APPLIED ORGANOMETALLIC CHEMISTRY
*Appl. Organometal. Chem.* 2002; **16**: 169–174

Environment, Biology and Toxicology AOC

# *In vitro* effects of tributyltin on functional responses of haemocytes in the clam *Tapes philippinarum*†

**Valerio Matozzo, Loriano Ballarin and Maria Gabriella Marin***

Department of Biology, University of Padua, Via Ugo Bassi 58/B, 35131 Padua, Italy

Received 8 June 2001; Accepted 5 December 2001

**The effects of tributyltin (TBT, as the chloride) on circulating cells from the clam *Tapes philippinarum* were investigated, in order to set up quick and reproducible *in vitro* bioassays to evaluate TBT toxicity in bivalve molluscs. Haemocytes, collected from the adductor muscle of clams, were exposed for 60 min at 25 °C to sublethal concentrations of TBT (0.01, 0.05, 0.1 μM) and the effects on uptake of the vital dye Neutral Red, and both superoxide dismutase (antioxidant enzyme) and lysozyme (bacteriolytic enzyme) activities were spectrophotometrically evaluated. Exposure of haemocytes to 0.05 μM TBT caused a significant increase ($P < 0.05$) in Neutral Red dye uptake compared with controls, whereas no differences resulted after exposure to 0.01 and 0.1 μM TBT. Enlarged lysosomes were observed in haemocytes exposed to 0.05 μM TBT. Moreover, in haemocytes treated with 0.05 μM and 0.1 μM TBT, superoxide dismutase activity significantly decreased ($P < 0.05$ and $P < 0.01$ respectively) with respect to that of controls. A significant decrease in lysozyme activity was also observed in haemocytes exposed to 0.05 ($P < 0.01$) and 0.1 μM TBT ($P < 0.001$). These results suggest a relationship between TBT exposure and alterations in functional responses of haemocytes in *T. philippinarum*. The proposed assays are sensitive, rapid and reproducible. They may be proposed as biomarkers, although their responsiveness needs to be more fully evaluated in haemocytes collected after clam exposure in both laboratory and field conditions.** 

**KEYWORDS:** tributyltin; bivalves; haemocytes; *in vitro* bioassays; Neutral Red uptake; superoxide dismutase; lysozyme

## INTRODUCTION

Tributyltin (TBT) compounds are among the most effective biocides used in antifouling paint and pesticide formulations.[1–3] Intense maritime and agricultural activities are the major sources of TBT input into aquatic ecosystems, where it shows a variable persistence in the water column (half-life from a few days to a few months), depending on the environmental conditions, such as pH, temperature, turbidity and light.[2,3] In sediments, TBT is more persistent, with a half-life of the order of months (see Maguire[3] for a review). As TBT is highly soluble in organic solvents and lipids,[4] TBT-contaminated sediments represent a risk for both benthic and pelagic organisms, which may rapidly accumulate it.[3] It is known that bivalve molluscs are able to accumulate very high levels of organotins,[5–8] showing the highest bioconcentration factor (BF) among aquatic organisms (900 000 and 500 000 for mussels and clams respectively, in comparison with 350 000 for algae and 50 000 for fish).[3] The absence of any cytochrome-P450-dependent system may explain the capacity of molluscs to accumulate high levels of TBT.[2]

Acute and chronic TBT toxicity is well known in several species of aquatic organisms.[9,10] The negative effects of TBT have been observed in the bivalve larval development of *Crassostrea gigas*,[11] *Mytilus edulis*[12] and *Mercenaria mercenaria*.[13] The embryotoxic effects of both TBT and its degradation products, resulting in altered or blocked embryonic development, have also been observed in the sea urchin *Paracentrotus lividus*, probably owing to the interference of organotins with intracellular calcium homeostasis during skeleton deposition.[14] Abnormal thickening of shells was first observed in the oyster *C. gigas* from the Bay of Arcachon.[15] Altered calcification mechanisms resulted in shell anomalies at TBT concentrations as low as 2 ng $l^{-1}$.[16] Recently, Ruiz *et*

*Correspondence to: M. G. Marin, Department of Biology, University of Padua, Via Ugo Bassi 58/B, 35131 Padua, Italy.
E-mail: mgmar@civ.bio.unipd.it
†This paper is based on work presented at the 5th International Conference on Environmental and Biological Aspects of Main-Group Organometals (ICEBAMO-5) held at Schielleiten, near Graz, Austria, 5–9 June 2001.

DOI:10.1002/aoc.278

*al.*[17] noted that the burying activity of the clam *Scrobicularia plana* is significantly reduced at TBT concentrations in sediments of 0.3 μg of tin per gram dry weight, with possible negative effects on the ability of the animal to avoid predators. However, the most well-known and -documented effect of TBT on marine organisms is imposex. This phenomenon, particularly investigated in the dogwhelk *Nucella lapillus*, leads to the development of male characters in females, with consequent sterility.[18–22] Imposex has been proposed as an effective biomarker of TBT exposure.

Organotin compounds, and TBT in particular, are also known to have immunotoxic effects on bivalve molluscs.[23–26] In *C. gigas, in vitro* spontaneous aggregation of haemocytes is altered by exposure to TBT, suggesting impairment of haemocyte functions in internal defence.[27] Inhibition of both phagocytic and β-glucuronidase activities have been observed in organotin-exposed haemocytes from the clam *Tapes philippinarum*.[25,26] TBT has also been shown to exacerbate the progress and lethality of *Perkinsus marinus* infections in *Crassostrea virginica*, presumably owing to impaired defence capability, as suggested by the suppression of chemiluminescence activity in *in vitro* TBT-exposed haemocytes,[28] whereas inhibition of NADH and NADPH cytochrome *c* reductase activity has been observed in TBT-treated haemocytes from *Mytilus galloprovincialis* and *Tapes decussata*.[29]

The deleterious effects of TBT have also been described in ascidian phagocytes: apoptotic events have been observed in TBT-exposed haemocytes from the colonial ascidian *Botryllus schlosseri*.[30] In that study, a relationship was suggested between increased calcium, TBT-mediated, and activation of endonucleases, $Ca^{2+}$-dependent DNA lytic enzymes. Alterations in the cytoskeletal organisation of *B. schlosseri* haemocytes have also been observed.[31]

In the present study, the effects of TBT on some functional responses of haemocytes from the clam *T. philippinarum*, a species ecologically and commercially important in the Lagoon of Venice, were studied. We evaluated the *in vitro* effects of TBT on Neutral Red (NR) uptake, and both superoxide dismutase (SOD) and lysozyme activities of haemocytes. The use of these cell responses as biomarkers of exposure in future research is discussed.

## MATERIALS AND METHODS

### Animals

Specimens of *T. philippinarum* were collected from the Lagoon of Venice, maintained in the laboratory in large aquaria provided with a sandy bottom and aerated sea water at salinity of 35 ± 1‰ and a temperature of 17 ± 0.5 °C, and fed with microalgae (*Isochrysis galbana*).

### Exposure concentrations

A stock solution of 10 mM TBT chloride (Sigma) was made by dissolving the chemical in 95% ethanol. Test solutions (0.01, 0.05, 0.1 μM) were obtained by diluting the stock solution in sea water filtered through a 0.45 μm glass microfibre filter (filtered sea water: FSW).

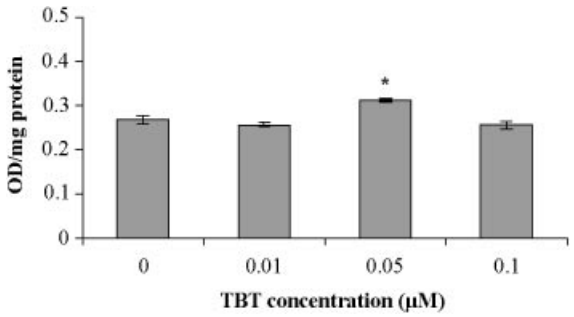


**Figure 1.** Effects of TBT exposure on NR uptake by haemocytes of *T. philippinarum*. Asterisks: significant results, compared with controls. Values are means ± SD ($n = 3$); * $P < 0.05$.

### Haemocyte collection and TBT exposure

Haemolymph was collected from the anterior adductor muscle with a 1 ml plastic syringe, placed in Eppendorf tubes, and centrifuged at 780*g* for 10 min. In each experiment, the haemolymph obtained from five clams was pooled. Haemocytes were resuspended in equal volumes of TBT test solutions at a final concentration of (8–10) × $10^6$ cells $ml^{-1}$, and incubated at 25 °C for 60 min. In controls, haemocytes were resuspended in FSW containing 0.1% of 95% ethanol. Both exposure times and concentrations were chosen on the basis of our previous data concerning TBT toxicity in bivalve haemocytes.[25,26]

### NR uptake spectrophotometric assay

We used the cationic probe NR to study the capability of haemocytes to carry out endocytosis (modified according to Coles *et al.*[32]). Alteration in this capability is commonly used as an indicator of cell damage in *in vitro* studies. Both TBT-treated and untreated cells were centrifuged at 780*g* for 10 min, resuspended in an equal volume of 8 mg $l^{-1}$ NR dye (Merck) solution in FSW, and incubated at 25 °C for 30 min. Then, haemocytes were centrifuged at 780*g* for 10 min, resuspended in distilled water, sonicated at 0 °C for 5 min with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12000*g* for 15 min at 4 °C. Supernatant, corresponding to cell lysate (CL), was collected for the NR uptake assay. 200 μl of CL were put in wells of a 96-well microplate and absorbance at 550 nm was recorded with a microplate reader. Results are expressed as optical density per milligram of protein (OD/mg protein). CL protein concentration was quantified according to Bradford,[33] using bovine serum albumin (BSA) as standard.

### NR uptake cytochemical assay

Short-term haemocyte cultures were prepared according to Ballarin *et al.*[34] After adhesion to coverslips, haemocytes

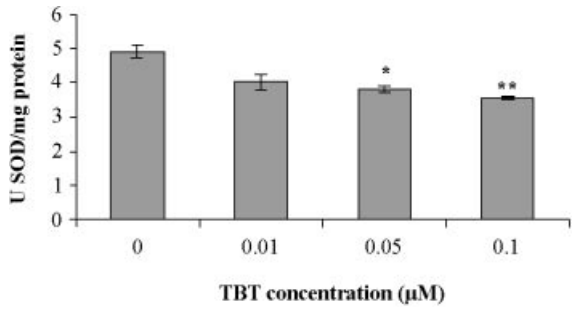


**Figure 2.** Effects of TBT exposure on SOD activity in haemocytes of *T. philippinarum*. Asterisks: significant results, compared with controls. Values are means ± SD ($n = 3$); * $P < 0.05$, ** $P < 0.01$.

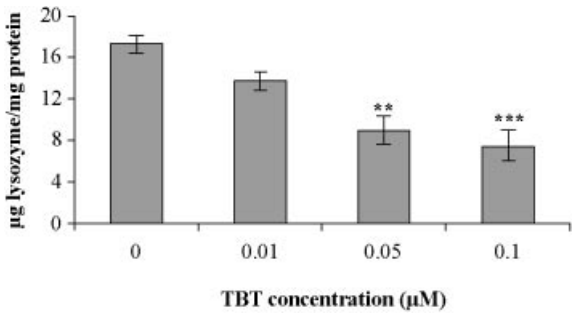


**Figure 3.** Effects of TBT exposure on lysozyme activity of haemocytes of *T. philippinarum*. Asterisks: significant results, compared with controls. Values are means ± SD ($n = 3$); ** $P < 0.01$, *** $P < 0.001$.

were incubated for 60 min at 25 °C in TBT test solutions; controls were incubated in FSW. The incubation media were then discarded from culture chambers and replaced with an equal volume of an 8 mg $l^{-1}$ NR solution in FSW. Haemocytes were incubated for 30 min at 25 °C and observed under a Leitz Dialux 22 light microscope at 1250×. Lysosomes of living cells appeared reddish-pink (Fig. 1).

## SOD activity assay

SOD activity was measured according to the method of Flohé and Ötting,[35] based on the reduction of cytochrome *c* by superoxide radical ($O_2^-$). Exposed and unexposed haemocytes (controls) were 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. 50 µl of CL were 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. One unit of SOD, according to Flohé and Ötting,[35] is defined as the amount of enzyme inhibiting the reduction of cytochrome *c* by 50% (Fig. 2).

## Lysozyme activity assay

Lysozyme activity was quantified according to Santarem *et al.*[36] 50 µl of CL, both from controls and TBT-treated haemocytes, were 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 ($\Delta A$ $min^{-1}$) was continuously recorded at 450 nm for 5 min at 20 °C. Standard solutions containing 1, 2.5, 5 and 10 µg lysozyme per millilitre 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 $\Delta A$ $min^{-1}$ 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.[36] Results are expressed as µg lysozyme/mg protein (Fig. 3).

## Statistical analysis

All experiments were performed in triplicate. The results of all assays were compared by one-way ANOVA and expressed as means ± SD.

## RESULTS

Adverse effects on the cell parameters analysed were observed after exposure of haemocytes to TBT. The differing capabilities of haemocytes to take up NR was recorded. No significant effect on NR uptake was observed in haemocytes exposed to 0.01 and 0.1 µM TBT, when compared with controls. Conversely, exposure to 0.05 µM TBT resulted in a significant increase ($P < 0.05$) in dye uptake (Fig. 1). Enlarged lysosomes were seen in haemocytes exposed to 0.05 µM TBT, with respect to both controls and 0.01 µM TBT-exposed cells. In 0.1 µM TBT-treated haemocytes, the dye spread throughout the cytosol (Fig. 4).

Significant negative effects on enzyme activities were also found, which decreased linearly with increasing TBT concentrations. Although a slight reduction in SOD activity was observed in cells exposed to 0.01 µM TBT, it was not significantly different from control values. Instead, in both 0.05 µM and 0.1 µM TBT-treated haemocytes, SOD activity decreased significantly ($P < 0.05$ and $P < 0.01$ respectively) with respect to controls (Fig. 2). In addition, a significant decrease in lysozyme activity was observed in haemocytes exposed to 0.05 ($P < 0.01$) and 0.1 µM TBT ($P < 0.001$) (Fig. 3).

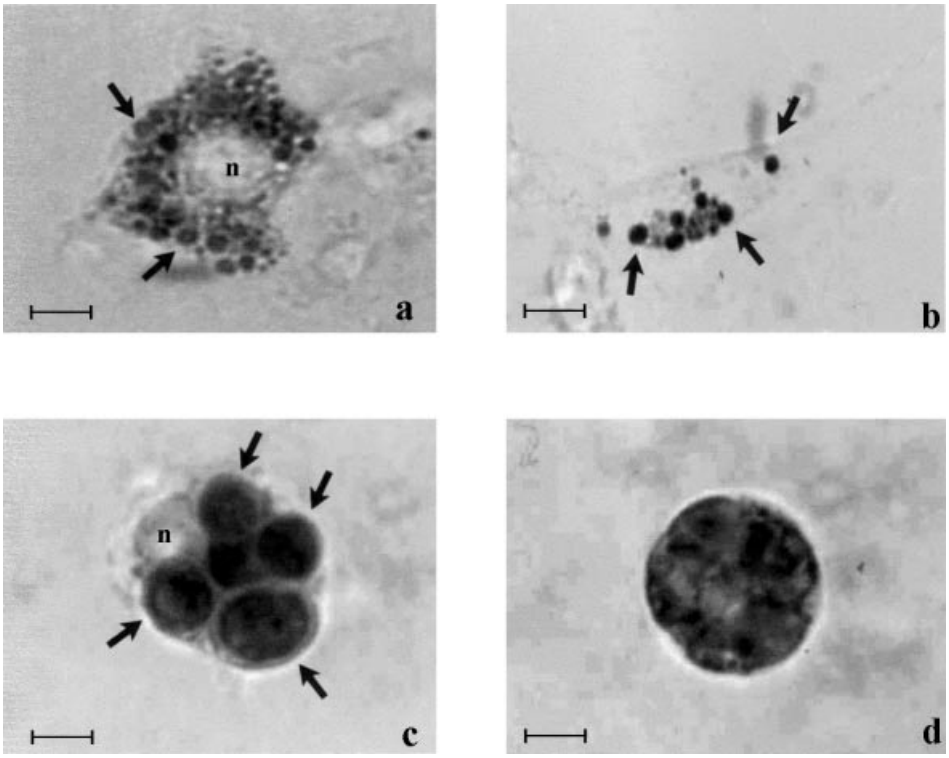


**Figure 4.** Haemocytes of *T. philippinarum* stained *in vivo* with NR; untreated haemocyte (a) showing obvious lysosomes (arrows); 0.01 μM TBT-treated haemocyte (b) showing few lysosomes (arrows); 0.05 μM TBT-treated haemocyte (c) showing enlarged lysosomes (arrows); 0.1 μM TBT-treated haemocyte (d) showing red cytosol. **n**: nucleus. Bar length: 3 μm.

## DISCUSSION

Bivalve haemocytes are known to be involved in immune defence reactions against foreign particulate and pathogens.[37] Consequently, the toxic effects of pollutants on haemocyte-dependent defence mechanisms potentially affects survival. In the last few years, many *in vitro* bioassays based on functional responses of bivalve circulating cells have been employed in biomonitoring studies to measure the deleterious effects of contaminants on both organisms and the quality of their habitat.[38,39]

In the present study, it was observed that TBT exposure affected the lysosomal compartments of *T. philippinarum* haemocytes. It is well known that lysosomes play an important role in the immune responses of bivalve molluscs: upon phagocyte stimulation, lysosomal hydrolases are released from cells to degrade foreign materials[40] or into phagosomes, thus participating in the degradation of internalized foreign particles.[37] Alteration of the integrity of lysosomal membranes may cause undesired release of hydrolases into the cytosol, with consequent damage for the cells themselves.[41] NR is commonly used in *in vitro* bioassays based on the accumulation of this cationic dye into the lysosomes of viable cells. Its uptake by haemocytes may occur by pinocytosis or passive diffusion across cell membranes.[32] Alteration in its uptake may reflect damage to the cell membrane or, alternatively, changes in the volume of the lysosomal compartments.[32] We observed an increase in NR uptake only by haemocytes exposed to 0.05 μM TBT; this may have been due to structural alterations of lysosomal membranes, with the consequent formation of enlarged lysosomes, as found in the mussel *Dreissena polymorpha* exposed to lead and zinc.[42] The results of our NR uptake cytochemical assay are in agreement with this hypothesis. In contrast, at the highest TBT concentration tested, NR appeared in the cytosol, and neither enlarged lysosomes nor obvious lysosomes were found. Presumably, at 0.1 μM TBT, the lysosomal membrane integrity was altered profoundly, causing dye loss. Lowe *et al.*[43] suggested that free passage of lysosomal contents, including NR, into the cytosol may be due to impairment of the lysosomal membrane proton pump. According to our results, an increase in NR uptake was demonstrated in haemocytes of the mussel *M. edulis* exposed to cadmium,[32] whereas exposure of mussels to copper did not result in significant differences in uptake of dye by haemocytes.[44] In brown cells of the clam *M. mercenaria*, exposure to heavy metals caused a decrease in NR uptake,[45] whereas no difference in NR uptake was found

in specimens of *M. galloprovincialis* collected from both polluted and clean sites, probably due to a situation of moderate pollution not causing serious damage in the cell membrane.[39]

In the present study, a significant decrease in SOD activity was found in haemocytes exposed to the highest TBT concentrations. Although little information concerning the molecular effects of organotin compounds is available, we suggest that the negative effects of TBT on SOD activity are due to the interaction of TBT with the enzyme, in view of the high affinity of organotins for the SH groups of proteins.[46,47] Antioxidant agents, such as catalase, SOD and glutathione, act against toxicity mediated by reactive oxygen species. In particular, SOD catalyses the dismutation of superoxide radicals ($O_2^-$)to hydrogen peroxide and oxygen. When antioxidant defences are impaired, oxidative stress may produce DNA damage, enzymatic inactivation and lipid peroxidation.[48] Regoli and Principato[49] observed that SOD activity remained constant in the digestive gland and increased in the gills of *M. galloprovincialis* exposed to copper. An increase in SOD activity was also reported for gills of mussels collected from a polluted site,[50] whereas SOD activity remained generally constant in the freshwater bivalve *Unio tumidus* after both experimental and field exposure to toxicants.[51] In a recent study, decreased SOD activity was found in specimens of *T. philippinarum* transplanted from a clean farming area into a heavily polluted area of the Lagoon of Venice.[52]

Significant inhibition of lysozyme activity has been observed in CL from cells exposed to 0.05 and 0.1 μM TBT, confirming previous data concerning the capability of organotins to alter enzymatic activity in fish cells,[53] urochordata ovaries[54] and bivalve haemocytes.[26] Moreover, our results support the hypothesis that TBT acts in the cytoplasm by causing inhibition of enzyme activities, probably through specific interactions with cellular targets. In bivalve immune responses, lysozyme is one of the most important bacteriolytic agents against several species of Gram-positive and Gram-negative bacteria.[55,56] Lysozyme is a lysosomal enzyme that may be secreted by haemocytes in the haemolymph during phagocytosis, thus participating in inactivating invading pathogens.[57] Therefore, reduced lysozyme activity suggests immunosuppression, resulting in lowered resistance to bacterial challenge. No significant effect of TBT on release of lysozyme into the haemolymph of either mussels or oysters was observed in some previous studies,[58,59] whereas Pickwell and Steinert[60] found an increase in the release of degradative enzymes into the haemolymph of *M. edulis* following exposure to copper. Exposure of mussels to 40 μg $l^{-1}$ cadmium significantly inhibited the release of *N*-acetyl-*β*-D-glucosaminidase into the haemolymph.[32]

In conclusion, our results demonstrate that *in vitro* exposure to TBT may affect cell responses of *T. philippinarum*, although improved knowledge of the mechanisms of action of TBT is required to explain better the observed effects on cell components. Moreover, the bioassays proposed here may be considered useful tools in biomonitoring studies to screen xenobiotic toxicity, because they are reproducible, rapid and sensitive. Nevertheless, they require further investigations using both field- and laboratory-exposed animals.

## REFERENCES


1. Champ MA and Seligman PF. An introduction to organotin compounds and their use in antifouling coatings. In *Organotin – Environmental Fate and Effects*, Champ MA, Seligman PF (eds). Chapman and Hall: London, 1996; 1–25.
2. Alzieu C. *Ecotoxicology* 2000; **9**: 71.
3. Maguire RJ. *Water Qual. Res. J. Can.* 2000; **35**: 633.
4. Laughlin RB, Guard HE and Coleman WM. *Environ. Sci. Technol.* 1986; **20**: 201.
5. Zuolian C and Jensen A. *Mar. Pollut. Bull.* 1989; **20**: 281.
6. Laughlin RB Jr, French W and Guard HE. *Environ. Sci. Technol.* 1986; **20**: 884.
7. Page DS, Dassanayake TM and Gilfillan ES. *Mar. Environ. Res.* 1995; **40**: 409.
8. Bressa G, Cima F, Fonti P and Sisti E. *FEB* 1997; **6**: 16.
9. Hall LW and Bushong SJ. A review of acute effects of tributyltin compounds on aquatic biota. In *Organotin – Environmental Fate and Effects*, Champ MA, Seligman PF (eds). Chapman and Hall: London, 1996; 157–190.
10. Laughlin RB, Thain J, Davidson B, Valkirs AO and Newton FC. Experimental studies of chronic toxicity of tributyltin compounds. In *Organotin – Environmental Fate and Effects*, Champ MA, Seligman PF (eds). Chapman and Hall: London, 1996; 191–217.
11. His E and Robert R. *Rev. Trav. Inst. Pěches Marit.* 1985; **45**: 117.
12. Lapota D, Rosemberg DE, Platter-Rieger MF and Seligman PF. *Mar. Biol.* 1993; **115**: 413.
13. Laughlin RB Jr, Gustafson R and Pendoley P. *Mar. Ecol. Prog. Ser.* 1988; **48**: 29.
14. Marin MG, Moschino V, Cima F and Celli C. *Mar. Environ. Res.* 2000; **50**: 231.
15. Alzieu C, Sanjuan J, Deltreil JP and Borel M. *Mar. Pollut. Bull.* 1986; **17**: 494.
16. Chagot D, Alzieu C, Sanjuan J and Grizel H. *Aquat. Living Resour.* 1990; **3**: 121.
17. Ruiz JM, Bryan GW and Gibbs PE. *Mar. Ecol. Prog. Ser.* 1994; **113**: 119.
18. Gibbs PE, Bryan GW, Pascoe PL and Burt GR. *J. Mar. Biol. Assoc. U.K.* 1987; **67**: 507.
19. Bryan GW, Gibbs PE and Burt GR. *J. Mar. Biol. Assoc. U.K.* 1988; **68**: 733.
20. Bailey SK. *Mar. Environ. Res.* 1991; **32**: 187.
21. Davies IM. *Mar. Environ. Res.* 1991; **32**: 201.
22. Gibbs PE, Bryan GW and Pascoe PL. *Mar. Environ. Res.* 1991; **32**: 79.
23. Anderson RS, Brubacher LL, Ragone Calvo LM, Burreson EM and Unger MA. *Environ. Res.* 1997; **74**: 84.
24. Bouchard N, Pelletier E and Fournier M. *Environ. Toxicol. Chem.* 1999; **18**: 519.
25. Cima F, Marin MG, Matozzo V, DaRos L and Ballarin L. *Chemosphere* 1998; **37**: 3035.
26. Cima F, Marin MG, Matozzo V, Da Ros L and Ballarin L. *Mar. Pollut. Bull.* 1999; **39**: 112.
27. Auffret M and Oubella R. *Comp. Biochem. Physiol. A* 1997; **118**: 705.


 

28. Anderson RS. *Biomarkers* 1999; **4**: 531.
29. Morcillo Y and Porte C. *Aquat. Toxicol.* 1997; **38**: 35.
30. Cima F and Ballarin L. *Appl. Organomet. Chem.* 1999; **13**: 697.
31. Cima F, Ballarin L, Bressa G and Burighel P. *Ecotoxicol. Environ. Saf.* 1998; **40**: 160.
32. Coles JA, Farley SR and Pipe RK. *Dis. Aquat. Organ.* 1995; **22**: 59.
33. Bradford MM. *Anal. Biochem.* 1976; **72**: 248.
34. Ballarin L, Cima F and Sabbadin A. *Dev. Comp. Immunol.* 1994; **18**: 467.
35. Flohé L and Ötting F. *Methods Enzymol.* 1984; **105**: 93.
36. Santarem MM, Robledo JAF and Figueras A. *Dis. Aquat. Organ.* 1994; **18**: 217.
37. Cheng TC. Bivalves. In *Invertebrate Blood Cells*, vol. 1, Ratcliffe NA, Rowley AF (eds). Academic Press: London, 1981; 233–300.
38. Anderson RS. *Am. Fish. Soc.* 1988; **18**: 238.
39. Cajaraville MP, Olabarrieta I and Marigomex I. *Ecotoxicol. Environ. Saf.* 1996; **35**: 253.
40. Mohandas A, Cheng TC and Cheng JB. *J. Invertebr. Pathol.* 1985; **46**: 189.
41. Lowe DM, Fossato VU and Depledge MH. *Mar. Ecol. Prog. Ser.* 1995; **129**: 189.
42. Giamberini L and Pihan JC. *Dis. Aquat. Organ.* 1997; **28**: 221.
43. Lowe DM, Moore MN and Evans BM. *Mar. Ecol. Prog. Ser.* 1992; **91**: 135.
44. Pipe RK, Coles JA, Carissan FMM and Ramanathan K. *Aquat. Toxicol.* 1999; **46**: 43.
45. Zaroogian G, Yevich P and Anderson S. *Ecotoxicol. Environ. Saf.* 1992; **35**: 41.
46. Chow SC and Orrenius S. *Toxicol. Appl. Pharmacol.* 1994; **127**: 19.
47. Musumeci MT, Madonia G, Lo Giudice MT, Silvestri A, Ruisi G and Barbieri R. *Appl. Organomet. Chem.* 1992; **6**: 127.
48. Halliwell B and Gutteridge JMC. *Free Radicals in Biology and Medicine*, 2nd edn. Clarendon Press: Oxford, 1989.
49. Regoli F and Principato G. *Aquat. Toxicol.* 1995; **31**: 143.
50. Regoli F. *Arch. Environ. Contam. Toxicol.* 1998; **34**: 48.
51. Doyotte A, Cossu C, Jacquin M-C, Babut M and Vasseur P. *Aquat. Toxicol.* 1997; **39**: 93.
52. Nasci C, Da Ros L, Nesto N, Sperni L, Passarini F and Pavoni B. *Mar. Environ. Res.* 2000; **50**: 425.
53. Bruschweiler BJ, Wurgler FE and Fent K. *Environ. Toxicol. Chem.* 1996; **15**: 728.
54. Puccia E, Mansueto C, Cangialosi MV, Fiore T, Di Stefano R, Pellerito C, Triolo F and Pellerito L. *Appl. Organomet. Chem.* 2001; **15**: 213.
55. Cheng TC and Rodrick GE. *Biol. Bull.* 1974; **147**: 311.
56. Allam B and Paillard C. *Dis. Aquat. Organ.* 1998; **33**: 123.
57. Cheng TC, Rodrick GE, Foley DA and Koehler SA. *J. Invert. Pathol.* 1975; **25**: 261.
58. Anderson RS, Unger MA and Burreson EM. *Mar. Environ. Res.* 1996; **42**: 177.
59. Pickwell GV and Steinert SA. *Mar. Environ. Res.* 1988; **24**: 215.
60. Pickwell GV and Steinert SA. *Mar. Environ. Res.* 1984; **14**: 245.

 