

IMMUNOTOXIC EFFECTS OF ORGANOTIN COMPOUNDS IN *Tapes philippinarum*

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ABSTRACT

One of the most harmful groups of coastal pollutants is the organotin compounds (OTCs) which have severe effects on both aquatic organisms and mammals including humans. The immunotoxic effects of OTCs were studied in the cultivated clam *Tapes philippinarum* by determining the immunosuppressant role on *in vitro* yeast phagocytosis at low doses (0.01, 0.05, 0.1 μ M). The phagocytic index was significantly reduced in an irreversible non-lethal manner depending on concentration and lipophilic affinity. The order of inhibition was TBT $\geq$ DBT>MBT for butyltins and TPTC>TPTA $\geq$ TPTH for triphenyltins.

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INTRODUCTION

Organotin compounds (OTCs) are commonly employed in industry and agriculture thus producing widespread ecotoxicological problems from both point and non-point (diffuse) sources. Today, they are mainly used as stabilisers for plastics and oils, biocide additives in antifouling paints and antiparasitic agents [1][2]. The input of OTCs from multiple sources to the water column and biota are at present difficult to estimate with consequent control and elimination problems [3]. Their acute and chronic toxicity has been

demonstrated in several freshwater and marine species [4][5], some of which have been successfully used in monitoring environmental OTC contamination [6][7].

The clam *Tapes philippinarum* is a venerid bivalve which has been introduced and bred in the lagoon of Venice and in the Northern Adriatic in the 1980s. This species, which is indigenous to the Pacific Ocean offers a better short-term quality yield than the autochthonous species *Tapes decussatus*, because it shows an adaptable nature, hardness and outstanding growth rate. Moreover, *T. philippinarum* offers the chance to start the breeding with homogeneous seed produced on a vast scale by specialized laboratories (hatcheries) in controlled conditions, thus increasing health guarantees not ensured by the introduction of specimens collected in various geographical areas.

Bivalve molluscs are sedentary organisms which feed by filtering suspended particulate organic matter, particularly phytoplankton, so they are placed at the low trophic levels as primary consumers. The occurrence of intense maritime activity in areas of mollusc culturing creates a potential for TBT contamination of the food chain and humans. OTCs can either be adsorbed to particulate matter of sediments or exist in a solution. Soluble pollutants are more mobile and biologically available and are consequently more likely to undergo bioaccumulation [8]. Bivalves such as *Mytilus edulis* and *Crassostrea virginica* accumulate OTCs to the greatest extent in gill tissue [9], but metabolise these pollutants more slowly in comparison with gastropods, crustaceans and fish [10][11]. OTC degradation appears to be relatively inefficient in *Mercenaria mercenaria* and *Mya arenaria* [12], thus bioaccumulation and bioavailability of these substances must be considered as a high potential risk for human health.

These contaminants cause morphological aberrations in bivalve molluscs and it is suspected that they may be responsible for a decline in the abundance of various bivalve populations, endangering the biodiversity of coastal ecosystems. Populations of *C. virginica* and *Ostrea edulis* in waters with relatively low levels (i.e. less than 10 ng/l) of OTCs show a high incidence of imposex, a condition in which female animals grow male sex organs and the population cannot reproduce. Histological observations indicate that OTCs may retard the hormonally determined change in sex from male to female that normally occurs during the gametogenic cycle [13][14]. For different species the demographic decrease is also caused by a chronic embryo-larval toxicity in parallel with increasing OTC concentrations, which is revealed by i) high embryonic mortality and malformation occurrence, ii) reduction of veliger and postlarvae length, and iii) absence of metamorphosis [7][15][16][17][18][19]. OTCs are the main causative agents of appreciable shell thickening and malformations encountered in *Crassostrea gigas*, *Crassostrea angulata* and the Australian *Saccostrea commercialis* [20][21][22], as they inhibit the H^+ transporting pump (coupled with the inhibition of bicarbonate transport into the haemolymph) and increase the diffusion of calcium across the outer mantle epithelium [23]. Finally, owing to their cosmopolitan distribution and ability to concentrate many different contaminants, bivalves may represent useful bioindicators of TBT contamination by quantifying the effects on survival, growth and bioaccumulation integrated by chemical measurement of seawater [7].

The objective of this research is to investigate the OTC immunotoxicity on *T. philippinarum*. Interference with the immune system by butylic and phenylic OTCs and their main derivatives that form during their transfer into the compartments of the coastal ecosystem has been widely described both in vertebrates [24][25][26][27][28] and marine invertebrates [29][30][31][32].

MATERIALS AND METHODS

Animals

Specimens of the clam *T. philippinarum* (Eulamellibranchia, Veneridae) were collected from the lagoon of Venice. They were placed and maintained in large aquaria and fed with unicellular algae (*Pheodactylum* sp.) and Liquifry Marine (Liquifry Co., Dorking, Surrey, U.K.).

Organotin compounds

All chemicals were purchased from Sigma. Monobutyltin chloride (MBT), dibutyltin chloride (DBT), and tributyltin chloride (TBT) were first dissolved at a 10 mM concentration in 95% ethanol. Triphenyltin acetate (TPTA), triphenyltin chloride (TPTC), and triphenyltin hydroxide (TPTH) were first dissolved at a 10 mM concentration in dimethylsulfoxide (DMSO). Stock solutions were then diluted in filtered sea water (FSW) at the final concentrations of 0.01, 0.05, and 0.1 μM . Exposure concentrations were selected on the basis of available experimental bioaccumulation data relating to TBT (7500 ppb) in *C. virginica* [33] and natural TBT (307 ppb) and TPT (246 ppb) bioaccumulation data in *M. edulis* collected from the lagoon of Venice [34].

Haemocyte and blood plasma collection

Clam blood samples were collected with a disposable plastic syringe from the posterior adductor muscle. These were then kept for a few minutes in FSW containing 10 mM L-cysteine adjusted to pH 7.0 to prevent clotting and washed at $780 \times g$ for 10 min. The pelleted haemocytes were resuspended in FSW to achieve a final concentration of 10^6 cells ml^{-1} . To obtain blood plasma (BP), the haemolymph from five specimens was separately collected in FSW without L-cysteine, pooled and centrifuged at $780 \times g$ for 15 min to collect the supernatant. The protein concentration of the BP pool, determined according to Bradford (1976) [35], was 0.6 mg/ml. Some fractions of the same BP pool were freshly used, while the remaining part was frozen.

Immunotoxicity assay

Short-term haemocyte cultures were prepared following the technique described for the ascidian *Botryllus schlosseri* haemocytes [31]. After adhesion of the haemocytes, debris containing FSW was discarded and replaced with equal volume of a suspension of yeast in FSW (yeast:haemocyte ratio = 10:1)

containing OTCs at several different concentrations and incubated for 60 min at 25 °C. OTCs were omitted in controls. After incubation, haemocyte monolayers were washed several times in FSW, fixed in a solution of 1% glutaraldehyde plus 1% sucrose in FSW at 4 °C for 30 min, stained with 10% Giemsa for 5 min, mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba) and observed with a Leitz Dialux 22 light microscope. To assess the effects of BP on phagocytosis, yeast cells were preincubated for 30 min in whole BP, thoroughly washed by three centrifugations at 1390 x g, and resuspended in FSW or OTC solutions before incubation with *T. philippinarum* haemocytes. These experiments were also carried out in parallel by using yeast cells preincubated in FSW. Viability was assessed by means of the Trypan Blue dye exclusion test.

Statistical analysis

All experiments were repeated five times ($n=5$) with 5 independent samples. Haemocytes were counted at a magnification of 1250x (at least 200 cells per monolayer in 10 fields). Data were expressed as mean values $\pm$ SD of the *phagocytic index* (i.e. the percentage of haemocytes containing ingested yeast particles) and were analysed using X^2 test.

RESULTS

T. philippinarum haemocytes *in vitro* recognised and phagocytised yeast cells, thus remarkably changing their own shape. As a consequence of yeast cell ingestion, amoeboidal phagocytes withdrew their pseudopodia, developed giant vacuoles and increased in size (Fig 1a,b). Preincubation of yeast with fresh BP significantly increased ($p < 0.001$) the phagocytic index with respect to controls preincubated in FSW (Fig. 2). As this BP activity was not altered by freezing and thawing (phagocytosis index 14.48 ± 1.8 versus 14.82 ± 1.18 in fresh BP-treated samples), yeast cells were preincubated in freezed BP in all the phagocytosis experiments.



Figure 1.

Light micrograph of *in vitro* cultured haemocytes of *Tapes philippinarum* (a). Control haemocytes containing phagocytized yeast cells (b) and altered haemocyte morphology after exposure to organotin compounds (c). Cells were fixed in 1% glutaraldehyde plus 1% sucrose in filtered sea water and stained with 10% Giemsa. Bar length: 10 μ m.

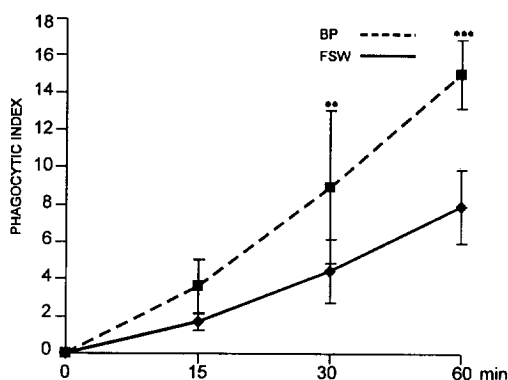


Figure 2.

Kinetics of yeast phagocytosis by *T. philippinarum* haemocytes. Effects of preincubation in filtered sea water (FSW) and blood plasma (BP) are compared. Asterisks indicate significant differences between treated and untreated yeast cells: ** $p < 0.01$; *** $p < 0.001$.

The phagocytic index was significantly reduced by OTCs in an irreversible manner and was dependant on concentration and lipophilic affinity. In particular, the phagocytic index was significantly reduced by butyltin compounds (Fig. 3) with $p < 0.01$ for $0.05 \mu\text{M}$ TBT and DBT, $p < 0.001$ for $0.1 \mu\text{M}$ MBT and by triphenyltin compounds (Fig. 4) with $p < 0.05$ for $0.01 \mu\text{M}$ TPTC and for $0.05 \mu\text{M}$ TPTA and TPTH. The preincubation of yeast cells in FSW showed the same significant pattern even though the control phagocytic indexes were lower (7.38 ± 0.72).

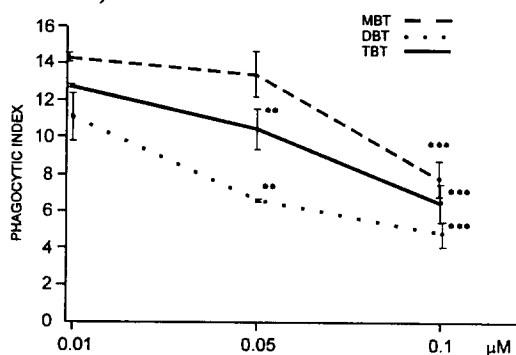


Figure 3.

Effects of monobutyltin trichloride (MBT), dibutyltin dichloride (DBT), and tributyltin chloride (TBT) on yeast phagocytosis by *T. philippinarum* haemocytes. Asterisks indicate the levels of significance with respect to controls: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

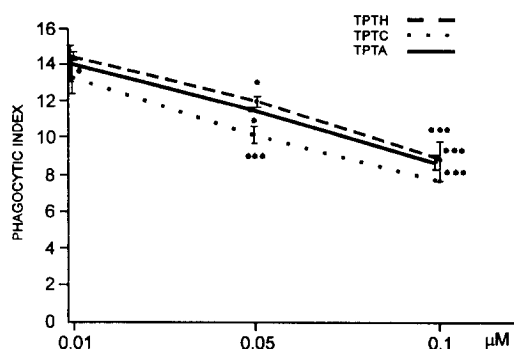


Figure 4. Effects of triphenyltin hydroxide (TPTH), triphenyltin chloride (TPTC), and triphenyltin acetate (TPTA) on yeast phagocytosis by *T. philippinarum* haemocytes. Asterisks indicate the levels of significance with respect to controls: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Neither cell mortality nor cytolysis occurred in the culture chambers and viability always exceeded 95%. In the presence of active doses of OTCs, severe alterations of haemocyte morphology were observed (i.e. all become spherical) (Fig 1c).

DISCUSSION

Interspecies comparisons of immunological effects due to chemical *in vitro* treatment can provide a better understanding of the relationships between animal and human data. The immune system is one of the first components which suffers stress conditions. In mammals, OTCs cause atrophy of the thymus, spleen and lymphonodes [36], lymphocyte depletion in peripheric blood [37], inhibition of both chemotaxis [38][39] and phagocytosis [40] in polymorphonuclear leukocytes. In filter feeding marine invertebrates, which potentially bioaccumulate great amounts of OTCs, a significant inhibition of phagocytic index occurs as reported in tunicates [31][32] and mussels [41]. Recently, Anderson *et al.* (1996) [33] have reported that *in vivo* and *in vitro* exposures of *C. virginica* specimens and haemocytes to environmentally relevant TBT levels enhance the progression of infection by the pathogenic protozoan *Perkinsus marinus*, causing a marked decline in oyster fishery. These authors also suggest that, although TBT has no significant effects on cell-mediated responses, its putative immunosuppressive activity could be attributed to the inhibition of the production of reactive oxygen intermediates.

Our results in immunotoxicity studies with haemocytes of the clam *T. philippinarum* make it possible to confirm the negative effects of OTCs on cell-mediated phagocytosis at extremely low doses. In the blood of this species at least four cell types are recognisable, i.e. small round cells, granulocytes, hyalinocytes, serous cells. The *in vitro* yeast phagocytosis is carried out by both granulocytes and hyalinocytes and is increased by preincubation of yeast with BP, probably reflecting the presence of an opsonin that makes the yeast surface more suitable for recognition. The OTC effects on phagocytosis are irreversible, dose-dependent, non-lethal within 60 min of acute exposure and show that TBT is the most toxic among butylic compounds. The toxicity order is strictly connected with OTC chemical structure and lipophily. The most toxic butyltin compounds are trisubstituted, followed by disubstituted, whereas monosubstituted compounds are the less toxic (TBT $\geq$ DBT $>$ MBT). Unlike TBT, which is the most studied OTC, ecotoxicity of triphenyltin compounds is still scarcely known. The toxicity of these compounds appears to show the same order of magnitude of TBT, but depends on lipophily of the anionic group. Therefore, the order of inhibition using the phagocytic index is TPTC $>$ TPTA $\geq$ TPTH.

T. philippinarum proved to be twenty times more sensitive than ascidians and may represent an effective bioindicator for organotin contaminants both in natural environments and mollusc cultures. Moreover, the phagocytosis assay proposed is rapid and easily reproducible. The stress induced by OTCs on the immune defences of this species is independent of BP presence, although BP could act as an opsonising agent for yeast phagocytosis.

Different mechanisms of action have been suggested to explain the biological effects and the toxicity of OTCs. In vertebrates, the main toxic action appears to be the inhibition of the oxidative phosphorylation in mitochondria [42] and, therefore, of the fundamental energy processes in cell systems [43][44]. These compounds also inhibit both the synthesis of proteins through coordination bonds with amino acids [45] and the activity of cytochrome P-450 in various organs [46]. Immunotoxicity may also be mediated by severe alterations of cytoskeletal organisation. OTCs interfere with the polymerisation of microtubules and microfilaments by altering the intracellular Ca^{2+} homeostasis both in mammal thymocytes [47] and ascidian phagocytes [48] and by direct interactions with cytoskeletal structures [49]. Such selective interactions also occur in the haemocytes of the clam *T. philippinarum* exposed to OTCs, as we observed irreversible morphological changes. Future efforts will be aimed at evaluating haemocyte damages detectable after exposure of clams to different OTC concentrations in sea water. In this way, the phagocytosis test could be effectively applied together with other ecotoxicological bioassays in evaluation studies of environmental impact by OTCs and may be useful in assessing the sensitivity of this species, improving its productivity in culture farms and controlling probable risks to human health.

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