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NOTE

In vitro toxicity of bithionol and bithionol sulphoxide to *Neoparamoeba* spp., the causative agent of amoebic gill disease (AGD)

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ABSTRACT: The objective of the present study was to evaluate the *in vitro* toxicity of bithionol and bithionol sulphoxide to *Neoparamoeba* spp., the causative agent of amoebic gill disease (AGD). The current treatment for AGD-affected Atlantic salmon involves bathing sea-caged fish in freshwater for a minimum of 3 h, a labour-intensive and costly exercise. Previous attempts to identify alternative treatments have suggested bithionol as an alternate therapeutic, but extensive *in vitro* efficacy testing has not yet been done. *In vitro* toxicity to *Neoparamoeba* spp. was examined using amoebae isolated from the gill of AGD-affected Atlantic salmon and exposing the parasites to freshwater, alumina (10 mg l^{-1}), seawater, bithionol or bithionol sulphoxide at nominal concentrations of 0.1, 0.5, 1, 5 and 10 mg l^{-1} in seawater. The numbers of viable amoebae were counted using the trypan blue exclusion method at 0, 24, 48 and 72 h. Both bithionol and bithionol sulphoxide demonstrated *in vitro* toxicity to *Neoparamoeba* spp. at all concentrations examined (0.1 to 10 mg l^{-1} over 72 h), with a comparable toxicity to freshwater observed for both chemicals at concentrations >5 mg l^{-1} following a 72 h treatment. Freshwater remained the most effective treatment, with only 6% viable amoebae seen after 24 h and no viable amoebae observed after 48 h.

KEY WORDS: Amoebic gill disease · AGD · Bithionol · *In vitro* · Toxicity · *Neoparamoeba* spp. · Atlantic salmon · Chemotherapy · Protozoan parasite

INTRODUCTION

Bithionol, 2,2′-thiobis (4,6-dichlorophenol), and bithionol sulphoxide, bis (2-hydroxy-3,5-dichlorophenyl) sulphoxide, are anthelmintics that are known to uncouple electron transport (Rew 1978), act on the mitochondrial respiratory chain (Iglesias et al. 2002) and aid in the suppression of adenosine-5′-triphosphate (ATP) synthesis via uncoupling of oxidative phosphorylation (Harder 2002). They are also reported to be halogenated anti-infective agents that are used against trematode and cestode infestations in humans (Harder 2002). Bithionol has been reported as effective for the treatment of metagonimiasis and paragonimiasis in humans, and for killing the trematodes *in vitro* (Yokogawa et al. 1961a,b, Sawatari & Hamajima 1967). Furthermore, bithionol is reported to kill the human parasite *Entamoeba histolytica in vitro* and was able to inhibit endogenous and 2-propanol-supported respiration, but not the formation of ethanol in the parasite (Takeuchi et al. 1984). Bithionol and bithionol sulphoxide have been examined as possible treatments for nat-

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ural rumen fluke infection in cattle and tapeworm infections in cats, dogs, sheep and chickens (Prasittirat et al. 1997).

Both bithionol and bithionol sulphoxide have been examined as treatments for numerous fish parasites and showed mixed results. Santamarina et al. (1991) observed limited toxicity and complete *in vitro* efficacy against *Gyrodactylus* sp. in rainbow trout *Oncorhynchus mykiss* at 12.5 mg l^{-1}, with a minimum 20 mg l^{-1} reported as efficacious *in vivo*. Tojo et al. (1994b) stated that bithionol was efficacious *in vivo* against *Ichthyobodo necator* in rainbow trout at 25 mg l^{-1} for a 3 h freshwater bath on 2 consecutive days; however, higher concentrations exhibited some host toxicity. Moreover, Madsen et al. (2000) determined that bithionol at 0.1 mg l^{-1} was an effective treatment against trichodiniasis in European eels *Anguilla anguilla*, but found bithionol to have a relatively narrow therapeutic index. Bithionol was identified as being a potential candidate for the control of neoparmoebiasis with a demonstrated toxicity *in vitro*, although only 2 concentrations were tested (Powell et al. 2003). More recently, bithionol has displayed efficacy as a bath and oral treatment for Atlantic salmon experimentally challenged with *Neoparamoeba* spp., resulting in amoebic gill disease (AGD), at arbitary concentrations and dosages of 1 to 10 mg l^{-1} for bath treatments and 25 mg kg^{-1} feed (Florent et al. 2007a,b, 2009), but more extensive *in vitro* efficacy testing is required for further development of this potential therapy.

In Tasmania, AGD is the primary disease affecting the production of Atlantic salmon *Salmo salar*, with the causative agent being the marine protozoan *Neoparamoeba perurans* (Young et al. 2007) which is cosmopolitan in its distribution (Powell et al. 2008). Commercial mitigation of AGD uses a freshwater bath for 2 to 3 h, which removes the amoeba and promotes improved gill health (Parsons et al. 2001). However, the frequency of freshwater bathing has increased as it appears that each bath is proving less effective (Parsons et al. 2001), and the search for alternative treatments is ongoing. Of all the alternative treatments screened to date, few have achieved comparable results to freshwater. These include chloramine-T (Harris et al. 2004, 2005), hydrogen peroxide (Powell & Clark 2003), levamisole (Findlay et al. 2000) and bithionol (Powell et al. 2003, Florent et al. 2007a,b, 2009). The familiarity of using freshwater baths and existing technology make commercial adoption of alternatives a slow process where no distinct advantage over the freshwater treatment is seen. However, the toxicity of bithionol and its sulphoxide derivative to the amoebae has not been clearly established, and lower concentrations may be effective at killing *Neoparamoeba* sp.

It is unknown if bithionol and bithionol sulphoxide exert the same toxic effects on *Neoparamoeba* spp. or whether they are capable of stimulating a therapeutic effect similar to that of freshwater by sloughing off affected gill tissue by effectively poisoning the epithelial tissue or if these chemicals are directly toxic to the amoeba. Bithionol is also known to interfere with oxidative metabolism and inhibition of NADH-fumerate reductase (Hamajima 1973, Reid et al. 2001); therefore, the potential to poison epithelia exists in addition to its effects on mitochondrial respiratory chain (Rew 1978, Harder 2002, Iglesias et al. 2002). Such treatments are beneficial if they are directly toxic to *Neoparamoeba* spp. at a lower concentration than is toxic to the fish. Therefore, the aim of the present study was to determine the effect of bithionol and bithionol sulphoxide on the survival of isolated gill amoebae *in vitro*. It was hypothesised that both bithionol and bithionol sulphoxide would decrease the survival of isolated gill amoeba when compared to seawater.

MATERIALS AND METHODS

Neoparamoeba spp. were isolated according to the frequently described method of Morrison et al. (2004). Briefly, the gills from donor AGD-affected Atlantic salmon were centrifuged at 400 × *g* for 2 min in distilled water and rinsed with clean seawater 3 times, dislodging amoebae from the gills. The amoebae were allowed to adhere to Petri dishes for approximately 2 h at 18°C before being washed with seawater. The amoebae were allowed to re-adhere to Petri dishes overnight at 18°C. The adherent cells were removed by the addition of 1 ml Hanks balanced salts trypsin EDTA, washed with seawater, centrifuged at 400 × *g* for 5 min and concentrated. An aliquot of amoebae isolate was stained with 0.5% trypan blue–seawater mix at a dilution of 1:1 and live amoeba (those not taking up the stain) counts were determined using a haemocytometer (Neubauer, BS 748). Three replicate counts were made, with 18 large squares counted per replicate.

The *in vitro* toxicity assay, modified from Powell et al. (2003), used isolated live amoebae that were adhered to flat-bottom, 96 well microtitre plates at a density of approximately 10 000 cells in 150 µl per well and allowed to adhere for 1.5 h at 18°C and then exposed to different treatments.

All test solutions were aerated to 100% air saturation and brought to 18°C before commencement of each experiment. Amoebae were exposed to either bithionol or bithionol sulphoxide (Sigma-Aldrich) at 0, 0.1, 0.5 1, 5 or 10 mg l^{-1} in triplicate, repeated 8 times (n = 24) per treatment over a 72 h exposure period. The number of

live amoebae was determined at 0, 24, 48 and 72 h of exposure using the trypan blue exclusion assay (described above). Bithionol and bithionol sulphoxide treatments were prepared making a stock solution using a mortar and pestle in order to create a suspension, as they are both insoluble in water, which was then diluted to make the necessary concentrations. Test concentrations are therefore reported as nominal concentrations based upon the amount of raw chemical added. There were several controls examined, including seawater (35‰, negative control), freshwater (dechlorinated municipal source, positive control) and alumina at 10 mg l^{-1} (Sigma-Aldrich), used to control for the low solubility of bithionol and bithionol sulphoxide in seawater and account for any surface effect the precipitates may have had on amoeba survival (after Powell et al. 2003).

A 1-way ANOVA was used to determine differences between assays. Where no significant differences were found, assays were pooled and a 2-way ANOVA was used to analyse means with time and treatment as factors. The interaction between time and treatment was examined first; if $p > 0.05$, there was an interaction and the factors of time and treatment were combined and a Tukey's post hoc test conducted on the combined variable to identify where the differences occurred. Homogeneity was determined using a residual plot and Levene's test; where the data were not normally distributed or variance homogeneous, a square root transformation was used. A result was considered significant at $p \leq 0.05$ and results are presented as means ± standard error of the mean (SEM). All statistical analysis was conducted using SPSS for Windows® (version 15.0). Survival of amoeba was calculated as a percentage of seawater control (conducted at the same time) to ensure consistency among treatments:

$$\%\ \text{of seawater control} = \left(\frac{\text{number of amoeba in treated group}}{\text{number of amoeba in sewater control group}}\right) \times 100 \quad (1)$$

RESULTS

Survival of amoebae in seawater controls was equal to or better than the initial concentrations observed at Time 0 and are given as 100% survival. Amoebae also survived when exposed to 10 mg l^{-1} alumina for the 72 h duration and were determined not to be significantly different from the seawater controls for both chemical treatments (bithionol: $F_{31,736} = 413.356$, $p < 0.001$; bithionol sulphoxide: $F_{31,736} = 280.358$, $p < 0.00.1$; Fig. 1). Amoebae numbers declined rapidly when exposed to freshwater, with a 94 and 96% relative reduction seen within the first 24 h when compared to the seawater control with bithionol and bithionol sulphoxide experimental treatments, respectively (Fig. 1).

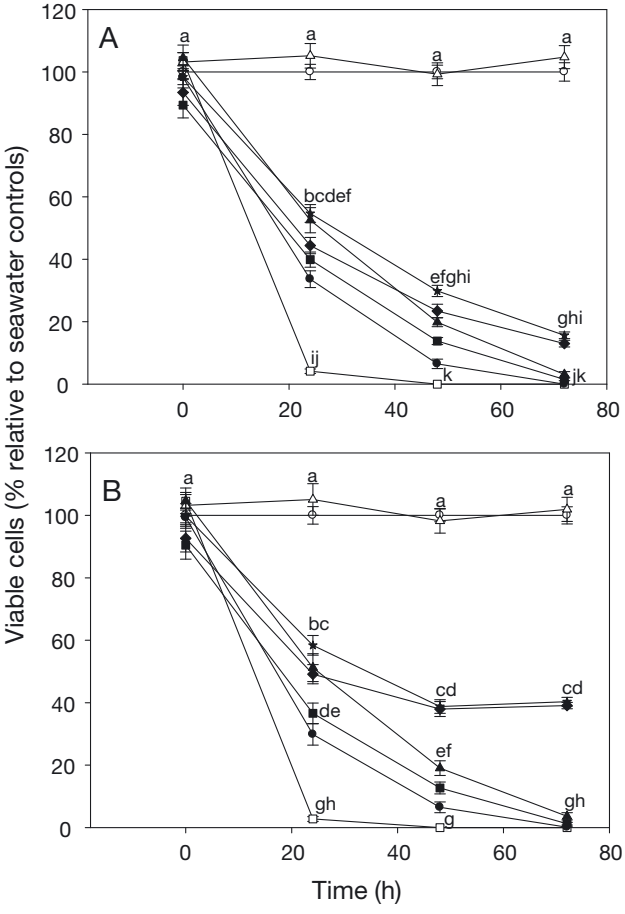

Fig. 1. *Neoparamoeba* spp. Effect of time and treatment on mean (±SEM) number of viable isolated amoeba (as a percentage of the seawater control) when exposed to freshwater (□), 10 mg l^{-1} alumina (△), seawater (○) or (A) bithionol or (B) bithionol sulphoxide at 0.1 (★), 0.5 (◆), 1 (▲), 5 (■) and 10 mg l^{-1} (●) (n = 24). Seawater controls remained at 10 000 cells $well^{-1}$ and are presented as 100% survival. Common letter across both time and treatment indicates no significant difference using a Tukey's test ($p > 0.05$)

Bithionol was effective at reducing amoebae numbers significantly at all concentrations and across all time points ($F_{31,736} = 413.356$, $p < 0.001$; Fig. 1). When amoebae were treated with 10 mg l^{-1} of bithionol there was a 53% relative reduction within the first 24 h compared to seawater, and there were no surviving amoebae observed after 72 h. Following 72 h of treatment, bithionol concentrations >1 mg l^{-1} resulted in at least a 92% relative reduction in amoebae numbers compared with seawater, the same level of reduction observed for the freshwater group (Fig. 1). At the culmination of the assay, the greatest reduction in amoebae numbers compared to the seawater control (100, 95 and 92%) was seen in the 10, 5 and 1 mg l^{-1} treat-

ments, respectively. The lower concentrations of 0.5 and 0.1 mg l^{-1} of bithionol also significantly reduced amoebae numbers compared to the seawater control at 72 h by 87 and 82%, respectively ($F_{31,736}$ = 413.356, p < 0.001; Fig. 1).

Similarly, bithionol sulphoxide reduced amoebae numbers significantly when compared to the seawater control at all concentrations and time points ($F_{31,736}$ = 280.358, p < 0.001; Fig. 1). When treated with the highest concentration of bithionol sulphoxide (10 mg l^{-1}), there was a 57% relative reduction in amoebae compared to seawater within the first 24 h. After 72 h, the relative reduction for the 10 mg l^{-1} treatment group was equal to that of the freshwater group at 99%. Following 72 h of treatment, bithionol sulphoxide concentrations >1 mg l^{-1} resulted in at least a 96% relative reduction in amoebae numbers compared with seawater, which was the same level of reduction observed for the freshwater group (Fig. 1). After 72 h exposure at the lower concentrations of bithionol sulphoxide of 0.1 and 0.5 mg l^{-1}, amoebae numbers were reduced to only 59 and 60%, respectively, significantly higher numbers than at the other bithionol sulphoxide concentrations ($F_{31,736}$ = 280.358, p < 0.001; Fig. 1).

DISCUSSION

The present study demonstrates that bithionol and bithionol sulphoxide used *in vitro* were successful at reducing the number of surviving amoebae relative to seawater controls over the 72 h assay. Across all of the *in vitro* toxicity assays, the seawater control groups were equal to or better than the initial amoebae counts, indicating that conditions were appropriate to observe growth. Moreover, the reduction in amoeba survival in the bithionol and bithionol sulphoxide treatments was unlikely to be a surface effect due to low chemical solubility, since the numbers of surviving amoeba in the alumina groups (particulate control) were equal to those in the seawater controls. This indicated that the particulate matter in the well did not adversely affect amoebae survival over the 72 h period, a result consistent with Powell et al. (2003). For bithionol and bithionol sulphoxide, the lowest concentration capable of killing a significant number of amoeba (>50%) over 72 h was 0.1 and 1 mg l^{-1}, respectively. Given the low apparent solubility of both bithinol and bithionol sulphoxide, it is likely that the present study overestimates the concentrations required to kill *Neoparamoeba* spp. However, based on a previous study, these concentrations are lower than those toxic to salmon in fresh or saltwater (Florent et al. 2007a).

When comparing freshwater treatments to the seawater controls, a 94 and 96% relative reduction in amoebae viability was observed, respectively. Amoebae numbers declined rapidly in freshwater, consistent with other studies (Howard & Carson 1993, Powell & Clark 2003), indicating that freshwater remains the most effective fast-acting treatment. However, following the 72 h toxicity assay, bithionol and bithionol sulphoxide both successfully reduced amoebae numbers to levels similar to those found with freshwater treatments at concentrations >1 mg l^{-1}. At lower concentrations (<1 mg l^{-1}), bithionol was successful in reducing amoebae numbers, albeit not as effectively as freshwater. Bithionol sulphoxide at the lower concentrations of 0.1 and 0.5 mg l^{-1} was the least effective treatment, reducing amoebae numbers by 50%. Furthermore, the results indicated that both chemicals were active at reducing the numbers of surviving amoeba throughout the entire 72 h time period.

To date, the only commercially used treatment for AGD is freshwater bathing. Several compounds have been screened for *in vitro* toxicity including various antimicrobials, antiparasitics, disinfectants and detergents with varying success. Of all the chemicals screened, only a small number have achieved comparable results to freshwater, including chloramine-T (Harris et al. 2004, 2005), hydrogen peroxide (Powell & Clark 2003), levamisole (Findlay et al. 2000) and bithionol (bath treatment, Florent et al. 2007a; oral treatment, Florent et al. 2007b, 2009). However, some of these treatments were most effective when added to a freshwater bath, hence, still maintaining reliance upon freshwater bathing to treat AGD.

Studies examining the chemical control and treatment of *Neoparamoeba* spp. (Powell et al. 2003), *Hexamita salmonis* (Tojo & Santamarina 1998a), *Gyrodactylus* spp. (Tojo & Santamarina 1998b), *Icthyobodo necator* (Tojo et al. 1994a, Tojo & Santamarina 1998c), *Microcotyle sebastis* (Kim & Choi 1998), *Pseudodctylogyrus* spp. (Buchmann et al. 1992) and *Trichodina jadranica* (Madsen et al. 2000) have all examined bithionol and, in some cases, bithionol sulphoxide *in vitro* and *in vivo* as bath and oral treatments. When examining theses studies, bithionol exhibited variable toxicity depending upon the parasite species being tested; however, in general it was reported to be effective *in vitro* and *in vivo*, as both bath and oral treatments reduced parasite loads dramatically. However, with the scale and intensity of salmonid farming occurring in Tasmania, a bithionol bath treatment would be impractical. The low solubility and high cost would require large quantities for effective treatment and pose difficulties with regard to disposal of the bath water. The low concentrations that appear toxic to amoebae in the present study (as low as 1 mg l^{-1}) make both bithionol and bithionol sulphoxide attractive as oral treatments. Florent et al. (2007b) have demon-

strated that oral treatment of Atlantic salmon affected by AGD with bithionol at a concentration of 25 mg kg^{-1} feed could reduce the severity of infection by approximately 50%. Bithionol and bithionol sulphoxide have both been used as a successful treatment for numerous human disease, including paragonimiasis (oriental lung fluke) and fasciolasis (liver fluke) (Yang & Lin 1967, Bacq et al. 1991), with bithionol sulphoxide having better anthelminthic activity in rats (Meshi et al. 1970). In the present study, bithionol and bithionol sulphoxide exhibited similar reductions at high concentrations; however, at concentrations of 0.5 mg l^{-1} or lower, bithionol sulphoxide was not as effective as bithionol.

Bithionol and bithionol sulphoxide are not currently licensed for use in fish as therapeutics in Australia. Bithionol is used as an ingredient in deodorants, shampoos and surgical soaps; however, this has been stopped by the US Food and Drug Administration for safety reasons (FDA 2002), even though bithionol is currently being investigated as a therapeutic for use in treating human paragonimiasis and fascioliasis (CDC 2008). Currently, the cost of bithionol is 8 times greater than that of bithionol sulphoxide and the likelihood of industry adoption should be considered prior to approaching regulatory bodies.

The use of this *in vitro* toxicity test has allowed the screening of compounds for potential toxicity toward *Neoparamoeba* spp. Stage I of drug screening, the development of single- and multi-day *in vitro* toxicity assays, has allowed for the bulk testing of numerous disinfectants, antibiotics and antiprotozoal drugs (Powell et al. 2003, 2005, 2007). To date, few candidate drugs have progressed to Stage II of testing, whereby salmon are either bathed or offered drug-coated feed and subsequently experimentally challenged with *Neoparamoeba* spp. to determine the fish toxicity and efficacy for reducing the onset of AGD (Powell et al. 2003, 2007). Bithionol has successfully completed both of these stages (Stage I, Powell et al. 2003, present study; Stage II, Florent et al. 2007a,b, 2009). A study has shown that bithionol may be more effective at high infection pressures, such as that in the laboratory trial or at times of a rapid onset of disease, as opposed to a low infection pressures seen typically with chronic conditions of disease on farms (Florent et al. 2009). Few treatments have moved on to Stage III testing involving field trials (Powell et al. 2007), except for chloramine-T (Harris et al. 2004, 2005) and Aquacite/ Betabec™ (Powell et al. 2008). None of the treatments tested have been adopted commercially to date. This 3-tiered approach has allowed for a strategic system of screening and identifying candidate drugs from a large pharmacopeia, whilst maintaining effective resource management. Bithionol and bithionol sulphoxide both demonstrate a high degree of toxicity to *Neopararmoeba* spp. *in vitro*, suggesting that sufficiently low concentrations as may be measured from the inclusion of this drug with feed are effectively toxic to the amoeba.

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