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Anti-phytoplankton therapy of finfish: the mucolytic agent L-cysteine ethyl ester protects coho salmon *Oncorhynchus kisutch* against the harmful phytoplankter *Chaetoceros concavicornis*

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ABSTRACT: The harmful diatoms *Chaetoceros concavicornis* and *C. convolutus* will kill salmonids such as rainbow trout *Oncorhynchus mykiss* and coho salmon *O. kisutch* if present at as few as 5 cells ml^{-1} seawater. Our previous research has shown that the barbed spines of these diatoms become wedged between the secondary lamellae of salmonids, where they cause excessive production of mucus by goblet cells. This mucus and the diatoms accumulate on and between the secondary lamellae to such an extent that the fish suffocate. A class of pharmacologically active compounds (mucolytic agents, e.g. L-cysteine ethyl ester) exists which decrease mucus production in mammals and humans when ingested. When coho salmon ingest this agent at up to 12 mg kg^{-1} biomass d^{-1}, mucus synthesis is reduced to such an extent that an accumulation of this material does not occur on and between the secondary lamellae; the fish remain viable in what would otherwise be a lethal exposure to *C. concavicornis* cells.

KEY WORDS: Anti-phytoplankton therapy · Mucolytic agent · Mucus · L-cysteine ethyl ester · Harmful phytoplankton · Salmon · *Chaetoceros concavicornis*

INTRODUCTION

In many regions of the world, finfish have been killed by harmful or toxic phytoplankters where they are farmed in coastal seawaters. Along the west coast of Canada and the northwest coast of the United States, there are 3 species of phytoplankters which most commonly kill penned salmonids. These are *Chaetoceros concavicornis*, *C. convolutus* and *Heterosigma akashiwo* (Gaines & Taylor 1986). Black (1991) has estimated that between 1986 and 1990 these phytoplankters killed between Can$ 0.5 and 4.2 million of farmed salmon annually along the British Columbia coast. Horner et al. (1991) estimated that toxic and harmful phytoplankton blooms in coastal seawaters of Washington State, USA, have killed more than 2 million farmed salmon, with monetary losses of approximately U.S.$10 million, in the period 1988 through 1990.

Measures to control phytoplankton-related finfish mortalities have been largely focused towards avoidance of culturing these fish in seawaters which are prone to harmful or toxic phytoplankton blooms. To date, control measures other than this are not available.

Chaetoceros concavicornis and *C. convolutus* are diatoms which possess siliconaceous spines which may be up to 200 µm in length. Along the length of each spine are numerous barbs. As water passes over the gills of finfish, the spined and barbed diatoms become trapped in large 'windrow-like' masses between the secondary lamellae (Fig. 1). These masses of spined and barbed diatoms irritate the goblet cells of the respiratory epithelium such that there is an accumulation of an excessive amount of mucus which restricts dissolved oxygen uptake (Yang & Albright 1992). If the uptake of oxygen is sufficiently restricted the fish dies (Yang & Albright 1992). *C. concavicornis* and *C. convolutus* can be overtly lethal

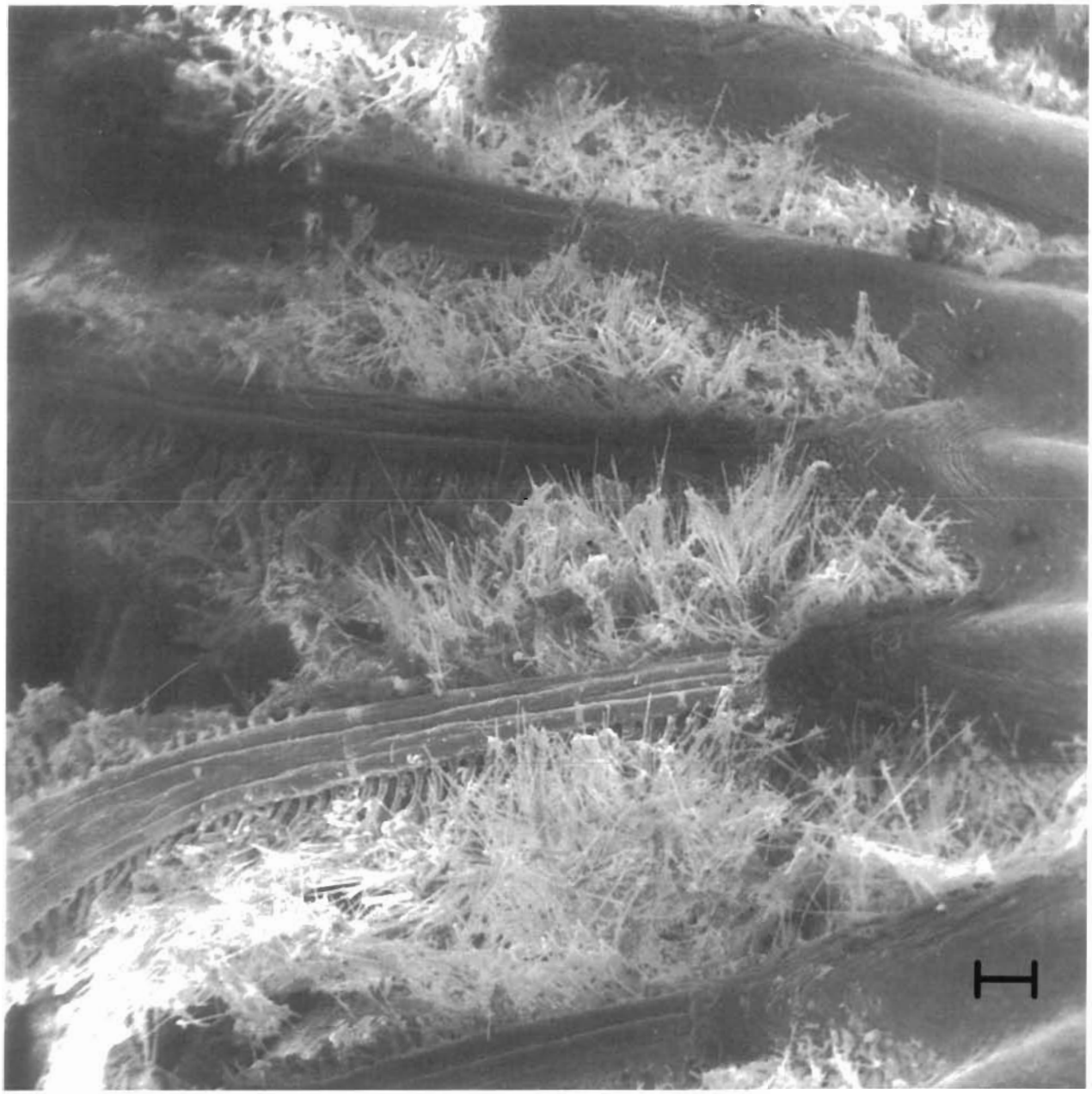

Fig. 1. *Oncorhynchus kisutch*. Scanning electron microscopic view of the primary and secondary lamellae of a juvenile coho salmon exposed to approximately 50 cells of *Chaetoceros concavicornis* ml $^{-1}$ seawater for 72 h. Scale bar = 100 µm

to penned salmon at concentrations as low as 5 cells ml^{-1} (Bell et al. 1974) and exacerbate pathogen-related mortalities at sublethal doses (Albright et al. 1993).

Mortalities related to excessive mucus production and accumulation on respiratory epithelia, from whatever cause, are also experienced by other animals such as rats and canines (Lightowler & Lightowler 1971, Martin et al. 1980, Servin et al. 1988, Ueno et al. 1989). Humans may also experience respiratory dysfunction due to overproduction and accumulation of mucus in lungs (Aylward et al. 1980).

A class of therapeutic drugs has been identified which alleviates diseases associated with excessive production and accumulation of mucus in the respiratory tract of domestic animals and humans. These are termed mucolytic agents. When administered orally to felines, canines and humans, they limit production of mucus in lungs, probably by acting as competitive inhibitors of mucus synthesis (e.g. Sheffner 1963, Martin et al. 1980).

When administered onto the lung surfaces as an aerosol, some mucolytic agents act as reducing agents, by cleaving s-s bonds of the mucus mucopolysaccharide strands (e.g. Majima et al. 1989). In this way the viscosity of the mucus is reduced. An example of such a mucolytic agent is L-cysteine ethyl ester.

Since goblet cells of teleost fish function in a way similar to those of mammals (Bird & Eble 1979), we felt that there was a good likelihood that a material such as L-cysteine ethyl ester may act as a mucolytic agent in salmon, as it does in mammals. Accordingly, we report here on the ability of: (1) L-cysteine ethyl ester to suppress mucus accumulation on the secondary lamellae of coho salmon and (2) the ability of these same L-cysteine ethyl ester-treated salmon to survive what would otherwise be a lethal challenge with *Chaetoceros concavicornis*.

MATERIALS AND METHODS

The culture of *Chaetoceros concavicornis* was obtained from the Northeast Pacific Culture Collection, Dept of Oceanography, University of British Columbia (B.C.). This harmful diatom was cultured using several 1 l quantities of Harrison's medium (Harrison et al. 1980) each contained in 2 l Fernback flasks at a temperature of 14°C and a 14 h light:10 h dark cycle. Cell concentrations were determined using the Utermöhl (1958) technique.

Coho salmon *Oncorhynchus kisutch* (mean weight 12 g) were obtained from the Capilano Fish Hatchery, Canada Dept of Fisheries and Oceans, West Vancouver, B.C. These fish were transported to Simon Fraser University and maintained in aerated seawater (salinity 10‰). At 30 d prior to the experiment, 200 fish were randomly divided into 4 groups of 50 fish each; each group was placed in a 200 l oval tank containing 100 l of seawater of 10‰ salinity, pH 6.7 to 7.5, temperature 17.5 to 18.0°C and oxygen content 10.0 to 10.5 mg l^{-1}.

L-cysteine ethyl ester (Sigma, St. Louis, MO, USA) was weighed and sprinkled onto standard fish feed pellets of 2 mm diameter such that final concentrations of 0.3, 0.6 and 0.9 mg L-cysteine ethyl ester g^{-1} of feed were attained. This treated feed was then mixed with several ml of herring oil such that the L-cysteine ethyl ester coated the pellets. The treated feed was stored at 5°C until used.

The fish in tanks 1, 2, 3 and 4 were fed with 8.0 g d^{-1} (1.33% body weight d^{-1}) of feed containing 0, 0.3, 0.6 and 0.9 mg L-cysteine ethyl ester g^{-1} feed respectively from 3 d prior to initiating the experiment (time 0) and for 2 d thereafter (Day 2). During this period, the fish in tanks 2, 3 and 4 ingested 4, 8, and 12 mg L-cysteine ethyl ester kg^{-1} body weight d^{-1} respectively. From Day 3 through Day 4, all fish were fed with a feed without L-cysteine ethyl ester.

On Day 0, *Chaetoceros concavicornis* was added to the water in all tanks such that the concentrations were maintained between 40 and 54 cells ml^{-1} for 4 d.

Gill arches were removed from moribund coho in tanks 1 to 3 and coho randomly selected from tank 4. Following incision, the gill arches were immediately immersed in 2.5% glutaraldehyde in a 0.1 M Na cacodylate buffer containing 2% (w/v) alcian blue (BDH, Toronto) (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

A total of 37, 8, 0 and 1 coho died in tanks 1, 2, 3 and 4 in which the fish were fed with 0, 4, 8 and 12 mg L-cysteine ethyl ester kg^{-1} biomass d^{-1} respectively during the 5 d treatment with *Chaetoceros concavicornis* (Fig. 2). The cumulative mortalities of the coho in tanks 1, 2, 3 and 4 were 57, 12, 0 and 2% repectively by Day 4 following their exposure to *C. concavicornis*.

A thick mucous layer overlaid the epithelia of the secondary lamellae of the coho treated with *Chaetoceros concavicornis* only, and a great deal of mucus accumulated between the adjacent secondary lamellae of these salmon (Fig. 3A, B). However, only a thin layer of mucus was observed on the epithelia of the secondary lamellae of control coho which were not exposed to cells of *C. concavicornis* and little or no

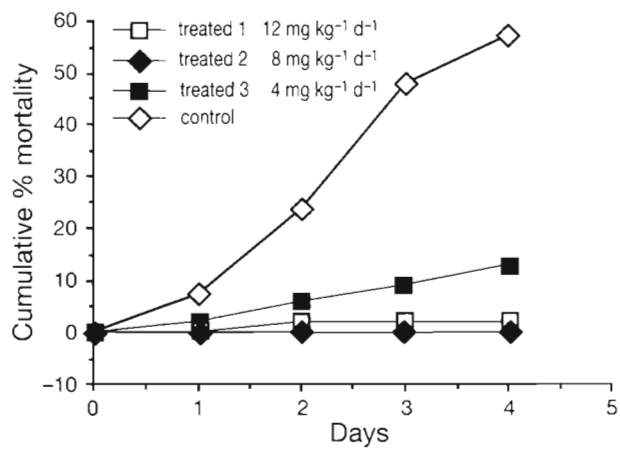

Fig. 2. *Onchorhynchus kisutch*. Cumulative mortalities of coho salmon (10 to 16 g) fed with 12, 8, 4 or 0 mg L-cysteine ethyl ester kg^{-1} biomass d^{-1} in the presence of between 40 and 54 cells of *Chaetoceros concavicornis* ml^{-1} water

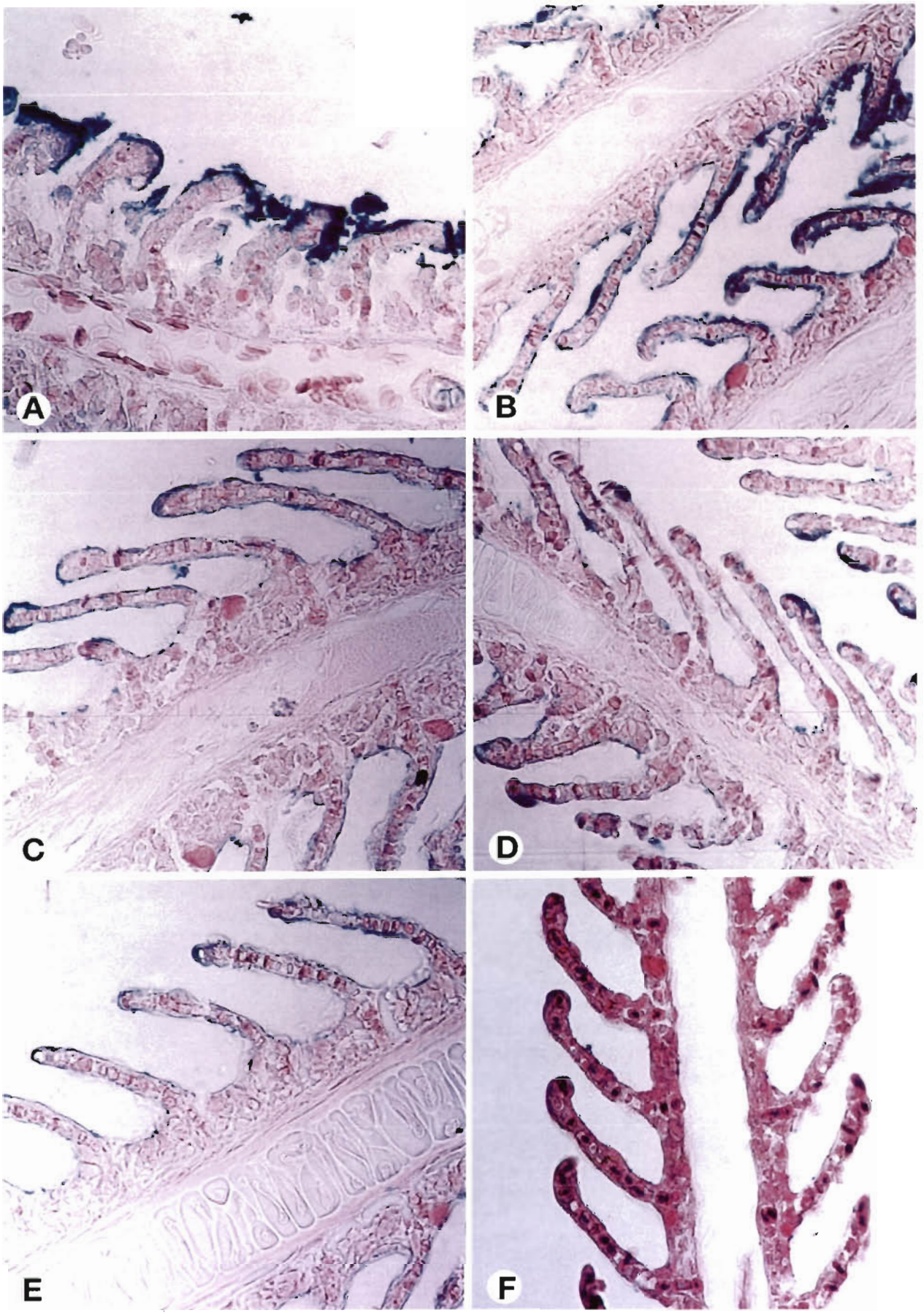
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Fig. 3. *Onchorhynchus kisutch*. Light microscopic views of 6 µm thick sections of alcian-blue-stained secondary lamellae of coho salmon: (A, B) treated with *Chaetoceros concavicornis* only; (C, D) treated with *C. concavicornis* and L-cysteine ethyl ester; (E, F) untreated

mucus was observed between adjacent lamellae of these fish (Fig. 3E, F). Similar small amounts of mucus were observed on the secondary lamellae of the coho treated with both L-cysteine ethyl ester and *C. concavicornis* (Fig. 3C, D).

DISCUSSION

Mucus is a term used to describe the secondary products of particular organs of animals with specific reference to products of mucous, goblet and serous cells (Charman et al. 1974). 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 high molecular weight glycoproteins which consist of a protein backbone with many oligosaccharide side chains, often called mucin. 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 disulfide bonds in the non-glycosylated region of each protein core, probably involving interaction between adjacent cysteine residues (Allen 1978) which results 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).

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 cells appears to be a direct consequence of irritation (Ziment 1978).

Sheffner (1963) reported that the L-cysteine analogue, N-acetylcysteine, was able to decrease human nasal mucus viscosity *in vitro* by reducing disulfide bonds of adjacent glycoproteins. Subsequent studies have confirmed this property (e.g. Lightowler & Lightowler 1971, Walters et al. 1985). N-acetylcysteine can also break the disulfide cross-linkage between proteins and DNA in the mucus by virtue of its free sulfhydryl group (Ziment 1978).

When ingested by 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, it also appears to be able to reduce disulfide bonds and break the macromolecular glycoprotein to smaller subunits when administered topically (Ueno et al. 1989). Cotgreave et al. (1987) proposed that N-acetylcysteine depresses mucus production by increasing the synthesis of glutathione.

When N-acetylcysteine 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 down the complex protein network into less viscous strands (Ziment 1978). Breakage of disulfide bonds results in a marked decrease in the viscosity of mucus; 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, Servin et al. 1988, Majima et al. 1989). 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, we decided to use L-cysteine ethyl ester as a mucolytic agent in the experiments described here.

The data show that L-cysteine ethyl ester suppresses mucus production and accumulation by the cells of the secondary lamellae of coho (Fig. 2). The specific cellular activity which is most likely inhibited by this therapeutant is the mucus secretory activity of the goblet cells of this tissue since mucolytic agents such as L-cysteine ethyl ester competitively inhibit mucus synthesis. The decomposition of mucoprotein likely resulted in a mucus of lower viscosity which was more easily washed off the primary and secondary lamellae by water passing over the gills of the fish.

Our data show that partial protection from the harmful effects of *Chaetoceros concavicornis* occurred at ingestion rates of L-cysteine ethyl ester of 4 and 8 mg kg^{-1} biomass d^{-1} and complete protection occurred at 12 mg kg^{-1} biomass d^{-1} (Fig. 1). Based upon these observations, it would appear that the most effective dosage is somewhere between 8 and 12 mg L-cysteine ethyl ester kg^{-1} d^{-1}.

The mucolytic agent L-cysteine ethyl ester is not destroyed by the temperature conditions used to produce commercial fish feed pellets, and the material is quite stable in the pellets. There is therefore a good possibility that treatment of farmed salmonids in seawater pens undergoing an exposure to harmful blooms of *Chaetoceros concavicornis* and/or *C. convolutus* with mucolytic-agent-treated feed would be effective in controlling mortality rates under farming conditions. It is also possible that the use of mucolytic agents in feed or water may be used to limit mucus accumulation on gills of aquatic animals such as finfish under conditions which would cause the excess production of this material by the animal, for example irritating chemicals in the water and bacterial gill disease.

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