Effects of tributyltin and other metals on the phenoloxidase activating system of the tunicate, *Styela plicata*

Niina Tujula, Jane Radford, Sham V. Nair, David A. Raftos *

Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia

Abstract

Toxic metals, such as tributyltin (TBT), contribute substantially to anthropogenic pollution in many estuarine environments. Animals that live in those environments, particularly invertebrate filter feeders like tunicates, are likely to be exposed to substantial metal contamination. This study investigates the effects of TBT and other metals on the phenoloxidase activity of the estuarine tunicate, *Styela plicata*, in an effort to identify a biochemical marker of metal pollution. Hemocytes harvested from *S. plicata* that were exposed to tributyltin or copper in aquaria had significantly enhanced phenoloxidase activities relative to non-exposed controls. This enhanced phenoloxidase activity could be explained by an increased frequency of morula cells, which contain high levels of phenoloxidase's proenzyme, prophenoloxidase. Unlike those from tunicates exposed to metals in aquaria, the phenoloxidase activities of hemocytes incubated with tributyltin in vitro were significantly reduced when compared with hemocytes cultured without tributyltin. The ability of tributyltin to decrease phenoloxidase activity in tissue culture may reflect its known inhibitory effects on calcium-dependent signaling systems such as those involved in the exocytosis of prophenoloxidase from morula cells. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Tributyltin (TBT) was used extensively during the last century to control the growth of sessile marine organisms on the hulls of pleasure craft and commercial shipping (Langston et al., 1987; Clarke et al., 1988). This biocide is now restricted in its use as the active component of anti-fouling paints in many countries (Evans et al., 1995). As a result, TBT concentrations have declined in the water column. However, TBT can remain stably bound to marine sediments for long periods and it is still used on larger vessels (> 25 m) (Evans, 1999; Evans et al., 1995). Potentially toxic contamination persists in many locations to the extent that tolerable average residue levels for TBT in a variety of marine organisms were exceeded in 9 out of 22 countries surveyed recently (Dowson et al., 1993; Hardiman and Pearson, 1995; Batley, 1996; Terlizzi et al., 1998; Venkatesan et al., 1998; Belfroid et al., 2000; Hall et al., 2000; Jacobsen and Asmund, 2000; Rilov et al., 2000).

* Corresponding author. Tel.: + 61-2-98508402; fax: + 61-2-985082451.

E-mail address: draftos@rna.bio.mq.edu.au (D.A. Raftos).

The effects of TBT have been studied in many aquatic animals including teleost fish, shrimp, crabs, mollusks and tunicates (Waldock and Thain, 1983; Bryan et al., 1986; Batley et al., 1989; Langston and Burt, 1991). In tunicates and mollusks, organotin complexes (mono-, di- and tri-butyltin) affect embryonic development, differentiation and host defence mechanisms (Mansueto et al., 1993; Cima et al., 1996a,b, 1998a; Gianguzza et al., 1996). Immunotoxic effects of TBT, reflected by altered phagocytic activity, chemotaxis or reactive oxygen species generation, have been identified in the bivalve mollusks, *Crassostrea gigas* (Anderson et al., 1997; Fisher et al., 1999), *Mytilus edulis*, *Mya arenaria*, *Mactromeris polynyma* (Bouchard et al., 1999) and *Tapes phillinarum* (Cima et al., 1998b, 1999), and in the tunicates, *Styela plicata* (Raftos and Hutchinson, 1997), *Ciona intestinalis* (Cooper et al., 1995) and *Botryllus schlosseri* (Cima et al., 1995, 1997). Copper, another biocidal metal often used as an antifoulant, has also been shown to suppress tunicate pharyngeal cell proliferation and to reduce the cytotoxic activity of tunicate hemocytes (blood cell equivalents) (Raftos and Hutchinson, 1997).

A number of authors have suggested that the detrimental effects of TBT or copper on tunicate host defence reactions arise from altered cellular structure and function (Raftos and Hutchinson, 1997; Cima and Ballarin, 2000; Cima et al., 1998c,d). Cima et al. (1998c,d) have shown that TBT alters the morphology and phagocytic activity of hemocytes from *B. schlosseri* by interfering with intra-cellular calcium homeostasis. Similarly, monoclonal antibodies against hemocyte surface antigens have been used in conjunction with histological analysis in *S. plicata* to demonstrate that TBT and copper affect the frequency and morphology of hemocytes, particularly morula cells (Radford et al., 2000). Morula cells are known to express mechanisms for sequestering iron or vanadium, which are essential for physiological purposes, within electron dense granules (Anderson and Swinehart, 1991; Taylor et al., 1994). TBT significantly increases the frequency and size of these electron dense granules implying that they may also be sites of TBT sequestration (Radford et al., 2000).

After exposure to TBT, morula cells in *S. plicata* become concentrated in the pharynx (the major feeding and respiratory organ of tunicates) where they congregate at the single layer pharyngeal epithelium. Electron dense vesicles also appear in the non-ciliated columnar epithelial cells that make up the immediate interface with the surrounding seawater (Radford et al., 2000).

Previous studies have shown that the granules within morula hemocytes also contain the pro-enzyme, prophenoloxidase (proPO) (Jackson et al., 1993; Arizza et al., 1995; Ballarin et al., 1998; Hata et al., 1998). ProPO is released from hemocytes by an active degranulation process that can be stimulated by inflammatory agents such as zymosan. Once released, proPO is proteolytically converted to its active form, phenoloxidase (PO), which is the central component of an enzyme cascade that has been identified in both crustaceans and tunicates (Söderhäll and Smith, 1985; Arizza et al., 1995; Hauton et al., 1997; Hughes, 1999). The major function of the PO cascade is deposition of the pigment, melanin, from tyrosine-based substrates such as L-dopa. Components of the PO cascade can also opsonize pathogens for phagocytosis, whilst others appear to be fungistatic or cytotoxic.

The apparent co-localization of proPO with metal induced, electron dense granules in morula cells suggests that metal contamination may affect PO activity. Here, we test that suggestion by assessing the PO activities of hemocytes that have been incubated with metals in vitro or harvested from tunicates exposed to TBT or copper in aquaria.

2. Materials and methods

2.1. Tunicates

Adult *S. plicata* (25–30 g wet weight) were collected from several locations in Sydney Harbor, New South Wales (NSW), Australia. The collecting locations were floating marinas mooring approximately 300 pleasure craft (< 25 m) at Birkenhead Point, shark netting at Balmoral Beach, boat mooring buoys at Sugarloaf Bay and a submerged rock platform at Quakers Hat Bay.

Tunicates were maintained in polyethylene aquaria filled with fresh seawater collected from The Spit, Sydney. A minimum of 10-l seawater was provided per tunicate, with a maximum of four tunicates per aquarium. Aquaria were held at 15°C under constant aeration. Tunicates were equilibrated in aquaria for at least 24 h prior to metal exposure, and were fed with 1 ml Invertebrate Food Supplement per aquarium every 48 h (Aquasonic Pty Ltd, Ingleburn, NSW).

Standard water quality parameters (pH, salinity, conductivity, turbidity, dissolved oxygen) were determined at all collecting sites and in aquaria using a Horiba Water Quality Checker U10 (Australian Scientific Pty. Ltd, Sydney, NSW). No substantial differences in these water quality parameters were detected between collecting sites and aquaria (data not shown).

2.2. Hemocyte collection

Tunicates were bled from incisions in their incurrent siphons. The exuding hemolymph was collected into ice chilled sterile tubes containing a 5-fold excess of filtered seawater from The Spit (FSW; 0.45 μm filter) or a 5-fold excess of tunicate tissue culture medium (T-RPMI). T-RPMI was prepared in FSW and contained 18% v/v MultiCel RPMI (without L-glutamine; Trace Biosciences, Sydney, NSW), 0.4% w/v NaCl, 40 μg/ml streptomycin sulfate and 100 IU/ml penicillin sulfate (Raftos and Cooper, 1990). Cell densities were determined using an Improved Neubauer counting chamber.

2.3. Cytology and cell sorting

To isolate different hemocyte sub-populations, 1×10^7 hemocytes were centrifuged ($200 \times g$, 5 min, 4°C) and resuspended in 1 ml marine anticoagulant (MAC; 26.3 g/l sodium chloride, 4.4 g/l tri-sodium citrate, 2.73 g/l citric acid, 3.72 g/l EDTA, pH 7.0). The cells were then sorted, based on their forward versus 90° light scatter characteristics, with a FACSCalibur cell sorter (Becton Dickinson, North Ryde, NSW). The parameters used for sorting are shown in Fig. 2A. Sorted cells were washed immediately by centrifugation, and then resuspended in FSW.

When required, 40-μl hemocytes or cell sorted hemocyte populations (2×10^5 cells per ml) were centrifuged (5 min, $100 \times g$) onto glass microscope slides using a cytocentrifuge. The cells were then stained with Giemsa so that the frequency of different hemocyte types could be determined by the classification scheme of Radford et al. (1998).

2.4. Exposure of hemocytes to metals in vitro

Hemocytes were harvested into T-RPMI (2×10^6 cells per ml) and aliquoted (100 μl per well) into 96 well flat-bottomed cell culture plates (Trace Biosciences, Sydney, NSW). Cultures were maintained at 15°C without CO_2 supplementation. Hemocyte viabilities were determined by ethidium bromide exclusion quantified with a single laser FACScan flow cytometer (Becton Dickinson) by the method of Raftos and Hutchinson (1997).

For in vitro exposures, a variety of potentially toxic metals (in 10 μl FSW per well) were added to cultured hemocytes. The metals were, TBT oxide (ICN Chemicals, Sydney, NSW; 0.001–10 μg/l), tin chloride (AJAX Chemicals, Sydney, NSW; 0.001–100 μg/l), copper sulfate (Jaeger Chemicals, Sydney, NSW; 0.001–1 mg/l), cadmium chloride (BDH Chemicals Ltd, Poole, England; 0.001–1 mg/l), mercuric chloride, lead nitrate (May and Baker Ltd, Dagenham, England; 0.001–1 mg/l) and zinc sulfate (AJAX Chemicals; 0.001–1 mg/l). After exposure to metals, cultured hemocytes were analyzed by the PO assay described below. All experiments also included hemocytes that were held in culture for 48 h in the absence of metals (designated 'nonexposed hemocytes').

2.5. Exposure of tunicates to TBT and copper in aquaria

Tunicates were exposed to TBT and copper in aquaria for 48 h according to the method of Raftos and Hutchinson (1997). The doses used (TBT, 1–5 μg/l; copper 1–25 mg/l) were sublethal over an 8-day period, but were known to induce immunotoxic effects (Raftos and Hutchinson, 1997).

TBT and copper were added to aquaria from concentrated stock solutions 48 h before tunicates to allow diffusion and partitioning throughout the tank. In all experiments, groups of tunicates were also held in aquaria containing seawater without added metals (designated 'nonexposed tunicates').

Water taken from aquaria 48 h after the addition of metals was analyzed by the CSIRO Centre for Advanced Analytical Chemistry (Lucas Heights, NSW). Atomic absorption spectroscopy implied that less than 2% of the original doses of TBT remained in solution after 48 h. At this time, approximately 0.2% of the initial TBT doses appeared as dibutyltin or monobutyltin. Water taken from aquaria without added metals contained 1.5 ng/l TBT. Approximately 10% of the initial doses of copper remained in solution after 48 h. Ten micrograms per liter copper were detected in water taken from aquaria with no added metals.

After exposure to metals, tunicates were incised and hemocytes were harvested into FSW. The cells were then washed by centrifugation ($300 \times g$, 5 min, 4°C), resuspended in FSW (2×10^6 cells per ml) and tested for PO activity (as described below).

2.6. Phenoloxidase assay

Phenoloxidase activity was quantified according to Aspán and Söderhäll (1995). In this assay, the exocytosis of proPO was elicited by zymosan and proPO was converted to active PO by trypsin. The results of optimization procedures to adapt this assay for *S. plicata* are shown in Fig. 1. In the optimized assay, hemocyte suspensions (100 μl per well, 2×10^6 cells per ml in either FSW or T-RPMI) were incubated with 10 μl zymosan A (from *Saccharomyces cerevisiae*; 1 mg/ml in FSW; Sigma Chemicals, St. Louis, MO) for 15 min (room temperature) in 96 well tissue culture plates (Trace Biosciences). Trypsin (10 μl per well, 1 mg/ml in FSW; Sigma Chemicals) was then added for 5 min, followed by L-β-3,4-dihydroxyphenylalanine (L-dopa; 65 μl per well; 3 mg/ml in FSW; ICN Biomedicals, Costa Mesa, CA).

Twenty minutes after the addition of L-dopa, color development, reflecting the formation of dopachrome, was measured spectrophotometrically at 490 nm. Phenoloxidase activity was taken to be absorbance at 490 nm less the absorbance of wells containing hemocytes, zymosan and trypsin but no L-dopa.
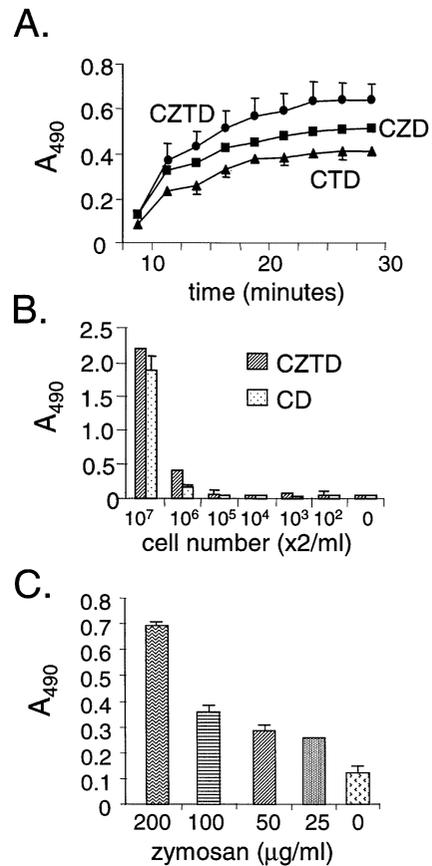

Fig. 1. Optimization of PO assay. (A) Formation of dopachrome (A_{490}) at various times after the addition of 250 μg/ml L-dopa to cells that had been incubated with 54 μg/ml zymosan + 54 μg/ml trypsin (CZTD), 54 μg/ml zymosan with no trypsin (CZD), or 54 μg/ml trypsin with no zymosan (CTD). Bars represent S.E.M., $n = 6$. (B) Effect of cell numbers on the formation of dopachrome (A_{490}) by hemocytes incubated with either 54 μg/ml zymosan + 54 μg/ml trypsin + 250 μg/ml L-dopa (CZTD) or 250 μg/ml L-dopa alone (CD). Absorbances were measured 20 min after the addition of L-dopa. Bars represent S.E.M., $n = 6$. (C). Effect of zymosan concentration on the formation of dopachrome (A_{490}) by hemocytes incubated with 2×10^6 cells per ml + 54 μg/ml trypsin + 250 μg/ml L-dopa. Absorbance was measured 20 min after the addition of L-dopa. Bars represent S.E.M., $n = 6$.

Hemocytes from at least three different tunicates were tested in each experiment. For in vitro exposures, triplicate wells were established from each tunicate. Controls included wells containing L-dopa but no hemocytes; hemocytes and L-dopa but no zymosan or trypsin; and hemocytes, zymosan and trypsin but no L-dopa.

2.7. Statistical analysis

Statistical analysis was performed with the Microsoft Excel software package. Mean values were calculated as averages of PO activities in ≥ 3 hemocyte suspensions taken from different tunicates. The statistical significance of differences between mean values were determined by Student's two-tailed *t*-test. Differences were considered to be significant for probabilities (P) < 0.05.

3. Results

3.1. Optimization of phenoloxidase assay

The effects of varying three different parameters of the PO assay (incubation time after the addition of L-dopa, cell density and zymosan concentration) are depicted in Fig. 1. Fig. 1A reveals that maximum PO activity was achieved when cells were incubated with zymosan, trypsin and L-dopa, relative to cells incubated with only zymosan and L-dopa or those incubated with only trypsin and L-dopa. Dopachrome formation (A_{490}) by all of these combinations became asymptotic within 25–30 min after the addition of L-dopa.

Maximum absorbance (A_{490}) was evident when assays were performed with 2×10^7 cells per ml (Fig. 1B). However, at this cell density spontaneous PO activation in wells containing only cells and L-dopa was almost equivalent to that of wells containing cells, zymosan, trypsin and L-dopa. In contrast, the spontaneous activation of PO in wells containing 2×10^6 cells per ml was only one-third that evident in wells containing 2×10^6

cells per ml in the presence of zymosan, trypsin and L-dopa.

Fig. 1C shows that PO activity was also dependent on zymosan concentration. PO activity did not vary substantially between lower zymosan concentrations (25–100 µg/ml), even though PO activities at all of these lower doses were significantly greater than those of controls performed in the absence of zymosan ($P < 0.05$). Far greater increases in PO activity were achieved at higher zymosan concentrations ($P < 0.05$ for 200 vs. 100 µg/ml).

The aim of this optimization procedure was to select zymosan concentrations, incubation times and cell densities that yielded intermediate PO activities so that both enhancement and suppression of PO activity could be detected in subsequent experiments. Hence, the optimized assay used 2×10^6 cells per ml, 54 µg/ml zymosan and was incubated with L-dopa for 20 min. Variation in trypsin and L-dopa concentrations had far less effect on PO activity than the other parameters (data not shown). Hence, the concentrations of trypsin and L-dopa used in other studies were adopted for the optimized assay (54 µg/ml trypsin, 250 µg/ml L-dopa; Aspán and Söderhäll, 1995).

3.2. Localization of PO activity

Three discrete hemocytes sub-populations were identified by flow cytometry based on their distinct forward versus 90° light scatter characteristics (Fig. 2A). Population 1 had high 90° scatter and variable forward scatter. Cytological analysis of sorted cells revealed that this sub-population was comprised of 79% morula cells (Fig. 2B). Population 2 was characterized by low 90° and forward scatter, whilst population 3 had variable forward scatter and intermediate 90° scatter. Neither population 2 nor 3 had substantial frequencies of morula cells (4 and 6%, respectively).

PO assays of sorted cells revealed that the vast majority of PO activity was associated with population 1, which also contained the majority of morula cells (Fig. 2B). Neither of the other cell populations had substantial PO activities.
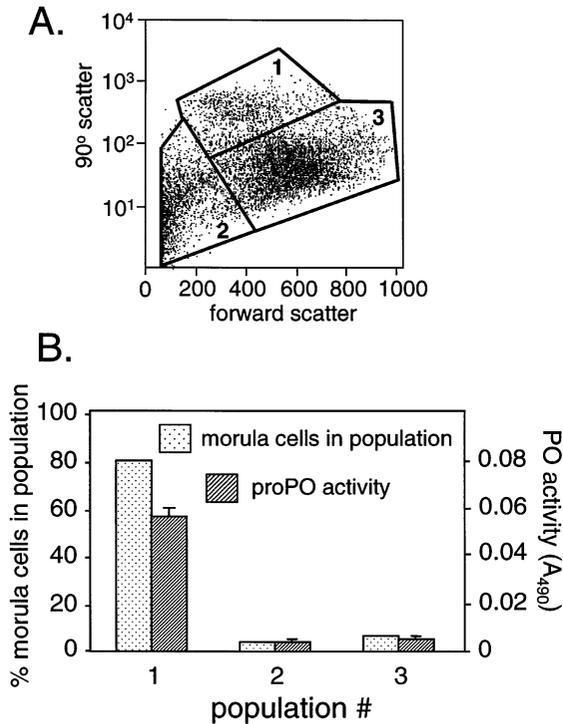
A.

B.

Fig. 2. (A) A forward vs. 90° light scatter dot plot of hemocytes from *S. plicata*. The gates used to sort the three distinct hemocytes sub-populations (designated 1–3) are shown. (B) The percentage of each of the hemocyte sub-populations identified in 2A that comprised morula cells and the PO activities of each of those sub-population measured after cell sorting. Bars represent S.E.M., $n = 3$.

3.3. In vitro exposure of hemocytes to metals

In vitro exposure of hemocytes to TBT resulted in a dose dependant decrease in PO activity (Fig. 3). Significant inhibition was evident at TBT concentrations > 0.1 μg/l ($P < 0.05$ vs. nonexposed hemocytes). The PO activity of cells exposed to 10 μg/l TBT was reduced to 40% of that evident in nonexposed hemocytes ($P < 0.05$). In contrast, copper, zinc, cadmium, lead and mercury did not significantly alter PO activity relative to nonexposed hemocytes at any of the doses tested ($P > 0.05$) (Fig. 4). Comparison of the effects of TBT and inorganic tin chloride indicated that only the organic form (TBT) significantly altered PO activity in vitro (Figs. 3 and 4). Tin chloride did not affect PO levels when compared with nonexposed

hemocytes at any of the concentrations tested ($P > 0.05$).

Fig. 5 shows that the inhibition of PO activity by TBT was dependent on the period of in vitro exposure. Significant differences between TBT-exposed and nonexposed hemocytes were evident after 24 h ($P < 0.05$). Cells exposed to TBT for 72 h had only 25% of the PO activity evident among nonexposed hemocytes.

Exposure to metals did not significantly ($P > 0.05$) alter the viability of *S. plicata* hemocytes held in culture for 72 h (data not shown).

3.4. Exposure of tunicates to TBT and copper in aquaria

Fig. 6 shows the pooled results from two aquarium exposures using tunicates collected from Sugarloaf Bay and Birkenhead Point. Animals exposed to TBT had PO levels four times greater than nonexposed tunicates ($P < 0.05$), whilst the PO activity of hemocytes from tunicates exposed to copper was 5.5 fold greater than nonexposed controls ($P < 0.05$). These differences were only evident when zymosan, trypsin and L-dopa were included in PO assays. No significant differences were observed between metal-exposed and nonexposed tunicates when zymosan and trypsin, or L-dopa, were excluded from PO assays.
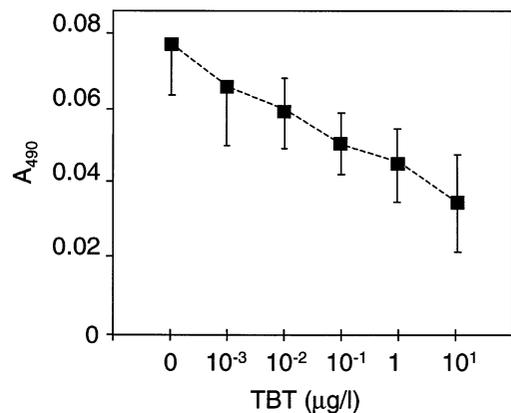

Fig. 3. The effect of incubation with varying concentrations of TBT for 72 h on the PO activity (A_{490}) of cultured *S. plicata* hemocytes. Bars represent S.E.M., $n = 6$.
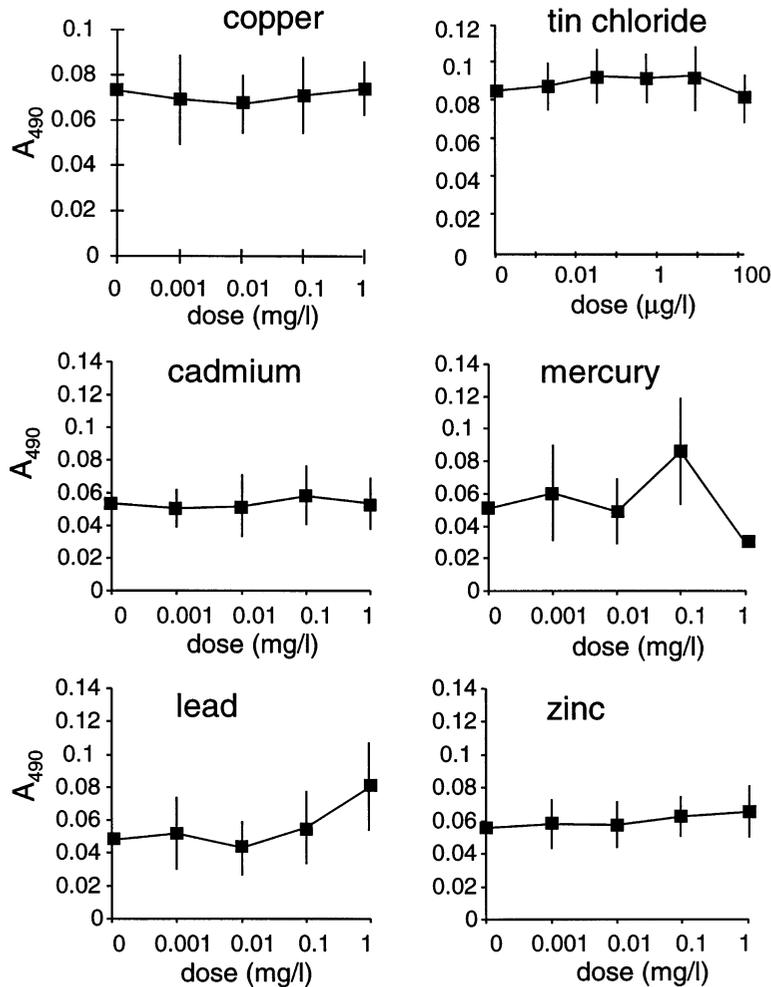
Fig. 4. The effect of incubation with varying concentrations of different metals for 72 h on the PO activity (A_{490}) of cultured *S. plicata* hemocytes. Bars represent S.E.M., $n = 6$.

The effects of exposure to both TBT and copper in aquaria were dose dependent (Fig. 7). PO activities in tunicates exposed to TBT increased with dose such that 2 and 5 µg/l TBT elicited PO levels that were significantly greater than those of nonexposed tunicates. In contrast, the PO activities of tunicates exposed to 1 µg/l TBT did not differ significantly from those of nonexposed tunicates. Similarly, tunicates exposed to 5 and 25 mg/l copper had significantly elevated PO activities relative to nonexposed animals ($P < 0.05$), whilst those exposed to 1 mg/l copper had PO activities that did not differ significantly from nonexposed controls ($P > 0.05$).

Fig. 8 shows that exposure to TBT in aquaria also increased both the number of hemocytes in the hemolymph and the relative frequency of morula cells. When compared with tunicates that had been held in aquaria without added metals, three times as many hemocytes per ml of hemolymph were evident in tunicates exposed to 5 µg/l TBT for 48 h ($P < 0.05$). The relative frequency of morula cells in the hemolymph was 2-fold greater in TBT-exposed tunicates than in untreated controls ($P < 0.05$).
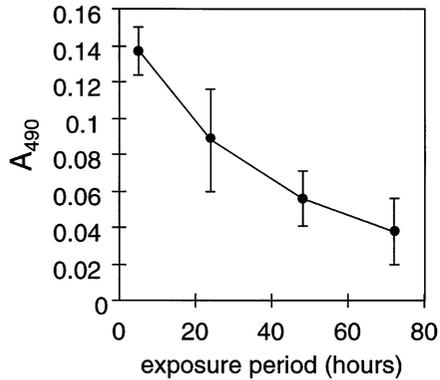
Fig. 5. PO activity (A_{490}) of cultured hemocytes incubated with 10 µg/l TBT for different periods. Bars represent S.E.M., $n = 6$.

4. Discussion

This study has shown that the PO activities of tunicate hemocytes are altered by exposure to some metal-based pollutants. Exposure of tunicates to TBT or copper in aquaria increased PO activity relative to nonexposed animals. Responses were dose dependent in the range 1–5 µg/l for TBT and 1–25 mg/l for copper. Analysis of water from aquaria revealed that only 2% of the TBT added to the aquaria was in solution and available to tunicates at the beginning of the
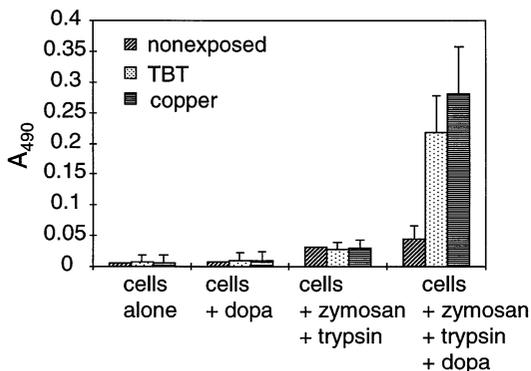

Fig. 6. PO activity (cells + zymosan + trypsin + L-dopa, A_{490}) of hemocytes from nonexposed tunicates or tunicates that had been exposed in aquaria to 5 µg/l TBT or 25 mg/l copper for 48 h. Also shown are absorbance values (A_{490}) for cells alone, cells incubated with L-dopa only and cells incubated with zymosan and trypsin but no L-dopa. Bars represent S.E.M., $n \geq 8$.
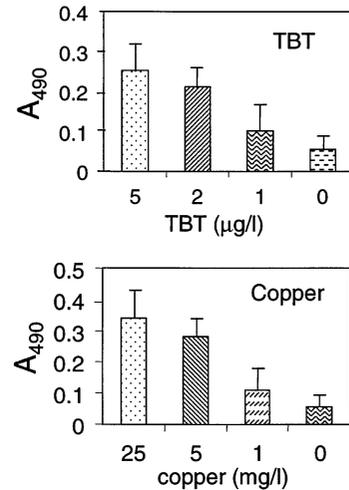

Fig. 7. PO activities (A_{490}) of hemocytes from tunicates that had been exposed in aquaria to varying concentrations of TBT or copper for 48 h. Bars represent S.E.M., $n = 3$.

exposure period. Hence, the dose range used here for TBT would yield soluble concentrations of TBT similar to those reported in some estuarine environments. TBT concentrations > 2 µg/l were reported before its use was restricted in the late 1980s, and levels > 100 ng/l still occur in some locations (Waldcock and Miller, 1983; Michel and Averty, 1999). This implies that effects of TBT on PO activity could be detected among tunicates in their native environment. Unlike TBT, the levels of copper remaining in solution at the beginning of exposures (approximately 10% of the initial dose) substantially exceed commonly reported environmental values (Batley, 1996).
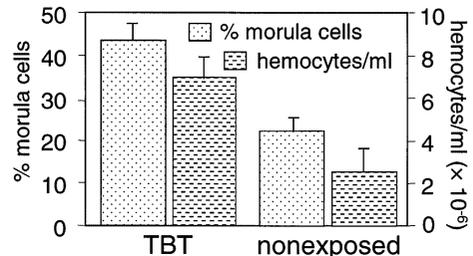

Fig. 8. Relative frequencies of morula cells (as percentages), and the total number of hemocytes per ml, in the hemolymph of either nonexposed tunicates or tunicates exposed to 5 µg/l TBT for 48 h. Bars represent S.E.M., $n = 6$.

The ability of TBT or copper exposure in aquaria to enhance PO activity is in direct contrast to data from in vitro experiments in which hemocytes from nonexposed tunicates were incubated with metals in tissue culture. TBT was found to significantly inhibit PO activity in vitro whilst copper and other metals had no discernible effect. Even though in vitro culture conditions may not have been amenable to the uptake of copper and other metals, the difference between aquarium and in vitro exposures can be most easily explained in terms of hemocyte frequencies. Ballarin et al. (1998) have demonstrated that proPO is localized in morula cells, and the current study has shown that maximum PO activity occurs in sorted cell populations that contain high frequencies of morula cells. We have also shown that exposure to TBT in aquaria significantly increases the relative frequency of morula cells in the hemolymph. This is in agreement with the observations of Radford et al. (2000) who have demonstrated that the relative frequency of morula cells, which contain both proPO and metal induced electron dense granules, is increased 2–3 fold by exposure to TBT in aquaria. Hence, it is possible that in vitro analysis of static hemocyte populations harvested from nonexposed tunicates could reveal a direct inhibitory effect of TBT on PO activity, whilst aquarium exposures, during which large numbers of morula cells are recruited into the circulating hemolymph, leads to an enhancement in overall PO level, despite the decreased PO activity of individual hemocytes.

The inhibitory effect of TBT on PO activity in vitro is probably related to its known sub-cellular toxicities. TBT interferes with cellular calcium homeostasis in tunicates by inhibiting the sub-cellular calcium regulator, calmodulin (Cima and Ballarin, 2000; Cima et al., 1998c,d). TBT's inhibitory effect on calmodulin disrupts sub-cellular signaling systems involving Ca^{2+}-ATPase activity and so can alter tubulin associated cytoskeletal events (Cima and Ballarin, 2000). Recent studies in our laboratory have shown that exocytosis by *S. plicata* hemocytes is dependent upon both Ca^{2+}-ATPase activity and tubulin microtubule assembly (Raftos, unpublished data). Hence, the effects of TBT on sub-cellular events are likely to disrupt the exocytosis of proPO containing granules, and in doing so, decrease the amount of proPO released per hemocyte.

5. Conclusion

This study has demonstrated that TBT and copper can affect the PO activity of tunicate hemocytes. Since the PO system has often been implicated in immunological defence in invertebrates, the effects of TBT and copper may have direct impacts on disease resistance (Fisher et al., 1999). The ability to detect significant alterations in PO activity among metal-contaminated tunicates also suggests that PO assays might provide rapid inexpensive tests for environmental stress.

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