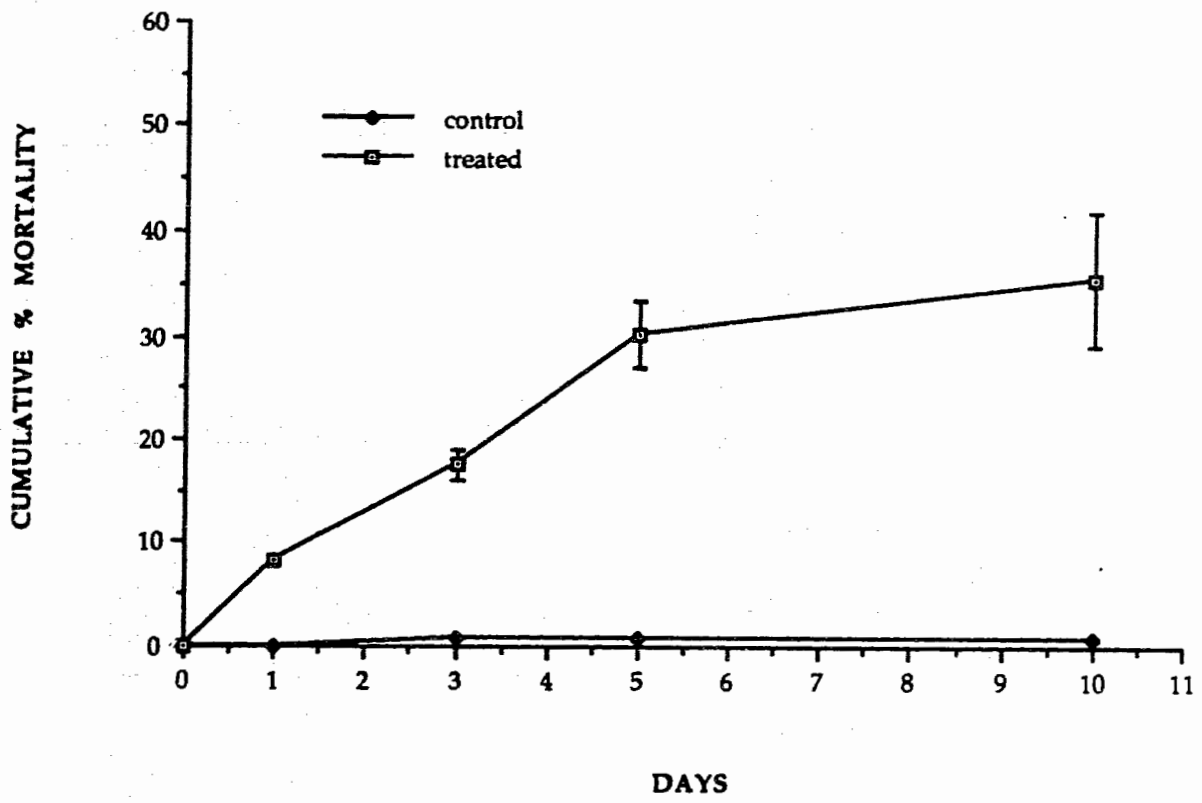
Fig. 27. The mean (of three trials) hematocrit values of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m. *, ** and *** indicate significant differences from the control values at the same sampling time (P<0.05), (P<0.01) and (P<0.001), respectively.
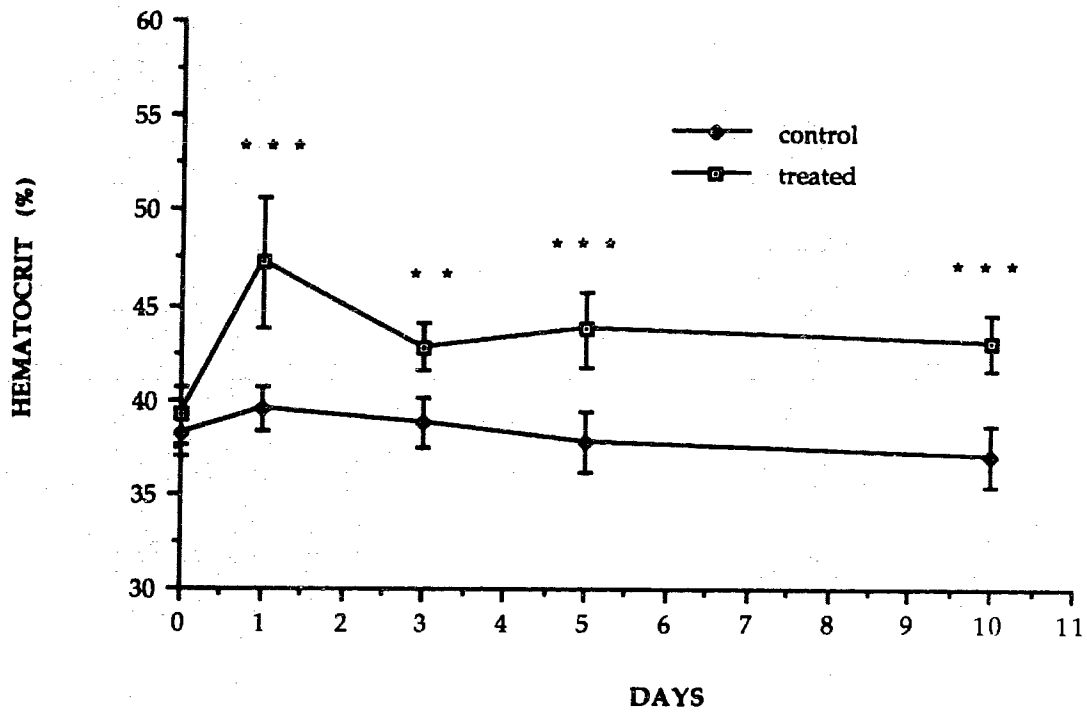
Although the hematocrit value of the treated fish decreased somewhat following the sharp rise at 24 h, the values of the treated fish remained above those of the control fish for the 10 days of the experiment. The hematocrit values in the treated fish were significantly different from those of the untreated control fish at day 1 ($P<0.001$), day 3 ($P<0.01$), day 5 ($P<0.001$) and day 10 ($P<0.001$).

*Blood erythrocyte concentrations.*

Immediately prior to *C. concavicornis* treatment the mean counts of erythrocytes in the blood of the treated and control fish were $7.64 \times 10^5$ and $7.76 \times 10^5$ $\mu L^{-1}$, respectively. While the concentration of erythrocytes remained approximately the same in the control fish, the concentration of erythrocytes in the blood of the treated fish greatly increased to $1.03 \times 10^6$ $\mu L^{-1}$ within 24 h of treatment with *C. concavicornis* (Fig. 28). Following this initial sharp increase in numbers, the erythrocyte concentration in the blood slowly decreased to day 10 at which time the experiment was stopped. During the entire 10 day treatment, the circulating erythrocyte concentrations in the treated fish were significantly different from those in the untreated controls at day 1 ($P<0.01$) and day 5 ($P<0.05$).

Fig. 28. The mean (of three trials) blood erythrocyte concentrations of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m. and * indicates significant difference from the control value at the same sampling time (P<0.05).
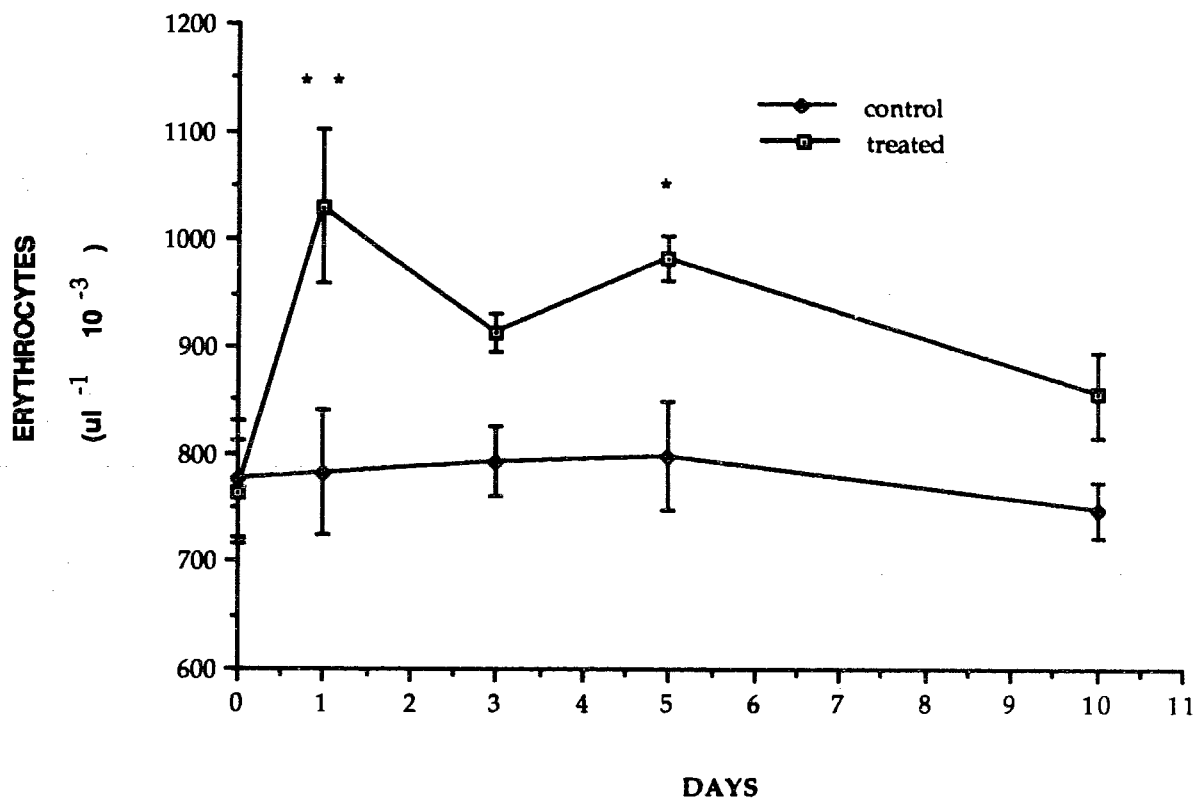
*Blood neutrophil concentrations.*

The neutrophil concentration of the blood of both the treated and untreated control fish decreased for the first five days of the experiment. The neutrophil concentration of the control fish regained its pre-treatment concentration, but that in the treated fish did not; the neutrophil concentrations in the treated fish remained depressed (Fig. 29). A significant difference between the treated and untreated fish occurred at day 10 (p<0.05).

*Blood lymphocyte concentrations.*

The mean concentrations of lymphocytes from blood of the treated and untreated fish immediately prior to *C. concavicornis* treatment were $1.1 \times 10^4$ and $1.15 \times 10^4$ $\mu L^{-1}$, respectively. During the 10 day course of the experiment, the lymphocyte concentrations in the control fish remained approximately constant while there was an initial (within 24 h) drop in the concentration of the treated fish to $4.9 \times 10^3$ $\mu L^{-1}$ (Fig. 30). Following this initial drop, the lymphocyte concentrations in the treated fish remained low. The mean lymphocyte concentrations in the circulating blood of the treated fish were significantly less than those of the control fish at days 1 (P<0.01), day 3 (P<0.01), day 5 (P<0.01) and day 10 (P<0.01).

*Blood thrombocytes*

The mean concentrations of thrombocytes from blood of treated and untreated fish immediately prior to *C. concavicornis* treatment were 1.1 x $10^4$ and 1.19 x $10^4$ $\mu L^{-1}$, respectively. Twenty-four h after treatment with *C. concavicornis* a sharp drop to 4.7 x $10^3$ thrombocyte cells $\mu L^{-1}$ occurred. Following this initial drop, the thrombocyte concentration continued to gradually decrease with time (Fig. 31).

The thrombocyte concentration of untreated fish showed a minor drop within 24 h; however, during the remainder of the experiment, there was a gradual increase in the concentration of these cells. The mean thrombocyte concentrations in the circulating blood in the treated fish were significantly less than those of the control fish at day 3 ($P<0.05$), 5 ($P<0.01$) and 10 ($P<0.001$).

In several cases, thrombocytes were observed to aggregate in the blood of treated fish. The number of thrombocytes per cluster varied from several to 30.

Fig. 29. The mean (of three trials) blood neutrophil concentrations of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m. and * indicates significant difference from the control value at the same sampling time (P<0.05).
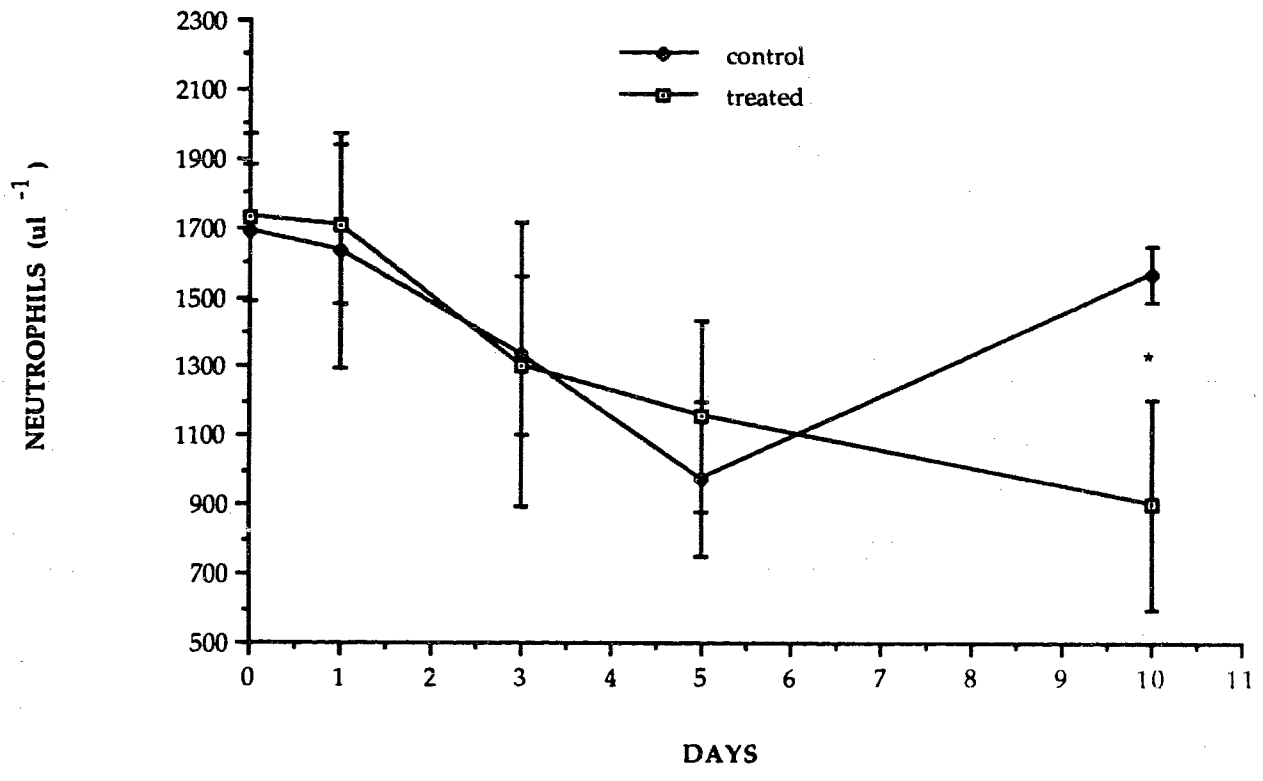
Fig. 30. The mean (of three trials) blood lymphocyte concentrations of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m.* and ** indicate significant differences from the control values at the same sampling time (P<0.05) and (P<0.01), respectively.
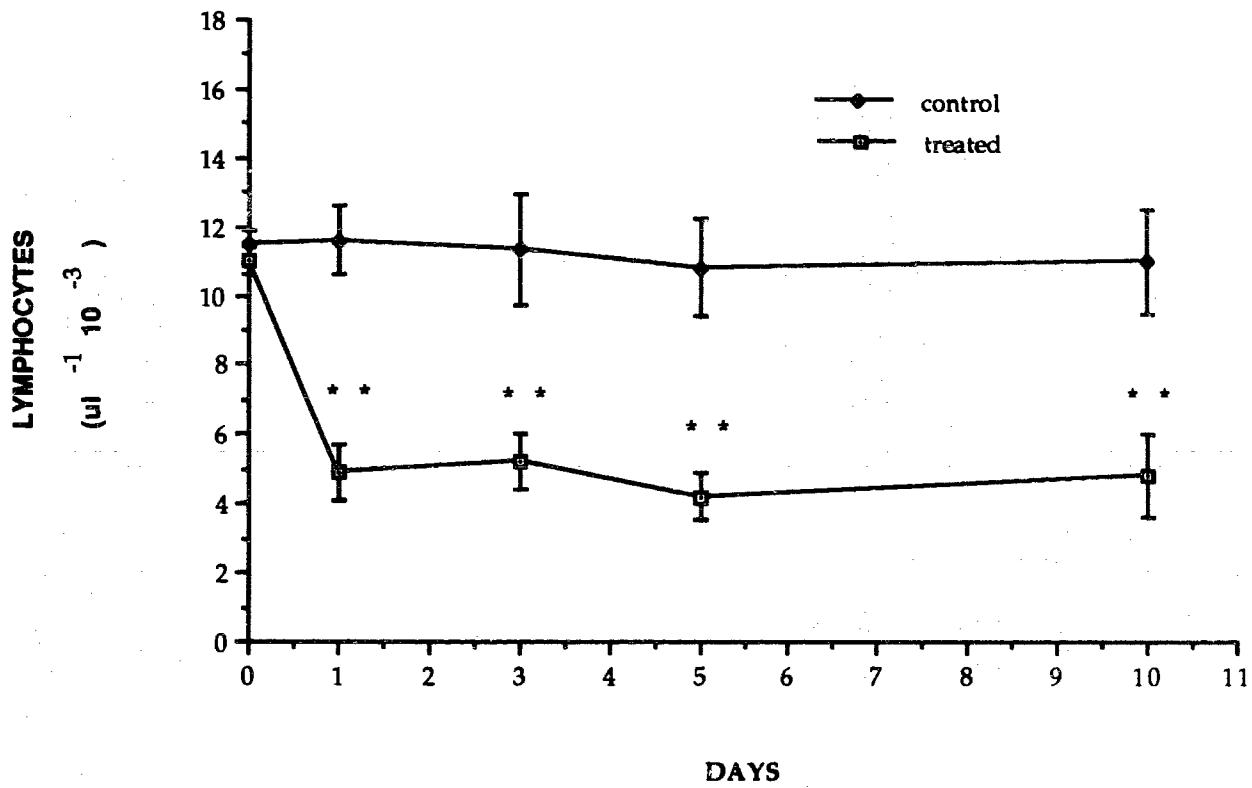
Fig. 31. The mean (of three trials) blood thrombocyte concentrations of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m.* and ** indicate significant differences from the control values at the same sampling time (P<0.05) and (P<0.01), respectively.
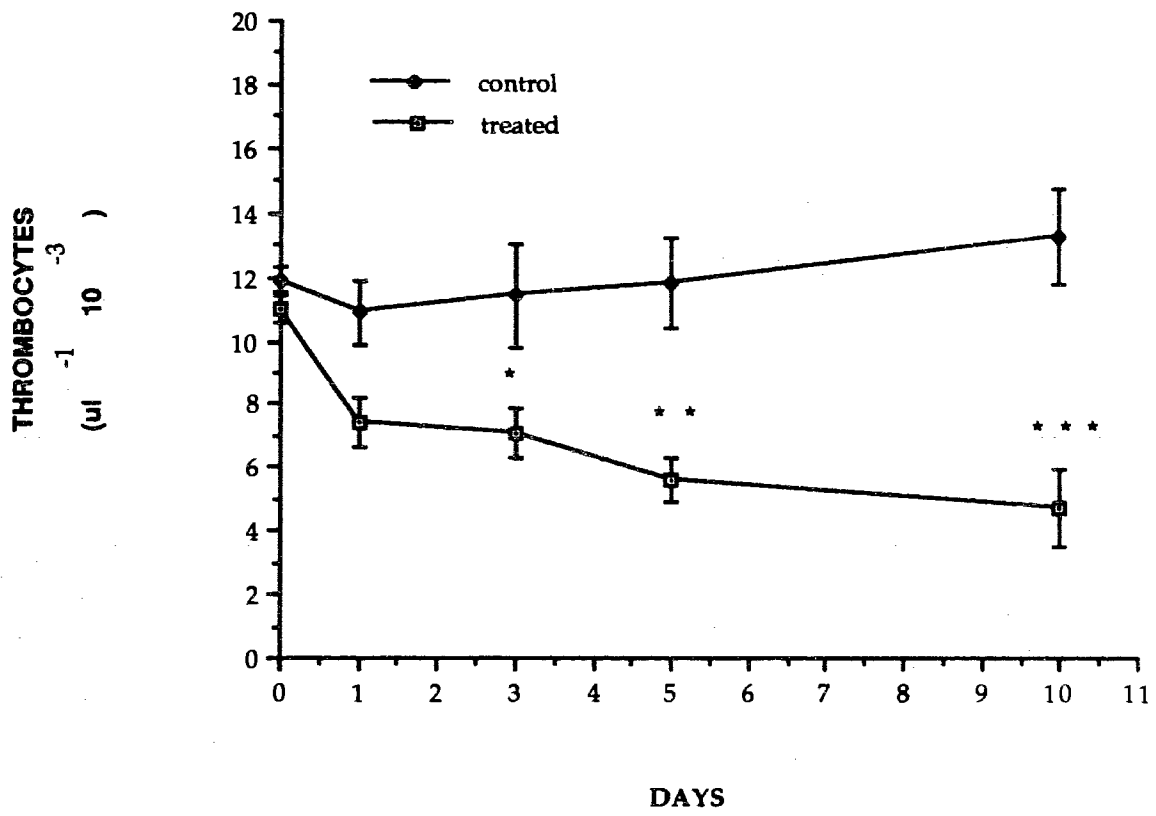
*Plasma glucose concentration*

The plasma glucose concentrations of the treated fish greatly increased from 57.0 mg dL$^{-1}$ immediately prior to *C. concavicornis* treatment to 83.8 mg dL$^{-1}$ within 72 h of the initiation of the treatment. After 72 h, the mean glucose concentration gradually decreased (Fig. 32). At time 0, the mean glucose concentration of the untreated control fish was 56.0 mg dL$^{-1}$. And, this concentration remained approximately the same during the 10 day duration of the experiment. The mean plasma glucose concentrations in the treated fish were significantly different from those of the untreated control fish at day 1 ($P<0.01$), day 3 ($P<0.001$), day 5 ($P<0.01$), and day 10 ($P<0.01$).

*Plasma lactate concentration*

The plasma lactate concentrations of the treated fish increased from 30.1 mg dL$^{-1}$ to 51.7 mg dL$^{-1}$ during the 10 day course of the treatment with *C. concavicornis* (Fig. 33). The mean lactate concentration within the blood of the control fish remained within the range of 31.1 to 33.8 mg dL$^{-1}$ during the course of the 10 day treatment. The plasma lactate concentrations in the treated fish were significantly different from those of the untreated control fish at day 1 ($P<0.05$), 3 ($P<0.01$), day 5 ($P<0.01$) and day 10 ($P<0.001$).

Fig. 32. The mean (of three trials) plasma glucose
concentrations of *C. concavicornis* treated and untreated
control fish. Error bar indicates +/- s.e.m.* and **
indicate significant differences from the control values at
the same sampling time (P<0.05) and (P<0.01), respectively.
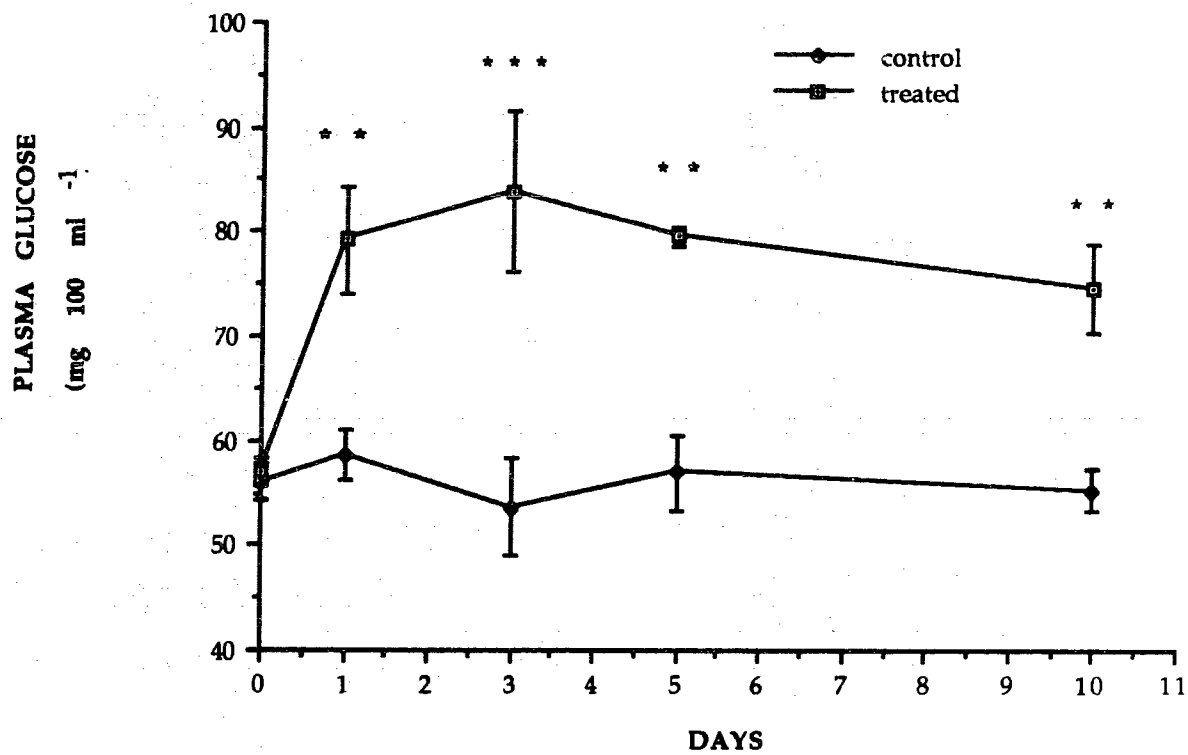
Fig. 33. The mean (of three trials) plasma lactate concentrations of *C. concavicornis* treated and untreated control fish. Error bar indicates +/- s.e.m.* and ** indicate significant differences from the control values at the same sampling time (P<0.05) and (P<0.01), respectively.
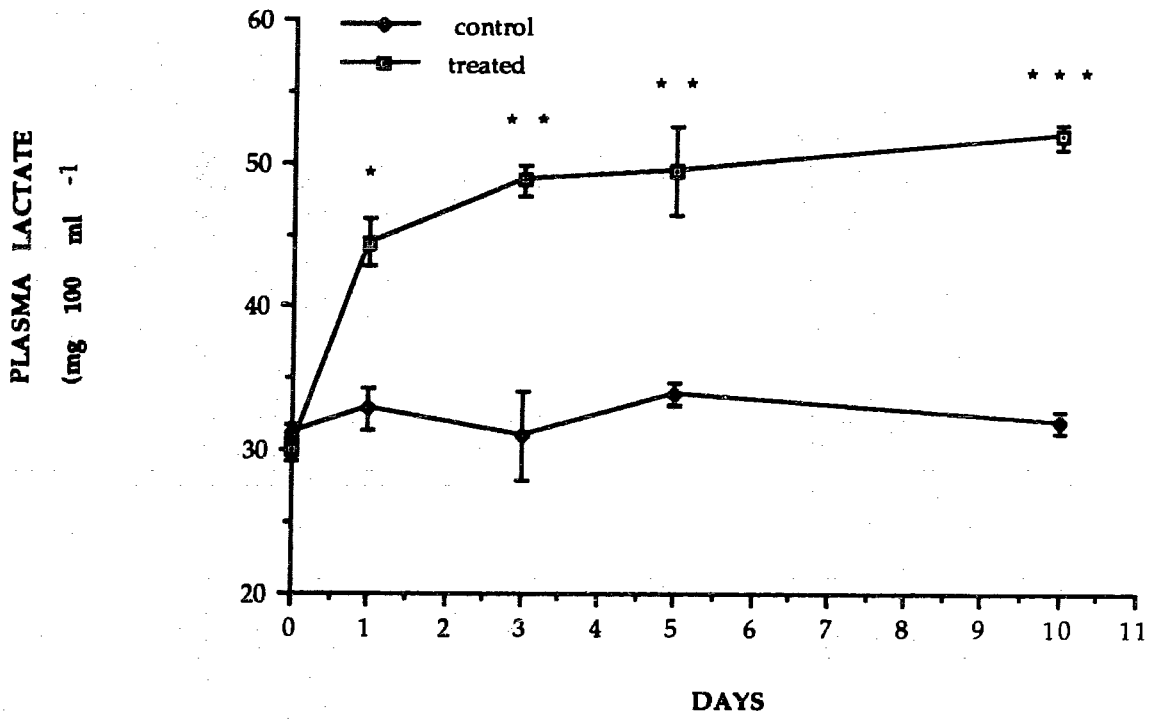
DISCUSSION

In the previous study on rainbow trout many cells of *C. concavicornis* became "wedged" in the inter-secondary and primary lamellar spaces. It was also noted that some of the barbed spines of this phytoplankter penetrated into the lamellar cells. In all cases where the trout were cultured in lethal concentrations of *C. concavicornis*, excessive amounts of mucus accumulated on and between the lamellae. In the investigation with chinook it was noted that there was a similar accumulation of large numbers of *C. concavicornis* cells in the inter-lamellar spaces of the secondary and primary lamellae along with an accumulation of mucus on the respiratory epithelium of the treated species occurred (Figs. 23 and 24). There also was a similar penetration of spines of *C. concavicornis* into the lamellar cells shown not only by S.E.M. (Fig. 24) but also by histopathological examination (Figs. 22A). Based upon these observations it would appear that the responses of the gill tissues of both the chinook salmon examined herein and the rainbow trout previously examined are similar.

When the topography of the lamellae surfaces was examined, harmful effects of *Chaetoceros* cells on microridges were also observed (Figs. 23 and 24). The microridges of the lamellae of untreated chinook salmon were well developed (Fig. 25A). However, following 72 h exposure to *C. concavicornis* the microridges became much less defined

(Fig. 25B). In addition to the apparent loss of microridges that occurred in the presence of *C. concavicornis* it was also noted that there was an increase in the prominence and number of goblet cells per unit area of the respiratory epithelium in the treated chinook salmon in comparison to that of the untreated salmon (Figs. 25A and 25B). Based upon these observations, it would appear that the microridges may become 'coated' and therefore "masked" by the accumulation of the appreciable amounts of mucus produced by the goblet cells which were more active in the presence of *Chaetoceros*. Since microridges are likely used to increase the surface area of the primary lamellae (Hughes, 1979; Fishelson, 1980), their diminution in the presence of harmful *Chaetoceros* spp. is another factor limiting the exchange of gases and metabolic wastes at the gills. Histological analysis of this tissue further shows degeneration, necrosis, exfoliation (Fig. 22B), intraepithelial edema, hypertrophy, and hyperplasia (Fig. 22A) of secondary lamellar cells. Unlike rainbow trout, the hemorrhages in the gills of treated chinook salmon were pronounced. The histopathological changes increased with the exposure time of fish to *C. concavicornis*.

The present investigation showed that the treatment with *C. concavicornis* caused mortality even at concentrations as low as about 15 cells $ml^{-1}$ seawater. The cumulative mortality rate of coho salmon was higher than that of chinook salmon when they were exposed to about the

same concentration of *C. concavicornis*. The death of the fish appeared to be from hypoxia, acid-base disturbance, and, eventually, respiratory failure which were caused by the accumulation of excessive amounts of mucus on the respiratory epithelium. The increased numbers of circulating erythrocytes in treated fish (Fig. 28) also support the observation that fish became hypoxic.

The increase in hematocrit values and erythrocyte concentrations of the yearling chinook that we observed upon treatment of the chinook salmon with the phytoplankter, *C. concavicornis* (Figs. 27 and 28), has also been observed by other investigators when they applied stressors other than harmful phytoplankton to finfish, including rainbow trout and salmon. Holeton and Randall (1967a) observed that the hematocrit value of rainbow trout arterial blood increased under the stress of hypoxia. This was apparently the result of cellular swelling, as it was not accompanied by an increase in the erythrocyte count. Swift and Lloyd (1974) observed that the elevation of hematocrits was seen for populations of rainbow trout exposed to crowded and high-temperature conditions. In this latter case, hemoconcentration was attributed to a stress-mediated release of erythrocytes from storage organs. Similarly, McLeay (1975) observed that erythrocyte counts in coho salmon held under crowded high-temperature conditions were also elevated. Privolnev (1954) and Ostroumova (1964) both found that erythrocytes entered into arterial circulation,

likely from the spleen, under the influence of hypoxia. Hall (1928) showed that in several marine fish, asphyxia caused contractions of the spleen.

Based upon these earlier observations and the present data, it would appear that the increases in hematocrit values and erythrocyte concentrations noted in Figs. 27 and 28 may be mainly due to entry of erythrocytes into the circulatory system, from storage organs, such as the spleen. Swelling of erythrocytes may have accounted, in part, for the increased hematocrits (Fig. 27) although it is unlikely that this was a major factor based upon the observations of others (see above) and the fact that the relative increase in the hematocrits was less than that of the increase in erythrocyte numbers (Fig. 28). It is possible that erythropoiesis contributed to the elevated hematocrits and erythrocyte values because these were still elevated after 24 hours.

The rather mild manipulations that the untreated control fish underwent during these experiments did not affect any of the blood components which were assayed, except for the neutrophil component (Fig. 29). The neutrophils of the control fish decreased for at least 5 days following the handling stress that occurred at time zero, when the experiment was initiated. Yet, by day 10, neutrophil concentration in the control fish had rebounded to the levels present prior to the initiation of the experiment (Fig. 29).

The literature is ambiguous with respect to the influence of stress on the concentration of circulating neutrophils in finfish. Lymphocytopenia and a simultaneous neutrophilia were demonstrated by Johansson-Sjobeck et al. (1978) and Ellsaesser and Clem (1987). Pickering (1984) and Pickering and Pottinger (1985) did not find any effect of transitory or chronic cortisol administration on the concentrations of circulating neutrophils in brown trout. However, Wiik et al. (1989) reported that treatment with cortisol for 1 day caused neutrophilopenia. It would appear that the neutrophilopenia in our control fish was a stress response to the manipulation of the fish needed to get the experiment underway. The occurrence of neutrophilopenia in the control fish probably explains why concentrations of neutrophils in the blood did not show significant difference between treated and control ones in the first several days during the experiment.

The present study revealed a marked and highly significant suppression in the concentration of circulating lymphocytes and thrombocytes in the C. concavicornis treated fish (Figs. 30 and 31). This type of lympho- and thrombocytopenia is a typical response of salmonids to a wide variety of acute and chronic stressors (Pickering et al., 1982; Pickering and Pottinger, 1987b). Evidence is now accumulating that lymphoid tissue and leucocyte activity in fish are strongly suppressed by corticosteroids (Pickering, 1984). Pickering (1986) reported that a marked

lymphocytopenia occurred during sexual maturation, which was closely correlated with elevated plasma cortisol levels in brown trout (*Salmo trutta*). Ellsaesser and Clem (1987) have demonstrated that both the number and the immunological competence of circulating lymphocytes in the blood of channel catfish, *Ictalurus punctatus*, are reduced during stress. Wiik *et al.* (1989) have shown that cortisol treatment caused a clear reduction of circulating lymphocytes and thrombocytes of Atlantic salmon (*Salmo salar*). Klinger *et al.* (1983) observed a decrease in plasma thrombocyte concentrations with increased stocking density of channel catfish. Pickering and Pottinger (1987b) showed that chronic crowding stress caused a marked and prolonged reduction in the circulating thrombocytes of both brown trout and rainbow trout. Pickering and Pottinger (1987a) also showed that a marked lymphocytopenia and thrombocytopenia occurred in Atlantic salmon S2 parr which had chronically elevated blood cortisol levels during the winter months. Wiik *et al.* (1989) observed that in the blood of Atlantic salmon (*Salmo salar*) treated with cortisol for four days, the thrombocytes became aggregated. They suggested that clustering was an effect of cortisol administration. They found that the fish which showed the clustering phenomenon were very susceptible to cold-water vibriosis. The thrombocyte clusters were also observed in the blood of coho exposed to *C. concavicornis*.

The neutrophils, lymphocytes, and thrombocytes of salmonids are significant components of the immune system of these fish (Anderson, 1974; Corbel, 1975; Post, 1978). Neutrophils play a role in non-specific defence system of fish. B-lymphocyte-like cells are involved with the humoral response and T-lymphocyte-like cells probably are involved in the cellular response. Thrombocytes mediate blood clotting. Because of this, the reduction in the quality and quantity of these cells would have a marked influence upon the immunocompetence of finfish. In this connection, the observations in chapter four are significant. When the water in which rainbow trout were suspended was treated with approximately 50 cells $mL^{-1}$ of *C. concavicornis* and 7.7 x $10^5$ cells $mL^{-1}$ of *V. anguillarum* all of the fish died with vibriosis within four days of what would otherwise have been a non-lethal challenge with this bacterium. The data reported herein suggest that this may be, at least in part, due to suppression of the neutrophil, lymphocyte, and thrombocyte components of the salmonid's immune system.

The increases in both glucose and lactate concentrations of the blood of both the chinook salmon (Figs. 32 and 33) and rainbow trout (chapter one) showed a very similar pattern: i.e. in all cases, there were sharp increases of the parameters within 72 h of treatment of the fish with harmful *C. concavicornis*. These observations, in addition to those noted above, indicate that the response of rainbow trout and coho (McLeay and Brown 1979) and chinook

salmon appear to be similar, at least with regard to these three parameters.

Hyperglycemia is, perhaps, the most frequently used indicator of stress responses in teleost fishes yet its magnitude and duration are strongly dependent upon the strain and nutritional status of the fish and on the environmental temperature (Nakano and Tomlinson, 1967; Wendt and Saunders, 1973; Wydoski et al., 1976). In the present study, treatment of chinook salmon with C. concavicornis caused increased plasma glucose concentrations (Fig. 32). Although the precise endocrine control in salmonids requires further study, there is evidence that the catecholamines are directly involved in regulating the concentration of blood glucose (Nakano and Tomlinson, 1967; Mazeaud et al., 1977) and the experimental administration of adrenaline to the carp induces a marked hyperglycemia. The corticosteroids are also indirectly implicated (Klinger et al, 1983) and cortisol injection causes a substantial elevation of blood glucose in the goldfish (Young and Chavin, 1965; Chavin and Young, 1970) but not, apparently, in the plaice, Pleuronectes platessa (Wardle, 1972). Short-term exposure of salmonids and other fish species to hypoxia has been shown to cause marked elevations in blood glucose and lactic acid levels and marked decreases in liver and muscle glycogen reserves (Heath and Prichard, 1965). Sub-acute or chronic (2 weeks - 9 months) exposure of salmonid and other teleost fishes to a variety of toxicants caused slight but

significant elevations in blood sugar levels (McLeay and Brown, 1979). The time taken for complete stabilization of blood glucose levels is influenced by the time course and severity of the stresses (Barton *et al.* 1986). Thus, the hyperglycemic response in coho salmon smolts to a chronic, crowding stress lasted more than 16 days (Wedemeyer, 1976), whereas the response of the same species to a mild, acute handling stress permitted recovery within approximately 24 h (Wedemeyer, 1972). In the present study, the *C.concavicornis* concentrations were continuously held at between 47 and 57 cells $mL^{-1}$ for 10 days. Thus, the hyperglycemic response of treated fish to this sub-acute stressor lasted for more than 10 days.

Under hypoxia, lactate is mainly produced by anaerobic muscle metabolism in the teleost fish (Joshi, 1978). Jorgensen and Mustafa (1980) reported that increased plasma lactate levels were found in different species of teleosts following hypoxia. Wendt(1967) observed that the incipient lethal level for lactate in the blood of Atlantic salmon is approximately 200 mg 100 $mL^{-1}$. The level of approximately 70 mg 100 $ml^{-1}$ observed in the present study makes it unlikely that this, in itself, would have constituted a severe threat to the survival of the yearling chinook salmon.

# CHAPTER THREE.


# The Elevation of Plasma Cortisol and the Hypertrophic Response of Interrenal and Chromaffin Tissues of Rainbow Trout (*Oncorhynchus mykiss*) to the Harmful Diatom, *Chaetoceros concavicornis*

# INTRODUCTION

As few as 5 cells of the harmful phytoplankters, *Chaetoceros concavicornis* and *C. convolutus* $mL^{-1}$ of seawater can kill salmonids (Bell *et al*. 1974). In chapter 1, the studies showed that the barbed spines of *C. concavicornis* irritate the goblet cells of the respiratory epithelium of rainbow trout (*Oncorhynchus mykiss*), such that excess mucus is produced and accumulates on and between the secondary lamellae. This accumulation of mucus limits oxygen uptake such that hypoxic conditions occur within the salmonids.

In chapter 2, the studies demonstrated that treatment of chinook salmon (*O. tshawytscha*) with *C. concavicornis* induced neutrophilo-, lymphocyto-, and thrombocytopenia. Coho salmon (*O. kisutch*) became exceedingly susceptible to bacterial disease under both laboratory and ocean-penned conditions (Albright *et al*, in press).

The observations described above suggest that salmonids which did not die in the presence of harmful concentrations of *Chaetoceros* spp. became immuno-suppressed. Because suppression of the immune system by sub-lethal concentrations of *C. concavicornis* may be significant, it was decided to investigate several important aspects of this system in rainbow trout in the presence of this phytoplankter. The studies focussed on the chromaffin and interrenal tissues as well as the plasma cortisol response

in rainbow trout exposed to harmful concentration of *C. concavicornis*.

Chromatin is a subunit of the chromosomes which, in the interphase cell nucleus, can exist in several condensation states. These condensation states are often divided into two broad categories which are characterized by a low density or diffuse component (euchromatin or decondensed chromatin) and a high density component, referred to as heterochromatin or condensed chromatin (Hsu 1962, Brown 1966, Kiefer *et al.*, 1973).

The general relationship between the condensation state of chromatin and its transcriptional activity has been the subject of many studies. Harris (1967) showed that the reactivation of the red blood cells of chicken (the mature nuclei are in a highly condensed, inactive state) was preceded by significant chromatin decondensation, with subsequent reactivation of both DNA and RNA synthesis. Electron and light microscopy, coupled with autoradiography (Hsu 1962, Littau *et al.* 1964), have shown that the incorporation of radioactively labeled RNA precursors generally occurs within the non-condensed chromatin (euchromatin) whereas the condensed chromatin shows few, if any signs of incorporation, suggesting that the euchromatin is transcriptionally active, whereas, the condensed chromatin is not. Panar and Nair (1975), using a combination of Feulgen-microspectrophotometry and autoradiography, have shown that later stages of locust light muscle

differentiation are associated with an increase in the condensation state of the nuclei, with a subsequent decrease in transcriptional activity. Ultrastructural studies (Lamb and Daneholt 1979, Hamkalo and Rattner 1980) have shown the transcriptional by active regions of *Chironomus tentants* appear to be more extended and to have fewer nucleosomes, whereas inactive regions show a higher density of nucleosomes in the higher order packing of nucleosomes and loop formation of chromatin fibers of the nucleus.

Autoradiographic studies of polytene chromosome puffs from *Drosophila* and *Chironomus tetans* (Ashburner, 1972; Daneholt, 1975), and lampbrush chromsomes from *Rana pipiens* (Rogers and Browder 1977) have also shown that transcription occurs in the decondensed puffs of the polytene chromosome and the extended loops of the lampbrush chromosome, but not in the highly condensed regions of these chromosomes.

DNAse I has been used to characterize differences between active and inactive chromatin. Weintraub and Groudine (1976) found that the globin gene (active in chicken erythrocytes) was selectively digested using DNAse I, whereas, the ovalbumin gene (inactive in chicken erythrocytes) was not.

Additional biochemical differences are known to exist between active and inactive chromatin, e.g. actively transcribed genes are undermethylated whereas inactive genes are usually methylated (Weisbrod, 1982). Davie and Candido

(1978) have shown that the nucleosome protein, $H_4$, is hyper-acetylated in active chromatin.

The classical stress response described in mammals by, Selye (1950) is essentially an endocrine response to external or internal stressors and the response to stress in the teleost appears to be patterned along the same lines. It appears to be centered around two endocrine systems: the chromaffin tissue, the homologue of the mammalian adrenal medulla, which produces catecholamines (Mazeaud and Mazeaud, 1981), and the interrenal tissue, the homologue of the mammalian adrenal cortex, which produces corticosteroid hormones (Donaldson, 1981). Donaldson et al., (1984) reported that the histological and biochemical techniques for assessing activity of interrenal and chromaffin tissue under conditions of stress are sensitive and reproducible. The mean nuclear diameter of interrenal cells increases during subacute and chronic stress. Another valuable assessment of interrenal activity is the measurement of plasma cortisol concentration, which is a widely used indicator of acute or medium term stress in teleosts (Goss and Wood, 1988; Donaldson et al., 1984). The determination of plasma catecholamine concentration provides a potential technique for assessment of the activity of chromaffin tissue.

In this study, a histological method was used to measure the interrenal nuclear and chromaffin diameters of rainbow trout for assessing their activities under C.

*concavicornis* stress. Computer-assisted Feulgen-microspectrophotometry was used, whereby a digitized image of a cell nucleus is generated. The image can be subjected to special pattern analysis, to compare the chromatin condensation states of interrenal and chromaffin nuclei of trout exposed to *C. concavicornis* with that of control trout and to assess the activity of interrenal and chromaffin cells under the *C. concavicornis* stress. The concentrations of plasma cortisol in circulating blood of rainbow trout under *C. concavicornis* stress was also determined

## MATERIAL AND METHODS

***Fish***. Experiments were carried out using 215 to 327 g rainbow trout, which were obtained from a commercial trout farm. All fish were transported to Simon Fraser University and placed in 20%o salinity water at least one month prior to cannulation. The oxygen concentrations, pH values and temperatures of the seawater in which the fish were placed varied from 9.9 to 10.7 mg $mL^{-1}$, 7.3 to 7.9 units and 10.5 to 11° C, respectively. Feeding was discontinued 3 days prior to cannulation.

***Anesthesia and surgical procedures***. To allow repetitive blood sampling, each fish was anesthetized using neutralized tricaine methanesulfonate (MS 222, 0.1 g $L^{-1}$). Following anesthetic administration, the fish were surgically fitted with dorsal aortic cannulae filled with heparinized physiological saline (1%). The anesthesia and surgical procedures have been described previously by Yang and Albright (1992).

***Experimental procedure***. Following cannulation, each fish (of ten) was placed in a metabolic chamber described by Maren *et al.* (1968). The chambers were then placed in two-75 x 60 x 22 cm black boxes: 5 chambers in the first box containing treated fish, 5 in the other box containing untreated control fish. Seawater of 20%o salinity was supplied and changed once each day. The pH, oxygen

concentrations, and temperature of the water varied between 7.4 - 7.8 units, 10.1 - 10.8 mg mL$^{-1}$, and 10.5 to 11° C, respectively. Forty-eight h after cannulation, *C. concavicornis* was added to the seawater of the first box. Blood samples from each fish were taken using the cannulae immediately prior to exposure and at 24, 48, 72, and 96 h after exposure to *C. concavicornis*. All arterial blood samples were immediately centrifuged for 10 min at 9000 x g at 4°C and plasma samples were stored at -70° C until cortisol analysis.

Cortisol concentrations were assayed in duplicate by $^{125}$I-radioimmunoassay using the "Clinical Assays$^{TM}$, Gammacoat$^{TM}$ [$^{125}$I] Cortisol Radioimmunoassay Kit" (Baxter Healthcare Corporation) according to the manufacturer's instructions.

At the end of the experiment, portions of the anterior kidneys were excised from the treated and control fish, fixed in Davidson's solution for 24 h, dehydrated in ethanol, impregnated with wax, and sectioned 5 μm thick. Following this, the tissue sections were stained using hematoxylin and eosin. Photomicrographs were taken using inverted light microscopy. The measurement of interrenal nuclear diameters used magnifications of these photomicrographs.

The remaining tissue sections were washed for 24 h in distilled water, put into xylene for 10 min and dehydrated in 70-100% ethanol. They were further processed using a

modification of the method of Fand (1972). This involved acid hydrolysis in 3.5 N HCl at 37 $^{\circ}$C, followed by staining in fresh Schiffs reagent, dehydrating, clearing and mounting in oil. A total of 100 interrenal nuclei were measured. Twenty interrenal nuclei were measured for each control or treated fish.

The Schiff-stained nuclear images were digitized using the Scanning Microscope Photometer (SMP, Carl Zeiss) which is connected (online) with MINC-11 (Digital) and IBM-3033 computers. The nuclei were scanned using a measuring aperture of 0.5 μm diameter at a wavelength of 575 nm and a stepsize of 0.5 μm.

Feature extraction began with a calculation of an initial boundary (IBND) which separated non-condensed chromatin from condensed chromatin using the procedure of Vidal *et al*. (1973). Following this, the average maximum OD (ODMAX) was obtained for the control group and the midpoint (ODMID) was located between IBND and ODMAX. Using these boundaries three optical density chromatin components were defined over an absorbance range of 0.02 to 2.0.

| Components | Boundary |
|---|---|
| 1. Low density component (LDC) | 0.02 to IBND. |
| 2. Medium density component (MDC) | IBND to ODMID. |
| 3. High density component (HDC) | ODMID to ODMAX. |

Two global features were extracted which were independent of boundary intervals. These features were as follows:

1. *Global features*:

    a. Total nuclear optical density (O.D): The sum of all O.D. values making up the digitized image. This sum is proportional to the total DNA content of the nucleus.

    b. Total nuclear area: The number of values making up the digitized image.

2. *Image features:*

    a. Total optical density (O.D). of the low, medium and high density components (TOLDC, TOMDC, TOHDC), expressed as percentages of total nuclear DNA.

    b. Total Area occupied by the low, medium and high density components (TALDC, TAMDC, TAHDC), expressed as percentages of the total nuclear area.

    Comparisons between the treated and control groups based on these features were analyzed for significance using the nonparametric Kruskal-Wallis test (Bartels, 1979).

The mean concentration of *C. concavicornis* cells remained at between 46 and 62 cells $mL^{-1}$ during the course of the experiments (Fig. 34).

The mean values of the total nuclear area of interrenal nuclei were 209 and 138 $\mu m^2$ in treated and control fish, respectively (Fig. 35). The mean values of total nuclear area of chromaffin nuclei were 200 and 158 $\mu m^2$ in the treated and control fish, respectively (Fig. 36). The total nuclear area of interrenal nuclei in the treated fish showed a significant increase in comparison with that in the control fish (p<0.001) (Fig. 35). The total nuclear area of chromaffin nuclei in the treated fish showed a significant increase in comparison with that of the control fish (P<0.001) (Fig. 36).

Fig. 37 shows the amounts of the low, medium, and high density components (LDC, MDC, HDC, respectively) of interrenal nuclei in control and treated fish as percentages of the total DNA in O.D. units whereas Fig. 38 gives the respective areas of these components of interrenal nuclei in control and treated fish as percentages of total nuclear area. The mean total area occupied by the LDC and the mean LDC optical density (O.D.) of interrenal nuclei were 86.8% and 74.9% in treated fish and 67.2% and 52% in control fish, respectively.

Both the mean total area occupied by the LDC and the mean LDC optical density (O.D.) of interrenal nuclei in treated fish showed significant increases in comparison with that in control fish: (p<0.001) and (p<0.001), respectively. The mean total area occupied by the MDC and the mean MDC optical density (O.D.) of interrenal nuclei were 10.7% and 19.1% in treated fish and 22.2% and 29.8% in the control fish, respectively.

Both the mean total area occupied by MDC and the mean MDC optical density (O.D.) of interrenal nuclei in treated fish showed significant decreases in comparison with that in control fish: (p<0.001) and (p<0.001), respectively. The mean total area occupied by HDC and the mean HDC optical density (O.D.) of the interrenal nuclei were 3.8% and 9.0% in the treated fish and 12.6% and 21.4% in the control fish, respectively. Both the mean total area occupied by the HDC and the mean HDC optical density (O.D.) of the interrenal nuclei in the treated fish showed significant decreases in comparison with that in control fish: (p<0.001) and (p<0.001), respectively.

Fig. 34. The mean concentration of *C. concavicornis* cells in the treated seawater. Error bar indicates +/- s.e.m.

Fig. 35. The mean of total nuclear area of interrenal nuclei of *C. concavicornis* treated and untreated control trout.*** indicates a significant difference (P<0.001)

GTA
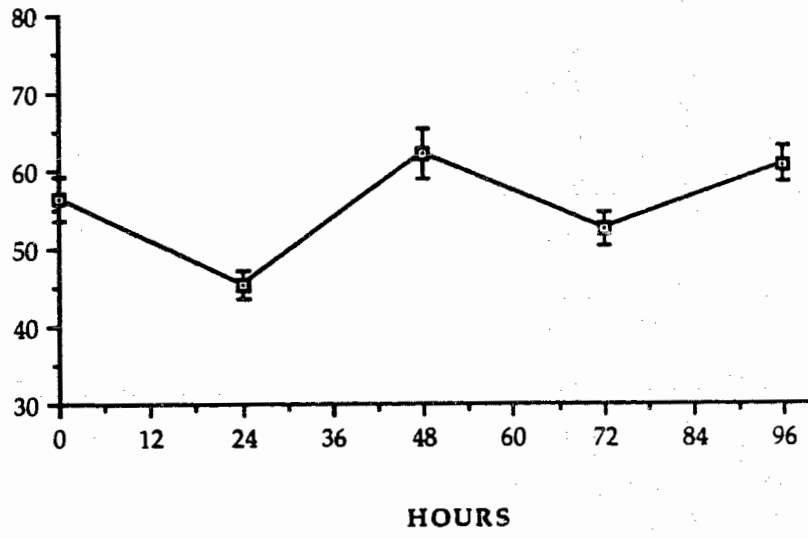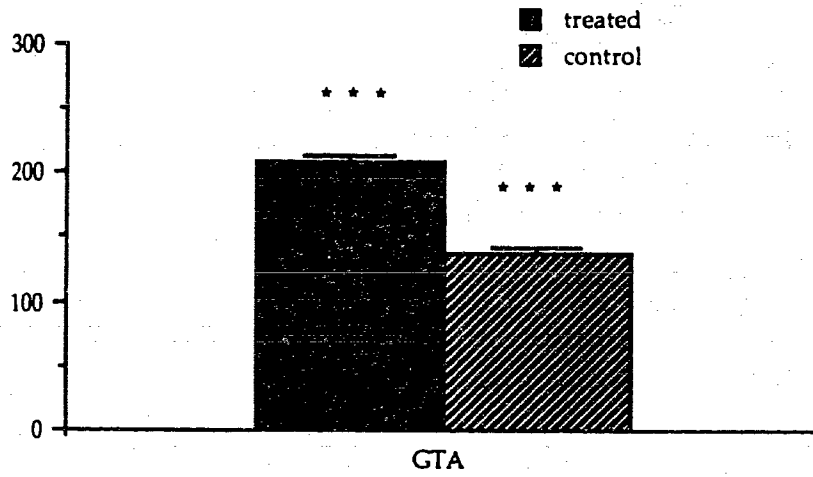
Fig. 36. The mean of total nuclear area of chromaffin nuclei of *C. concavicornis* treated and untreated control trout.*** indicates a significant difference (P<0.001).
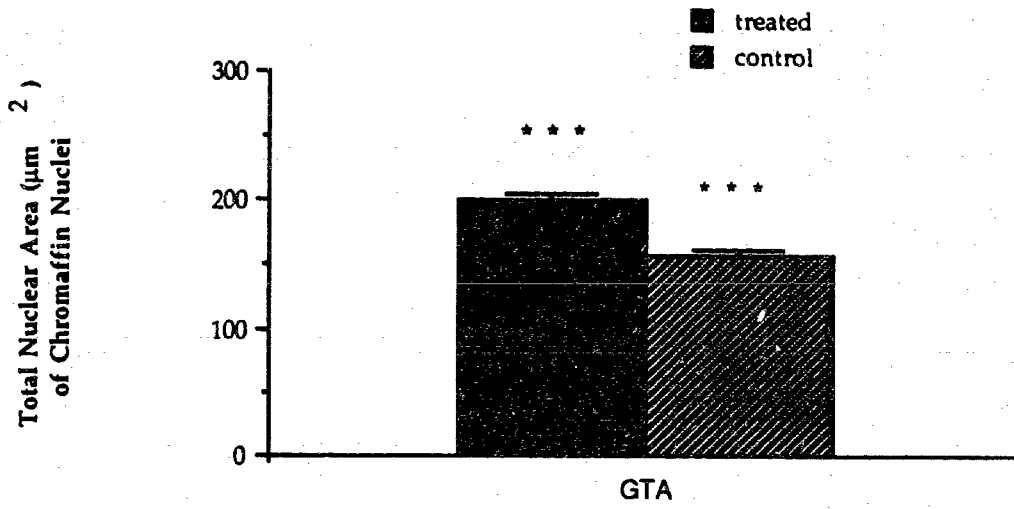
Fig. 37. The amounts of low, medium, and high density
components (TOLDC, TOMDC, TOHDC, respectively) in interrenal
nuclei of *C. concavicornis* treated and untreated control
trout as percentages of total DNA in O.D. units.***
indicates a significant difference (P<0.001).
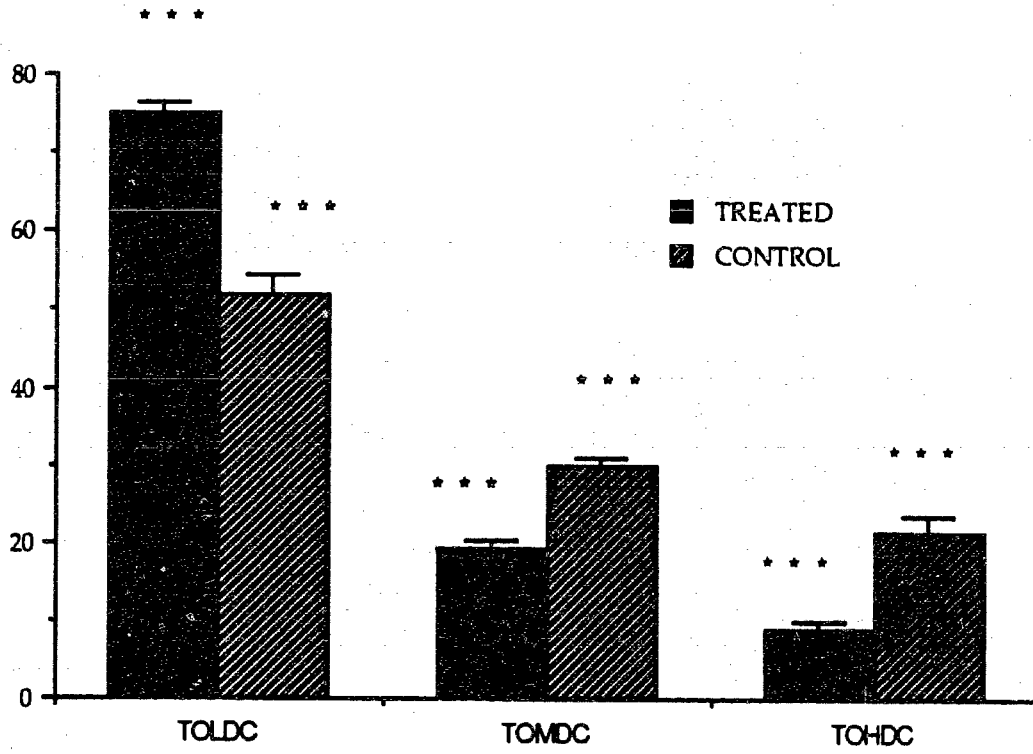
Fig. 38. Areas of low, medium, and high density components
(TALDC, TAMDC, TAHDC, respectively) of interrenal nuclei in
*C. concavicornis* treated and untreated control trout as
percentages of total nuclear area.*** indicates a
significant difference (P<0.001).
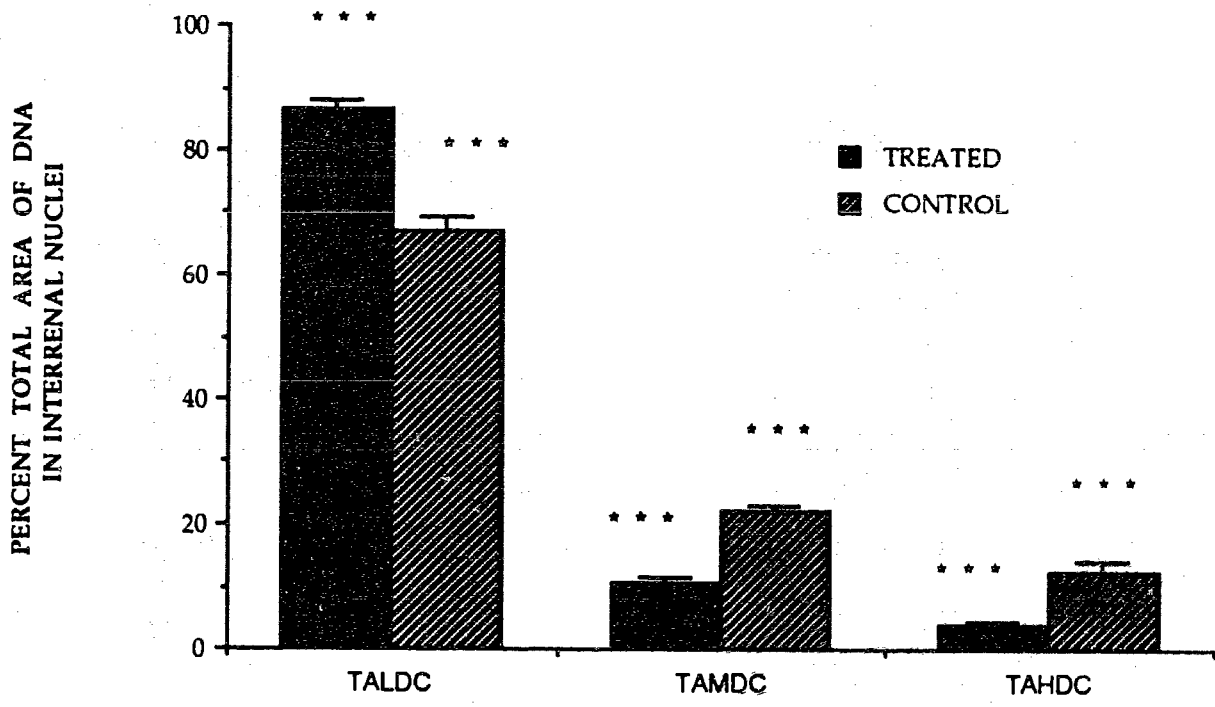
Fig. 39 shows the amounts of the low, medium, and high density components (LDC, MDC, HDC) of chromaffin nuclei in control and treated fish as percentages of total DNA in O.D. units whereas Fig. 40 gives the respective areas of these components of chromaffin nuclei in control and treated fish as percentages of total nuclear area. The mean total area occupied by LDC and the mean LDC optical density (O.D.) of chromaffin nuclei were 77% and 62.7% in treated fish and 70.1% and 54.9% in control fish, respectively. Both the mean total area occupied by LDC and the mean LDC optical density (O.D.) of chromaffin nuclei in treated fish showed significant increases in comparison with that in control fish ($p < 0.01$) and ($p < 0.01$), respectively. The mean total area occupied by MDC and the mean MDC optical density (O.D.) of chromaffin nuclei were 18.1% and 27.2% in treated fish and 22.8% and 31.9% in control fish, respectively. Both the mean total area occupied by MDC and the mean MDC optical density (O.D.) of chromaffin nuclei in treated fish showed significant decreases in comparison with that in control fish ($p < 0.005$) and ($p < 0.01$), respectively. The mean total area occupied by HDC and the mean HDC optical density (O.D.) of chromaffin nuclei were 5.7% and 11.7% in treated fish and 7.9% and 14.8% in control fish, respectively.

Fig. 39. The amounts of low, medium, and high density components (TOLDC, TOMDC, TOHDC, respectively) of chromaffin nuclei in *C. concavicornis* treated and untreated control trout as percentages of total DNA in O.D. units.** indicates a significant difference (P<0.01).

PERCENT TOTAL OPTICAL DENSITY OF DNA IN CHROMAFFIN NUCLEI

Fig. 40. Areas of low, medium, and high density components (TALDC, TAMDC, TAHDC, respectively) of chromaffin nuclei in *C. concavicornis* treated and untreated control trout as percentages of total nuclear area.** indicates a significant difference (P<0.01).
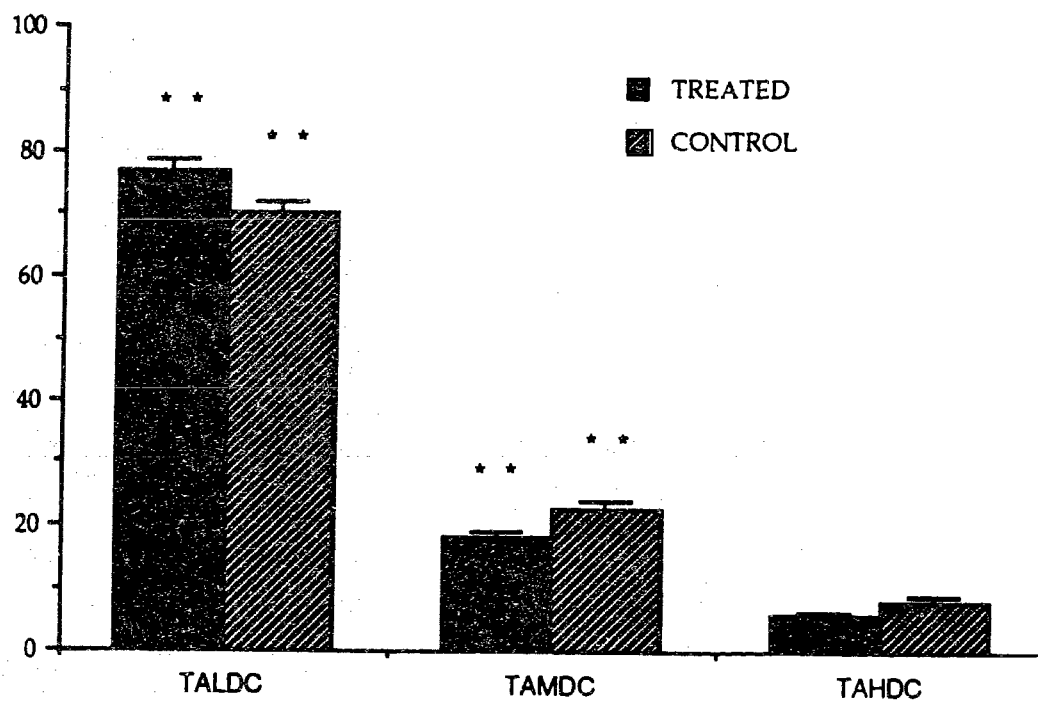
There was no significant difference between the mean total area occupied by HDC and the mean HDC optical density (O.D.) of chromaffin nuclei in treated and control trout.

Fig. 41 shows computerized images from treated interrenal nuclei group (41a), control interrenal nuclei group (41b), treated chromaffin nuclei group (41c) and control chromaffin nuclei group (41d). These nuclei were selected by a program which evaluates all nuclei in each group with respect to how well they represent the group mean in all features considered. The general trends involving total area, LDC, MDC, and HDC are clearly evident.

In untreated trout, the interrenal tissue consisted of large groups of closely packed small cells (Fig. 42a). The cytoplasm of these cells was finely granulated. Their nuclei were usually spherical with a mean diameter of 6.25 μm +/- 0.062 μm and contained inconspicuous nucleoli. In treated fish, the interrenal tissue exhibited striking changes. Groups of cells were larger and more abundant than in the control fish (Fig. 42b). The cytoplasm of these cells was vaculated. Their nuclei showed prominent nucleoli and had a mean diameter of 7.81 μm +/- 0.151 μm. A significant increase in mean nuclear size was evident (Fig. 42b).

Fig. 41. Computerized images of interrenal nuclei from *C. concavicornis* treated trout (a) and untreated control trout (b) as well as computerized images of chromaffin nuclei from *C. concavicornis* treated trout (c) and untreated control trout (d). ❑⊞▧ represent low, medium and high density DNA and are occupied by low, medium and high density DNA respectively.

Treated · Control

a · b
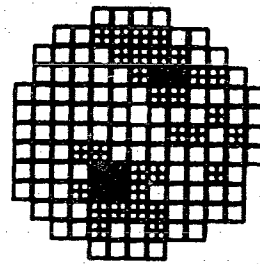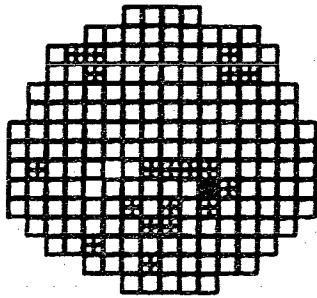
Interrenal cells

c · d
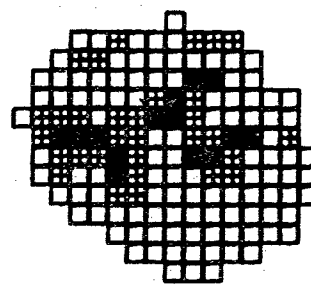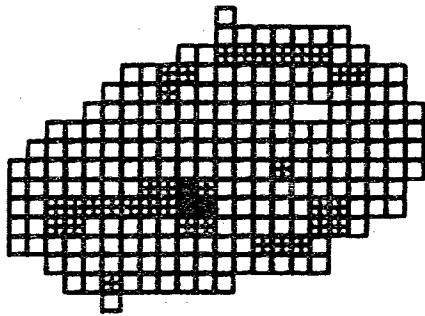
Chromaffin cells

Fig. 42. Interrenal tissue of *C. concavicornis* untreated
control (a) and treated trout (b). H & E 560 x
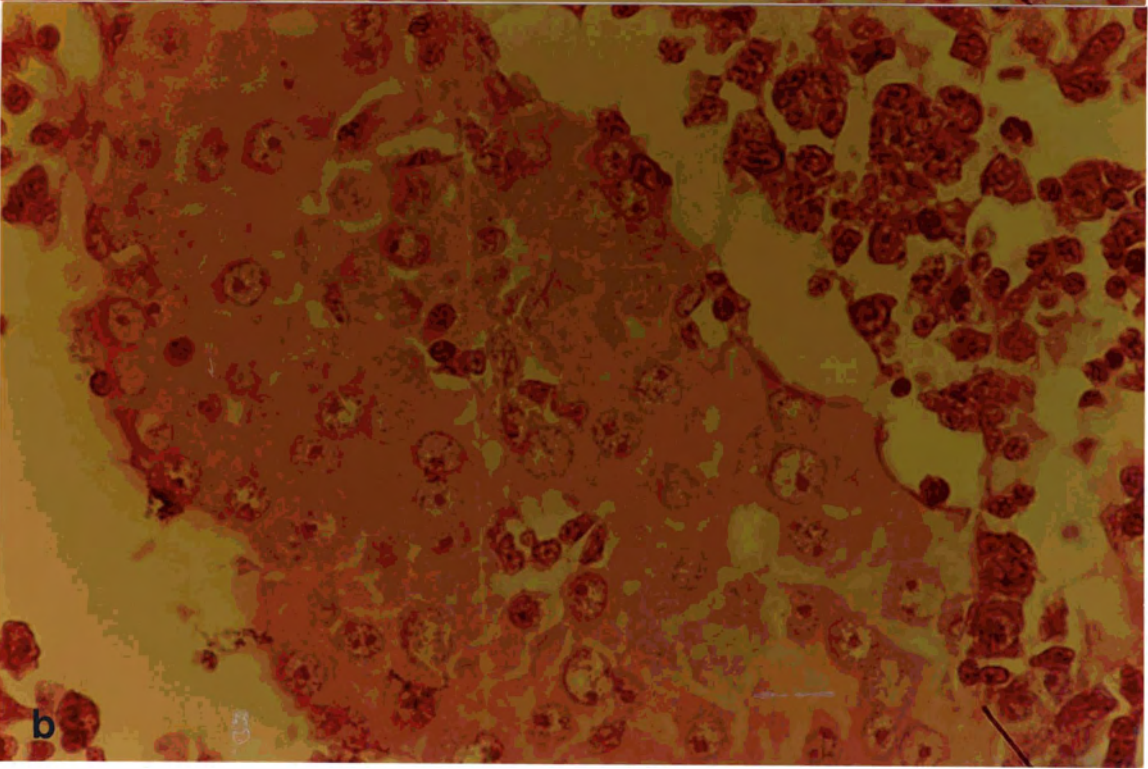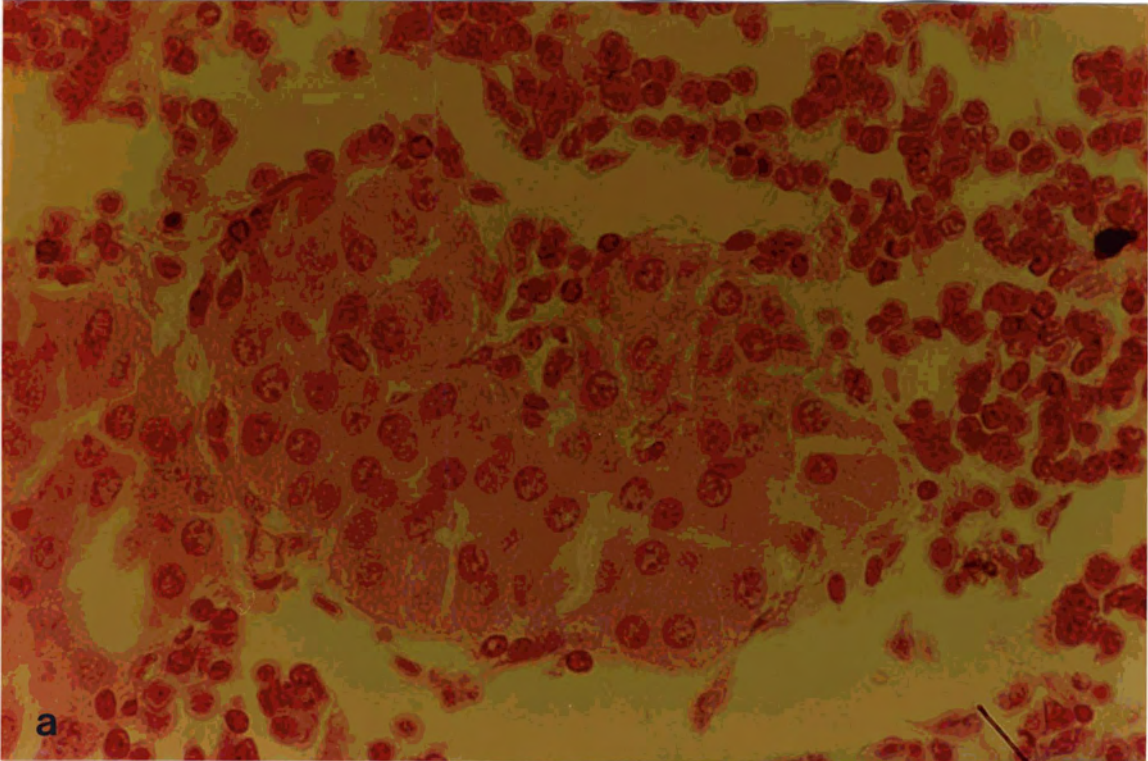
In untreated control trout, the chromaffin tissue consisted of closely packed small cells (Fig. 43a). The cytoplasm of these cells was finely granulated. Their nuclei were usually present in different shapes with triangular, pear and spherical shapes prevailing. In treated trout, the chromaffin tissue exhibited striking changes in the chromaffin tissue. Groups of cells were hypertrophied and their nuclei were larger than in the untreated control trout(Fig. 43b).

Circulating plasma cortisol levels of the untreated control fish did not significantly change during the course of the experiment (Fig. 44). The plasma cortisol level of fish exposed to *C. concavicornis* was not significantly different from the untreated control fish prior to *C. concavicornis* exposure but it increased by 10-fold after 48 h. Thereafter, it remained high until the experiment was terminated, indicating a severe stress response (Fig. 44). The cortisol levels in the treated fish were significantly different from those of the untreated control fish at hour 24 (P<0.01), hour 48 (P<0.01), hour 72 (P<0.01) and hour 96 (P<0.05).

Fig. 43. Chromaffin tissue of untreated control trout (a) and trout treated with *C. concavicornis* (b). H & E 560 x
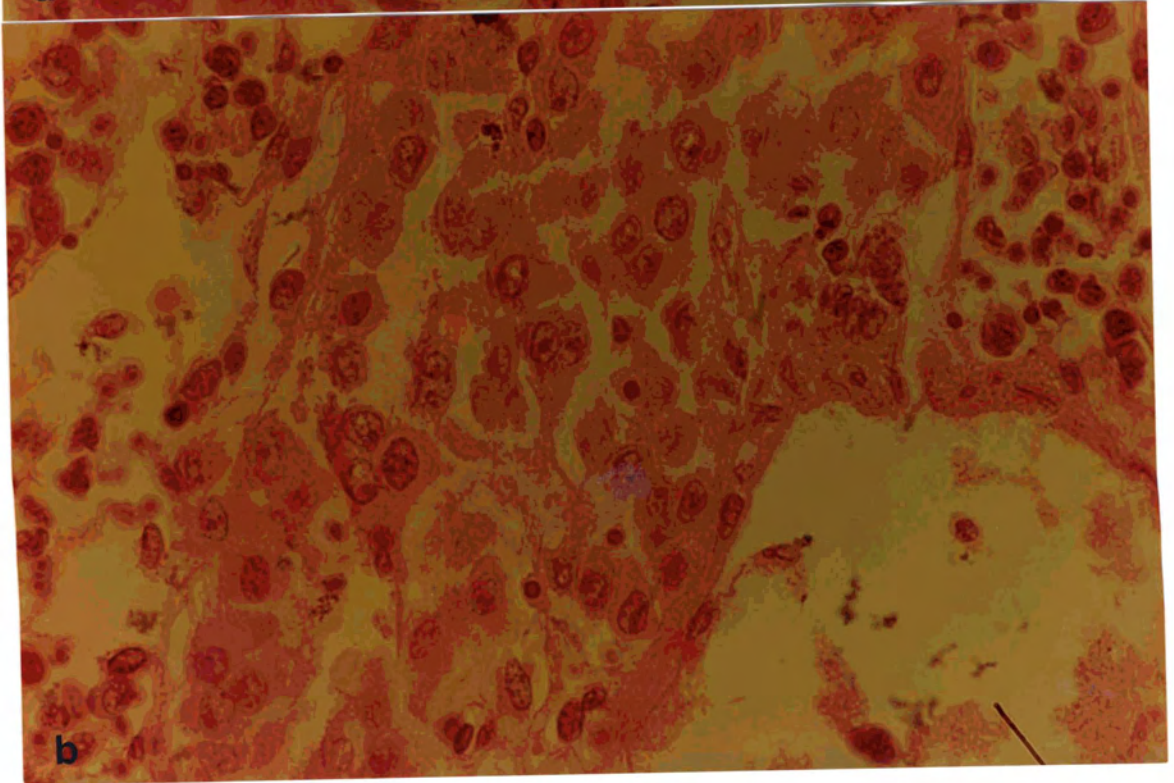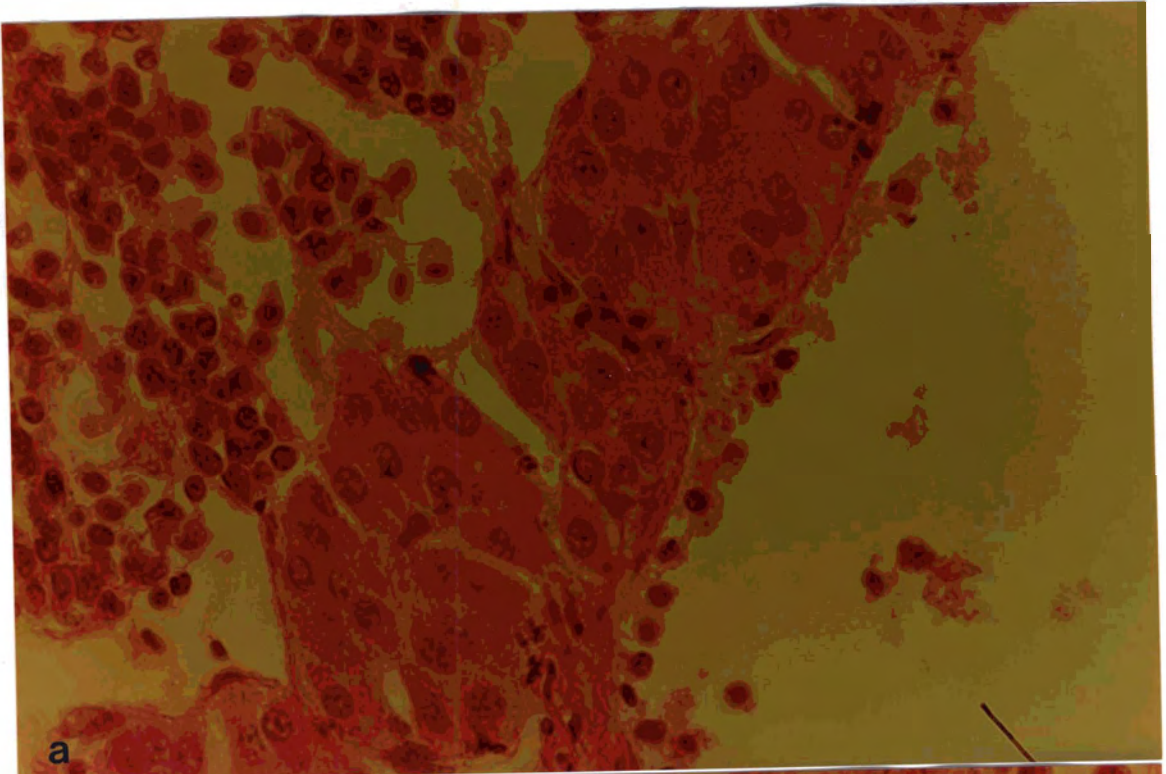
Fig. 44. The mean plasma cortisol concentrations of *C. concavicornis* in treated and untreated control fish. Error bar indicates +/- s.e.m.* and ** indicate significant differences (P<0.05) and (P<0.01), respectively.
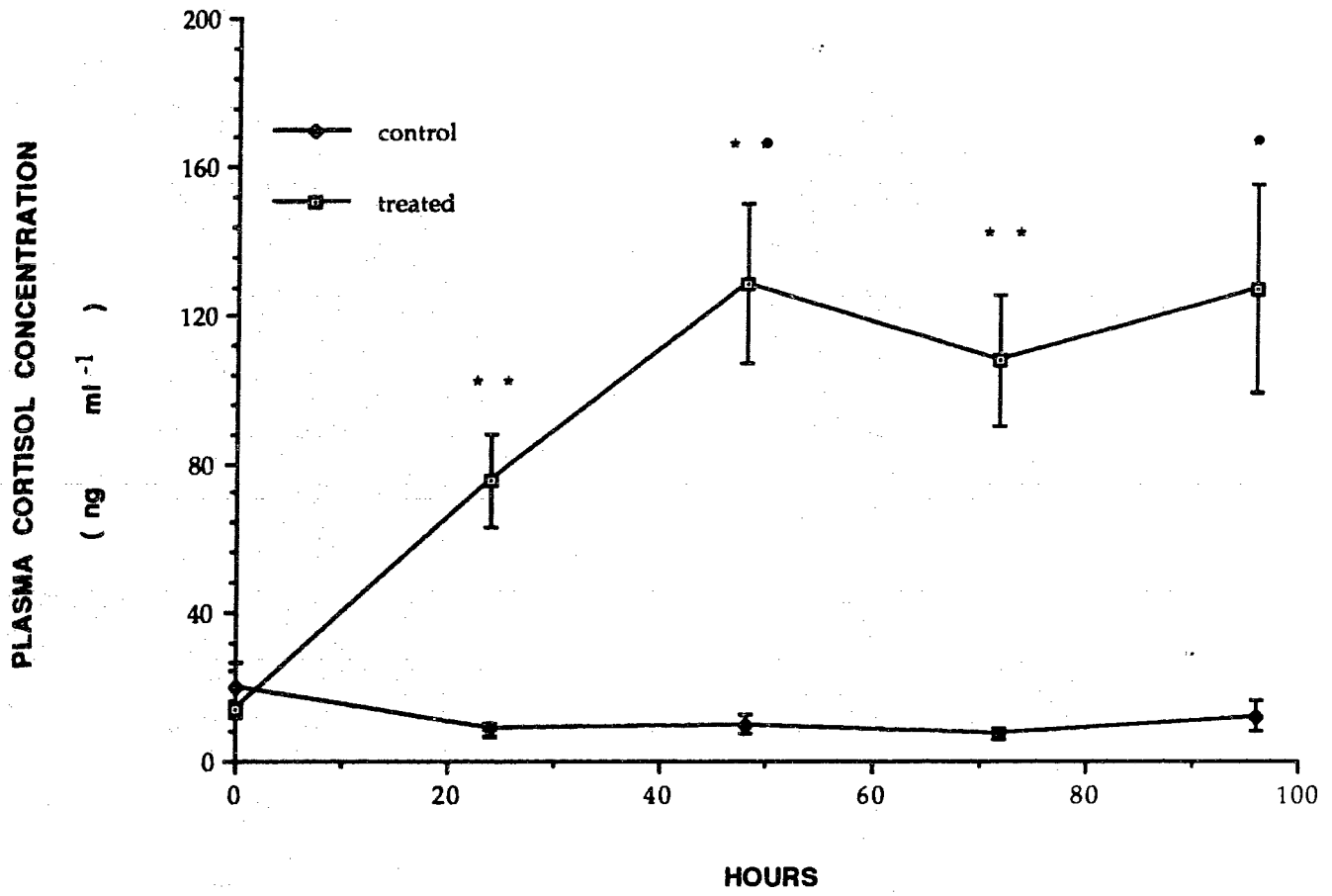
DISCUSSION

Pattern analysis showed significant *C. concavicornis* exposure-related changes in the interphase chromatin condensation patterns in both interrenal and chromaffin nuclei (Figs. 35-41), but the total DNA content of the nuclei remained unchanged. These changes related primarily to the amount and distribution of low density chromatin component (LDC) and medium density chromatin component (MDC).

The general relationship between the degree of chromatin condensation and the cell activity examined in interphase nuclei has been shown to have an inverse relationship when examined at the microscopic, ultrastructural, and molecular levels (Harris, 1967; Hamkalo and Rattner, 1980; Weintraub and Groudine, 1976; Weisbrod, 1982). Our pattern analysis showed that the chromatin densities of interrenal and chromaffin nuclei in treated trout (Figs. 37 and 39) decreased significantly in comparison with those in the control trout. By dividing the total nuclear O.D. into low, medium, and high density components (LDC, MDC, HDC) we were able to show that the decrease of chromatin density is due to a reciprocal exchange among LDC, MDC, and HDC of interrenal and chromaffin nuclei in the treated trout. This reciprocal exchange involved an increase in the amount of LDC and a

decrease in the amount of MDC and HDC of interrenal and chromaffin in treated fish. Chromatin decondensation affected total nuclear area and total area occupied by the LDC, MDC and HDC. Our results showed the total nuclear areas of the interrenal and chromaffin cells in treated trout increased in comparison with those in the control trout (Figs. 35 and 36). The increases in nuclear areas of interrenal and chromaffin cells in treated fish occurred as the chromatin becames less compact which increased the nuclear volume and consequently the total areas. The total areas occupied by the LDC of interrenal and chromatin nuclei in treated trout increased while that occupied by the MDC and HDC of interrenal and chromaffin nuclei in treated fish decreased in comparison with those in control fish (Figs. 38 and 40). These results indicated decondensation of interrenal and chromaffin nuclei and therefore increased activities of interrenal and chromaffin tissues in treated trout.

Although the changes of interrenal and chromaffin nuclei in treated trout in comparison with control trout were similar with regard to the general form of the decondensation event, the results show that the rates of changes of the two cell types were different. The interrenal nuclei of treated trout changed at a higher rate than the chromaffin nuclei of treated trout.

Using classical histological methods, several authors have observed increases in interrenal nuclear diameters in

salmonids exposed to different stressors, such as kraft pulp mill effluent (Dye and Donaldson, 1974), copper (Donaldson and Dye, 1975), sanitary landfill leachate (McBride et al., 1979), ammonia (Donaldson, 1981), accompanied by increased cortisol level. In the present study, using histological methods, the increased nuclear diameter in trout exposed to *C. concavicornis* was also observed. This indicated that the treated trout suffered from stress and that interrenal cells of trout exposed to *C. concavicornis* became hypertrophic. These results confirm the observations that were obtained by nuclear chromatin analysis.

Cortisol is released from the interrenal tissue as teleost fish attempt to cope with stress (Donaldson, 1981). Measurements of the plasma levels of cortisol, the major corticosteroid in many teleosts (Idler and Truscott, 1972), have been used to assess the magnitude of stress responses (Donaldson, 1981). In this study, the plasma cortisol level of trout exposed to *C. concavicornis* was more than 10 times greater than that of the control trout. Clearly, the trout were experiencing a severe physiological disturbance although no mortality of treated fish was observed. Higher plasma cortisol levels have been reported in other salmonids which were lethally stressed (Fagerlund, 1967; Goss and Wood, 1988). It is well established that increased cortisol concentrations suppress the immune systems of salmonids (Ellsaesser and Clem 1987; Maule et al. 1987; Tripp et al., 1987). The increased cortisol levels (Fig. 44) reported in

this investigation may help to explain the increased

susceptibility of coho salmon (*Oncorhynchus kisutch*) to

bacterial pathogens under field and laboratory conditions

when they were exposed to harmful concentrations of

*Chaetoceros* spp. (chapter 4).

# CHAPTER FOUR

Susceptibility of Coho Salmon (*Oncorhynchus kisutch*) to
Infection with the Bacterial Pathogen, *Vibrio anguillarum*,
during a Harmful *Chaetoceros concavicornis* Exposure

# INTRODUCTION

In an earlier investigation, Albright *et al.* (in press) found that although the harmful diatoms, *Chaetoceros concavicornis* and *C. convolutus* are lethal to salmon at concentrations in excess of approximately 5 cells $mL^{-1}$, sub-lethal concentrations at between approximately 0.40 and 5 cells $mL^{-1}$ appear to cause mortalities of both coho (*Oncorhynchus kisutch*) and chinook salmon (*O. tshawytscha*) due to bacterial diseases, such as vibriosis and bacterial kidney disease (BKD) under both field and laboratory conditions. These authors speculated that sub-lethal concentrations of these harmful *Chaetoceros* spp. may sufficiently suppress the salmonid's defence mechanism such that they express a disease (Albright, *et al.* in press).

In chapter 2, the neutrophils, lymphocytes, and thrombocytes of chinook salmon were found to become depleted in the presence of harmful concentrations of *C. concavicornis*. These data suggested that immuno-suppression occurred in these fish. Suppression of at least a portion of the immune system may partially explain the observation that salmon cultured in the presence of harmful *C. concavicornis* concentrations became more susceptible to diseases, including vibriosis and BKD.

The above observations support the view that the presence of the harmful barbed diatoms of *C. concavicornis*

and *C. convolutus*, even in low concentrations, act as stressors. The association between stress and outbreak of disease in teleost fish is well established and evidence for a role of the HPI-axis in this response is accumulating (Snieszko, 1974, Pickering and Duston, 1983, Pickering and Pottinger, 1987a). As finfish try to adapt to the stressors, interrenal and chromaffin cell activities as well as diameters increase (Donaldson, 1981, McBride *et al.* 1979, Dye and Donaldson, 1974) and cortisol concentrations increase (Goss and Wood, 1988, Strange and Carl, 1978). One of the effects of the increase in cortisol concentration is lymphocytopenia; the reduction in the numbers of circulating lymphocytes may be an important link between acute stress responses and the onset of disease.

Pickering and Duston (1983) have shown that a prolonged elevation of plasma cortisol levels in excess of 30 ng mL$^{-1}$ is sufficient to increase the susceptibility of brown trout (*Salmo trutta*) to furunculosis. Maule *et al.* (1987) reported that fish with cortisol implants had higher plasma cortisol titers, reduced numbers of splenic antibody-secreting cells, splenic lymphocytes, and circulating leucocytes as well as greater mortality when exposed to *V. anguillarum*. Loss of mitogen responsiveness was also observed with macrophages isolated from fish previously implanted with cortisol (Ellsaesser and Clem, 1987).

Based upon the above observations, it was decided to investigate a possible link between a salmonid's exposure to

harmful concentrations of *C. concavicornis*, its plasma cortisol levels, and its susceptibility to a pathogenic bacterium, *V. anquillarum*, a causative agent of vibriosis.

## MATERIALS AND METHODS

All experiments were carried out using 9 to 18 g small coho salmon, which were obtained from Capilano Fish Hatchery in West Vancouver, B.C. All fish were transported to Simon Fraser University and supplied with aerated, 20‰ (in experiment 1) or 27 to 30 ‰ (in experiment 2) salinity water for more than one month prior to each experiment.

The bacterial fish pathogen *Vibrio anguillarum* (strain R20) was obtained from Pacific Biological Station, Canada Dept. of Fisheries and Oceans, Nanaimo, B.C.

### Experiment 1

Two-hundred fifty coho were randomly divided into five groups of 50 fish each. Each group was then placed in a separate 200-L tank and fed commercial feed daily for one month. The salinity, pH, temperature, and dissolved oxygen contents of the water were 20‰, 7.0-7.6, 12.5-13.5° C, and 7.9-10.2 mg $L^{-1}$, respectively during the experiment.

Standard curves of *V. anguillarum* were generated by plotting the number of viable cells of the bacterium (determined using standard plate counts on Trypticase Soy Agar, TSA) against absorbance. Trypticase Soy Broth log-phase cultures of the bacterium were centrifuged at 3.500 *x* g for 20 min. and the resultant pellet resuspended in phosphate-buffered saline, pH 7.4. The desired numbers of

bacteria for challenging the fish were then obtained by diluting the bacterial suspension to the appropriate absorbance value. The bath challenge was done using 40 L of water. Bacteria were added to the water to yield a concentration of $4.5 \times 10^4$ $mL^{-1}$. The fish were challenged for 30 min.

The fish in tank 1 were treated with *C. concavicornis* for the entire 10 day experiment; on day 3 a culture of *V. anguillarum* was added to attain a concentration of $4.5 \times 10^4$ viable cells $mL^{-1}$. The fish in tank 2 were treated in a similar fashion except that the concentration of *C. concavicornis* was 1/3 lower than that of tank 1. The fish in tank 3 were untreated with *C. concavicornis* but were treated with a similar concentration of *V. anguillarum* on day 3 as described above. The fish in tank 4 were untreated with *V. anguillarum* but were treated with a similar concentration of *C. concavicornis* with tank 1 for the entire 10 day experiment. The untreated fish in tank 5 served as a control.

All moribund fish from tanks 1, 2, 3 and 4 were sampled to verify the cause of death. Each fish was blotted dry and sprayed with 70% ethanol to disinfect its surface. The gills, kidneys, and spleens were then sampled using a sterile loop and the material was streaked onto TSA supplemented with 1% NaCl. The plates were incubated at $20^{\circ}$ C for 48 h.

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The presumptive evidence of *V. anguillarum* infection was whether growth recovered from the organs of the dead fish was inhibited by novobiocin (Sigma) and the vibriostatic agent 0/129 (2,4-diamino-6,7-di-isopropyl pteridine,) (Sigma) and exhibited the *V. anguillarum* characteristic colony morphology. Twenty % of the presumptive *V. anguillarum* isolates were also assayed for cytochrome oxidase activity (Sigma), Gram stain reaction and rapid agglutination response with specific *V. anguillarum* antiserum.

*Experiment 2*

One hundred eleven coho were randomly divided into three groups of 37 fish each and each group placed in an oval tank containing 200 L of seawater. The oxygen concentrations, pH values, salinities, and temperatures of the seawater in which the fish were placed varied from 9.3 to 10 mg mL $^{-1}$, 7.1 to 7.8 units, 27%o to 30%o, and 8.5 to 9.5° C, respectively during the course of the experiment. The fish in tank 1 were treated with *C. concavicornis* for 7 days. Approximately 7.7 X 10$^5$ *V. anguillarum* cells mL$^{-1}$ were added to the water on day 3. The fish in tank 2 were treated with 7.7 X 10$^5$ *V. anguillarum* cells mL$^{-1}$ only which were added to the water on day 3. The fish in tank 3 were untreated. They were not exposed to *C. concavicornis* or to *V. anguillarum* cells and served as control fish.

Three fish from each of tanks 1, 2 and 3 were sampled at 6, 24, 48, 72, 96, and 120 h after being challenged in tanks 1 and 2 with *V. anguillarum*. At each sampling, fish were anesthetized lethally using 2-phenoxyethanol and aseptically dissected. Portions of gill, kidney, and spleen tissue were obtained, homogenized in 1.1% physiological saline, diluted in the same solution, and inoculated on TSA (Difco) plates immediately. The plates were incubated at 21 to 22° C. Three different kinds of colored and shaped colonies were observed on plates inoculated with gill tissue while two different kinds of colored and shaped colonies were observed on plates inoculated with kidneys and spleens. Twenty percent of the colonies isolated from each sample were tested to confirm they were *V. anguillarum* according to the method described in experiment 1. Total colony units of *V. anguillarum* were counted after 48 h incubation.

Values are expressed as the arithmetic mean +/- standard error (S.E.). All comparisons between treated and control fish were made by a two-way analysis of variance (ANOVA).

# RESULTS

*Experiment 1*

The concentrations of *C. concavicornis* varied between 24 and 31 cells mL$^{-1}$ in tank 1, between 14 and 20 cells mL$^{-1}$ in tank 2 and between 25 and 32 cells ml$^{-1}$ in tank 4 for the entire 10 day experiment (Fig. 45).

No untreated control fish died during the course of the experiment in tank 5 (Fig. 46). Forty-one of the coho died in the tank 1 treated with both *C. concavicornis* at approximate 30 cells mL$^{-1}$ seawater and *V. anquillarum* over the 10 day period of the experiment (Fig. 46) (three of the coho died in the 2 day period after *C. concavicornis* treatment and before *V. anguillarum* challenge whereas 38 coho died following the *V. anguillarum* challenge). A total of 23 coho died in tank 2 during the treatment with *C. concavicornis* at approximately 20 cells mL$^{-1}$ seawater and *V. anguillarum* (two died in tank 2 following *C. concavicornis* treatment and before *V. anguillarum* challenge; twenty-one coho died in tank 2 following the *V. anguillarum* challenge). A total of 6 fish treated with *V. anquillarum* only died in tank 3. A total of 12 fish treated with *C. concavicornis* only died in tank 4. The cumulative mortality rates of the coho in tanks 1, 2, 3, and 4 were 82, 46, 12 and 24%, respectively (Fig. 46). None of the untreated coho died in tank 5.

Fig. 45. The concentrations of *C. concavicornis* cells in the seawater of treated tanks 1, 2 and 4 in experiment 1.

CHAETOCEROS CELLS ml $^{-1}$
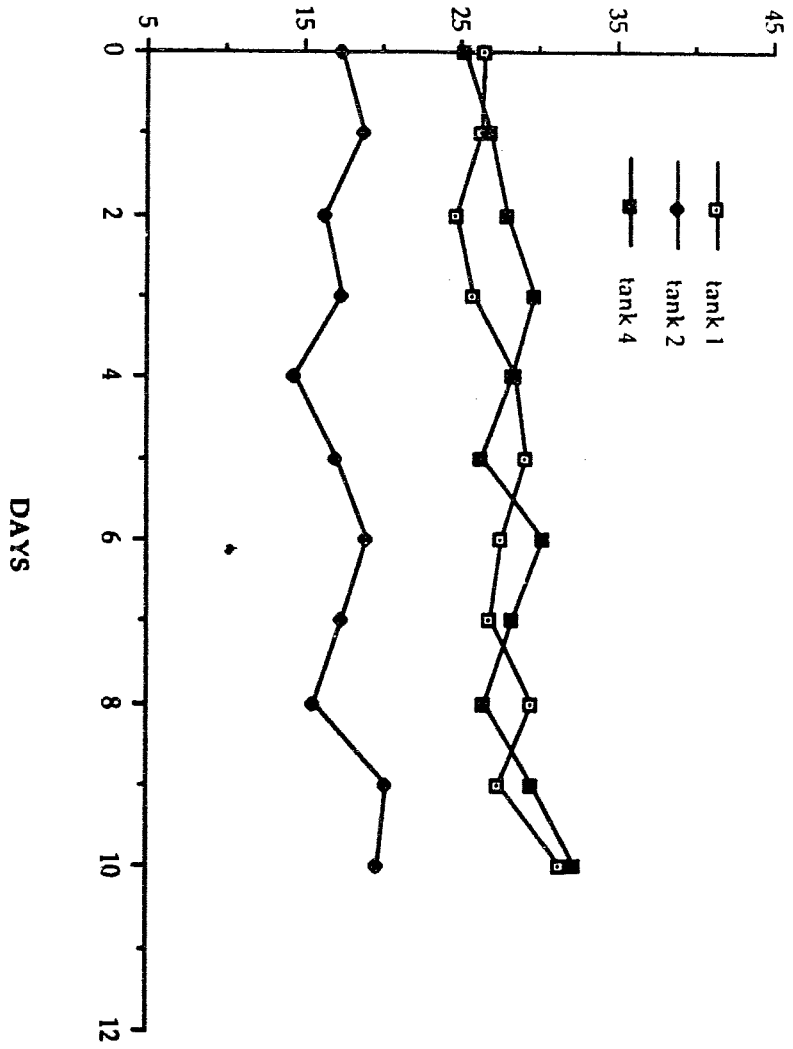
Fig. 46. The cumulative mortalities of coho salmon in tank 1 (30 *C. concavicornis* cells mL$^{-1}$ and *V. anguillarum*), tank 2 (20 *C. concavicornis* cells mL$^{-1}$ and *V. anguillarum*), tank 3 (*V. anguillarum* only), tank 4 (30 *C. concavicornis* cells mL$^{-1}$ only) and tank 5 (untreated control) for the 10 day period of the experiment. All *V. anguillarum* concentrations were 4.5 x 10$^4$ cells mL$^{-1}$ seawater.
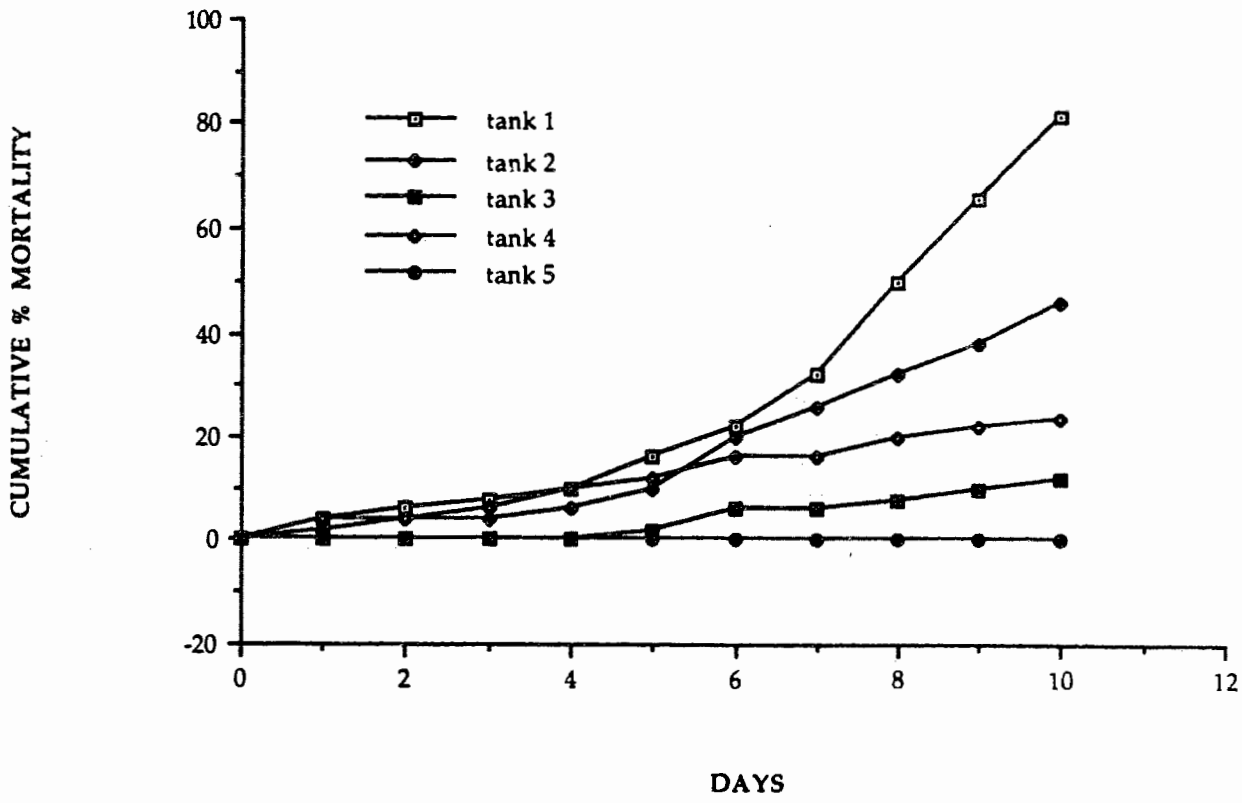
*Experiment 2*

The concentration of *C. concavicornis* varied between 14 and 37 cells $mL^{-1}$ for the 7 day duration of the experiment (Fig. 47).

No untreated control fish died while a total of 18 fish in tank 1 treated with both *C. concavicornis* and *V. anguillarum* died during the course of the experiment from day 0 to 7 (Fig. 48) Of these a total of 5 fish died during the *C. concavicornis* treatment while 13 fish died following the *V. anguillarum* challenge in tank 1. The cumulative mortality of the fish in tank 1 was 100% by day 7 following the *V. anguillarum* challenge (Fig. 48). No fish died in tank 2 prior to and following the *V. anguillarum* treatment (The cumulative mortality was calculated by the method described in chapter 2, experiment 2).

Colony forming units (CFU, i.e., the number of microorganisms that can form colonies in spread plates or pour plates, an indication of the number of viable microorganisms in a sample) $g^{-1}$ of excised gill tissue of live fish in tank 3 after *V. anguillarum* challenge were significantly different from those of the fish in tank 1 at 12 h (P<0.001), and in tank 1 and 2 at 24 h (P<0.001, P<0.001), 48 h (P<0.001, P<0.001), 72 h (P<0.001, P<0.001), 96 h (P<0.001, P<0.001) and 120 h (P<0.001, P<0.001), respectively (Fig. 49). The CFU $g^{-1}$ gill tissue of the fish in tank 1 were significantly different from those of the fish in tank 2 at 72 h (P<0.001) (Fig. 49).

Fig. 47. The concentrations of *C. concavicornis* cells in the treated seawater in experiment 2. *C. concavicornis* was added daily to maintain a concentration of approximately 37 cells $mL^{-1}$ seawater at the beginning, dropping to approximately 14 cells $mL^{-1}$ seawater by day 4.
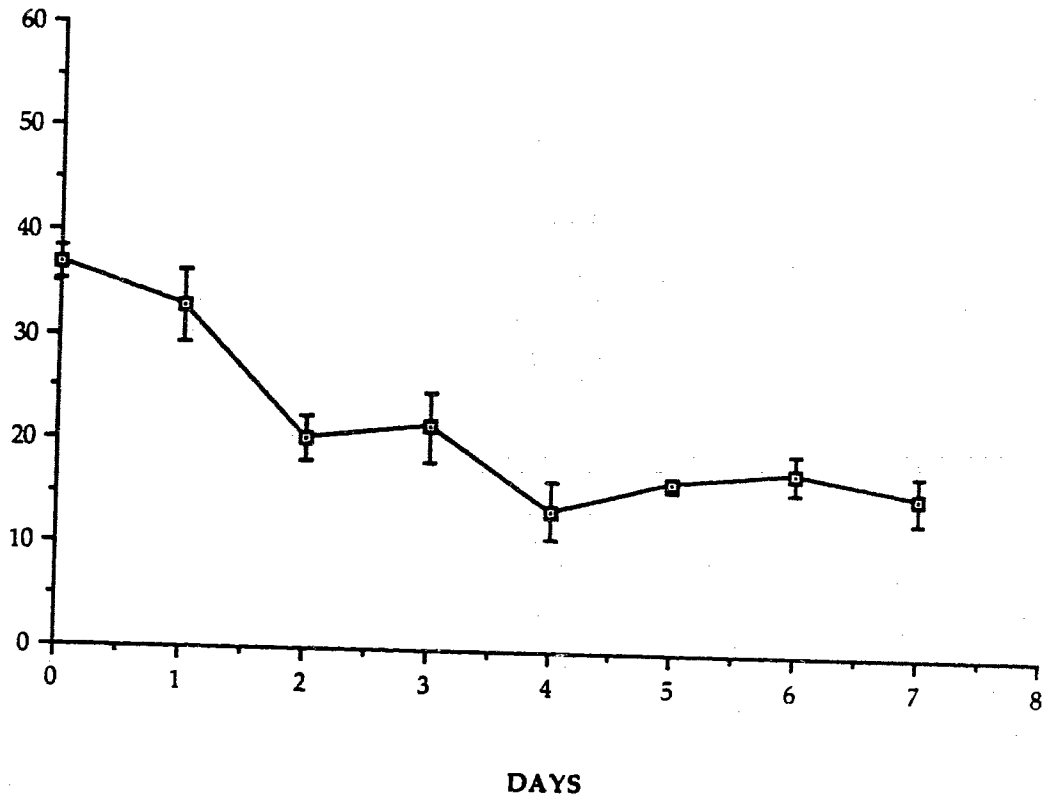
Fig. 48. The mean cumulative mortalities of coho salmon treated with between 14 and 37 *C. concavicornis* cells $mL^{-1}$ in tank 1 for the 7 day period of the experiment. At day 3 the coho in tanks 1 and 2 were treated with *V. anquillarum* to a final concentration of $7.7 \times 10^{-5}$ cells $mL^{-1}$ seawater.

CUMULATIVE % MORTALITY



DAYS
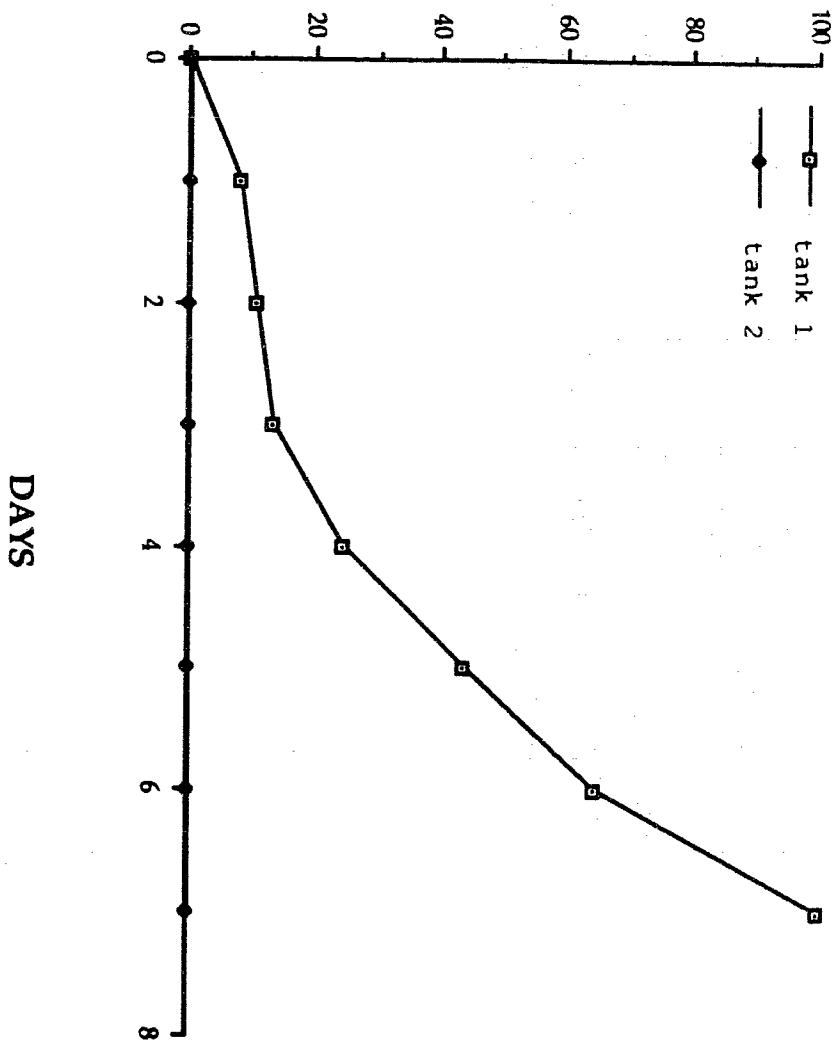
Fig. 49. *V. anquillarum* content (CFU g$^{-1}$) of gill tissue of coho in tank 1 (*C. concavicornis* and *V. anquillarum*), tank 2 (*V. anquillarum* only) and tank 3 (untreated control) at various times following a challenge with this bacterium at time 0. Error bar indicates +/- s.e.m.and *** indicate significant difference from the control value at the same sampling time (P<0.001).
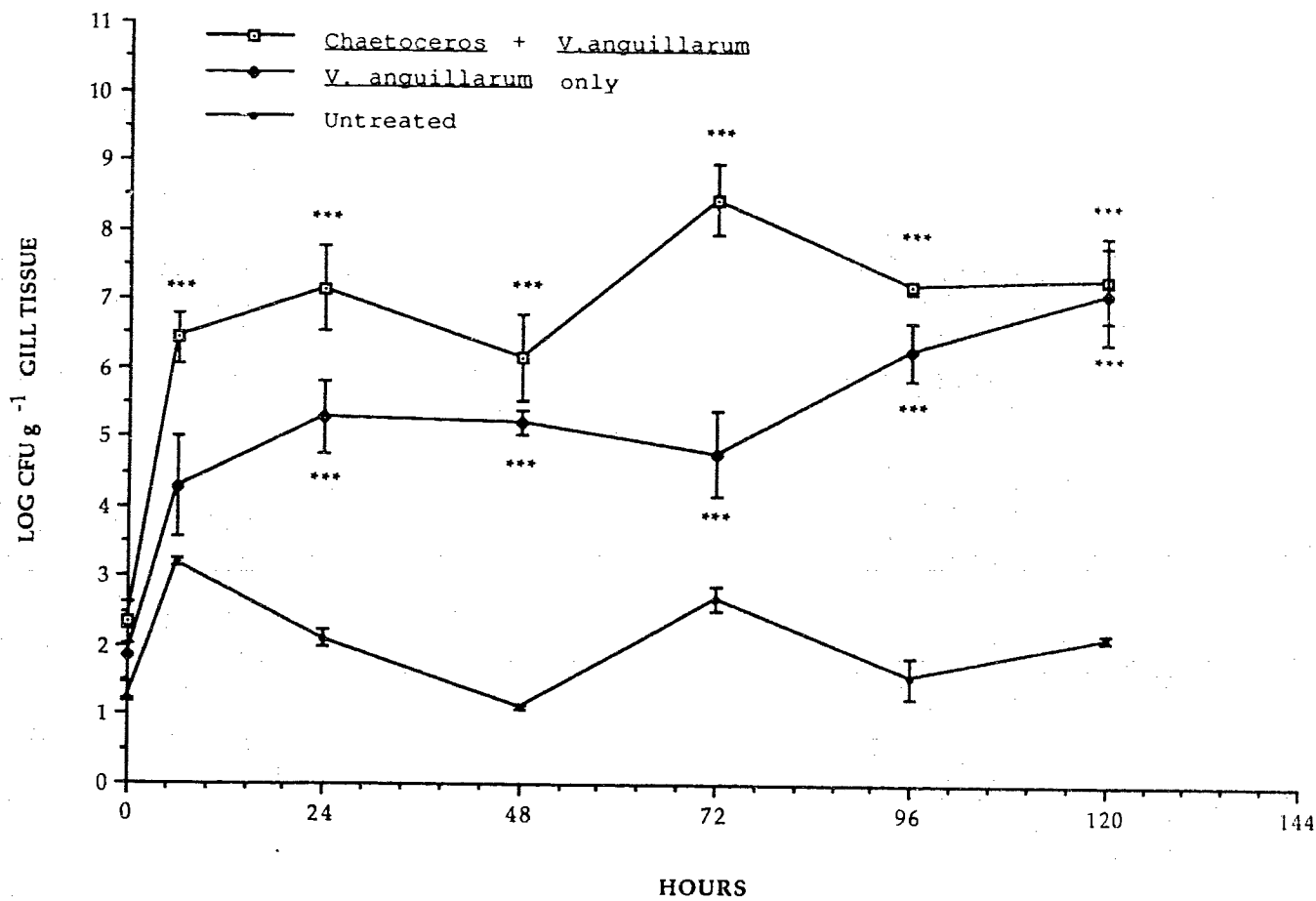
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The CFU g$^{-1}$ of kidney tissue of the fish in tank 3 were significantly different from those of the fish in tank 1 and 2 at 12 h (P<0.01, P<0.01), 24 h (P<0.001, P<0.001), 48 h (P<0.001, P<0.001), 72 h (P<0.001, P<0.001), 96 h (P<0.001, P<0.001) and 120 h (P<0.001, P<0.001) respectively (Fig. 50). The CFU g$^{-1}$ of kidney tissue of the fish in tank 1 were significantly different from those of fish in tank 2 at 24 h P<0.001) (Fig. 50).

The CFU g$^{-1}$ of spleen tissue of the fish in tank 3 were significantly different from those of the fish in tank 1 at 12 h (P<0.01) and tank 2 at 24 h (P<0.001, P<0.001), 48 h (P<0.001, P<0.001), 72 h (P<0.001, P<0.001), 96 h (P<0.001, P<0.001) and 120 h (P<0.001, P<0.001) respectively (Fig. 51).

Gross pathology included petechial hemorrhages of the fins, vents, and gills. Large diffuse hemorrhages on the body surface occurred (Fig. 52) as well as hemorrhages within muscle tissue. The epidermis around each ulceration showed necrosis and greyish-white coloration. Hemorrhages occurred around the periphery of each ulceration. Hemorrhages occurred in the livers and intestines. The spleens were enlarged and there was a moderate amount of ascites in the peritoneal cavity.

Fig. 50. *V. anguillarum* content (CFU $g^{-1}$) of kidney tissue of coho in tank 1 (*C. concavicornis* and *V. anquillarum*), tank 2 (*V. anquillarum* only) and tank 3 (untreated control) at various times following a challenge with this bacterium at time 0. Error bar indicates +/- s.e.m.** and *** indicate significant differences from the control values at the same sampling time (P<0.01) and (P<0.001) respectively.
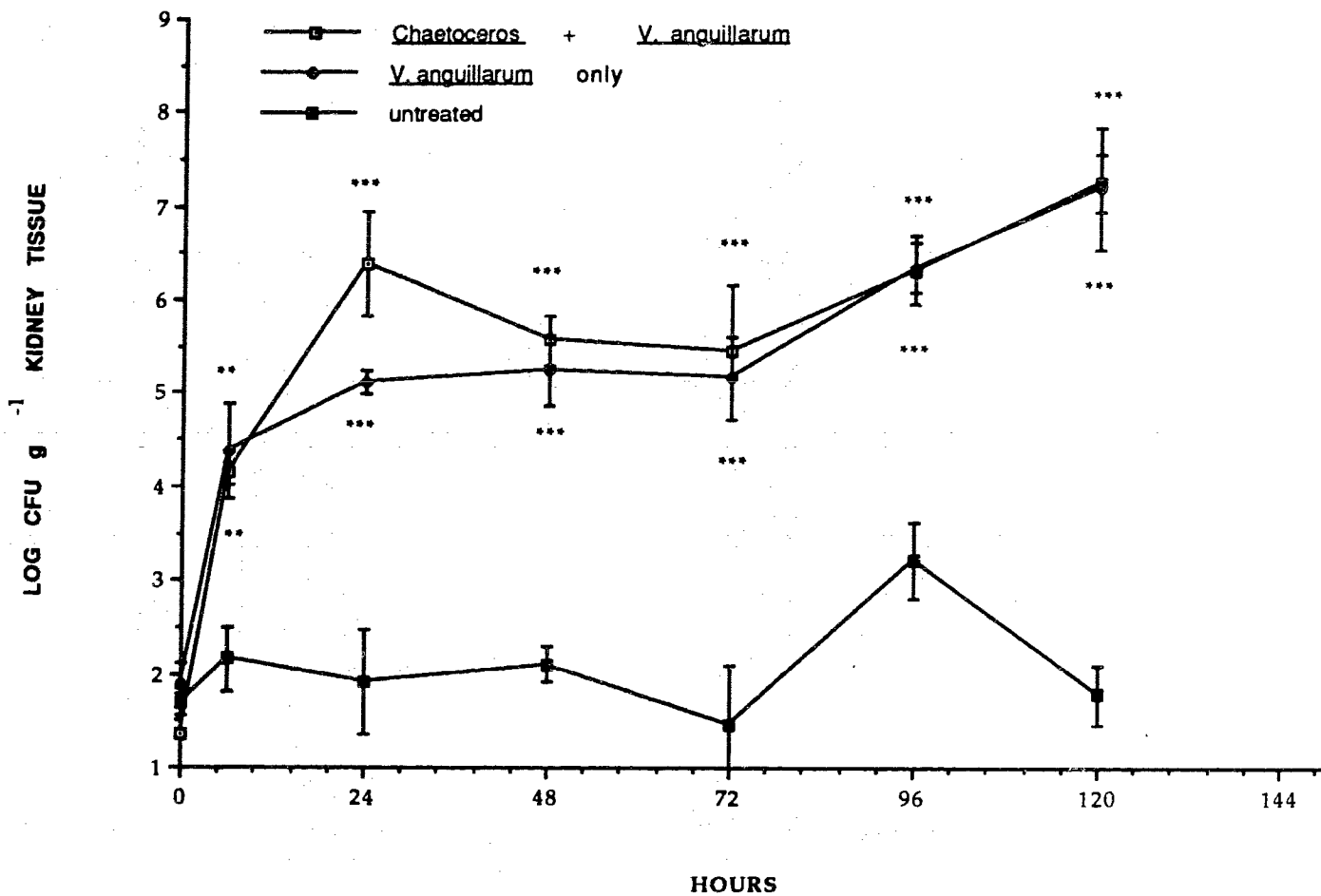
Fig. 51. *V. anguillarum* content (CFU $g^{-1}$) of spleen tissue of coho in tank 1 '(*C. concavicornis* and *V. anquillarum*), tank 2 (*V. anquillarum* only) and tank 3 (untreated control) at various time following a challenge with this bacterium at time 0. Error bar indicates +/- s.e.m.** and *** indicate significant differences from the control values at the same sampling time (P<0.01) and (P<0.001) respectively.

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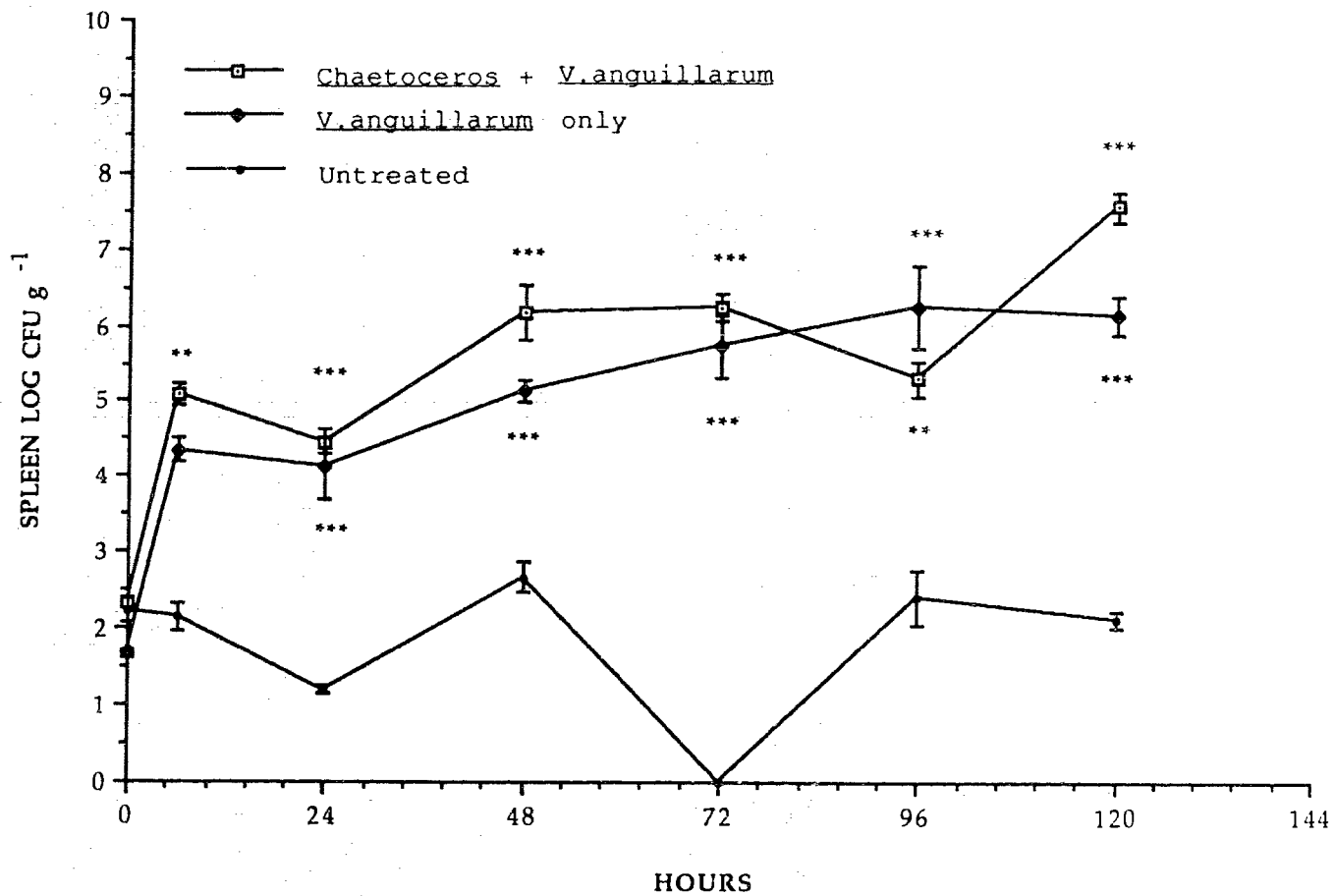
Fig. 52. Gross pathological view showing hemorrhages and excessive grey to yellow mucus mixture on surface of the coho gill (a) and large diffuse hemorrhages, swelling, and ulcerations on the surface of coho body (b).

A



B
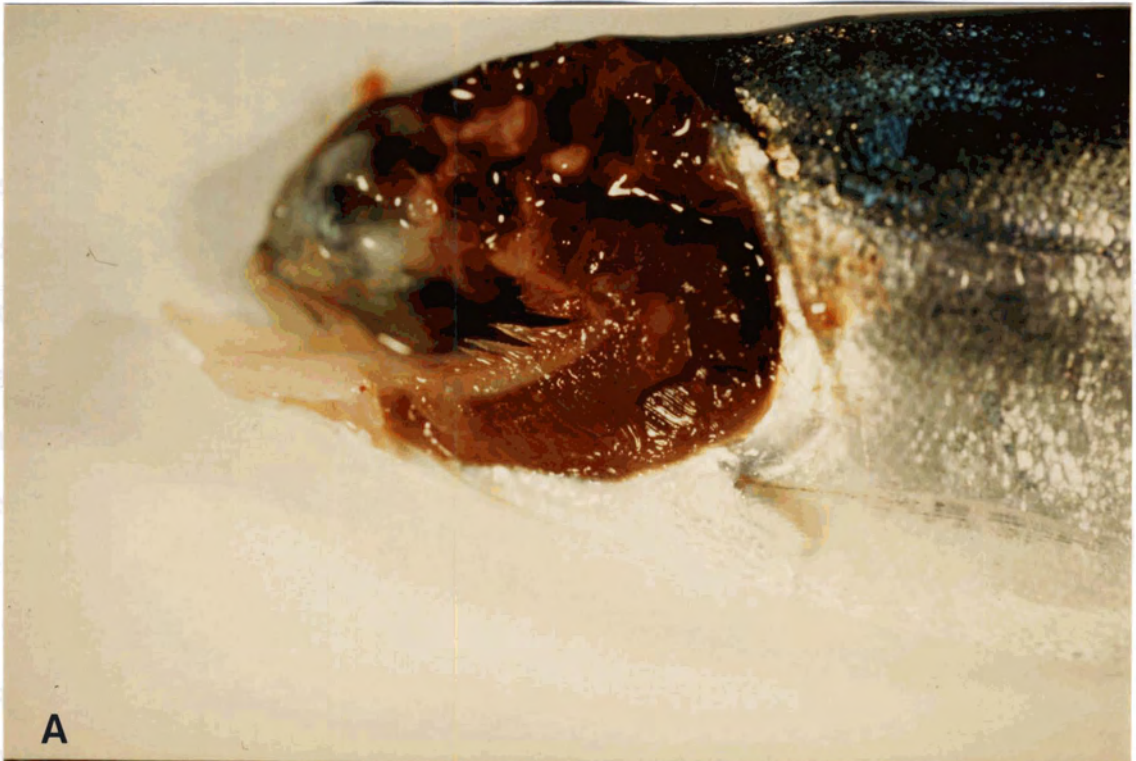
# DISCUSSION

In experiment 2, *C. concavicornis* was added to the seawater to a concentration of approximately 37 cells $mL^{-1}$ in day 0 (Fig. 47). the *C. concavicornis* concentration was then allowed to droop to approximately 33, 21 and 22 cells $mL^{-1}$ on days 1, 2 and 3 respectively (Fig. 47). From day 4 the concentrations were maintained at approximately 14 cells $mL^{-1}$. The greater concentrations of *C. concavicornis* were used from day 0 to day 3 to help initiating vibriosis infection. The lower *C. concavicornis* concentrations from day 4 were used to ensure that mortalities were vibriosis-related rather than *C. concavicornis*-related.

In the previous two chapters, it was determined that several components of the immune system of both chinook salmon and rainbow trout were affected when *C. concavicornis* was present at harmful concentrations. In chapter 2 it was observed that the numbers of neutrophils, lymphocytes, and thrombocytes of chinook salmon decreased in the presence of harmful concentrations of *Chaetoceros* spp. In chapter 3 it was demonstrated that cortisol levels were significantly increased. Other authors have shown that these changes were associated with immuno-suppression in salmonids (Roth, 1972; Robertson *et al.*, 1963; Maule *et al.*, 1987; Wiik *et al.*, 1989). In chapter three it was also observed that harmful concentrations of *C. concavicornis* caused increases in the

interrenal and chromaffin nuclear and cell diameters in rainbow trout. Several authors have noted a correlation between increased diameters of the cells and their nuclei, and the susceptibility of salmonids to diseases (Wedemeyer, 1970; Snieszko, 1974; Wedemeyer and McLeay, 1981).

The present investigation showed that the mortality of the fish treated with *C. concavicornis* only was much lower than that of fish treated with a similar concentration of *C. concavicornis* and *V. anguillarum* cells (Fig. 46). Because the concentrations of *V. anguillarum* were added to ensure low-level challenges the *V. anguillarum* treated fish was displayed low mortality rates due to vibriosis. However, in the presence of between approximately 15 and 25 cells of *C. concavicornis* mL$^{-1}$ seawater, mortality rates due to vibriosis greatly increased (Figs. 46). This indicated that the presence of *C. concavicornis* stressed the coho, reduced their resistance to *V. anguillarum* and caused an increased susceptibility to infection with this bacterium. Indeed, there appeared to be a concentration dependence of mortality and susceptibility of coho to this phytoplankter, because in the presence of higher concentrations of *C. concavicornis*, the mortality rates and susceptibility to this pathogen were greater than those expressed in lower concentration of this phytoplankter (Fig. 46).

Several colonies were observed on the Petri plates inoculated with kidney, spleen, and gill tissues of the coho prior to the challenging of *V. anguillarum*. Although one

would expect the first two mentioned organs to test negative for the presence of heterotrophic bacteria, the manipulations involved in removing these organs from the fish's bodies appeared to contaminate them with small numbers of bacteria (Figs 49-51). The gills, being exposed to bacteria in the seawater, not surprisingly had larger numbers of different types of heterotrophic bacteria.

The presence of *C. concavicornis* caused the numbers of *V. anguillarum* in and on the kidney, spleen and gill tissues to increase greatly (Figs. 49-51). It is probable that the bacteria gained easier access to tissues of the coho when they were treated with *C. concavicornis*. This is perhaps because that the immune system of coho salmon might have been impaired and the damage to the gills caused by the penetration by spines may have allowed bacteria to enter the fish in the presence of *C. concavicornis*. These increases probably explain why the mortalities of coho treated with both *C. concavicornis* and bacteria were much higher than those of coho treated with bacteria only.

Bacterial adherence to tissue surfaces is believed to be the initial step in the initiation of bacterial infections (Horne and Baxendale, 1983). Krovacek *et al.* (1987) have reported that some strains of *V. anguillarum* isolated from finfish possessed an adherence factor(s). They showed that vibrio strains adhered to liver and embryo tissue cultures of rainbow trout as well as to fish mucus coated glass surfaces. They suggested that this adherence

factor(s) may cause the attachment to specific receptors on the fish cells and that mucus may possess this receptor. They also suggested that presence of mucous material in the environment might enhance accumulation of *V. anguillarum*.

In the present study, approximately 100-fold greater concentrations of *V. anguillarum* accumulated in the gills of fish treated with *C. concavicornis* as compared to those treated with the bacteria only (Fig. 50). There were masses of *C. concavicornis* cells and mucus on and between the primary and secondary lamellae of coho treated with the phytoplankter. This excessive amount of mucus may have acted as a filtering mechanism to trap the added *V. anguillarum*.

# CHAPTER FIVE

The Use of Mucolytic Agents for Decreasing the Mortality
Rates of Coho Salmon Exposed to Lethal Concentrations of the
Harmful Phytoplankter, *Chaetoceros concavicornis*

# INTRODUCTION

Previous work (chapter 1) has shown that when the spines of *C. concavicornis* become trapped between the primary and secondary lamellae of rainbow trout and chinook salmon, they cause the production and accumulation of such an excessive amount of mucus that oxygen uptake by the gills is greatly inhibited. The finfish become hypoxic. As few as five cells $mL^{-1}$ of seawater can cause mortality of salmon (Bell *et al.*, 1974). At concentrations less than five cells $mL^{-1}$, the salmonids become sufficiently stressed that they becomes prone to diseases (Albright *et al.* in press).

Because the primary factor initiating salmon mortality appears to be the secretion and accumulation of excess mucus, any substance(s) that (1) prevented or slowed secretion of mucus and (2) limited its accumulation should reduce salmon mortalities when harmful *Chaetoceros* spp. were present.

Fortunately, such substances exist. These are mucolytic agents. Mucolytic agents, such as N-acetylcysteine and L-cysteine ethyl ester act *in vitro* by destroying the binding between adjacent mucus strands and *in vivo* by serving as competitive inhibitors of mucus synthesis (Sheffner, 1963; Walters *et al.* 1985; Ueno *et al.* 1989).

Accordingly, the mucolytic agent, L-cysteine ethyl ester, was used to treat salmon subjected to a lethal

concentration of *C. concavicornis* in the suspending seawater to determine if an enhanced mucus production and accumulation is indeed the primary factor causing increased mortalities by this phytoplankter.

*Experiment 1*

All experiments were carried out using yearling coho salmon (*O. kisutch*) which were obtained from the Capilano Hatchery, Canada Dept of Fisheries and Oceans in West Vancouver, B.C. All fish were transported to Simon Fraser University and maintained in aerated, 10‰o salinity water for more than one month prior to each experiment. A total of 200 coho, weighing between 10 to 16 g, were randomly divided into four groups of 50 fish each. Each group was placed in a 200 L oval tank, each of which contained 100 L of seawater of 10‰o salinity. The pH, temperature and dissolved oxygen content were maintained at between 6.8-7.2, 10.0-10.5° C, and 9.8-10.5 mg $L^{-1}$, respectively. The water in each tank was changed daily.

The mucolytic agent, L-cysteine ethyl ester (Sigma, St. Louis, MO), was sprayed onto 2 mm diameter standard salmon feed pellets (Murray Elevatores, Murray, UT) such that concentrations of 0.3, 0.6, and 0.9 mg were added $g^{-1}$ feed.

The coho in tanks 1, 2, and 3 were fed a ration which contained 0.3, 0.6, and 0.9 mg L-cysteine ethyl ester $g^{-1}$ feed, respectively for 3 days prior to *C. concavicornis* treatment and for another 2 days following *C. concavicornis* treatment. The coho in tank 4 were fed feed without L-

cysteine ethyl ester. All four groups of coho were fed at the rate of 8.0 g feed $d^{-1}$ $tank^{-1}$. The coho in tanks 1, 2, and 3 were therefore calculated to ingest 12, 8, and 4 mg L-cysteine ethyl ester $kg^{-1}$ biomass $d^{-1}$, respectively.

Following these L-cysteine ethyl ester treatments, the coho in each of the four tanks were treated with cells of *C. concavicornis* such that a concentration of this harmful diatom was maintained at between 22 and 28 cells $mL^{-1}$ seawater for 4 days. Mortalities in each tank were retrieved and recorded daily.

*Experiment 2*

The material and methods were similar to experiment 1, except for the following minor changes.

Two-hundred sixty coho each weighing between 10 to 16 g were randomly divided into four groups of 65 fish each. Each group was placed in each of four-200 L tanks. The pH, temperature, and dissolved oxygen content were maintained at between 6.7-7.5, 17.5-18.0° C and 10.0-10.5 mg $L^{-1}$, respectively. Following three days of L-cysteine ethyl ester treatment (in feed), the fish in all four tanks were treated with cells of *C. concavicornis* such that the concentrations of the harmful diatom were maintained between 40 and 54 cells $mL^{-1}$ in the 10 ‰ salinity water for 4 days.

*Histological methods*

Gill arches were removed from moribund coho in tanks 1, 2 and 3 and randomly from coho in tank 4 and immediately immersed in 2.5% glutaraldehyde in a 0.1M sodium cacodylate buffer containing 2% (w/v) alcian blue (BDH Chemicals, Toronto, ON) (Powell, *et al.* 1992). Tissues were maintained at room temperature in the fixative for 24 h and then dehydrated in ethanol, embedded in paraffin wax, sectioned, stained with Giemsa and examined by light microscopy.

RESULTS

*Experiment 1*

The concentrations of *C. concavicornis* in all tanks were successfully maintained at between 22 and 31 cells $mL^{-1}$ for the duration of the experiment (Fig. 53). One fish died in tank 1 in which the fish were fed with 12 mg of L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$ during the 5 day experiment. Three fish died in tank 2 in which the fish were treated with 8 mg of L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$ during the 5 day experiment. A total of 4 fish died in the tank 3 in which the fish were treated with 4 mg of L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$ during the 5 day experiment. A total of 13 fish died in the control tank in which the fish were not treated with L-cysteine ethyl ester in the presence of *C. concavicornis* during the 5 day experiment. Although all coho were moved into *C. concavicornis*-free water at the end of day 4, mortalities of all groups of fish were assayed to day 5 (Fig. 54). The cumulative mortalities of the coho in tanks 1, 2, 3, and 4 were 2%, 6%, 8% and 26%, respectively by day 5 (Fig. 54).

*Experiment 2*

The concentrations of *C. concavicornis* in all tanks were successfully maintained at between 40 and 54 cells $mL^{-1}$

seawater for the four days of the experiment (Fig. 55). One fish died in tank 1 in which the fish were fed with 12 mg of L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$ during the 4 day experiment. No fish died in tank 2 which were treated with 8 mg of L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$ during the 4 day experiment. A total of 8 fish died in tank 3 in which the fish were treated with 4 mg L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$ during the 4 day experiment. A total of 37 fish died in the untreated control tank 4 during 4 day experiment. The cumulative mortalities of the coho in tanks 1, 2, 3, and 4 were 2%, 0%, 12%, and 57%, respectively by day 4 (Fig. 56).

*Histological results*

A very thick mucous layer overlayed the epithelia of the secondary lamellae of coho treated with *C. concavicornis* only. A great deal of mucus accumulated between the adjacent secondary lamellae of these salmon. (Figs. 57a and 57b). However, only a thin layer of mucus was observed on the exterior of the epithelia of the secondary lamellae of control coho which were not exposed to cells of *C. concavicornis*. Little to no mucus was observed between adjacent secondary lamellae of these fish (Figs. 57e and 57f). Similar results were observed on the secondary lamellae of coho treated with both L-cysteine ethyl ester and *C. concavicornis* (Figs. 57c and 57d).

Fig. 53. The mean concentration of *C. concavicornis* cells in tanks 1, 2, 3, and 4. Error bar indicated +/- s.e.m.

# CHAETOCEROS NUMBER

(cells ml$^{-1}$)



DAYS

treated 1   12 mg kg$^{-1}$ day$^{-1}$

treated 2   8 mg kg$^{-1}$ day$^{-1}$

treated 3   4 mg kg$^{-1}$ day$^{-1}$

control
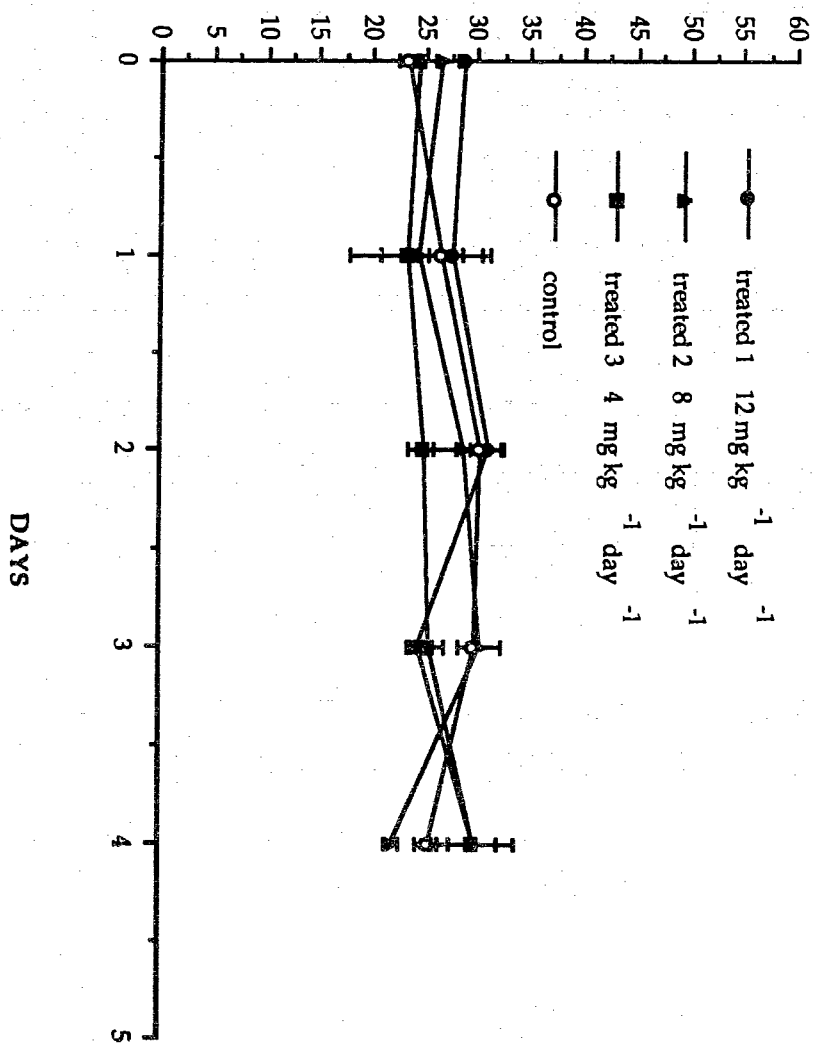
Fig. 54. The cumulative mortalities of coho salmon treated with 12, 8, 4, and 0 mg L-cysteine ethyl ester $kg^{-1}$ biomass $day^{-1}$, respectively, in tanks 1, 2, 3, and 4. All coho salmon were exposed to 22-30 *C. concavicornis* cells $mL^{-1}$ seawater for a 4 day period.

CUMULATIVE % MORTALITY
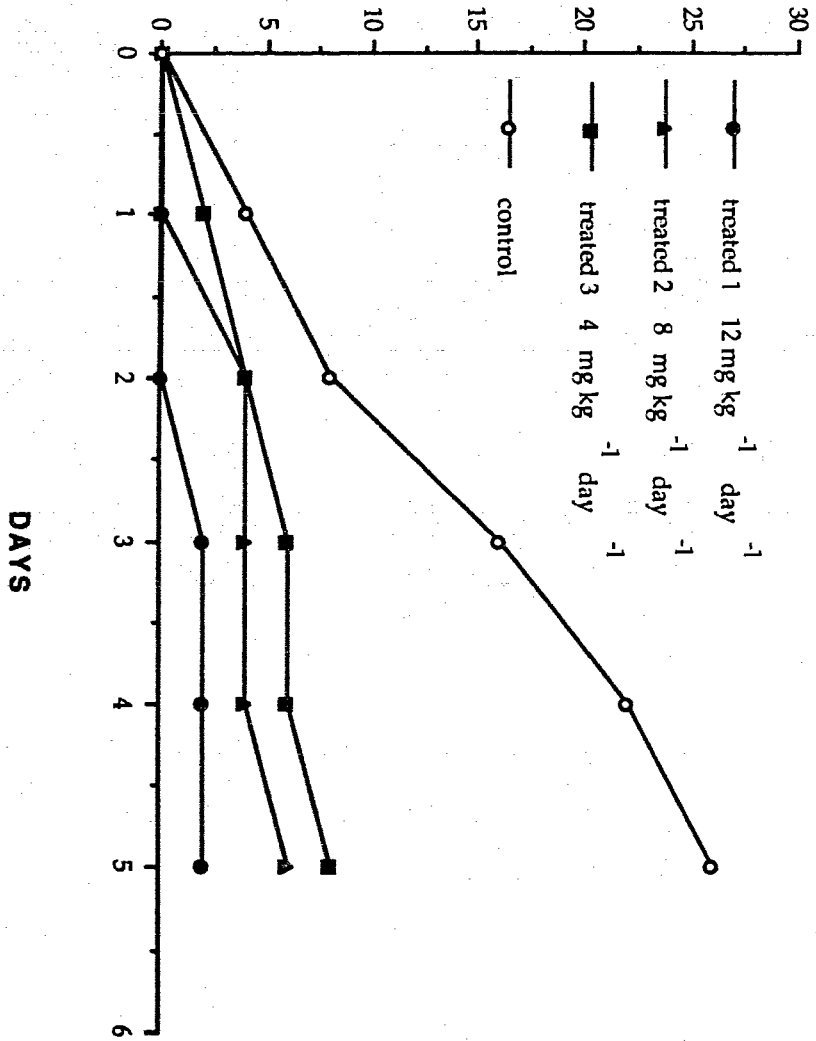
treated 1  12 mg kg⁻¹ day⁻¹

treated 2  8 mg kg⁻¹ day⁻¹

treated 3  4 mg kg⁻¹ day⁻¹

control

DAYS

Fig. 55. The mean concentration of *C. concavicornis* cells in tanks 1, 2, 3 and 4. Error bar indicated +/- s.e.m.

CHAETOCEROS NUMBER

(cells    ml $^{-1}$ )

treated 1   1.2 mg kg $^{-1}$ day $^{-1}$

treated 2   8 mg kg $^{-1}$ day $^{-1}$

treated 3   4 mg kg $^{-1}$ day $^{-1}$

control

DAYS
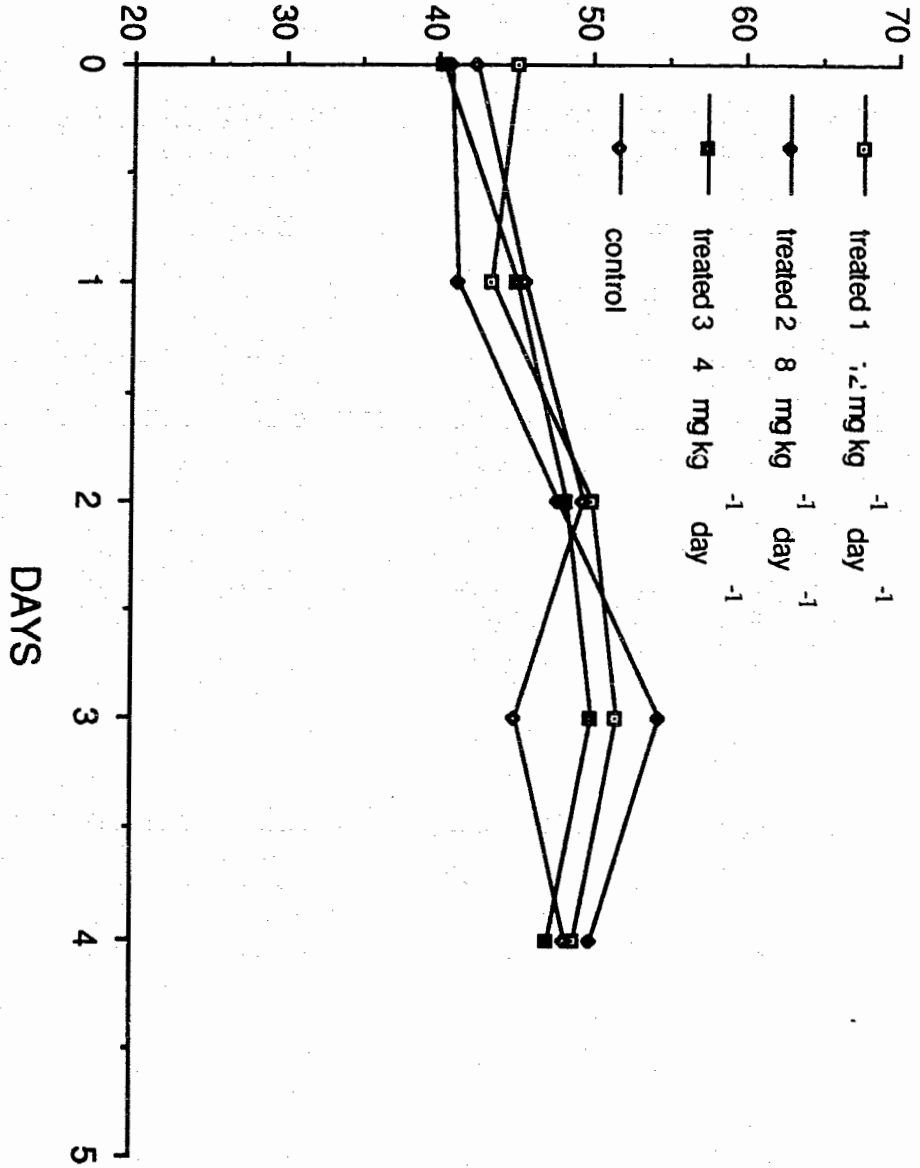
Fig. 56. The cumulative mortalities of coho salmon treated with 12, 8, 4, and 0 mg L-cysteine ethyl ester kg$^{-1}$ biomass day$^{-1}$ in tanks 1, 2, 3, and 4, respectively. All coho salmon were exposed to 40-54 *C. concavicornis* cells mL$^{-1}$ seawater.

CUMULATIVE % MORTALITY

treated 1  12 mg kg$^{-1}$ day$^{-1}$

treated 2  8 mg kg$^{-1}$ day$^{-1}$

treated 3  4 mg kg$^{-1}$ day$^{-1}$

control

DAYS
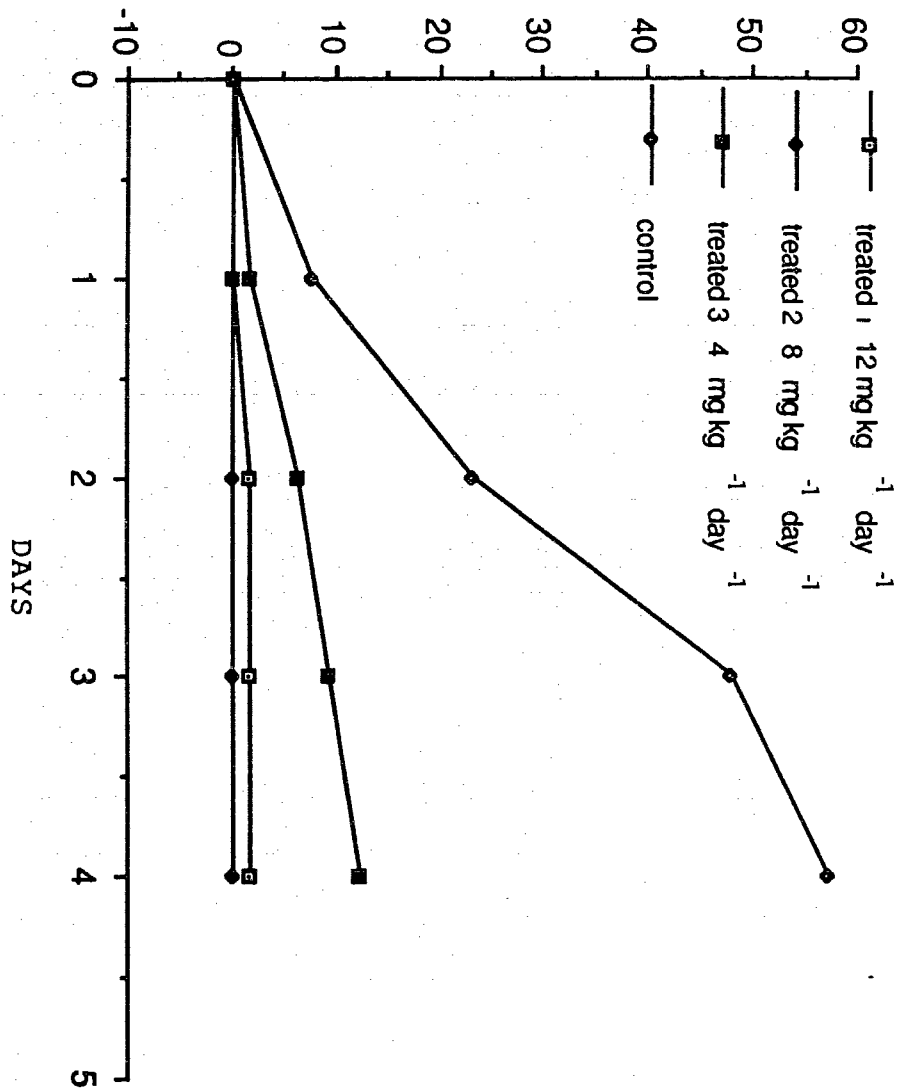
Fig. 57. Light microscopic views of 6 μm thick sections of alcian blue stained secondary lamellae of coho salmon: A and B, treated with *C. concavicornis* only; C and D, treated with *C. concavicornis* and L-cysteine ethyl ester and E and F, untreated control
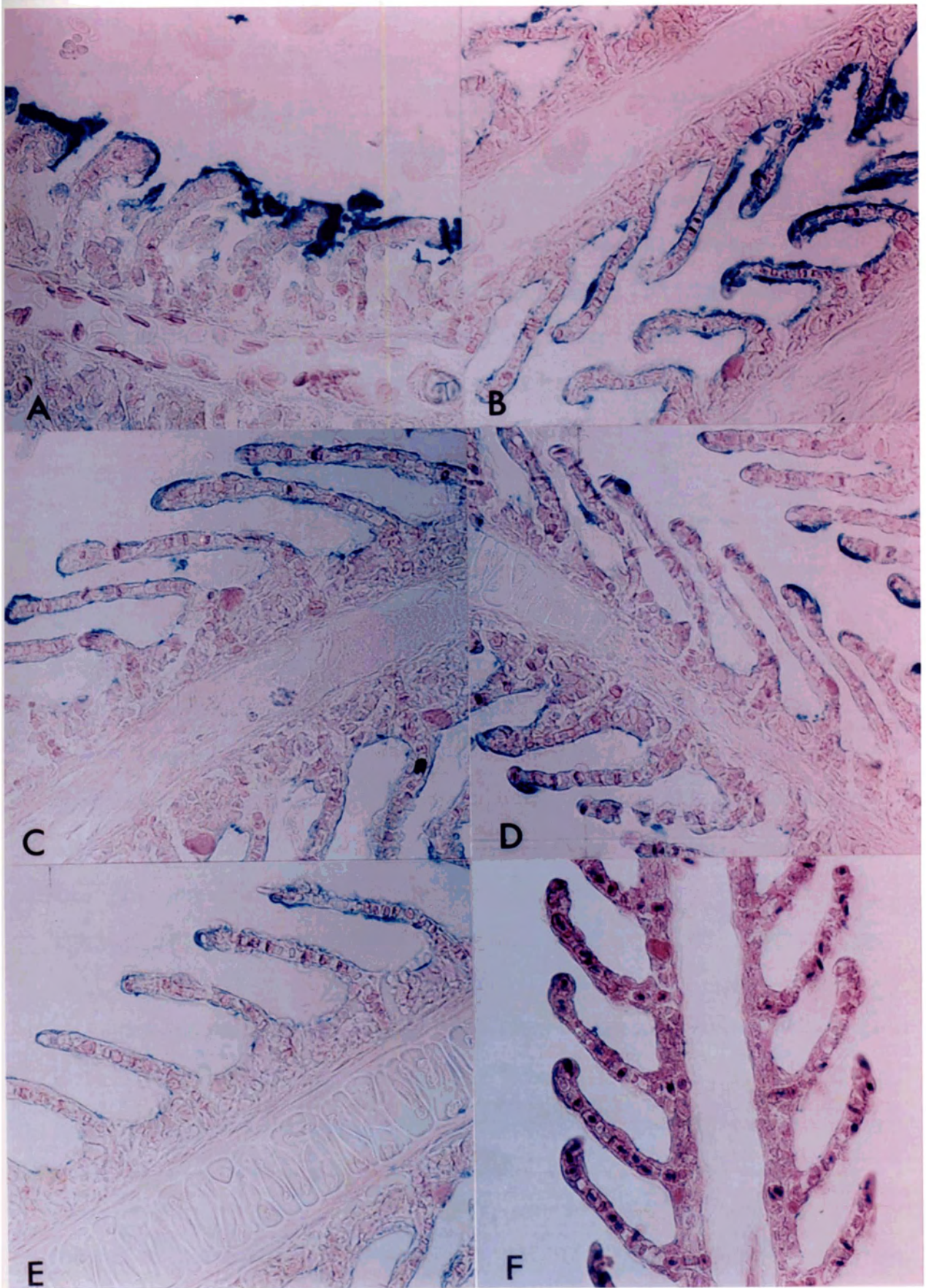
# DISCUSSION

Mucus is a term used to describe the secretory products
of particular organs of animals with specific reference to
products of mucous, goblet and serous cells (Charman et al.,
1974). The terms, glycoproteins, mucoprotein,
mucopolysaccharides and mucin have been used to describe
mucus. Mucus is a proteinaceous material which consists
mainly of mucopolysaccharides, with the long,
interconnected, fibrous molecules occurring within a gel
(Ziment, 1978). The physical properties of mucous secretions
are largely determined by the large molecular weight
glycoproteins (mucins) which consist of a protein backbone
with many oligosaccharide side chains. The peptide chain of
mucin contains some non-glycosylated regions; these regions
contain many cysteine residues. Many mucous glycoproteins
are composed of polymerized glycoprotein subunits through
the formation of disulphide bonds in the non-glycosylated
region of each protein core, probably involving interaction
between cysteine residues (Allen, 1978) and between the long
mucopolysaccharide strands, resulting in a network of matted
molecules. Other intermolecular bonds, such as ionic and
hydrogen bonds as well as London-van der Waals forces, also
bind glycoprotein subunits to each other (Majima et al.
1989).

Both acidic and neutral glycoproteins occur (Charman *et al*. 1974), although the former is probably the more important determinant of the rheological behavior of these types of secretion (Gibbons and Glover, 1959). The absolute level of viscosity appears to be mainly related to the concentration of acid glycoproteins. Fig. 58 illustrates the general makeup of a human nasal acid glycoprotein. It consists of a polypeptide core with branching oligosaccharide side chains, which include fucose- a 6-deoxyhexose sugar, and a sulphate residue or sialic acid, probably in terminal positions on the side chains. Sialic acid contains an amino group and carboxylic acid (Gottschalk, 1966). Calcium ions, as well as other proteins and DNA may contribute to high viscosity of mucus (Ziment, 1978). In many respiratory diseases, the goblet cells increase in number and, perhaps, in activity (Ziment, 1978). As a result, an excessive amount of viscous mucus is secreted. Unfortunately, the goblet cells do not appear to be under nervous or hormonal control, and therefore they are not readily susceptible to pharmacologic therapy (Ziment, 1978). The overactivity of goblet cell appears to be a direct consequence of irritation (Ziment, 1978).

In 1963, Sheffner reported that the L-cysteine analogue, N-acetylcysteine, was able to decrease human nasal mucus viscosity *in vitro* by reducing disulphide bonds of adjacent glycoproteins.

Fig. 58. Structure of mucus. The mucopolysaccharide chains are interconnected by numerous chemical and physical bonds (cited from Ziment, 1978).

MUCOPROTEIN STRAND

AMIDES (e.g., urea) — Hydrogen bond

NATURAL PROTEASES (i.e., proteolytic enzymes)

Electrophysical bonds

REDUCING AGENTS (e.g., thiols)

Ca⁺⁺

Disulfide bridge

SURFACE-ACTIVE AGENTS (e.g., detergents, wetting agents)

DNA

Ca⁺⁺

Calcium ion bond

NUCLEASES (e.g., deoxyribonuclease)

DNA

DISSOCIATING AGENTS (e.g., hypertonic saline)

CHELATING AGENTS (e.g., L-arginine)

IONS (e.g., hypertonic saline)

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Subsequent studies have confirmed this property (e.g. Lightowler and Lightowler, 1971; Walters *et al.*, 1985). N-acetylcysteine also can break down the disulphide cross-linkage between proteins and DNA in the mucus by virtue of its free sulphydryl group (Ziment, 1978).

When administered *in vivo* to humans (Aylward *et al.*, 1980) and canines (Martin *et al.*, 1980) N-acetylcysteine decreases the production and secretion of mucus by goblet cells. In addition to this, it also appears to be able to reduce disulphide bonds and break the macromolecular glycoprotein to smaller subunits *in vivo* (Ueno *et al.*, 1989). Cotgreave *et al* (1987) proposed that N-acetylcysteine depresses mucus production by increasing the synthesis of glutathione.

As a reducing agent, N-acetylcysteine is broken down by oxidizing reagents. When it reacts with mucoproteins, it decomposes to acetate and cysteine with liberation of hydrogen sulfide. The free sulfhydryl group of cysteine reacts with the disulfide bridges of mucoprotein, thereby breaking the complex protein network into less viscous strands (Ziment, 1978). Breakage of disulfide bonds results in a marked decrease in the viscosity of mucus, and the most successful mucolytic agents that have been investigated appear to produce their effects by this mechanism (Ziment, 1978).

The important mucolytic agents are cysteine analogues such as N-acetylcysteine and L-cysteine ethyl ester (Martin

*et al.*, 1980, Flora *et al.*, 1985, Majima *et al.*, 1989, Servin *et al.*, 1988). N-acetylcysteine has an undesirable feature in that it possesses a strong odor, whereas L-cysteine ethyl ester is odorless. Because finfish, including salmonids, have a strong olfactory avoidance response to certain odors, it was decided to use L-cysteine ethyl ester as a mucolytic agent in the experiments described here.

The data clearly show that L-cysteine ethyl ester suppresses mucus production by the cells of the secondary lamellae and decomposed mucoprotein (Figs. 57c and 57d). The specific cellular activity which is most likely inhibited by this therapeutant is the mucus secretory activity of the goblet cells of this tissue. The decomposition of mucoprotein likely resulted in a mucus of lower viscosity which was likely more easily washed off by water passing through the gills of the fish.

The data also show that the suppression of mucus production by L-cysteine ethyl ester in the diet at either 4, 8, or 12 mg kg$^{-1}$ biomass d$^{-1}$ was able to reduce fish mortality (Figs. 55 and 57). Partial protection occurred at ingestion rates of 4 and 8 mg L-cysteine ethyl ester kg$^{-1}$ biomass d$^{-1}$ and complete protection occurred at 12 mg kg$^{-1}$ biomass d$^{-1}$ (Figs. 55 and 57).

In the present investigation, coho were treated with mucolytic agent before exposure of the fish to *C. concavicornis*. If the fish were fed with feed containing mucolytic agent starting one day after exposure to *C.*

*concavicornis*, it is probable that a protective effect would also occur. The mucolytic agent will remove excess mucus from secondary lamellae when administered prior to or after exposure of fish to *C. concavicornis*.

The oral treatment of the fish with L-cysteine ethyl ester will also likely cause suppression of skin mucus production. It has been suggested that the mucous layer on fish skin acts as a mechanical protective barrier and limits colonization by parasites, fungi and bacteria (e.g. Arillo and Melodia, 1990). Therefore suppression of skin mucus production may allow greater colonization and invasion of this tissue by pathogens. This is not foreseen as a major problem since no fish died in any of the experiments following removal of *C. concavicornis*.

The observations presented herein are direct proof that the third of three hypotheses presented in chapter 1 is indeed correct, ie. that "suffocation (of salmonids) from excessive mucus production at the sites of penetration of the gills by the (*C. concavicornis*) spines" is the cause of salmonid mortalities in the presence of overtly harmful concentrations of the phytoplankter.

GENERAL CONCLUSION

This study has focussed on the influence of the harmful phytoplankter, *Chaetoceros concavicornis*, on the survival and physiology of several salmonid species. The results of scanning and light microscopic examination of gill tissues showed that large numbers of *C. concavicornis* cells and their spines became lodged between secondary lamellae with some of the spines penetrating the lamellar cells. The presence of these irritating barbed spines stimulated the production and accumulation of excess mucus on and between the secondary lamellae. The goblet cells of the gills of treated fish became more numerous and prominent. Similar changes were observed in gills of rainbow trout and chinook and coho salmon among different sized fish of between 5-300 g weights.

The data presented in chapter 1 clearly indicate that the barbed spines of the harmful diatom *C. concavicornis* damaged the physical integrity of the respiratory epithelium of salmonids. The salmonids responded to this physical injury of the gill tissue by producing excessive amounts of mucus (chapters 1 and 2). The accumulation of this mucus on and between the secondary lamellae inhibited the gill functions of $O_2$ diffusion (and likely of metabolic waste products release). The hypoxic conditions that resulted

within the fish then caused a cascade of events, including decreased oxygen consumption, increased hematocrits, increased ventilation volume and ventilation frequency, and anaerobic metabolism, as the trout attempted to compensate for loss of arterial blood $O_2$ (chapter 1). The observations are direct proof that the third of the hypothesis presented in chapter 1 is indeed correct: i.e, "suffocation (of salmonids) from excessive mucus production at the sites of penetration of the gills by the (C. concavicornis) spines" causes salmonid mortalities in the presence of overtly harmful concentrations of the phytoplankter.

It was also noted that there were several subtle effects of harmful Chaetoceros cells treatment on the surface of the cells of this organ. One was its effect on the gill microridges. The microridges of the lamellae of untreated chinook salmon were well developed. However, following 72 h of exposure to C. concavicornis the microridges became much less well defined (chapter 2). Since microridges are likely used to increase the surface area of the primary lamellae (Hughes, 1979; Fishelson, 1980), their diminution in the presence of harmful Chaetoceros spp. is very likely another factor limiting the exchange of gases and metabolic wastes at the gills.

Lactate and glucose concentrations have been shown by many investigators to be sensitive indicators of hypoxic stress in rainbow trout (Holeton and Randall, 1967a) as well as several other fresh and seawater species (Hattingh, 1967;

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Yu and Woo, 1987). The pattern of lactate and glucose concentration increases noted in chapters 1 and 2 indicates that low blood $O_2$ concentration likely triggered anaerobic metabolism in the rainbow trout and chinook salmon treated with *C. concavicornis.*

The increase in blood hematocrit values and erythrocyte concentrations of the yearling chinook and rainbow trout which were observed upon treatment of these two species with the stressor, *C. concavicornis* (chapters 1 and 2), has also been observed by other investigators when they applied various other stressors to finfish, including rainbow trout and salmon (Holeton and Randall, 1967a; Swift and Lloyd, 1974; McLeay, 1975; Ostroumova, 1964). Based upon the observations of these workers and our data it would appear that the increases in erythrocyte concentrations and hematocrit values noted in chapters 1 and 2 may be mainly due to entry of erythrocytes into the circulatory system from storage organs, such as the spleen. Although there may be some influence on the hematocrits due to swelling of erythrocytes it is unlikely that swelling was a major factor in the increase based upon the observations of others and my data. Erythropoiesis was likely the major factor explaining the elevated hematocrits because erythrocyte numbers increased significantly (chapter 2).

The study in chapter 2 revealed a marked and highly significant suppression of the concentration of circulating neutrophils, lymphocytes, and thrombocytes in the *C.*

*concavicornis*-treated fish. Neutrophilo-, lymphocyto-, and thrombocytopenia is typical of the response of salmonids to a wide variety of acute and chronic stressors (Pickering *et al.* 1982; Pickering and Pottinger, 1987a). Evidence is now accumulating that lymphocyte and thrombocyte activities in fish is strongly suppressed by corticosteroids (Pickering, 1984; Pickering and Pottinger, 1987a; Wiik *et al.* 1989). Ellsaesser and Clem (1987) have demonstrated that both the number and the immunological competence of circulating lymphocytes in the blood of channel catfish, *Ictalurus punctatus*, were reduced during stress. The neutrophils, lymphocytes, and thrombocytes of salmonids are significant components of the immune system of these fish (Andersen, 1974; Corbel, 1975; Post, 1978). Because of this, the depletion in the quality and quantity of these cells would have a marked influence upon the ability of the finfish to become or to remain immunocompetent. Therefore, the result of leucocytopenia observed in chapter 2 indicates the immuno-suppression of a portion of the chinook's immune system.

Because suppression of the immune system by sub-lethal concentrations of *C. concavicornis* might be significant, it was decided to further investigate several important aspects of this system in rainbow trout in the presence of lethal concentrations of this phytoplankter. These were the study of chromaffin and interrenal cell morphologies as well as

plasma cortisol concentrations of rainbow trout exposed to harmful concentration of *C. concavicornis*. By employing computer-assisted Feulgen-microspectrophotometry, a digitized image of a cell nucleus was generated. This image was subjected to special pattern analysis. The chromatin condensation states of interrenal and chromaffin nuclei of trout exposed to *C. concavicornis* were compared with that of control trout. It was shown that the interrenal and chromaffin cells became hypertrophic, and activities of these two kinds of tissues were increased under the *C. concavicornis* stress. Cortisol is released from the interrenal tissue as teleost fish attempt to cope with stress (Donaldson, 1981). Measurements of the plasma levels of cortisol have been used to assess the state of teleost immune systems (Ellsaesser and Clem 1987; Maule *et al.* 1987) and the magnitude of the stress responses (Donaldson, 1981). In chapter 3, the plasma cortisol levels of trout exposed to *C. concavicornis* were more than 10-fold greater than that of the control trout. Clearly, the trout were experiencing a severe physiological disturbance.

In chapter 2 and 3, it was shown that the immune system of salmonids exposed to *C. concavicornis* was suppressed, as measured by the lymphocyto-, thrombocyto, and neutrophilopenia, elevated cortisol levels, and increases in interrenal and chromaffin cells and cell nucleus diameters. It is the suppression of the immune system that predisposes salmonids to bacterial pathogens under field and laboratory

conditions when they are exposed to sub-lethal

concentrations of *Chaetoceros* spp.(Albright *et al.* in press;

chapter 4).

The association between stress and outbreak of disease

in teleost fish is well established and evidence for a role

of the hypothalamic-pituitary-interrenal axis in this

response is accumulating (Snieszko, 1974; Pickering and

Duston, 1983 and Pickering and Pottinger, 1987a). In

chapter 4, it was observed that the mortality rate of coho

salmon exposed concurrently to a harmful concentration of *C.*

*concavicornis* and to a concentration of the bacterial

pathogen, *V. anguillarum*, was greatly increased in

comparison to that for fish exposed to *V. anguillarum* only.

This indicated that the presence of the *C. concavicornis*

stressor reduced the resistance of coho to *V. anguillarum*

and caused increased susceptibility to this bacterial

infection. Indeed, there appeared to be a concentration

dependence of mortality on this phytoplankter, because in

the presence of higher concentration of *C. concavicornis*,

the mortality rates to this diatom were greater than with

the lower one. By comparing the time course appearance of *V.*

*anguillarum* in the kidneys, gills, and spleens of coho

salmon simultaneously treated with harmful concentrations of

*C. concavicornis* and *V. anguillarum*, it appeared that the

damaged gills were the sites for the initial entry of this

pathogen. This may have been due to the secondary lamellae-

*C. concavicornis*-mucus matrix acting as a trap for the pathogenic bacterium, *V. anguillarum* (chapter 4).

Mucus is a proteinaceous material which consists mainly of mucopolysaccharides, with long, interconnected, fibrous molecules (Ziment, 1978). Important mucolytic agents are cysteine analogs, such as N-acetylcysteine and L-cysteine ethyl ester. These agents are able to decrease human and canine mucus viscosity *in vitro* and *vivo* (Sheffner, 1963; Walters *et al.*, 1985; Martin *et al.*, 1980; Majima *et al.*, 1989, Servin *et al.*, 1988). When N-acetylcysteine reacts with mucoproteins as a reducing agent, the liberated hydrogen sulfide reduces the disulfide bridges of mucoprotein, thereby breaking the complex protein network into less viscous mucus (Ziment, 1978). Breakage of disulfide bands results in a marked decrease in the viscosity of mucus and most mucolytic agents appear to produce their effects by this mechanism (Ziment, 1978). The data in chapter 5 clearly show that L-cysteine ethyl ester suppressed mucus production by the cells of the secondary lamellae of coho salmon. The specific cellular feature which was most likely inhibited by this therapeutant was the mucus secretory activity of the goblet cells of this tissue. The data in chapter 5 also show that the mucus production by yearling coho was suppressed by the induction of L-cysteine ethyl ester in the diet at either 4, 8 or 12 mg $kg^{-1}$ biomass $d^{-1}$ for several days prior to and during a treatment with

lethal concentration of *C. concavicornis*. The suppression of mucus production significantly reduced fish mortality rates.

In summary, at concentration higher than 15 cells $mL^{-1}$ of harmful *C. concavicornis*, mortalities of salmonids occurred. This was due to suffocation of fish caused by excess mucus production and accumulation on the respiratory epithelia. At sub-lethal concentrations of harmful *Chaetoceros* spp., the immune system may be sufficiently suppressed to predispose the salmonids to bacterial pathogens. Oral treatment of salmon with L-cysteine ethyl ester, a mucolytic agent, greatly reduced mucus production and significantly lowered fish mortality rates when they were cultured in the presence of lethal concentrations of *C. concavicornis*. Although most of the stress indicators showed significant differences between controls and treated fish, the best criteria for judging stress in these fish was the $Pao_2$.

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