Vol. 36: 213–219, 1999 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published May 31

Environmental factors and chemical agents affecting the growth of the pathogenic marine ciliate *Uronema nigricans*

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ABSTRACT: The scuticociliate *Uronema nigricans* is an opportunistically parasitic marine ciliate known to cause disease in some aquacultural environments with epizootics documented from marine larval rearing systems, marine aquaria and in southern bluefin tuna *Thunnus macoyii* growout enclosures. This study examined growth responses of laboratory cultures of the ciliate and prey bacteria to variations in temperature and salinity, and the efficacy of potential chemotherapeutants for control of *U. nigricans* infections. Differences in ciliate growth responses were marginal at temperatures of 10 to 25°C and at salinities between 15 and 35 ppt, though 3.5 ppt or less was lethal. Ciliates were found to be sensitive to fluctuations in bacterial densities, which may be a factor in the seasonal occurrence of the ciliate-related disease in tuna. Commonly used chemotherapeutants such as formalin, malachite green and hydrogen peroxide were all effective against the ciliate during *in vitro* trials.

KEY WORDS: *Uronema nigricans* · Opportunistic pathogen · Growth response · Chemotherapeutants

INTRODUCTION

Until recently, infections of fish with *Uronema* sp. (Order: Scuticociliatida) had been mainly reported in aquarium species in which they were invasive and resulted in severe tissue damage and subsequent mortalities (Cheung et al. 1980, Bassleer 1983). More recently the potential for scuticociliatids to be a problem in mariculture has been highlighted by reports of serious disease of larval and juvenile fish in Japan and Spain attributed to infection with *Uronema* sp. (Yoshinaga & Nakazoe 1993, Dykova & Figueras 1994) and, of mature sea bass in the Mediterranean, where the parasite was described as *Philasterides dicentrarchi* (Dragesco et al. 1995). In Australia mortalities of southern bluefin tuna *Thunnus maccoyii* in growout seacages have also been reported with the causative agent being identified as *Uronema nigricans* (Munday et al. 1997).

This paper consists of 2 parts. The first reports the *in vitro* growth responses of *Uronema nigricans* to variations in temperature and salinity which were investigated to gather fundamental information on environmental parameters influencing growth of this ciliate. The second provides information on potential chemotherapeutants for the control of *U. nigricans* infections of fish in closed larvae-rearing systems and aquaria.

MATERIALS AND METHODS

Stock cultures of ciliates and bacteria. Stock cultures of *Uronema nigricans* were laboratory cultures held by the School of Aquaculture, University of Tasmania, and were originally isolated from infected tuna tissue (Munday et al. 1997). These cultures were grown with a variety of marine bacteria as a food source.

Cultures of *Vibrio anguillarum* for use as one of the bacterial food sources for the ciliates were obtained from the microbiology laboratory within the Department of Applied Biomedical Science, University of Tasmania.

Maintenance of ciliate cultures. Ciliates were cloned using the silicon oil plate procedure detailed by Soldo & Brickson (1993) and were maintained and sub-

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cultured every 5 to 6 wk by procedures described by Watts (1995). Briefly, culture vessels were 25 ml McCartney bottles containing a 1 in 10 dilution of brain heart infusion (Oxoid®) broth supplemented with 25 µl l^{-1} of both defibrinated horse blood and multi vitamin supplement (Accomin®). The bacterial load of 1 isolate was suppressed by a process involving the use of antibiotics, repeated centrifugation and exploitation of the ciliates' geotactic nature, then maintained at room temperature in a marine axenic medium (MAM) as modified from Messick & Small (1996).

Growth rate estimations of ciliates and bacteria. Ciliate growth rates were measured in response to variations in temperature (10, 15, 20, 25 and 30°C) or salinity (0, 15, 25 and 35 ppt, all incubated at 20°C) in quadruplicate batch cultures. Cultures were performed in 25 ml McCartney bottles containing 20 ml of 0.2 µm filtered seawater, 2 ml 1% peptone and 1 ml *Vibrio anguillarum* suspended in 1% peptone (for an initial density of 10^8 cells ml^{-1}). Growth trials were initiated by an inoculation with a ciliate suspension drawn from stock cultures in exponential growth phase (subcultured from MAM broth to 1% peptone seeded with *V. anguillarum* approximately 48 h prior to initiation of the experiment) to give an initial density of approximately 100 to 200 ciliates ml^{-1}. At appropriate time intervals (approximately every 10 to 14 h over 7 d) all cultures were sampled to enumerate ciliates. For counting, 0.5 ml samples were taken from thoroughly mixed suspensions and fixed with 0.5 ml of 10% formalin. Densities were estimated as the average of quadruplicate counts of ciliates in 20 µl sub-samples in a Sedgewick-Rafter counting chamber. However, when ciliate densities were low, 50 to 200 µl sub-samples were used. Culture volumes were never diminished by more than half in the course of sampling.

Optimal conditions for the ciliate in terms of temperature and salinity were determined from growth curves and, in particular, net ciliate production which was calculated by: $N - N_0$ where N_0 and N are ciliate numbers at the beginning and end of exponential growth phase respectively.

To investigate ciliate tolerance to lower salinities between 3.5 and 10 ppt, culture methods based on those of Novotny et al. (1996) were used. Seven day old ciliate cultures were concentrated by centrifugation (3000 rpm, 500 × *g*, for 5 min) then re-suspended in filtered seawater. Effects of salinities 3.5, 5, 7.5 and 10 ppt were observed in individual wells of 6-well tissue culture trays (Greiner Labortechnik®) when 200 µl of ciliate culture was added to 1.8 ml of appropriately diluted seawater. The procedure was performed twice to ensure that it was repeatable.

Bacteria were enumerated as colony-forming units (CFUs) on 1% peptone agar using the spread plate viable count method. Where morphologically different cells were present, indicating mixed bacterial populations, elementary identification methods (i.e. Gram stains and isolation onto thiosulphate citrate bile salt media) were used to determine whether or not that the organisms were *Vibrio* sp.

Determination of the efficacy of therapeutants. Methods used to appraise therapeutants were similar to those used to assess reduced salinity tolerance. The effects were observed using 1.8 ml of ciliate suspension (centrifuged and re-suspended in 2 µm filtered seawater as before) with 200 µl of suitably diluted therapeutants to give the desired final concentration. Ciliate densities for each trial ranged from 1.7×10^4 to 4.4×10^4 cells ml^{-1}. Compounds tested for efficacy in killing the ciliates were formalin (25, 50, 100, 200 ppm), malachite green (1, 2, 4, 6, 8 ppm) and hydrogen peroxide (250, 500, 1000, 1500, 2000 ppm). All trials were performed in triplicate wells for each compound and concentration and with appropriate controls (200 µl of seawater in lieu of therapeutants). Effects of therapeutants on motility and morphology were observed periodically by scanning several fields of view at 100 and 200× magnification using an inverted microscope (Zeiss® Invertoscope ID 02) and assigning scores, interpretations of which are outlined in Table 1. Assay temperatures ranged from 18 to 20°C and maximum period for observation was set at 180 min.

Statistical analysis. Cell density data were analysed with a 1-way analysis of variance (ANOVA). Significant differences were highlighted as a multiple com-

Table 1 Scoring system for appraising the effects of various compounds on motility and morphology of *Uronema* sp. (Modified from Novotny et al. 1996)

Score	Interpretation
Motility	
4	No effect; motility normal
3	Motility slowed in more than 50% of ciliates
2	Approximately 50% of ciliates stationary, but cilia still beating
1	More than 50% of ciliates stationary and cilia still beating
0	No sign of motility or cilia movement
Morphology	
4	No change; cells elliptical
3	Less than 50% of cells round or irregular shape
2	Approximately 50% of cells round or irregular shape
1	More than 50% of cells round or irregular and lysis evident
0	Extensive lysis with few cells intact

parisons of means (Tukey-Kramer HSD test) was carried out. For all tests a significance level of $p < 0.05$ was adopted.

RESULTS

Growth trials at varying temperature and salinity

There appeared a general trend in all *Uronema nigricans* growth curves of a slowing of growth at the end of exponential growth phase, followed by a decline, then an increase. Growth curves for ciliates and bacteria (Fig. 1) at 10 and 30°C only are presented here as these are representative of the data at all incubation temperatures. There was no indication of a final death phase at any of the incubation temperatures over a period of 185 h. The data generally show typical predator-prey oscillations in bacterial and ciliate populations. However, at 10 and 15°C there was a sharp decline in bacterial numbers after 25 h which did not quite coincide with the initial surge in ciliate numbers.

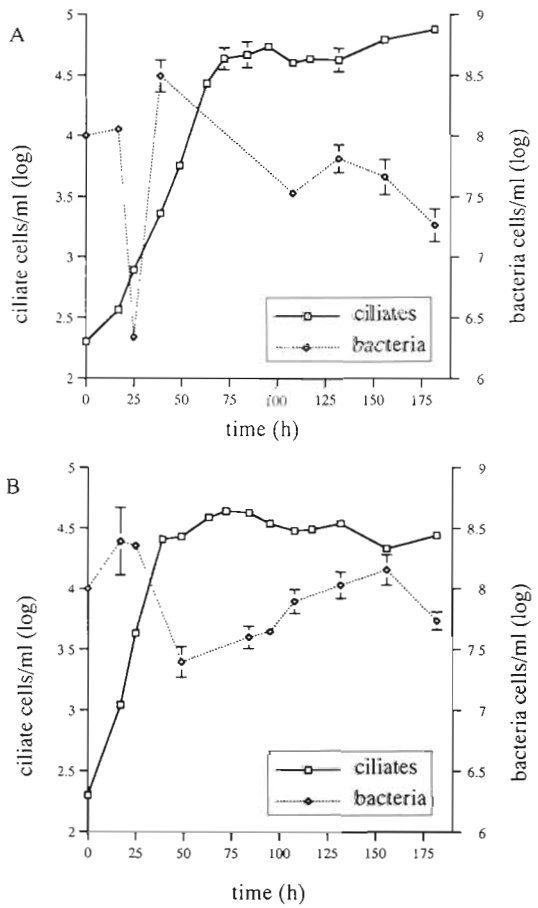

Fig. 1. Growth curves for the ciliate and bacteria incubated at (A) 10°C and (B) 30°C. All values are means ± SE (n = 4 except for some bacteria counts where n = 2 or 3)

Table 2. *Uronema nigricans*. Maximum cell densities achieved during exponential phase and for the duration of the growth trial (185 h) where ciliates were cultured at a range of salinities. All values are means ± SE (n = 4) and those sharing a common superscript are not significantly different ($p > 0.05$, Tukey-Kramer HSD test)

Temperature (°C)	Maximum densities × 10^4 (cells ml^{-1}) Exponential phase	Duration of trial
10	$5.138^{ab} \pm 0.1093$	$7.656^{a} \pm 0.1663$
15	$5.081^{ab} \pm 0.1634$	$8.781^{a} \pm 0.4641$
20	$5.863^{a} \pm 0.7631$	$8.700^{a} \pm 0.2963$
25	$4.688^{ab} \pm 0.5447$	$7.275^{a} \pm 0.4147$
30	$2.731^{b} \pm 0.1637$	$4.788^{b} \pm 0.4421$

Table 3. *Uronema nigricans*. Maximum cell densities achieved during exponential phase and for the duration of the growth trial (185 h) where ciliates were cultured at a range of salinities and at 20°C. All values are means ± SE (n = 4) and those sharing a common superscript are not significantly different ($p > 0.05$, Tukey-Kramer HSD test)

Salinity (ppt)	Maximum densities × 10^4 (cells ml^{-1}) Exponential phase	Duration of trial
15	$4.475^{a} \pm 0.211$	$6.838^{a} \pm 0.419$
25	$4.288^{a} \pm 0.372$	$7.288^{a} \pm 0.225$
35	$5.863^{a} \pm 0.763$	$8.700^{b} \pm 0.296$

Maximum ciliate densities achieved during exponential growth phase and for the duration of the trial (Table 2) were significantly lower at 30°C.

Attempts to culture *Uronema nigricans* in freshwater failed as cells immediately swelled and lysed. However, there was little difference in growth curves at salinities of 15, 25 and 30 ppt (Fig. 2, Table 3) with the exception being that the maximum yield over the

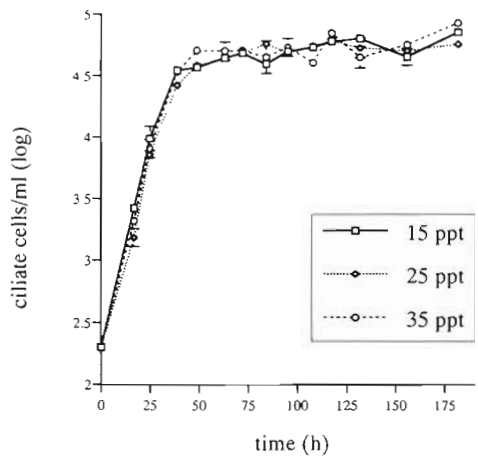

Fig. 2. *Uronema nigricans*. Growth curves forciliates incubated at 20°C and cultured in a range of salinities. All values are means ± SE (n = 4)

course of the trial was significantly higher in full-strength seawater. It was also found that salinities of 3.5 ppt or less are lethal to *U. nigricans* but there was significant ciliate survival at salinities higher than these (data not presented), with no impact observed at 10 ppt.

Two morphologically distinct groups of bacteria were present on some plates up to approximately 66 h post incubation. Both were regarded as *Vibrio* spp. as they grew on TCBS plates within 24 h. Towards the end of the trial period different bacteria were evident as red, translucent colonies (Gram negative and no growth on TCBS). However, these were a relatively small proportion of the total bacterial population.

Effects of therapeutants

All the therapeutants tested were lethal to the ciliates with responses characterised by changes in motility and morphology. Changes in motility were reasonably constant for each compound; cells would slow down, become stationary and cilia would cease beating. The ciliates were assumed to be dead at this point. Effects on cell morphology were more varied. Prior to treatment the protozoans were ovoid in shape. Organisms exposed to hydrogen peroxide developed bulging of the cytoplasm through the oral cavity before swelling to a round or irregular shape and lysing. Formalin and malachite green induced cell rounding prior to eventual lysis. Assigned scores for all compounds at the various concentrations appear in Figs. 3, 4 & 5.

The most effective concentrations of formalin were 100 and 200 ppm where total cell lysis occurred after 120 and 60 min respectively. At these concentrations the ovoid cell shape was maintained for 20 to 40 min after motility had ceased in the majority of cells. Lower concentrations of formalin did not result in 100% mortality, with no real effect noted at 25 ppm, and the response to 50 ppm varied slightly between trials but indicated significant ciliate survival after 180 min. Hydrogen peroxide was lethal to the ciliate at all concentrations. There was slight variation in cell response between each concentration 30 min after treatment, but mortality was complete for all after 60 min. With this compound morphological changes occurred within 10 min of cessation of motility in the majority of cells. Malachite green was also lethal at each concentration

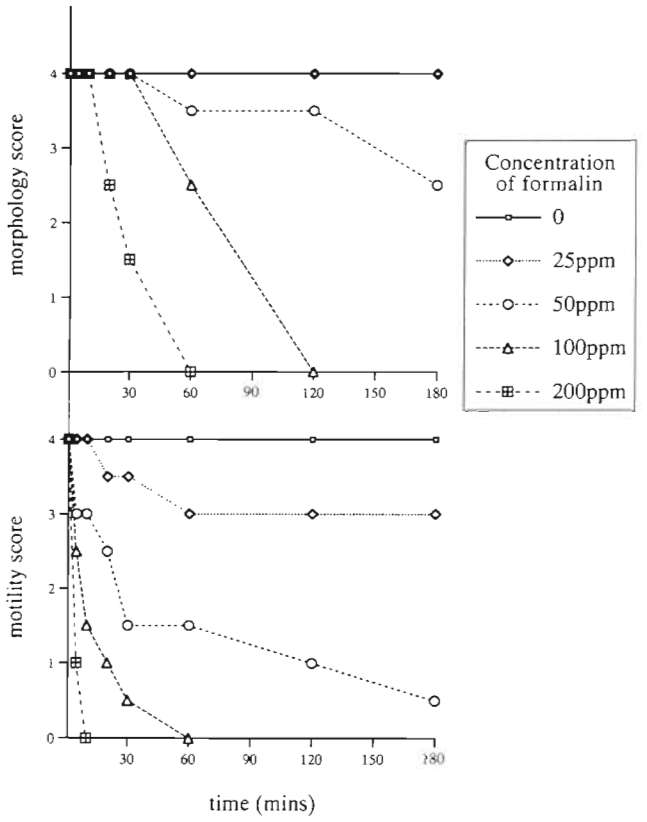

Fig. 3. *Uronema nigricans*. Observed effects of a range of concentrations of formalin on morphology and motility. Each data point represents the mean of 2 replicate experiments

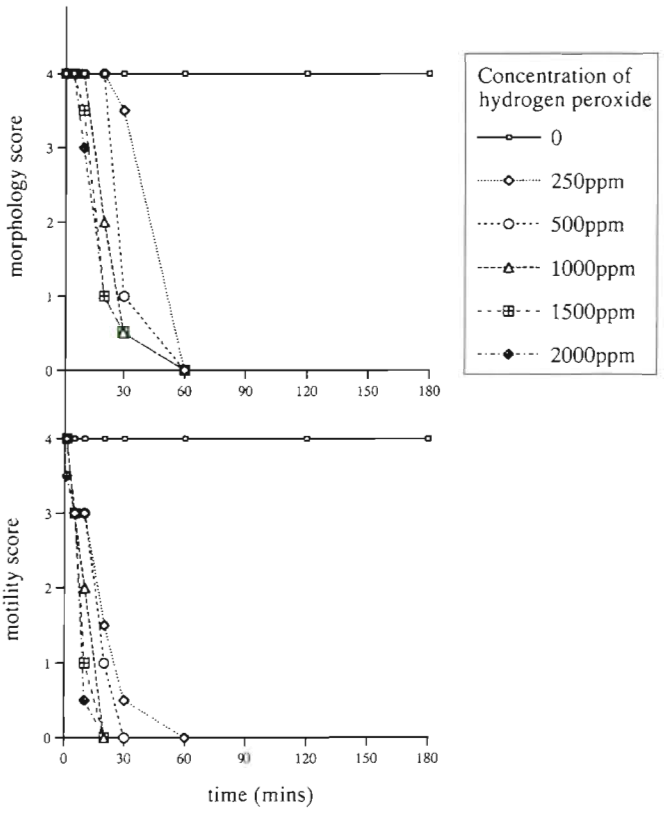

Fig. 4. *Uronema nigricans*. Observed effects of a range of concentrations of hydrogen peroxide on morphology and motility. Each data point represents the mean of 2 replicate experiments

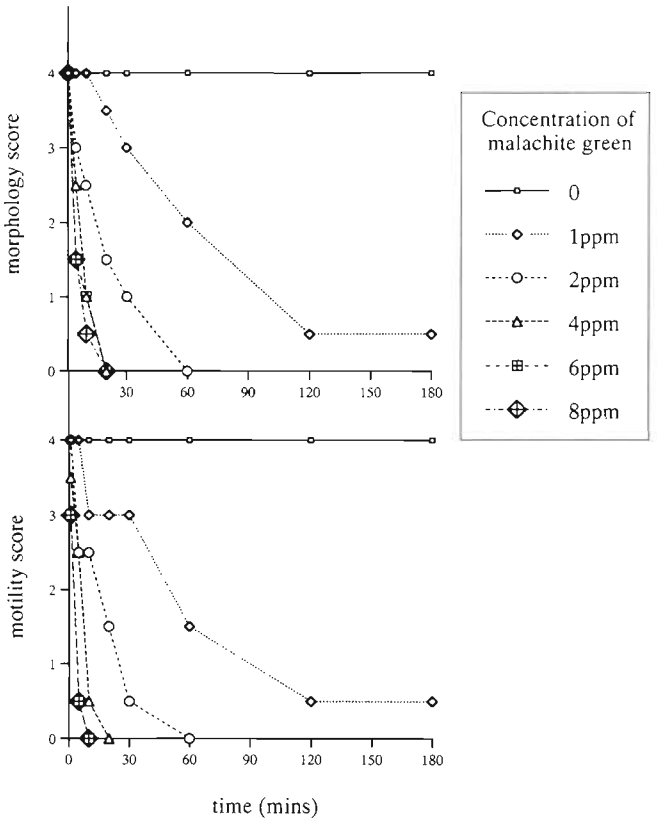

Fig. 5. *Uronema nigricans*. Observed effects of a range of concentrations of malachite green on morphology and motility. Each data point represents the mean of 2 replicate experiments

used although there was some variation at 1 ppm with cells surviving after 180 min for 1 trial. Higher concentrations, i.e. 4, 6 and 8 ppm, all resulted in total mortality within 20 min.

DISCUSSION

It is notable that the maximum ciliate densities achieved during exponential phase and for the duration of the observations varied little with temperature although the highest concentrations tended to be at 25 and 30°C. This suggests that ciliates can grow and be maintained across a wide temperature range (in the order of 10 to 25°C).

Generally, growth curves for both ciliates and bacteria illustrated typical predator-prey relationships though at 10 and 15°C there were 2 distinct troughs in the growth curves for bacteria. These troughs occurred before the ciliates entered exponential phase and thus appeared due to a temperature effect on the bacteria rather than a grazing effect by the ciliates. Bacterial numbers recovered over the next 14 h, suggesting a change in species of the dominant bacteria. For this experiment it was possible that changes in proportions of different bacterial species may have introduced another variable which may have influenced the results. However, as one of the aims of this experiment was to identify the ranges of temperatures and salinities in nature under which the ciliates thrive, and the possible effects of total bacterial load on growth, we believe that our system reflected the situation likely to be encountered in aquaculture settings.

A direct temperature effect on the ciliates may be marginal as the probable permissive temperatures established by this study are in the range of 10 to 25°C. It is probably pertinent that ciliate densities are very sensitive to changes in bacterial densities. Thus, variations in total bacterial load or, in proportions of different bacteria, due to a change in temperature would logically impact on the ciliate population by affecting their food supply. Even though *Uronema* spp. are bacterivorous, they are also opportunistic pathogens and may become histophagous (Lom & Dykova 1992). Changes in bacterial diversity or load in cooler temperatures may represent a scarcity of food for the ciliates and result in the typical ciliate response to starvation, which is rapid cell division with a subsequent decrease in cell volume, rapid motility and swarming behaviour (Fenchel 1990). Thus, in the absence of sufficient bacteria, tuna may be preferentially colonised during the colder months of the year (water temperatures less than 18°C), especially as their internal body temperature will be above ambient (Brill et al. 1994) and they may be immuno-compromised by confinement and possible nutritional deficiencies as noted by Munday et al. (1997).

Growth responses of *Uronema nigricans* to salinities of 15 and 25 ppt did not vary from those in full-strength seawater. Hamilton & Preslan (1969) noted the same lack of variation in growth rates of a *Uronema* sp. at salinities of 17 and 43 ppt. Similarly, Cheung et al. (1980) noted a wide range of both salinity (20 to 31 ppt) and temperature (8 to 28°C) during an epizootic attributed to *Uronema marinum* at the New York aquarium. Although *U. nigricans* is vulnerable to salinities lower than 3.5 ppt, the use of freshwater or low salinity baths, from a surface disinfection perspective, are probably impracticable for controlling *U. nigricans* epizootics in ornamental aquaria, especially as significant ciliate survival was noted at 5 ppt and freshwater treatment would be detrimental to fish once the ciliate has invaded and damaged tissue (Bassleer 1983).

In the second part of the experiment all 3 potential therapeutants investigated proved lethal to the ciliates. Generally, effectiveness was determined by concentration but hydrogen peroxide was an exception with all concentrations trialed taking the same time to pro-

duce end-point. This was also found for malachite green at concentrations above 4 ppm. Formalin was most effective at concentrations of 100 to 200 ppm, which concurs with effective concentrations identified by Novotny et al. (1996) during *in vitro* trials on the ciliate *Anophryoides haemophila*, a pathogen of the American lobster. This chemical is widely used in aquaculture as a fungicide and parasiticide, typically at 25 to 50 ppm for extended periods (Tonguthai & Chanratchakool 1992) and, at 167 to 250 ppm for 60 min baths (Poupard 1978). In our studies lower concentrations (25 to 50 ppm) had no perceptible effect on the ciliates.

All concentrations of malachite green were effective against the ciliate, with total mortality recorded in virtually every case. This chemical is also widely used in aquaculture, principally as a fungicide for incubating fish eggs, but also as a treatment against ectoparasites on fish (Gerundo et al. 1991). Typical applications are as a 1 min immersion at 67 ppm or as a bath/flush treatment at 1 to 2 ppm for 60 min (Poupard 1978). A synergistic effect of combinations of formalin and malachite green as demonstrated by Gilbert et al. (1979) on the ciliate *Tetrahymena pyriformis* was not investigated here but would be worth future study. Hydrogen peroxide also was effective against the ciliate, with total mortality observed within 60 min at concentrations 250 to 2000 ppm. However, this compound causes mortality in salmon at concentrations of 200 to 300 ppm at 17°C for an exposure time of 2 h (Cameron 1994) and therefore may be of limited applicability. At concentrations of 100 ppm for 3 h and 300 ppm for 30 min at 15°C salmon survival was not affected (Cameron 1994) but it is not known whether these concentration/time regimes will kill *Uronema nigricans*. However, since no difference was recorded in times taken to reach end-points for concentrations ranging from 250 to 2000 ppm, it is possible that lower concentrations would be effective, albeit over a longer period. *In vivo* efficacy of these chemicals in controlling *U. nigricans* infections can only be confirmed by trials on infected fish, although formalin at concentrations ranging from 75 to 200 ppm has been used successfully to treat *Uronema* sp. infestations of seahorses at a commercial facility (N. Forteath pers. comm.).

The effect of the various compounds tested on ciliate morphology was varied and presumably relates to the killing mechanisms of the chemicals, but was more consistent on motility. *Uronema nigricans* is highly motile both at 4°C (culture storage temperatures) and at 18 to 20°C (temperatures at which trials were carried out) and a slowing in motility was the first noticeable effect. Similar observations were recorded by Novotny et al. (1996) in trials on the ciliate *Anophryoides haemophila*.

These experiments give useful preliminary results which will aid future investigations into both the ecology of *Uronema nigricans* and to treatment options to control infections in enclosed aquaculture systems.

Acknowledgements. Dr Tiina Hawkesford assisted in ciliate culture and suppression of bacterial load for one isolate. Ms Marianne Watts maintained and supplied original ciliate isolates and advised on the culture techniques.

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Editorial responsibility: Wolfgang Körting, Hannover, Germany

Submitted: November 23, 1998; Accepted: February 8, 1999
Proofs received from author(s): May 17, 1999