APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2002; **16**: 163–168

Environment, Biology and Toxicology AOC

Effect of different organotin compounds on DNA of gilthead sea bream (*Sparus aurata*) erythrocytes assessed by the comet assay†

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Received 13 August 2001; Accepted 10 December 2001

The 'comet' assay appears to be a promising tool for estimating DNA damage at the single cell level. We used this test to evaluate the effect of organotin compounds on sea bream nucleated erythrocytes. The tributyltin chloride (TBTC), dibutyltin chloride (DBTC) and monobutyltin chloride (MBTC) employed in this study show different genotoxicities. TBTC and DBTC have pronounced effects on tail length, tail intensity and tail moment, though TBTC is more efficient in producing DNA damage. MBTC leads to a fast genotoxic effect that does not change with the incubation time. The data obtained are important for the analysis of the environmental risks produced by organotin compounds used as antifouling agents in marine paints and as biocides in agriculture. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: gilthead sea bream erythrocytes; DNA; comet assay; organotins

The increasing use of organotin compounds has produced an ubiquitous contamination in aquatic ecosystems. Their presence in the environment is a consequence of their use in agriculture (as fungicides, preservative biocides, etc.) and in industry [as wood preservatives, marine antifouling paints (tributyltin; TBT), etc., and as stabilizers for PVC (dibutyltin: DBT; monobutyltin: MBT)].[1] Many studies have described the nature and the source of these toxic agents in the environment.[2] In particular, they are known as contaminants of marine and freshwater ecosystems, this leads to bioaccumulation and concentration in sediments, and, because of their solubility in lipids, they accumulate in the food chain.[1,2] The biochemical and toxicological properties of these compounds have been studied extensively,[1–4] and their toxicity can be related to the number and the nature of organic substituents on tin(IV). Trisubstituted compounds were more cytotoxic than disubstituted and tetrasubstituted organotins. The lowest cytotoxicity was detected for monosubstituted moieties and inorganic tin. Studies on fish cell lines in the presence of various trisubstituted organotin compounds causing membrane damage showed that the sequence of cytotoxicity among butyltins was TBT > bisTBT > DBT > tetrabutyltin > MBT > tin(IV);[5] for phenyltins the sequence was triphenyltin > diphenyltin > phenyltin > tin(IV).[5] It has also been reported that toxic effects for organotin compounds decrease with increased length of the groups of the organic moiety.[6,7] Very important for the environment is the high toxicity of TBT, triphenyltin and tricyclohexyltin derivatives of tin[1] and the toxicologic potency decreases for organotins in the order ethyl > methyl > propyl > phenyl, based on the type of organic ligand bound to tin.[2] The toxicity of triorganotins is due to their ability to bind cysteine and histidine residues of proteins[8] and their liposolubility induces cytogenetic damage and apoptosis.[9–14] Previously, our studies on erythrocytes from rainbow trout (*Salmo irideus*) have shown that these organotin compounds produce plasma membrane perturbations[15] and hemoglobin (Hb) destabilization,[16] as well as having hemolytic[15] and genotoxic effects.[17] Since organotin compounds are present in marine sediments, food-chain accumulation of TBT has been demonstrated in crabs, in

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†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.

Contract/grant sponsor: MURST; Contract/grant number: MM05033722.

DOI:10.1002/aoc.275

marine mussels and in the muscle of chinook salmon.[2] In recent years, the increased use of these compounds has led to a large number of studies aimed at controlling their possible environmental and health effects. The present study was undertaken to investigate the effect of these compounds on a seawater fish. In particular, the effect of TBT chloride (TBTC), DBT chloride (DBTC) and MBT chloride (MBTC) on DNA from gilthead sea bream (*Sparus aurata*) nucleated erythrocytes was analyzed using the comet assay. The single-cell gel (SCG) test, or comet assay, is a rather new test with widespread potential applications in genotoxicity testing and biomonitoring.[18,19] The commonly used alkaline version of the test detects DNA strand breaks and alkali-labile lesions with sensitivity.[20] In this assay, cells are embedded in agarose, followed by lysis, electrophoresis and staining to visualize DNA damage using fluorescence microscopy. Relaxed and broken DNA fragments stream further from the nucleus than intact DNA, so the extent of DNA damage can be evaluated by the length of the stream. With this technique it is possible evaluate the damage even at low levels in the single cell using a very small sample of cells.

MATERIALS AND METHODS

Organotin compounds were obtained from Aldrich. All reagents were of analytical grade. Blood from sea bream *Sparus aurata* was drawn from the caudal vein with heparinized syringes into an isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA, pH 7.8), where a film of eparin was added as anticoagulant. After removal of the plasma and buffy coat by centrifugation, the erythrocytes were washed three times with isotonic phosphate buffer. After washing, the erythrocyte suspension was adjusted to a concentration of Hb 60 mg ml^{-1} and divided into different aliquots (the concentration of Hb was determined spectrophotometrically using an $E^{1\%}_{540} = 8.5$ for the oxygenated derivative). All manipulations were carried out at 4 °C. Organotin compounds dissolved in ethanol (100%) were added to the erythrocytes (4 µl ml^{-1} of erythrocyte suspension) to a final concentration of 10 µM; the choice of this organotin concentration derives from the fact that, in our experimental conditions, hemolysis is absent. In addition, previous studies with lower concentrations of organotin did not show any DNA damage, whereas too much damage (typical of apoptotic death) was observed at higher concentrations. Control experiments were performed by adding an equal volume of ethanol. The erythrocytes were tested immediately after addition of organotin (incubation time 0 min) and after incubation at 27 °C for 30 min. DNA damage in the erythrocytic suspension was evaluated using the alkaline single-cell microgel electrophoresis (comet assay), basically according to Singh *et al.*,[20] with minor modifications.[21] This technique permits quantification of DNA damage by evaluating three different parameters of the comet assay, namely the tail length (measured in Micrometers from the head center), tail intensity (percentage of fluorescence in the comet tail) and tail moment (TM).[22,23] Tail length considers DNA migration by measurement of the length of the comet; the tail intensity is the nuclear material that has migrated out from the comet head into the comet tail. The TM considers both the tail length and the fraction of the DNA in the comet tail,[20] and is defined as product of DNA in the tail and is calculated according the following formula:

$$\mathrm{TM} = (\text{tail intensity}/\text{total comet intensity}) \times (\text{tail center of gravity} - \text{head center})$$

where the percentage amount of DNA migrated in the tail (i.e. tail intensity/total comet intensity) was multiplied by the mean distance of migration in the tail (i.e. the distance between the tail centre of gravity, which is the sum of tail positions divided by the number of points and the head center.[24,25] Around 2×10^5 cells were mixed with 65 µl of 0.7% low melting agarose (LMA) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) to form a cell suspension. The cell suspension was rapidly spread over a precleaned microscope slide previously conditioned by spreading a 1 ml aliquot of 1% normal melting agarose (NMA) in Ca^{2+}- and Mg^{2+}-free PBS. After solidification the cells were protected with a top layer of 75 µl of 0.7% LMA. To lyse the embedded cells and to permit DNA unfolding, the slides were immersed in freshly prepared ice-cold lysis solution (1% sodium *N*-lauroyl-sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl pH 10, with 1% Triton X-100 and 10% dimethylsulfoxide added just before use) for 1 h at +4 °C in the dark. After the lyse the slides were placed on a horizontal electrophoresis box. The unit was filled with freshly made alkaline buffer (300 mM NaOH, 1 mM Na_2EDTA pH >13) and, to allow DNA unwinding and expression of alkali-labile damage, the embedded cells were left in the solution for 20 min. Electrophoresis was performed for 20 min by applying an electric field of 25 V and adjusting the current to 200 mA. After the electrophoresis, the slides were washed gently with 0.4 M Tris–HCl buffer pH 7.5 to neutralize the excess alkali and remove detergents. Slides were stained by adding 100 µl of ethidium bromide (2 µg ml^{-1}) and the rate of DNA damage was evaluated. In each experiment images of 150 randomly selected cells (50 cells from each of three replicate slides) were analyzed from each sample using an epifluorescent microscope (Leitz) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A × 50 immersion objective (Fluotar) was used with an ocular to project the comet cell image into a high-sensitivity CCD camera. Imaging was performed using a specialized analysis system (Comet Assay II, Perceptive Instruments Ltd, Suffolk, UK) that acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components, head and tail, and evaluates a range of derived parameters. Experiments were replicated three times and data (at least

Table 1. Observed distributions of comet parameter tail length (mean ± SEM) in gilthead sea bream *Sparus aurata* erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C. Data (at least 150 scores per sample) are mean values of three replicated experiments. Organotin compounds were dissolved at a final concentration of 10 μM

Sample	Tail length (μm)	
	t 0 min	*t* 30 min
Control	12.74 ± 0.22	12.75 ± 0.55
TBTC	12.80 ± 0.18	15.07 ± 0.26***
DBTC	12.99 ± 0.16	14.94 ± 0.21***
MBTC	14.25 ± 0.18***	14.37 ± 0.25***

*** $p < 0.001$.

Table 3. Observed distributions of comet parameter TM (mean ± SEM) in gilthead sea bream *Sparus aurata* erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C. Data (at least 150 scores per sample) are mean values of three replicated experiments. Organotin compounds were dissolved at a final concentration of 10 μM

Sample	TM	
	t 0 min	*t* 30 min
Control	0.73 ± 0.06	0.75 ± 0.07
TBTC	0.66 ± 0.06	1.10 ± 0.08***
DBTC	0.69 ± 0.05	0.99 ± 0.07***
MBTC	0.89 ± 0.06	0.80 ± 0.06

*** $p < 0.001$.

150 scores per sample) are the mean values plus/minus the standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test and Pearson's χ^2 test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

We performed the comet assay on gilthead sea bream erythrocyte suspensions incubated in the presence of 10 μM organotin compounds at 27 °C and pH 7.8 for 30 min.

Table 1 shows the tail length mean values of different samples. The values of these parameters increase in the presence of both DBTC and TBTC after 30 min of incubation. A rapid and significant increment at 0 min, which remains constant after 30 min could be observed when MBTC was added.

The results referring to tail intensity are shown in Table 2. Considering this parameter, a different pattern with respect to the tail length was observed. In fact, the percentage of DNA in the tail was significantly increased after the same incubation time in the presence of TBTC ($p < 0.01$) and DBTC, though the extent of DNA damage is less when DBTC is present ($p < 0.05$). On the contrary, in the presence of MBTC the tail intensity does not change with respect to the control.

Table 2. Observed distributions of comet parameter tail intensity (mean ± SEM) in gilthead sea bream *Sparus aurata* erythrocyte suspension after incubation in phosphate buffer, pH 7.8, at 27 °C. Data (at least 150 scores per sample) are mean values of three replicated experiments. Organotin compounds were dissolved at a final concentration of 10 μM

Sample	Tail intensity (%)	
	t 0 min	*t* 30 min
Control	8.17 ± 0.66	8.77 ± 1.09
TBTC	7.91 ± 0.78	11.25 ± 0.87**
DBTC	7.53 ± 0.61	10.62 ± 0.78*
MBTC	9.33 ± 0.70	8.34 ± 0.67

* $p < 0.05$.
** $p < 0.01$.

The TM mean values (Table 3) show that TBTC produces a greater effect on erythrocytes from gilthead sea bream after 30 min of incubation compared with DBTC. Both organotins display significant DNA damage, though TBTC is more effective. MBTC does not lead to any significant change in TM after 30 min of incubation.

We also considered threshold levels indicating the cells with abnormal size tails (AST),[26,27] and the 95th percentile for the tail parameters considered (tail length, tail intensity, and TM) in the control cells (untreated cells, incubation time 0 min) was used as a cut-off point. Cells with tail parameters values below the cut-off were classified as 'undamaged', and those with higher values as 'damaged' (AST). In this study, cut-off values were: tail length 16.67 μm (Fig. 1), tail intensity 25.30% (Fig. 1), and TM 2.14 (Fig. 2). Figure 2 shows the distribution of cells according to DNA TM values (plots are drafted using cumulative data from the three replicated experiments). Following the incubation time of 30 min a shift toward higher TM values is always evident. However, the differences were statistically significant only for DBTC and TBTC (with 11.3% and 13.7% of AST respectively at 30 min). The trend of DNA damage is particularly evident for MBTC, because when MBTC was added to erythrocytes (incubation time 0 min) an increased proportion of cells showing high TM values was immediately observed (with 7.7% of AST). However, the instantaneous increase in the extent of DNA damage is not significant. Following 30 min incubation, this situation was only slightly modified, with 10.0% of AST. Figure 1 shows the correlation between percentage of migrated DNA and tail length for each experimental set.

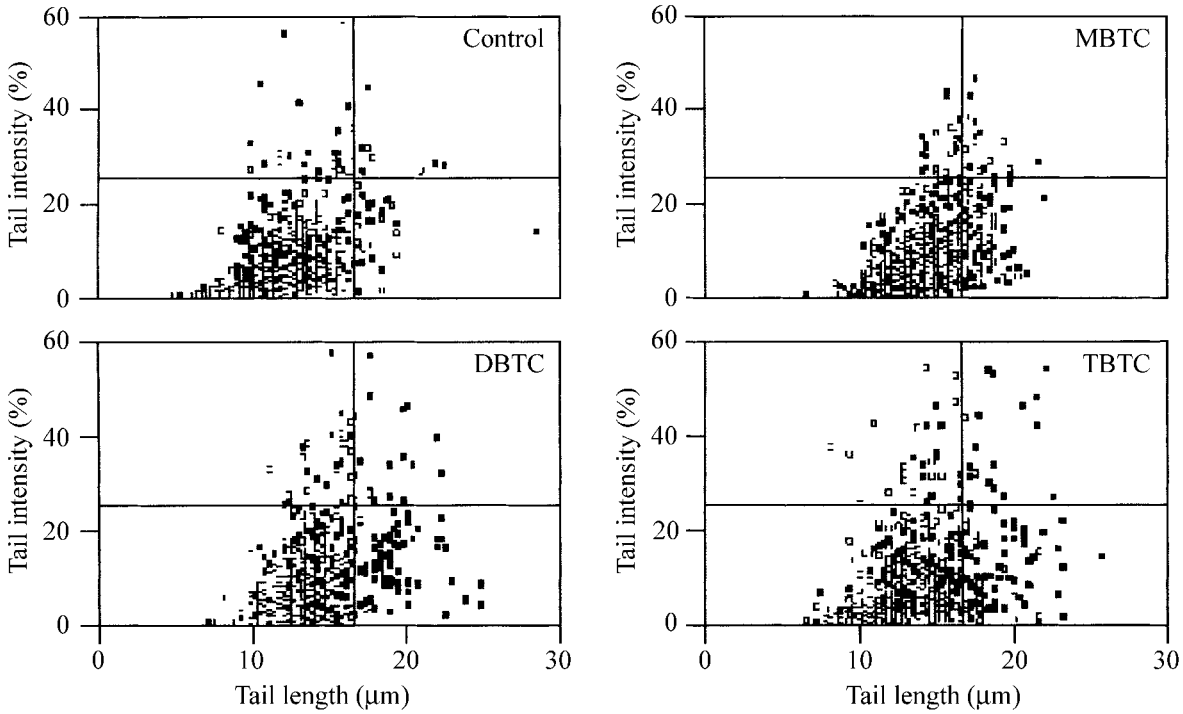

Figure 1. Correlation between percentage of migrated DNA and tail length in gilthead sea bream erythrocytes following 0 min (□) and 30 min (■) incubation time. Plots are drafted using cumulative data from the three replicated experiments. The dotted lines indicate the 95th percentile values for tail length (horizontal) and tail intensity (vertical). Percentile values were calculated in the control cells (untreated cells, incubation time 0 min) and were used as cut-off points to classify the cells as 'undamaged' or 'damaged' (AST).

DISCUSSION

The widespread use of organotin compounds has caused severe environmental pollution and consequent potential health hazards. In coastal areas, these compounds are released from harbor operations, or transported by rivers from industry and agriculture, finally reaching the oceans. These pollutants are distributed, transferred and accumulated through trophic chains, threatening the health of marine organisms and are taken up by humans in their diet. This increased presence of contaminants has resulted in the necessity for a sensitive assay to monitor the genotoxicity of these compounds. Evaluation of DNA damage on fish nucleated erythrocytes using the comet assay could give important information on the alterations induced by these compounds. Previous studies on rainbow trout erythrocytes (*Salmo irideus*) have shown that TBTC has a marked genotoxic effect, whereas DBTC produces less DNA damage and DNA damage is completely absent for MBTC.[17] In the present paper, we studied the effect of these organotin compounds on DNA of erythrocytes from gilthead sea bream, *Sparus aurata*. The comet assay was employed to determine DNA damage since it gives indications on the state of DNA. In particular, with this test it is possible to evaluate the genotoxic effect of these organotins by measuring three parameters: tail length, tail intensity and TM. As shown in Figs 1 and 2, the greatest DNA damage, after 30 min of incubation, can be observed in the presence of TBTC, which leads to a significant increase in the extent of DNA damage as measured by the three tail parameters considered (tail length, tail intensity, and TM). The increased level of damage is also evident as an increased proportion of AST (Fig. 2), as well as an increased number of cells showing both tail length and tail intensity values higher than the 95th percentile.

A similar behavior is obtained with DBTC, although the increase in these parameters is lower. MBTC produces a rapid increase in tail length (Table 1), but not in tail intensity (Table 2) or TM (Table 3). In fact, at 0 min a higher value of tail length, compared with the other samples at the same time of incubation, was measured. This effect could possibly be linked with the different size of the molecule that could enter more easily into the erythrocytes. This effect does not increase with incubation time and it is, however, of less intensity compared with the damage induced by TBTC and DBTC. The behavior observed is quite similar to that reported for trout erythrocytes exposed to the same amount of organotins and experimental conditions.[17] It is important to consider that other differences (Hb stability, hemolytic effect) characterize the interaction of trout erythrocytes[15,16] and sea bream erythrocytes with organotins (work in progress in our

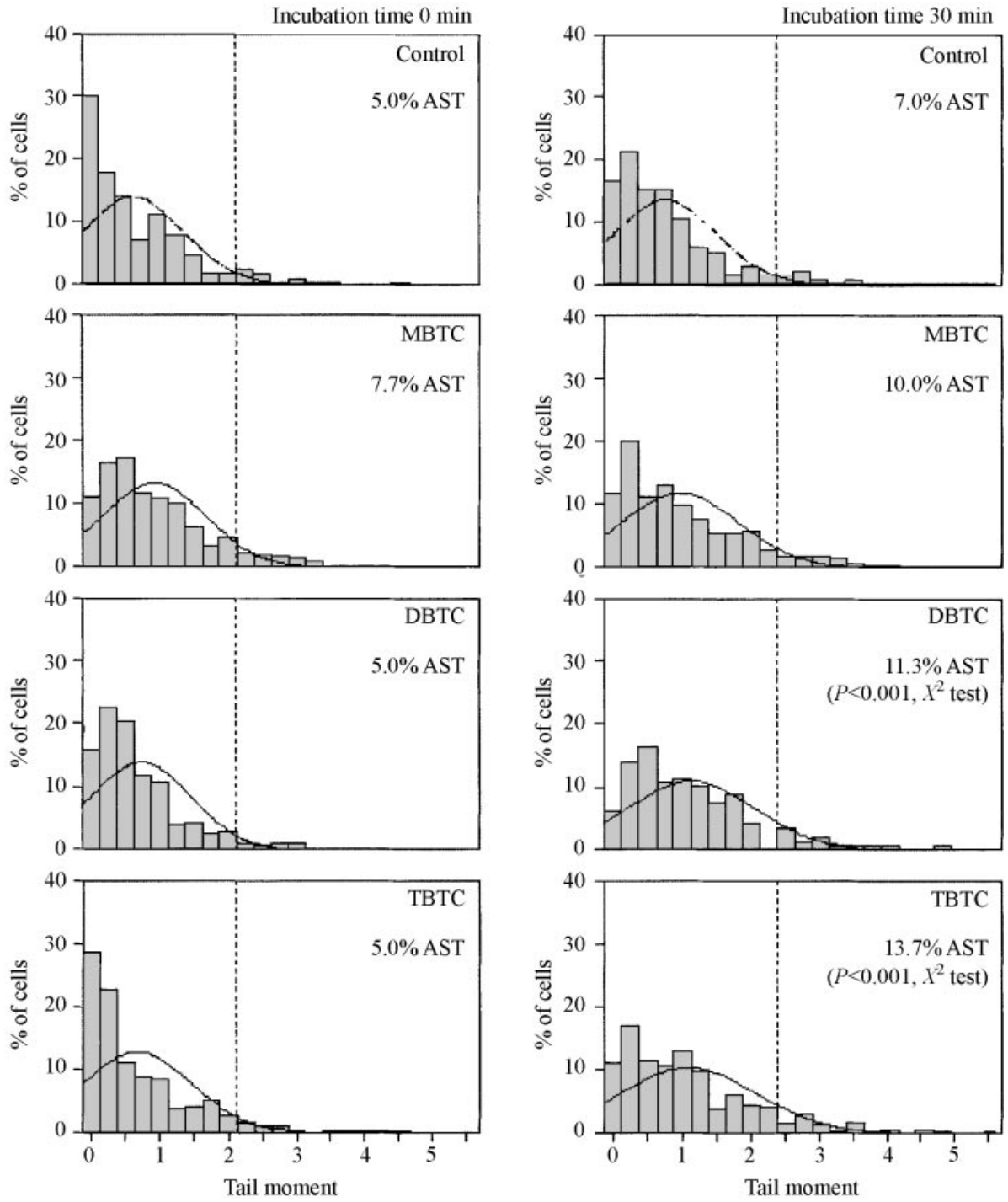

Figure 2. Percentage distribution of cells (gilthead sea bream erythrocytes) as a function of DNA damage (TM) after incubation (0 and 30 min) in phosphate buffer (pH 7.8, 27 °C) with 10 μM MBTC, DBTC, and TBTC. Plots are drafted using cumulative data from the three replicated experiments. The dotted line indicates the 95th percentile value calculated in the control cells (untreated cells, incubation time 0 min), which was used as a cut-off point to classify the cells as 'undamaged' or 'damaged' (AST).

laboratory). Preliminary results show that these two types of fish behave differently towards pollutants. Nevertheless, the data obtained are in agreement with the general toxicity of these compounds, since this is a maximum for TBTC.[1,2] This compound was shown to affect the chromosome structures of Mollusca and Isopoda.[28,29]

In the same way, organotin compounds have immunotoxic and neurotoxic properties and inhibit phagocytosis and

exocytosis in rats.[30–32] In addition, tributyltin compounds influence the hormonal metabolism leading to an increase in the level of testosterone,[11] new findings indicate that hormonal metabolites, for both *in vivo* and *in vitro* conditions, can initiate the multistage process of carcinogenesis.[33–35]

It is known that these compounds can bind cellular macromolecules that include thiol groups,[1] and that some metallic compounds have the capacity to bind macromolecules such as DNA and repair enzymes inducing genetic damage.[11] TBT is capable of inducing cytogenetic damage in *Mytilus edulis*,[28] and, like other organotin compounds (MBTC, DBTC and dimethyltin), is mutagenic on *Salmonella typhimurium* TA100.[10] Apoptosis and cytogenetic damage have been induced by organotin compounds on eukaryotic cell models.[11–13] An increase in cytosolic free Ca^{2+} from intracellular stores was measured using 1–10 μM TBTC.[13] This could induce DNA cleavage typical of apoptotic death in thymocytes and mammalian cell lines.[13]

Of particular interest is the behavior shown by MBTC, which produced rapid DNA damage but which did not increase with incubation time. This result is different to that previously reported for trout erythrocytes,[17] where the incubation with MBTC did not show any effect. Probably this different behavior observed for the effect of MBTC on marine and freshwater fish erythrocytes could be due to a different uptake of the mono-organotin derivative by the cellular membrane. Transport mechanisms of organotin derivatives across the plasma membrane are assumed to be taken up via passive diffusion processes by partitioning into hydrophobic biological membranes. Physicochemical properties, including molecular size, electric charge and chemical lipid speciation, which influence solubility in plasma membranes, are probably very important for organotins. In addition, in contrast to toxic interactions of organotins with components of the plasma membrane, the molecular uptake and transfer mechanisms into cells is not yet understood. To this end, evaluation of the permeability of MBTC in trout and *Sparus aurata* erythrocytes needs to be correlated in order to understand the rapid toxicity observed (this study is in progress).

Evaluation of the effects of these organotin compounds is important, since further studies may show that sub-lethal effects can produce serious long-term consequences in various processes that will ultimately affect the survival and propagation of the species. The environmental risks from the use of these compounds should be evaluated in order to control biological damage to marine fish, and the method presented in this study could be used to monitor the environmental risk linked with these pollutants.

Acknowledgements

This work was supported by MURST 40% no. MM05033722 to G. Lupidi.

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