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Effects of tributyltin and hypoxia on the progression of *Perkinsus marinus* infections and host defence mechanisms in oyster, *Crassostrea virginica* (Gmelin)

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Abstract

Oysters, *Crassostrea virginica* (Gmelin), naturally infected by the protozoan parasite *Perkinsus marinus* in the field were exposed for 6 weeks to tributyltin (TBT), hypoxia, or to both stressors simultaneously. The TBT-exposed oysters continuously bioaccumulated TBT, reaching about 4 mg kg^{-1} dry weight by 6 weeks; hypoxic oysters were exposed to water containing an average dissolved oxygen level of about 3 mg L^{-1}. Untreated control oysters suffered about 30% cumulative mortality by 6 weeks as a result of the progression of their *P. marinus* infections. The TBT treatment alone produced no additional mortality; however, cumulative mortality in hypoxic oysters was elevated. Mortality among oysters receiving both TBT and hypoxia significantly exceeded that caused by either stressor alone, suggesting a synergistic effect. In an attempt to identify immunotoxicological mechanisms underlying stress-related augmentation of *P. marinus* infections, defence-related immune functions were measured at 3 and 6 weeks in control and treated oysters. In general, the total number of haemocytes increased as the infections progressed, and the TBT and hypoxic treatments also caused significant additional increments in some samples. However, oxygen-dependent (reactive oxygen species) and oxygen-independent (lysozyme) antimicrobial host defence mechanisms appeared to be largely unaffected by TBT and/or hypoxia. This may be explained by the death of those oysters with marked immunological lesions prior to sampling or by the actual lack of treatment effects.

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Introduction

Oysters and other estuarine species are often exposed to various anthropogenic and/or natural environmental stressors. It is possible that a consequence of such exposures is increased susceptibility to infectious disease via suppression of immunity. The present study was designed to test this hypothesis. Specifically, the effects of chronic exposure to tributyltin (TBT) and/or hypoxia on the progression of *Perkinsus marinus* infections and selected defence parameters were measured in the oyster, *Crassostrea virginica* (Gmelin). Organotin compounds are used as industrial catalysts, industrial and agricultural biocides, and components of antifouling marine paints. Seasonal depletion of oxygen in bottom waters is one of the more devastating stresses being placed on the biota of the Chesapeake Bay, USA. *P. marinus*, the aetiological agent of dermo disease, is a parasitic protozoan which is responsible for the virtual decimation of the *C. virginica* fishery in many areas of the East Coast of the USA (Burreson & Ragone Calvo 1996). As the disease progresses, the numbers of parasites in the haemolymph increase markedly, both free in the serum and contained within the haemocytes.

Haemocytes of *C. virginica* are known to possess potential defence mechanisms involving lysozyme,

other lysosomal hydrolases, stress response proteins, prophenoloxidase system, agglutinins and reactive oxygen species (ROS) (Anderson 1996). These mechanisms are apparently insufficient to control advanced *P. marinus* infections, which become systemic and lethal, but might influence the course of early infections. The presence of dead, fragmented *P. marinus* cells within oyster haemocytes suggests at least a limited ability to destroy these pathogens (Bushek, Allen, Alcox, Gustafson & Ford 1994; LaPeyre, Chu & Vogelbein 1995). Previous studies have shown that exposure of *C. virginica* to chemical stressors such as mammalian carcinogens (Winstead & Couch 1988), polycyclic aromatic hydrocarbon and metal-containing sediments (Chu & Hale 1994), and TBT (Fisher, Oliver, Sutton, Manning & Walker 1995; Anderson, Unger & Burreson 1996) enhances the progression and severity of dermo disease. Since TBT treatment of *C. virginica* haemocytes *in vitro* is known to reduce their ability to respond to ROS stimulators (Fisher, Wishkovsky & Chu 1990; Anderson, Brubacher, Ragone Calvo, Burreson & Unger 1997), the present authors hoped to find decreased ROS-generating capacity in haemocytes withdrawn from TBT-treated oysters that have apparently reduced resistance to dermo disease. Knowledge of the particular immune activities that are impaired by xenobiotic exposure and/or hypoxia might provide mechanistic understanding of the role of immunomodulation in the progression of *P. marinus* infections.

Materials and methods

Oysters

Native, naturally infected oysters were collected from the James River (Horsehead Rock), Virginia, USA, on 26 September 1995. An initial sample of 25 of these oysters was shown to be 100% infected with *P. marinus* by the thioglycollate tissue assay (Ray 1966); 15 oysters had light infections, six were moderate and four were scored as heavy.

Treatment groups

The oysters were separated into control, TBT-exposed, hypoxic or TBT-exposed plus hypoxic groups. Four replicate 30-L tanks, each containing 25 oysters, were established for each treatment and the control group. Tanks were maintained at 15 (the salinity at the collection site) and 25 °C in 1 μm of filtered water from the York River, Virginia. Algal paste (0.1 g oyster^{-1}) was added to the water daily (Monday to Friday), and the water was replaced three times a week (Monday, Wednesday and Friday). The TBT treatment tanks received daily (Monday to Friday) additions of tributyltin chloride (Aldrich Chemical Co., Milwaukee, WI, USA) to maintain an average nominal level of ≈ 50–100 ng L^{-1}. The TBT solutions to be added to the tanks were obtained by serial dilution of nearly saturated TBTCl solutions in water (no carrier solvent was used). Water samples were collected weekly immediately following and 24 h after TBT addition to monitor TBT exposure levels. Hypoxic conditions were produced by reducing aeration to the appropriate tanks; dissolved oxygen (DO) levels were monitored daily in all tanks using an YSI oxygen meter. On water renewal days, DO was measured before and after water renewal. Daily mortality logs were also kept for all tanks. The oysters were sampled at the initiation of the experiment, and after 3 and 6 weeks of treatment. On each date, oysters were removed from each replicate tank to form a composite sample of 20 individuals from each experimental group. The experiment was conducted at the Virginia Institute of Marine Science (VIMS), and on each sample date, oysters were removed from the aquaria, placed in a cooler with ice and transported to CBL (< 6 h) for assessment of haemolymph defence functions and initiation of thioglycollate cultures for *P. marinus* diagnosis. Oysters were maintained at CBL in aquaria (15 and 25 °C) until haemolymph sampling was completed (3–4 days). After haemolymph sampling was completed, whole oysters were frozen. Frozen oysters and thioglycollate cultures were subsequently transported to VIMS for determination of TBT tissue concentrations and diagnosis of *P. marinus*, respectively.

Tributyltin analysis

Water and tissue TBT concentrations were quantified by the methods of Unger, MacIntyre, Greaves & Huggett (1986) and Rice, Espourteille & Huggett (1987). Samples were diluted to 500 mL with deionized water and spiked with tripentyltin chloride internal standard. Samples were extracted with hexane/tropolone and the resulting extracts were derivatized with hexyl magnesium bromide,

cleaned up by open column chromatography with florisil and adjusted to final volume under dry nitrogen. Laboratory blanks were analysed with each batch of media samples. Extracts were analysed with a Varian model 3300 gas chromatograph equipped with a modified flame photometric detector. Standards were analysed daily prior to the samples to assure gas chromatograph performance.

Perkinsus marinus diagnosis

The *P. marinus* levels in haemolymph samples were determined by a method based on that of Gauthier & Fisher (1990). Haemolymph samples were withdrawn from the adductor muscle sinuses of individual oysters. Haemolymph (300 μL) was mixed with 1 mL fluid thioglycollate medium containing 500 U each of penicillin and streptomycin. The samples were transported to VIMS and incubated (7 days, 25 °C), the resultant hypnospores removed by centrifugation, incubated in 2 M NaOH (30 min at room temperature), resuspended in water and stained with Lugol's iodine solution. Samples were transferred to 24-well culture plates and the numbers of hypnospores mL^{-1} haemolymph sample were determined by light microscopy. The level of infection in each oyster was scored according to an arbitrary scale: (0) uninfected, (1) < 10 000, (3) 10 000–100 000 and (5) > 100 000 *P. marinus* cells mL^{-1} haemolymph. Samples with high abundance of hypnospores were serially diluted prior to quantification.

Haemocyte preparation

Haemolymph was withdrawn with a syringe from the adductor muscle sinus and placed in a small covered glass culture dish. The supernatant was decanted after 15 min and centrifuged to yield cell-free haemolymph (serum). The attached cells were gently washed with ≈ 5 mL ambient estuarine water which had been passed through a 0.2 μm filter (FA). The haemocyte monolayer was overlaid with ≈ 20 mL FA and incubated for 2.5 h at 20 °C. The supernatant was then poured off and the cells resuspended in FA by aspiration. The cells were concentrated by centrifugation (200 *g*, 15 min, 20 °C) and resuspended in a small volume (≈ 0.1 mL) of cell support medium. Cell support medium (CSM) contained 5% foetal bovine serum, 0.5% antibiotic/antimycotic solution (10 000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B mL^{-1}), and 1 mg mL^{-1} d-glucose in FA. The haemocyte density was determined in a haemocytometer and additional CSM was added as required to attain the desired final concentration for the assay in question.

Haemolymph defence functions

The total circulating haemocyte count (cells mL^{-1} haemolymph) was determined using a haemocytometer. Since haemocytes are thought to be of central importance to bivalve internal defence, the total number in circulation at any given time could be taken as an indication of the host's ability to resist infection.

Nitroblue tetrazolium (NBT) reduction was used to quantify intracellular superoxide anion (O_2^-) production by haemocytes. A haemocyte suspension (2×10^6 mL^{-1} 15 CSM) was prepared as described above. The NBT (0.2% in PBS) solution was passed through a 0.45 μm filter and used only on the day of preparation. Phorbol 12-myristate 13-acetate (PMA) stock (2 mM) in dimethylsulfoxide (DMSO) was diluted with 15 CSM to give a 1 μM working stock. A DMSO/KOH solution was made by mixing 6 mL 7 mL DMSO plus 2 M KOH. The NBT reduction was carried out in 15 mL conical glass centrifuge tubes as follows. The PMA-stimulated NBT reduction (in duplicate) was quantified after mixing 1 mL of haemocytes (2×10^6 cells in CSM), 1 mL 0.2% NBT, 22 μL 1 μM PMA and 178 μL CSM. The NBT reduction in the absence of PMA (in duplicate) was measured after mixing 1 mL of haemocytes (2×10^6 cells in CSM), 1 mL 0.2% NBT and 200 μL CSM. Reagent controls consisted of 1 mL 0.2% NBT, 22 μL 1 μM PMA and 1.178 mL CSM (stimulated control), and 1 mL 0.2% NBT and 1.2 mL CSM (unstimulated control). The tubes were incubated (20 °C, 60 min) with agitation at 15-min intervals. The tubes were then centrifuged (540 *g*, 20 °C, 10 min), supernatants discarded and 2 mL 70% methanol was added to each tube. After vigorous mixing, the tubes were centrifuged (540 *g*, 20 °C, 10 min), 1.25 mL DMSO/KOH solution was added and the cells were resuspended by vortexing. The tubes were again centrifuged (540 *g*, 20 °C, 10 min) and the OD of the supernatants read versus a DMSO/KOH blank at 625 nm. The PMA-stimulated and unstimulated cellular NBT reduction values were

corrected by subtracting the values from the corresponding reagent (cell-free) controls. The PMA-stimulated NBT reduction by the haemocytes was determined by subtracting the corrected unstimulated from the corrected stimulated values.

Lysozyme levels were measured in fresh cell-free haemolymph (serum) samples or in frozen samples after storage at –70 °C. A 0.25 mL serum sample was mixed with 2.0 mL of a *Micrococcus luteus* (Sigma Chemical Co., St Louis, MO, USA) suspension (0.2 mg mL^{-1} 0.05 M HEPES, pH 6.8). The OD was measured at 1-min intervals for 5 min at 540 nm versus 0.05 M HEPES and the δOD min^{-1} was recorded. The lysozyme concentration was determined from a standard curve of δOD min^{-1} for egg white lysozyme concentrations from 0 to 20 μg mL^{-1}.

Statistics

The data were statistically analysed by a Kruskal–Wallis non-parametric analysis of variance (ANOVA), followed by Dunn's multiple comparisons test or Fisher's exact test, as indicated in the 'Results' section. Differences of $P < 0.05$ or less were considered significant.

Results

Tributyltin levels

Tributyltin levels in the water in the control tanks remained at ≈ 2 ng L^{-1} throughout the study. In the TBT-dosed tanks, the initial aqueous concentrations after the addition of TBT ranged from 67 to 140 ng L^{-1} and the mean value throughout the study was ≈ 50% of these maximal values.

Oyster body burdens (μg kg^{-1} dry weight) were measured at the start of the experiment, and at 3 and 6 weeks post-exposure (Table 1). Tributyltin (≈ 400 μg kg^{-1} dry weight) was present in the control oysters as a result of existing environmental contamination. Throughout the present paper, the term 'TBT-exposed' will refer to oysters experimentally exposed to TBT, while it is understood that all oysters had a background level of the compound in their tissues. Hypoxic conditions produced slightly elevated background TBT tissue levels at both sample times, but this was significant only in the 3-week sample ($P < 0.05$). Tributyltin exposure produced large increases in body burden and continued uptake over the course of the experiment resulted in a significantly higher level at week 6 ($P < 0.05$). Hypoxic, TBT-exposed oysters accumulated higher mean TBT levels than aerated, TBT-exposed oysters, but this effect was not significant ($P = 0.064$).

Dissolved oxygen levels

Reduced DO conditions were achieved by reducing aeration to a low rate for the hypoxic group and the hypoxic TBT-exposed group. Immediately after water changes, DO levels were saturated in all treatment and control tanks; however, within 4 h, the DO in the air-flow-restricted tanks fell to a low level which was maintained until the next water change. Control and TBT-exposed groups had mean DOs over the 6-week study of 7.036 ± 0.471 ng L^{-1} (92 readings) and 6.983 ± 0.532 (92), respectively. However, the average DOs for the hypoxic and TBT/hypoxic groups were 2.859 ± 1.578 (92) and 3.209 ± 1.740 (79), respectively. Therefore, this variable aeration procedure resulted in statistically significant hypoxia ($P < 0.0001$).

Intensity of *P. marinus* infections and mortality

The oysters used in the present study were infected by *P. marinus* (2.3 ± 1.6, mean diagnostic score) at the start of the experiment (Table 2). The water for

Table 1 Oyster tissue tributyltin (TBT) levels. Values are mean ± SD μg kg^{-1} dry weight (*n*)

	Sample time		
	Prior to exposure	3 weeks	6 weeks
Control	453.3 ± 35.1 (3)	410.0 ± 36.5 (4)	427.5 ± 50.6 (4)
Hypoxia	–	647.5 ± 97.8 (4)	517.5 ± 239.8 (4)
TBT	–	1450.0 ± 173.2 (4)	3000.0 ± 890.7 (4)
TBT/hypoxia	–	2750.0 ± 1870.0 (4)	5000.0 ± 1539.5 (4)

Table 2 Progression of *Perkinsus marinus* infection in oyster haemolymph samples, as influenced by tributyltin (TBT) and/or hypoxia. Diagnostic scores (intensity of infection) reflect the number of *P. marinus* mL^{-1} haemolymph: (0) uninfected; (1) < 10000, 3 = 10 000–100 000; and (5) > 100 000. Values are mean scores ± SD (*n*)*

	Sample time		
	Prior to exposure	3 weeks	6 weeks
Control	2.3 ± 1.6 (21)	2.3 ± 1.5 (18)[a, b]	4.1 ± 1.3 (24)[a]
Hypoxia		3.2 ± 1.1 (19)[b]	3.4 ± 1.6 (21)
TBT		2.7 ± 1.6 (20)[c]	3.8 ± 1.3 (21)[c]
TBT/hypoxia		2.8 ± 1.4 (18)	3.6 ± 1.6 (21)

*Numbers followed by the same superscript letter are statistically different from each other ($P < 0.05$).

all groups was maintained at 15 and 25 °C for the course of the experiment; these conditions favour progression of the infection. In fact, the intensity of infection increased between 3 and 6 weeks for all groups; these increases were statistically significant for the control and TBT-treated groups ($P < 0.01$). Within samples, the only treatment that significantly affected intensity of infection was hypoxia, which stimulated *P. marinus* progression at 3 weeks.

A rising level of mortality was also recorded for the oysters during the present study (Fig. 1). In the untreated (control) group, cumulative mortality increased from 2% at week 1 to 33% by week 6. Statistical analysis of cumulative mortality data was carried out using Fisher's exact test. Significant mortality differences between the controls and TBT-treated oysters were not observed. Mortality was higher in the hypoxic group than in the controls, but this difference was significant only at 28 and 35 days ($P < 0.05$). However, oysters in the TBT-exposed plus hypoxia group consistently had a much higher mortality than the controls ($P < 0.05$ on day 14; $P < 0.001$ on all other days).

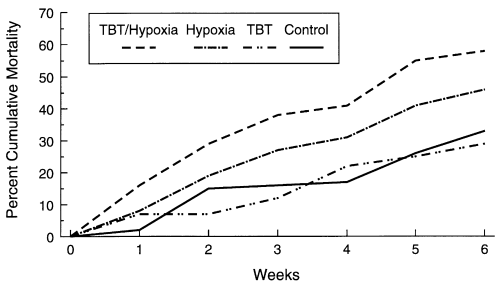

Figure 1 Effects of hypoxia and/or tributyltin on percentage cumulative mortality of *Crassostrea virginica* associated with the progression of *Perkinsus marinus* infection.

Haemocyte counts, intracellular O_2^- production, and serum lysozyme levels

Data on these defence-related parameters are presented in Table 3. The total haemocyte counts for each of the groups at 6 weeks exceeded the counts recorded for those groups at 3 weeks; however, the increases were only significant ($P < 0.05$) for the control and TBT-treated oysters. All treatments caused apparent haemocyte recruitment, but significant increases relative to the control within each sample date were seen only in hypoxic and TBT-treated oysters at 3 and 6 weeks, respectively. The NBT reduction by the haemocytes and serum lysozyme levels showed no significant changes between 3 and 6 weeks. The experimental treatments did not affect these parameters, with the exception of the TBT treatment group which showed significantly reduced O_2^- generation after 3 weeks of exposure.

Discussion

A previous study of the effects of sublethal TBT exposure on laboratory-initiated *P. marinus* infections indicated chemically induced disease enhancement, but produced little direct evidence of immunotoxicity (Anderson *et al.* 1996). The present paper describes the effects of sublethal TBT and/or hypoxia on field-initiated *P. marinus* infections, and uses different methods to quantify potentially antimicrobial immune mechanisms. Tributyltin is a recognized toxicant with important biological impacts and has been included on the *Toxics of concern list* (U.S. Environmental Protection Agency, Chesapeake Bay Program 1991). Tributyltin exposure produces immunotoxicity in mammals (Snoeij, Penninks & Seinen 1987), as

Table 3 Defence-related activities in oysters exposed to tributyltin (TBT) and/or hypoxia. Values are mean scores ± SD (*n*)

	Total haemocyte count ($\times 10^6$)	PMA-stimulated NBT reduction (Abs_{625})	Serum lysozyme ($\mu g\ mL^{-1}$)
Prior to exposure	3.16 ± 1.90 (22)	0.058 ± 0.021 (8)	14.3 ± 5.6 (13)
Samples at 3 weeks			
Control	2.74 ± 0.82 (20)[a, b]	0.072 ± 0.036 (12)[e]	11.8 ± 7.1 (12)
Hypoxia	3.99 ± 1.44 (19)[b]	0.067 ± 0.047 (11)	10.7 ± 3.9 (12)
TBT	3.55 ± 1.83 (20)[c]	0.025 ± 0.038 (11)[e]	13.3 ± 4.7 (12)
TBT/hypoxia	3.72 ± 2.81 (17)	0.051 ± 0.035 (8)	7.7 ± 4.8 (12)
Samples at 6 weeks			
Control	3.75 ± 1.99 (24)[a, d]	0.033 ± 0.028 (12)	11.1 ± 3.9 (12)
Hypoxia	4.64 ± 2.32 (22)	0.048 ± 0.029 (12)	12.1 ± 4.2 (12)
TBT	5.57 ± 3.65 (20)[c, d]	0.056 ± 0.027 (12)	10.6 ± 5.0 (12)
TBT/hypoxia	4.58 ± 2.60 (20)	0.033 ± 0.028 (12)	0.4 ± 5.2 (12)

*Numbers followed by the same superscript are statistically different from each other ($P < 0.05$).

well as ROS inhibition in blood phagocytes of fish (Rice & Weeks 1990) and shellfish (Fisher *et al.* 1990; Anderson *et al.* 1997). Low dissolved oxygen levels, sometimes < 1 mg L^{-1} in bottom waters during the summer months, in the Chesapeake Bay and its tributaries have caused declines in habitat for living resources, as well as direct toxic effects on fish and shellfish. Although the effect of hypoxia on dermo disease progression in oysters has not previously been experimentally examined, several lines of evidence suggest a possible relationship. During exposure to low oxygen, oyster tissues become hypoxic, hypercapnic and acidotic. The normal pH of *C. virginica* haemolymph is ≈ 7.7, but declines to ≈ 7.2 when infected with *P. marinus*; minimal hypoxic stress for 5 h caused a rapid decline in pH to 6.7 in infected oysters versus 7.3 in healthy oysters (Dwyer & Burnett 1996). Oysters rendered hypoxic by removal from water eventually lose their ability to hold their valves closed; this 'time to gaping' is reduced in *P. marinus*-infected animals (Paynter 1996). These data suggest a correlation between hypoxia, acid-base physiology and *P. marinus* infection in oysters.

In the current study, oysters exposed to TBT showed tissue levels ≈ 10-fold higher than pre-exposure after 6 weeks. Hypoxic conditions resulted in an average oxygen level of ≈ 3 mg L^{-1}, while non-hypoxic tanks had ≈ 7 mg L^{-1}. While not significantly different, oysters in hypoxia treatment groups accumulated higher TBT body burdens than oysters in the respective aerated treatment groups. Similar trends have been described for the uptake of endrin across the gills of brook trout under reduced dissolved oxygen conditions (McKim 1994). As oxygen levels decreased, pumping rates increased to maintain oxygen consumption, and thereby, more contaminant-containing water was passed over the gills.

The effects of these treatments, alone or in combination, on mortality as well as levels of infection and immune parameters of survivors were measured. At the start of the experiment, all of the oysters had *P. marinus* infections, resulting in ≈ 30% mortality in the controls by week 6. The TBT alone treatment had no additional effect on mortality; however, hypoxia produced higher mortality after 4 weeks exposure, and cumulative mortality in the TBT plus hypoxia group was elevated compared to controls for the entire 6 week course of the study. These results suggest that hypoxic conditions enhance dermo disease lethality and that TBT, at sublethal concentrations, exacerbates this effect. Thus, the concept of synergistic effects of multiple stressors seems to be reinforced.

The intensity of infection in all groups of the surviving oysters increased during the present study, indicating disease progression, as reflected by the increasing mortality. However, treatment effects between groups at the sample times were not pronounced, probably because of the death of heavily infected oysters. A positive correlation between disease progression and numbers of circulating haemocytes had been noted previously (Anderson, Burreson & Paynter 1995). This effect was also seen here where the cell counts in all groups tended to rise with time. Furthermore, the average cell counts in all of the treatment groups in each of the two samples were greater than controls; this difference was significant for the hypoxic group at 3 weeks and the TBT group at 6 weeks.

In mammalian cells, ROS are involved in antimicrobial defence mechanisms and are thought to play a major role in the control of infectious diseases (Klebanoff 1985; Babior 1988). Similar functions have been ascribed to ROS generated by oyster haemocytes, although direct evidence for this is limited. The level of ROS production by oyster haemocytes is very low in comparison to vertebrate leucocytes, and may be relatively ineffective in oyster antimicrobial responses to bacteria (Bramble & Anderson 1998). However, appropriate immunological stimulation of *C. virginica* haemocytes will trigger a large burst of ROS activity (Adema, van der Knaap & Sminia 1991; Anderson 1994), thus making ROS assays common components of suites of tests of immunocompetence. It should be noted that, whereas treatment of haemocytes with classical mammalian leucocyte stimuli such as zymosan or PMA will trigger ROS, phagocytosis of *P. marinus* by *C. virginica* haemocytes does not (LaPeyre *et al.* 1995). In a previous paper (Anderson *et al.* 1996), ROS production by zymosan-stimulated haemocytes from TBT-exposed oysters was measured by luminol-augmented chemiluminescence (LCL). In mammalian leucocytes, this technique measures activity of the myeloperoxidase (MPO)-halide-hydrogen peroxide (H_2O2) antimicrobial system (Dahlgren & Stendahl 1983; Albrecht & Jungi 1993). The same pathway is probably present in bivalve haemocytes which have been shown to contain MPO (Schlenk, Garcia Martinez & Livingstone 1991; Coles & Pipe 1994) and produce H_2O2 upon stimulation (Nakamura, Mori, Inooka & Nomura 1985; Pipe 1992; Friedl & Alvarez 1992). Furthermore, LCL activity is reduced in haemocytes treated with MPO inhibitors (Noël, Bachère & Mialhe 1993). The present authors have previously reported that, although TBT exposure increased the severity and lethality of experimental *P. marinus* infections, it had little effect on LCL responses; the only significant LCL modulation was a function of the level of *P. marinus* infection (Anderson *et al.* 1996). In the present paper, ROS production was measured using a different stimulus and by another method. Immunological stimulation was provided by PMA, a soluble ligand that reacts with cell surface receptors; ROS production was measured by NBT reduction. This method specifically quantifies the level of intracellular O generation, and like LCL, has been directly correlated with bactericidal capacity of leucocytes (Anclair & Voisin 1985). The current results showed no effect of hypoxia on NBT reduction and TBT exposure produced a transient decrease in NBT reduction at 3 weeks; however, no change was seen in the TBT/hypoxia exposure group. Increased NBT reduction was not associated with increased severity of *P. marinus* infection, as had been expected based on previous studies of LCL responses under similar conditions. This may be explained by the differences in infection levels seen in these two studies: average diagnostic score ranged from 0 to 3.5 in the first study and from 2.3 to 4.1 in the present research. ROS responses differ more significantly between haemocytes from uninfected and moderately to heavily infected oysters than between lightly to moderately infected oysters (Anderson *et al.* 1995).

The antimicrobial enzyme lysozyme can be detected in *C. virginica* serum and haemocytes, and has been implicated as playing a role in surviving *P. marinus* infections (LaPeyre, Chu & Ragone 1989; Chu & LaPeyre 1993; Chu, LaPeyre & Burreson 1993). In the present study, no significant changes in serum lysozyme levels were associated with experimental treatments or intensity of *P. marinus* infections. However, in the dual stressor (TBT and hypoxia) groups, the lysozyme concentrations appeared lower than controls ($P < 0.07$), these stressed animals also showed significantly higher mortality than the controls. The TBT treatment alone had no effect on lysozyme, in agreement with earlier findings (Anderson *et al.* 1996).

In conclusion, while it seems reasonable to speculate that the functional capacity of the immune system of *C. virginica* plays a role in host responses to *P. marinus* (Cheng 1987), it was difficult to demonstrate this with the experimental approaches used in the present study. It seems clear that exposure to chemical stressors (i.e. TBT) can enhance natural or experimental *P. marinus* infections and that exposure to multiple stressors (TBT and hypoxia) can exacerbate this effect. Furthermore, oyster haemocytes show various forms of immunosuppression after *in vitro* exposure to sublethal levels of several environmental contaminants (including TBT). However, the same signs of immunotoxicity are apparently lacking in surviving oysters which had been exposed *in vivo* to stressors at concentrations that increase the progression and lethality of dermo disease. It is possible that oysters with pronounced immunological lesions succumb

to the lethal effects of the disease, and thus, are excluded from further analysis.

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