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Gustavo W. Calvo
Virginia Institute of Marine Science

Eugene M. Burreson
Virginia Institute of Marine Science

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IN VITRO AND *IN VIVO* EFFECTS OF EIGHT CHEMOTHERAPEUTANTS ON THE OYSTER PARASITE *PERKINSUS MARINUS* (MACKIN, OWEN, AND COLLIER)

GUSTAVO W. CALVO AND EUGENE M. BURRESON
School of Marine Science
Virginia Institute of Marine Science
The College of William and Mary
Gloucester Point, Virginia 23062

ABSTRACT Eight therapeutants were tested for *in vitro* inhibition of *Perkinsus marinus* (Mackin, Owen, and Collier) enlargement and *in vivo* control of established infections. In addition, acute toxicity of six anticoccidials to oysters was determined. For *in vitro* experiments 0.2 ml aliquots of infected hemolymph were exposed to 5 concentrations (100 mg/l, 10 mg/l, 1 mg/l, 0.1 mg/l and 0.01 mg/l) of amprolium, arprinocid, cycloheximide, lasalocid, malachite green, monensin, sulfadimethoxine, and a potentiated sulfadimethoxine. Exposure lasted 1 day and was followed by incubation in fluid thioglycollate medium. Lasalocid and malachite green were the most effective compounds, showing significant anti-*P. marinus* activity at concentrations as low as 10 mg/l. Cycloheximide, monensin, and to a lesser extent sulfadimethoxine, were also effective but only at the highest concentration tested (100 mg/l). At concentrations lower than 10 mg/l, no compound tested had a significant effect on *P. marinus*. Lasalocid, monensin, and malachite green, were toxic to oysters at concentrations below 10 mg/l. The 96-hr LC50 for lasalocid was 0.59 mg/l. No median lethal dose was obtained for monensin or malachite green, but oyster mortality resulted from exposures ranging from 1 mg/l to 10 mg/l of either compound. In two *in vivo* experiments, infected oysters were exposed to amprolium, cycloheximide, malachite green, and sulfadimethoxine at various concentrations. Only cycloheximide was effective in reducing *P. marinus* infections. After 15 days of exposure to 10 mg/l of cycloheximide, weighted prevalence significantly declined from 3.78 in untreated controls to 2.10 in treated oysters. In addition, infections as measured by repeated hemolymph samples from individual oysters, significantly decreased after treatment. Extension of cycloheximide exposure to 30 days similarly reduced disease prevalence and weighted prevalence. Infections, however, were not completely eliminated even after 30 days of exposure to 10 mg/l of cycloheximide. Furthermore, infections progressed after treatment was discontinued as indicated by an increase in weighted prevalence from 0.71 at the end of treatment to 1.31 one month later.

KEY WORDS: *Perkinsus*, oyster, chemotherapeutants, Apicomplexa

INTRODUCTION

The oyster industry in Virginia has been in serious decline since an MSX epizootic caused large-scale mortality to oysters planted on private leased grounds in the lower portion of the Chesapeake Bay in 1960 (Burreson 1991, Haskin and Andrews 1988, Hargis and Haven 1988). Between 1960 and 1985 *Haplosporidium nelsoni*, the causative agent of MSX disease, was responsible for most of the disease-induced mortality in Chesapeake Bay oysters. However, since 1985, *Perkinsus marinus* (Mackin, Owen, and Collier) has gradually replaced *H. nelsoni* as the most important pathogen in Chesapeake Bay (Burreson 1991, Burreson and Andrews 1988, Andrews 1988a). In addition to disease, overharvesting and water quality deterioration have contributed to the decline of the oyster resource and industry (Hargis and Haven 1988). Nevertheless, disease-induced mortality and reduced plantings in private grounds, because of fear of high losses due to disease, have been cited as the primary factors involved in the rapid decrease in oyster landings during the 1960s and 1980s (Burreson, 1991).

P. marinus has proven to be extremely difficult to eradicate once it has invaded an area and no treatment for individual oysters is available. Low temperature and salinity has been shown to arrest infections, but not in a manner that could be applicable for treatment (Andrews 1988b). Unlike *H. nelsoni*, which can be eliminated by a 2 week exposure to salinities of 10 ppt or less (Ford 1985), *P. marinus* persists after 8 weeks of exposure to salinities as low as 6 ppt (Ragone and Burreson 1993). Control measures that could possibly prevent or reduce the impact of disease on oyster populations in Virginia include management strategies (Andrews and Ray 1988), breeding programs for increased disease resistance (Burreson 1991), and introduction of a more resistant oyster species (Mann et al. 1991). None of these strategies have been fully tested yet, and it is unlikely that their application would exclude the potential usefulness of chemotherapy to treat small lots of oysters that could be held in a bath regime. Applications of such an approach would include treatment of seed and broodstock oysters, as well as oysters held in trays or aquaria for use in aquaculture and research.

There have been only two studies that explored the effects of chemotherapeutics on *P. marinus*. At a time when the parasite was classified as a fungus, Ray (1966a) determined that among 12 antifungal antibiotics, cycloheximide was the most effective compound, completely inhibiting *P. marinus* enlargement when added at 25 mg/l to pieces of infected oyster tissues in seawater. That result prompted an investigation on the effect of cycloheximide on infected oysters (Ray 1966b), which showed that cycloheximide was effective in prolonging the life of *P. marinus*-infected oysters for several weeks longer than untreated controls. Higher concentrations or longer exposure times increased survival time and decreased infection intensities. Conclusions, however, were limited since sample sizes were small (N = 5 in most cases), and no statistical analysis was performed on the data.

The present study focuses on identifying efficacious anti-*P. marinus* compounds that could be used on oysters. The experimental approach was to test chemicals against *P. marinus* cells (*in vitro* experiments), and against infections in oysters (*in vivo* experiments). Chemicals were selected based on existing information indicating efficacy against *P. marinus*, efficacy against coccidians, potential for use in aquatic organisms, and safety considerations. A total of eight compounds were tested. Six of them (amprolium, arprinocid, lasalocid, monensin, sulfadimethoine, and potentiated sulfadimethoxine) are anticoccidials widely used

in the poultry industry. It was reasonable to expect that anticoccidial compounds would be effective in controlling *P. marinus* infections, since *P. marinus* has more recently been classified within the phylum Apicomplexa, which contains coccidians (Perkins 1976, Levin 1978). Indeed, chemicals effective against avian coccidiosis have been especially recommended for use in fish (Meyer and Schnick 1989), and oysters (Perkins 1979). Nevertheless, there is very little information on recommended doses for treatment of aquatic organisms, particularly bivalves. Therefore, *in vivo* studies of the anticoccidials included a toxicity experiment to determine possible lethal concentrations to oysters. In addition to anticoccidials, cycloheximide and malachite green were included at a later stage, after the toxicity study had been conducted. These latter compounds have the disadvantage of being potentially harmful to human health or non target organisms (Bower 1989, Meyer and Schnick 1989). However, they have successfully been used against aquatic pathogens and they constitute good reference standards. Cycloheximide has been used to control *Labyrinthuloides haliotidis*, a pathogen of juvenile abalone (Bower 1989), in addition to its use against *P. marinus*. Bower found that exposure of infected abalone to 1 mg/l of cycloheximide for 5 consecutive days reduced infection prevalence and abalone mortality; exposure to 100 mg/l for 10 days did not affect abalone survival. Malachite green has been included since it has been the most extensively used parasiticide of aquatic organisms (Meyer and Schnick 1989). Exposure of rainbow trout to 1.5 mg/l of malachite green for 1 day prevented the development of proliferative kidney disease (PKD) (Clifton-Hadly and Alderman 1987).

MATERIALS AND METHODS

In Vitro Experiments

Two experiments were conducted to examine the effect of chemicals on *P. marinus* enlargement in fluid thioglycollate medium (FTM). The first experiment consisted of exposing hemolymph from infected oysters to chemical baths containing 100 mg/l of active ingredients of amprolium, arprinocid, cycloheximide, lasalocid, malachite green, monensin, sulfadimethoxine, and potentiated sulfadimethoxine in 0.22 μm filtered York River water (FYRW) of 23 ppt salinity for one day. If 100 mg/l baths significantly inhibited the number of parasite cells enlarging to the prezoosporangia (hypnospore) stage, infected hemolymph was then exposed to 10 mg/l, 1 mg/l, 0.1 mg/l, and 0.01 mg/l baths during a second experiment.

The methodology was based on the hemolymph technique (Gauthier and Fisher 1990), modified to test for effects of chemicals on parasite cells. Infected hemolymph was withdrawn with a syringe from the adductor muscle sinuses of at least 10 oysters (selected from a heavily infected group) until a 10 ml stock was obtained. After mixing the stock, 0.2 ml aliquots were dispensed into 1.5 ml microcentrifuge tubes. Infected hemolymph aliquots were then exposed to 1 ml chemical baths for one day. After exposure to chemical baths, hemolymph aliquots were centrifuged at 265 × g for 15 minutes to concentrate parasite cells in pellets and discard cell free serum. The pellets, containing merozoites, meronts and schizonts of the parasite, were further washed in FYRW and then resuspended in 1 ml FTM containing 0.05 ml of penicillin and streptomycin (2500 units/ml). The inoculated media was incubated at 20°C in the dark for 5–7 days, then samples were centrifuged at 265 × g for 15 minutes and the supernatant was discarded. Pellets were resuspended in 1 ml 2 M NaOH for 15 minutes and then washed twice in distilled water. Pellets were then stained with 2 drops of Lugol's iodine working solution (Gauthier and Fisher 1990). After staining, the volume of each sample was raised to 1 ml with distilled water and dispensed into a separate well of a 24-well tissue culture plate. Enumeration of the total number of recognized prezoosporangia per well was made on an inverted scope at 100× magnification.

Chemical baths containing amprolium, sulfadimethoxine, cycloheximide, or malachite green were freshly prepared by dissolving 10 mg of the chemical in 10 ml of FYRW of 23 ppt salinity and then serially diluting in FYRW to the desired concentration. In the case of highly water insoluble compounds (arprinocid, lasalocid, monensin, potentiated sulfadimethoxine) 10 mg of the chemical was first dissolved in 1 ml of dimethylsulfoxide (DMSO) and then diluted in FYRW.

The experimental design consisted of triplicate treatments and included an untreated control group, and a solvent control group run at 1% DMSO (the highest concentration of solvent used). A one way ANOVA was used to examine differences in mean number of cells between treatments. When differences were detected, a Dunnett's test was performed to examine differences between individual treatments and controls.

Toxicity Experiment

For this experiment, *P. marinus*-free oysters (70 mm mean shell height, 32 g mean whole weight) were obtained from the Wye River in Maryland. After a 2 week acclimation to 25°C and 17 ppt in 1 μm FYRW, a sample of 25 oysters was taken for diagnosis of *P. marinus* in a combined sample of rectal, gill, and mantle tissue taken from each oyster following the method of Ray (1952). Subsequently, the remaining oysters were randomly assigned to aquaria for exposure to chemical baths.

The experimental design consisted of duplicate treatments of 5 oysters per 10 l bath. Oysters were exposed to 4 daily renewal baths of amprolium, arprinocid, lasalocid, monensin, potentiated sulfadimethoxine, or sulfadimethoxine at four concentrations: 0.01 mg/l, 0.1 mg/l, 1 mg/l, and 10 mg/l. In addition, there were 2 control baths, one untreated control and one solvent control at 0.1% DMSO. Stocks of chemical solutions were freshly prepared each day by dissolving 100 mg of active ingredients in 10 ml of DMSO and then adjusting to the desired concentration by dilution in FYRW. Sulfadimethoxine, a highly water soluble compound, was directly dissolved in FYRW.

Oysters were fed a daily ration of diatoms (*Chaetoceros calcitrans* and *Thallasiosira pseudonana*) and/or flagellates (*Isochrysis galbana*) at an approximate concentration of 5×10^8 to 5×10^9 cells/l. Chemical solutions were dispensed into aquaria, following the addition of food, using a 100 ml beaker. Aeration was continuously provided to aquaria. Aquaria were inspected daily, at the time of water change, for dead oysters. Oysters that remained open, after the tanks were emptied for water exchange, were considered dead. When appropriate, the concentration of exposure associated with a 50% mortality at 96 hr (LC50) was estimated by graphic and binomial methods (Gelber et al. 1985).

In Vivo Experiments

There were 2 experiments designed to determine the effect of chemicals on *P. marinus* infections in oysters. Experiment 1 was conducted to determine if amprolium, cycloheximide, malachite green, and/or sulfadimethoxine baths were effective in reducing *P.*

marinus infections. Adult oysters (>50 mm shell height), were collected from Point of Shoals in the James River in September 1992, cleaned of fouling organisms, individually labeled using a water proof marker, and subsequently maintained in a static tank filled with 1 μm FYRW at 20°C and 20 ppt for one week. Water was renewed daily. During that time, 0.3 ml hemolymph samples were withdrawn from each oyster and diagnosed for *P. marinus* following the hemolymph technique (Gauthier and Fisher 1990).

One hundred and eighty oysters, most with light infections, were selected and randomly assigned to 10 l aquaria in groups of ten. Concentrations of chemical baths ranged from 100 mg/l to 1 mg/l. Compounds considered safe, namely amprolium and sulfadimethoxine, were applied as 100 mg/l and 10 mg/l baths. Cycloheximide and malachite green were applied as 10 mg/l and 1 mg/l baths. Since oysters did not survive exposure to 10 mg/l or 1 mg/l of malachite green, another experiment was set up to test 0.1 mg/l and 0.01 mg/l.

All baths, including untreated controls, were tested in duplicate. Stock solutions of the chemicals were prepared immediately prior to use by adding 10 mg, 100 mg, or 1000 mg of active ingredients to approximately 10 ml of FYRW in 50 ml centrifuge tubes. Thorough mixing was accomplished with a vortex blender. There was no need for solvents during *in vivo* experiments since all compounds tested, including amprolium which is 20% water soluble, could be dissolved in FYRW. Daily rations of microalgae suspensions were added to each tube until the volume was raised to 50 ml. Then, the chemical-algae suspension was mixed as before and added to aquaria every other day for 2 weeks.

Dilution water consisted of 1 μm FYRW with a salinity of 22 ppt warmed to 20°C. Aeration was provided continuously. Aquaria were covered with sheets of Plexiglas to avoid possible loss of chemicals at the air interface. On days when chemicals were not added to aquaria, oysters were fed an algae diet as in the previous experiment.

After the 2 week treatment, *P. marinus* diagnosis was performed on a second hemolymph sample and on a tissue sample taken from each oyster. Tissue samples were assayed for *P. marinus* as previously described. Only tissue diagnosis was performed on oysters exposed to the lower concentrations of malachite green, during the follow-up experiment. Infection intensity in tissue samples was determined as described by Mackin (1962), and categorized as negative, light, moderate, or heavy. To calculate weighted prevalence, the following code numbers were assigned to the intensity (I) categories: 0 = negative, 1 = light, 3 = moderate, and 5 = heavy (Paynter and Burreson 1991). Weighted prevalence was calculated as the average value of infection intensity for number of oysters diagnosed in the treatment replicate (Σ In_I/N).

A one way ANOVA was used to examine differences in log transformed weighted prevalence between treatments. Differences in log transformed prezoosporangia counts were examined by a repeated measure ANOVA. A Dunnett's test followed to identify treatments that differed from controls. In addition, a paired *t*-test was used to compare pre-treatment and post-treatment abundance of *P. marinus* cells in the hemolymph samples of oysters from selected treatments.

In experiment 2 the ability of a 30 day cycloheximide exposure to reduce infections in oysters was tested. In addition, the subsequent progression of infections was monitored for 30 days after treatment. Two hundred and fifty oysters (66 ± 7 mm mean shell height) were collected from Point of Shoals in the James River in November 1992, cleaned and 25 oysters were sacrificed for *P. marinus* diagnosis. A second diagnostic sample was taken after 10 days of acclimation to 20°C. The diagnosis was performed on a combined tissue sample as previously described. Subsequently, the remainder of the oysters were labeled, and randomly assigned to one of four treatments: 30 day exposure to 10 mg/l cycloheximide bath, 30 day untreated control, 30 day exposure to 10 mg/l cycloheximide followed by 30 days without treatment, and 60 day untreated control. All treatments were tested in duplicate 25 l aquaria containing 25 oysters each. Chemicals were mixed with algae and added to aquaria, as in experiment 1, for 30 days. Oysters were fed an algae diet, and aeration was provided, as in previous experiments. Dilution water consisted of 1 μm FYRW with a salinity of 20 ppt heated to 20°C. Aquaria were covered as in the previous experiment. Disease prevalence and intensity were determined, as described before, for all treatment replicates at 30 days and 60 days. The effects of treatment and time on transformed prevalence and transformed weighted prevalence were examined by two way ANOVAS.

For statistical analysis of results from *in vitro* and *in vivo* experiments, data were transformed, if necessary, using SuperAnova software (Abacus Concepts Inc. 1989). Prezoosporangia count data and weighted prevalence data were log $(x + 1)$ transformed, and prevalence was arcsin transformed. Normality and homogeneity of variance of untransformed and transformed data were examined, using $SPSS^X$ software (SPSS Inc. 1986), by the Kolmogorov-Smirnov test and Cochran's C test respectively. Data used for subsequent analysis was significantly normal and homogeneous at a confidence level above 90%. ANOVA and Dunnett tests were performed using SuperANOVA software (Abacus Concepts Inc. 1989), *t*-tests were performed using Statview software (Abacus Concepts Inc. 1992).

Amprolium was obtained from MSD-AGVET, St. Louis, MO; lasalocid, sulfadimethoxine, and potentiated sulfadimethoxine from Roche Vitamins and Fine Chemicals, Nutley, NJ; lasalocid, monensin, and arprinocid from Lilly Research Laboratories, Greenfield, IN; cycloheximide from Sigma Chemical Company, St. Louis, MO; and malachite green from Argent Chemical Laboratories, Redmond, WA.

RESULTS

In Vitro Experiments

At 100 mg/l exposure, there was a significant ($P = 0.0001$) difference in prezoosporangia abundance among treatments (Fig. 1). Solvent controls had a higher abundance of prezoosporangia than untreated controls. Therefore, only untreated controls were included in the statistical analysis in order to give conservative results. Results of Dunnett's test indicate that 5 treatments: lasalocid, malachite green, cycloheximide, monensin, and sulfadimethoxine were associated with significantly lower prezoosporangia abundance ($P < 0.05$) than the untreated control. At 10 mg/l exposure, there was also a significant ($P = 0.0001$) difference in prezoosporangia abundance between treatments (Fig. 2). At this concentration, however, only lasalocid and malachite green were associated with a significantly ($P < 0.05$) lower prezoosporangia abundance than untreated controls. At concentrations lower than 10 mg/l, no significant ($P > 0.05$) difference was found among treatments.

Toxicity Experiment

Oysters ($N = 25$) from the baseline diagnostic sample were all negative for *P. marinus*. All solvent control oysters survived 96

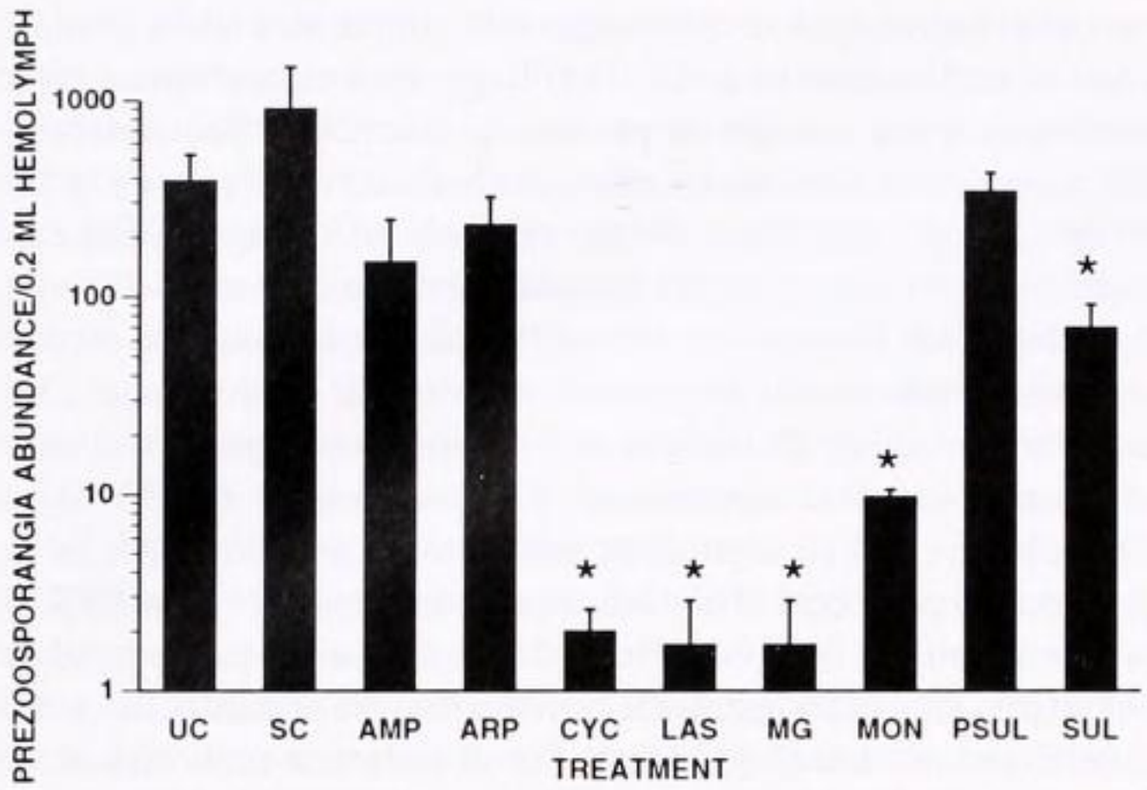

Figure 1. ***In vitro*** **experiment. Mean number of prezoosporangia after exposure to 100 mg/l chemical treatments averaged over 3 replicate 0.2 ml hemolymph samples. Abbreviations: UC = Untreated control, SC = Solvent control, AMP = Amprolium, ARP = Arprinocid, CYC = Cycloheximide, LAS = Lasalocid, MG = Malachite green, MON = Monensin, PSUL = Potentiated sulfadimethoxine, SUL = Sulfadimethoxine. * = Significantly lower ($P < 0.05$) than untreated control (using transformed data). Error bars = Standard errors.**

hour exposure to 0.1% DMSO. Among the chemicals tested in this experiment, only lasalocid and monensin were lethal to oysters. The LC50 for lasalocid was 0.51 mg/l as determined by the graphic method, and 0.59 mg/l with a 95% confidence interval ranging from 0.1 to 10 mg/l by the binomial method. Exposure to 1 mg/l and 10 mg/l of monensin for 96 hours resulted in 10% and 40% mortality respectively.

In Vivo Experiments

At the start of experiment 1, most oysters had light infections (<1000 *P. marinus* cells per 0.3 ml of hemolymph). Comparison of parasite cell abundance in hemolymph samples taken before and after treatment and among treated and untreated groups, revealed that only oysters exposed to cycloheximide had lower infections after treatment than they had before treatment (Fig. 3). Tissue samples showed an overall agreement with hemolymph samples, but intensity estimates were generally higher for tissue samples within each oyster. At the end of the 15 day period, weighted prevalence was significantly ($P < 0.05$) lower in oysters exposed to 10 mg/l of cycloheximide than in untreated controls. Average weighted prevalence was 2.10 and 3.78 respectively. In hemolymph samples, there was a significant ($P = 0.0151$) effect of treatment on prezoosporangia abundance averaged over time. The effect of time alone however, averaged over treatment, was not significant ($P = 0.8674$). Nevertheless, the pattern of change over time was different for different treatments, as indicated by the significant ($P = 0.0001$) effect of the interaction term. In agreement with results from tissue diagnosis, only oysters exposed to 10 mg/l of cycloheximide had a significantly lower ($P < 0.05$) number of prezoosporangia per unit hemolymph than untreated controls. There were no oysters surviving exposure to 10 mg/l or 1 mg/l of malachite green after 15 days. More than 50%, 21 in 40, of the oysters exposed to malachite green were dead after chemicals were renewed four times. During the follow-up experiment, all oysters survived exposure to 0.1 mg/l and 0.01 mg/l of malachite green, but no effect on disease prevalence or infection intensity was detected at those concentrations.

In experiment 2, the first diagnostic sample indicated a 68% prevalence of mostly light infections (weighted prevalence = 0.92) at the time oysters were collected. Ten days after acclimation to 20°C, prevalence rose to 95% and intensities had increased (weighted prevalence = 1.85). Following 30 days of exposure to cycloheximide, infection prevalence and weighted prevalence declined to 67% and 0.71 respectively (Figs. 4 and 5). At that time, oysters exposed to cyclohexmide had fewer and lighter infections than untreated control oysters. The magnitude of the decline in infections, as measured by the reduction in weighted prevalence

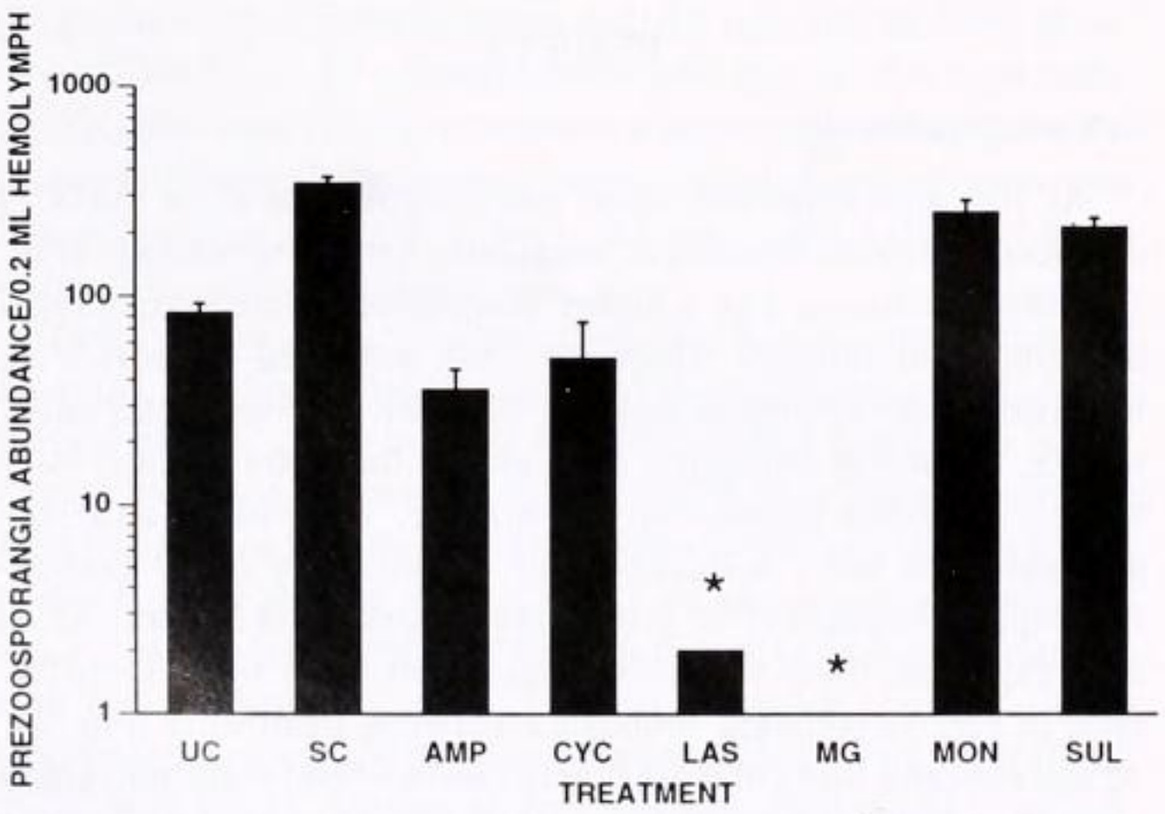

Figure 2. ***In vitro*** **experiment. Mean number of prezoosporangia after exposure to 10 mg/l chemical treatments averaged over 3 replicate 0.2 ml hemolymph samples. Abbreviations: UC = Untreated control, SC = Solvent control, AMP = Amprolium, CYC = Cycloheximide, LAS = Lasalocid, MG = Malachite green, MON = Monensin, SUL = Sulfadimethoxine. * = Significantly lower ($P < 0.05$) than untreated control. Error bars = Standard errors. No error bars shown when replicate samples were equal.**

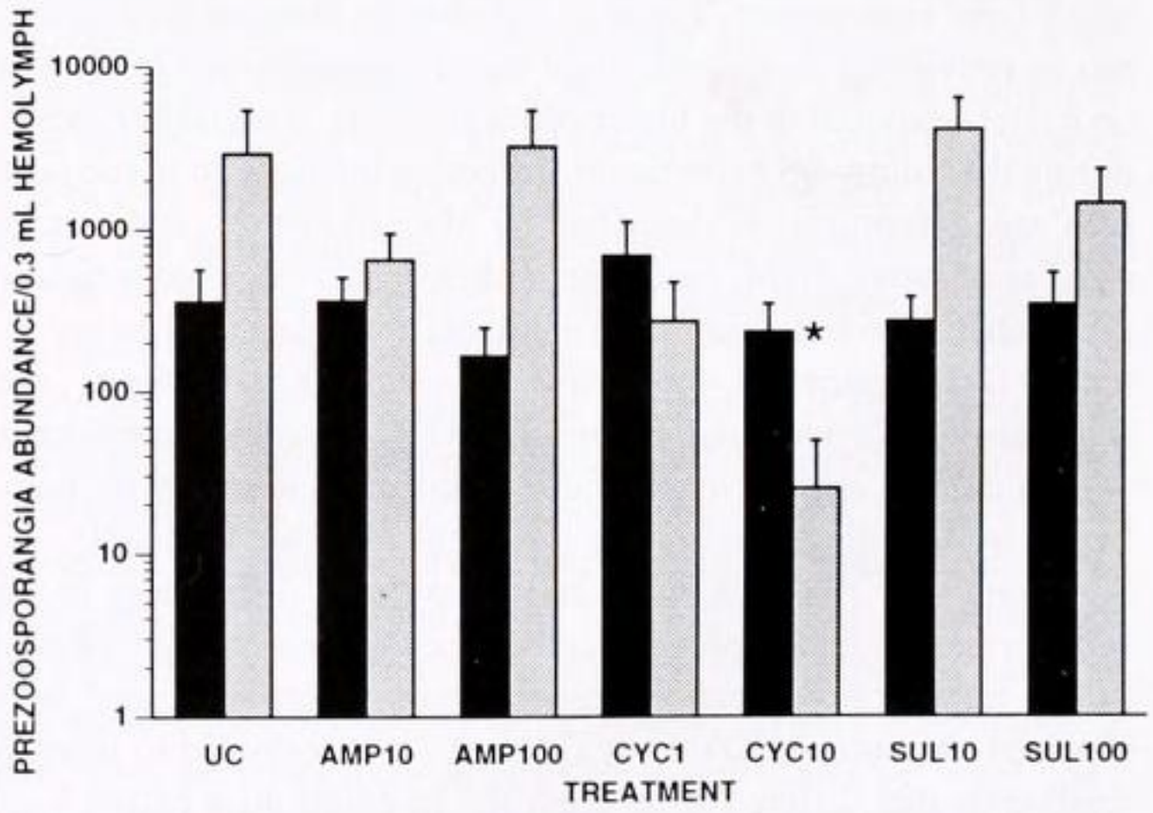

Figure 3. ***In vivo*** **experiment 1. Mean number of prezoosporangia, before and after treatment, per 0.3 ml hemolymph samples averaged over 16–20 oysters. Dark bars indicate pre-treatment values. Light bars indicate post-treatment values. Abbreviations: UC = Untreated control, AMP = Amprolium, CYC = Cycloheximide, SUL = Sulfadimethoxine. Numbers following acronyms indicate concentration of exposure in mg/l. One sample of the CYC1 group had more than 100,000 cells after treatment and was excluded from this chart. * = Final value significantly lower ($P < 0.05$) than initial value, and overall value significantly lower ($P < 0.05$) than untreated control.**

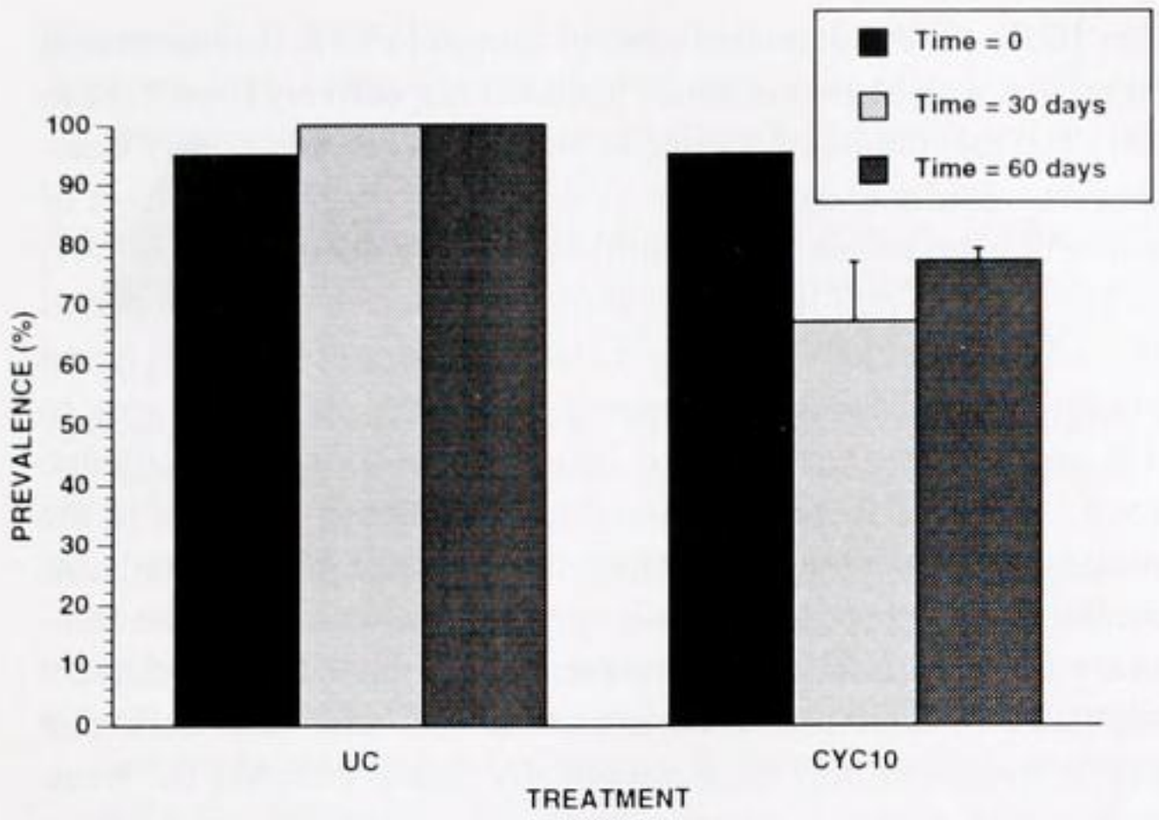

Figure 4. *In vivo* experiment 2. Effect of treatment on prevalence. UC = Untreated control treatment, CYC10 = 10 mg/l cycloheximide treatment. The bar to the left of each group corresponds to the diagnostic sample (N = 25 oysters) taken immediately prior to the initiation of the experiment. Other bars indicate average of 2 replicate groups of 25 oysters. Error bars = Standard deviation. No error bars shown when replicate samples were equal.

between treated oysters and untreated control oysters after the treatment period, in this experiment, was similar to the one in the previous experiment (i.e. a 1.50 decline as compared to a 1.68 decline, respectively). Two way ANOVAs indicated a significant effect of treatment on prevalence and weighted prevalence ($P = 0.0001$ and $P = 0.0011$ respectively). There was no significant ($P = 0.2350$) effect of time on prevalence (prevalence was already at the maximum at 30 days in controls), but there was a significant ($P = 0.0143$) effect of time on weighted prevalence as weighted prevalence rose both in control and treated oysters alike (Figs. 4 and 5). The interaction effect of treatment and time on prevalence and weighted prevalence was not significant ($P = 0.2350$ and $P = 0.9118$ respectively). There was no adverse effect of cycloheximide on oyster survival. Among 100 oysters treated, only 8 died as compared to 4 that died among 100 untreated controls. Even though sublethal effects were not measured, no overall differences in soft tissue appearance or wet tissue weight between oysters exposed to cycloheximide and untreated controls were observed.

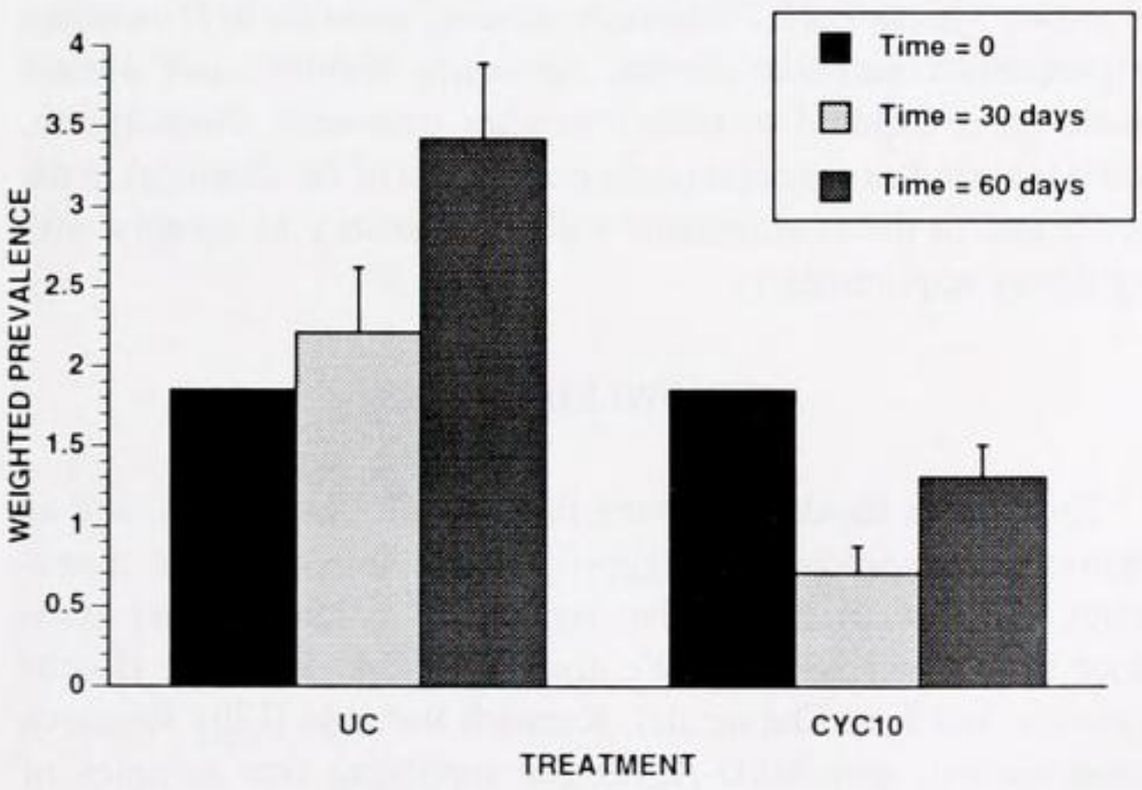

Figure 5. *In vivo* experiment 2. Effect of treatment on weighted prevalence. UC = Untreated control treatment, CYC10 = 10 mg/l cycloheximide treatment. The bar to the left of each group corresponds to the diagnostic sample (N = 25 oysters) taken immediately prior to the initiation of the experiment. Other bars indicate average of 2 replicate groups of 25 oysters. Error bars indicate standard deviation. No error bars shown when replicate samples were equal.

DISCUSSION

In Vitro Studies

Of the initial eight compounds tested, five (i.e. cycloheximide, lasalocid, malachite green, monensin, and sulfadimethoxine) showed efficacy in inhibiting *P. marinus* cells from enlarging in FTM. Malachite green and lasalocid, followed by cycloheximide, were the most effective compounds. We speculate that the mechanism by which chemicals inhibited *P. marinus* enlargement is related to the general mode of chemical action. Lasalocid, monensin, and malachite green may have been lethal to *P. marinus* cells. Polyether ionosphorus compounds (including lasalocid and monensin) are known to disrupt the electrochemical balance of ions, such as Na^+ and K^+, which are involved in maintaining the selective permeability of membranes thereby having a cytocidal effect (McDougald 1982). Malachite green is a respiratory enzyme poison known to destroy mitochondria (Alderman 1985). In the case of cycloheximide, a protein synthesis inhibitor highly active against a large number of yeasts including fungal pathogens (Ennis and Lubin 1964), a cytostatic effect is more likely to have occurred in the short term.

Inhibition of *P. marinus* enlargement by cycloheximide has been reported by Ray (1966a). In Ray's study, exposure of infected tissues to 25 mg/l of cycloheximide in FTM completely inhibited prezoosporangia development. In this study, exposure of infected oyster hemolymph to 100 mg/l of cycloheximide in FYRW of 23 ppt salinity for 1 day drastically inhibited subsequent prezoosporangia development in FTM, and exposure to 10 mg/l resulted in some inhibition. Adding chemotherapeutants into FTM, as in Ray's (1966a) study, may result in greater inhibition of prezoosporangia development given the longer time of exposure in FTM (at least 5 days) as compared to the 1 day exposure used in the present investigation. Further comparison of results is complicated because Ray used infected tissues and categorized the degree of inhibition on an ordinal scale.

Perhaps incorporation of chemicals into the culture medium would be a better way for screening compounds *in vitro*, but the biochemical action of antimetabolite anticoccidials such as amprolium and sulfadimethoxine may be neutralized by aminoacids and vitamins present in the medium. Amprolium and sulfadimethoxine are analogs of thiamine and p-aminobenzoate respectively, and presence of the latter compounds inhibits the action of the referred anticoccidials (McDougald 1982).

To some extent, anticoccidials (particularly the polyether ionosphore lasalocid and monensin) showed activity against *P. marinus* as expected based on the proposed affinities of this parasite to the coccidians. However, the fact that compounds that are mostly considered antifungals (malachite green and cycloheximide) were even more effective, indicates that perhaps other antifungals should be tested.

It should be noted that no adverse effect of DMSO was detected on *P. marinus* cells. To the contrary, it appears that DMSO may have prevented cell deterioration, as suggested by the higher cell counts in the solvent control than in the untreated (FYRW) control. Control of microbial contamination by DMSO could account for the increased cell counts in solvent controls. This observation

is supported by the indication that DMSO may not only act as a solvent without interfering with parasite development or host cell integrity, but it may also sterilize *in vitro* assays (Ryley and Wilson 1976).

Toxicity Studies

Among the six anticoccidials tested in the toxicity experiment, only lasalocid and monensin caused oyster mortality when applied at 10 mg/l or 1 mg/l. Oyster mortality due to lasalocid and monensin exposure is not entirely surprising since both chemicals are polyether ionosphorus compounds known for their toxic effects associated with their mode of action (McDougald 1982). It should also be noted that there was no oyster mortality associated with exposure to 0.1% DMSO, suggesting that DMSO can be used as solvent at that concentration or at lower concentrations.

In addition to anticoccidials affecting oyster survival in the toxicity experiment, malachite green was lethal to oysters in experiment 1 (*in vivo*). Similar to concentrations of exposure of monensin and lasalocid, 10 m/l and 1 mg/l of malachite green resulted in oyster mortality while 0.1 mg/l and 0.01 mg/l did not. Even though experiment 1 was not designed to determine median lethal doses, results suggest that acute toxicity of malachite green to oysters may be in the range of that for most freshwater invertebrates (0.51 mg/l–3.45 mg/l) but lower than that for the Asiatic clam *Corbicula leana* (122 mg/l), as reported by Bills et al. (1977). In addition to its destructive action against mitochondria, malachite green is known to be teratogenic in laboratory animals, and its use in aquaculture in the US has not been approved by FDA (Meyer and Schnick 1989).

In Vivo Studies

Among all compounds tested, only cycloheximide was effective in controlling *P. marinus* infections, while not adversely affecting oyster survival. In contrast to exposure to low salinity (Ragone and Burreson 1993), infections did not merely stop progressing, but regressed significantly after exposure to cycloheximide. Both hemolymph and tissue samples confirmed reduction of infections, indicating that chemical exposure had some parasiticidal effect. Short term medication may arrest parasite development whereas long term medication may kill the parasite. This conclusion agrees with results reported for other antiparasitic drugs (McDougald 1982).

Tissue samples generally showed higher infection intensities than hemolymph samples suggesting perhaps that established infections in the tissues were not as easily affected by the chemical as parasites circulating in the hemolymph. Alternatively, higher parasite abundance in the tissues as compared to hemolymph may only be indicative of a difference in sensitivity of the two assays; the tissue diagnostic assay being more sensitive than the hemolymph one, as proposed by Burreson and Ragone (1993).

In agreement with Ray (1966b), experiment 2 showed that exposure of infected oysters to 10 mg/l of cycloheximide for 30 days results in a decrease in infection prevalence and intensity. Experiment 2 clearly defined the effect of cycloheximide on disease prevalence and weighted prevalence. Prevalence declined from 100% (in the untreated control group) to 67% (in the treated group) and weighted prevalence declined respectively from 2.21 to 0.71. The magnitude of decline in weighted prevalence after treatment was similar to experiment 1, suggesting that 15 more days of treatment were not enough to further reduce infections. However, the fact that several light infections of treated oysters comprised only a few cells may have prevented a greater effect from being detected. Thirty days after stopping treatment, prevalence rose to 77% and weighted prevalence increased to 1.31 in the treated group. A similar increase in weighted prevalence occurred in the untreated control group. The mode of action of cycloheximide, as an inhibitor of protein synthesis, probably accounts for the temporary regression of infections during treatment and subsequent recurrence of infections once treatment was removed. Cells that survive treatment may be metabolically inactive during the treatment period. Perhaps a better strategy to control *P. marinus* infections with cycloheximide would be to treat infected oysters for 1–2 weeks to kill metabolically active parasites, withdraw treatment for 1 week to allow "dormant" parasites to develop, followed by another cycloheximide treatment. This schedule could be repeated until infections were eliminated or greatly reduced.

In summary, cycloheximide baths of at least 10 mg/l renewed every other day for 2 weeks are necessary to reduce *P. marinus* infections in oysters held at 20°C and 20 ppt. Extension of treatment for 2 more weeks may not result in eradication of parasites. Infections may relapse once treatment is stopped and mortalities will most likely follow. Given its potential to cause harmful effects in humans, the use of cycloheximide is currently restricted to laboratory applications. All other uses have been canceled by EPA (Carl Grable, Environmental Protection Agency, pers. comm.).

In the future, other effective anti-*P. marinus* compounds that could be used on oysters may be found using the basic approach developed in this study. The recently developed *P. marinus* cell cultures (La Peyre et al. 1993) would facilitate screening of compounds *in vitro*. Studies that follow this line of research, however, should note that the potential applications of chemotherapy to *P. marinus*-infected oysters may be restricted to small lots of oysters, for example important broodstock. It is unlikely that this approach would be practical for aquaculture situations where oysters must be grown "in the field." In nature, disease pressure to *P. marinus* is probably continuous during the warm months, and oysters would be re-exposed to infections after treatment. Furthermore, additional studies concerning the persistence of the chemical in the oyster and in the environment will be necessary to comply with regulatory requirements.

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