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In vitro and *in vivo* effects of chemotherapeutants on the oyster parasite, *Perkinsus marinus*

Calvo, Gustavo W., Ph.D.

The College of William and Mary in Virginia, 1994

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IN VITRO AND *IN VIVO* EFFECTS OF CHEMOTHERAPEUTANTS ON THE OYSTER PARASITE, *PERKINSUS MARINUS*

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

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by
Gustavo W. Calvo
1994

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

Gustavo W. Calvo

Approved, May 1994

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ABSTRACT

To investigate the potential of chemotherapeutants to control the oyster pathogen *Perkinsus marinus*, anticoccidial and antifungal compounds were tested *in vitro* on infected hemolymph and cultured *P. marinus* cells and *in vivo* on infected oysters. In addition, acute toxicity to oysters was determined for six anticoccidials.

In vitro experiments with infected hemolymph consisted of 24 h exposure of 0.2 mL hemolymph aliquots to concentrations ranging from 100 mg/L to 0.01 mg/L of amphotericin-B, amprolium, arprinocid, cycloheximide, lasalocid, malachite green, monensin, sulfadimethoxine, and a potentiated sulfadimethoxine, followed by incubation in fluid thioglycollate medium (FTM) to determine prezoosporangia abundance. Lasalocid, malachite green, and amphotericin-B were the most effective compounds reducing prezoosporangia abundance, relative to the untreated control group, 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, none of the compounds tested had a significant effect on abundance of prezoosporangia.

In vitro experiments with cultured *P. marinus* consisted of 24 h exposure of 10^5 cells to 100 mg/L, 10 mg/L, and 1 mg/L of amphotericin-B, and 100 mg/L of cimetidine, cycloheximide, fumagillin, 5-fluorocytosine, ketoconazole, lasalocid, and monensin, followed either by incubation in FTM to determine abundance and size of prezoosporangia, or by addition of Neutral Red to determine cell viability. Amphotericin-B, lasalocid, and monensin were effective in reducing prezoosporangia abundance, size, and/or cell viability. No effects of cycloheximide on cultured cells were apparent.

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 determined for monensin or malachite green, but oyster mortality resulted from exposures ranging from 1 mg/L to 10 mg/L of either compound.

In three *in vivo* experiments, infected oysters were exposed to amprolium, arprinocid, cycloheximide, lasalocid, monensin, malachite green, potentiated sulfadimethoxine, 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 *P. marinus* 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.

In summary, cycloheximide baths of at least 10 mg/L renewed every other day for 2 weeks were necessary to reduce *P. marinus* infections in oysters held at 20 °C and 20 ppt. Continuation of the treatment schedule for 2 additional weeks did not result in eradication of parasites, and infections relapsed once treatment stopped.

IN VITRO AND *IN VIVO* EFFECTS OF CHEMOTHERAPEUTANTS ON THE OYSTER PARASITE, *PERKINSUS MARINUS*

INTRODUCTION

The oyster industry in Virginia experienced serious decline since an MSX epizootic caused large scale mortality to oysters in the lower portion of the Chesapeake Bay in 1960 (Burreson 1991a, Haskin & Andrews 1988, Hargis & 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. Since 1985, however, *Perkinsus marinus* has gradually replaced *H. nelsoni* as the most important pathogen in Chesapeake Bay. Estimates of up to 50% total annual oyster mortality have been attributed to *P. marinus* disease (Andrews, 1988a). Although over harvesting and water quality deterioration have contributed to the decline of the oyster resource and industry (Hargis & Haven 1988), disease mortality and decreased investment in private farming, because of the fear of losses to disease, are believed to be primarily responsible for much of the decline in oyster landings (Burreson, 1991a).

Sublethal effects of *P. marinus* on oyster growth and reproduction have also been proposed as additional factors impacting production. Ray et al. (1953) reported an average of 33% reduction in condition index of heavily infected oysters compared to uninfected oysters. Andrews (1961), and Menzel and Hopkins (1955) found that *P. marinus* infection reduces oyster shell growth. Similarly, Paynter and Burreson (1991) correlated shell growth reduction and oyster condition reduction with the acquisition and progression of *P. marinus* infections. Mackin (1962) reported that infections disrupted gonadal tissue and impaired gonad development. Decrease in oyster setting as a result of lethal and sublethal effects of disease on broodstock has been proposed as an additional factor contributing to the decline of oyster populations (Haven & Fritz 1985, Ford & Figueras 1988).

Perkinsus marinus is endemic in most of the lower Chesapeake Bay including the lower sections of all rivers in Virginia. The parasite also now occurs in the upper parts of

the rivers, but abundance is low and little disease mortality occurs there. *Perkinsus marinus* has thrived in densely planted private beds, but it has persisted even in sparsely populated public beds. It is not clear how oyster density affects disease transmission, but denser beds seem to have had the highest mortality (Andrews 1988a). Infections follow a seasonal pattern of maximum prevalence and intensity during summer and fall and minimum prevalence and intensity during winter and spring. Temperature and salinity modulate the seasonal pattern of infections and play a major role in determining the distribution and abundance of the parasite. At temperatures above 20 °C and salinities above 12 ppt *P. marinus* develops rapidly and can be lethal to oysters within a month. In natural beds most oysters die after two consecutive summers of exposure (Andrews, 1988a). In response to above normal seasonal temperature and salinity regimes such as the persistent droughts in 1985-1988, *P. marinus* increased its distribution and abundance (Burreson and Andrews 1988). Below normal seasonal salinities, such as the ones registered in 1989 and 1990 due to abnormally high rainfall, however, did not eradicate the pathogen (Burreson 1991b). Therefore, for *P. marinus* disease to disappear, below average temperature and salinity conditions may need to persist for a very long time.

The taxonomy of this parasite is still controversial. Initially, *P. marinus* was placed in the fungal genus *Dermocystidium* on the basis of the characteristic eccentric nucleus and vacuole with refringent vacuoplast observed in hematoxylin and eosin stained sections of infected oyster tissue (Mackin et al. 1950). Later, it was transferred to the fungal genus *Labyrinthomyxa* by Mackin and Ray (1966). More recently, electron microscopic observations of the apical complex of zoospores by Perkins (1976a) led Levine (1978) to create the class Perkinsea and the genus *Perkinsus* within the protozoan phylum Apicomplexa to accommodate *P. marinus*. Although this latter classification has been endorsed by the Society of Protozoologists (Levine 1980), the taxonomic status of *P. marinus* and indeed of the phylum Apicomplexa, has not been settled. Since 1980, there have been 3 independent revisions of the phylum Apicomplexa, reviewed by Canning

(1986), which propose to re-establish Sporozoa as the phylum name. Two revisions, by Krylov & Kostenko (1981) and Corliss (1984), propose to include *P. marinus* within the sporozoans under a new class or order. The third proposal, by Vivier (1982), disregards the similarities of the apical complex in *P. marinus* with that of other sporozoans, and suggests placing the species within the flagellates based on the lack of a sexual phase in its life cycle. Recent biochemical studies of *Perkinsus sp.* indicate that the genus is phylogenetically closer to dinoflagellates and to coccidian and piroplasm apicomplexans than to fungi or flagellates (Goggin & Barker 1993).

In oysters, *P. marinus* can be detected by direct microscopic examination of tissue or hemolymph smears (Mackin, 1962). Those methods, however, are not suitable for routine diagnosis given the difficulty in observing a parasite of such a small size. The smallest meronts found in oyster tissue are 2-4 μm in diameter (Perkins, 1988). Histological sectioning and staining with hematoxylin and eosin can also be used for diagnosis but these are time consuming methods (Johnson and Sparrow, 1961). A rapid diagnostic method currently used is the thioglycollate culture method developed by Ray (1952). Ray's method consists of incubating whole animal tissues or pieces of infected oyster tissues in fluid thioglycollate medium (FTM). This procedure results in enlargement of *P. marinus* meronts (called prezoosporangia or hypnospores after enlargement) to sizes that are easily observed by light microscopy, especially after staining with iodine. The oyster tissues that are usually examined are mantle, gill, and rectum. Extensive use of the thioglycollate culture method by oyster biologists since the 1950s has shown that *P. marinus* is an important agent of oyster mortality along the east and gulf coasts of the United States (Ray 1954, Mackin 1962, Andrews 1976). For assessment of *P. marinus* infection intensity, Ray (1954) and later Mackin (1962), developed ordinal scales which rate the relative abundance and distribution of the parasite in pieces of oyster tissue. More quantitative methods based on counting the number of prezoosporangia per unit area and including the use of replicates were developed by Scott et al. (1985). The number of

prezoosporangia per unit weight after extraction from oyster tissue has also been used as a measure of infection intensity (Choi et al., 1989). More recently, a hemolymph assay has been developed for diagnosis. The "hemolymph technique", which is based on incubation of hemolymph in FTM, has the advantage of allowing for repeated diagnosis of the same individual without having to sacrifice the oyster (Gauthier and Fisher, 1990).

Despite recent developments that allow culture of the parasite continuously *in vitro* (La Peyre et al. 1993), the life cycle of *P. marinus* is still not well understood. The known life stages of *P. marinus* in oysters are: meronts (2-4 µm coccoid uninucleate cells), mature meronts (10-20 µm cells containing an eccentrically situated vacuole), and schizonts typically containing 8-32 merozoites (Perkins, 1988). These life stages have been extensively observed and described from live smears and oyster tissue sections using light (Mackin et al. 1950, Mackin 1951, Mackin and Wray 1952, Mackin 1962) and electron microscopy (Perkins 1966, 1969, 1976a, 1988). Based on those observations, the following sequence of events has been postulated. Immature meronts, also called merozoites, develop into meronts which then form multicellular schizonts by successive bipartitioning or progressive cleavage of the protoplasm. Subsequent rupture of the schizont releases immature meronts (Perkins 1976a, 1988). The rest of the life cycle, especially what happens outside the oyster is unclear.

It should be noted that incubation in FTM results in 15 µm to greater than 100 µm enlarged cells called prezoosporangia or hypnospores, but these cells do not multiply and do not survive in FTM for more than 10 days (Perkins 1966, 1988, Perkins and Menzel 1966). Transfer of prezoosporangia into seawater, however, results in production and release of biflagellated zoospores from zoosporangia (Perkins 1966, Perkins and Menzel 1966).

Perkinsus marinus zoospores have been shown to infect oyster tissue in laboratory experiments. Only light infections resulted even when tissues were exposed to millions of zoospores (Perkins, 1988). In contrast, heavy infections have easily been obtained by

exposing live oysters to tissue minces from infected oysters (Ray 1952, Mackin 1962). This suggests that meronts and not zoospores may be the primary infective cell type (Perkins, 1988).

Infections can be initiated in the gill, mantle, or gut epithelia, or in connective tissue near the basement membrane (Perkins, 1976b). Most initial infections have been observed in the digestive epithelium from the stomach to the rectum (Mackin, 1951). Early stages of infection are believed to occur in the hemolymph (Gauthier and Fisher, 1990). In fact, immature meronts have often been found within oyster hemocytes (Perkins, 1988). Parasites may grow and multiply within hemocytes or in the intercellular spaces of the hepatopancreas and gut (Mackin 1962, Perkins 1976b). Since oysters have an open circulatory system, it has been hypothesized that hemocytes phagocytize the pathogens and convey them all over the body (Mackin 1962, Andrews 1988a).

Advanced infections are characterized by abscesses of the epithelia, massive concentration of parasites throughout the connective tissue, and occlusion of hemolymph sinuses by parasites and hemocytes as well (Mackin 1951, Perkins 1976b). Rapid multiplication of the parasites results in destruction of hemocytes and tissues and usually causes death in about 1 month at 25-30 °C (Mackin 1962, Andrews 1988a).

Perkinsus marinus can be transmitted directly from oyster to oyster. Experimental infection in the laboratory is usually carried out by adding a few infected oysters to an aquarium where a group of about 25 uninfected oysters are maintained ("proximity method"), by adding minced tissue from infected oysters to the aquarium ("feeding method"), or by injecting a suspension of parasites in seawater into the mantle cavity of individual oysters ("injection method"). Comparison of these methods indicates that injection produces mortality in the shortest time (90% mortality in a group of 25 oysters in 6 weeks) and also ensures simultaneous infections in the group. Injection, however, has the disadvantage of introducing another stress to the oysters. By the feeding method, 90% mortality occurs in about 9 weeks, but the additional stress is eliminated (Ray, 1954). The

proximity method is seldom used since it is more time consuming.

The effect of dosage of *P. marinus* infective cells on oyster mortality was studied by Mackin (1962) using the injection method. His work indicated that oysters (N = 25) receiving a single 1.0 mL injection containing 5×10^4- 5×10^6 parasite cells per oyster start to die after 3 weeks at a rate of 2% per day. Oysters injected with 5×10^3 cells start to die after 4 weeks at a rate of 0.3-1.0% per day, and oysters injected with less than 100 cells were not affected after 6 weeks when the experiment was terminated.

Andrews and Hewatt (1957) investigated the effects of low temperature on the establishment and progression of infections. Oysters injected with *P. marinus* extracted from infected tissues did not develop infections when maintained at temperatures lower than 15 °C for six weeks while oysters initially maintained at 28 °C for one week and subsequently transferred to 15 °C conditions developed only light infections during the same period. Oysters held continuously at 28 °C for six weeks experienced 90% mortality.

Perkinsus 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 have been shown to arrest infections, but not in a manner that could be applicable for treatment (Andrews 1988b). Unlike *Haplosporidium nelsoni*, which can be eliminated by a 2 week exposure to salinities of 10 ppt or less (Ford 1985), *P. marinus* persisted after 8 weeks of exposure to salinities as low as 6 ppt (Ragone & Burreson 1993).

Control measures that could possibly prevent or reduce the impact of disease on oyster populations in Virginia include management strategies and breeding programs to increase oyster survival after exposure to disease agents. Andrews and Ray (1988) proposed management strategies for private oyster beds based on isolation of beds, harvesting early and cleaning beds, and closely monitoring disease. Implementation of such proposals, however, seems to be constrained by the potential risk of transmission of infections from contiguous public areas.

Breeding programs to increase oyster survival involve selecting surviving oysters

from disease endemic areas and breeding them over successive generations. Such an approach has been used successfully to increase survival of oysters exposed to *H. nelsoni* over eight generations of selection (Ford and Haskin 1987). Improvement in survival of oysters challenged with *H. nelsoni* , however, has not been correlated to particular cellular or humoral defense mechanisms of disease resistance (Ford 1986), but appears to result from an overall physiological superiority that makes cultured oysters more tolerant to the effects of disease (Myhre 1973). Stocks selected for increased survival when subjected to *H. nelsoni* challenge, however, have not attained increased survival when challenged with *P. marinus* suggesting that the physiological requirements of the two pathogens are different (Burreson, 1991a). A continuing effort to develop oysters with increased survival to *P. marinus* using native strains is underway at VIMS but it is unlikely that substantial progress can be made within a few years.

Another strategy to circumvent disease affecting oysters in the Chesapeake Bay would be to introduce another oyster species more resistant and/or tolerant to diseases than *C. virginica.* The proposed introduction of *C. gigas* has been considered by Mann et al. (1991). Arguments that support the proposed introduction of *C. gigas* are based on results from laboratory challenges with *P. marinus* that showed a lower prevalence and intensity of infections and lower oyster mortality in *C. gigas* than in *C. virginica* (Meyers et al. 1991). Proposals to introduce *C. gigas* in the Chesapeake Bay, however, are controversial due to potential parallel introduction of foreign disease agents and/or displacement of local biota, including extant *C. virginica* populations, by *C. gigas* . Nevertheless, carefully controlled introduction of *C. gigas* for research is possible and has recently been effected in Virginia for challenge studies with *H. nelsoni* and *P. marinus* following the proposal of Mann et al. (1991).

Chemotherapy, the focus of this study, is another approach that could be used to control *P. marinus* infections in oysters. Applications of this approach would include treatment of seed and broodstock oysters as well as oysters held in trays or aquaria for use

in aquaculture and research. To date, there have been only two studies that explored the effects of chemotherapeutants on *P. marinus*. At a time when the parasite was classified as a fungus, Ray (1966a) examined the effects of 12 antifungal antibiotics on *P. marinus*-infected oyster tissue. Initially, antifungal agents were directly added to FTM containing pieces of infected tissues. Compounds that markedly inhibited *P. marinus* enlargement at concentrations of 50 mg/L or less were further tested by adding them to autoclaved sea water aliquots containing infected oyster tissues. At weekly intervals, pieces of gill tissue were checked for ciliary activity and then tissues were rinsed in sterile seawater and transferred for incubation in FTM. Among the 12 compounds tested, only cycloheximide and to a lesser extent streptimidone, which is structurally related to cycloheximide, were effective when added to FTM or seawater cultures at concentrations ranging from 10 to 50 mg/L.

The results presented above prompted Ray (1966b) to expose *P. marinus*-infected oysters to cycloheximide baths in seawater of 25 ppt and 25 °C. In a first experiment, oysters were exposed to 1- 50 mg/L cycloheximide baths renewed weekly for periods of 45 to 164 days. In a second experiment, oysters were exposed to a 10 mg/L cycloheximide bath for 30 days (renewed weekly). In the first experiment, prevalence (collectively for all treated oysters), declined from 70% before treatment to 50% after treatment. In the second experiment, weighted prevalence declined from 3.00 before treatment to 1.25 after treatment. In both experiments, survival was greater for treated oysters than for untreated oysters. In the first experiment, there was 100% survival of treated oysters and only 20% survival of untreated oysters after 90 days of treatment. In the second experiment, there was 93% survival of treated oysters and 70% survival of untreated oysters after 28 days of treatment. A trend of increased survival with increasing concentrations and longer exposure time was reported. Furthermore, the intensity of the infections appeared to be lower as treatment time increased. Treatment appeared to arrest mortalities associated with moderate-heavy infections. In addition, infection intensities seemed to be reduced during

treatment, although they may have later relapsed. Conclusions were limited because sample sizes were small (N = 5 in most cases), and no statistical analysis was performed on the data.

Other studies on the effects of chemicals on *P. marinus* include Scott et al. (1985) who found that exposure of infected oysters to a combination of low salinity and chlorine-produced oxidants (CPO) significantly decreased *P. marinus* related mortalities as compared to untreated controls held at low salinities. *In vitro* studies by Goggin et al. (1990) indicate that *Perkinsus* spp. prezoosporangia free of host tissue die within 30 minutes when exposed to chlorine solutions of 6 ppm or higher.

Since the work of Ray in 1966, there have been several developments that allowed for re-examination of the effects of chemotherapeutants on the oyster pathogen *P. marinus*. The referred developments include ultra structural studies of *P. marinus* leading to the present classification of the parasite within the Apicomplexa, extension of the thioglycollate method to diagnose *P. marinus*-infected hemolymph, and the recent formulation of a medium that supports *P. marinus* cultures, as previously mentioned. In addition, anticoccidials of wide use against avian coccidiosis have more recently been recommended for use in fish (Meyer & Schnick 1989).

Criteria to select chemotherapeutants for the present investigation included prior information concerning efficacy against *P. marinus*, efficacy against parasites taxonomically related to *P. marinus*, use on and toxicity to aquatic organisms and food animal species, water solubility, mode of chemical action, availability and cost. Similar criteria have been used before to select chemicals for investigations on control of parasitic diseases (Griffin 1989). It was very difficult to identify compounds that met all or most of the aforementioned criteria, but there was at least one reason to include each compound chosen.

Very little information could be found on the use of antifungals and anticoccidials in marine organisms. Disease control research in freshwater aquaculture has been conducted

for several decades but advances in understanding and control of disease in marine aquaculture is recent, largely within the last 20 years. Reports on chemotherapy of marine organisms are mostly restricted to bacterial diseases affecting finfish, crustaceans, and larval stages of bivalves (Sindermann & Lightner 1988). Moreover, treatments for internal infections in aquatic organisms, including freshwater ones, are not well established (Meyer & Schnick 1989). Therefore, background information for the present study was mostly limited to freshwater or terrestrial organisms.

Considering the new classification of *P. marinus* within the Apicomplexa, and the fact that chemotherapeutants have been widely developed against coccidian parasites in poultry, the following anticoccidial compounds were selected. Amprolium, arprinocid, and sulfadimethoxine were selected because they are fairly safe anticoccidial agents. Sulfadimethoxine has also been used in fish as antibacterial agent. The latter compounds have been without adverse effects even when administered at several times their therapeutic dose in poultry or fish (McDougald 1982, Maestrone et al. 1984). The water solubility of sulfonamide compounds, which include sulfadimethoxine, has made them more useful for treatment of established infections than other less water soluble compounds. The mode of action of sulfadimethoxine, amprolium, and arprinocid is by competitive inhibition of metabolites leading to the formation of vitamins and nucleic acids. Sulfadimethoxine is an antagonist of para-aminobenzoic acid (PABA) which is involved in the formation of amino acids. Amprolium is a partially water soluble analog of the vitamin thiamine, and arprinocid blocks formation of nucleic acids (McDougald 1982, Gutteridge and Coombs 1977). Chemical structures of anticoccidial and antifungal compounds used in this study are given in Appendix A.

Unlike the anticoccidial agents described above, the polyether ionosphores lasalocid and monensin are not water soluble and have a narrow margin of safety. In embryonic chick kidney cell cultures inoculated with *Eimeria tenella* sporozoites, monensin was toxic to kidney cells at concentrations ranging from 100 mg/L to less than 1 mg/L and was active

against *E. tenella* sporozoites at concentrations lower than 0.1 mg/L (Strout & Ouellettte 1973). The LD_{50} for monensin ranges from 2 mg/kg in horses to 185 mg/kg in chickens (McDougald 1982). Despite their narrow margin of safety and their mediocre performance in laboratory tests, polyether antibiotics including lasalocid and monensin, have shown results superior to other compounds when tested under field conditions. Efficacy and toxicity of polyether ionosphorus antibiotics is related to their ability to disrupt the selective permeability of membranes (McDougald 1982, 1990).

Toltrazuril, a synthetic anticoccidial compound developed in Europe, has also shown considerable activity against coccidians, microsporidians, and myxozoan parasites of fish and insects (Melhorn et al. 1988, Schmahl et al. 1989). Toltrazuril, however, could not be used in the present study because it is not available in the U.S. market.

Based on Ray's (1966 a,b) success with cycloheximide, it as well as the other antifungal agents were selected. Cycloheximide and malachite green were included even though they are potentially harmful to human health or non-target organisms (Bower 1989, Meyer & Schnick 1989). They have been successfully used against various aquatic pathogens and therefore constitute good reference standards. In addition to the use against *P. marinus*, cycloheximide has been used to control *Labyrinthuloides haliotidis*, a pathogen of juvenile abalone (Bower, 1989). Bower found that exposure of infected abalone to 1 mg/L of cycloheximide for 5 consecutive days reduced infection prevalence and abalone mortality. No lethal effects on abalone survival were reported after a 10 day exposure to 100 mg/L of cycloheximide.

In vitro exposure of yeasts, fungal pathogens of man, and bacteria to cycloheximide by Whiffen (1948) showed that cycloheximide is highly active against certain yeasts and fungal pathogens. Among the yeasts and fungal pathogens exposed to cycloheximide by Whiffen, *Cryptococcus neoformans*, *Nematospora phaseoli*, *Pichia membranaefasciens*, several species of *Saccharomyces* and other yeasts species were inhibited by concentrations of less than 1 mg/L. None of the tested bacteria were inhibited when exposed to

cycloheximide concentrations as high as 1000 mg/L. Median lethal dose of cycloheximide by intravenous injection ranged from 2.7 mg/kg in rats to 150 mg/kg in mice (Whiffen 1948).

Malachite green was included because it has been the most extensively used external parasiticide of aquatic organisms (Meyer & Schnick, 1989). It is an aryl-methane dye with wide antimicrobial activity against bacteria, fungi, and protozoa. Aryl-methane dyes are particularly active against Gram-negative bacteria, and also against oomycete fungi responsible for saprolegniasis in freshwater fish and other fungal infections in marine crustacea (Alderman 1985). The use of malachite green as an internal chemotherapeutant, however, has not been widely investigated. 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 & Alderman (1987). Acute toxicity of malachite green to freshwater invertebrates, as reported by Bills et al. (1977), varies from 0.51 mg/L - 122 mg/L. Malachite green is a respiratory enzyme poison known to destroy mitochondria (Alderman 1985). 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 therefore not been approved by FDA (Meyer & Schnick 1989).

Despite the fact that malachite green is known to cause adverse effects, it is still used as a reference standard against which new antifungal agents are compared. In an effort to find a substitute for malachite green to treat saprolegnial fungi infecting fish, Bailey and Jeffrey (1989) tested cycloheximide, amphotericin-B, ketoconazole, 5-fluorocytosine, as well as 200 or more other antifungal agents on two species of *Saprolegnia ,in vitro*, and on infections in rainbow trout eggs. Minimum inhibitory concentrations and toxicity to eggs were determined in short term exposures (< 1 hr). Cycloheximide showed moderate antifungal activity *in vitro* but was toxic to eggs at exposures of 25 mg/L. *In vitro* exposures to > 1000 mg/L of amphotericin-B or 300 mg/L of ketoconazole had low antifungal activity. Toxic effects of amphotericin-B and

ketoconazole, however, were not reported. In Bailey and Jeffrey's paper, 5-Fluorocytosine was referred to as being either ineffective, toxic to non-target organisms, or otherwise unsuitable for further testing. No further details were given, however, to ascertain the specific reason for removing 5-Fluorocytosine from further testing. Exposure of infected eggs to 5 mg/L of malachite green was effective without causing acute mortality to eggs.

Amphotericin-B has been effective in treatment of systemic mycosis in humans and domestic animals. Grayson (1982) reported that amphotericin-B was active *in vitro* against pathogenic yeasts at concentrations of 0.01-2.0 mg/L. Its activity against opportunistic, subcutaneous and dermatophytic fungi was less, 3.12-30 mg/L. The mode of action of amphotericin-B is by preferentially binding to sterol components in the membranes of fungi. The LD_{50} in laboratory animals ranges from 4-6 mg/kg by intravenous administration to > 8000 mg/kg by oral administration. Systemic mycosis caused by fungi of the genera *Aspergillus, Blastomyces, Candida, Cryptococcus*, and *Histoplasma* can be controlled by amphotericin-B (Deacon 1984). Similar to amphotericin-B, ketoconazole interferes with membrane function in fungi by blocking the synthesis of ergosterol. The LD_{50} of ketoconazole ranges from 210 mg/kg in rats to > 900 mg/kg in dogs by oral administration (Janssen 1989). 5-fluorocytosine has been used against yeast infections caused by *Candida albicans* and *Cryptococcus neoformans*, but it has little effect against other mycotic fungi. Its mode of action is by interfering with RNA and protein synthesis in fungi. It is almost completely non-toxic to mammals (Deacon 1984, Merck & Co. 1986).

Two other compounds were selected for *in vitro* testing. Fumagillin has shown significant activity on preventing development of *Pleistophora sp.*, a microsporidian parasite of eels (Kano et al. 1982). Cimetidine inhibits gastric acid secretion and is used as an anti-ulcerative (Merck & Co. 1989). It should be noted that arprinocid, lasalocid, monensin, and potentiated sulfadimethoxine are almost insoluble in water. Therefore, the use of a solvent is necessary. Dimethyl sulfoxide (DMSO) has been used successfully to dissolve anticoccidial agents, and, provided that its final concentration was less than 1.0%,

it did not interfere with parasite development or host cell integrity (Ryley and Wilson, 1976). Furthermore, toxicity tests on several species of fish showed that more than 3% of DMSO is required to produce mortality in a 96h test (Willford, 1968).

Methods to search for chemotherapeutants in animals have largely relied on empirical testing. Theoretical models that relate the structure of a chemical to its biological activity have been developed, but their role in drug discovery has been minimal. Therefore, *in vitro* and *in vivo* tests are still the preferred methods for detection of antiparasitic activity (Lichtfield 1958, Schnitzer and Hawking 1963, Ryley and Wilson 1976, Grayson 1982). The objective of *in vitro* and *in vivo* is to detect antiparasitic activity of compounds at levels non toxic to the host. In the long term, effective compounds are expected to produce no side effects on the host and to be purged or removed prior to harvest when the animal is used for human consumption.

Based on Ray's results with antibiotic antifungal agents and considering the new classification of *P. marinus* within the Apicomplexa I hypothesized that antifungal and/or anticoccidial compounds would be active against *P. marinus* and could possibly be used to control established infections in oysters. It was reasonable to expect that anticoccidial compounds would be effective in controlling *P. marinus* infections, since *P. marinus* has recently been classified within the phylum Apicomplexa, which contains coccidians (Levine 1978). Given that several anticoccidial agents have already been approved by the Federal Drug Administration (FDA) for use in poultry, their registration for use in aquaculture would be facilitated (Meyer & Schnick, 1989).

The experimental approach of the present investigation was to test chemicals against *P. marinus*-infected hemolymph and cultured *P. marinus* cells (*in vitro* experiments), and against infections in oysters (*in vivo* experiments). Since there very little information was found on recommended doses of anticoccidial agents for treatment of aquatic organisms (particularly bivalves), the *in vivo* studies included a toxicity experiment to determine possible lethal concentrations to oysters. *Perkinsus* cell cultures and most antifungal agents

only became available towards the end of the investigation. Therefore, initial experiments focused on anticoccidial agents and testing *in vitro* was accomplished on infected hemolymph whereas later experiments focused on antifungal agents using *P. marinus* cultures preferentially for later *in vitro* tests.

METHODS

In vitro experiments

The first *in vitro* experiments consisted of adding hemolymph from *P. marinus*-infected oysters to chemical baths containing 100 mg/L of active ingredients of amprolium, arprinocid, cycloheximide, lasalocid, malachite green, monensin, sulfadimethoxine, and potentiated sulfadimethoxine 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 subsequent experiment. Amphotericin-B was tested in a separate trial at 100 mg/L, 10 mg/L and 1 mg/L.

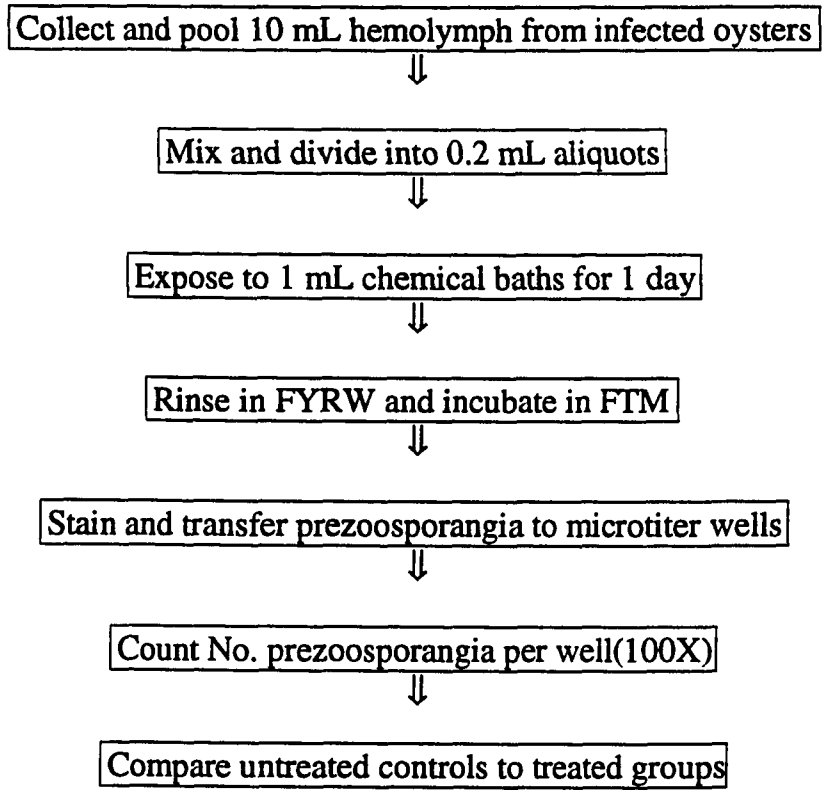
This methodology was based on the hemolymph technique (Gauthier & Fisher 1990), modified to test the effects of chemicals on parasite cells (Table 1). Oyster shells were notched adjacent to the adductor muscle, using a lapidary saw. Infected hemolymph was withdrawn with a syringe from the adductor muscle sinus of at least 10 oysters (selected from a heavily infected group) until a 10 mL stock was obtained. After mixing the stock by vortex, 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 at room temperature (20°C). After exposure to chemical baths, hemolymph aliquots were centrifuged at 265 x g for 15 minutes to concentrate parasite cells in pellets; cell free serum was discarded. The pellets, containing merozoite, meront and schizont stages of the parasite, were further washed once in 0.22 µm-filtered York River water (FYRW) at 23 ppt salinity and resuspended in 1 mL FTM containing 0.05 mL of penicillin and streptomycin (2500 units/mL). The inoculated media was incubated at 25 °C in the dark for 5-7 days, then samples were centrifuged at 265 x 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 stained with 2 drops of Lugol's iodine working solution (Gauthier & Fisher 1990). After staining, the volume of each sample was raised to 1 mL with distilled water and dispensed into separate wells 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 X magnification. Criteria to identify prezoosporangia included size, color, and overall appearance. In general, coccoid cells greater than 6 μm in diameter and/or mostly blue in color were recognized as prezoosporangia.

Chemical baths containing amprolium, sulfadimethoxine, cycloheximide, or malachite green were freshly prepared by dissolving 10 mg of the chemical in 10 mL FYRW at 23 ppt salinity and then serially diluting to the desired concentration. When amphotericin-B, arprinocid, lasalocid, monensin, and potentiated sulfadimethoxine were used, 10 mg of the chemical was first added to 1 mL of dimethylsulfoxide (DMSO), thoroughly mixed using a vortex blender, and then diluted in FYRW at 23 ppt salinity.

The experimental design consisted of triplicate treatments and included an untreated control group, and a solvent control group tested at 1% DMSO (the highest concentration of solvent used). Since Amphotericin-B became available at a later stage, it was tested separately using a new set of solvent and untreated controls. Therefore, results for Amphotericin-B on infected hemolymph are presented separately. A one-way ANOVA was used to examine differences in mean number of cells between treatments. When significant F-ratios were detected, a Dunnett's test was performed to examine the data for differences between individual treatments and controls.

Table 1. Flow chart of methods for *in vitro* experiments with infected hemolymph. Note: In the hemolymph technique (Gauthier & Fisher 1990), hemolymph is withdrawn from individual oysters to assess *P. marinus* infections. In the present technique, hemolymph was pooled from several oysters (> 10) and exposed to treatment prior to incubation in FTM to test for chemical effects.



In addition to experiments in which hemolymph was used as a source for *P. marinus*, other *in vitro* experiments involved testing chemicals on cultured-*P. marinus* cells (Table 2). Stocks of the serologically identified *Perkinsus marinus* isolate; *Perkinsus-1* was supplied by Dr. J. F. La Peyre at VIMS and maintained in 35 cm^2 tissue culture flasks (Corning Glass Works, Corning NY) in 22 ppt JL-ODRP medium at 28°C (La Peyre et al., 1993). Cell density in stock cultures was 5 x 10^6 cells/mL. In all experiments, log-phase cultures were used.

Aliquots containing approximately 10^5 cells were removed from stock cultures with a micropipette and exposed for 1 day to 1 mL chemical solutions in 1.5 mL microcentrifuge tubes. After exposure to chemical baths, treated cells were centrifuged at 265 x g for 15 minutes to concentrate cell pellets and the supernatant was discarded. Pellets were rinsed once in 22 ppt artificial seawater (ASW) and incubated in FTM as in the previous experiment. ASW was prepared by dissolving seawater synthetic basal salt mixture (Sigma Chemical Co.) in distilled water. After incubation, pellets were placed on 24 well tissue culture plates. Prezoosporangia were counted from 3 replicate fields at 100 X in each of three replicate wells. Cell diameter was measured at 320 X on 25 haphazardly selected cells in each replicate.

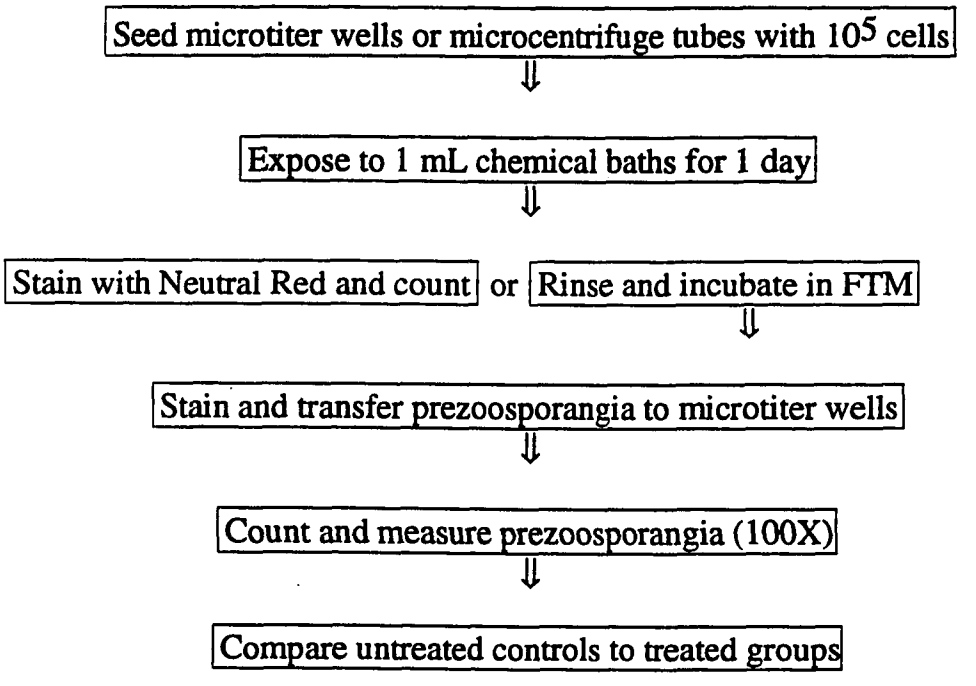
In a separate assay, cells were tested for viability after exposure to chemicals. Instead of using microcentrifuge tubes, cells were seeded directly on 24 well plates and exposed to 1 mL chemical baths for 1 day. Then, 0.10 mL of neutral red stain (0.05%) was added to each well. Neutral red is a vital stain. Live cells uptake the stain while dead cells do not. After 15 minutes, stained and unstained cells were counted at 320 X using an inverted microscope. Counts were made on 2 replicate fields in each of two wells.

The choice of using prezoosporangia abundance and size as endpoints of chemical activity was based on the prior use of inhibition of prezoosporangia enlargement as an indicator of anti-*P. marinus* activity by Ray (1966a). Other possible endpoints such as zoospore formation could have been used on non-cultured cells but development of

zoospores in the laboratory has not been consistently achieved. Viability was selected as an endpoint because the procedure for its determination has been well established and is reliable, and because cytocidal effects could be expected at least from those compounds that disrupt membrane function such as amphotericin-B, lasalocid, and monensin.

Eight chemicals (amphotericin-B, cimetidine, cycloheximide, 5-fluorocytosine, fumagillin, ketoconazole, lasalocid, and monensin) were tested on cultured-*P. marinus* cells. In addition, there was a solvent control (1% DMSO) and a 22 ppt ASW control. All compounds were dissolved in DMSO by dissolving 10 mg of each chemical in 1 mL DMSO and mixing with a vortex. The 22 ppt ASW was added to bring the final volume to 10 mL. The resulting solution was diluted in ASW until the concentration of the chemicals was 100 mg/L and the concentration of the solvent was 1% . Lasalocid and monensin were tested at a later stage than the other compounds. Therefore, results for lasalocid and monensin are presented separately. A list of compounds tested on infected hemolymph (prior experiment) and on cultured cells (this experiment) is provided in appendices B and C.

Table 2. Flow chart of methods for *in vitro* experiments with cultured-*P. marinus* cells.



Toxicity experiment

For this experiment, *P. marinus*-free oysters (70 mm mean shell height, 32 gm mean whole weight) were obtained from the Wye River in Maryland in May 1991. After a 2 week acclimation to 25°C and 17 ppt in 1.0 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 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. Each bath was renewed daily for 4 days. Stocks of chemical solutions were freshly prepared each day by adding 100 mg of active ingredients to 10 mL of DMSO, thoroughly mixing using a vortex blender, and adjusting to the desired concentration by dilution in FYRW. Sulfadimethoxine, a highly water soluble compound, was dissolved directly 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 x 10^8 to 5 x 10^9 cells/L. Chemical solutions were dispensed into aquaria, following the addition of food. 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 (LC_{50}) was estimated by graphic and binomial methods (Gelber et al. 1985).

In vivo experiments

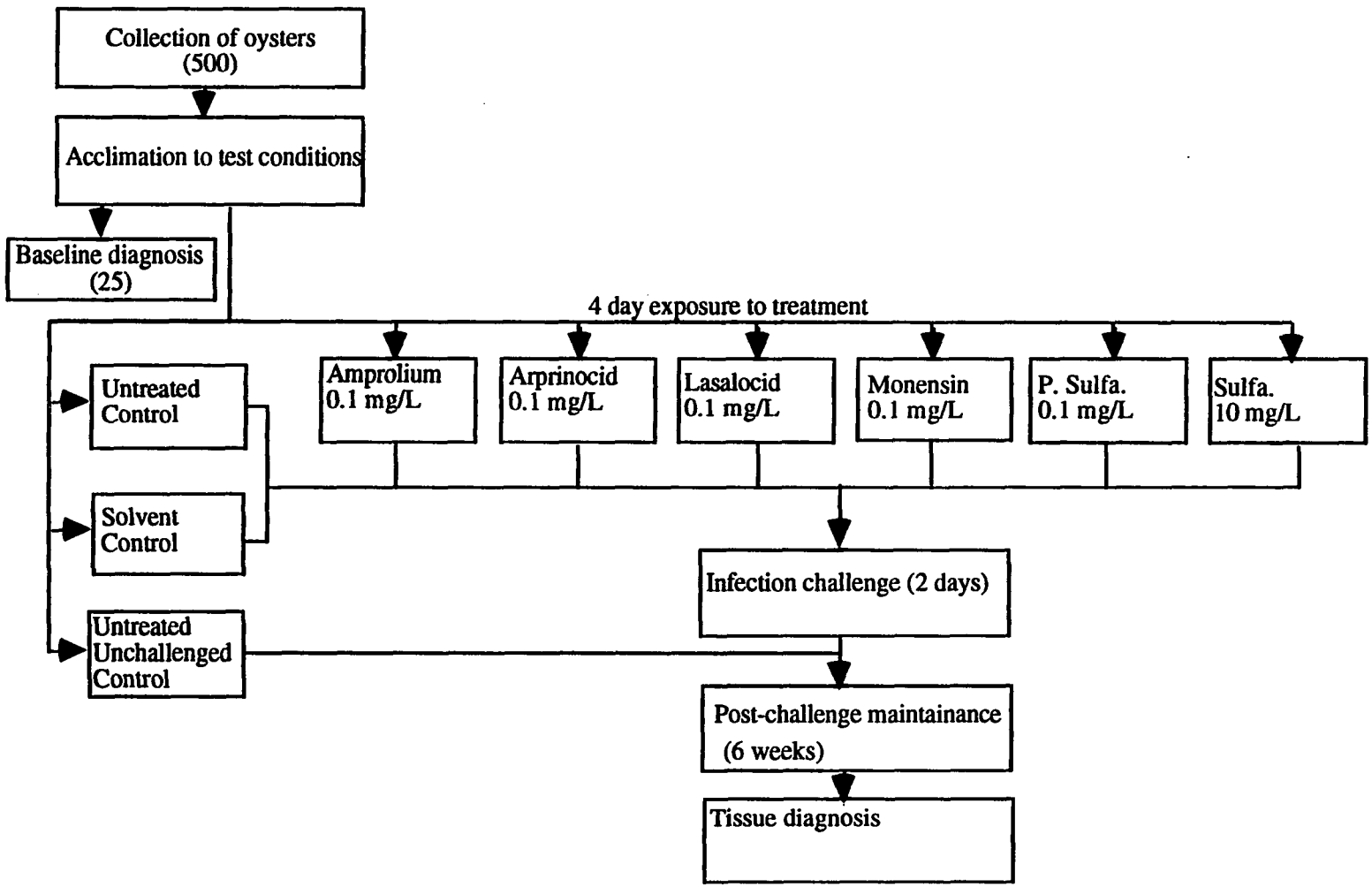
There were 3 experiments designed to determine the effect of chemicals on *P. marinus* infections in oysters. Experiment 1 was designed to determine the effect of 4 day chemical baths on preventing infections (Table 3). Adult oysters (>50 mm shell height) were obtained from the Wye River in Maryland, in August 1991, cleaned of fouling organisms, and acclimated to 25°C and 20 ppt in 1 µm FYRW for one week. Then, FTM tissue diagnosis for *P. marinus* was performed, as previously described, on 25 oysters. The remaining oysters were randomly assigned to 25 L aquaria in groups of 25. Treatment consisted of 4 day static renewal baths of 0.1 mg/L of amprolium, arprinocid, lasalocid, monensin, potentiated sulfadimethoxine, and 10 mg/L of sulfadimethoxine. There were six treatments (one for each chemical) and three controls (untreated unchallenged, untreated challenged, solvent challenged) all tested in duplicate. Chemicals were prepared and added to aquaria, as in the toxicity experiment. In the present experiment, however, amprolium (which is 20% water soluble) was directly dissolved in water without previous dissolution in DMSO. The final concentration of DMSO in exposure media was kept below 0.1%.

Following exposure to chemicals, oysters (N = 25) were challenged to daily consecutive doses of 1×10^7 *P. marinus* cells per 25 L aquarium for 2 consecutive days. To obtain parasite cells for challenge, tissues from 20 oysters (selected from a source showing 100% prevalence), were minced in a blender with 250 mL of 0.22 FYRW and then filtered through a 300 µm, 75 µm, 53 µm, and 20 µm sieve set. Parasite cell abundance was estimated as follows from three 1-mL subsamples taken from the resultant suspension. Each subsample was incubated in 10 mL FTM containing 0.5 mL of penicillin and streptomycin at 25 °C in the dark for 1 day. After incubation, samples were centrifuged at 265 x g for 15 minutes to discard the FTM supernatant. Pellets, containing parasite cells, were washed twice in distilled water and resuspended in 1 mL distilled water. Aliquots of the resulting cell suspensions were then counted using a

hemocytometer, and then the stock suspension was adjusted to yield the desired concentration.

Oysters were fed an algae diet in aerated aquaria as in the previous experiment. Water was maintained at acclimation conditions throughout the experiment and changed once a day. Six weeks after challenge, all oysters were sacrificed and diagnosed for disease as before. Prevalence was calculated by dividing the number of infected oysters by the total number of oysters diagnosed for each treatment replicate. 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 & 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). Where In_i = sum of prevalences for each intensity category, and N = total number of oysters diagnosed. Differences in average prevalence and weighted prevalence between treated and untreated groups were examined by one way ANOVAs.

Table 3. Flowchart of methods for experiment 1. Number of oysters in parenthesis. Treatment groups consisted of 2 replicate aquaria containing 25 oysters each. P.Sulfa.=Potentiated sulfadimethoxine, Sulfa.=Sulfadimethoxine.



Experiment 2 was conducted to determine if amprolium, cycloheximide, malachite green, and/or sulfadimethoxine baths were effective in reducing *P. marinus* infections in oysters (Table 4). 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 1.0 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 & 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 non toxic to oysters, 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 setup to test that compound at 0.1 mg/L and 0.01 mg/L.

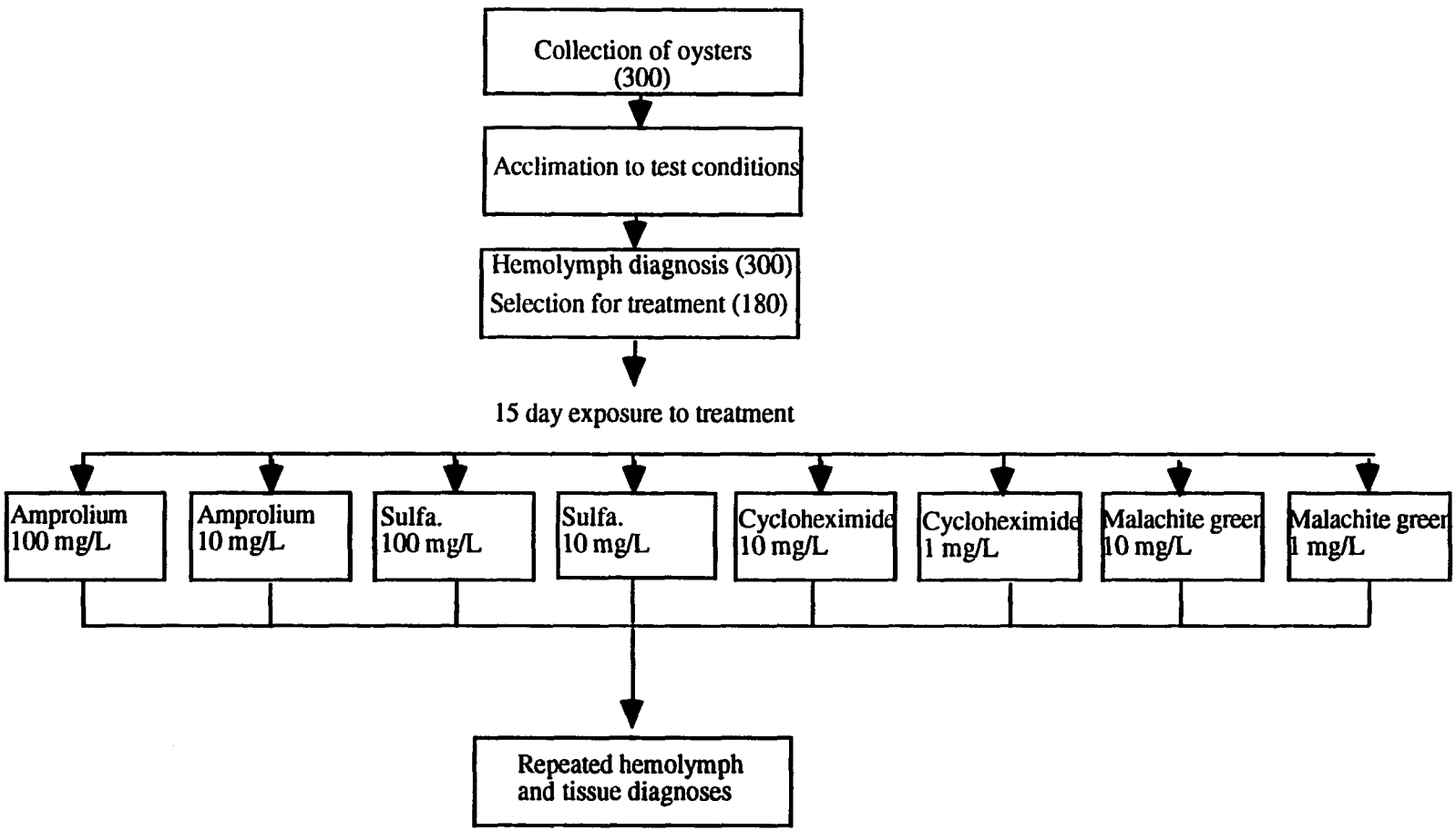
All baths, including untreated controls, were tested in duplicate. Stock solutions of the chemicals were prepared immediately prior to use by adding 10 mg, 100 mg, or 1000 mg of active ingredients to 10 mL of FYRW in 50 mL centrifuge tubes. Thorough mixing was accomplished with a vortex blender. All compounds tested in this experiment were directly 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 1.0 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. Water was changed prior to addition of chemicals. 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* in FTM as previously described. Only tissue diagnosis was performed on oysters exposed to the lower concentrations of malachite green during the follow-up experiment.

Log transformed prezoosporangia count data was examined by a repeated measure ANOVA. If the ANOVA indicated significant differences, 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's of *P. marinus* cells in the hemolymph samples of oysters from selected treatments. Differences in log transformed weighted prevalence between treatments were examined by a one way ANOVA. For all statistical tests alpha was set at 0.05.

Table 4. Flow chart of methods for experiment 2. Number of oysters in parenthesis. Treatment groups consisted of 2 replicate aquaria containing 10 oysters each. Sulfa.= Sulfadimethoxine



In experiment 3, the effect of a 30 day cycloheximide exposure on *P. marinus* infections in oysters was examined. In addition, the subsequent progression of infections was monitored for 30 days after treatment (Table 5). 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 and an ambient salinity of 20 ppt. 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 2, for 30 days. Oysters were fed an algae diet, and aeration was provided, as in previous experiments. Dilution water consisted of 1.0 µm-FYRW with an ambient salinity ranging from 16 to 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.

Statistical Analysis

For statistical analysis of results from *in vitro* and *in vivo* experiments, data were transformed, if necessary, following Gagnon et al. (1989). Prezoosporangia count data and weighted prevalence data were log (x+1) transformed, and prevalence(%) was arcsin transformed. Normality was examined by the Kolmogorov-Smirnov test, and homogeneity of variance by the Cochran and Bartlett/Box tests, using SPSS^X software (SPSS Inc. 1986). Data used for subsequent analysis were normal and homogeneous at a

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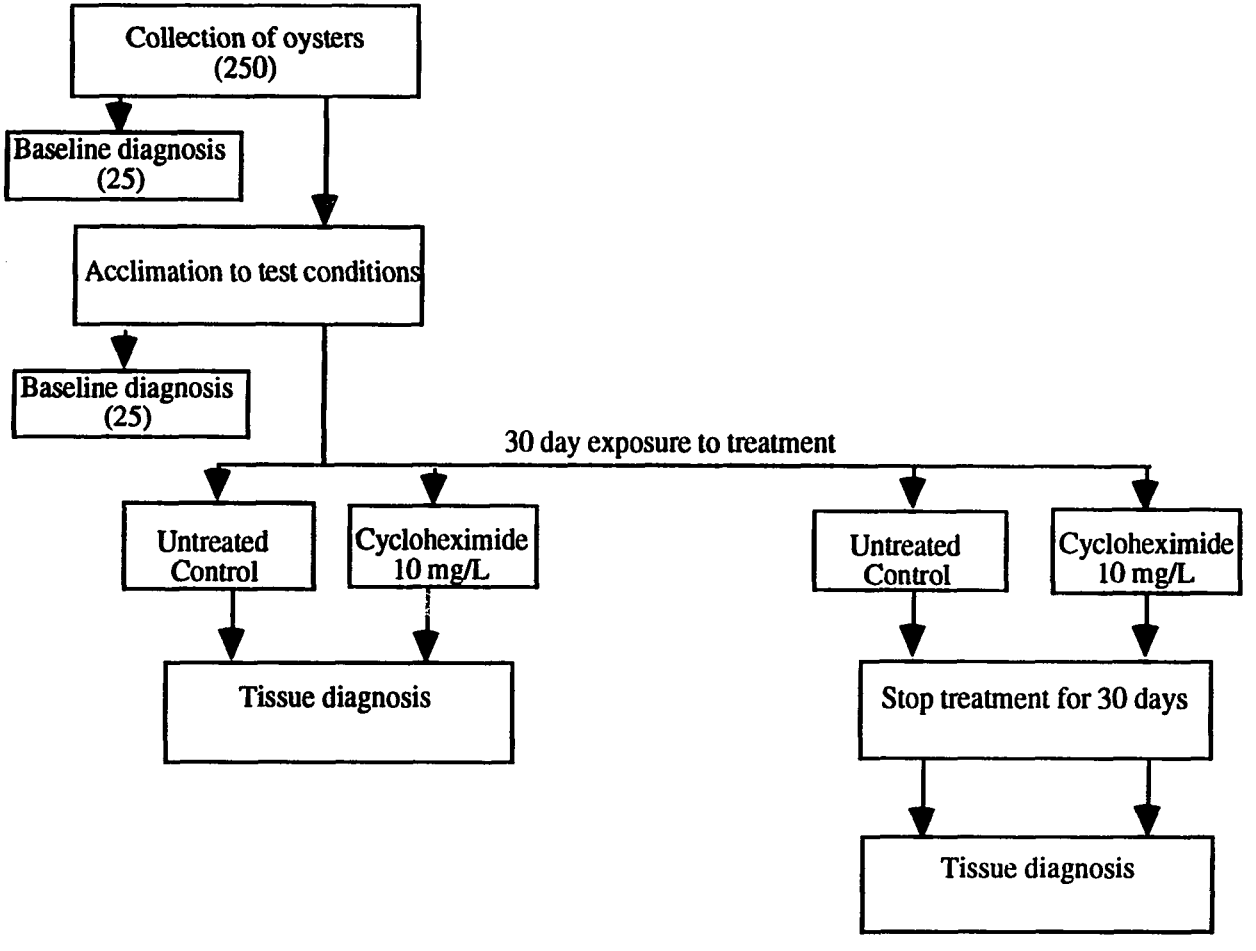
confidence level above 90% (Appendices D and E). ANOVA, Dunnett, and t-tests were performed using SuperAnova software (Gagnon et al. 1989). Only one control group, either untreated control or solvent control, was included in the statistical analysis depending on which gave the more conservative results.

Source of chemicals

Amphotericin-B, cycloheximide, and 5-fluorocytosine were obtained from Sigma Chemical Company, St. Louis, MO; Amprolium from MSD-AGVET, St. Louis, MO; cimetidine from SmithKline Beecham Pharmaceuticals, King of Prussia, PA; ketoconazole from Research Diagnostics Inc., Flanders, NJ; lasalocid, sulfadimethoxine, and potentiated sulfadimethoxine from Roche Vitamins and Fine Chemicals, Nutley, NJ; lasalocid, monensin, and arprinocid from Lilly Research Laboratories, Greenfield, IN; and malachite green from Argent Chemical Laboratories, Redmond, WA.

Table 5. Flow chart of methods for experiment 3. Number of oysters in parenthesis. Treatment groups consisted of 2 replicate aquaria containing 25 oysters each.



RESULTS

In vitro experiments

In experiments using infected hemolymph as a source of *P. marinus* cells, there was a significant (P = 0.000) effect of exposure to 100 mg/L on prezoosporangia abundance. Six chemicals, amphotericin-B, cycloheximide, lasalocid, malachite green, monensin, and sulfadimethoxine, yielded significantly lower ($P < 0.05$) prezoosporangia abundance than the untreated control group or the solvent control group (Fig. 1 and Tables 6 and 7A). Average prezoosporangia abundance decreased from 387 cells in the untreated control group to less than 10 cells in the cycloheximide, lasalocid, and malachite green groups respectively (Table 8); and from 413 cells in the untreated control group to less than 1 cell in the amphotericin-B group (Table 7B).

At 10 mg/L exposure, there was also a significant (P = 0.000) effect of treatment on prezoosporangia abundance (Fig. 2 and Tables 7A and 9). At this concentration, amphotericin-B, lasalocid, and malachite green treatments had significantly ($P < 0.05$) lower prezoosporangia abundance than the untreated control group. Average prezoosporangia abundance decreased from 83 cells in the untreated control group to less than 3 cells in lasalocid and malachite green groups respectively (Table 10) and from 413 cells in the untreated control group to 25 cells in the amphotericin-B group (Table 7B). At concentrations lower than 10 mg/L, the effect of treatment on prezoosporangia abundance was not significant ($P > 0.05$).

Figure 1. Mean abundance of prezoosporangia in infected hemolymph samples after exposure to 100 mg/L chemical treatments. 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. Bars = means of 3 replicate 0.2 mL hemolymph samples. Error bars = standard errors. * = significantly lower ($p < 0.05$) than untreated control.

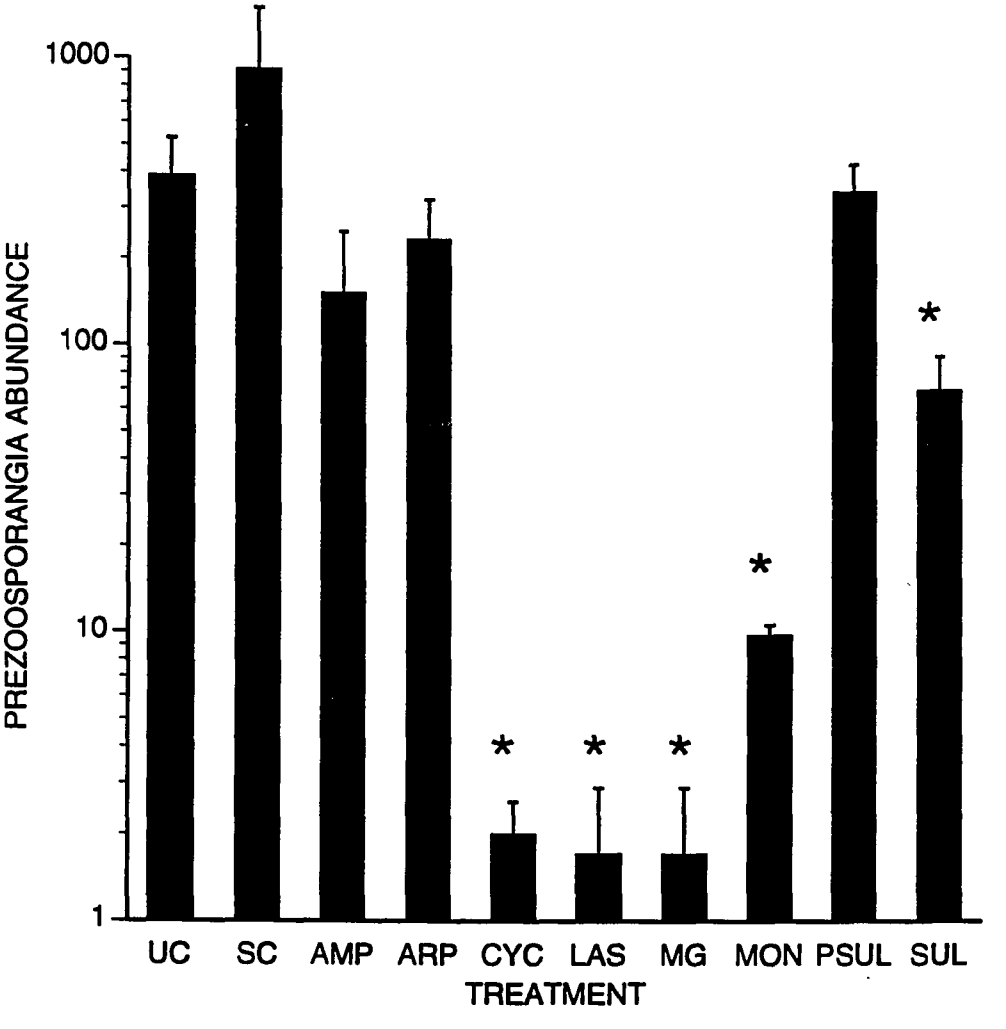

1000
100
10
1
PREZOOSPORANGIA ABUNDANCE
*
*
*
*
*
UC
SC
AMP
ARP
CYC
LAS
MG
MON
PSUL
SUL
TREATMENT

Table 6. One way ANOVA and Dunnett tests describing the effect of 100 mg/L chemical treatments on mean prezoosporangia abundance in infected hemolymph samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	8	21.080	2.635	26.758	0.000
Residual	18	1.773	0.098		

Dunnett's test for mean < control. Critical difference = 0.661.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Lasalocid	-2.200	significant
	Malachite green	-2.200	significant
	Cycloheximide	-2.073	significant
	Monensin	-1.508	significant
	Sulfadimethoxine	-0.724	significant
	Amprolium	-0.599	not significant
	Arprinocid	-0.228	not significant
	P. sulfadimethoxine	-0.031	not significant

Table 7A. One way ANOVA and Dunnett tests describing the effect of amphotericin-B treatments on mean prezoosporangia abundance in infected hemolymph samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	4	14.081	3.520	158.229	0.000
Residual	10	0.222	0.022		

Dunnett's test for mean < control. Critical difference = 0.301.

	COMPARISON	DIFFERENCE	SIGNIFICANCE
Control vs.	Amphotericin-B (100 mg/L)	-2.427	significant
	Amphotericin-B (10 mg/L)	-1.112	significant
	Amphotericin-B (1.0 mg/L)	-2.073	not significant
	Amphotericin-B (0.1 mg/L)	-1.508	not significant

Table 7B. Mean abundance of prezoosporangia in infected hemolymph samples after exposure to amphotericin-B treatments. Values indicate average of three replicate counts per 0.2 mL pooled hemolymph sample. Standard deviation in parenthesis.

	PREZOOSPORANGIA ABUNDANCE	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	413.67 (97.76)	2.61 (0.11)
Solvent control	356.00 (148.01)	2.53 (0.18)
Amphotericin-B (100 mg/L)	0.33 (0.57)	0.10 (0.17)
Amphotericin-B (10 mg/L)	25.33 (5.03)	1.41 (0.08)
Amphotericin-B (1.0 mg/L)	323.67 (115.63)	2.49 (0.16)

Table 8. Mean abundance of prezoosporangia in infected hemolymph samples after exposure to 100 mg/L treatments. Values indicate average of three replicate counts per 0.2 mL pooled hemolymph sample. Standard deviation in parenthesis.

	PREZOOSPORANGIA ABUNDANCE	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	387.00 (241.63)	2.53 (0.27)
Solvent control	902.33 (1012.18)	2.73 (0.56)
Amprolium	150.00 (166.96)	1.93 (0.61)
Arprinocid	231.33 (155.03)	2.30 (0.27)
Cycloheximide	2.00 (1.00)	0.46 (0.15)
Lasalocid	1.67 (2.08)	0.33 (0.35)
Malachite green	1.67 (2.08)	0.33 (0.35)
Monensin	9.67 (1.53)	1.02 (0.06)
P. sulfadimethoxine	339.33 (147.55)	2.50 (0.20)
Sulfadimethoxine	69.33 (37.90)	1.81 (0.22)

Figure 2. Mean abundance of prezoosporangia in infected hemolymph samples after exposure to 10 mg/L chemical treatments. Bars = means of 3 replicate 0.2 mL hemolymph samples. Error bars = standard errors. No error bars shown when replicate samples were equal. * = significantly lower ($P < 0.05$) than untreated control. Abbreviations: UC = untreated control, SC = solvent control, AMP = amprolium, CYC = cycloheximide, LAS = lasalocid, MG = malachite green, MON = monensin, SUL = sulfadimethoxine.

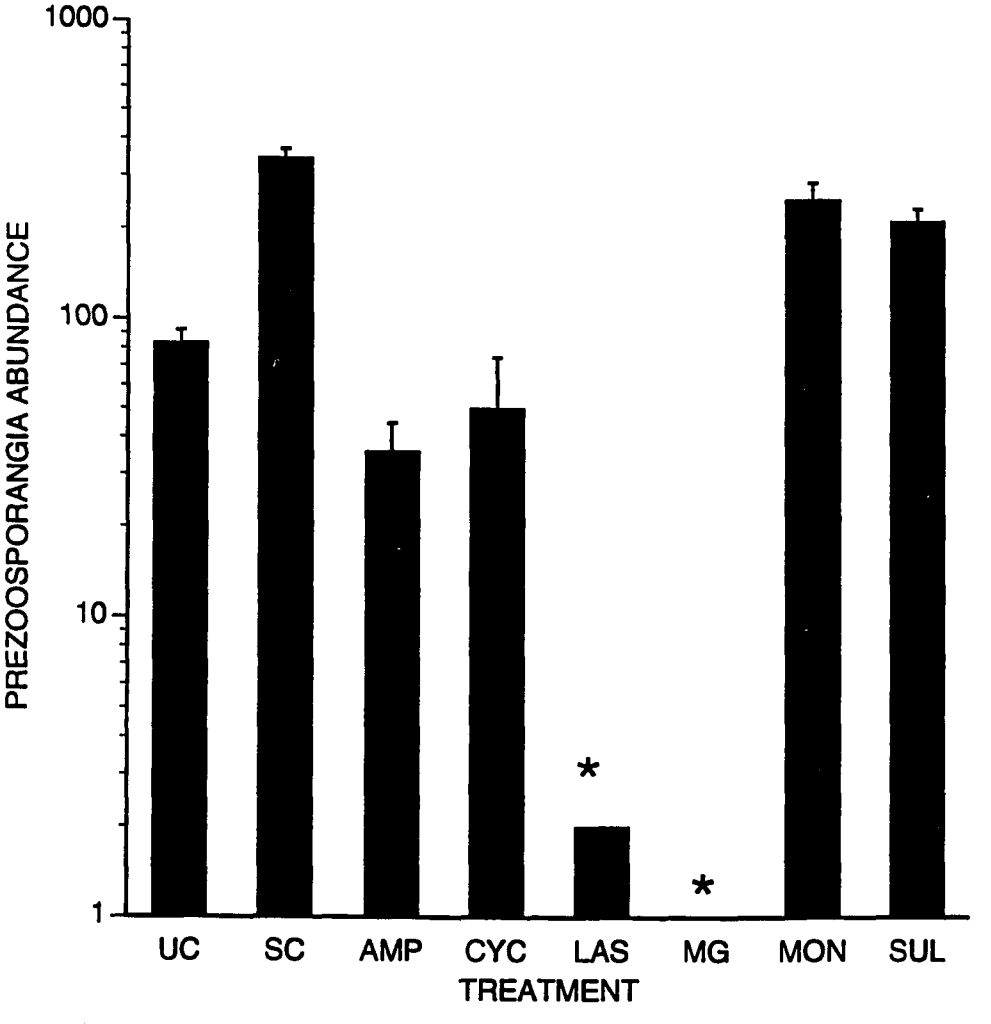

1000
100
10
1
PREZOOSPORANGIA ABUNDANCE
*
*
UC
SC
AMP
CYC
LAS
MG
MON
SUL
TREATMENT

Table 9. One way ANOVA and Dunnett tests describing the effects of 10 mg/L chemical treatments on mean prezoosporangia abundance in infected hemolymph samples. Significance level = 0.05

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	6	14.732	2.455	33.233	0.000
Residual	14	1.034	0.074		

Dunnett's test for mean < control

COMPARISON		DIFFERENCE	CRITICAL DIFFERENCE	SIGNIFICANCE
Control vs.	Malachite green	-1.919	0.562	significant
	Lasalocid	-1.442	0.562	significant
	Cycloheximide	-0.446	0.562	not significant
	Amprolium	-0.381	0.562	not significant
	Sulfadimethoxine	0.400	0.562	not significant
	Monensin	0.468	0.562	not significant

Table 10. Mean abundance of prezoosporangia in infected hemolymph samples after exposure to 10 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L chemical treatments. Values indicate average of three replicate counts per 0.2 mL pooled sample. Standard deviation in parenthesis.

TREATMENT	PREZOOSPORANGIA ABUNDANCE	
10 mg/L EXPOSURE	UNTRANSFORMED	TRANSFORMED
Untreated control	83.00 (15.59)	1.92 (0.09)
Solvent control	341.33 (47.71)	2.53 (0.06)
Amprolium	35.67 (15.82)	1.54 (0.18)
Cycloheximide	50.00 (42.57)	1.47 (0.67)
Lasalocid	2.00 (0.00)	0.48 (0.00)
Malachite green	0.00 (0.00)	0.00 (0.00)
Monensin	248.00 (63.41)	2.39 (0.11)
Sulfadimethoxine	210.00 (40.29)	2.32 (0.08)
1.0 mg/L EXPOSURE		
Amprolium	138.33 (81.38)	2.10 (0.23)
Cycloheximide	100.33 (20.03)	2.00 (0.08)
Lasalocid	393.67 (51.60)	2.59 (0.06)
Malachite green	86.33 (28.94)	1.92 (0.16)
Monensin	297.33 (28.88)	2.47 (0.04)
Sulfadimethoxine	322.67 (48.76)	2.51 (0.06)
0.1 mg/L EXPOSURE		
Amprolium	130.00 (23.64)	2.11 (0.08)
Cycloheximide	127.67 (21.57)	2.10 (0.08)
Lasalocid	350.00 (209.01)	2.48 (0.31)
Malachite green	101.67 (32.19)	2.00 (0.13)
Monensin	270.00 (76.22)	2.42 (0.14)
Sulfadimethoxine	349.00 (106.30)	2.53 (0.14)
0.01 mg/L EXPOSURE		
Amprolium	180.00 (102.81)	2.21 (0.24)
Cycloheximide	126.67 (24.21)	2.10 (0.09)
Lasalocid	269.33 (168.36)	2.34 (0.39)
Malachite green	172.33 (97.68)	2.19 (0.26)
Monensin	399.00 (68.09)	2.60 (0.07)
Sulfadimethoxine	359.33 (105.10)	2.54 (0.14)

In tests with cultured *P. marinus* cells, there was a significant (P = 0.000) effect of treatment on prezoosporangia abundance (Fig. 3 and Table 11). Amphotericin-B and lasalocid treatments had significantly ($P < 0.05$) lower prezoosporangia abundance than the control treatment (Table 11). Average prezoosporangia abundance decreased from 102 cells in the untreated control group to 35 cells in the amphotericin-B group, and from 124 cells in the untreated control group to 2 cells in the lasalocid group (Table 12).

The effect of treatment on size of prezoosporangia was also significant (P = 0.000) (Fig. 4 and Table 13). Again, only cells exposed to amphotericin-B or lasalocid were significantly ($P < 0.05$) smaller in diameter than control cells. Mean diameter of prezoosporangia in the control groups ranged from 7.8 to 10.2 μm. Amphotericin-B treated cells averaged 6.7 μm, and lasalocid treated cells averaged 3.6 μm (Table 14).

There was also a significant (P = 0.000) effect of treatment on percent cell viability (Fig. 5 and Table 15). Among all compounds tested, only amphotericin-B, lasalocid, and monensin significantly ($P < 0.05$) reduced cell viability when compared to the control group. Mean viability of cells in control and amphotericin-B groups was 97% and 79% respectively (Table 16).

For all *in vitro* experiments, abundance and size of prezoosporangia were greater in solvent control groups than in untreated control groups. Cell viability was slightly lower for solvent control groups than for untreated control groups.

Figure 3. Mean abundance of prezoosporangia per sample using cultured *P. marinus* cells after exposure to 100 mg/L chemical treatments. Abbreviations: UC = untreated control, SC = solvent control, AMB = amphotericin-B, CIM = cimetidine, CYC = cycloheximide, FLU = 5-flourocytosine, FUM = fumagillin, KET = ketoconazole. Bars = means of 3 replicate counts. Error bars = standard errors. * = significantly lower ($p < 0.05$) than untreated control.

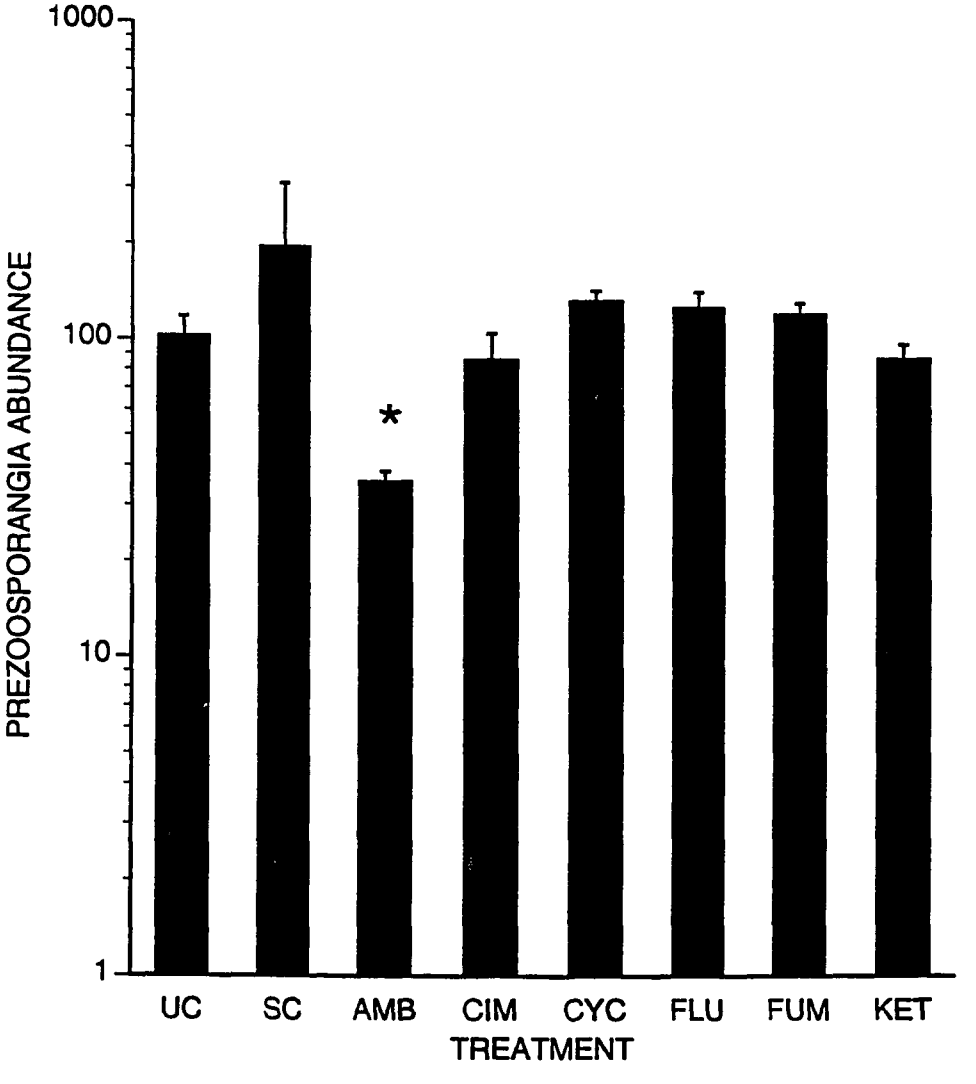

1000
100
10
1
PREZOOSPORANGIA ABUNDANCE
*
UC
SC
AMB
CIM
CYC
FLU
FUM
KET
TREATMENT

Table 11. One way ANOVA and Dunnett tests describing the effect of 100 mg/L chemical treatments on prezoosporangia abundance in cultured *P. marinus* samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	6	58012.825	9668.804	6.817	0.000
Residual	56	79423.111	14818.270		

Dunnett's test for mean < control. Critical difference = 0.184.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Amphotericin-B	-0.414	significant
	Cimetidine	-0.098	not significant
	Ketoconazole	-0.046	not significant
	Fumagillin	0.097	not significant
	Fluorocytosine	0.103	not significant
	Cycloheximide	0.142	not significant

One way ANOVA and Dunnett tests describing the effect of 100 mg/L lasalocid and monensin treatments on prezoosporangia abundance in cultured *P. marinus* samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	3	20.519	6.840	455.367	0.000
Residual	32	0.481	0.015		

Dunnett's test for mean < control. Critical difference = 0.122.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Lasalocid	-1.607	significant
	Monensin	0.169	not significant

Table 12. Mean abundance of prezoosporangia in cultured *P. marinus* samples after exposure to 100 mg/L chemical treatments. Values indicate mean of three replicate counts. Standard deviation in parenthesis.

	PREZOOSPORANGIA ABUNDANCE	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	102.11 (46.70)	1.97 (0.21)
Solvent control	194.89 (341.44)	1.99 (0.46)
Amphotericin-B	35.67 (8.34)	1.55 (0.09)
Cimetidine	86.22 (53.77)	1.87 (0.25)
Cycloheximide	131.33 (30.52)	2.11 (0.10)
Fluorocytosine	124.78 (45.42)	2.07 (0.17)
Fumagillin	119.11 (30.87)	2.07 (0.12)
Ketoconazole	86. 56 (28.97)	1.92 (0.13)

Mean abundance of prezoosporangia in cultured *P. marinus* samples after exposure to 100 mg/L lasalocid and monensin treatments. Values indicate mean of three replicate counts. Standard deviation in parenthesis.

	PREZOOSPORANGIA ABUNDANCE	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	124.67 (26.18)	2.09 (0.08)
Solvent control	183.00 (28.22)	2.26 (0.06)
Lasalocid	2.33 (1.50)	0.48 (0.19)
Monensin	205.56 (46.63)	2.30 (0.11)

Figure 4. Mean size of prezoosporangia in cultured *P. marinus* samples after exposure to 100 mg/L chemical treatments. Abbreviations: UC = untreated control, SC = solvent control, AMB = amphotericin-B, CIM = cimetidine, CYC = cycloheximide, FLU = 5-fluorocytosine, FUM = fumagillin, KET = ketoconazole. Bars = means of 75 measurements. Error bars = standard errors. * = significantly lower ($p < 0.05$) than untreated control.

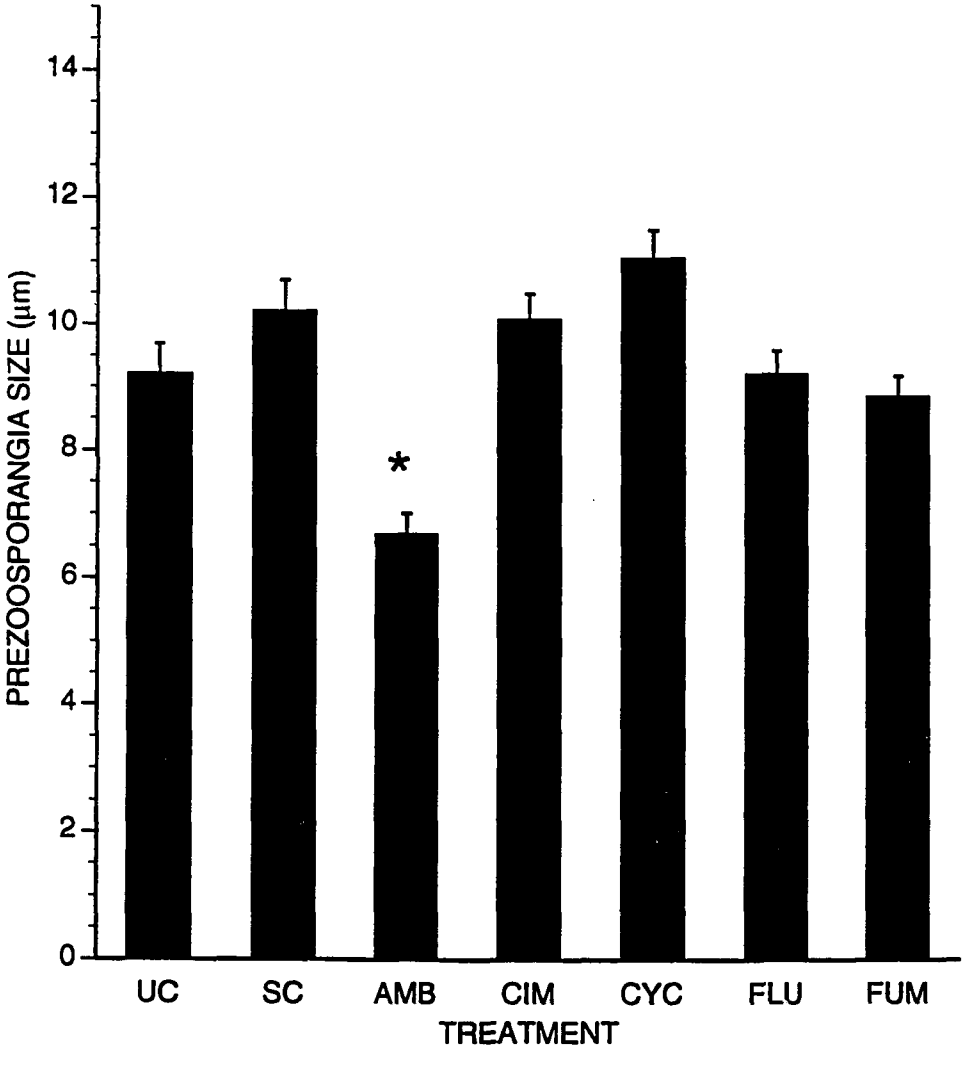

PREZOOSPORANGIA SIZE (µm)
0
2
4
6
8
10
12
14
*
UC
SC
AMB
CIM
CYC
FLU
FUM
TREATMENT

Table 13. One way ANOVA and Dunnett tests describing the effects of 100 mg/L chemical treatments on mean prezoosporangia size in cultured *P. marinus* samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	6	1.953	0.325	12.643	0.000
Residual	518	13.335	0.026		

Dunnett's test for mean < control. Critical difference = 0.060.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Amphotericin-B	-0.107	significant
	Ketoconazole	-0.037	not significant
	Fumagillin	0.011	not significant
	Fluorocytosine	0.023	not significant
	Cimetidine	0.056	not significant
	Cycloheximide	0.098	not significant

One way ANOVA and Dunnett tests describing the effects of 100 mg/L lasalocid and monensin treatment on mean prezoosporangia size in cultured *P. marinus* samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	3	2.145	0.715	45.545	0.000
Residual	251	3.941	0.016		

Dunnett's test for mean < control. Critical difference = 0.056.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Lasalocid	-0.279	significant
	Monensin	-0.005	not significant

Table 14. Mean size of prezoosporangia in cultured *P. marinus* samples after exposure to 100 mg/L treatments. Values indicate mean of 75 measurements Standard deviation in parenthesis.

	PREZOOSPORANGIA DIAMETER (µm)	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	9.20 (4.25)	0.96 (0.21)
Solvent control	10.20 (4.36)	1.01 (0.21)
Amphotericin-B	6.68 (2.86)	0.85 (0.16)
Cimetidine	10.08 (3.58)	1.02 (0.16)
Cycloheximide	11.04 (3.92)	1.06 (0.13)
Fluorocytosine	9.20 (3.32)	0.98 (0.14)
Fumagillin	8.84 (2.87)	0.97 (0.13)
Ketoconazole	8.04 (3.39)	0.93 (0.21)

Mean size of prezoosporangia in cultured *P. marinus* samples after exposure to 100 mg/L lasalocid and monensin treatments. Values indicate mean of 75 measurements. Standard deviation in parenthesis.

	PREZOOSPORANGIA DIAMETER (µm)	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	7.88 (2.74)	0.93 (0.12)
Solvent control	8.12 (2.35)	0.95 (0.11)
Lasalocid	3.60 (1.22)	0.65 (0.10)
Monensin	8.44 (9.72)	0.92 (0.15)

Figure 5. Mean percent cell viability in cultured *P. marinus* samples after exposure to 100 mg/L treatments. Abbreviations: UC = untreated control, SC = solvent control, AMB = amphotericin-B, CIM = cimetidine, CYC = cycloheximide, FLU = 5-fluorocytosine, FUM = fumagillin, KET = ketoconazole. Bars = means of 2 replicate counts. Error bars = standard errors. * = significantly lower ($p < 0.05$) than untreated control.

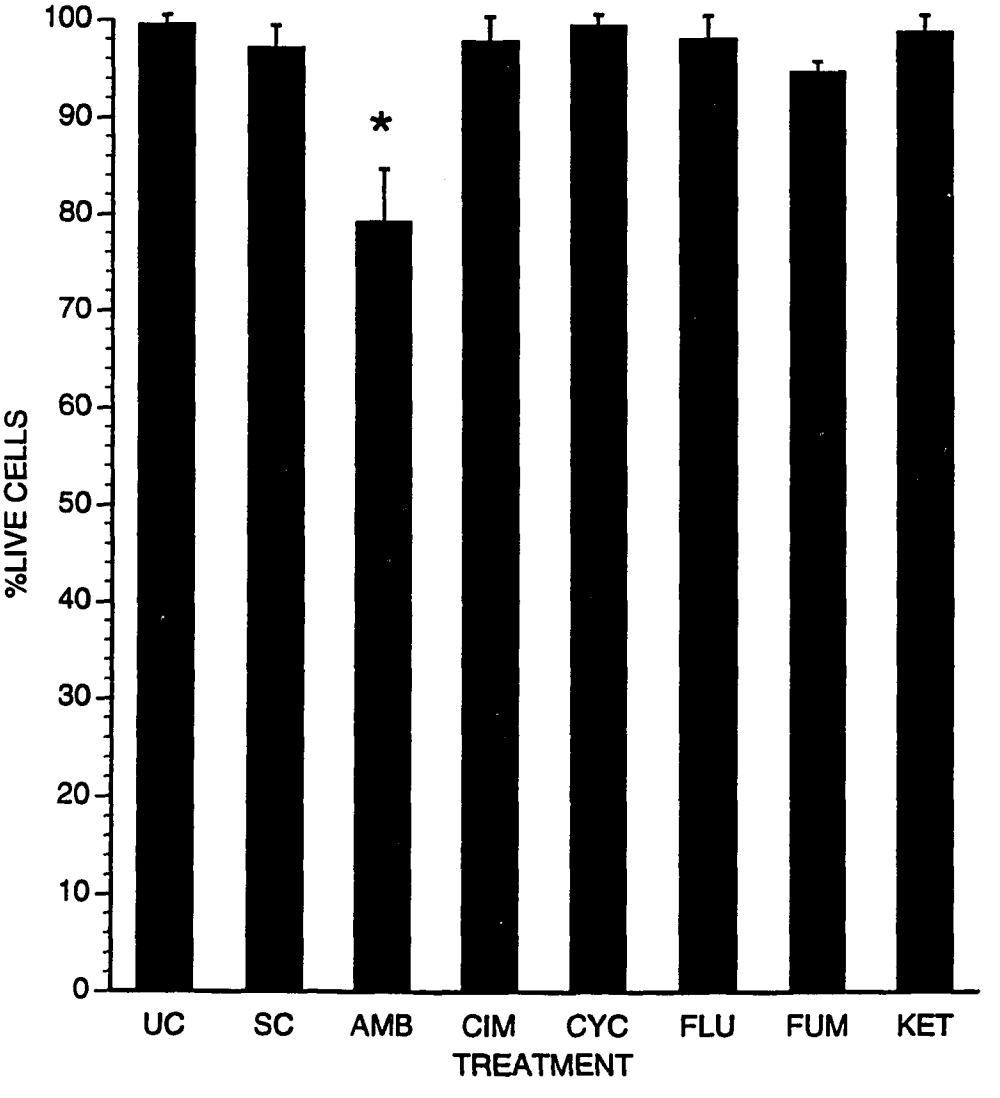

100
90
80
70
60
50
40
30
20
10
0
%LIVE CELLS
*
UC
SC
AMB
CIM
CYC
FLU
FUM
KET
TREATMENT

Table 15. One way ANOVA and Dunnett tests describing the effects of 100 mg/L chemical treatments on percent cell viability in cultured *P. marinus* samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	6	0.520	0.087	10.147	0.000
Residual	21	0.179	0.009		

Dunnett's test for mean < control. Critical difference = 0.160.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Amphotericin-B	-0.325	significant
	Fumagillin	-0.087	not significant
	Cimetidine	0.041	not significant
	Fluorocytosine	0.045	not significant
	Ketoconazole	0.070	not significant
	Cycloheximide	0.107	not significant

One way ANOVA and Dunnett tests describing the effects of 100 mg/L lasalocid and monensin treatments on percent cell viability in cultured *P. marinus* samples. Significance level = 0.05.

One way analysis of variance

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	2	6.580	3.290	1.707×10^{19}	0.000
Residual	9	1.735×10^{-18}	1.927×10^{-19}		

Dunnett's test for mean < control. Critical difference = 6.768×10^{-10}.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Lasalocid	-1.571	significant
	Monensin	-1.571	significant

Table 16. Mean percent viability in cultured *P. marinus* samples after exposure to 100 mg/L chemical treatments. Values indicate average of 2 replicates. Standard deviation in parenthesis.

	PERCENT VIABILITY	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	99.50 (1.00)	1.53 (0.07)
Solvent control	97.12 (2.31)	1.42 (0.10)
Amphotericin-B	79.20 (5.55)	1.10 (0.70)
Cimetidine	97.84 (2.54)	1.47 (0.12)
Cycloheximide	99.39 (1.22)	1.53 (0.78)
Fluorocytosine	97.98 (2.44)	1.47 (0.12)
Fumagillin	94.63 (1.14)	1.34 (0.03)
Ketoconazole	98.77 (1.78)	1.49 (0.09)

Mean percent viability in cultured *P. marinus* samples after exposure to 100 mg/L lasalocid and monensin treatments. Values indicate average of 2 replicates. Standard deviation in parenthesis.

	PERCENT VIABILITY	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated control	100 (0.00)	1.57 (0.00)
Solvent control	100.00 (0.00)	1.57 (0.00)
Lasalocid	0.00 (0.00)	0.00 (0.00)
Monensin	0.00 (0.00)	0.00 (0.00)

Toxicity experiment

Oysters from the baseline diagnostic sample were all negative for *P. marinus*. All solvent control oysters survived 96 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 (Table 17A). Exposure to 1 mg/L and 10 mg/L of monensin for 96 hours resulted in 10 % and 40 % mortality respectively (Table 17B).

In vivo experiments

At the start of experiment 1, more than half the oysters had light infections (prevalence = 56%, weighted prevalence = 0.76). By the end of the experiment, prevalence had increased to more than 80% for treatments and controls alike (Table 18). There was no significant effect of treatment on prevalence or weighted prevalence $P = 0.369$, $P = 0.632$ respectively (Tables 18 and 19). Comparison of solvent challenged control groups and untreated challenged control groups did not reveal any effect of solvent on prevalence or weighted prevalence. However, prevalence and weighted prevalence were lower for challenged controls than for unchallenged controls (Tables 18 and 19).

At the start of experiment 2, 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. 6 and Table 20). 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 = 0.015) effect of treatment on prezoosporangia abundance averaged over time. The effect of time alone, however, averaged over treatment, was not significant (P = 0.867). Nevertheless, the pattern of change over time was different for different treatments, as indicated by the significant (P = 0.000) effect of the interaction term (Table 21). 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.

No oysters survived 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 3, 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 and 20 ppt, prevalence had risen 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. 7 and 8). At that time, oysters exposed to cycloheximide had fewer and lighter infections than untreated control oysters. The reduction in weighted prevalence between treated oysters and untreated control oysters after the treatment period, in this experiment, was similar to that in the previous experiment (i.e., a 1.50 decline as compared to a 1.68 decline respectively). The effects of treatment on prevalence and weighted prevalence were both significant (P = 0.000 and P = 0.001 respectively) (Tables 22 and 23) . There was no significant (P = 0.235) effect of time on prevalence (prevalence was already at the maximum at 30 days in controls), but there was a significant (P = 0.014) effect of time on weighted prevalence as it increased both in

control and treated oysters after treatment was halted (Fig. 8 and Table 23). The interaction effects of treatment and time on prevalence and weighted prevalence were not significant ($P = 0.235$ and $P = 0.912$ 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.

Table 17A. Toxicity of lasalocid to oysters

Nominal concentration of exposure (mg/L)	Mortality (%)			
	24 hr.	48 hr.	72 hr.	96 hr.
0.00	0	0	0	0
0.01	0	0	0	0
0.10	0	0	0	0
1.00	50	70	70	70
10.00	70	90	100	100

Table 17B. Toxicity of monensin to oysters

Nominal concentration of exposure (mg/L)	Mortality (%)			
	24 hr.	48 hr.	72 hr.	96 hr.
0.00	0	0	0	0
0.01	0	0	0	0
0.10	0	0	0	0
1.00	0	0	10	10
10.00	0	0	30	40

Table 18. *In vivo* experiment 1. Mean prevalence and one way ANOVA describing the effect of chemical treatment on prevalence.

Prevalence after exposure of oysters to amprolium, arprinocid, lasalocid, monensin, potentiated sulfadimethoxine, and sulfadimethoxine. Values indicate means of two replicates. Standard deviations in parenthesis.

	PREVALENCE(%)	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated unchallenged	96 (0.00)	1.37 (0.00)
Untreated challenged	88 (5.66)	1.22 (0.09)
Solvent challenged	94 (2.83)	1.33 (0.06)
Amprolium	96 (5.66)	1.43 (0.20)
Arprinocid	94 (2.83)	1.33 (0.06)
Lasalocid	100 (0.00)	1.57 (0.00)
Monensin	80 (22.63)	1.15 (0.31)
Potentiated sulfadimethoxine	92 (0.00)	1.28 (0.00)
Sulfadimethoxine	94 (8.45)	1.39 (0.25)

One way ANOVA on transformed prevalence

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	6	0.236	0.039	1.292	0.369
Residual	7	0.213	0.030		

Table 19. *In vivo* experiment 1. Mean weighted prevalence and one way ANOVA describing the effect of chemical treatment on weighted prevalence.

Weighted prevalence after exposure of oysters to amprolium, arprinocid, lasalocid, monensin, potentiated sulfadimethoxine, and sulfadimethoxine. Values indicate means of two replicates. Standard deviations in parenthesis.

	WEIGHTED PREVALENCE	
TREATMENT	UNTRANSFORMED	TRANSFORMED
Untreated unchallenged	2.80 (0.79)	0.57 (0.09)
Untreated challenged	1.84 (0.17)	0.45 (0.03)
Solvent challenged	1.82 (0.24)	0.45 (0.02)
Amprolium	2.80 (0.62)	0.58 (0.07)
Arprinocid	2.22 (0.71)	0.50 (0.10)
Lasalocid	2.94 (1.27)	0.58 (0.14)
Monensin	1.92 (0.91)	0.45 (0.14)
Potentiated sulfadimethoxine	1.76 (0.28)	0.44 (0.04)
Sulfadimethoxine	2.76 (1.19)	0.56 (0.14)

One way ANOVA on transformed weighted prevalence

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	6	0.048	0.008	0.745	0.632
Residual	7	0.075	0.011		

Figure 6. *In vivo* experiment 2. Mean abundance of prezoosporangia in repeated hemolymph samples, before and after a 15 day exposure of infected oysters to chemical treatments. Abbreviations: UC = untreated control, AMP = amprolium, CYC = cycloheximide, SUL = sulfadimethoxine. Numbers following acronyms indicate concentration of exposure in mg/L. Note: One sample of the CYC1 group had more than 100,000 cells after treatment and was excluded from this chart. Bars = means per 0.3 mL hemolymph samples averaged over 16-20 oysters. Dark bars indicate pre-treatment values. Light bars indicate post-treatment values. Error bars = standard errors. * = final value significantly lower ($p < 0.05$) than initial value, and overall value significantly lower ($p < 0.05$) than untreated control.

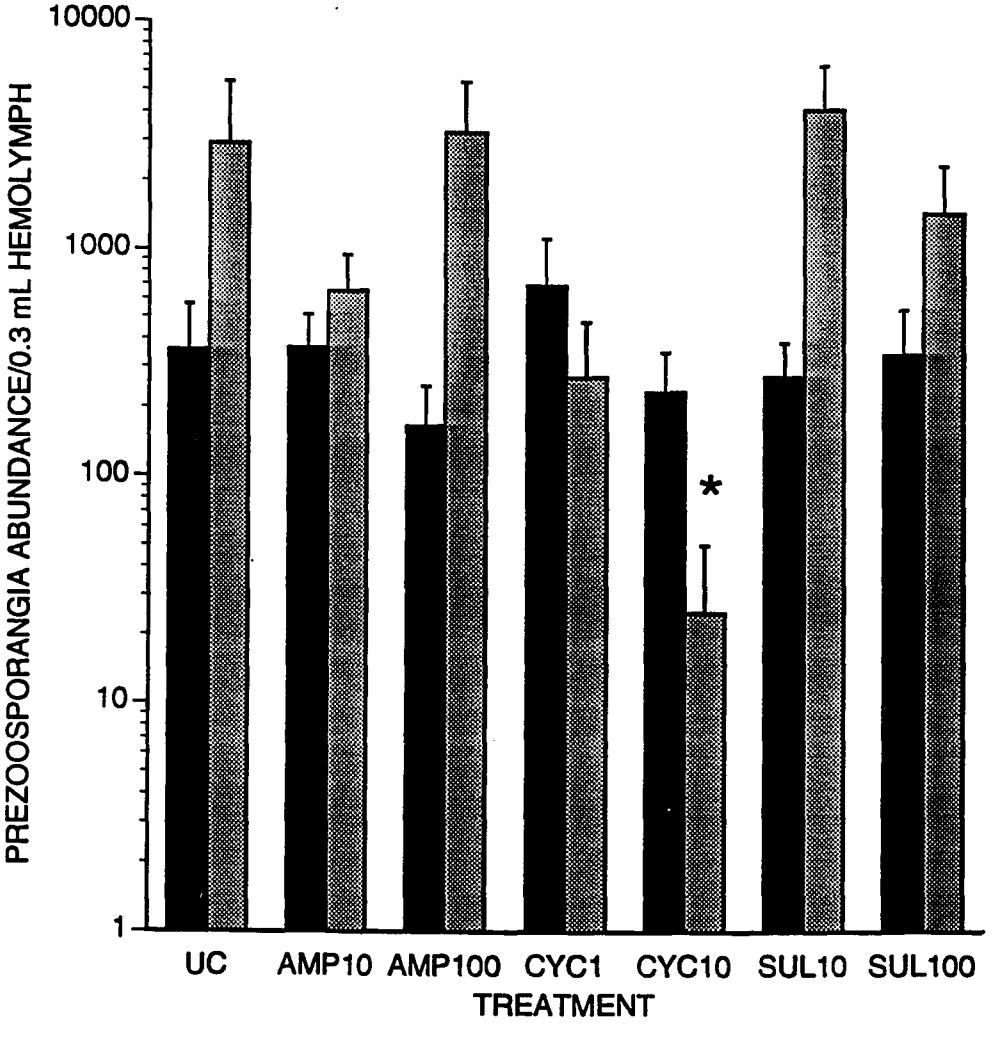

10000
1000
100
10
1
PREZOOSPORANGIA ABUNDANCE/0.3 mL HEMOLYMPH
*
UC
AMP10
AMP100
CYC1
CYC10
SUL10
SUL100
TREATMENT

Table 20. *In vivo* experiment 2. Mean abundance of prezoosporangia in repeated hemolymph samples before and after exposure of oysters to amprolium, cycloheximide, and sulfadimethoxine treatment. Values indicate means per 0.3 mL hemolymph samples averaged over 16-20 oysters. Standard deviation in parenthesis.

	PREZOOSPORANGIA ABUNDANCE			
	UNTRANSFORMED		TRANSFORMED	
TREATMENT	INITIAL	FINAL	INITIAL	FINAL
Untreated control	358 (893)	2913 (10630)	1.57 (1.00)	1.75 (1.34)
Amprolium 10	364 (580)	648 (1173)	1.66 (1.05)	2.15 (1.04)
Amprolium 100	165 (357)	3233 (9323)	1.32 (0.89)	2.38 (1.15)
Cycloheximide 1	788 (1835)	5769 (24605)	1.88 (1.02)	1.05 (1.44)
Cycloheximide 10	233 (519)	25 (111)	1.38 (0.98)	0.22 (0.61)
Sulfadimethoxine 10	272 (495)	4080 (10096)	1.52 (1.00)	1.78 (1.41)
Sulfadimethoxine 100	341 (811)	1440 (3627)	1.62 (0.89)	1.73 (1.25)

Table 21. *In vivo* experiment 2. Repeated measures ANOVA, Dunnett and t-tests describing the effects of chemical treatments on prezoosporangia abundance in hemolymph samples. Significance level = 0.05.

Repeated measure ANOVA

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
TREATMENT	6	31.442	5.240	2.756	0.015
SUBJECT(GROUP)	123	233.901	1.902		
TIME	1	0.015	0.015	0.028	0.867
TIME*TREATMENT	6	34.151	5.692	10.953	0.000
TIME*SUBJECT(GROUP)	123	63.918	0.520		

Dunnett's test for mean < control. Critical difference = 0.735.

COMPARISON		DIFFERENCE	SIGNIFICANCE
Control vs.	Cycloheximide 10	-0.861	significant
	Cycloheximide 1	-0.201	not significant
	Sulfadimethoxine 10	-0.011	not significant
	Sulfadimethoxine 100	0.011	not significant
	Amprolium 100	0.185	not significant
	Amprolium 10	0.240	not significant

Paired t-test for selected treatments. Hypothesized difference < or = 0.

TREATMENT	MEAN DIFFERENCE	DF	t-VALUE	P-VALUE
Cycloheximide 10	1.167	19	5.045	0.000
Cycloheximide 1	0.777	19	2.944	0.004

Figure 7. *In vivo* experiment 3. Prevalence after a 30 day exposure of oysters to cycloheximide, and prevalence 30 days after stopping exposure. 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.

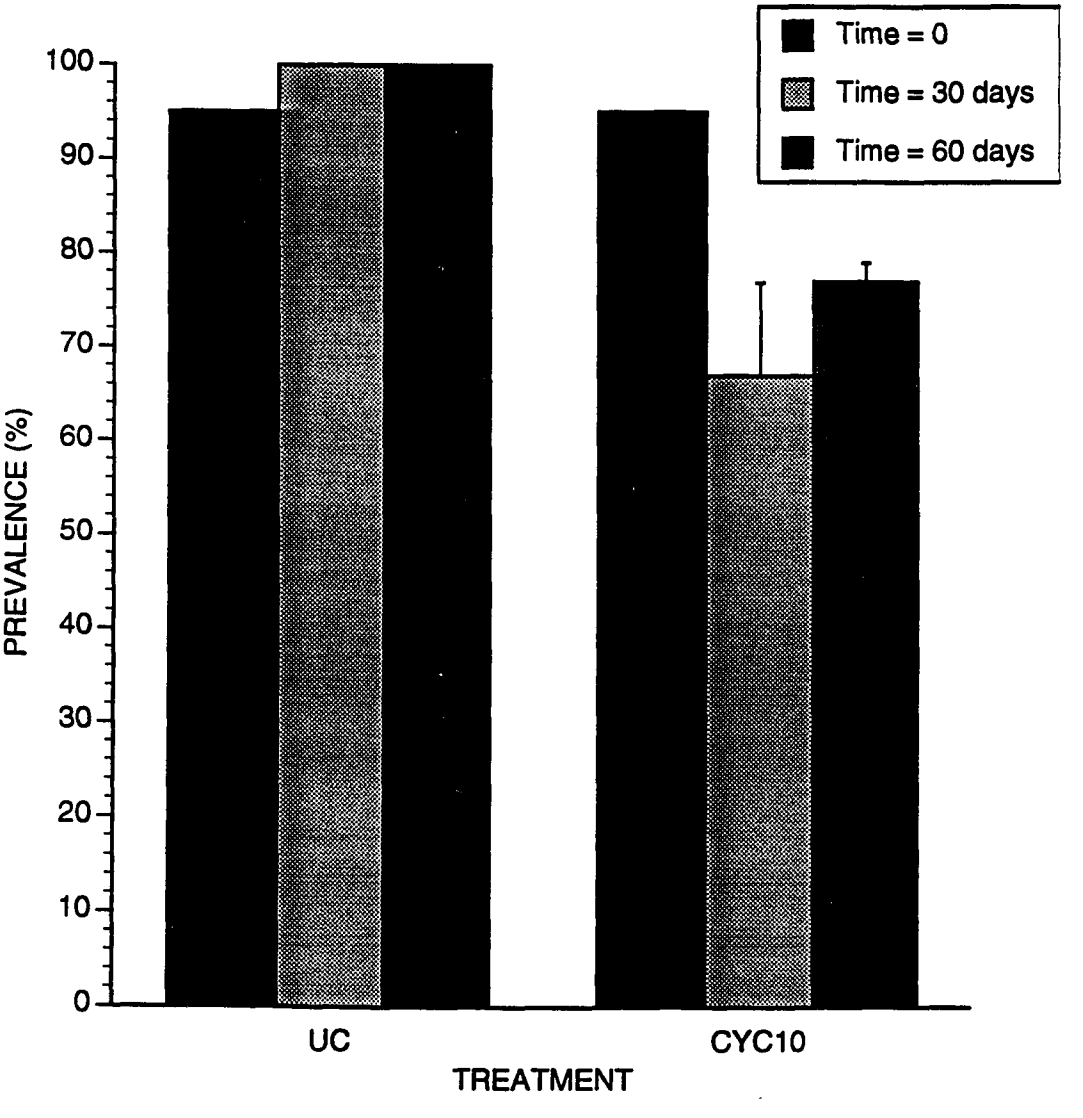

Time = 0
Time = 30 days
Time = 60 days
100
90
80
70
60
50
40
30
20
10
0
PREVALENCE (%)
UC
CYC10
TREATMENT

Figure 8. *In vivo* experiment 3. Weighted prevalence after a 30 day exposure of oysters to cycloheximide, and weighted prevalence 30 days after stopping exposure. Abbreviations: 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.

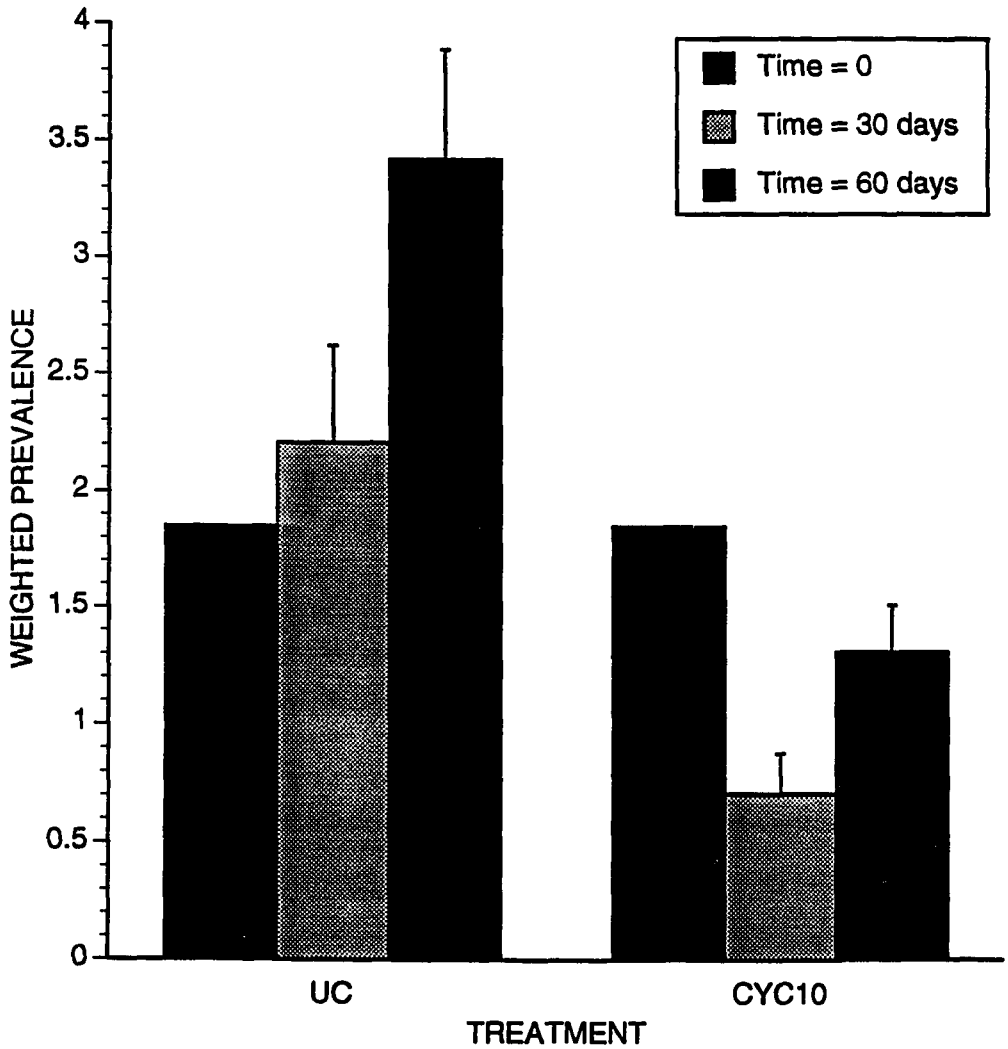

Time = 0
Time = 30 days
Time = 60 days
WEIGHTED PREVALENCE
4
3.5
3
2.5
2
1.5
1
0.5
0
UC
CYC10
TREATMENT

Table 22. *In vivo* experiment 3. Mean prevalence and two-way ANOVA describing the effects of cycloheximide treatment and time on prevalence.

Mean prevalence before and after exposure of oysters to cycloheximide. Values indicate mean of two replicates. Standard deviation in parenthesis.

TREATMENT	PREVALENCE (%)		TRANSFORMED PREVALENCE	
	time = 30 days	time = 60 days	time = 30 days	time = 60 days
Untreated control	100.00 (0.00)	100.00 (0.00)	1.571 (0.00)	1.571 (0.00)
Cycloheximide 10	72.00 (8.29)	77.00 (2.83)	0.961 (0.11)	1.071 (0.03)

Two way ANOVA on transformed prevalence

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	1	0.616	0.616	198.694	0.000
Time	1	0.006	0.006	1.951	0.235
Treatment*Time	1	0.006	0.006	1.951	0.235
Residual	4	0.012	0.003		

Table 23. *In vivo* experiment 3. Weighted prevalence and two-way ANOVA describing the effects of cycloheximide treatment and time on weighted prevalence.

Mean weighted prevalence before and after exposure of oysters to cycloheximide. Values indicate mean of two replicates. Standard deviation in parenthesis.

TREATMENT	WEIGHTED PREVALENCE		TRANSFORMED WEIGHTED PREVALENCE	
	time = 30 days	time = 60 days	time = 30 days	time = 60 days
Untreated control	2.21 (0.41)	3.42 (0.47)	0.51 (0.06)	0.64 (0.05)
Cycloheximide 10	0.71 (0.17)	1.31 (0.20)	0.23 (0.04)	0.36 (0.04)

Two way ANOVA on transformed weighted prevalence

SOURCE	DF	SS	MS	F-VALUE	P-VALUE
Treatment	1	0.153	0.153	71.583	0.001
Time	1	0.037	0.037	17.204	0.014
Treatment*Time	1	2.9×10^{-5}	2.9×10^{-5}	0.0141	0.912
Residual	4	0.009	0.002		

DISCUSSION

In vitro studies

Among the nine compounds tested on *P. marinus*-infected hemolymph, six (i.e., amphotericin-B, cycloheximide, lasalocid, malachite green, monensin, and sulfadimethoxine) showed efficacy in inhibiting *P. marinus* cells from enlarging in FTM. Malachite green, amphotericin-B, and lasalocid, followed by cycloheximide, were the most effective compounds. The concentrations of malachite green active against *P. marinus*-infected hemolymph (100 mg/L to 10 mg/L) in the present study were higher than the range of 2 mg/L to 4 mg/L reported for effective concentrations against the model protozoan *Tetrahymena pyriformis* (Griffin 1989).

The results for cycloheximide were similar to those obtained by Ray (1966a). In Ray's study, exposure of infected oyster tissue to 25 mg/L of cycloheximide for several weeks completely inhibited parasite enlargement. In the present study, exposure of infected oyster hemolymph to 100 mg/L for 1 day significantly inhibited the number of *P. marinus* cells enlarging in FTM, and exposure to 10 mg/L resulted in some inhibition. Further comparison of results is not possible because Ray used pieces of infected tissues and described the degree of inhibition only as slight, marked, or complete.

Among the eight compounds tested using cultured *P. marinus* cells, only two (i.e., amphotericin-B and lasalocid showed efficacy in inhibiting the number and size of cells enlarging to prezoosporangia stage. In addition to the above compounds, monensin reduced cell viability.

Four chemotherapeutants were tested both on *P. marinus*-infected hemolymph and cultured *P. marinus* cells. Three of them, amphotericin-B, lasalocid and monensin, had similar effects on parasites regardless of their source. Cycloheximide, however, was effective against parasites from infected hemolymph but not against cultured forms. The

lack of detectable activity of cycloheximide on cultured parasites may be related to the presence of nutrients in the culture medium. It is possible that cultured cells eluded the adverse effects of cycloheximide on growth and viability by relying on amino acids present in the culture medium (La Peyre et al. 1993). Composition of culture media can alter not only susceptibility of parasites to chemotherapeutants but also their temperature tolerance, protein profile, and virulence (Cline et al. 1983, Marciano-Cabral & Toney 1994). Alternatively, effects of cycloheximide on cultured cells may have occurred but were not detectable by the assay used. An assay based on amino acid incorporation into cultured cells would be more specific and sensitive for the determination of activity with cycloheximide than the one used in the present study based on enlargement or viability.

The concentrations of exposure of amphotericin-B active against *P. marinus* in the present study, 100 mg/L - 10 mg/L, are in agreement with results by Ray (1966a). In Ray's study, *P. marinus* cells were markedly inhibited after exposure of infected tissues to 50-100 mg/L of amphotericin-B. Exposure of infected tissues to less than 50 mg/L of amphotericin-B resulted only in slight inhibition. Concentrations in the range of 10 mg/L to 100 mg/L of amphotericin-B are, however, well beyond the recommended dose of 2.5 mg/L for antifungal treatment of *in vitro* cultures (Sigma Chemical Co. 1993).

The concentrations of exposure of lasalocid and monensin active against *P. marinus* in the present study (100 mg/L to 10 mg/L) were much higher than the range 0.001 mg/L to 0.005 mg/L found active against the coccidian *Eimeria tenella* by Ryley and Wilson (1976). A longer exposure duration (4 days in Ryley & Wilson's study as compared to 1 day in the present study) and possibly a more sensitive endpoint for activity (clusters indicating growth of specific life stages) may account for some of the difference.

The mechanism by which chemicals inhibit *P. marinus* is probably related to the general mode of chemical action. Amphotericin-B, cycloheximide, lasalocid, and monensin may be lethal to *P. marinus* cells. Amphotericin-B is a polyene antifungal

antibiotic. The mode of action and toxicity of the polyenes have been attributed to their ability to bind to sterols in cell membranes producing alterations in permeability (Grayson, 1982). 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. It is not known how coccidia are preferentially affected by polyether ionosphorus compounds, but it has been proposed that the lack of active transport mechanisms in coccidia may allow for selective activity of these compounds (McDougald 1982). Cycloheximide is a protein synthesis inhibitor highly active against a large number of yeasts including fungal pathogens (Ennis & Lubin 1964), but a cytostatic effect is more likely to have occurred in short term exposures. The mode of action of cycloheximide is by inhibiting protein synthesis in eukaryotic ribosomes. The inhibition may involve blocking one or more steps in the initiation and elongation of peptide chains (Deacon 1984, Obrig et al. 1971).

To some extent, anticoccidial agents (particularly 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 usually considered antifungal agents (amphotericin-B, malachite green and cycloheximide) were also effective, does not support the conclusion that anticoccidial compounds are necessarily the most effective ones against *P. marinus*.

The implications of these results for the phylogenetic affiliation of *P. marinus* with fungi and apicomplexan organisms is difficult to assess because the spectrum of action of chemotherapeutants often ranges beyond a single group of organisms. Polyether antibiotic anticoccidial compounds such as lasalocid and monensin, for instance, have shown activity against Gram-positive bacteria, mycobacteria, and fungi (Grayson 1982). Aryl-methane dyes, such as malachite green, have a broad spectrum of action against Gram-positive bacteria, fungi (especially oomycetes), and external

protozoan parasites of fish (Alderman 1985). Cycloheximide activity has been reported mainly in reference to fungi (Whiffen 1948, Grayson 1982), but it is active against eukaryotes in general (Deacon 1984). Polyene antibiotics such as amphotericin-B possess broad antifungal activity and some antiprotozoal activity. Activity of amphotericin-B, however, is mostly restricted to yeasts, fungi, and molds including *Candida spp.*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatidis* (Merck & Co. 1986).

It should be noted that no adverse effects of DMSO were detected on *P. marinus*. To the contrary, it appears that DMSO may have prevented cell deterioration, as suggested by the higher prezoosporangia abundance in several solvent control groups as compared to untreated (FYRW) control groups. Control of microbial contamination by DMSO could account for the increased cell counts in solvent controls. This suggestion is supported by the indication that DMSO may not only act as a solvent without interfering with parasite development or host cell integrity, but by the fact that it may also serve to sterilize *in vitro* assays (Ryley & 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 related to lasalocid and monensin exposure is not entirely surprising since both chemicals are polyether ionosphorus compounds known for toxic effects associated with their mode of action (McDougald 1982). It is difficult to compare acute toxicity of anticoccidials by bath administration in oysters with that by injection or oral administration in terrestrial organisms. In mice, the 24 h median lethal dose of lasalocid ranges from 64 mg/kg (intraperitoneal) to 146 mg/kg (oral) (Grayson 1982). In oysters , the 96 h LC_{50} for lasalocid by bath administration was 0.56 mg/L (this study). Assuming one 24 h exposure of 1 mg/L of lasalocid to 10 oysters weighing an arbitrary average wet weight

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of 5 g per oyster, in a 10 L bath, and assuming that all the available compound was taken up in equal parts by oysters and no losses occurred, would result in a tissue concentration of 1 mg of the compound per 5 g wet tissue weight, or a dose equivalent to 200 mg/kg which is comparable to the acute toxic dose of lasalocid by oral administration for mice as previously noted. Most likely, however, only some fraction of the compounds was taken up by oysters and even less was available to the parasites. To better compare acute toxicity of compounds to aquatic organisms with that of terrestrial organisms, perhaps the preferred mode of administration of compounds would be by injection. 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 a solvent at that or 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 monensin and lasalocid, 10 mg/L and 1 mg/L exposures 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). Malachite green is a respiratory enzyme poison known to destroy mitochondria (Alderman 1985). 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 therefore not been approved by the FDA (Meyer & Schnick 1989).

In vivo studies

Experiment 1, designed to determine the effect of chemical baths on preventing infections, had some drawbacks and its results should be interpreted with caution. Despite the fact that oysters were collected from an area that had traditionally been free

from *P. marinus*, the Wye River in Maryland, the baseline diagnostic sample showed 56% prevalence of mostly light infections. Therefore, treatment was not strictly preventive in the sense that some infections were already established prior to exposure of oysters to chemical baths. This experiment was not repeated because, at the time, no source of *P. marinus*- free oysters was known.

Anticoccidial compounds, including lasalocid and monensin that were active *in vitro* at concentrations ranging from 10 mg/L to 1 mg/L, were not effective *in vivo* at 0.1 mg/L. It is possible that exposure of oysters to 0.1 mg/L of lasalocid and monensin for 4 days was not sufficient to deliver therapeutic levels of these compounds to parasites. Exposure of oysters to lasalocid or monensin at concentrations above 1 mg/L, however, was lethal to oysters, as indicated in the toxicity experiment. Therefore, for lasalocid and monensin the difference between a therapeutic dose and a toxic dose in oysters may be, if existent, between 0.1 and 1 mg/L. Small differences between therapeutic and toxic doses of lasalocid and monensin were typical in poultry and other animals (McDougald 1982 and 1990).

Oysters (N = 25) challenged with one daily dose of 1×10^7 *P. marinus* cells from minced oyster tissues for two consecutive days did not exhibit increase infections relative to unchallenged control oysters. Therefore this challenge was not effective. Mantle cavity injections containing $> 5 \times 10^4$ *P. marinus* cells per oyster resulted in disease-induced mortality after 3 weeks (Mackin 1962). Considering that the "injection method" is more effective in producing *P. marinus* infections than the "feeding method" (Ray 1954), the dose and duration of the challenge in experiment 1 may have been below that required to detect an increase in the number infections. Prior studies using the "feeding method" with *P. marinus*-infected tissue minces (Ray 1954, Mackin 1962) have not reported infective doses in terms of cells but in terms of number of infected oysters used as a source of parasites, usually ranging from 1 -10.

In poultry, preventive anticoccidial treatment has been preferable to therapeutic treatment because coccidiosis is not clinically recognizable until tissue damage associated with second or third generation schizonts and mortality occurs soon after those signs appear. Initiation of treatment as soon as clinical signs appear is of value because not all individuals in a group become infected at the same time (McDougald 1982). Similarly, preventive or early treatment of *P. marinus* infections may be more effective than therapeutic treatment of advanced infections.

Among all compounds tested in experiment 2, designed to examine the effect of chemical baths on established infections, only cycloheximide was effective in controlling *P. marinus* infections while not adversely affecting oyster survival. In contrast to what has been reported for low salinity exposures (Ragone & 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 the 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 compounds (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 assay, as proposed by Burreson and Ragone-Calvo (1993). On the basis of the *in vitro* efficacy of amphotericin-B, both on hemolymph and cultured cells, *in vivo* trials may be conducted in the future. No attempts to test amphotericin-B *in vivo* were carried out during the present investigation because the compound was tested

during the last part of the study. In addition, amphotericin-B is a relatively expensive compound.

Experiment 3 clearly defined the effect of cycloheximide on prevalence and weighted prevalence. Prevalence declined from 95% (prior to treatment) to 67% (after 30 days of exposure to cycloheximide) and weighted prevalence declined from 0.92 to 0.71. These results are in general agreement with those reported by Ray (1966b). In Ray's study, however, prevalence only slightly declined from 95% (prior to treatment) to 80% (after 30 days of exposure to cycloheximide) while weighted prevalence was drastically reduced from 3.00 to 1.25. Greater sample size in experiment 3 (N = 25) as compared to Ray's study (N = 10) may provide more confidence to differences detected in experiment 3. The magnitude of decline in weighted prevalence after treatment was similar to experiment 2, suggesting that 15 more days of treatment were not enough to further reduce infections. However, the fact that several light infections in 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 agreement with Ray (1966b), experiment 3 showed that exposure of infected oysters to 10 mg/L of cycloheximide for 30 days resulted in a decrease in infection

prevalence and intensity. In experiment 3, prevalence declined from 100% (in the untreated control group) to 67% (in the treated group) and weighted prevalence declined from 2.21 to 0.71 respectively.

The magnitude of decline in weighted prevalence in experiment 2 was also similar to the one observed in experiment 3, 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 in experiment 3 may have prevented a greater effect from being detected. The increase in prevalence and weighted prevalence after treatment stopped in experiment 3 suggests that the parasiticidal effect of cycloheximide did not persist beyond the exposure period.

In vitro assays have proven to be useful to screen anti-*P. marinus* compounds, but *in vivo* tests are necessary to identify suitable compounds for treatment of oyster diseases. Not only does a compound need to be active against the parasite but it also needs to be available to established infections and not harmful to the host. This agrees with conclusions for testing of antiparasitic compounds in other animals (Lichtfield 1958, Schnitzer & Hawking 1963, Ryley & Wilson 1976, Grayson 1982). Comparison of *in vitro* activity with *in vivo* efficacy in the present study indicates that even though several compounds were active *in vitro*, only cycloheximide was efficacious *in vivo*. Lasalocid and monensin, which were also active *in vitro*, were toxic to oysters *in vivo*. Difficulties in correlating *in vitro* activity with *in vivo* efficacy are not unusual and limit the utility of *in vitro* tests for predicting *in vivo* efficacy. Compounds that are very active *in vitro* may not be active *in vivo*, and compounds with minimal *in vitro* activity may be efficacious *in vivo* (Ryley & Wilson 1976).

Sources of discrepancies between *in vitro* and *in vivo* tests may be attributed in part to pharmacodymamics and bioavailability of test compounds to parasites in their host environment. In the present investigation, delivery of compounds via water baths must have provided enough quantity of lasalocid and monensin to oysters on the basis of the

recorded toxic effects . Similarly, enough cycloheximide must have reached established parasites in oysters given the observed decrease of infections after treatment. It was assumed that delivery of chemicals mixed with algae or delivered at the same time algae were provided to aquaria would facilitate uptake by oysters. No measurements were taken, however, to validate this assumption. Availability of chemicals to oysters may in fact have been altered by factors such as uptake by algae and loss of chemicals to the air interface.

In solution, polyether antibiotic agents such as lasalocid and monensin assume a specific configuration with the center negatively charged and the exterior neutral and hydrophobic (McDougald 1982). The exterior hydrophobicity of polyether compounds may facilitate their interaction with organic matter including algae. If so, mixing polyether compounds with algae may be a good way to administer these compounds to oysters. With micro-encapsulation techniques (Langdon & Bolton 1984) it may be possible to improve the delivery of lasalocid and monensin to parasites while avoiding probable toxic effects to oysters. A recently developed liposomal amphotericin-B formulation has shown superior efficacy in the treatment of leishmaniasis and no toxicity to humans. A similar approach may be appropriate for use on oysters, even though the referred liposomal amphotericin-B formulation is too expensive even for use in humans (Olliaro & Bryceson 1993).

Cycloheximide is known to be toxic to algae (Bower 1989), but whether uptake by algae would alter the availability of cycloheximide to oysters is open for speculation. In aquatic organisms, uptake of chemicals may occur directly from the water through absorptive epithelia or from the food via the digestive tract. Uptake of compounds adsorbed to food or sediment particles has generally been considered negligible compared to uptake from water, but studies including those for bivalves have been contradictory (Langston 1978, Pruell et al. 1986, and Williams 1989)

Loss of chemicals to the air interface may have occurred in the present study considering that a continuous supply of air bubbles was being delivered to aquaria by air stones. Use of Plexiglas covers, such as the ones used in the present study, can prevent chemicals form escaping from aquaria into the surrounding space to some extent, but offer no assurance against losses of chemicals to the air.

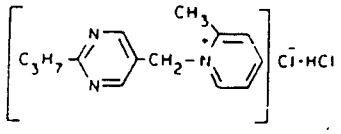
Very little is known about the effects of seawater on the activity of anticoccidial and antifungal compounds because the compounds have usually been used on terrestrial or freshwater organisms. Malachite green in alkaline waters rapidly transforms to the carbinol form which has greater liposolubility and may be more easily absorbed by skin and gills of fish (Alderman 1985). Cycloheximide may lose its activity in seawater (Ray 1966). Therefore, direct measurements of the chemicals in the water and in oyster tissues would be necessary to determine the availability of the chemicals to oysters and parasites.

The recently developed *P. marinus* cell cultures (La Peyre et al. 1993) facilitates screening of compounds *in vitro*, but the advantage of using propagated cells may be offset if, as in the case of cycloheximide, no activity can be detected. Further studies are needed to compare and refine tests using culture and non-cultured *P. marinus* cells. Studies that may follow this line of research, however, should also note that the potential applications of chemotherapy to *P. marinus*-infected oysters may be restricted to small lots of oysters maintained in closed systems. It is debatable whether this approach would be practical for aquaculture situations where oysters must be grown "in the field." In nature, disease pressure from *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 would be necessary to comply with regulatory requirements.

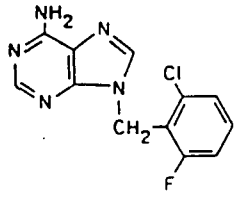
In summary, cycloheximide baths of at least 10 mg/L renewed every other day for 2 weeks are necessary to reduce *P. marinus* infections in oysters held at 20 °C and 20 ppt.

Continuation of treatment for 2 additional weeks may not result in eradication of the 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.).

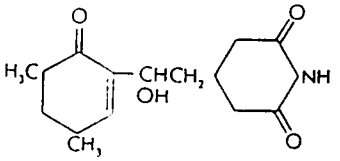
Appendix A. Structure of chemotherapeutants used in the present investigation. Note: Potentiated sulfadimethoxine, a compound based on sulfadimethoxine is not shown.



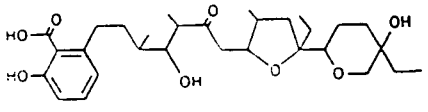
amprolium



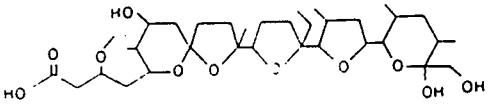
arprinocid



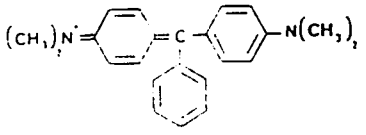
cycloheximide



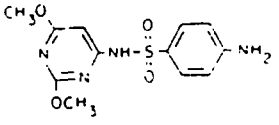
lasalocid



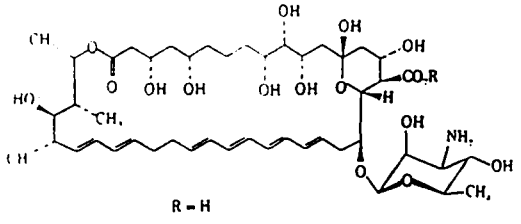
monensin



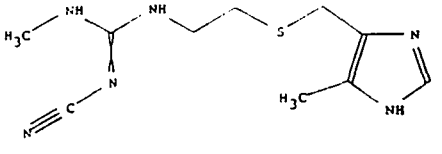
malachite green



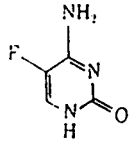
sulfadimethoxine



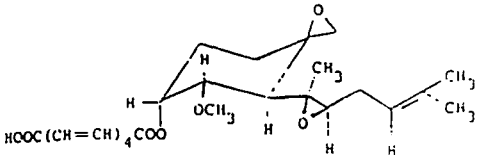
amphotericin-B



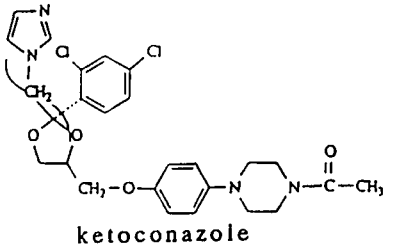
cimetidine



5-fluorocytosine



fumagillin



ketoconazole

Appendix B. List of compounds tested *in vitro* on oyster hemolymph infected with *P. marinus* and on cultured *P. marinus* cells. * = Compounds tested both on infected hemolymph and cultured cells.

Compounds tested on infected hemolymph	Compounds tested on cultured cells
*Amphotericin-B	*Amphotericin-B
Amprolium	Cimetidine
Arprinocid	Cycloheximide
*Lasalocid	Fumagillin
Malachite green	5-Fluorocytosine
*Monensin	Ketoconazole
Potentiated sulfadimethoxine	*Lasalocid
Sulfadimethoxine	*Monensin

Appendix C. Summary of *in vitro* experiments. IH= Experiments in which hemolymph from infected oysters was used. CC = experiments in which *P. marinus* cultured cells were used.

Experiment	Compounds tested	Concentration of exposure	Effects measured on
IH 1	amprolium, arprinocid, cycloheximide, lasalocid, malachite green, monensin, potentiated sulfadimethoxine, and sulfadimethoxine	100 mg/L	prezoosporangia abundance
IH 2	amprolium, cycloheximide, lasalocid, malachite green, monensin, and sulfadimethoxine	10 mg/L, 1 mg/L, 0.1 mg/L, and 0.01 mg/L	prezoosporangia abundance
IH 3	amhotericin-B	100 mg/L, 10 mg/L, 1 mg/L	prezoosporangia abundance
CC 1	amphotericin-B, cimetidine, cycloheximide, 5-fluorocytosine, fumagillin, and ketoconazole	100 mg/L	prezoosporangia abundance and size
CC 2	amphotericin-B, cimetidine, cycloheximide, 5-fluorocytosine, fumagillin, and ketoconazole	100 mg/L	cell viability
CC 3	lasalocid and monensin	100 mg/L, 10 mg/L, 1 mg/L	prezoosporangia abundance and size
CC 4	lasalocid and monensin	100 mg/L, 10 mg/L, 1 mg/L	cell viability

Appendix D. Summary of toxicity and *in vivo* experiments.

Experiment	Compound(s) tested	Concentration and duration of exposure	Effects measured on
Toxicity	amprolium, arprinocid, lasalocid, monensin, potentiated sulfadimethoxine, and sulfadimethoxine	10 mg/L, 1 mg/L, 0.1 mg/L, and 0.01 mg/L (4 day exposure, daily renewal)	oyster mortality
In vivo 1	amprolium, arprinocid, lasalocid, monensin, potentiated sulfadimethoxine, and sulfadimethoxine	0.1 mg/L, except for sulfadimethoxine (10 mg/L) (4 day exposure, daily renewal)	prevalence and weighted prevalence
In vivo 2	amprolium, sulfadimethoxine; cycloheximide, and malachite green	100 mg/L, 10 mg/L; 10 mg/L, 1 mg/L (15 day exposure, every other day renewal)	prevalence and weighted prevalence
In vivo 3	cycloheximide	10 mg/L (30 day exposure, every other day renewal)	prevalence and weighted prevalence

Appendix E. *In vitro* experiments. Normality (KSZ = Kolmogorov-Smirnov statistic). For P > 0.05 accept hypothesis of normal distribution of data. Homogeneity of variance (Cochran and Bartlett tests). For P > 0.05 accept hypothesis of homogeneous variance of data. *= Bartlett/Box test cannot be performed. Abbreviations: IH = Experiments with infected hemolymph. CC = Experiments with cultured cells .

Experiment	Variable	KSZ	P-value	Cochran C	P-value	Bartlett/Box F	P-value
IH 1	log(prezoosporangia abundance+1)	0.562	0.910	0.315	0.332	1.110	0.355
IH 2	prezoosporangia abundance	0.841	0.480	0.393	0.242	*	
CC 1	log(prezoosporangia abundance+1)	0.491	0.970	0.410	0.014	2.003	0.052
CC 1	log(size+1)	0.381	0.999	0.367	0.325	0.881	0.522
CC 2	arcsin(percent cell viability)	0.638	0.810	0.238	0.958	0.999	0.431

Appendix F. *In vivo* experiments. Normality test (KSZ = Kolmogorov-Smirnov statistic). For P > 0.05 accept hypothesis of normal distribution of data. Homogeneity of variance tests (Cochran and Bartlett). For P > 0.05 accept hypothesis of homogeneous variance of data. * = Bartlett/Box test cannot be performed.

Experiment	Variable	KSZ	P-value	Cochran C	P-value	Bartlett/Box F	P-value
In vivo 1	arcsin(prevalence)	0.750	0.627	0.459	0.359	*	
In vivo 1	log(weighted prevalence+1)	0.570	0.902	0.283	>0.999	0.300	0.951
In vivo 2	log(weighted prevalence+1)	1.116	0.166	0.647	0.112	0.702	0.649
In vivo 3	arcsin(prevalence)	0.567	0.905	0.908	0.049	*	
In vivo 3	log(weighted prevalence+1)	0.795	0.553	0.314	>0.999	0.029	0.993

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