Hemocyte functions and bacterial clearance affected *in vivo* by TBT and DBT in the blue mussel *Mytilus edulis*

S. D. St-Jean^{1,2}, É. Pelletier^{2,*}, S. C. Courtenay¹

¹Fisheries and Oceans Canada, Gulf Fisheries Centre, PO Box 5030, Moncton, New Brunswick E1C 9B6, Canada

²Institut des sciences de la mer à Rimouski (ISMER), Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski, Québec G5L 3A1, Canada

ABSTRACT: The effects of tributyltin (TBT) and dibutyltin (DBT) on the hemocyte functions of the blue mussel *Mytilus edulis* were investigated in 2 separate experiments at 2 different cold periods of the year. In Expt 1, we exposed mature mussels to waterborne TBT or DBT (from 5 to 80 ng Sn l⁻¹) under flow-through conditions. The mussel hemocytes were monitored for membrane injury (MI), phagocytic activity (PA), lysosome retention (LR) and hemocyte count (HC) over 32 d. Both TBT and DBT significantly affected all cellular functions measured. MI was present as early as Day 1 for TBT doses >10 ng Sn l⁻¹ and DBT >20 ng Sn l⁻¹ in Expt 1, and by Day 32 all doses of TBT and all DBT doses >5 ng Sn l⁻¹ had produced significant MI increases. PA was reduced by all doses of TBT and DBT in a time- and dose-responsive manner from Day 1 for TBT >10 ng Sn l⁻¹ and by Days 18 to 32 for all doses of both chemicals. LR was significantly elevated by DBT dose of 80 ng Sn l⁻¹ between Days 1 and 4 but not thereafter. Significant elevations and depressions in HC were observed in mussels exposed to both butyltins, although effects were highly variable and not clearly related to either dose or time of exposure. To confirm our first observations, Expt 2 was carried out using mussels exposed to 40 and 80 ng Sn l⁻¹ TBT or DBT over 13 d under the same experimental design. The hemocytes were monitored for: (1) PA using both microscopic and spectrophotometric observations; (2) LR using 2 incubation times; and (3) HC in hemolymph from individual and pooled mussel samples. The general trends of Expt 1 results were confirmed. In addition to hemocyte assays, mature mussels from Expt 1 as well as mature and juvenile mussels from Expt 2 were injected with the common bacterium *Listonella* (= *Vibrio*) *anguillarum* after 32 and 13 d of exposure, respectively, to TBT or DBT concentrations described above. For both butyltins, a significant dose-related impairment in clearing *Vibrio* from the hemolymph was observed for all doses in both experiments. Clearance of bacteria was slower for the juveniles than for the adults suggesting that the early life stages of mussels may be more affected by butyltin exposure than adults. This study established strong and sustained responses of hemocyte functions of blue mussels exposed to environmentally relevant, waterborne concentrations of TBT and DBT (0.04 to 0.67 nM as Sn) which is interpreted as an increase of their vulnerability to other environmental stressors and pathogens, even at low seawater temperatures, (<5°C) often present in high latitude coastal waters.

KEY WORDS: Blue mussels · Butyltins · TBT · DBT · Hemocytes · Membrane injury · Bacterial clearance · Phagocytosis · Lysosome retention · Bioaccumulation

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INTRODUCTION

Internal defense in bivalves relies largely on circulating hemocytes and a wide range of nonimmuno-

globulin serum proteins to identify and eliminate potential pathogens. Hemocytes are often the primary targets of many contaminants, which has serious consequences for their homeostatic capacity (Auffret &

Oubella 1997). As a consequence, any toxicant that interferes with hemocyte functions may increase vulnerability of targeted organisms to other environmental stressors and ultimately expression of disease. Therefore, the study of the physiology and defense mechanism of this system is crucial in assessing the toxicity associated with xenobiotics in the coastal environment, where bivalves often constitute a major contribution to the local and national economy.

Tributyltin (TBT) is still used worldwide as an antifoulant in marine paints and is accumulated within the tissues of biota (Fent 1996). Despite a partial ban imposed on boats <25 m in most industrialized countries during the 1980s, hotspots still remain in areas of intense shipping activity (Waite et al. 1991). TBT concentrations up to 133 ng Sn l⁻¹ were reported for Danish coastal waters (Kure & Depledge 1994), between 14 and 28 ng Sn l⁻¹ in Alexandria Harbor (Abd-Allah 1995) and up to 105 ng Sn l⁻¹ in wastewater from Zurich, Switzerland (Fent & Hunn 1995). Some French coastal locations still showed TBT levels of >36 ng Sn l⁻¹ in 1997 (Michel & Averty 1999). Concentrations of butyltins in the order of ng l⁻¹ are known to cause deleterious effects to marine biota. Best-documented examples include: mussel larval mortality (Beaumont & Budd 1984); shell abnormalities in the oyster *Crassostrea gigas* (Alzieu et al. 1981); imposex in neogastropod molluscs (Bryan et al. 1987); and disruption of oxidative phosphorylation in all animals tested (Fent 1996).

Only a few studies have related immunomodulations in bivalves to TBT exposure. Chemiluminescence (an indirect indicator of phagocytosis), locomotion of hemocytes and time to spread were shown to be modulated by *in vitro* exposure to TBT (Fisher et al. 1990). Furthermore, Fisher et al. (1995) showed the virulence of the protozoan pathogen *Perkinsus marinus* in the oyster *Crassostrea virginica* to be correlated to TBT exposure. Anderson et al. (1996) also reported similar results. In addition, hemocyte aggregation, which is associated with the internal defense of bivalves, was disrupted in *C. gigas* (Auffret & Oubella 1997) as well as *Mytilus edulis* (Oubella, 1997) following *in vitro* exposure to TBT. Although these and other studies have provided important information, the relationships between *in vivo* exposure to butyltins and immunocompetence or immune functions over time in bivalves have not yet been described.

Beyond exploring the impacts of the butyltins TBT and DBT on hemocyte functions and comparing their relative toxicities to blue mussels, we were interested in the field relevance of these changes and in determining whether butyltin-induced alterations of hemocyte functions translate into a reduced ability to clear bacteria from the hemolymph. Various marine *Vibrio* spp. are known to be a threat to mollusk husbandry (Elston

1984). Jeffries (1983) reported that pathogenic *Vibrio* could cause up to 90% mortalities of oyster larvae within 24 h. It is generally accepted that infection of adult bivalves by bacteria, including *Vibrio*, in the natural environment is rare (Nottage & Birkbeck 1990, Lambert & Nicholas 1998). However, McHenery & Birkbeck (1986) reported that 8 *Vibrio* species rapidly inhibited filtration by adult *Mytilus edulis* while *Escherichia coli* and *Pseudomonas* and 7 other bacterial species were readily ingested by the mussels. More recently, it was shown that a range of *Vibrio* strains induced an inhibition of chemiluminescence, associated with the respiratory burst utilized in bacterial degradation, in adult mussel hemocytes (Lambert & Nicholas 1998). Finally, Lane & Birkbeck (1999) reported that very small numbers of *Vibrio* induced cell rounding, thus preventing movement, in adult mussel hemocytes that had previously been allowed to attach to the plastic surface of a tissue culture plate. Although *Vibrio* spp. are not recognized as a pathogen of the adult mussel, we selected *Listonella* (= *Vibrio*) *anguillarum* as a model invader for our bacterial challenge.

The 3 main objectives of this study were: (1) to describe changes in hemocyte functions in blue mussels exposed continuously to various doses of waterborne TBT and DBT over 2 and 4 wk periods; (2) to compare the relative toxicity of TBT and DBT; and, (3) to describe the relationships between altered hemocyte functions of mussels exposed to TBT and DBT and their ability to clear bacteria from their hemolymph. To achieve these objectives while permitting repeated sampling of the same individuals over an extended period of time, end-points measured were: hemocyte count (HC), phagocytic activity (PA), lysosome retention (LR), membrane injury (MI) and clearance of bacteria. A first experiment was carried out for a 32 d period with doses of TBT and DBT ranging from 5 to 80 ng Sn l⁻¹. To confirm the observed results, and also to examine possible bias introduced by analytical techniques used in Expt 1, Expt 2 was carried out in which mussels were exposed to 40 or 80 ng Sn l⁻¹ TBT or DBT over 13 d. Specifically, we examined: (1) the effects of pooling hemolymph from different mussels on HC and MI; (2) the difference between spectrophotometric and microscopic techniques on PA; (3) the influence of incubation time on LR; and (4) the responses of adult and juvenile mussels to the bacterial inoculation. Chemical analyses were carried out at the end of both experiments to quantify bioaccumulation of butyltin species.

MATERIALS AND METHODS

All chemicals were certified grade for the dyes or pesticide grade for the solvents and were purchased

from Sigma Chemical. A stock solution of Tris-buffered saline (TBS), pH 8.4, was used as the dilution medium for the dyes, and consisted of 17.3 ml of 0.1 N HCl made up to 100 ml with distilled water added to 25 ml of 0.2 M aqueous Trizma base. NaCl (2.5% w/v) was added to maintain osmolarity between 1000 and 1200 mOsm (Grundy et al. 1996). The TBS solution was filtered (0.2 μ m) before each sampling day.

Expt 1. Mussels: Mature blue mussels *Mytilus edulis* between 6.5 and 8.5 cm in shell length were obtained in March 1998 from a local fisherman at Grande Digue Bay (southern Gulf of St. Lawrence, NB) and immediately transported under optimum conditions to the laboratory facilities in Rimouski, Qc. Mussels were then cleaned of all epibionts and allowed to acclimatize at 4 to 5°C in a flow-through aquarium for 2 wk. Two d prior to the beginning of the exposure, the mussels were divided among 11 flow-through aquaria (7.5 l) supplied with non-filtered seawater pumped from St. Lawrence Estuary (mean salinity 28) and flow rate was established at 13 l h⁻¹ ensuring a rapid renewal of water in each aquarium. Thirty mussels were placed in each aquarium, 5 groups of 4 animals were randomly selected and put into separate mesh bags (labeled for subsequent identification). The other 10 mussels remained untouched for the duration of the experiment and were used only for the bacterial challenge.

Exposure: Stock solutions of 2.68 μ g Sn l⁻¹ in ethanol were prepared for each butyltin species and from these stock solutions, distribution of contaminants to each aquarium was made with peristaltic pumps to obtain final concentrations of 5, 10, 20, 40 and 80 ng Sn l⁻¹ for TBT and DBT corresponding to molar concentrations of 0.04, 0.08, 0.16, 0.33 to 0.67 nM as Sn. To minimize adsorption of the butyltins on tubing and aquarium walls and maximize bioavailability to mussels, dried Spiruline (Sigma) was added to the stock solution in a 10:1 ratio (ng of algae:ng of butyltin). This addition contributed very little to feed the mussels and seawater pumped from the St. Lawrence Estuary contained no phytoplankton cells during the course of this experiment, which is considered normal for that winter period of the year. Mussels were sampled on Days 0, 1, 4, 11, 18, 25 and 32.

Hemolymph extraction: Preliminary sampling indicated that an average of 2.5 ml of hemolymph per mussel could be extracted in total. Therefore, to minimize experimental stress, no more than 0.5 ml was extracted from each mussel at each sampling. The mussel valves were gently pried open around the byssus, any mantle fluid was drained, a sterile 20 gauge needle was inserted directly in the posterior adductor muscle sinus and 0.5 ml hemolymph was extracted into a dry 3 ml syringe and divided into 4 subsamples: 0.1 ml for the MI assay, 0.1 ml (fixed immediately with Baker's for-

mol) for the hemocyte counting with a hemocytometer, 0.1 ml for the LR assay and 0.2 ml for the phagocytosis assay. The operation was repeated for the 4 mussels contained in each bag and hemolymph was then pooled for a total of 5 pools of 4 individuals each per aquarium and each sampling day.

Lysosome retention (LR): LR was evaluated by using neutral red dye following the technique described by Lowe & Pipe (1994). Aliquots of 100 μ l pooled hemolymph were added to 200 μ l 2% neutral red dye in TBS, in a sterile centrifuge tube, and incubated at room temperature for 5 h. The cells were then washed with TBS, fixed 2% glutaraldehyde in TBS, and centrifuged at 800 $\times g$ for 10 min. The fixative solution was then removed with a Pasteur pipette, 1 ml of TBS was added and the preparation was stored cold in the dark. Prior to the readings, TBS was removed and 1 ml extraction solution consisting of 1% (v/v) acetic acid plus 50% (v/v) ethanol in distilled water was added for 30 min (Grundy et al. 1996). Neutral red retained by the hemocytes was displaced by the addition of the extraction solution and measured at 540 nm on a Perkin-Elmer spectrophotometer, calibrated with the extraction solution. Data reported are optical densities and were standardized to 10⁶ cells.

Membrane injury (MI): Quantification of MI to hemocytes was carried out with a nigrosine dye test. The dark blue dye enhances the contrast of the outer cell membrane and enables detection of abnormal cell shape. Pooled hemolymph (100 μ l) was placed in a clean well, overlaid with an equal volume of 0.2% nigrosine in TBS and incubated at room temperature for 1 to 5 min. The percentage of damaged cells was calculated from 150 cells examined under a phase contrast microscope at 40 $\times$ magnification.

Phagocytic activity (PA) and hemocyte count (HC): The fluorescent dye, fluorescein-isothiocyanate (FITC) suspended in TBS solution, was added to the yeast zymosan type 1 in excess, and incubated for 2 h at room temperature with periodic mixing. The labeled yeast were then washed repeatedly with TBS until the supernatant contained no yellow fluorescence. Subsamples of 1 ml were then frozen at -20°C until used. Densities of the labeled yeast were ascertained in all samples with a fluorescence microscope and ranged from 4 to 5 $\times 10^7$ ml⁻¹.

Hemocyte numbers in 0.4 ml hemolymph, pooled from mussels at 0.1 ml each and adjusted to 1 ml, were determined using an Improved Neubauer hemacytometer. Fluorescent yeast were then added to the hemocyte solution in a 100:1 density ratio and left to incubate at room temperature in the dark for 5 h, after which they were evaluated for PA. To prevent dehydration of samples, 0.5 ml of a 4% Bovine Serum Albumin (BSA) solution diluted in RPMI 1640 (Sigma) was

added to each sample. To distinguish yeast that had been phagocytosed from yeast attached to the external cell membrane, 1 ml of a 0.05 % ethidium bromide (EB) solution was added to the sample just prior to microscope observation. The EB reacted quickly with the FITC of unphagocytized yeast, which lowered its wavelength from a bright green to a mat green; all other cells and debris were colored bright red. Fluorescent zymosans inside phagocytes were protected by the membrane and thus remained bright green. The activity of the first 300 hemocytes encountered was characterized as either active (phagocyte full of zymosans with little free space in cytoplasm) or inactive (<10 % of cytoplasm contains zymosans). Results are expressed as a percentage of active cells. The presence of aggregated cells was also recorded.

Bacterial challenge: Preparation of stock bacteria: A pure strain of *Listonella* (= *Vibrio*) *anguillarum* was obtained from the University of Prince Edward Island (PEI, Canada) and maintained on marine agar. To ascertain that the bacteria counted after the challenge were *L. anguillarum* and not from the natural bacterial population, we progressively rendered the original strain resistant to 2 antibiotics: streptomycin and rifampicin, and then maintained the resistant strain on marine agar containing 500 mg l⁻¹ of streptomycin and 100 mg l⁻¹ rifampicin. Prior to inoculation of the mussels, an inoculum of *L. anguillarum* was transferred into an antibiotic-free solution of marine broth (Difco) and left to grow at room temperature for 24 h, at which point the bacterial culture had reached the exponential growth phase.

Bacterial number estimation: The concentration of the bacteria (10⁷ ml⁻¹) at the exponential phase was established by using the most probable number (MPN) technique. This technique consists of preparing a decimal dilution series from a known volume of bacterial solution in a 96 multi-well sterile microplate. The wells were examined visually for the lowest concentration to contain bacteria. To prevent contamination of the wells, 250 mg l⁻¹ of streptomycin, 100 mg l⁻¹ rifampicin and 10 mg l⁻¹ of cyclohexamide, a fungicide, were added. To verify that only *Vibrio* could grow in the wells, we intentionally contaminated several culture tubes containing the same antibiotic/fungicide concentration. No growth was noted in the test tubes after 7 d of incubation at room temperature.

Bacterial clearance experiment: Tests were carried out on 10 mussels in each aquarium that had been exposed to various doses of TBT or DBT for 32 d or not exposed (controls), but not manipulated in any previous assay. An optimal volume of 0.5 ml bacterial suspension (5 × 10⁶ bacteria) was injected in the visceral mass of each mussel. The mussels were then closed tightly with elastic bands to avoid any leaking and left

out of the water at cool room temperature (12°C) for 2 h. The mussels were then freed and permitted to purge themselves in clean flow-through aquaria for 4 d, before sampling commenced. The mussels were tested for bacterial clearance 4 and 14 d after the inoculation by drawing 0.5 ml of hemolymph from each individual. From each sample, 3 aliquots of 20 µl were used to assess the bacterial levels remaining in each mussel by using the MPN technique, and the mean of these samples was used for the statistical analysis.

Chemical analysis: At the end of the experiment, soft tissue from 20 mussels in each aquarium was blended and the homogenate was divided into 3 subsamples, which were analyzed separately. The results are reported as an average ±1 SD of 3 replicates for each sample. The techniques used to quantify butyltin concentrations have been developed previously by the same laboratory (St-Louis et al. 1997, St-Jean et al. 1999). A Finnigan MAT ion-trap detector (ITD) splitless coupled to a Perkin-Elmer Model 8500 gas chromatograph (GC) were used with a DB5 fused silica capillary column (30 m × 0.25 mm in diameter). Butyltins were extracted from dry samples by hexane/tropolone solution, ethylated with NaBEt₄, purified on a silica gel column and quantified from a standard curve established with known concentrations of ethylated TBT and DBT.

Expt 2. Mussels: Mature blue mussels (100) between 6.5 and 8 cm in shell length and 50 juvenile blue mussels between 1 and 2 cm in shell length were obtained in December 1999 from the same location as the previous samples and acclimatized at 2 to 3°C in a flow-through aquarium for 4 d. One day prior to the beginning of the experiment, mature and juvenile mussels were divided equally among 5 aquaria (7.5 l). The experimental design was identical to that previously described, with the addition of an ultraviolet sterilizer on the incoming water, intended to ensure that incoming bacteria would not be introduced to the system. In each aquarium, 10 mature mussels were randomly selected, separated from the others within a mesh bag and left untouched for the final bacterial clearance.

Hemolymph extraction was performed as previously described with the following modifications: 0.6 ml hemolymph was extracted from each of the 10 remaining adult mussels with a sterile 23 gauge needle into a 3 ml syringe containing 0.6 ml of TBS. The hemolymph/TBS solution was then divided into 6 subsamples: 0.1 ml for HC with pooled (2 mussels) hemolymph, 0.1 ml for HC with non-pooled hemolymph, 0.1 ml for PA using microscopic technique, 0.3 ml for PA using spectrophotometric technique, 0.3 ml for LR with 4 h incubation time and finally 0.3 ml for LR with 30 min incubation time. Spectrophotometric analyses were made in triplicate and the mean of these results

was used in the statistical analysis. Precision of each assay was expressed as the mean coefficient of variation for the 10 mussels and was 10.2% for PA with spectrophotometer and 6.4% with fluorescence, 13.1% for LR 30 min incubation and 12.6% for 4 h incubation, and finally 27.2% for HC of pooled cells and 19.5% for non-pooled samples. Results were expressed as a mean average.

Exposure was carried out as previously described, with the following modifications: only 2 nominal concentrations were used (40 and 80 ng Sn l⁻¹ for TBT or DBT) and no dried spiruline was added to the stock solution assuming adsorption was minimal after conditioning of tubes and glassware for 24 h. As in Expt 1, mussels were not fed during the exposure period. Mussels for assays were sampled on Days 0, 1, 6 and 13. Although MI was not quantified, cells were observed for the presence of any deformity.

Lysosome retention (LR): Three aliquots of 100 µl hemolymph per mussel were added to 200 µl of 2% neutral red dye in TBS in microplate wells and left to incubate in the dark at room temperature for 4 h. Cells were then washed by centrifugation with TBS (100 × *g* for 5 min), fixed in Baker's formol (4% [v/v] formaldehyde, 2% [w/v] sodium chloride and 1% [w/v] calcium acetate) for 30 min (Pipe et al. 1995, Dyrinda et al. 1998). The neutral red was then extracted by the addition of 100 µl acetic acid/ethanol solution to each well, which was left to stand for 30 min, after which the plates were quantified at 490 nm with a Tecan fluorospectrophotometer. Three hemolymph samples per mussel were analyzed and the mean of these samples was used for statistical analyses. To test the effect of the incubation period on the LR response, the same protocol was replicated using only 30 min incubation in dark at room temperature.

Phagocytic activity (PA): Microscopic observation: Microscopic observations of PA were performed as previously described for Expt 1.

Spectrophotometric observations: Aliquots of 100 µl of hemolymph/TBS were added to microplates. Twenty µl of neutral-red stained zymosan type 1 yeast, 2.3 × 10⁸ particles ml⁻¹, were added to each well for 30 min incubation, after which 100 µl of fixative was added for 30 min (Pipe et al. 1995, Dyrinda et al. 1998). The plates were washed twice with TBS by centrifugation (1000 × *g* for 5 min) and the neutral red was solubilized by adding 100 µl of extraction solution for 30 min. The microplates were read at 490 nm, using a spectrophotometer (Molecular Devices). All readings were adjusted for cell numbers. Three hemolymph samples per mussel were analyzed and the mean of these samples was used for statistical analysis.

Bacterial challenge: The bacterial challenge was performed after a 13 d exposure with the following

modifications from Expt 1. The inoculation was performed in a cold room (12°C) to minimize temperature shock for the mussels. The inoculum (0.5 ml for adults and 0.1 ml for juveniles) was injected directly into the hemolymph for adults and into the visceral mass for juveniles. After inoculation, the mussels were put back into their aquaria (previously drained) without elastic bands. After 2 h, the aquariums were slowly refilled with clean, UV-sterilized, flow-through seawater. Three separate hemolymph samples per mussel were taken on Day 6, analyzed for bacterial number as described above and the mean of these 3 counts was used for the statistical analysis.

Statistical analysis. Statistical analysis used SPSS and Systat softwares for a PC. Distributions of variables were examined for normality in probability plots and tested with Levine's test. Homogeneity of variances among groups to be compared were tested by *F*-test. Where required, data were transformed to their logarithms except for the percentage data, which were transformed using the arcsine square root.

Expt 1: Differences among treatment and control groups for all end-points (MI, LR, PA and HC) were determined using a repeated measure analysis of variance (ANOVA) with time (0, 1, 4, 11, 18, 25 and 32 d) and TBT or DBT concentrations (0, 5, 10, 20, 40 and 80 ng Sn l⁻¹) as independent variables. Where significant effects (*p* < 0.05) of dose were detected, or dose interacted significantly with sampling day, days were analyzed separately by 1-way ANOVA followed by an *a posteriori* test (least significant difference of means) to determine the doses or days on which treatment and control groups differed.

To establish whether the 2 chemicals, TBT or DBT, affected the end-points differently, we pooled doses (omitting the 0 dose) and did an ANCOVA with day as the covariate and chemical as the principal variable. If the analysis showed significant interactions (i.e. chemicals had different effects over days), ANCOVAs were performed on each dose separately to determine whether all doses or only some doses differed over time. Where interactions were found in dose-specific analyses, they were described. Where no interactions were found, the intercept was tested to determine whether or not the chemicals differed in effect over all days.

In addition, to determine whether both chemicals were capable of achieving the same maximal effect (i.e. a threshold) impacts of TBT and DBT on the day of greatest effect were compared in 2 ways. First, chemical (TBT vs DBT) and dose were compared in a 2-way ANOVA. Second, maximal responses directed by each chemical were compared by 1-way ANOVA. The first analysis tested whether both chemicals were effective at a similar dose. The second analysis tested whether

both chemicals achieved the same maximal effect. Significant differences are reported where $p < 0.05$.

Differences in bacterial counts among treatment and control groups for bacterial challenge were determined by a 2-way ANOVA with time (4 and 14 d) and TBT or DBT concentrations (0, 5, 10, 20, 40 and 80 ng Sn l⁻¹) as independent variables. As for the previous assays, where significant effects ($p < 0.05$) of dose were detected, or dose interacted significantly with day, days were analyzed separately by 1-way ANOVA followed by an *a posteriori* test (least significant difference of means) to determine the doses or days on which treatment and control groups differed. To compare TBT to DBT impacts, the ANCOVA was performed exactly as described above with the exception that no maximal effect impacts of TBT or DBT were compared.

Expt 2: Differences among treatment and control groups (LR, PA and HC) were determined by a 2-way ANOVA with time (0, 1, 6 and 13 d) and TBT or DBT concentrations (0, 40 and 80 ng Sn l⁻¹) as independent variables. All other analyses were performed as described above.

Differences among treatment and control groups for bacterial challenge were determined as previously described with age (juvenile and mature) and TBT or DBT concentrations (0, 40 and 80 ng Sn l⁻¹) as independent variables. Where significant effects ($p < 0.05$) of dose were detected or dose interacted significantly with age, ages were analyzed separately by 1-way ANOVA followed by an *a posteriori* test (least significant difference of means) to determine the doses for which treatment and control groups differed.

RESULTS

Membrane injury (MI)

When observed microscopically, we detected 3 general shapes of hemocytes grading from normal to severely damaged: (1) normal, with filipod projections and visible ectoplasm; (2) naivelike (rounded) with no filipod; and (3) short, with blunt projections either tightly spaced or interspersed with rough, blebbing membrane, resembling hemocytes kept in hypo-osmotic conditions. In addition, cellular debris and dark unidentified small bodies were present in all pooled or exposed samples.

For both TBT and DBT, MI over time differed with dose (time $\times$ dose interaction for TBT and DBT, respectively: $F_{30,144} = 6.43$, $p < 0.001$; $F_{30,144} = 7.56$, $p < 0.001$; Fig. 1). Therefore, effects of dose were examined on each sampling date separately. For TBT, doses ≥ 10 ng Sn l⁻¹

produced significant elevation in MI by Day 1, which was maintained through the end of the experiment on Day 32. Furthermore, within the first 4 d of exposure, the degree of MI was generally dose-responsive (e.g. 40 and 80 ng Sn l⁻¹ produced significantly higher MI than the lower doses). By Day 11, significant MI elevation was also seen in the 5 ng Sn l⁻¹ treatment and by Day 18 the MI produced by all doses was similar and plateaued at 40 to 50 % of hemocytes showing MI.

DBT had less impact on MI than TBT (Fig. 1). By Day 1, mussels exposed to ≥ 20 ng Sn l⁻¹ DBT showed significantly higher MI than unexposed mussels or those exposed to lower doses. By Day 4, MI was significantly elevated in all groups exposed to DBT and this was maintained through to the end of the exposure. Within mussels exposed to DBT, degree of MI was related to dose. On Day 11, the 80 ng Sn l⁻¹ group showed a significantly higher MI than groups exposed to the lower doses; on Days 18 and 32, mussels exposed to ≥ 10 ng Sn l⁻¹ DBT showed higher MI than those exposed to 5 ng Sn l⁻¹. On Day 25, the 40 and 80 ng Sn l⁻¹ groups showed higher MI than the lower dose groups.

Statistical comparison of the impacts of TBT and DBT on MI revealed that all doses of TBT produced more rapid MI than DBT (chemical $\times$ time interaction for dose-specific analyses: $F_{6,56} = 2.59$ to 5.51, $p = 0.027$ to < 0.001). To determine whether or not the magnitude of MI differed between TBT and DBT, responses at the end of the exposure were examined by 2-way ANOVA (i.e. chemical and dose effects on MI). Over all doses, TBT did not produce more MI than DBT ($F_{1,48} = 2.11$, $p = 0.080$). A maximum limit of MI appeared with 80 ng Sn l⁻¹ of TBT and DBT as both produced the same degree (ca. 44 %) of MI ($F_{1,8} = 0.84$, $p = 0.39$).

Fig. 1. Membrane injury (MI) observed from 5 pools of 4 mussels sampled after 0, 1, 4, 11, 18, 25 and 32 d of exposure to tributyltin (TBT) and dibutyltin (DBT). Asterisks represent individual or groups of values (in brackets) significantly different from controls. SD are not indicated for clarity of all figures

Phagocytic activity (PA)

As for MI, the effect over time of both butyltins on phagocytosis, as determined by microscopic observation in Expt 1, differed with dose (time $\times$ dose interaction for TBT: $F_{30,144} = 4.24$, $p < 0.001$; for DBT: $F_{30,144} = 4.27$, $p < 0.001$). The day-by-day analysis of the trends for TBT are shown in Fig. 2.1a. By Day 1, PA was significantly reduced in the 3 highest dose groups (20, 40 and 80 ng Sn l⁻¹) relative to the lower doses and control. By Day 4, all TBT doses except 5 ng Sn l⁻¹ showed significant reduction in phagocytosis, and by Day 18, even 5 ng Sn l⁻¹ was significantly depressed relative to the control. Reduced phagocytosis persisted through to the end of the experiment and degree of reduction was dose-responsive. The highest dose of TBT tested (80 ng Sn l⁻¹) produced a significantly higher reduction in phagocytosis than the 2 lowest doses (5 and 10 ng Sn l⁻¹) by Day 32.

DBT appeared slower to affect phagocytosis than TBT but ultimately had the same result (Fig. 2.1b). No significant depression of phagocytosis was observed until Day 18, from which time until the end of the experiment all doses of DBT significantly reduced PA.

Comparison of the impacts of TBT and DBT on phagocytosis revealed that for all doses greater than 5 ng Sn l⁻¹, TBT produced more rapid reduction in phagocytosis than DBT (chemical $\times$ time interaction for dose-specific analyses: $F_{6,56} = 3.00$ to 5.09, $p = 0.012$ to < 0.001). To determine whether the magnitude of reduction in phagocytosis differed between TBT and DBT, responses at the end of the exposure were examined by 2-way ANOVA (i.e. chemical and dose effects on phagocytosis). Over all doses, there was no difference between the effects of TBT and DBT on PA ($F_{1,40} = 2.25$, $p = 0.081$) and the maximal reduction in PA (only 53% of cells exposed to 80 ng Sn l⁻¹ TBT or DBT actively phagocytosing) did not differ between TBT and DBT ($F_{1,8} = 2.45$, $p = 0.156$).

In Expt 2, the effect over time of both butyltins on phagocytosis, as determined by microscopic evaluation, differed with dose (time $\times$ dose interaction for TBT: $F_{6,48} = 5.98$, $p < 0.001$; for DBT: $F_{6,48} = 6.52$, $p < 0.001$). The daily analysis showed that by Day 1, PA was significantly reduced by both doses (40 and 80 ng Sn l⁻¹) of TBT but did not differ between them and that was maintained until the end of exposure (Fig. 2.2a). Significant depression of phagocytosis was observed on Day 1 for DBT (Fig. 2.2b), but only for the 80 ng Sn l⁻¹ group. For Days 6 and 13, both doses of DBT significantly reduced PA. Comparison of the impacts of TBT and DBT on PA did not show any difference between the butyltins for all doses combined.

The effect over time of both butyltins on phagocytosis, as determined by spectrophotometric tech-

Fig. 2. Phagocytic activity (PA) measured by microscopic observations. In Expt 1, PA was measured from 5 pools of 4 mussels sampled after 0, 1, 4, 11, 18, 25 and 32 d of exposure to TBT (1a) and DBT (1b). In Expt 2, PA was measured from 10 mussels sampled after 0, 6 and 13 d of exposure to TBT (2a) and DBT (2b). Asterisks represent individual or groups of values (in brackets) significantly different from controls

nique, differed with dose (time $\times$ dose interaction for TBT: $F_{6,108} = 7.17$, $p < 0.001$; for DBT: $F_{6,108} = 2.19$, $p = 0.049$; results not shown). The day-by-day analysis of the trends for TBT revealed the following. On Days 6 and 13, both 40 and 80 ng Sn l⁻¹ groups were significantly elevated in comparison to the control group but significantly different from each other only on Day 13. A significant elevation of PA on Day 1 compared to the other groups was induced by 80 ng Sn l⁻¹. From Day 6, both doses of DBT significantly increased PA over controls and to a degree not significantly different. The apparent discrepancy between the results obtained by microscopic and spectrophotometric methods are discussed below. Comparison of the impacts of TBT and DBT on PA revealed no significant difference between the 2 toxicants.

Lysosome retention (LR)

In Expt 1, the impact of the butyltins on LR (Fig. 3.1) was not monotonic over time, resulting in a significant

Fig. 3. Lysosome retention (LR). In Expt 1, LR was measured from 5 pools of 4 mussels sampled after 0, 1, 4, 11, 18, 25 and 32 d of exposure to TBT (1a) and DBT (1b). In Expt 2, LR was measured after 4 h incubation time from 10 mussels sampled after 0, 1, 6 and 13 d of exposure to TBT (2a) and DBT (2b). Asterisks represent individual or groups of values (in brackets) significantly different from controls

interaction between time and dose for DBT ($F_{30,144} = 2.53$, $p < 0.001$) and time effect for TBT ($F_{6,144} = 6.74$, $p < 0.001$). Exposure to higher doses of TBT (40 and 80 ng Sn l⁻¹) had a tendency to raise LR on Days 1 and 4 followed by a reduction below control levels by Days 18 through to 32 (Fig. 3.1a). However, none of these differences was statistically significant. DBT also produced a rise and fall in LR, but with a greater amplitude than TBT (Fig. 3.1b). On Days 1 and 4, the 80 ng Sn l⁻¹ group showed significant elevation in LR, which disappeared by Day 18. Levels of LR then continued to fall in the 2 highest doses of DBT reaching significantly lower levels than the 20 ng Sn l⁻¹ treatment by Day 25 and remaining low through Day 32, although differences were no longer significant. No significant differences were detected between the effects over time of TBT and DBT on LR.

As in Expt 1, the impact of the butyltins on LR in Expt 2 was not monotonic over time, resulting in significant interactions between time and dose for both TBT and DBT: TBT ($F_{6,108} = 7.17$, $p < 0.001$) and ($F_{6,108} = 4.22$, $p < 0.001$) for the 30 min and 4 h assays, respectively; DBT ($F_{6,108} = 3.45$, $p < 0.001$) and ($F_{6,108} = 2.66$, $p = 0.019$) for the 30 min and 4 h assays, respectively. As results were similar for incubation times of 30 min

and 4 h, only the 4 h data are shown in Fig 3.2 for direct comparison with Expt 1. TBT significantly raised LR on Day 1 for the 40 ng Sn l⁻¹ group and on Day 6 for both 40 and 80 ng Sn l⁻¹ groups for the 4 h incubation assay (Fig. 3.2a). On Day 13, there was no significant difference between the groups anymore. Similarly, exposure to doses of 40 and 80 ng Sn l⁻¹ TBT significantly raised LR, but with a lesser magnitude, on Days 6 and 13 for the 30 min incubation assay (results not shown). DBT also produced a rise and fall in LR (Fig. 3.2b). LR was significantly elevated on Day 6 by 40 and 80 ng Sn l⁻¹ DBT. By Day 13, no significant difference was detected among treatment groups. Exactly the same patterns were observed with the 30 min incubation as with the 4 h incubation time.

The ANCOVA for the 4 h incubation assay revealed no difference in the effect of TBT and DBT in the 80 ng Sn l⁻¹ groups (intercept: $F_{1,72} = 0.006$, $p = 0.941$). However, in the 40 ng Sn l⁻¹ groups, TBT had a different effect over time from DBT (intercept: $F_{3,72} = 5.21$, $p = 0.003$). The maximal effect on LR achieved by 40 ng Sn l⁻¹ DBT was smaller than the maximal effect achieved by 40 ng Sn l⁻¹ TBT (ANOVA on TBT Day 1 vs DBT Day 6: $F_{1,18} = 45.56$, $p < 0.001$). Finally, it should be mentioned that for the 30 min incubation assay, the ANCOVA revealed that overall TBT had a greater impact on LR than DBT (intercept: $F_{1,157} = 5.55$, $p = 0.020$).

Hemocyte counts (HC)

Effects of butyltins on HC in Expt 1 were less clear than effects on MI, phagocytosis or LR. The effect of TBT on HC over time differed with dose (time $\times$ dose interaction: $F_{30,144} = 2.14$, $p = 0.002$). DBT effects on HC differed with dose ($F_{5,24} = 3.54$, $p = 0.015$) and time ($F_{6,144} = 8.33$, $p < 0.001$) without interaction ($F_{30,144} = 1.44$, $p = 0.080$). Daily comparisons of HC in mussels exposed to different doses of TBT (Fig. 4.1a) revealed significant HC depression in the 20 ng Sn l⁻¹ group on Day 1. By Day 4, the 80 ng Sn l⁻¹ group was elevated over 0 to 20 ng Sn l⁻¹ groups. Similarly, 40 ng Sn l⁻¹ TBT elevated HC over all groups except for the 10 ng Sn l⁻¹ on Day 4. Only the 40 ng l⁻¹ group was above all other groups on Day 18. On day 32, HC were significantly lower than control levels in all TBT-exposed groups except for the highest dose (80 ng Sn l⁻¹).

For DBT, ANOVA failed to detect any significant difference in the HC between treatment groups on Days 1, 4, 18 and 25. HC was elevated on Day 11 for the 80 ng Sn l⁻¹ group relative to all other groups except the 40 ng Sn l⁻¹. On Day 32, the HC was significantly lower in the 10, 20 and 40 ng Sn l⁻¹ treatments than in the control. However, the HC in the 80 ng Sn l⁻¹

Fig. 4. Hemocyte counts (HC). In Expt 1, hemocytes were counted from 5 pools of 4 mussels sampled after 0, 1, 4, 11, 18, 25 and 32 d of exposure to TBT (1a) and DBT (1b). In Expt 2, hemocytes came from 5 pools of 2 mussels sampled after 0, 1, 6 and 13 d of exposure to TBT (2a) and DBT (2b). Asterisks represent individual or groups of values (in brackets) significantly different from controls

group was similar to the control level and significantly higher than in the 40 ng Sn l⁻¹ group (Fig. 4.1b). No significant differences were detected in comparing the effects of TBT and DBT on HC over time.

As reported for Expt 1, effects of butyltins on HC in Expt 2 were obscured by a large variability within each group (Fig. 4.2). The effect of TBT on HC over time differed with dose in both assays (time × dose interaction: $F_{6,48} = 2.45$, $p = 0.038$ for the pooled hemolymph and $F_{6,106} = 1.85$, $p = 0.047$ for the non-pooled). Daily comparisons of HC in mussels exposed to TBT in the pooled samples revealed significant depression of HC in the 80 ng Sn l⁻¹ group on Day 1 (Fig. 4.2a). The non-pooled samples revealed a significant depression in HC on Day 6 for the 80 ng Sn l⁻¹ group and both 40 and 80 ng Sn l⁻¹ groups on Day 13 (results not shown).

DBT effects on HC over time also differed with dose for both the pooled hemolymph ($F_{6,48} = 7.01$, $p < 0.001$) and the non-pooled hemolymph ($F_{6,105} = 3.71$, $p = 0.002$). HC in the pooled samples was elevated by both doses of DBT on Day 6 and was significantly more elevated by 40 than 80 ng Sn l⁻¹ (Fig. 4.2b). For the non-pooled hemolymph, the 40 and 80 ng Sn l⁻¹ groups showed a significant elevation in HC by Day 1 which was maintained to Day 13 (results not shown).

In the pooled samples, DBT had a more pronounced effect than TBT on HC (chemical × time interaction: $F_{3,32} = 4.33$ to 4.21, $p = 0.013$ to 0.011) for 40 and 80 ng Sn l⁻¹, respectively. The timing of TBT and DBT action on HC was different for both doses; for both doses there was a rise between Days 1 and 6 in DBT, which was absent for TBT. Furthermore, HC in mussels exposed to both doses of DBT, was significantly higher on Day 6 than HC in mussels exposed to comparable levels of TBT (ANOVA: $F_{1,8} = 12.06$ to 11.45, $p = 0.008$ to 0.010 for 40 and 80 ng Sn l⁻¹, respectively). In the non-pooled samples, the ANCOVA revealed that DBT overall had a greater impact on HC than TBT (intercept: $F_{1,154} = 6.57$, $p = 0.011$).

Bacterial clearance

Clearance of labeled *Listonella anguillarum* in Expt 1 was impeded by a previous 32 d exposure to TBT or DBT. Four d after the inoculation, counts of *L. anguillarum* were higher in mussels pre-exposed to 10,

Fig. 5. Effects of pre-exposure of blue mussels to butyltins on their bacterial clearance capacity. In Expt 1, mussels were exposed for 32 d to various doses of TBT (1a) or DBT (1b) doses before the bacterial test. Bacteria were counted 4 d (light gray bars) and 14 d (dark gray bars) after the injection of the common bacterium *Listonella anguillarum* (*Vibrio*) at the end of the pre-exposure. In Expt 2, mussels were exposed for 13 d to TBT (2a) or DBT (2b) doses before the challenge test. Bacteria were counted for adults (light gray bars) and juveniles (dark gray bars) 6 d after injection of the common bacterium *L. anguillarum* (*Vibrio*). Bars are mean responses of 10 mussels. Asterisks indicate significant elevation over respective control

40 and 80 ng Sn l⁻¹ TBT than in the control and the 5 ng Sn l⁻¹ groups (Fig. 5.1a). Though bacterial counts dropped between Days 4 and 14 in mussels exposed to TBT ($F_{1,90} = 11.532$, $p = 0.036$), they were still significantly elevated over controls in mussels exposed to ≥ 20 ng Sn l⁻¹ TBT and were significantly higher in the 40 and 80 ng Sn l⁻¹ groups than in groups exposed to lower doses. Mussels exposed to all doses of DBT showed significantly higher counts of *Listonella anguillarum* than controls 4 and 14 d after infection ($F_{1,90} = 72.691$, $p = 0.012$) (Fig. 5.1b). Furthermore, by Day 14, groups exposed to 20 to 80 ng Sn l⁻¹ still maintained significantly higher levels of bacteria than controls and the 5 ng Sn l⁻¹ group. No significant differences in chemical species on bacterial clearances were detected over time.

The effect of TBT dose and age of mussel (adult vs juvenile) on clearance of *Listonella anguillarum* in Expt 2 were analyzed using 2-way ANOVA. The effect of TBT dose differed with age of mussel (interaction term: $F_{2,54} = 7.76$, $p = 0.001$); juveniles showed a greater effect than adults (Fig. 5.2a). Both age groups showed significantly elevated bacterial counts 6 d after inoculation when pre-exposed to 40 or 80 ng Sn l⁻¹ TBT and this effect was greater in the 80 ng Sn l⁻¹ than the 40 ng Sn l⁻¹ group ($F_{2,27} = 68.26$ to 48.25 , $p \leq 0.001$ for mature and juvenile mussels, respectively).

Bacterial levels were higher in juvenile than in mature mussels exposed to DBT (Age: $F_{1,54} = 41.09$, $p < 0.001$) (Fig. 5.2b). Both age groups showed significantly higher *Listonella anguillarum* counts 6 d after inoculation when previously exposed to 40 or 80 ng Sn l⁻¹ DBT, with levels in the 80 ng Sn l⁻¹ being higher than in the 40 ng Sn l⁻¹ group ($F_{2,27} = 10.87$ to 23.23 , $p < 0.001$ for mature and juvenile mussels, respectively). As for Expt 1, no significant differences were detected between the *L. anguillarum* clearance in all groups previously exposed to either TBT or DBT.

Table 1. Tributyltin (TBT) and dibutyltin (DBT) accumulation in blue mussels exposed to waterborne TBT or DBT for 32 d for Expt 1 and 13 d for Expt 2. Each value is the mean of 3 replicate analysis of the pooled soft tissues of 20 mussels (Expt 1) or 10 mussels (Expt 2) $\pm$ SD. Results expressed as ng Sn g⁻¹ of tissue (dry wt). Traces represent detected TBT or DBT between the instrumental detection limit (1 ng Sn g⁻¹) and the quantification limit (5 ng Sn l⁻¹)

Doses (ng Sn l ⁻¹)	Treatments					
	Expt 1			Expt 2		
	TBT as TBT	as DBT	DBT as DBT	TBT as TBT	as DBT	DBT as DBT
0	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
5	≤ 1	≤ 1	≤ 1	–	–	–
10	Traces	≤ 1	Traces	–	–	–
20	Traces	≤ 1	Traces	–	–	–
40	50 ± 13	Traces	50 ± 13	23 ± 1	Traces	Traces
80	45 ± 12	25 ± 9	45 ± 12	248 ± 34	Traces	Traces

Chemical analysis

Concentrations of butyltins found in each group of mussels at the end of the 2 experiments are presented in Table 1. TBT and DBT were detected but not quantified (traces) in mussels exposed to concentrations of 10 and 20 ng Sn l⁻¹ in Expt 1. TBT was present in mussels exposed to 40 and 80 ng Sn l⁻¹, but DBT was also present in these mussels indicating *in vivo* dealkylation in progress. In Expt 2, TBT was present in mussels exposed to TBT with only traces of DBT present. Mussels exposed to DBT showed only traces of the compound, indicating a fast elimination process.

DISCUSSION

This study demonstrated time- and dose-responsive effects of environmentally relevant concentrations of TBT and DBT on the internal defense of blue mussels, as well as a dose responsive impairment in clearing *Listonella anguillarum* from the hemolymph. The 2 different experiments conducted at different periods of the year, using different techniques for certain endpoints, gave generally comparable results as did a lowest observed effect level (LOEL) experiment reported in St-Jean et al. (2002, this issue).

Butyltin concentrations accumulated by blue mussels in this study were environmentally realistic for coastal areas. Tissue concentrations of TBT in mussels are considered moderately high in the 170 to 609 ng Sn g⁻¹ dry wt range and chronically below 170 ng Sn g⁻¹ dry wt (Page & Widdows 1991). The highest level of TBT accumulation reached in this study was 45.2 ng Sn g⁻¹ dry wt for mussels exposed to 80 ng Sn l⁻¹ TBT-Sn in Expt 1 and 248 ng Sn g⁻¹ dry wt for Expt 2. Differences observed in TBT and DBT accumulation between the 2 experiments might be attributed to the longer exposure period in

Expt 1, and to the *in vivo* dealkylation of TBT, clearly observed in Expt 1 but not yet in place after 13 d in the Expt 2. Mussels exposed to DBT showed no detectable accumulation after 13 d but showed clear evidence of accumulation after 32 d, indicating a faster clearing rate for DBT than TBT.

In both experiments, we observed MI in hemocytes from all groups following exposure to butyltins. MI was particularly severe in Expt 1 including control groups, suggesting an influence of external stressors in addition to butyltins. MI was also observed in pooled hemolymph samples of Expt 2, albeit at lower

incidence than Expt 1, but was much less common in non-pooled samples. No MI was observed in the control mussels. Between the 2 protocols used, the pooled samples consistently showed greater MI than the non-pooled samples. However, the behavior of allogenic hemolymph transfer in bivalves is unknown. Ey & Jenkin (1982) have shown non-self recognition directed towards invading microorganisms, damaged tissue and foreign material. In addition to greater MI, the pooled samples always showed more debris and lower HC than non-pooled samples. This suggests that MI was enhanced in Expt 1 by the pooling of hemocytes from 4 different individuals. Although it remains to be verified, these hemocytes may have reacted to foreign hemocytes by membrane attack.

Previous studies have reported that hemocytes exposed to organotin compounds sustain varying degrees of membrane damage ranging from cell rounding, interfering with aggregation and locomotion (Auffret & Oubella 1997, Oubella 1997) to cytoskeletal damage causing important shape changes (Porvaznik et al. 1986). Our results are consistent with these reports; butyltin-exposed samples showed less aggregation than the controls. Levels of MI were smaller and occurred more slowly in our study than those found in ascidian hemocytes (Cima et al. 1998) or human erythrocytes (Gray et al. 1986, 1987, Porvaznik et al. 1986) exposed to TBT concentrations much higher than ours. However, various levels of MI have been observed at comparable butyltin concentrations in blood cells of rainbow trout *Oncorhynchus mykiss* (C. Ribiero, J. Padros & É. Pelletier unpubl. results) and amaebocytes of starfish *Leptasterias polaris* (É. Pelletier unpubl. results).

Triorganotins are known to be membrane-active molecules (Zucker et al. 1988) which bind to membrane proteins and phospholipids and modify the permeability (Oubella 1997). The action of butyltins on MI could be explained by the following. First, Ambrosini et al. (1996) suggested that organotin compounds dissolved in aqueous solution form different cations, depending on the number of ligands. These differences (types and number of ligands) influence the polarity and the structural characteristics of each organotin and could explain the different actions at the phospholipid-membrane level. These authors showed that a triorganotin (triphenyltin: TPT) affects the membrane lipids at the hydrophobic core of the membrane bilayer while DBT affects lipids on the membrane's polar surface. In addition, Akhtar et al. (1987) showed TBT to bind to an anion exchanger with greater affinity than DBT, while DBT has been shown to have a higher affinity than TBT with sulphidryl groups associated with membranes or cytoskeleton (Boyer 1989, Saboori & Newcombe 1992). Therefore, it is hypothesized that

the organotins influence different domains of the membrane lipids and thus have different effects on membrane fluidity and the degree of damage to cell function.

Secondly, both organotins, but especially DBT, were also shown to shut down the Ca^{2+} pump (Gray et al. 1987, Cima et al. 1998). Bouchard et al. (1999) hypothesized that DBT appears to be a better competitor for calcium sites than TBT because the ionic form of DBT is a double-charged cation like Ca^{2+} and is a much smaller moiety than TBT, which brings only 1 positive charge. Finally, TBT was shown to reduce the blood osmolality of flounder *Platichthys flesus* (Hartl et al. 2000) and to lower intracellular pH (Gray et al. 1986). Our study shows that organotins, in addition to causing MI, interfered with both phagocytosis and LR. Since the cell communicates with its environment through the plasma membrane, alteration of its composition or structure might well result in disruption of crucial functions like phagocytosis, lysosomal retention or basic cellular maintenance.

Both organotins in both experiments modulated PA. When PA was measured microscopically, all DBT and TBT doses significantly reduced PA. The reduction was more severe in Expt 1, especially with TBT. In the DBT-exposed groups, while no significant differences were found before Day 18 in Expt 1, higher doses resulted in PA reduction, in both experiments. These results are in accordance with other studies using microscopic evaluation of PA of cells under chemical stress (Fries & Tripp 1980, Cheng 1988a, Grundy et al. 1996). In contrast, when PA was measured spectrophotometrically, organotins produced a significant increase in phagocytosis. Although these results appear contradictory, the difference likely resides in the techniques used. Microscopic observations focused mainly on very active mature granulocytes and did not detect small and weak phagocytes like hyalinocytes. However, spectrophotometric measurements included all types of phagocytes and thus yielded a measure of the total PA of the mussels. Our results strongly suggest that the cumulative response of the phagocytic hemocytes was stimulated by organotins while the number of very active cells was reduced, which is consistent with both types of measurements. Such differences in the response of PA using those 2 techniques were also reported in the literature: when measured microscopically, $1 \mu\text{g l}^{-1} \text{Cu}^{2+}$ inhibited PA (Cheng 1988b) whereas $5 \mu\text{g l}^{-1} \text{Cu}^{2+}$ (Cheng & Sullivan 1984) or $0.2 \mu\text{g l}^{-1} \text{Cu}^{2+}$ (Pipe et al. 1999) enhanced PA when measured spectrophotometrically. Clearly, more spectrophotometric studies are needed to investigate the PA response of different hemocyte populations to environmental toxicants.

Phagocytosis is a complex process and could possibly be modulated by several mechanisms. Recently,

cytokines (IL-1 α , IL-2 and TNF- α) and pro-opiomelanocortin (POMC)-derived peptides such as ACTH, β -endorphin and α -MSH were shown to be present in molluscan hemocytes (Ottaviani et al. 1997a). In addition, these molecules were also shown to increase PA in mollusks (Ottaviani et al. 1995, 1997b). Since these molecules are known to be involved in stress response, it is possible that butyltins promoted their release and stimulated the hyalinocytes, although this remains to be demonstrated.

Several mechanisms may be involved in butyltin-related inhibition of phagocytosis. According to Cheng (1983) and Renwanz (1990), the phagocytic process can be subdivided into 4 steps: chemotaxis, recognition, internalization and intracellular degradation. Phagocytosis is, therefore, fundamentally dependent on membrane communication, for chemotaxis, fluidity of movement and internalization, as well as maintenance of internal ionic balance for the biochemical degradation. PA could have been disrupted through an impediment of cellular movement. While both butyltins can promote shape transformation by the disruption of the proton pump (Akhtar et al. 1987), TBT has a stronger affinity for the anion exchanger and could have interfered more with the integrity of the membrane than DBT. On the other hand, DBT could have interfered more than TBT with the locomotion of the cell via DBT's greater affinity for the plasma membrane-associated or cytoskeleton-associated sulfide groups (Ambrosini et al. 1996). Since cellular locomotion and attachment to substrates (including other cells) are controlled by proteins that join the underlying cytoskeleton to the plasma membrane (Stryer 1988), disruption of the sulfide groups would modify membrane fluidity and, thus, interfere with cellular locomotion. In addition, cell spreading, necessary to cellular locomotion, requires hemocytes to be isoosmotic or slightly hyperosmotic (Fisher 1986). By reducing the osmolalities, TBT could have inhibited cell spreading, promoting cell rounding and therefore reducing PA of larger and older cells but not smaller ones.

Finally, organotins could disrupt PA through disruption of the calcium pumps. Chemotaxis is dependent upon membrane receptors (lectins) (Mullainadhan & Renwanz 1986) while trans-membrane movements are facilitated by annexins (Creutz 1992). Both processes are Ca²⁺-dependent and situated predominantly on the polar surface of the membrane. DBT, having a greater affinity than TBT for the polar side of membranes and, possibly, being a better competitor for Ca²⁺, would have more of an impact on those processes than TBT. On the other hand, cellular locomotion is also closely associated with the cytoskeleton, which is anchored to the membrane through the anion exchanger, which is more susceptible to TBT than DBT. It

is therefore possible that both TBT and DBT interfere with phagocytosis, in different ways or levels.

In Expt 1, the DBT-exposed groups retained more neutral red dye, on average, than the control groups. By Day 18, however, the trend reversed. Similarly, in Expt 2, the exposed groups showed an increase in neutral red retention, with the greatest difference from the controls on Day 6, followed by a decreasing difference in retention to the end of the experiment. In Expt 2, the incubation time did not appear to significantly change the degree of retention of the dye, however, the changes in methods used did reduce variability. Recently, Viarengo et al. (2000) reported the effects of some heavy metals (Hg, Cd and Cu) on the lysosomes of mussel hemocytes using a neutral red retention technique by microscopy with short incubation times ranging from 15 to 60 min. This technique provided clear responses from lysosomes, but metal concentrations used where 1000 to 10000 times higher than those used in present experiments with TBT and DBT. The optimal response time is most probably a function of the concentrations of toxicants and seawater temperature, but this aspect of the lysosomal mechanism deserves further research.

The pattern of elevation in dye retention, followed by a decrease, may be due to the intrinsic ability of lysosomes to concentrate a wide range of contaminants including lipophilic xenobiotics (Moore 1990). Winston et al. (1996) reported that, under persistent xenobiotic challenge, oxidant stress can exceed normal antioxidant defenses and lead to release of hydrolytic enzymes into the cytosol. When the capacity to store butyltins is exceeded, the lysosomal membrane destabilizes, changing its permeability characteristics, and hydrolases are released into the cytoplasm (Anderson 1993). In the present study, we suggest that, after an initial increase in permeability, the lysosomes became overloaded, antioxidant protection was impaired and damage resulted. This would agree with other studies where xenobiotics were found to cause loss of lysosomal membrane integrity (Nott & Moore 1987, Grundy et al. 1996), but using much higher concentrations of toxicants. According to Gundy et al. (1996), hemocytes exposed *in vitro* to 8.3 $\mu\text{g l}^{-1}$ anthracene showed an augmentation of lysosome permeability (more dye retained) while exposure to anthracene at 50 $\mu\text{g l}^{-1}$ *in vitro* or 14 d exposure to 250 ng l^{-1} *in vivo* showed a decrease in membrane integrity (less dye retained) indicative of leakage. In addition, Moore & Farrar (1985) reported that mussels exposed to concentrations of 0 to 200 $\mu\text{g l}^{-1}$ anthracene showed a linear decrease in LR while an exposure to 0 to 200 $\mu\text{g l}^{-1}$ phenanthrene showed a sigmoidal relationship. These authors suggested that a critical concentration for a given compound is required for lysosome membrane damage.

Furthermore, DBT could have enhanced pinocytosis, which could have resulted in an augmentation of the membrane permeability, as reflected by the fact that the DBT-exposed groups retained more neutral red dye than TBT and the control in Expt 1. These results are in agreement with earlier *in vitro* studies on mussel hemocytes (É. Pelletier & I. Taupeau unpubl. data) showing that neutral red entered cells previously exposed to DBT more rapidly and in a greater volume than TBT-exposed groups.

Changes in immune parameters measured in this study were also reflected in the HC. For both experiments, a small increase was observed in HC in mussels exposed to either butyltin, but especially high doses of DBT early in the experiment. According to Pipe & Coles (1995), the most commonly observed change in total HC following laboratory exposure of bivalves to toxicants is an increase in the number of cells. A number of studies reported such elevation in HC for bivalves under numerous conditions (Anderson 1981, Cheng 1988b, Renwratz 1990, Auffret & Oubella, 1994, Pipe & Coles 1995, Coles et al. 1994, 1995). In addition, our results showed the pooled samples to present a greater variability than the non-pooled samples. This could be a result of the allogenic interaction postulated above, although as for the MI, this remains to be demonstrated.

Listonella anguillarum clearance was impeded by both butyltins following both experiments, and the effects were greater in juveniles than adults. Although *Vibrio* are not normally considered to be pathogens of adult bivalves, they are a good model for pathogenic bacterial clearance. Relationships between enhanced severity and incidence of disease and the presence of toxicants in fish are well known (Sinderman 1993), but a similar relationship in bivalves is only starting to emerge (Anderson 1981, Chu & Hale 1994, Pipe & Coles 1995, Fisher et al. 1995, Anderson et al. 1996, 1998). Anderson (1981) reported that the majority of clams *Mercenaria mercenaria* exposed to 200 ng l⁻¹ benzo[a]pyrene, hexachlorobenzene or pentachlorophenol showed an impaired ability or even failure to clear injected *Flavobacterium* sp., while untreated clams cleared >90%. This author also showed that resistance to bacterial infection was decreased by exposure to xenobiotics and was directly proportional to tissue levels of the test pollutants. Winstead & Couch (1988) showed that, after sublethal exposure to *n*-nitrosodiethylamine, the oyster *Crassostrea virginica* developed heavy infections of the protistan pathogen *Perkinsus marinus*, and the virulence of *P. marinus* was enhanced by exposure to TBT (Fisher et al. 1995, Anderson et al. 1996) and water-soluble fractions of PAH-contaminated sediments (Chu & Hale 1994). Pipe & Coles (1995) determined that a 7 d pre-exposure to

<0.5 ng l⁻¹ waterborne copper impaired the immune function of adult *Mytilus edulis* so that when challenged with the pathogenic bacterium *Vibrio tubiashii*, mortality rate increased. These authors suggested that pre-exposure of mussels to contaminants can influence the immune response when challenged with a potential pathogen, and can increase the pathogenicity of normally benign opportunistic strains (Pipe & Coles 1995).

To date, 3 factors have been identified in the ability of bivalves to fight infection. First, lectins (agglutinins) react with bacteria and stimulate phagocytosis uptake (Olafsen 1986, Anderson et al. 1996) by facilitating bacterial aggregation or binding of bacteria to hemocytes (Renwratz & Stahmer 1983). Secondly, lysosomal enzymes, which when released may act on the surface of bacteria and promote their recognition or destruction (Anderson et al. 1996). Finally, reactive oxygen intermediates (ROI) are generated with the respiratory burst associated with PA (Anderson 1996). Which, if any, of these defense mechanisms is impaired by the butyltins remains to be investigated.

Our short-term exposure to environmentally realistic butyltin concentrations associated with the use of a novel bacterial clearance test showed a clear relationship between immunomodulations of the internal defense of the mussels and an impairment in clearing *Vibrio* from their hemolymph. Therefore, chronic exposure of mussels to butyltins may alter their capability in terms of immune response to an opportunistic pathogen or exacerbate an existing infection, with obvious population consequences. In that respect, further work is needed to verify if a correlation exists between butyltin-contaminated sites and higher disease incidence.

In conclusion, this study showed that both butyltins affect each immunological end-point measured at most doses tested. In fact, impairment of cell function was observed at levels well below the accumulation threshold of the mussels for this study. In addition, this study showed DBT to modulate the internal defense of mussels at similar concentrations and, it appears, in different ways from TBT. These observations raise the following questions: Since DBT is a degradation product of TBT, are animals subjected to the damage inflicted from TBT and then from the by-product DBT? Are their inherent toxicities additive or synergistic? This study established strong and sustained responses of hemocyte functions of blue mussels exposed to environmentally relevant waterborne concentrations of TBT and DBT (0.04 to 0.67 nM as Sn), which is interpreted as an increase of their vulnerability to other environmental stressors and pathogens. In addition, what are the lowest TBT or DBT concentrations that could trigger the hemocyte functions of blue mussels under *in vivo* laboratory conditions similar to those described in the pre-

sent work? That question was addressed by our laboratory and results are discussed in a LOEL study (St-Jean et al. 2002). There is no doubt that results obtained in controlled experiments are capable of establishing relationships between xenobiotics, immunomodulations and disease susceptibility. The results obtained in such experiments could eventually contribute greatly to a better understanding of field observations and ultimately facilitate biomonitoring and environmental assessment of locations potentially exposed to very low concentrations of butyltins.

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