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Effects of triclosan on growth, viability and fatty acid synthesis of the oyster protozoan parasite *Perkinsus marinus*

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ABSTRACT: *Perkinsus marinus*, a protozoan parasite of the Eastern oyster *Crassostrea virginica*, has severely impacted oyster populations from the Mid-Atlantic region to the Gulf of Mexico coast of North America for more than 30 yr. Although a chemotherapeutic treatment to reduce or eliminate *P. marinus* from infected oysters would be useful for research and hatchery operations, an effective and practical drug treatment does not currently exist. In this study, the antimicrobial drug triclosan 5-chloro-2-(2,4 dichlorophenoxy) phenol, a specific inhibitor of Fab1 (enoyl-acyl-carrier-protein reductase), an enzyme in the Type II class of fatty acid synthetases, was tested for its effects on viability, proliferation and fatty acid synthesis of *in vitro*-cultured *P. marinus* meronts. Treatment of *P. marinus* meront cell cultures with concentrations of ≥2 µM triclosan at 28°C (a temperature favorable for parasite proliferation) for up to 6 d stopped proliferation of the parasite. Treatment at ≥5 µM at 28°C greatly reduced the viability and fatty acid synthesis of meront cells. Oyster hemocytes treated with ≥20 µM triclosan exhibited no significant ($p < 0.05$) reduction in viability relative to controls for up to 24 h at 13°C. *P. marinus* meronts exposed to ≥2 µM triclosan for 24 h at 13°C exhibited significantly ($p < 0.05$) lower viability relative to controls. Exposure of *P. marinus* meronts to triclosan concentrations of ≥20 µM resulted in >50% mortality of *P. marinus* cells after 24 h. These results suggest that triclosan may be effective in treating *P. marinus*-infected oysters.

KEY WORDS: Triclosan · Dermo disease · *Perkinsus marinus* · Eastern oyster · Fatty acid synthesis

INTRODUCTION

The protozoan parasite *Perkinsus marinus*, causative agent of Dermo disease in the Eastern oyster *Crassostrea virginica*, has decimated oyster populations along the Atlantic coast of North America and the Gulf of Mexico for at least 30 yr. One element critical to both the restoration of wild oyster populations and the development of commercial aquaculture operations is a practical method for reducing or eliminating *P. marinus* from infected oysters. Currently, virtually all oysters in the mid-Atlantic region are infected with *P. marinus* (Burreson & Ragone-Calvo 1996), which has severe ramifications for restoration efforts, oyster disease research and commercial oyster aquaculture.

Potential benefits from the development of a protocol to eliminate *Perkinsus marinus* from infected oysters include (1) increasing the quantity and quality of gametes of oysters conditioned for hatchery spawns; (2) a reduction in the frequency of spawning required to maintain specific strains of oysters; (3) production of disease-free spat; (4) production of disease-free oysters for use in research on host–parasite interactions.

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To be practical for use in controlling *Perkinsus marinus* infection, a drug therapy needs to have several important features: (1) high specific action against the parasite; (2) low toxicity to the host; (3) application through direct addition to water in holding containers; (4) low/no toxicity to humans; (5) low cost and ready availability. Several studies evaluating potential chemotherapeutic agents for treatment of Dermo disease have been published, but the protocols so far developed to successfully treat *P. marinus* infection in oysters fail to meet several of these criteria (Ray 1966a,b, Calvo & Burreson 1994, Faisal et al. 1999, Delaney et al. 2003).

Parasitic protozoans must either acquire lipids from their host or synthesize lipids de novo to produce the new cell membrane necessary for cell replication. Inhibiting the ability to synthesize new membrane prevents the parasite from increasing in surface area, thereby halting cell proliferation and disease progression.

The antimicrobial drug triclosan, 5-chloro-2-(2,4 dichlorophenoxy) phenol, has been used in a wide array of consumer products such as soaps, toothpastes and household plastics for the last 30 yr. Triclosan inhibits fatty acid synthesis and stops the proliferation of protozoan parasites including species of the genera *Plasmodium*, *Toxoplasma*, and *Trypanosoma in vitro* and *in vivo* (Beeson et al. 2001, McLeod et al. 2001, Surolia & Surolia 2001, Roberts et al. 2003). The purpose of the present study was to determine whether triclosan may be a useful chemotherapeutic agent to treat oysters infected with *Perkinsus marinus*. The effectiveness of triclosan against *P. marinus* was evaluated by measuring the effect of the drug on the growth, viability and fatty acid synthesis of *in vitro* parasite cultures. To address concerns about potential toxic effect on host cells, the effect of triclosan on oyster hemocyte viability was also assessed.

MATERIALS AND METHODS

Culture conditions. *Perkinsus marinus* meront cells were cultivated as previously described (Chu et al. 2002) in medium defined by La Peyre et al. (1993). Medium was prepared with artificial seawater (ASW) and adjusted to an osmolarity of 590 (equivalent to a salinity of 20, Lund et al. 2004). The medium was then sterilized by 0.2 µm filtration and stored at 4°C until use. Meronts were inoculated at a concentration of 1×10^6 ml^{-1} and cultivated in 10 ml aliquots of medium in T-10 tissue-culture flasks at 28°C. Meronts in exponential growth phase (7 d old) were harvested and used for all assays.

Expt 1. Effect of triclosan on growth, viability and fatty acid synthesis by *Perkinsus marinus*. The effects of triclosan on the fatty acid synthesis rates of *P. marinus* were tested by measuring the incorporation of exogenous ^{13}C sodium acetate into fatty acids of *P. marinus* meront cells. We added 10 ml aliquots of fresh media containing 6 mM sodium acetate 1,2 $^{13}C_2$ (Cambridge Isotope Laboratories) to culture flasks and inoculated those with 2.5×10^7 cells and 0, 2, 5 or 10 µM triclosan using ethanol (5 µl ml^{-1}) as a carrier (n = 3). After 72 h at 28°C, cultures were harvested and cell density and percentage of viable cells for each culture flask was determined using a neutral red assay (Borenfreund & Puerner 1985), and fatty acid synthesis rates were determined by gas chromatography/mass spectroscopy (GC/MS) (Chu et al. 2002).

Expt 2. Viability and cell proliferation of *Perkinsus marinus* cultures exposed to different levels of triclosan. To test a wide range of triclosan concentrations, 21 T-10 tissue-culture flasks containing 10 ml aliquots of medium were inoculated with 1 ml (0.25×10^6 cells) of 4 d old *P. marinus* culture. Triclosan solutions of 0, 2, 5, 10, 20, 50 and 100 µM were added with ethanol (5 µl ml^{-1}) to the culture flasks (3 replicates per treatment). Aliquots of 1.2 ml from each culture flask were collected after 3 h, 24 h, 3 d and 6 d incubation at 28°C for determination of cell culture density and viability measurements by flow cytometry (FCM). All the suspensions were filtered through 80 µm mesh into FCM tubes and maintained on ice until FCM analysis.

Expt 3. Viability of oyster hemocytes exposed to triclosan. Adult oysters, *Crassostrea virginica*, were supplied by Pemaquid Oyster Company (Waldoboro) and maintained in a flow-through seawater system at ambient temperature (13 to 14°C) and salinity (15 to 20). They were processed as follows: hemolymph was withdrawn from the adductor muscle through a notch previously (24 h) ground in the oyster shell, using a 1 ml plastic syringe with a 25-gauge needle. Hemolymph from each oyster was transferred into an individual Eppendorf tube held on ice. Only individual samples confirmed by microscope observation to be free of contaminating particles were used. Hemolymph from 30 individuals was pooled to constitute 3 replicates of 9 ml of fresh hemolymph. Pooled hemolymph samples were filtered through 80 µm mesh prior to use. We combined 1 ml of hemolymph with 1 ml of anti-aggregant hemocyte solution (AAHS, Auffret & Oubella 1995) in 21 Falcon vials (7 vials per hemolymph pool), and then added 0, 2, 5, 10, 20, 50 and 100 µM of the ethanol (5 µl ml^{-1}) triclosan solutions to the 2 ml hemolymph + AAHS mix (3 replicates per ttreatment). The hemolymph was continuously maintained on ice until initiation of incubation. All the Falcon tubes were thus incubated at 13°C (the temper-

ature at which, the oyster were maintained prior to bleeding). After 24 h incubation, 0.6 ml of each cell suspension was collected and filtered into FCM tubes for subsequent FCM analysis.

Expt 4. Viability of *Perkinsus marinus* meronts exposed to triclosan at temperature similar to that in oyster hemocyte challenge. To compare triclosan treatment of *Perkinsus marinus* meronts with oyster hemocytes, meronts were cultured at 13°C for 2 wk prior to triclosan challenge. Cultures were concentrated by centrifugation at 800 × *g* for 20 min, and then used to inoculate 5 ml of fresh medium into each flask to a final concentration of 10^6 cells ml^{-1}. Cultures were exposed to 0, 2, 5, 10, 20, 50 and 100 µM triclosan at 13°C using ethanol (5 µl ml^{-1}) as a carrier (n = 3). The number of viable meronts after 24 h was determined using a neutral red viability assay (Borenfreund & Puerner 1985). The percentage of viable cells was determined by dividing the number of viable cells in a treatment by the number of viable cells in controls (0 µM triclosan).

Measurement of *Perkinsus marinus* fatty acid synthesis rates by GC/MS. Fatty acid synthesis rates as measured by ^{13}C incorporation into meront fatty acids were determined as described previously (Chu et al. 2002). Briefly, cell pellets were freeze-dried and lipids extracted according to the method of Bligh & Dyer (1959). An aliquot of approximately 500 µg of lipids from each sample and 20 µg internal standard (23:0) were transesterified by addition of 1 ml of 10% BF_3 (w/w) in methanol followed by heating for 15 min at 95 to 100°C (Metcalfe & Schmitz 1961). After cooling, the fatty acid methyl esters (FAME) were extracted with carbon disulfide (Marty et al. 1992). The organic phase was evaporated, and the samples dissolved in hexane.

Separation of FAME was carried out on a GC/FID (gas chromatograph equipped with a flame ionization detector) (Varian 3300), using a DBWAX capillary column (J & W, 25 m × 0.32 mm; 0.2 µm film thickness). The column temperature was programed from 60 to 150°C at 30°C min^{-1} and 150 to 220°C at 2°C min^{-1}, injector and detector temperatures were 230 and 250°C, respectively, and the flow rates of compressed air and hydrogen were 300 and 30 ml min^{-1}. Helium was used as the carrier gas (2 ml min^{-1}). Identification of FAMEs was based on the comparison of their retention times with those of authentic standards and confirmed by GC/MS. The quantity of each component was calculated based on the internal standard.

To detect incorporation of ^{13}C acetate, FAMEs were further analyzed qualitatively and quantitatively by mass spectroscopy with a Varian 3400 gas chromatograph equipped with a Varian Saturn 4D GC/MS/MS detector. Methane was used as the reagent gas for positive chemical ionization (CI). The same column as that used for GC/FID analysis of the FAME samples (J & W DB-WAX, 25 m × 0.32 mm; 0.2 µm film thickness) was used for GC/MS analysis. Carrier gas (helium) flow rate was 1 ml min^{-1}. The temperature of the injection port was 230°C and that of the interface 250°C. The column temperature was programed for a 4 min hold at an initial temperature of 60°C, followed by a 30°C min^{-1} increase to 150°C and a subsequent 2°C min^{-1} increase to 220°C. Data were collected and processed using Varian Saturn GC/MS software Version 5.2. FAMEs were identified by retention time relative to known standards, fragmentation pattern and mass of the molecular ion. FAMEs containing ^{13}C derived from acetate were quantified using standard curves constructed for each FAME standard and ratio with an internal standard (23:0). The molecular ions in the spectra of each FAME were used to quantify masses containing exogenous ^{13}C relative to the native molecule. Samples from cultures containing unlabeled sodium acetate were also analyzed in an identical manner, and served as negative controls for ^{13}C incorporation. Data were expressed as micrograms of fatty acids containing stable isotope g^{-1} dry cell pellet h^{-1}.

Determination of cell concentration and viability by flow cytometry. FCM analysis of *Perkinsus marinus* meronts and *Crassostrea virginica* hemocyte viability were carried out using a Coulter®, EPICS®, Altra™ flow cytometer. Briefly, cells were stained with 2 nucleic acid fluorescent dyes: the permeant SYBR Green I ($_{excitation}$ = 497 nm, $_{emission}$ = 520 nm, commercial stock solution at 10 000×, molecular probes, S-7563) and the impermeant propidium iodide, PI, ($_{excitation}$ = 535 nm, $_{emission}$ = 617 nm, Sigma, P4170) at the respective final concentrations of 1× of stock solution (10 000×) and 10 µg ml^{-1}. SYBR Green I stains both live and dead cells and PI stains only cells that have lost membrane integrity (dead cells). The principle of this assay is that SYBR Green I fluorescence (FL1) differentiates live and dead cells from other particles (e.g. cell debris) present in the medium and from instrument 'noise', while PI allows detection of dead cells on the FL3 detector. Samples were incubated with the dyes for 15 min at room temperature prior to analysis. A data acquisition time of 30 s was sufficient to obtain between 2000 and 10 000 analyzed cells. Data acquisition was in logarithmic mode (4 decades). To calculate *P. marinus* meront cell concentration, the flow rate of the instrument was measured using a known concentration of 10 µm diameter fluorescent microspheres (Coulter PN6605359).

Treatment of the FCM data was performed with the software WinMDI Version 2.8 (Joseph Trotter©). *Perkinsus marinus* meronts and oyster hemocytes were first identified and selected according to 3 FCM para-

meters: forward scatter (FSC), side scatter (SSC) and yellow-green fluorescence (FL1). FSC corresponds to the diffracted light on the small angle (detected in line with the incident light source) and is proportional to size. SSC corresponds to the diffracted light on the right angle and is proportional to the cell complexity or granular content. FL1 allowed visualization of the SYBR-green fluorescence of live and dead cells. After selection, red fluorescence allowed visualization of the PI fluorescence of the dead cells and calculation of the percentage of viable cells in the sample.

Table 1. *Perkinsus marinus.* Proliferation, viability and fatty acid synthesis of cultures after 3 d exposure to triclosan. Initial cell concentrations were 2.5×10^6 cells ml^{-1}. Cell viability measured by uptake of neutral red, fatty acid synthesis by incorporation of ^{13}C-labeled sodium acetate (6 mM) from the medium into *Perkinsus marinus* fatty acids using gas liquid chromatography/mass spectroscopy. Different superscripts indicate treatments that differed significantly at the $p < 0.05$ level. All values are mean ± SD (n = 3)

Parameter	Control	Triclosan (2 µm)	(5 µm)	(10 µm)
Cell conc. (10^6 cells ml^{-1})	12.7 ± 0.9^a	10.2 ± 0.6^{ab}	7.7 ± 1.7^b	1.0 ± 0.2^c
% viable cells	85.4 ± 1.3^a	84.1 ± 0.5^a	74.0 ± 2.4^b	11.8 ± 1.7^c
µg 16:0 g dry wt h^{-1}	70.7 ± 6.1^a	55.0 ± 2.4^b	14.3 ± 1.7^c	8.6 ± 2.1^c
µg 20:4(n-6) <µg dry wt h^{-1}	38.8 ± 3.0^a	19.1 ± 2.2^b	8.8 ± 0.6^c	5.7 ± 1.9^c

Statistical analysis. Analysis of variance (ANOVA) was used to compare the differences in proliferation and fatty acid synthesis rates for *Perkinsus marinus* meronts exposed to different concentrations of triclosan (Expt 1) and FCM measurements of cell concentration of *P. marinus* meronts exposed to different concentrations of triclosan for 3 h, 24 h, 3 d and 6 d (Expt 2). When significant ($p < 0.05$) treatment effects were found, individual comparisons were conducted using Tukey's Honestly Significant Difference (HSD) test.

Differences between percentage of viable *Crassostrea virginica* hemocytes (Expt 3) and *Perkinsus marinus* meronts (Expt 4) exposed to different concentrations of triclosan for 24 h (Expt 3) at 13°C were determined using ANOVA. When significant ($p < 0.05$) treatment effects were found, individual comparisons were conducted using Tukey's HSD test. All percentage data were arcsine-transformed prior to statistical analysis. Differences were considered significant when $p < 0.05$.

RESULTS

Expt 1

Perkinsus marinus cell cultures incubated with 5 and 10 µM triclosan exhibited lower cell densities and lower cell viability than controls (Table 1). The 2 µM triclosan treatment produced a similar trend, but cell density and viability were not significantly lower than in the controls. After 3 d incubation, the control cultures had increased from an initial concentration of 2.5×10^6 cells ml^{-1} to $12.7 \pm 0.9 \times 10^6$ cells ml^{-1} while the 2, 5 and 10 µM triclosan treatments had cell densities of 10.2 ± 0.6, 7.7 ± 1.7 and $1.0 \pm 0.2 \times 10^6$ cells ml^{-1}, respectively (Table 1). Cell viability was also significantly ($p < 0.05$) lower in the 5 and 10 µM treatments relative to the controls and 2 µM treatment (Table 1).

Incorporation of ^{13}C from acetate into the initial product of fatty acid synthesis, palmitic acid (16:0) (Table 1), was significantly reduced in all triclosan treatments relative to the controls. Controls had a synthesis rate for palmitic acid of 70.7 ± 6.1 µg g^{-1} dry wt h^{-1}, which was significantly higher than that of the 2, 5 and 10 mM triclosan treatments (55.0 ± 2.4, 14.3 ± 1.7 and 8.6 ± 2.1 µg g^{-1} dry wt h^{-1}, respectively) (Table 1). Synthesis of 20:4(n-6) (arachidonic acid), an important prostaglandin precursor, was also significantly inhibited in all triclosan treatments relative to the controls (Table 1).

Expt 2

Proliferation rates of *Perkinsus marinus* cultures were inhibited by increasing dosages of triclosan relative to the controls (Fig. 1). At 3 h post-inoculation the control already had the highest cell density ($1.48 \pm 0.68 \times 10^5$ cells ml^{-1}), but was only significantly higher than the 5 and 10 µM treatments (1.08 ± 0.05 and $1.13 \pm 0.11 \times 10^5$ cells ml^{-1}, respectively) (Fig. 1A). From 24 h on through the 6 d experiment, no treatment above 2 µM had a cell density higher than 1.3×10^5 cells ml^{-1} (Fig. 1B–D). In contrast, the control treatment increased from the initial inoculation density of 1×10^5 cells ml^{-1} to $2.32 \pm 0.22 \times 10^5$ cells ml^{-1} at 24 h, $19.31 \pm 1.09 \times 10^5$ cells ml^{-1} at 3 d, and $37.40 \pm 4.60 \times 10^5$ cells ml^{-1} at 6 d (Figs. 1B–D). These values were significantly higher ($p < 0.05$) than in any of the triclosan treatments. The culture in the 2 µM treatment proliferated slightly during the course of the experiment, increasing to 1.67 ± 0.08, 3.76 ± 0.15 and $3.69 \pm 0.61 \times 10^5$ cells ml^{-1} at 24 h, 3 d and 6 d, respectively, but growth was significantly attenuated relative to the controls (Fig. 1B–D).

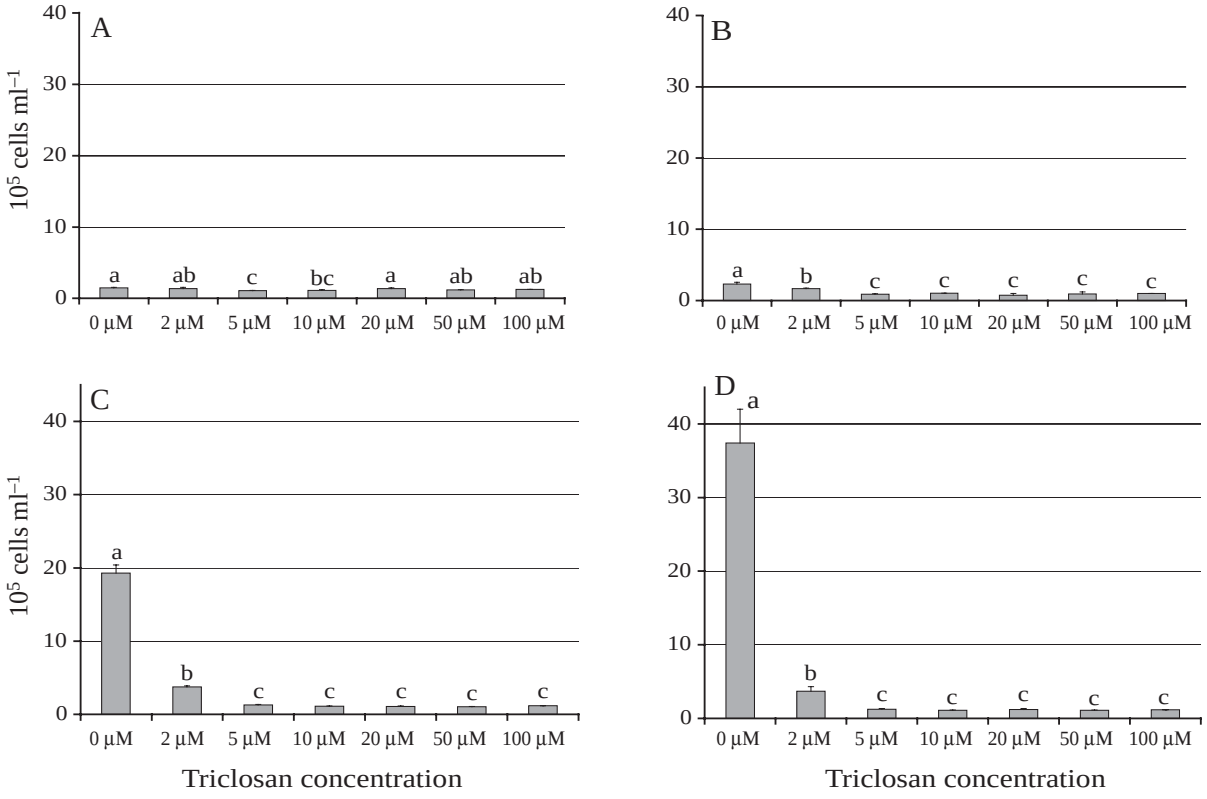

Fig. 1. *Perkinsus marinus.* Proliferation of *in vitro* cultures exposed to triclosan for (A) 3 h, (B) 24 h, (C) 3 d, (D) 6 d measured by flow cytometry using SYBR Green I/propridium iodide labeling (n = 3). Different letters indicate significant differences at the $p < 0.05$ level

Expt 3

Oyster hemocytes treated with triclosan exhibited mortalities of less than 10% at triclosan concentrations of <20 µM for up to 24 h (Fig. 2). No significant ($p < 0.05$) reduction in viability relative to the controls occurred at triclosan concentrations below 50 µM.

Expt 4

After 24 h triclosan exposure at 13°C, all *Perkinsus marinus* treatments exhibited significantly ($p < 0.05$) lower viability relative to the controls (Fig. 3). Incubation at triclosan concentrations ≥20 µM resulted in >50% mortality of *P. marinus* cells, and incubations at concentrations of ≥50 µM in almost 100% mortality after 24 h.

DISCUSSION

The results of the current study are very encouraging, since triclosan has been shown to inhibit proliferation of *Perkinsus marinus in vitro* at 28°C (a temperature that favors parasite proliferation) at concentrations as low as 2 to 5 µM (Table 1, Fig. 1), and to reduce cell viability at concentrations as low as 5 µM at both 28 and 13°C (Table 1, Fig. 2). In contrast, oyster hemocytes exhibited less than 20% mortality after 24 h exposure to triclosan concentrations below 50 µM at

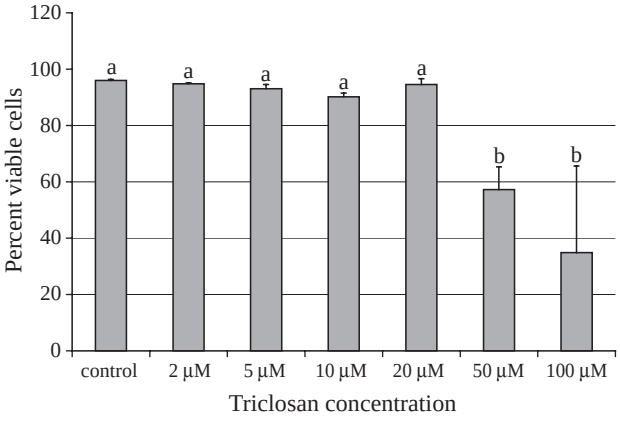

Fig. 2. *Crassostrea virginica.* Viability of *in vitro*-cultured oyster hemocytes exposed to triclosan for 24 h at 13°C (n = 3). Viability of hemocytes was determined using SYBR Green I/propridium iodide labeling. Different letters indicate significant differences in viability of treatments at the $p < 0.05$ level

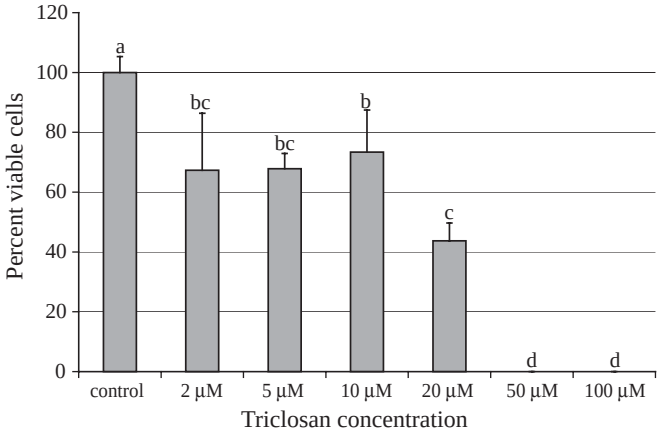

Fig. 3. *Perkinsus marinus.* Viability of *in vitro*-cultured meronts exposed to triclosan for 24 h at 13°C (n = 3). Viability of cultures determined using neutral red assay. Different letters indicate significant differences in viability of treatments at the $p < 0.05$ level

13°C (Fig. 2). This suggests that it may be possible to treat *P. marinus*-infected oysters with a concentration of triclosan that will kill the parasite without substantially harming the host oyster. To the best of our knowledge, no studies have yet been conducted on the toxicity of triclosan to any molluscan species. However, the 48 h EC_{50} of triclosan for *Ceriodaphnia dubia* has been determined to be 400 µg l^{-1}, and triclosan can significantly reduce survival of rainbow trout fry at a concentration of 71 µg l^{-1} (Orvos et al. 2002). As both these levels are below those tested in the current study (1.5 to 75 mg l^{-1}), long-term treatment may be potentially harmful to oysters. However, the high survival rate of oyster hemocytes exposed to triclosan for 24 h suggests that this drug may prove effective in treating this disease.

Although the results of Expts 1 and 2 revealed a similar pattern of decreasing cell proliferation and viability with increasing triclosan concentration, the threshold concentration for effective repression of *Perkinsus marinus* proliferation appeared to vary between the 2 experiments. In Expt 1, after 3 d, cell concentration and viability in the 2 µM treatment did not differ significantly from control values, and cell concentrations in the 5 µM treatment were still above inoculum level (Table 1). Only at 10 µM was proliferation completely inhibited (Table 1). In contrast, at Day 3 in Expt 2, all treatments with triclosan concentrations of 5 µM were equally effective in suppressing cell proliferation (Fig. 1). These apparent discrepancies may be the result of the lack of synchronicity of the *P. marinus* cultures used in the experiments and/or the different techniques used to measure cell concentrations in Expts 1 and 2. Since the goal of this study was to generate *in vitro* information for design of *in vivo* experiments using infected oysters, small inter-experiment variation in the effective minimum concentration does not diminish the significance of the results.

The attenuated activity of triclosan against *Perkinsus marinus* at 13°C (Fig. 3) relative to its activity at 28°C may be a function of either lower metabolic activity, or lower drug solubility at colder temperatures. While it may have been preferable, from the perspective of experimental design, to conduct the hemocyte assays in Expt 3 at 28°C, practical issues precluded this. The oysters used for this study had been procured from a cold-water environment and maintained at ambient water temperatures during the winter. Also, it is doubtful that the hemocytes could have been maintained *in vitro* for 24 h at elevated temperatures. Phagocytic activity of oyster hemocytes is reduced at temperatures >25°C (Chu & LaPeyre 1993). The greater tolerance of oyster hemocytes at 13°C relative to the level of triclosan tolerance of the parasite (Figs. 2 & 3) is encouraging and will stimulate further investigations at higher temperatures.

Previously, several studies have tested different chemotherapeutic agents for treatment of Dermo disease. Ray (1966a) tested 12 fungicides against *Perkinsus marinus* in infected oyster tissue by measuring chemotherapeutic inhibition of parasite enlargement in fluid thioglycolate medium (FTM). The most promising drug, cycloheximide, completely inhibited enlargement of *P. marinus* in FTM at concentrations between 0.25 and 1.0 µg ml^{-1}. Subsequent testing of cycloheximide on *P. marinus*-infected oysters demonstrated suppression of *P. marinus* proliferation in oysters at dosages of 1 to 50 µg ml^{-1} wk^{-1} and complete elimination of the parasite from infected oysters at 50 µg ml^{-1} wk^{-1} after 164 d (Ray 1966b). Calvo & Burreson (1994) screened 8 antifungal antibiotics, including cycloheximide, both *in vitro* with FTM and with *P. marinus*-infected oysters. Among the drugs tested, only treatment with cycloheximide (10 µg ml^{-1}) reduced parasite prevalence. Unfortunately, cycloheximide is now classified by the US Environmental Protection Agency as a suspected mutagen, making it unsuitable for use in treating *P. marinus*-infected oysters.

Faisal et al. (1999) were able to kill *Perkinsus marinus in vitro* and *in vivo* using Bacitracin. However, they encapsulated the Bacitracin in liposomes for drug delivery to the oysters, and a very high dosage of the drug (10 mg ml^{-1}) was required to be effective. Since liposomes are time-consuming to prepare and have a useful shelf-life of only a few days, this methodology may not be practical for use in hatchery or aquaculture operations. Furthermore, the large quantities of Bacitracin that would be required to treat hundreds or thousands of oysters would make the procedure prohibitively expensive.

Delaney et al. (2003) tested the effects of 2 N-halamine compounds, 1,3-dichloro-2,2,5,5-tetramethyl-4-imidaolidinone (DC) and 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC) on the viability of *Perkinsus marinus* cells in seawater. These compounds apparently damage the outer membrane of *P. marinus* cells, thereby inhibiting osmoregulation. DC induced 100% mortality of *in vitro*-cultured *P. marinus* cells after exposure to 44.6 mg l^{-1} for 8 h, while MC induced 98.6% mortality after 8 h exposure.

Most recently, Elandalloussi et al. (2005) tested the effects of 9 drugs on the viability of *Perkinsus olseni*, a parasite of the clam *Ruditapes decussatus*, *in vitro* and *in vivo*. Of the drugs tested, only deferoxamine (DFO), an iron chelator, was effective in reducing, but not eliminating, *P. olseni* infection levels in clams when applied at a concentration of 1 mg l^{-1} or higher. The effects of DFO on *P. marinus* and *Crassostrea virginica* are not known, although this would appear to be a promising drug therapy for Dermo disease worthy of further pursuit.

While the results of these previous studies are encouraging, the effective dosage levels of drugs in all these studies, with the exception of Elandalloussi et al.'s (2005) study, were considerably higher than those effective for triclosan. For comparison purposes, the 5, 10 and 20 µM triclosan concentrations used in the present study equate to 1.5, 3 and 6 µg ml^{-1}, respectively. These are >1000-fold lower than the lowest effective concentration of Bacitracin (10 mg ml^{-1}) reported by Faisal et al. (1999), and 1 order of magnitude lower than the effective dosages of the N-halamine compounds tested by Delaney et al. (2003). None of the drugs tested by Calvo & Burreson (1994) were effective against *Perkinsus marinus* at concentrations <10 mg l^{-1}. Furthermore, none of the previous studies, except that of Elandalloussi et al. (2005), tested whether the applied chemicals and dosages have toxic side-effects on the host cells.

Enzymes involved in the acquisition and synthesis of lipids in parasitic protozoans are particularly attractive drug targets. Several of the pathways used for lipid synthesis and acquisition by protozoan parasites differ from those utilized by their hosts (Coombs & Mueller 2002). For example, animals and some fungi use Type I fatty acid synthetase enzymes for fatty acid synthesis, while bacteria and plants employ Type II fatty acid synthetase enzymes. The Type I class differs from the Type II class in that the former comprise a single multienzyme complex, whereas the latter function as separate enzymes. This affords the potential of creating drugs that specifically target the parasite with minimal impact on the host.

Triclosan offers a high degree of pathogen-specificity because animals use the Type I class of fatty acid synthetase enzymes for fatty acid synthesis. Recent studies on triclosan have shown that the drug is a specific inhibitor of Fab1 (enoyl-acyl-carrier-protein reductase), an enzyme in the Type II class of fatty acid synthetases that are found in bacteria and plants, but not animals (Heath et al. 1998, Suguna et al. 2001, Perozzo et al. 2002). This property makes triclosan a potentially attractive chemotherapeutic agent that can kill pathogens that utilize Type II fatty acid synthetase enzymes without harming the host animal. *Perkinsus marinus* is unique among well-studied parasitic protozoans in that it synthesizes a wide range of polyunsaturated fatty acids (Chu et al. 2002). Some metabolites of these de novo-produced fatty acids apparently contribute to the virulence of this parasite. Its ability for de novo fatty acid synthesis and its morphological characteristic (the presence of a non-photosynthetic plastid in the biflagellate zoospore stage) suggest that this parasite uses Type II class synthetases (Soudant & Chu 2001, Chu et al. 2002). The finding that triclosan inhibits fatty acid synthesis by *P. marinus* at concentrations as low as 5 µM is consistent with the presence of a Type II fatty acid synthetase system in this parasite. Although the genus *Perkinsus* has been assigned to the phylum Apicomplexa (Levine 1988), recent genetic analysis and reexamination of the ultrastructure of the parasite suggest that its phylogenetic position is closer to the dinoflagellates than to apicomplexans (Fong et al. 1993, Flores et al. 1996, Reece et al. 1997, Siddall et al. 1997, Leander & Keeling 2004). Several recent studies have shown that the Type II fatty acid synthetases are also found in some protozoan parasites, including the apicomplexans *Plasmodium falciparum*, *Toxoplasma gondii* and *Trypanosoma* sp. (Waller et al. 1998, Kapoor et al. 2001, McLeod et al. 2001, Suguna et al. 2001, Surolia & Surolia 2001, Roberts et al. 2003). Triclosan has been demonstrated to inhibit both fatty acid synthesis and the proliferation of these parasites *in vitro* and *in vivo* (Beeson et al. 2001, McLeod et al. 2001, Surolia & Surolia 2001). Similarly, the present study has demonstrated that *P. marius* meront cells fail to proliferate at triclosan concentrations at or above 5 µM.

In summary, triclosan inhibits growth and greatly reduces cell viability of *in vitro*-cultured *Perkinsus marinus*. These effects are consistent with a mode of action in which triclosan acts as an inhibitor of Type II fatty acid synthetase which has been demonstrated in bacteria and several Apicomplexan protists (Heath et al. 1998, Kapoor et al. 2001, McLeod et al. 2001, Suguna et al. 2001, Surolia & Surolia 2001, Perozzo et al. 2002). The observation that triclosan kills *P. marinus* at low (5 to 20 µM) concentrations that have minimal effect on oyster hemocyte viability suggests that this drug may be useful in treating infected oysters. Further toxicology studies with infected and uninfected

oysters are required to ultimately determine the utility of using triclosan to treat Dermo infections in hatchery and research environs.

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