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4-Nonylphenol induces immunomodulation and apoptotic events in the clam *Tapes philippinarum*

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ABSTRACT: The effects of 4-nonylphenol (NP) on functional responses of haemocytes from the clam *Tapes philippinarum* were investigated after 7 d exposure to sublethal NP concentrations (0, 0 + acetone, 0.025, 0.05, 0.1 and 0.2 mg l^{-1} NP). Haemocytes from both controls and exposed clams were collected, and the effects of NP on uptake of the vital dye Neutral Red (NR), superoxide dismutase (SOD) and lysozyme activities, total haemocyte count (THC) and volume of circulating cells were evaluated. The capability of NP to induce apoptosis was also investigated. Exposure of clams to 0.2 mg l^{-1} NP significantly increased ($p < 0.05$) NR uptake when compared with controls, suggesting that NP caused alterations in cell membrane stability. Significant decreases in both SOD and lysozyme activity were observed from 0.05 mg l^{-1} NP ($p < 0.01$ and $p < 0.05$, respectively) with respect to controls, indicating that NP causes oxidative stress and reduces the immunocompetence of the exposed clams. From 0.05 mg l^{-1} NP, the apoptotic index (percentage of haemocytes showing positivity to the TUNEL reaction) significantly increased ($p < 0.001$). Apoptotic haemocytes showed shrinkage and lost their amoeboid shape. Moreover, NP exposure significantly increased THC ($p < 0.05$ at 0.2 mg l^{-1} NP) and caused a different distribution in size frequency. On the basis of the threshold concentration values, the most sensitive endpoints for NP were SOD, lysozyme and apoptosis assays. Our results highlight a relationship between NP exposure and changes in the functional responses of haemocytes, suggesting that the contaminant induces immunomodulation in *T. philippinarum*, mainly by altering cell morphology, cell membrane stability and enzymatic activities, and by promoting oxidative stress and apoptotic events.

KEY WORDS: Nonylphenol · Xenoestrogens · Bivalves · Haemocytes · Immunotoxicity · Apoptosis · Cell volume

INTRODUCTION

Numerous studies have demonstrated that contaminants can induce immunomodulation in bivalve molluscs by altering the functional responses of haemocytes, circulating cells known to play an important role in the internal defence of these animals. Alterations in immunocompetence have been reported for bivalves after exposure, both *in vitro* and *in vivo*, to organic and inorganic contaminants, such as heavy metals (Cheng & Sullivan 1984, Cheng 1988, Viarengo et al. 1994, Coles et al. 1995, Pipe et al. 1999, Matozzo et al. 2001, Sauvé et al. 2002), organotins (Fisher et al. 1990, Auffret & Oubella 1997, Cima et al. 1998, Matozzo et al. 2002), fungicides (Alvarez & Friedl 1992), polycyclic aromatic hydrocarbons (PAHs) (Sami et al. 1992, Coles et al. 1994, Grundy et al. 1996, Gómez-Mendikute et al. 2002) and chlorinated phenols (Florence et al. 1997). Among the functional responses of bivalve circulating cells, total haemocyte count (THC), viability, motility, aggregation and adhesion capability, phagocytosis, production of reactive oxygen species (ROS), hydrolytic and oxidative enzyme activities, and lysosomal and cell membrane stability are all generally recognised as useful biomarkers in immunotoxicity studies.

At the present time, limited data are available on nonylphenol (NP)-mediated immunomodulation in bivalves. NP is used in the production of nonylphenol ethoxylates (NPEs), NP phosphites and insecticide

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sprays (Maguire 1999). NP phosphites are used as stabilisers and antioxidant agents in both rubber and plastic industries, and NPEs are nonionic surfactants commonly employed in plastics, latex paints, lubricating oils, emulsifiers, household and industrial detergents, and paper and textile industries (Lee 1999). As a consequence of their widespread use, NPEs are discharged in large quantities into aquatic environments, either directly from untreated effluents or indirectly from sewage treatment plants (STPs) (Ekelund et al. 1993, Maguire 1999). NPEs are not persistent in aquatic ecosystems but biodegrade to de-ethoxylated intermediates, of which NP is the final product (Maguire 1999). In water and sediment, NP appears to be more persistent to degradation than NPEs (Brunner et al. 1988, Ekelund et al. 1993), and, owing to its higher lipophilicity, can be accumulated by aquatic organisms (Ekelund et al. 1990, Ahel et al. 1993). The adverse effects of both NP and NPEs have been reported in fish and aquatic invertebrates, although NP appears to be acutely toxic at lower concentrations with respect to NPEs, their toxicity reducing with decreasing ethoxylate unit chain lengths (Servos 1999). It has been shown that NP affects population growth rates in invertebrates, as observed in the infaunal polychaete *Capitella* sp. and the marine copepod *Tisbe battagliai* (Bechmann 1999, Hansen et al. 1999). Moreover, NP reduced the scope for growth in the Manila clam *Tapes philippinarum* (Matozzo et al. 2003a) and the mussel *Mytilus edulis* (Granmo et al. 1989). NP and NPEs also alter hormonal functions in various aquatic organisms, being able to mimic the action of endogenous estrogens, such as 17β-estradiol (E_2), by binding estrogen receptors (Arukwe et al. 1997, Madigou et al. 2001). As a consequence, these estrogenic compounds induce vitellogenin synthesis (vitellogenins are the major precursor of the egg-yolk proteins in oviparous females) in exposed males (Christiansen et al. 1998, Christensen et al. 1999). Indeed, as the vitellogenin gene (normally silent) is also present in males, it may be activated by (xeno-) estrogens (Flouriot et al. 1995).

Since data about the immunotoxicity of NP in bivalves are lacking, the present study was addressed to assess NP's effects on the functional responses of haemocytes from a widespread and economically relevant clam species in the Lagoon of Venice (Italy). In water samples from various stations of the Lagoon, the mean concentration values of NPEs, including NP, peak at 39 µg l^{-1} (Marcomini et al. 2000). Similarly, in estuarine areas of Croatia and England, NP levels as high as 2.3 and 5.2 µg l^{-1}, respectively, have been detected (Kvestak & Ahel 1994, Blackburn & Waldock 1995). A number of other reports reviewed by Ying et al. (2002) indicate NP levels ranging from the limit of detection to 644 µg l^{-1} in surface waters around the world. However, NP levels in the sediments are much higher than in the corresponding surface waters, thus representing an important source for future contamination (Bennie 1999, Ying et al. 2002).

In this study, specimens of *Tapes philippinarum* were exposed to sublethal and environmentally realistic NP concentrations to evaluate the effects on uptake of the vital dye Neutral Red (NR) (as an index of cell membrane stability), activity of superoxide dismutase (SOD, antioxidant enzyme) and lysozyme (bacteriolytic enzyme), THC, and the volume of circulating cells. The capability of NP to induce apoptotic events in haemocytes from NP-exposed clams was also investigated.

MATERIALS AND METHODS

Animals. Specimens of *Tapes philippinarum* were collected from a reference site located inside a licensed area for clam culture in the southern basin of the Lagoon of Venice (Italy) and acclimatised in the laboratory for 7 d before exposure to NP. The clams were kept in large aquaria provided with a sandy bottom and aerated sea water (salinity of 35 ± 1‰, temperature of 17 ± 0.5°C) and fed with the microalgae *Isochrysis galbana*.

4-Nonylphenol solutions. NP, a mixture of p-isomers, was purchased from Fluka Chemika. Owing to its low solubility in water, a stock solution of NP was prepared in acetone and stored at room temperature for the duration of the experiments. Working solutions were prepared daily by diluting the stock solution in sea water.

Exposure to NP. To evaluate NP effects on functional responses of haemocytes, 2 series of experiments were performed. In the former, 15 animals per concentration were exposed for 7 d to 0, 0 + acetone, 0.025, 0.05, 0.1 and 0.2 mg l^{-1} NP to evaluate NP effects on NR uptake, SOD and lysozyme activities, and apoptosis. In the latter, 20 clams per concentration were exposed to the same NP concentrations to study the effects on both THC and volume of circulating haemocytes. The nominal exposure concentrations were chosen on the basis of the LC_{50} value (1.12 mg NP l^{-1}) recorded in *Tapes philippinarum* (Matozzo et al. 2003a). In acetone controls, solvent was added at the highest concentration (16 µl l^{-1}) used in NP treatments. Clams were maintained in glass aquaria (without sediment) containing aerated sea water (1 l per animal), in the same thermohaline conditions used in the acclimatisation period. Water was changed every day, and NP and microalgae added (*Isochrysis galbana*, at an initial concentration of about 100 000 cells l^{-1}).

Haemolymph collection. After NP exposure, haemolymph was collected from the anterior adductor muscle in a 1 ml plastic syringe and placed in Eppendorf tubes at 4°C. In the first series of experiments, 3 pools of haemolymph obtained from control and NP-exposed clams (5 animals per pool) were used, in the second series, 4 pools of haemolymph (5 animals per pool) were used.

NR uptake assay. The cationic probe, NR (Merck), was used to evaluate haemocyte membrane stability after NP exposure. Alterations in its uptake are commonly recognised as an indicator of cell damage in *in vitro* studies (Gómez-Mendikute et al. 2002, Matozzo et al. 2002). Haemolymph (final volume = 1 ml) from both NP-treated and untreated clams (controls) was centrifuged at 780 × *g* for 10 min, haemocytes were resuspended in an equal volume of 8 mg l^{-1} NR dye solution in filtered seawater (FSW) and then incubated at 25°C for 30 min. They were then centrifuged at 780 × *g* for 10 min, resuspended in distilled water, sonicated at 0°C for 1 min with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12 000 × *g* for 15 min at 4°C. Supernatant, corresponding to cell lysate (CL), was collected for the NR uptake assay. CL (200 µl) was put in the wells of a 96-well microplate and absorbance at 550 nm was recorded with a microplate reader (Reader SR400, Techno Genetics). Results were expressed as optical density per mg protein (OD mg $protein^{-1}$). CL protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as standard.

SOD activity assay. SOD activity was measured according to the method of Flohé & Ötting (1984), based on the reduction of cytochrome *c* by the superoxide radical (O_2^-). Haemolymph from exposed and unexposed clams was centrifuged at 780 × *g* for 10 min, resuspended in distilled water, sonicated and centrifuged as described above. CL was collected and used for the SOD activity assay. CL (50 µl) was added to 900 µl of solution A (5 µM xanthine [Sigma] in 1 mM NaOH, 2 µM cytochrome *c* [Sigma] and 0.1 mM EDTA in 50 mM phosphate buffer, pH 7.8 [2.72 g l^{-1} KH_2PO_4 and 5.34 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$ in distilled water]) and to 50 µl of solution B (0.45 U xanthine oxidase [Sigma], 1 ml 0.1 mM EDTA in 50 mM phosphate buffer, pH 7.8). Changes in absorbance at 550 nm were continuously recorded for 2 min with a Uvikon 930 spectrophotometer. Results were expressed as U SOD mg $protein^{-1}$. According to Flohé & Ötting (1984), 1 unit of SOD is defined as the amount of enzyme inhibiting the reduction of cytochrome *c* by 50%.

Lysozyme activity assay. Lysozyme activity was quantified according to Santarem et al. (1994). CL (50 µl), obtained as described above, was added to 950 µl of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2, and the decrease in absorbance (ΔA min^{-1}) was continuously recorded at 450 nm for 5 min at 20°C. Standard solutions containing 1, 2.5, 5 and 10 µg lysozyme per ml of 66 mM phosphate buffer, pH 6.2, were prepared from crystalline hen egg white lysozyme (Sigma). The average decrease in absorbance per minute was determined for each enzyme solution and a standard curve of enzyme concentration versus ΔA min^{-1} was drawn. A unit of lysozyme was defined as the amount of enzyme producing an activity equivalent to 1 µg of lysozyme, in the conditions described above. Results were expressed as µg lysozyme mg $protein^{-1}$.

Short-term haemocyte cultures and apoptosis assay. DNA fragmentation, a biochemical feature of apoptosis, was investigated in cultured haemocytes using the TUNEL reaction mixture (*In Situ* Cell Death Detection Kit, Boehringer Mannheim). After NP exposure, haemolymph from both controls and NP-exposed clams was collected in a 1 ml plastic syringe containing 300 µl of 10 mM L-cysteine in FSW, pH 7.5, to prevent clotting, and centrifuged at 780 × *g* for 10 min. Haemocytes were then resuspended in FSW at a final concentration of 10^6 cells ml^{-1} to prepare short-term haemocyte cultures, according to Ballarin et al. (1994). Haemocyte suspensions (60 µl) were placed in the centre of culture chambers made by a teflon ring (15 mm internal diameter and 1 mm thick), smeared with vaseline, glued to a siliconised glass slide and covered with a coverslip. Chambers were kept upside down for 30 min at 25°C to allow haemocytes to settle and adhere to coverslips. After adhesion to coverslips, haemocytes were fixed in a 4% paraformaldehyde solution in ISO buffer (Tris 20 mM, NaCl 0.5 M, pH 7.5) for 30 min at 25°C, rinsed with phosphate buffer (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.2) and incubated for 2 min at 4°C in a permeabilisation solution containing 0.1% Triton X-100 (Merck) in 0.1% sodium citrate. Haemocytes were rinsed in PBS and then incubated in the TUNEL reaction mixture for 1 h at 37°C. During this phase, the enzyme deoxynucleotidyl transferase (TdT) catalyses the attachment of fluorescein isothiocyanate-labelled dUTP to free 3'-OH DNA ends. Haemocytes were then observed under a fluorescence microscope and the apoptotic index expressed as the percentage of haemocytes (at least 500 cells in 10 fields per slide were counted) showing positivity to the TUNEL reaction (apoptotic nuclei appeared greenish-yellow).

THC and haemocyte volume determination. Both THC and haemocyte volume were measured by an electronic particle counter/size analyser (Coulter Counter, Model Z2). Pooled haemolymph (500 µl) was added to 19.5 ml of 0.45 µm-FSW, and both total numbers and volume of circulating haemocytes were

immediately estimated. THC and haemocyte volume results were expressed as number of haemocytes ($\times 10^6$) ml haemolymph^{-1} and femtolitre (fl), respectively.

Statistical analysis. For all indices, data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test). Results of NR uptake, lysozyme and SOD activity assays were compared using a 1-way ANOVA, followed by a post hoc test (Duncan's test). When ANOVA assumptions were not fulfilled (e.g. THC results), the non-parametric Mann-Whitney *U*-test was used to perform pairwise comparisons between treatments. The results of the apoptosis assay were compared by the χ^2 test. All results were expressed as means ± SD. For all indices, threshold concentrations were evaluated as the geometric mean of the highest tested concentration that produced no statistically significant observable effect (NOEC) and the lowest tested concentration that produced a statistically significant observable adverse effect (LOEC). The software packages STATISTICA 5.5 (StatSoft) and SAS (SAS Institute) were used for statistical analyses.

RESULTS

Alterations were observed in all the cell parameters analysed. Indeed, exposure to 0.2 mg l^{-1} NP resulted in significant increases ($p < 0.05$) in NR uptake, when compared with controls (Fig. 1).

The effects of NP on enzyme activities are shown in Figs. 2 & 3, respectively. SOD significantly decreased from 0.05 mg l^{-1} NP ($p < 0.01$), with respect to controls.

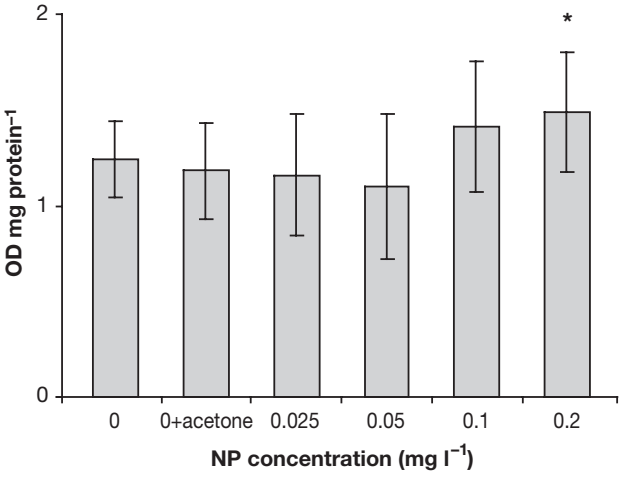

Fig. 1. *Tapes philippinarum*. Effects of NP on the capability of haemocytes to take up Neutral Red dye. Results are expressed as OD mg protein^{-1}. Asterisks: significant results in comparison with controls. Values are means ± SD; n = 3; *p < 0.05

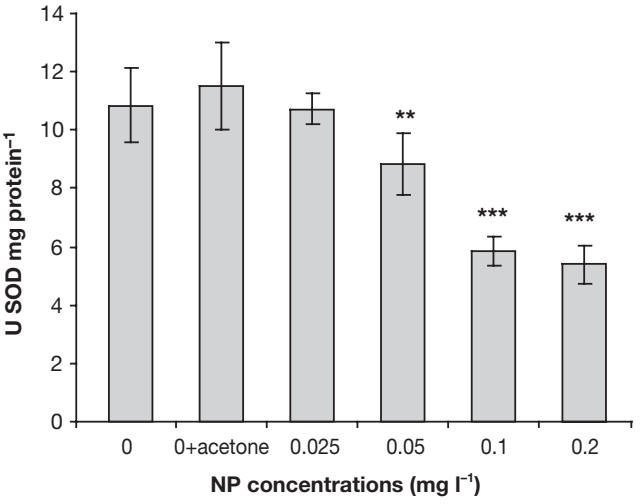

Fig. 2. *Tapes philippinarum*. Effects of NP on SOD activity, expressed as U SOD mg protein^{-1}, in haemocytes. Asterisks: significant results in comparison with controls. Values are means ± SD; n = 3; **p < 0.01, ***p < 0.001

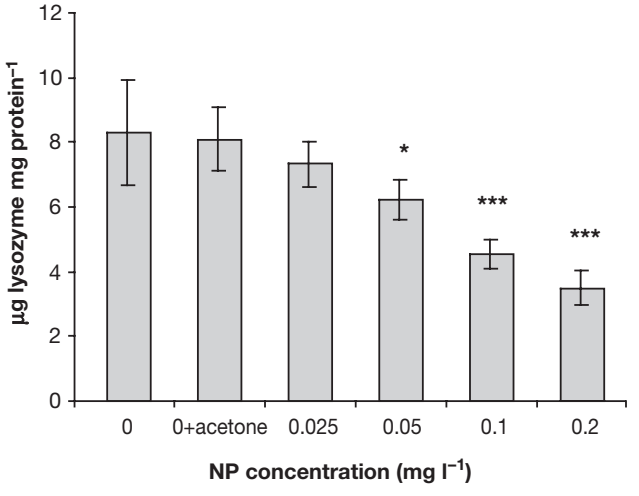

Fig. 3. *Tapes philippinarum*. Effects of NP on lysozyme activity, expressed as µg lysozyme mg protein^{-1}, in haemocytes. Asterisks: significant results in comparison with controls. Values are means ± SD; n = 3; *p < 0.05, ***p < 0.001

In 0.05 mg l^{-1} NP-exposed clams, SOD activity was also significantly lower when compared with that in 0.025 mg l^{-1} NP-exposed animals ($p < 0.05$). Moreover, clams exposed to 0.1 and 0.2 mg l^{-1} NP showed lower SOD activity ($p < 0.001$) when compared with 0.025 and 0.05 mg l^{-1} NP-exposed clams. Lysozyme activity was significantly reduced ($p < 0.05$) in haemocytes from clams exposed to 0.05 mg l^{-1} NP, with respect to controls. At 0.1 and 0.2 mg l^{-1} NP, lysozyme activity significantly decreased when compared with 0.025 mg l^{-1} NP ($p < 0.01$ and $p < 0.001$, respectively) and 0.05 mg l^{-1} NP-exposed animals ($p < 0.05$ and $p < 0.01$, respectively).

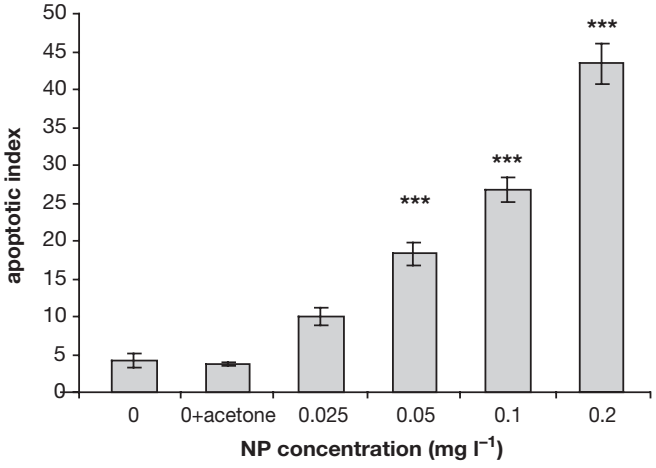

Fig. 4. *Tapes philippinarum*. Apoptotic index, expressed as percentage of haemocytes positive to TUNEL reaction after exposure to NP. Asterisks: significant results in comparison with controls. Values are means ± SD; n = 3; ***p < 0.001

A significant increase ($p < 0.001$) in the apoptotic index was observed in haemocytes from bivalves exposed to 0.05 mg l^{-1} NP (Fig. 4). Haemocytes from NP-exposed clams underwent a series of morphological changes: apoptotic haemocytes generally showed a marked decrease in cell volume and lost their characteristic amoeboid shape, as cells withdrew their pseudopodia (Fig. 5).

Exposure to NP also resulted in a dose-dependent different haemocyte size frequency distribution: the fractions of both smaller (<100 fl) and larger haemocytes (>400 fl) appeared increased at the highest NP concentrations tested with respect to controls (Fig. 6). Moreover, at the end of NP exposure, a significant increase ($p < 0.05$) in THC was recorded, ranging from a mean of 2.9×10^6 cells ml^{-1} in controls to 6.4×10^6 cells ml^{-1} in 0.2 mg l^{-1} NP-exposed clams (Fig. 7).

The threshold concentration was 0.14 mg l^{-1} NP in NR uptake and THC assays, and 0.035 mg l^{-1} NP in SOD, lysozyme and apoptosis assays.

DISCUSSION

As reported by various authors, bivalve haemocytes play a key role in internal defence against pathogens and foreign materials. Consequently, the adverse effects of contaminants on haemocyte functionality may increase the susceptibility of animals to diseases and reduce their survival capability. In the present study, marked alterations in the functional responses of haemocytes were observed after 7 d exposure of *Tapes philippinarum* to sublethal NP concentrations, indicating a decrease in their immunocompetence and, thus, highlighting a condition of potential risk for the well-being of clam populations in estuarine areas.

NR is commonly used in *in vitro* bioassays based on the accumulation of this cationic dye into the lysosomes of viable cells. Uptake by haemocytes generally occurs either by pinocytosis or passive diffusion across

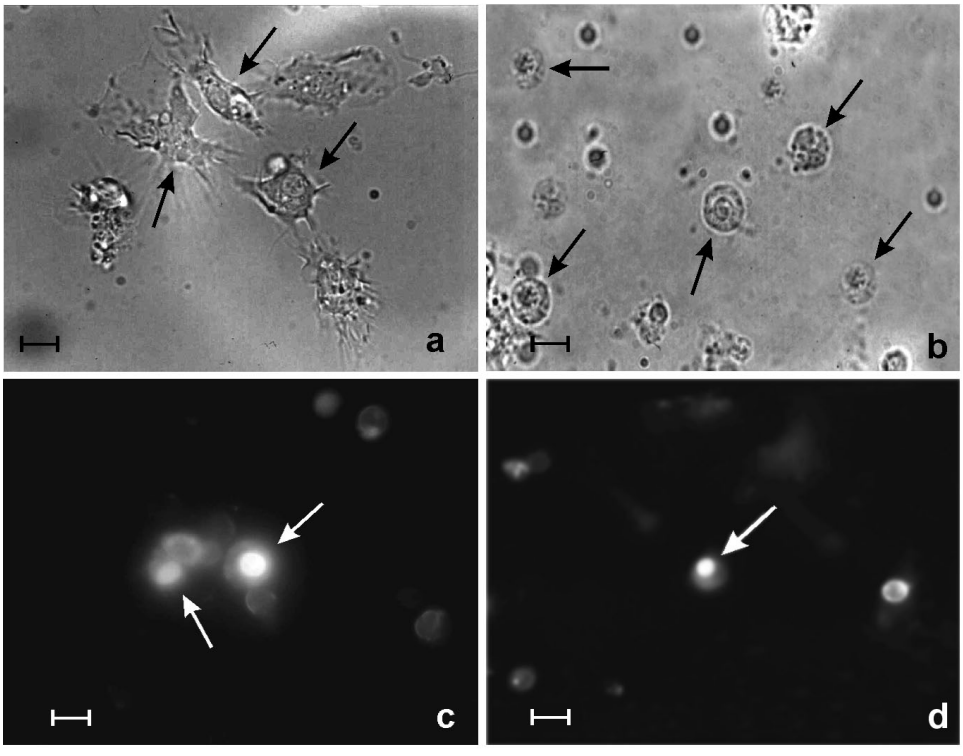

Fig. 5. *Tapes philippinarum*. Haemocytes after exposure to NP. Phase-contrast micrographs: control haemocytes showing amoeboid shape (arrows) (a), and haemocytes from 0.2 mg l^{-1} NP-exposed clams showing spherical shape (arrows) (b). Fluorescence micrographs: haemocytes from 0.1 mg l^{-1} (c) and 0.2 mg l^{-1} (d) NP-exposed clams showing positivity to TUNEL reaction (apoptotic cells showed greenish-yellow nuclei) and spherical shape (arrows). Scale bars = 3 µm

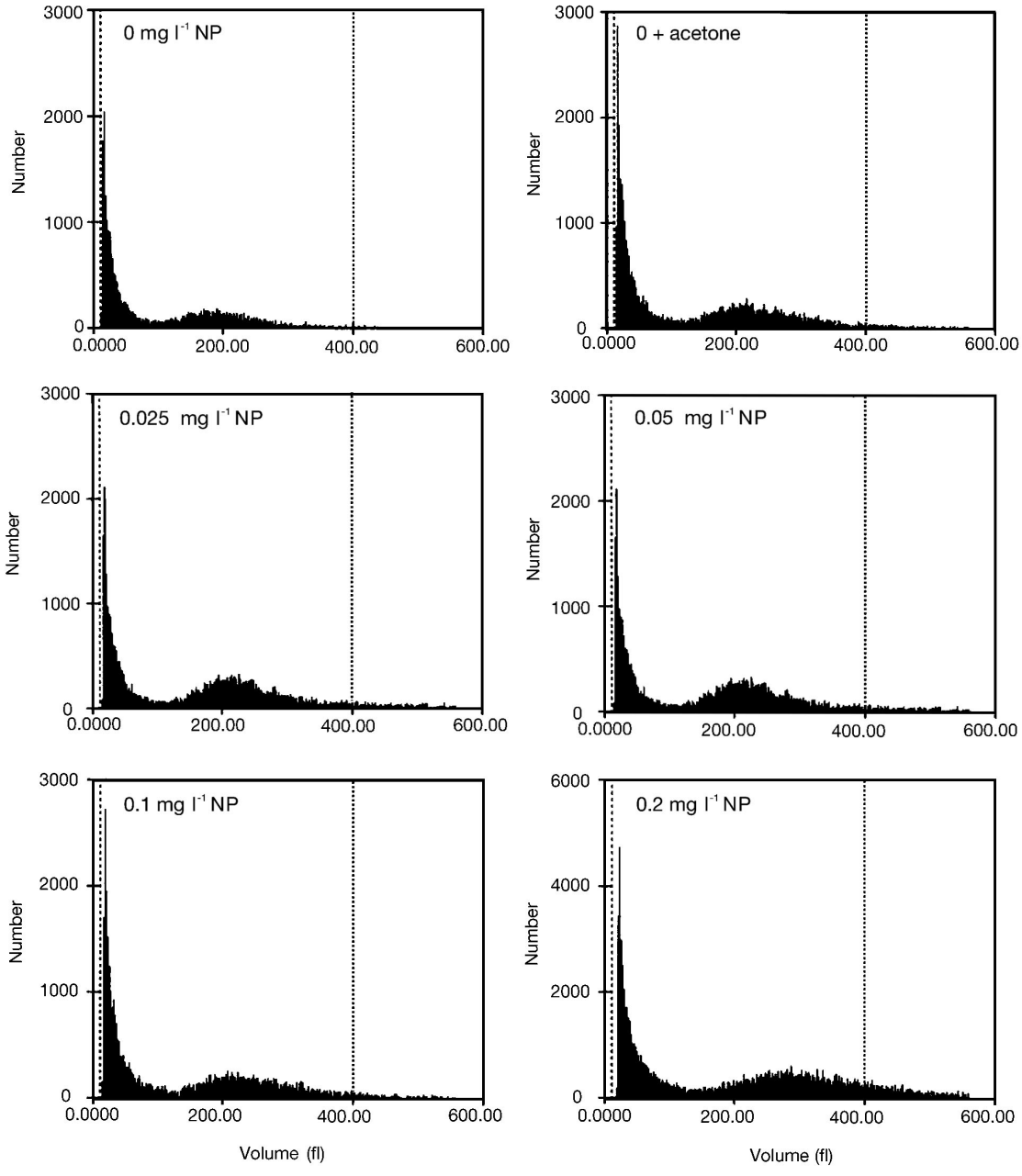

Fig. 6. *Tapes philippinarum*. Haemocyte size frequency distribution (volumes expressed as femtolitre) after exposure to NP

cell membranes (Coles et al. 1995). Alterations in dye uptake may, therefore, reflect damage to cell membranes, as well as changes in number and/or volume of lysosomes. Our results showed an increase in NR uptake by haemocytes from clams exposed to the highest NP concentration tested. As a first hypothesis, we suggest that changes in haemocyte membrane permeability occurred. Indeed, increased membrane permeability in haemocytes from NP-exposed clams presumably determined the higher NR assumption with respect to haemocytes from control clams. Although this needs to be verified, NP may act as a membrane-active molecule able to bind membrane proteins and phospholipids, similarly to what has been suggested for other lipophilic compounds. Indeed, in a recent study, severe haemocyte membrane injury, measured by a nigrosine dye test, was observed in haemocytes from *Mytilus edulis* exposed to tributyltin (TBT) and dibutyltin (DBT) (St-Jean et al. 2002). An increase in NR uptake was also demonstrated in haemocytes from cadmium-exposed mussels, *M. edulis* (Coles et al. 1995), whereas no significant differences in dye uptake were

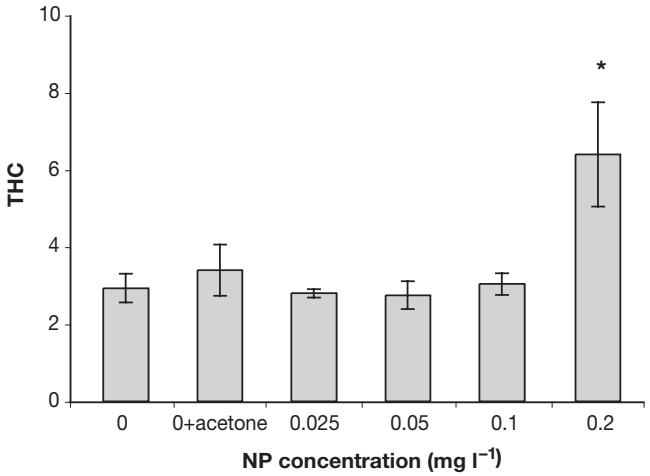

Fig. 7. *Tapes philippinarum*. Total haemocyte count (THC), expressed as number of haemocytes ($\times 10^6$) ml haemolymph^{-1}, after exposure to NP. Asterisks: significant difference in comparison with controls.Values are means ± SD; n = 4; *p < 0.05

recorded in haemocytes from copper-exposed mussels (Pipe et al. 1999). Haemocyte membrane destabilisation has also been revealed using other experimental methods. Significant increases in the uptake of ethidium homodimer-1 were recorded in haemocytes from *Mya truncata* after 2 wk exposure to PAH-contaminated sediments (Camus et al. 2003). As a second hypothesis, it cannot be excluded that, in haemocytes of exposed animals, NP caused an increase in the number and/or volume of lysosomes, which probably took up more NR than those of cells from control clams. This is in agreement with our previous findings. Indeed, increased NR uptake and enlarged lysosomes were observed in haemocytes from *Tapes philippinarum* exposed *in vitro* to TBT (Matozzo et al. 2002).

Haemocytes from NP-exposed clams showed a marked reduction in enzymatic activities. Anti-oxidant agents, such as SOD, catalase, glyoxalase, glutathione peroxidases and glutathione protect cells against ROS-mediated oxidative damages (Di Giulio et al. 1989, Regoli & Principato 1995). An important role in the anti-oxidant defence of aquatic invertebrates is mainly played by SOD and CAT (Winston 1991, Livingstone 2001). SOD, in particular, catalyses the dismutation of the superoxide radical (O_2^-) to oxygen and hydrogen peroxide. It is well-known that increases in ROS levels may be due to exposure of animals to several contaminants, such as heavy metals, PAHs, polychlorobiphenyls and dioxins (Livingstone 2001, Geret et al. 2002, Orbea et al. 2002). Recently, it has been observed that NP may also enhance ROS production in human blood neutrophils (Okai et al. 2004). As a consequence, exposure to ROS-generating contaminants may increase or inhibit anti-oxidant enzyme activities in organisms. Griveau et al. (1995) observed that ROS can inactivate anti-oxidant enzymes, such as glutathione peroxidase, glucose-6-phosphate dehydrogenase and SOD. In this study, the significant inhibition of SOD in NP-exposed clams may reflect the inability of cells to eliminate O_2^-, an oxygen metabolite responsible for oxidative stress in cells. NP exposure also decreased SOD activity in the digestive gland and gills of *Tapes philippinarum* (Matozzo et al. 2004). Decreased SOD activity was previously observed in haemocytes from *T. philippinarum* after 7 d exposure to copper (Matozzo et al. 2001), and in *in vitro* TBT-exposed haemocytes (Matozzo et al. 2002). We hypothesise that the inhibition of SOD observed in the present study is due to ROS-mediated oxidation of the enzyme SH groups. According to Roche & Bogé (2000) and Okai et al. (2004), phenols increase ROS levels, which in turn cause inactivation of enzymes, determining oxidative damage.

In the immune system of bivalves, lysozyme is one of the most important bacteriolytic agents against several species of Gram-positive and Gram-negative bacteria (Cheng & Rodrick 1974). Lysozyme is a lysosomal enzyme secreted by haemocytes in the haemolymph during phagocytosis, thus participating in the inactivation of invading pathogens. Reduced lysozyme activity, therefore, suggests immunosuppression, resulting in lowered resistance to bacterial challenge. In this study, significant inhibition of lysozyme activity was observed in haemocytes from 0.05 mg l^{-1} NP-exposed clams, confirming previous data concerning the capability of lipophilic contaminants to alter enzymatic activity in fish cells (Brüschweiler et al. 1996) and bivalve haemocytes (Cima et al. 1999, Matozzo et al. 2002). Although the mechanisms of action of the contaminant need to be more fully studied, we suggest that NP causes haemocyte degranulation, followed by a decrease in intracellular lysozyme activity, as well as an increase in extracellular enzyme activity. As in our previous observations in haemocytes of *Tapes philippinarum*, higher lysozyme levels than in the haemolymph were found (Matozzo et al. 2003b); in the present study, lysozyme activity was determined in haemocyte lysate. The lower intracellular lysozyme activity observed in NP-exposed animals may, therefore, be due to increased enzyme secretion from haemocytes in the haemolymph. Interestingly, induction of haemocyte degranulation, followed by an increase in extracellular lysozyme activity, has recently been observed in haemocytes from *Mytilus galloprovincialis* exposed *in vitro* to E_2 (Canesi et al. 2004). In this regard, it is important to highlight that NP can act by mimicking endogenous estrogens such as E_2.

Apoptosis, or programmed cell death, is a physiological process by which useless cells are eliminated during normal cell turnover. Cells undergoing apop-

tosis show peculiar morphological and biochemical features, such as shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation, and formation of apoptotic bodies containing cellular organelles. *In vivo*, apoptotic bodies are generally recognised and phagocytised by either macrophages or active phagocytes. In the present study, both DNA fragmentation and cell shrinkage were observed in haemocytes from NP-exposed clams. Increased cellular apoptosis and shrinkage have also been found in spermatocytes and Sertoli cells from medaka *Oryzias latipes* exposed to NP, indicating testicular degeneration via testicular cell apoptosis (Weber et al. 2002). Similarly, apoptosis of cultured Sertoli cells was proposed as the major reason for the adverse effects of NP in rats (Wang et al. 2003). Apoptotic events have also been observed in haemocytes from the ascidian *Botryllus schlosseri* after *in vitro* exposure to TBT: haemocytes positive to the TUNEL reaction significantly increased, cell volume decreased, and plasma membranes acquired a blebbed appearance (Cima & Ballarin 1999). Although the mechanisms by which apoptotic processes are regulated in bivalve molluscs are still unknown, DNA damage observed in haemocytes from NP-exposed clams was probably mediated by increases in cytosolic Ca^{2+} levels, which in turn can activate Ca^{2+}-dependent DNA lytic enzymes, such as endonucleases. Apoptotic events mediated by a cytosolic Ca^{2+} increase (caused by inhibition of Ca^{2+}-ATPase activity) have been proposed in previous studies (Cima & Ballarin 1999, Hughes et al. 2000). In particular, among the compounds tested, NP has been shown to be the most potent inhibitor of the Ca^{2+} pump, and the resulting mobilisation of intracellular Ca^{2+} was associated with apoptotic cell death (Hughes et al. 2000). NP capability to disrupt Ca^{2+} homeostasis was also observed by Michelangeli et al. (1990).

NP also causes changes in both volume and morphology of haemocytes from *Tapes philippinarum*. According to Cima & Ballarin (1999), haemocyte shrinkage is due to the release of fluid from cells after contaminant exposure. Indeed, higher haemocyte fractions showing smaller cell volume were found in NP-exposed clams with respect to controls, confirming our previous findings concerning haemocyte shrinkage (see the results of the apoptosis assay). In addition, the lost of amoeboid shape of haemocytes from *T. philippinarum* may be a consequence of ROS-mediated alterations in cytoskeleton organisation. Gómez-Mendikute et al. (2002) found that oxidative stress mediated by increased levels of ROS (O_2^-, in particular) causes modifications in the cytoskeletal proteins of haemocytes from *Mytilus galloprovincialis* exposed *in vitro* to PAHs, provoking the disorganisation of the cytoskeleton. In this regard, it is important to emphasise that a significant inhibition of SOD activity was recorded in haemocytes from NP-exposed clams, suggesting that cells were unable to eliminate O_2^-. Moreover, as reported above, ROS have been shown to inactivate anti-oxidant enzymes, such as SOD (Griveau et al. 1995). It is, therefore, suggested that changes in the amoeboid shape of haemocytes from NP-exposed clams are a consequence of ROS-mediated oxidative stress.

Lastly, exposure to NP results in significant increases in the number of circulating haemocytes in *Tapes philippinarum*. Alterations in both the total numbers of circulating haemocyte and the relative proportion of cell types are generally considered a response to environmental stress (Pipe & Coles 1995, Pipe et al. 1995, Matozzo et al. 2003b). Increases in the total numbers of circulating haemocytes may be due to either proliferation or movement of cells from tissues into the haemolymph, whereas decreases in numbers are considered to be a consequence of cell lysis (Pipe & Coles 1995). In previous studies, exposure to contaminants, such as heavy metals, phenol and fluoranthene (Renwrantz 1990, Coles et al. 1994, Pipe et al. 1999), also caused increases in THC. According to Pipe et al. (1999), increased haemocyte numbers in NP-exposed clams are due to stimulation of haemocyte mobilisation from tissues to haemolymph during exposure; however, it cannot be excluded that the increased fraction of smaller circulating haemocytes (which may correspond to apoptotic bodies arising from NP-affected haemocytes) enhanced THC. Conversely, the increased fraction of larger haemocytes observed in clams exposed to the highest NP concentration tested also suggests the presence of haemocytes phagocytising apoptotic bodies.

CONCLUSIONS

Our results reveal that NP causes alterations in the functional responses of haemocytes from the clam *Tapes philippinarum*, indicating reduced immunocompetence in NP-exposed animals, even at NP concentrations similar to environmentally realistic levels (see Ying et al. 2002, for a review); however, a lack of data concerning the mechanisms of action of NP at the cellular level in bivalves makes a full evaluation of our results difficult. We hypothesise that NP acts negatively in the cytoplasm of bivalve haemocytes, affecting enzyme activities, whereas apoptotic events and changes in haemocyte morphology are probably caused by NP-mediated alterations in Ca^{2+} homeostasis and oxidative stress, respectively. For more information on the mechanisms of action of NP at the cellular level, as well as on increased susceptibility of

NP-exposed clams to diseases, future research will be performed. The results already obtained also indicate that haemocytes represent a suitable model for evaluating NP toxicity in aquatic invertebrates. On the basis of the threshold concentration values, the most sensitive endpoints for NP are the SOD, lysozyme and apoptosis assays. Further studies in field conditions are, however, required to validate functional responses of haemocytes, such as those measured in clams exposed to NP in the laboratory.

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