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CYTO-GENOTOXIC EFFECTS OF SOME PHARMACEUTICAL AND PERSONAL CARE PRODUCTS (PPCPs) ON THE FRESHWATER BIVALVE ZEBRA MUSSEL (*Dreissena polymorpha*)

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ABSTRACT

Pharmaceutical and personal care products (PPCPs) are an emerging class of environmental pollutants that are extensively and increasingly being used in human and veterinary medicine. Due to their continuous production, consumption and often abuse, many studies have shown worldwide measurable concentrations of about 100 of these drugs in the aquatic environment in the high ng L^{-1} to low µg L^{-1} range. Among these, antimicrobial, antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) are three of the most used and frequently detected PPCP classes in aquatic environments. Since these chemicals have physico-chemical characteristics similar to those of harmful xenobiotics, they could be potentially dangerous to aquatic non-target organisms, as they are exposed to contaminants over their whole life span. At present, few studies were carried out in order to evaluate the PPCPs potential toxicity on aquatic organisms. The major part of these researches was aimed to acute toxicity evaluation of few drugs on organisms belonging to different trophic levels. Nonetheless, considering the current low environmental levels, these data are not suitable for an accurate risk assessment since chronic effects are much more probable. In order to enlarge this topic, we decided to evaluate the potential cyto-genotoxicity of a common antimicrobial agent (triclosan; TCS), a widespread antibiotic (trimethoprim; TMP) and three largely used NSAIDs (paracetamol; PCM – diclofenac; DCF – ibuprofen; IBU) on the freshwater bivalve zebra mussel (*Dreissena polymorpha*). This mussel was chosen as biological model because the invertebrates constitute more the 90% of living species, they play an important role in freshwater ecosystems and are particularly susceptible to environmental stressors. Additionally, previous studies have revealed that *D. polymorpha* is useful and sensible organism capable of highlighting sub-lethal effects when exposed to synthetic chemicals. In detail, a stepwise *in vitro-in vivo* multi-biomarker approach was planned to study the potential environmental risk due to these PPCPs. The first screening evaluation was carried out by using an *in vitro* approach on the mussel hemocytes. Genotoxicity was evaluated by SCGE (single cell gel electrophoresis) and DNA diffusion assay, while cytotoxicity was checked by Neutral Red Retention Assay (NRRA). By analyzing the obtained results we drawn the first toxicity scale for zebra mussel hemocytes (TCS<PCM< DCF<IBU<TMP).

These data lay the groundwork for *in vivo* exposures, which will allow for a better definition of the observed cyto-genotoxicity of these molecules in a setting miming real environmental exposure. Starting from the most toxic PPCP, an *in vivo* multi-biomarker battery was applied on *D. polymorpha* to evaluate their real potential sub-lethal effect. In order to give a marked ecological relevance to our research, mussels were exposed for 96 h to increasing environmentally relevant drug concentrations. Cyto-genotoxicity was determined in mussel hemocytes by the lysosome membrane stability (NRRA), the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and the assessment of the apoptotic frequency (DNA diffusion assay). Moreover, the probable unbalance of mussels' oxidative status was evaluated by analyzing the activity of three antioxidant phase I enzymes, namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), as well as the detoxifying phase II enzyme glutathione S-transferase (GST) in the cytosolic fraction extracted from a pool of entire mussels. By analyzing the final biomarker data, the PPCP *in vitro* toxicity scale was partially confirmed. In fact, according to *in vitro* screening results, TCS was the most toxic drug, followed by PCM, IBU and TMP. On opposite, nonetheless its remarkable *in vitro* cyto-genotoxicity, *in vivo* results noticed that current DCF levels do not induce significant adverse effects on *D. polymorpha*.

Our data point out that the use of a tied *in vitro/in vivo* approach is a useful method to study in depth the toxicity of new environmental pollutants. *In vitro* studies, thanks to their simplicity, reliability and speed, provide basic information on the toxicity of xenobiotics and often assist in hypothesizing or testing the probable mechanism of action of pollutants. In addition, they can act as a guide to direct additional research and could be used in first environmental risk assessment (ERA) evaluations. On this basis, *in vivo* approach, miming the real environmental conditions of exposure (concentrations, uptake pathways, defense mechanisms, metabolism) allow us to evaluate the effective environmental hazard of single PPCPs and/or their mixtures and it furnish more precise and in-depth information of their toxicity on aquatic species for further ERA operations.

Considering the relevance of *in vitro* tests in ecotoxicological preliminary analyses, *in vitro* cultures of zebra mussel hemocytes, gill and digestive gland cells were developed in collaboration with the Irish Centre for Environmental Toxicology (ICET - Galway, Ireland). These innovatory techniques were applied to investigate the potential *in vitro* cytotoxicity of atenolol (ATL), carbamazepine (CBZ), diclofenac (DCF) and gemfibrozil

(GEM) and to discriminate the most sensitive cell type for further applications. On overall, DCF was the more cytotoxic compound for zebra mussel cells, followed by GEM, CBZ and ATL, while gill cells and hemocytes seemed to be the most sensitive targets for tested PPCPs.

Chapter 1 – STATE of ART

1.1 Pharmaceutical and Personal Care Products (PPCPs) as new environmental pollutants

Pharmaceutical and Personal Care Products (PPCPs) are an emerging class of environmental pollutants that are extensively and increasingly being used both in human and veterinary medicine (Fent et al., 2006). Their growing occurrence in ecosystems is causing increasing concern, so that the improvement of their ecological and human risk assessment constitutes a new challenge for the scientific community (Carlsson et al., 2006). These chemicals has been designed to have a specific mode of action, targeting specific organs, metabolic pathways and receptors in order to modulate physiological functions of the organism so that a disease can be treated and the healthy state restored. According to their marked daily utility, the consumption of PPCPs is substantial. In the European Union (EU), about 3,000 different substances are commonly used in human therapy such as anti-inflammatory drugs, contraceptives, antibiotics, β-blockers, lipid regulators, neuroactive compounds and many others (Fent et al., 2006). Similarly, a large number of these molecules are used in veterinary applications.

Sales data report a relatively high PPCP consumption in several countries. For instance, in England, Germany and Australia the amount of the most frequently used drugs is in the hundreds of tons per year (Jones et al., 2002; Huschek et al., 2004; Khan and Ongerth, 2004), while in Italy their annual consumption is in the dozen of tons (Calamari et al., 2003). The pharmaceutical consumption pattern for different countries is not identical and some drugs may be forbidden or replaced by new related ones. However, some therapeutic molecules are regularly monitored within the most frequently applied range as reported in Germany for the class of non-steroidal anti-inflammatory drugs (NSAIDs), including paracetamol (622 t in 2001), ibuprofen (345 t in 2001) and diclofenac (86 t in 2001). Unfortunately, data representing the annual sales or consumption include mainly prescribed drugs, while pharmaceutical mixtures, over the counter and internet sales are often not considered. Therefore, the real amount of applied drugs is uncertain, but probably significantly underestimated, since the consumption, and sometimes the abuse of pharmaceuticals could be much higher than that described in current reports. Hence, figuring out the real annual consumption of a certain drug is very difficult and often based on estimates.

After their assumption and the explication of their therapeutic action, pharmaceutical compounds are excreted in their native form or as active metabolites, reaching the aquatic system via different ways. The main pathway in human application comprehends ingestion, following excretion and disposal via wastewaters. Municipal sewage is therefore the main route that leads human drugs after therapeutic use and disposal of unused medicines into the environment. In addition, hospital and manufacturers wastewaters, as well as landfill leachates (Holm et al., 1995) may contain significant concentrations of pharmaceutical compounds (Figure 1).
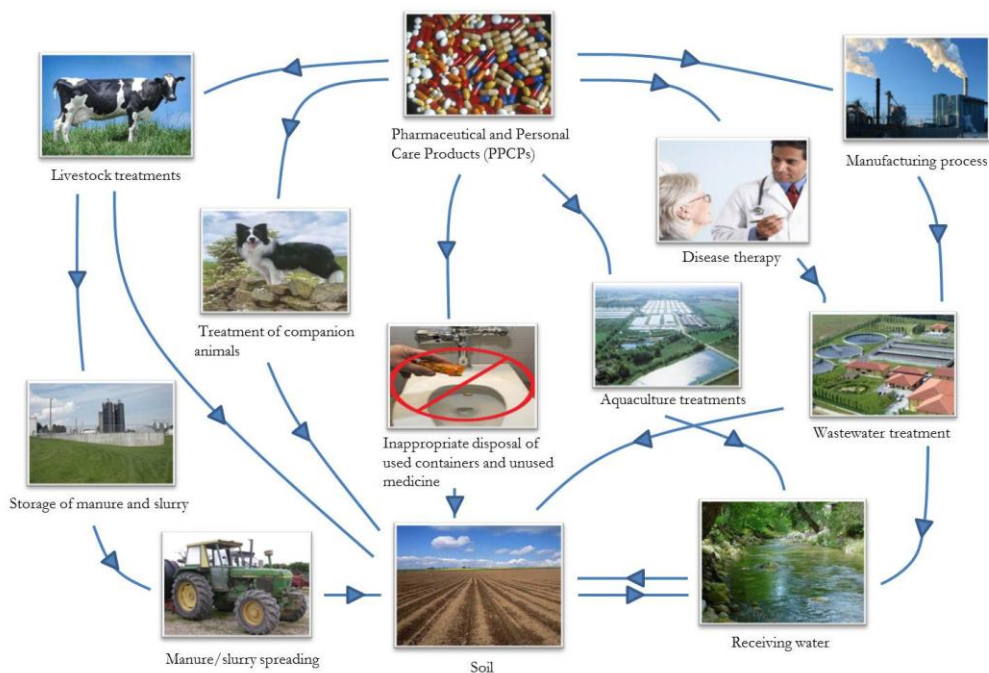
Figure 1: Pharmaceutical and Personal Care Products (PPCPs) environmental fate

Pharmaceuticals are not readily and completely degraded in wastewater treatment plants (WWTPs), so they are discharged in treated effluents resulting in contamination of rivers, lakes, estuaries and, rarely, groundwater and drinking water. Sewage sludge from WWTPs can be considered a secondary PPCP contamination source, since after its application in agricultural fields, contamination of soils, runoff into surface water but also drainage may occur.

In addition, veterinary pharmaceuticals can enter aquatic ecosystems via manure application to field and the following runoff, but also via direct application in aquaculture. Finally, inappropriate disposal of used containers and unused medicine are sources that cannot be neglected. Nonetheless the input sources are well-known, the behavior and the fate of these molecules and their metabolites in the environment is largely unknown.

Considering these issues, an increasing number of studies were aimed to the quali-quantitative evaluation of PPCPs in aquatic environments. Occurrence of pharmaceuticals was first reported in USA treated wastewater, where clofibric acid was found in concentration ranging between 0.8-2 µg/L (Garrison et al., 1976). Afterward in 1981, some drugs were detected in some UK rivers up to 1 µg/L (Richardson and Bowron, 1985), and ibuprofen and naproxen were identified in Canadian wastewater (Rogers et al., 1986). In the last decade, after the establishment and the improvements of chemical analysis methods able to determine polar compounds, many environmental analyses were performed in several countries. These monitoring researches demonstrated that drug residues in the aquatic system are widespread. Many reports reported measurable concentrations of a wide range of about 80-100 pharmaceutical compounds belonging to different therapeutic classes, and some of their metabolites in sewage, rivers, creeks, seawater, groundwater and even drinking water from many European and US countries. Moderate concentrations of pharmaceuticals were mainly reported in WWTP effluents and in surface waters located near sewage treatment plants (Halling-Sorensen et al., 1998; Kolpin et al., 2002; Ashton et al., 2004; Gross et al., 2004), where a huge number of dissimilar drugs occur at levels generally ranging between ng/L to low µg/L. Among these, ibuprofen and its metabolites were commonly revealed in WWTP effluents (Ternes, 1998; Boyd et al., 2003; Wiegel et al., 2004a, b). In a monitoring study carried out in the United Kingdom, propranolol (median level 76 ng/L) was always found in sewage treatment plants effluents, while diclofenac (424 ng/L) was detected in 86%, ibuprofen (3086 ng/L) in 84%, mefenamic acid (133 ng/L) in 81%, dextropropoxyphene (195 ng/L) in 74% and trimethoprim (70 ng/L) in 65% of analyzed samples (Ashton et al., 2004).

In the corresponding receiving streams, fewer compounds and lower levels were measured. Notwithstanding, pharmaceuticals were commonly, and always more frequently, detected also in surface waters. Ternes (1998) reported the occurrence of 32 therapeutic compounds belonging to different medicinal classes in German municipal WWTP effluents, rivers and

stream waters. Twenty drugs and four correspondent metabolites including anti-inflammatory drugs (salicylic acid, ibuprofen, diclofenac, naproxen), lipid regulators (bezafibrate, gemfibrozil, clofibric acid, fenofibric acid), β-blockers (metoprolol, propranolol) and the antidepressant carbamazepine were ubiquitously found both in stream and river water in the ng/L range. Recently, an analysis of drug distribution in the German Elbe River and its tributaries, carried out in the tract between the source and the city of Hamburg, revealed the presence of many pharmaceuticals. Diclofenac, ibuprofen, carbamazepine, as well as various antibiotics and lipid regulators, were the substances mainly detected (Wiegel et al., 2004a, b). A similar contamination pattern was also found in two Italian streams, the Po and Lambro Rivers (Calamari et al., 2003), where in all the sampling sites atenolol, bezafibrate, furosemide and some antibiotics were detected in ng/L concentrations. About the US situation, Kolpin et al. (2002) detected measurable concentrations of 95 micro-pollutants in samples from 139 US water courses downstream urban areas and livestock production. In some sampling sites as many as 38 of targeted 95 compounds were detected. Steroids, an insect repellant (N,N-diethyltoluamide), caffeine, triclosan, antibiotics, 4-nonylphenol and pharmaceuticals were the most frequently detected compounds. A subsequent study (Kolpin et al., 2004) have monitored the concentrations of pharmaceuticals and other aquatic pollutants in water samples upstream and downstream of selected towns and cities in Iowa (USA) during different flow conditions, revealing that prescription drugs were only detected during low-flow conditions.

Among the common detected drugs, non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most revealed pharmaceutical classes; ibuprofen, diclofenac, naproxen, paracetamol and some of their metabolites are often found in surface water in up to µg/L range. β-blockers, blood lipid lowering agents, neuroactive compounds and low levels of antineoplastic agents were detected, as well. Particular attention should be to give to the antidepressant carbamazepine that has been proposed as an anthropogenic marker in water bodies (Clara et al., 2004) since its commonly revealed in surface waters in concentrations exceeding 1 µg/L (Fent et al., 2006). At last, also drinking water (Putschew et al., 2000; Zuccato et al., 2000; Stackelberg et al., 2004), groundwaters (Ternes et al., 2001) and landfill leachates (Holm et al., 1995) contain pharmaceuticals in ng/L range, in some cases up to µg/L. For instance, phenazone, propiphenazone and clofibric acid were found in

sample of potable water collected in the vicinity of Berlin (Germany; Heberer and Stan, 1997; Reddersen et al., 2002).

In spite of the sizeable amount of human and veterinary drugs released and detected into environment, concise regulation for environmental risk assessment is deeply missing. Only in the last years, regulatory agencies have drawn detailed guidelines on how pharmaceutical compound should be assessed for possible unwanted deleterious effects on the environment. In 1980, the United States Food and Drug Administration (FDA) required an environmental assessment of pharmaceutical compounds applied in veterinary medicine (Boxall et al., 2003). Only in 1995, the European Union (EU) Directive 92/18 EEC and the corresponding "Note for Guidance" (EMEA, 1998) decided that ecotoxicity testing are a necessary prerequisite for registration of veterinary pharmaceuticals. Afterwards, the European Commission released a draft guideline (Directive 2001/83/EC) specifying that an authorization for a medicinal product for human use must be accompanied by an environmental risk assessment analysis (EMEA, 2005). Similarly, the FDA previously published a guidance for the assessment of human drugs. According to this, pharmaceutical applicants are required to furnish an environmental risk assessment report when the expected concentration of the active compound introduced in the aquatic system is higher than 1 µg/L (FDA-CDER, 1998), which corresponds to about 40 t as a trigger level.

The guidelines mentioned above were drawn because several studies found that the risk tied to pharmaceutical exposures is higher for aquatic species in comparison with human.

Pharmaceuticals in fact are designed to target specific metabolic and molecular pathways in humans and animals, but often they have important side effects too. In the environment, they can affect the same pathway in animals having identical or similar target organs, tissues, cells or biomolecules (Fent et al., 2006). Certain receptors in lower animals resemble those in humans, but others are different or completely lacking, which means that dissimilar modes of actions may occur in lower organisms. Moreover, it is important to highlight that the mode of action is not well known for some drugs and sometimes they possess many different mechanisms of action. According to these reasons, specific toxicity analyses in lower animals are difficult to perform and, at present, only little is known on ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms and wildlife.

Aquatic organisms are particularly important targets, since they are exposed to several of these drugs for their whole life. An increasing number of researches are carrying out in order to evaluate the potential dangerousness of these new environmental pollutants on the biocoenosis. At present, above all pharmaceuticals' acute toxicity was tested by traditional standard tests according to established guidelines on organisms belonging to different levels of biological organization, such as algae (Yang et al., 2008), cnidarians (Quinn et al., 2008), crustaceans (Haap et al., 2008) mussels (Canesi et al., 2007) and fish (Choi et al., 2008). By comparing these different trophic levels, Webb (2001) suggested that algae are more sensitive to pharmaceuticals than the crustacean *Daphnia magna*, and fish are even less sensitive. However, these tests do not focus enough on the different modes of action of different administered drugs, and hence, differences in toxicity among the phyla are not easily explainable. In addition, by comparing the different classes of pharmaceuticals in terms of acute toxicity, Webb (2001) noted that the most toxic classes were antidepressant, antibacterial and antipsychotic drugs, but the range of responses within each of these categories was large, comprehended in several orders of magnitude. According to data currently available in scientific literature reported by Fent et al. (2006), 17% of tested pharmaceuticals displayed an acute toxicity below 100 mg/L, even if fluoxetine toxicity values were below 10 mg/L for each biological model. On the other hand, 38% drugs such as acetylsalicylic acid, betaxolol, sotalol, bezafibrate, gemfibrozil, cimetidine and ranitidine showed LC_{50} values higher than 100 mg/L. These drugs, according to the EU Directive 93/67/EEC (Commission of the European Communities, 1996), are classified as not being harmful for aquatic organisms. Other pharmaceuticals (45%) displayed a wide variability of acute toxicity values that makes the classification difficult. In addition, according to other findings, compounds such as ibuprofen, paracetamol, amoxicillin, oxitetracyclin and mephenamic acid pose a not negligible risk for aquatic organisms (Jones et al., 2002).

Unfortunately, available datasets were built only on a limited number of drugs, while in the environment recent studies showed hundreds of potentially hazardous therapeutic drugs. Moreover, these data alone may not be suitable for specifically addressing the question of effects in real environment, and subsequently the hazard and risk assessment (Fent, 2003). In conclusion, according to the evidences mentioned above, acute toxicity to aquatic organisms is unlikely to occur at current measured environmental levels, as acute effect concentrations are 100-1000 fold higher than residues found in surface and wastewaters.

Hence, according to the lowest concentrations, pharmaceuticals in environment are suggested to pose only a low risk for acute toxicity. On the basis of all the previous considerations, the high activity of pharmaceuticals and the continuous exposure of aquatic organism to them over long time periods or over their entire life cycle, may suggest that chronic effects are absolutely more probable. Nonetheless, at present, they have only very rarely been investigated, with a subsequent substantial lack of information.

The available chronic data refer to only few molecules, limited biological models and often do not investigate important key targets. Moreover, life-cycle analyses are not reported and toxicity to benthic and soil organisms has very rarely been evaluated. Currently, the best knowledge on chronic effects related to pharmaceutical compounds exists for the synthetic steroid 17α-ethinylestradiol (EE2) contained in contraceptive pills. Several studies showed its estrogenic effects on different fish species at extremely low and environmentally relevant concentrations (Lange et al., 2001; Brian et al., 2005; Parrot and Blunt, 2005; Fenske et al., 2005). Some other researches were carried out in order to evaluate the possible sub-lethal effects induced by NSAIDs (Ferrari et al., 2003, 2004; Schwaiger et al., 2004; Triebskorn et al., 2004; Hallare et al., 2004), β-blockers (Haider and Baqri, 2000; Huggett et al., 2002), blood lipid lowering agents (Ferrari et al., 2003; Mimeault et al., 2005) and neuroactive compounds (Ferrari et al., 2003; Pascoe et al., 2003; Thaker et al., 2005) on some fish species (*Oryzias latipes*, *Danio rerio*, *Oncorhynchus mykiss*, *Salmo trutta*, *Carassius auratus*), crustaceans (*Daphnia magna*, *Ceriodaphnia dubia*) and algae (*Hyalella azteca*). Nonetheless the increased efforts to enlarge this crucial environmental topic, the knowledge on drug-induced sub-lethal responses and involved mechanism of action on aquatic species are still very inadequate.

Hence, the use of biomarkers on a reference biological model is strongly recommended as sensitive approach to study in-depth the environmental hazard of pharmaceutical compounds (Bottoni and Fidente, 2005).

1.2 Drugs monitored in this study

In order to investigate on the potential ecotoxicity of PPCPs on freshwater non-target organisms, we decided to evaluate the potential cyto-genotoxic effect induced on zebra mussel (*Dreissena polymorpha*) specimens by some of the most common drugs currently revealed in the aquatic system. We decided to assess the onset of sub-lethal effects caused by a common antimicrobial agent (triclosan; TCS), a widespread antibiotic (trimethoprim; TMP) and three largely used non-steroidal anti-inflammatory drugs (paracetamol; PCM – diclofenac; DCF – ibuprofen; IBU).

1.2.1 Triclosan

Among the emerging class of environmental pollutants of PPCPs, one of the most widely used groups is that of antibacterial agents, which are used not only for human and veterinary medication, but also for the promotion of growth in livestock and aquaculture species (Daughton and Ternes, 1999). While antibacterial agents at low concentrations are probably not pharmaceutically active in humans, they may still be potential pollutants in aquatic environments (Yang et al., 2008).

Triclosan (TCS; 2,4,4'-trichloro-2'-hydroxydiphenyl ether) is one of the main known antibacterial agents and it is added to a wide range of consumer products (e.g. toothpastes, soaps, deodorants, textiles, shoes, toys and cosmetics). It has been marketed for over 30 years, and its use has increased over time. The UK Environment Agency (2004) estimated that about 350 tons of this chemical are used every year in the EU. TCS is a lipophilic compound (log Kow = 4.8) that has the potential to bioaccumulate and affect non-target organisms. It has been found in four fish species from Sweden, ranging from 0.24 to 120 mg/kg fresh weight (Adolfsson-Erici et al., 2002), and in algae *Cladophora* spp. at 100–150 ng/g fresh weight (Coogan et al., 2007). Wilson et al. (2003) showed that TCS may influence both the structure and the function of algal communities in stream ecosystems that receive treated wastewater effluent, and there is a huge amount of documentation on the impact of antibacterial residues on microbial processes and the consequential effects on the whole ecosystem (Yang et al., 2008). Many studies showed that TCS is a biodegradable and photo-unstable compound, which continues to breakdown following its release into the aquatic system.

TCS has been reported to exist also in a methylated form, methyl-TCS, more stable and toxic with respect to the parental compound (Lindstrom et al., 2002), and can be converted into a dioxin congener (2,8-dichlorodibenzo-*p*-dioxin, DCDD) by photolytic degradation under laboratory conditions (Aranami and Readman, 2007). Recently, Buth et al. (2009) demonstrated that the photochemical conversion of three chlorinated TCS derivatives to three polychlorodibenzo-*p*-dioxins that possess higher toxicity than 2,8-dichlorodibenzo-*p*-dioxin, the sole TCS photoproduct previously known. At present, the onset of this degradation process into natural environments is completely unknown, and therefore it is difficult to assess the environmental significance of this finding (UK Environment Agency, 2004). Recently, Yang et al. (2008) measured the growth-inhibiting effects of 12 different antibacterial agents and showed that TCS is the most toxic antibacterial compound (NOEC = 200 ng/L) for the freshwater microalga *Pseudokirchneriella subcapitata*. A previous study carried out by Orvos et al. (2002) revealed a NOEC of 700 ng/L for the algae *Scenedesmus subspicatus*. On the basis of this result, the most conventional PNEC (Predicted No Effect Concentration) of 70 ng/L for freshwater species was derived. Several studies found TCS concentrations exceeding this value in German, English and Swiss rivers (Singer et al., 2002; Lindstrom et al., 2002; Sabaliunas et al., 2003; Wind et al., 2004), with levels ranging from 10 ng/L to 100 ng/L measured downstream of sewage treatment plants. Moreover, Reiss et al. (2002) found TCS concentrations in US wastewater effluents ranging from 200 ng/L to 2700 ng/L. Capdevielle et al. (2007), by using the Geography-referenced Regional Exposure Assessment Tool for European Rivers (GREATER), estimated a PEC (Predicted Environmental Concentration) for the UK that rarely exceeded 200 ng/L of TCS. For North America, the PhATE (Pharmaceutical Assessment and Transport Evaluation) model suggested that TCS concentrations could be as high as 850 ng/L without in-stream removal and 250 ng/L with in-stream removal. These authors calculated a chronic PNEC of 1550 ng/L by using the species sensitivity distribution (SSD) approach derived from toxicity data on 14 different aquatic species. The great amount of NOEC and PNEC values that have been calculated for aquatic organisms highlights the species-specific effects of TCS and that toxicity studies of different classes of antibacterial agents are urgently required to assess their potential impact on aquatic ecosystems (Yang et al., 2008). However, very scarce data are available on other sub-lethal effects (genotoxicity,

cytotoxicity, impairment of cell signaling, oxidative stress), which can also demonstrate the possible mechanisms of TCS action.

1.2.2   Trimethoprim

Antibiotics are considered molecules having high priority in environmental risk assessment because of their extensive use in humans and animals. Moreover, these drugs can potentially cause damage to the ecosystem by affecting key-species and by promoting the development and spread of resistant genes in the environment (Costanzo et al., 2005). Moreover, the use of antibiotics is constantly increasing, as indicated by recent research carried out by the European Surveillance of Antimicrobial Consumption (ESAC, 2007). Among the plethora of these therapeutic products, trimethoprim (TMP; 5-[3,4,5-trimethoxybenzyl]pyrimidine-2,4-diamine) is a largely used wide spectrum antimicrobial drug and one member of the family of synthetic 2,4-diaminopyrimidines. TMP has a potent microbicidal activity against a wide variety of bacterial species (Lampert and O' Grady, 1992), and forms metal complexes with useful antimalarial properties (Ajibade and Kolawole, 2008). It inhibits the enzyme dihydrofolate reductase that is involved in blocking of the synthesis of tetrahydrofolate, an essential precursor in the synthesis of thymidine. Loss of this nucleoside ultimately impacts DNA, RNA and protein synthesis, resulting in stasis or cell death (Baccanari, 1995). Halling-Sørensen et al. (2000) investigated the acute effects of TMP on some non-target organisms. They found an EC_{50} value of 110 mg/L in the green freshwater microalga *Pseudokirchneriella subcapitata*, very similar to that observed in the cyanobacterium *Microcystis aeruginosa* (EC_{50}=112 mg/L) and crustacean *Daphnia magna* (48hEC_{50}=123 mg/L). However, these values are several orders of magnitude higher than environmental concentrations found both in wastewaters and freshwaters. Chang et al. (2008) measured levels of TMP ranging from 0.09 to 0.34 ng/L in four Japanese rivers, while this drug was detected at a concentration up to 546 ng/L in the South Korean Han River (Choi et al., 2008). With regard to European contamination, Kasprzyk-Hordern et al. (2008) measured TMP concentrations up to 183 ng/L in the River Ely (South Wales, UK), while average concentrations ranging from12 to 27 ng/L were found in five different sampling sites of the Seine (France; Tamtam et al., 2008). These values are much lower than the two available PNECs of 180 μg/L (Halling-Sørensen et al., 2000) and 16 μg/L (Grung et al., 2008), calculated by using either acute toxicity results or a

combination of classical acute and chronic toxicity data, respectively. By contrast, few data are available on other sub-lethal effects, coupled with considerable controversy about its potential cytotoxicity and genotoxicity. This is largely due to conflicting reports from the scientific literature and from the manufacturers that describe both positive and negative effects within the same bacterial and mammalian cell models (Abou-Eisha, 2006).

1.2.3   Paracetamol

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most important therapeutic groups revealed in the aquatic ecosystems. With an annual production of several kilotons (Cleuvers, 2004), NSAIDs are the sixth most sold drugs worldwide (Langman, 1999). Additionally, since some of these pharmaceuticals can be purchased without medical prescription, their consumption could be even higher. NSAIDs are commonly used to cure pain and inflammation, since they inhibit the synthesis and release of prostaglandins acting as non-selective inhibitors of the enzyme cyclooxygenase and inhibiting both the cyclooxygenase-1 (COX-1) and the cyclooxygenase-2 (COX-2) isoenzymes (Gierse et al., 1995). Due to the continuous and increasing application, as well as their pharmacokinetic properties (half-life, urinary and fecal excretion, metabolism, etc.), NSAIDs can reach detectable concentrations both in sewage and in surface water (Cleuvers, 2004). Many authors have reported levels of these drugs exceeding 1 µg/L in wastewaters and in the effluents of sewage treatment plants (STP), while lower concentrations have been found in surface waters (Stumpf et al., 1996; Ternes et al., 1998; Farré et al., 2001; Heberer, 2002; Metcalfe et al., 2003). Although the analgesic and antipyretic agent paracetamol (PCM; N-(4-hydroxyphenyl)acetamide) does not possess a real anti-inflammatory action, it is usually considered an NSAID in toxicology due to its very similar mode of action (Misra et al., 1990). Since it is considered a safe drug at therapeutic doses, it can be purchased as an over-the-counter preparation in most countries, and it is currently the most widely used drug worldwide (An et al., 2009). Due to the huge production and quantity of use, it is reported as one of the most frequently detected pharmaceuticals to be found in surface waters, wastewaters and drinking water. Kolpin et al. (2002) detected PCM in 24% of samples from a survey of 139 US streams, at a median concentration of 0.11 µg/L, with a maximum detection level up to 10 µg/L. These concentrations are perfectly in agreement with the PEC value calculated by Kim et al. (2007) for Korean waters (16.5 µg/L). The

median concentration of this compound measured in surface waters worldwide is 0.055 ± 0.051 µg/L (Bound and Voulvoulis, 2006; Gros et al., 2006), while in raw wastewaters was detected at a higher median concentration of 48 ± 75 µg/L (Gros et al., 2006; Han et al., 2006). Due to its widespread presence in aquatic ecosystems, PCM is one of the possible dangerous compounds for the entire aquatic biocoenosis (Crane et al., 2006; Schulte-Oehlmann et al., 2007). Although very few studies have been carried out to evaluate its environmental risk, Henschel et al. (1997) classified this drug as harmful to aquatic organisms on the basis of some ecotoxicological tests carried out on different biological models including bacteria, algae, crustaceans and fish embryos. According to this assumption, on the basis of the PNEC value (9.2 µg/L) found by Kim et al. (2007), a PCM hazard quotient (1.8) was calculated, suggesting potential adverse ecological consequences.

1.2.4   Diclofenac

Diclofenac (DCF; 2-[(2,6-dichlorophenyl)amino] phenylacetic acid) is a well-known phenylacetic acid NSAID, commonly used to reduce inflammation and pain associated with arthritis and other conditions, such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis (Todd and Sorkin, 1988). Because of its huge worldwide selling, often over-the-counter and large prescription volume, DCF has been identified as one of the main pharmaceuticals present in the aquatic ecosystems. Dermal application results the main emission pathway of the active compound (Heberer and Feldmann, 2005) due to its relative small absorption on skin (5−10%) while the remaining is released into water by washing (Letzel et al., 2009). For this reason, DCF is recognized as a very important pollutant due to its low removal rate during the wastewater treatment process. It is frequently detected at low µg/L range in STP effluents of Europe and North and South America (Deng et al., 2003; Koutsouba et al., 2003; Ashton et al., 2004; Lindqvist et al., 2005; Roberts and Thomas, 2006; Gómez et al., 2007). Accordingly, DCF is commonly found also in surface waters in concentrations covering a wide range from few ng/L up to the µg/L (Metcalfe et al., 2003; Ashton et al., 2004; Lindqvist et al., 2005; Gros et al., 2006). Nonetheless, these findings, its environmental fate, and above all its ecotoxicological effects on non-target organism are only poorly understood. Although the recent decline of three Indian gyps vulture populations (Indian White-rumped Vulture, *Gyps bengalensis*; Indian Vulture, *Gyps indicus*; and Slender-billed Vulture, *Gyps tenuirostris*) has been related to diclofenac-

induced renal failures (Oaks et al., 2004; Taggart et al., 2007), at present still few investigations report the adverse effects of DCF on aquatic organisms. Some studies were carried out on *Vibrio fischeri* (Zhang et al., 2008a), several algal species, *Daphnia magna* (Cleuvers, 2003; Ferrari et al., 2003), and *Danio rerio* (Dietrich and Prietz, 1999) which demonstrated that this drug has a low acute toxicity as short-term EC_{50}, with tested concentrations much higher than those currently present in the surface waters. However, continuous release of DCF may lead to chronic exposure of aquatic organisms and consequently lower effect concentrations. Triebskorn et al. (2004) highlighted that 1 µg/L DCF was able to cause harmful effects on liver, kidney, and gills of rainbow trout, while Hong et al. (2007) showed that the expression of biomarker genes was related to cellular toxicity, genotoxicity, and estrogenic effects in Japanese medaka fish (*Oryzias latipes*) exposed to low DCF concentration. Notwithstanding these evidences, at present, no study was carried out on invertebrate species to study the possible DCF sub-lethal effects.

1.2.5   Ibuprofen

Ibuprofen (IBU; ((+/-)-2-(p-isobutylphenyl) propionic acid with R and S isomers) is one of the most sold NSAID worldwide and it is widely used to relieve symptoms of arthritis, rheumatic disorders, pain, and fever (Hayashi et al., 2008). It is one of the core medicines in "Essential Drug List" of World Health Organization, and therefore produced in large amounts worldwide (Heckmann et al., 2007). Because of its considerable selling, often over-the-counter, large prescription volume, and high excretion degree (70-80% of the therapeutic dose) as parent compound and/or as metabolites, IBU has been identified as one of the main pharmaceuticals present in the aquatic ecosystems. Moreover, it has relatively high mobility into aquatic environments, but a low persistence compared to other pharmaceuticals (Buser et al., 1999) even if its half-life has been estimated to be 32 days in the field (Tixier et al., 2003). IBU has been detected in moderate concentrations (ranging between ng/L and µg/L) both in European (Ashton et al., 2004; Thomas and Hilton, 2004; Weigel et al., 2004a, b) and North American (Kolpin et al., 2002; Metcalfe et al., 2003) rivers and estuaries, as well as in effluents of wastewater treatment plants (WWTPs).

As in the case of many other human pharmaceuticals, nonetheless its widespread diffusion in many aquatic ecosystems, at present there is limited ecotoxicological information

available in the literature regarding the potential chronic impact of IBU on the biocoenosis (Fent et al., 2006). Some acute studies have been carried out on the cladoceran crustacean *Daphnia magna*, which report that the 48 h EC_{50} for immobility ranges between 10 and 100 mg/L IBU (Halling-Sørensen et al., 1998; Cleuvers, 2003, 2004; Heckmann et al., 2005; Han et al., 2006). Fewer researches were carried out to investigate IBU chronic effects on invertebrate aquatic species. In *D. magna*, reproduction and population growth were investigated after long-term exposures to environmentally relevant IBU concentrations, reporting a 14 days reproduction EC_{50} and survival NOEC of 13.4 mg/L and 20 mg/L IBU, respectively (Heckmann et al., 2007). Pascoe et al. (2003) found that IBU, as well as acetylsalicylic acid and paracetamol, had no effect on the cnidarian *Hydra vulgaris* at concentrations up to 1 mg/L following 7 days exposure. However, 1 mg/L was sufficient to produce a 25% growth reduction of duckweed *Lemna minor*, with a 7-day EC_{50} of 4 mg/L (Pomati et al., 2004). Conversely, the same authors found that the growth of the cyanobacteria *Synechocystis* sp. was stimulated at 10 μg/L IBU. The few aforesaid chronic-effects studies indicate that IBU probably has very little impact on aquatic biota. However, more in-depth studies are needed to enlarge the knowledge of its possible sub-lethal effects on the aquatic biocoenosis.

1.3 Role of biomarkers in PPCPs environmental risk assessment

Considering the huge lack of information on the potential sub-lethal effects induced by PPCPs on non-target aquatic species, in-depth researches are absolutely needed in order to enlarge this topic and elucidate the possible mechanisms involved in the onset of serious damage to each level of biological organization. To reach this goal, the application of biomarker techniques on an appropriate reference biological model is strongly recommended as a sensitive approach to investigate the environmental hazard of several classes of pollutants, including pharmaceutical and personal care products (Bottoni and Fidente, 2005). A biomarker can be defined as "the measurement of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response" (Bodin et al., 2004). A more generalized definition which would also accommodate whole animal studies would include "measurements on whole animals" and "indicate in physiological, behavioral or energetic terms" (Ross et al., 2002;

Magni et al., 2005). In conclusion, any biological response to an environmental chemical at the sub-individual level, measured inside an organism or in its products (urine, faeces, hair, feathers, etc.), indicating a deviation from the normal status that cannot be detected in the whole organism can be considered a biomarker (van der Oost et al., 2003). These assays have their origins in human toxicology, in which they have proven to be very useful as measures of human exposure to specific chemicals or as early warning indicators of specific diseases or syndromes. Their primary strength in this context is that they permit nondestructive sampling of tissues or body fluids, thereby providing accurate estimates of internal or effective dose, organ function, and in some cases, individual susceptibility to disease. During the past 20 years, the biomarker approach has received considerable attention in ecotoxicology as a new and potentially powerful informative tool for detecting and documenting exposure to, and effects of, environmental contamination (Newman and Jagoe, 2006).

According to the National Research Council (NRC, 1987) and World Health Organization (WHO, 1993), biomarkers can be divided into three classes:

- ✓ *biomarkers of exposure*: covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;
- ✓ *biomarkers of effect*: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease;
- ✓ *biomarkers of susceptibility*: indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure.

The responses of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects. Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance, providing a link between external exposure and internal dose. Biomarkers of effect can be used to document either

preclinical alterations or adverse health effects due to external exposure and absorption of a chemical. Biomarkers of susceptibility help to elucidate variations in the degree of responses to toxicant exposure observed between different individuals. These biochemical assays are generally considered more sensitive than bioindicators at higher levels of the biological hierarchy, such as the organ, the individual or the population (Mayer et al., 1992; Stegeman et al., 1992). Effects at higher hierarchical levels in fact, are always preceded by earlier changes in biological processes (Figure 2), allowing the development of early-warning biomarker signals of effects at later response levels (Bayne et al., 1985).
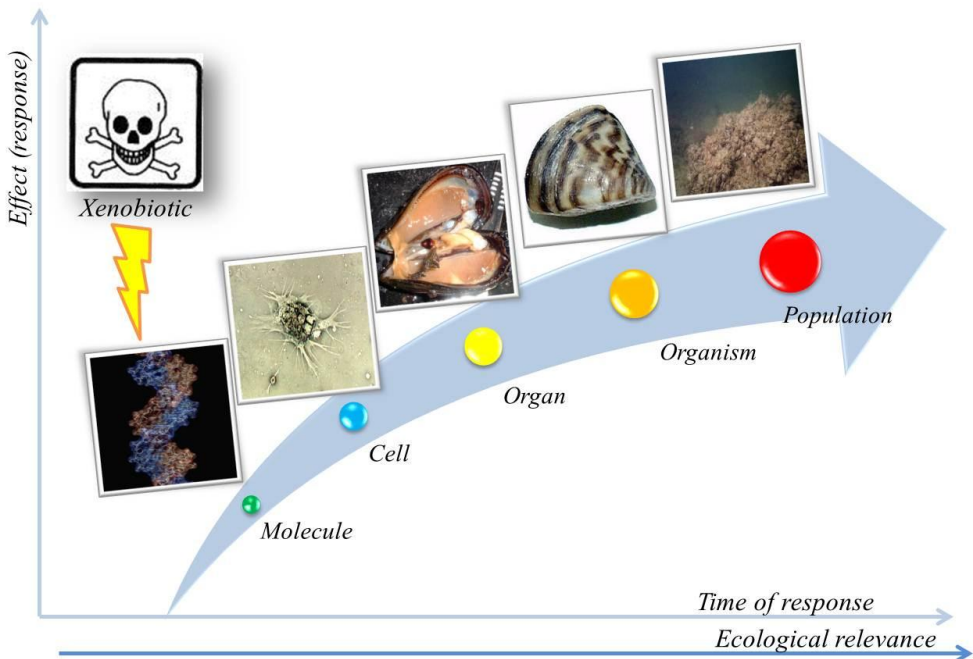
Figure 2: Effects of a xenobiotic to different levels of hierarchical organization

In contrast to the simple measurement of contaminants accumulation in body tissues, biomarkers can offer complete and biologically more relevant information on the potential impact of toxic pollutants on the health of organisms (Van der Oost et al., 1996).

A pollutant stress situation normally triggers a cascade of biological responses, each of which may, in theory, serve as a biomarker (McCarthy et al., 1991). As the primary effect of xenobiotics was first revealed at the sub-cellular level (as biochemical and molecular

variations, enzymatic activity modifications, DNA alterations), its rapid evaluation can allow the activation of some procedures for the pollutant impact reduction before the damage reaches the higher hierarchical levels (Figure 3).

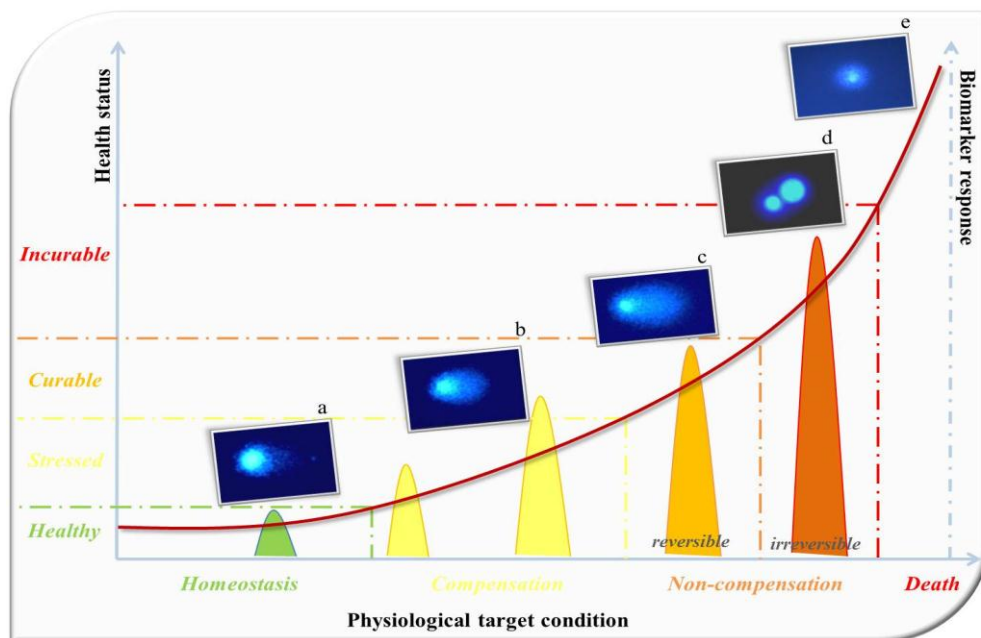*Figure 3*: Relationship between exposure to pollutant, health status and biomarker responses. The exponential magenta line shows the progression of the health status of an individual in relationship with increase of exposure time and pollutant concentration. Green broken line represents the threshold between homeostasis and compensation processes; yellow broke line represents the rise of non-compensatory processes; the orange broken line: the limit beyond which the pathological damage is irreversible by repair mechanisms; the red broken line: the threshold after with the biological target die. Histograms and pictures show the hypothetical response of three different genotoxicity biomarkers used to assess the evolution of genetic damage into biological target (cell). a: very low DNA damage; b: moderate DNA damage; c: extreme DNA damage; d: fixed DNA damage (micronuclei production); e: cell death (apoptosis).

Moreover, biomarkers may provide insight into the potential mechanisms of contaminant effects. By screening multiple biomarker responses, important information will be obtained about organism toxicant exposure and stress. Historically, biomarkers were used to assess the biological and ecological significance of environmental contaminants as complementary approach to chemical analysis and they become an important component of many

environmental monitoring programs. Notwithstanding, they have seldom been used for decision making in regulatory ecotoxicology, but they could play a crucial role in assessing risks of some human and veterinary pharmaceuticals. In particular, molecular and/or biochemical responses, specific for certain drug mode/mechanism of action (MoA) in non-target species may serve as powerful tools for ecotoxicological risk assessment (Williams et al., 2005; Crane et al., 2006). In addition, several recent biomarker discovery researches have been fueled by new "omic techniques" (genomic, proteomic, trascrittomic, glycomic, metabolomics and lipidomic) that enable scientist to simultaneously measure literally thousands of genes, proteins, and metabolites in organisms exposed to different types of chemical stressors (Ankley et al., 2006). Some authors recommended the use of sub-organismal biomarkers for detecting either exposure to, or the effects of, human and veterinary pharmaceuticals. They suggested the use of these techniques because one of their major values may be as rapid screens to help prioritize further in depth studies (Dinan et al., 2001). At present, many authors applied both traditional enzymatic and cellular biomarkers (Jos et al., 2003; Nunes et al., 2004; Canesi et al., 2007) and newer biomarkers born from the field of genomics, proteomics and metabolomics (Miracle et al., 2003; Viant et al., 2003; Snape et al., 2004). Among these numerous techniques, an excellent example of a biomarker used in fish and other oviparous animals in order to detect pharmaceutical MoA is vitellogenin (egg yolk protein), which is normally present in reproductively mature females but which can be induced in males exposed to estrogens, as EE2, or similar-estrogen xenobiotics. Gene expression profiles have also been used to assess the exposure to largemouth bass (*Micropterus salmoides*) to EE2 (Larkin et al., 2003), and the proteomics of similar estrogenic exposures were reported by Shrader et al. (2003) for zebrafish. Many other biomarker techniques (i.e., comet assay, DNA damage assays, cDNA microarrays) were used to assess the potential toxicity of different chemicals on several species. However, none of these approaches has been extensively used for detecting or assessing human/veterinary pharmaceutical effects, although their use is absolutely recommendable. Biomarkers may be very useful in helping efficiently to direct research and testing towards substances with biological activity and MoA that are relevant to particular taxonomic groups (Henshel et al., 1997). They may also demonstrate possible cause and effect relationships both in field survey and in laboratory experiments.

However, they are unlikely to be useful as unique endpoints in pharmaceuticals (or other chemicals) environmental risk assessment. Their use in this field of application remains uncertain, particularly with regard to what molecular or biochemical changes might mean to the types of endpoints on which environmental regulations typically are based (for instance, survival, growth, reproduction).

1.4 Mussels as biological models in ecotoxicology

Since the aquatic environment is often the ultimate ecological compartment of anthropogenic pollutants, such as PPCPs, either due to direct discharge or to hydrologic and atmospheric processes, it is not surprising that there has been an increasing interest regarding the possible adverse effect that exposure to these numerous xenobiotic may cause to aquatic organisms. By far the most popular organism used in biomonitoring studies has been the mussel, and hence the term "Mussel Watch" was coined. Since the mid-1970s, scientists of several countries have used bivalve-filter feeding mollusks to monitor selected contaminants, from heavy metals to pesticides and other organic pollutants, in coastal marine waters. The 1990s saw a rapid increase in use of mussels as tools for measuring POP (Persistent Organic Pollutants) loads in the environment. Mussels or other bivalves are commonly preferred for biomonitoring operations of aquatic ecosystems because of their many advantages over other organisms (Goldberg, 1975). There are several attributes that make mussels superior to other organisms for use as "sentinel" or "indicator" organisms in environmental monitoring programs throughout the world (Tanabe, 2000). According to Saiz-Salinas et al., (1996) the contamination of these organisms provides a time-integrated measure of contaminant bioavailability, responding essentially to the fraction of the total environmental load that is of direct ecotoxicological relevance (Rainbow and Phillips, 1993). Given the relevance of mussels as sentinels of aquatic pollution, it is pivotal to develop useful methods to quantify not only the exposure to chemicals, but also the potential adverse effects caused by xenobiotics. To fill this gap, in recent years an increasing number of studies were carried out in order to develop, improve and apply several biomarker techniques to different mussel species. Among these, there has been considerable interest in the use of biomarkers within bivalve mollusks, which might provide greater sensitivity and/or more information on how these animals interact with environmental pollutants (Peakall, 1994).

Marine (*Mytilus* spp., *Crassostrea* spp., *Ruditapes decussatus, Chamelea gallina*) and freshwater (*Dreissena polymorpha*, *Unio* spp.) bivalves were used in field surveys to evaluate the potential dangerous effects caused by the exposure to a complex environmental mixture of pollutants. Moreover, thanks to their peculiar physiological characteristics, among which the high filtration rate which favors a rapid accumulation of pollutants, they were always more considered in studies carried out in controlled laboratory condition to assess the potential toxicity of several aquatic pollutants, including heavy metals (Munari et al., 2007; Faria et al., 2009), pesticides (Binelli et al., 2008), halogenated hydrocarbons (Barreira et al., 2007; Binelli et al., 2008).

1.4.1 Zebra mussel (*Dreissena polymorpha*)

Dreissena polymorpha (Pallas, 1771) is a relatively widespread macrobenthic bivalve species having a striped coloration of the shell which confers to it the common name "zebra mussel" (Figure 4). It lives both in lentic and lotic freshwaters, nonetheless it is able to survive also in estuarine environments and tolerate salinity up to about 5 g/L.

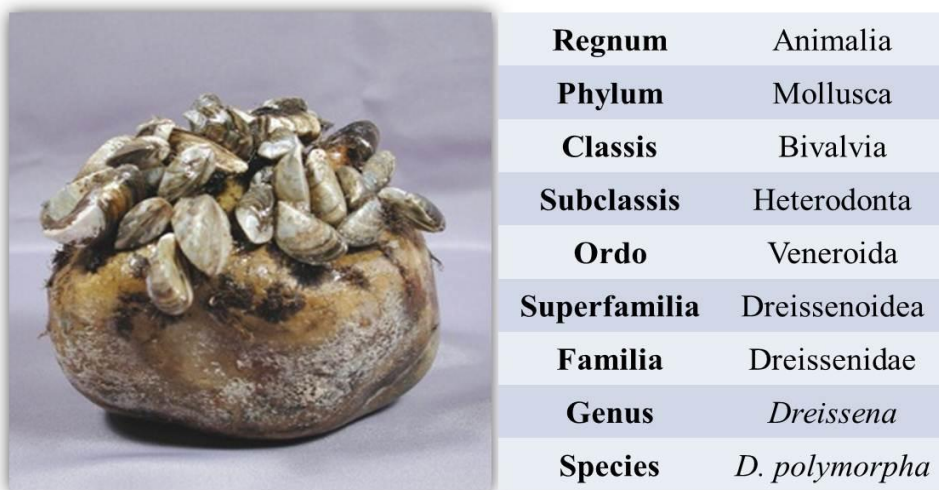| Regnum	Animalia
Phylum	Mollusca
Classis	Bivalvia
Subclassis	Heterodonta
Ordo	Veneroida
Superfamilia	Dreissenoidea
Familia	Dreissenidae
Genus	*Dreissena*
Species	*D. polymorpha*

Figure 4: Zebra mussel (*Dreissena polymorpha*) specimens and their systematic classification

This mussel comes from the Aral-Caspian area and it was found across Europe before the last glacial period (Starobogatov and Andreeva, 1994).

The Caspian Sea, the Black Sea Basin, the Azov Sea, and lower parts of rivers feeding them formed the postglacial distribution of the zebra mussel. Since the first years of the past century it was the object of several researches because of its fast diffusion in many European aquatic environments, and its remarkable damages caused to ecosystems and economy. The expansion of this species into other areas is a result of unintentional introductions and invasions mediated by shipping canals for transportation and commerce. Man-mediated invasions of zebra mussel from the Ponto-Caspian basin through Europe have been reported since the eighteenth century (Nowak, 1971; Starobogatov and Andreeva, 1994). *Dreissena polymorpha* first escaped from the catchment basin of the Black Sea effluents around 1760 and rapidly began to occupy the northern part of Central and Western Europe (Kinzelbach, 1992). In the 20th century, a new period of expansion occurred. Zebra mussels were reported in alpine and sub-alpine European regions in 1960s (Pollux et al., 2003).

In Italy, this invasive bivalve has been found in Lake Garda since 1969 (Giusti and Oppi, 1972) and has invaded all aquatic environments in the Po River basin. Moreover, the presence of *D. polymorpha* has been also found in 2000 in Lake Trasimeno (Spilinga et al., 2000), located in Central Italy, indicating that it can be properly considered as a typical representative of Italian malacofauna. About the global diffusion of the species, North American presence of zebra mussel was first reported in 1988 in Lake St. Clair, a moderate-sized lake in the Great Lakes region between Lake Huron and Lake Erie (Hebert et al., 1989). A rapid spread was noted during the 1990s and it colonized many US environments, including many Great Lakes and Hudson and Mississippi watersheds. Nowadays it is also found in Africa and Asia. *D. polymorpha* possesses a triangular–shape shell that reaches a maximum length of about 30-36 mm (Karataev et al., 1994). It is a typical and obligate seston-feeder. Filtration normally occurs at a temperature of 5-30 °C (Kondratiev, 1969) and selection of filtered particles occurs on the epithelium of the gills and labial palps. All rejected materials are collected in the mantle cavity and then expelled via the inhalant siphon as pseudofaeces, and only 10% of filtered material is found in the stomach. Dreissenids are unisexual with iteroparous reproduction, and populations have equal proportions of males and females.

Its annual cycle (Figure 5) can be divided in three periods: a pre-reproductive period (autumn-May) when the maturation of gametes occurs, a reproduction period, and a short post-reproductive period.
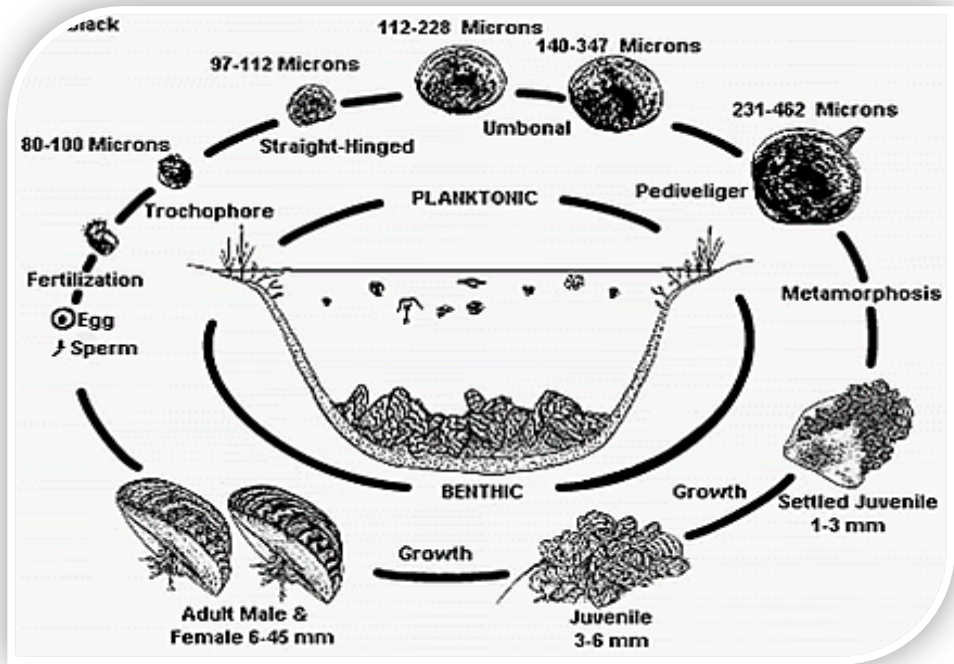
Figure 5: Zebra mussel (*Dreissena polymorpha*) life cycle

Temperature and trophic conditions are the two variables that mainly influence these biologic phases (Binelli et al., 2001). A temperature threshold of about 12-13 °C seems to synchronize the annual reproductive cycle of this mussel. Gonad growth and gametogenesis continue during wintertime and the release of the first ripe oocytes and sperm begins in spring at water temperatures above 12°C and continues until late summer (Sprung, 1987, 1989; Borcherding and De Ruyter van Staveninck, 1992). Zebra mussels are unusual among freshwater mollusks in that they have planktonic larval stages (trochophore, veliconcha, pediveliger) that more closely resemble marine than freshwater bivalves (Neumann and Jenner, 1992). The mean life of *D. polymorpha* ranges between 3 and 5 years, while the sexual maturity is reached when specimens are 2 years old.

Its ecological success is due mostly to its ability of attachment by byssus threads on hard substrates. This appears to be one of the principal factors affecting its spatial distribution. Abundant populations have been recorded on rocks, plants (reeds, flooded forests, and submerged aquatic plants), shells and valves of mollusks and crustaceans, also interfering with their movements (Lyakhnovich et al., 1994). The greatest abundances of *D. polymorpha* have also been observed on artificial substrata such as water pipes of power plants where they can reach a maximum abundance of 4,107,000 ind/m^2 (Protasov et al., 1983). Zebra mussel plays an important role in various freshwater ecosystems because it can be considered the link between the aqueous matrix and the organism belonging to higher trophic levels. In fact, it is a source of food for benthivorous fish, such as the roach (*Rutilus rutilus* and *Rutilus pigus*), and for diving ducks (Stanczykowska, 1977; Suter, 1982). Moreover, zebra mussels may also modify some trophic parameters such as chlorophyll, phosphorus and nitrogen concentrations and transparency (Binelli et al., 1997) by its filtration activity. This mussel possess all the characteristics of an appropriate bioindicator organism (wide geographical distribution, continuous availability throughout the year, adequate body size, ease of sampling, sessile, good tolerance to salinity, relatively high longevity in laboratory conditions), and for this reason, it has been commonly used in the biomonitoring of POPs (Binelli et al., 2001, 2004; Riva et al., 2007a, 2010), trace metals (Gundacker, 1999; Camusso et al., 2001) and radionuclides (Garnier-Laplace et al., 1998) in freshwater ecosystems.

1.4.2 Biomarkers applied on zebra mussel specimens

Since mussels have acquired a global importance as sentinel organisms in aquatic environments, in recent years they have also been used for the assessment of biological effects of pollution (Cajaraville et al., 2000; Livingstone et al., 2000; Oehlmann et al., 2002; Roméo et al., 2003; Smolders et al., 2003; Petrović et al., 2004). Although the application of different types of biomarkers has been intensively investigated in the genus *Mytilus*, there are few reports on its freshwater counterpart *D. polymorpha*. Nevertheless, thanks to the peculiar physiological characteristics, zebra mussel is always more used in laboratory researches aimed to the evaluation of the potential toxicity of different xenobiotics. An increasing number of biomarkers was adapted and applied to *Dreissena polymorpha* specimens to evaluate different notable endpoints. For instance, as reported by many authors (Riva et al., 2007b; Binelli et al., 2008, 2009a,b; Parolini et al., 2009, 2010; Faria et al., 2010), cyto- and genotoxicity assays are commonly used both in field and in laboratory researches in order to assess the hazard of single or mixtures of pollutants. In laboratory studies, the potential toxicity of a xenobiotic can be evaluated through a stepwise experimental design by using a multi-biomarker approach.

The first step of analysis consists in applying *in vitro* methods, which are highly recommended but rarely considered in ecotoxicology (Laville et al., 2004). A number of ethical, scientific and economic reasons support the efforts to develop and apply *in vitro* approaches in aquatic ecotoxicology. Firstly, the application of *in vitro* models reduces the use of test organisms, which aims to reduce the use of experimental animal research. Cells represent a key level of biological organization and *in vitro* methods can be used as useful rapid, low cost and reliable screening tools to evaluate the toxic effect of many chemicals with great precision and reproducibility (Olabarrieta et al., 2001). Moreover, they allow a further classification of the chemical toxicity potential and the comparison of the effects caused by pollutants to different species at the cellular level under equivalent conditions of toxicant exposure. Lastly, the major application of *in vitro* methods is the understanding of mechanisms involved in cellular and molecular responses to environmental pollutants (Gagnaire et al., 2004) to justify more intensive *in vivo* studies with whole organisms or mesocosms (Blaauboe, 2008; Gura, 2008) that mimes the natural environmental situation, providing a more general ecotoxicological screening (Ching et al., 2001). It faithfully

reproduces both the uptake pathways and the bioavailability in water of chemicals, and it allows to the organism to put in action all its defense mechanisms. Although these approaches can be successfully used separately, their complementarity can give a more complete and exhaustive report of the pollutant's toxicity (Hartmann et al., 2004).

1.4.3 *in vitro* biomarker battery

The Single Cell Gel Electrophoresis (SCGE) assay and the DNA diffusion assay were applied as biomarker of genetic damage on zebra mussel hemocytes exposed by an *in vitro* approach. In addition, cytotoxicity was evaluated by the Neutral Red Retention Assay (NRRA), which evaluating the degree of destabilization of the lysosome membranes highlights an eventual cellular stress induced by xenobiotic to the cell/organism (Lowe et al., 1995).

The **Single Cell Gel Electrophoresis (SCGE) assay**, also known as comet assay, is a rapid, reliable and sensitive method for evaluating DNA damage induced in individual cells by physical and chemical agents (Kim et al., 2002). It was first introduced by Östling and Johanson (1984) as an assay for detecting DNA double-strand breaks in irradiated mammalian cells under neutral conditions. Singh et al. (1988) and Olive et al. (1990) independently modified the assay by developing alkaline versions (pH 13 and pH 12.3, respectively). Since the introduction of the alkaline modification, the breadth of applications and the number of investigations using this assay have significantly increased. The alkaline comet assay is able to detect a wide variety of DNA damage such as DNA single-strand breaks, double-strand breaks, DNA-DNA/DNA-protein cross-links, oxidatively-induced base damages, alkali-labile sites, and sites undergoing DNA repair (Mitchelmore and Chipman, 1998a; Tice et al., 2000). It has also been employed to identify DNA degradation due to necrosis and apoptosis (Kizilian et al., 1999; Singh, 2000). The comet assay is recognized as one of the most sensitive methodologies available for DNA strand break detection (Collins et al., 1997) and is distinguished by being simple, fast, and effective, even for extremely small samples of cells, and applicable to cells from any organ of eukaryotic organisms (Mitchelmore and Chipman, 1998b). Briefly, this method measures the electrophoretic migration of relaxed or fragmented DNA away from the nuclei of cells immobilized in agarose gel. The DNA is stained with a fluorescent nucleic acid stain and

25

viewed using a fluorescent microscope. The distance and/or amount of DNA migration (Figure 6) from individual nuclei are indicative of the number of strand breaks. The microscopic determination of DNA migration can be evaluated by using either an ocular micrometer or by using image analysis software. By using this system, it is possible to measure the fluorescence intensity and distribution of DNA in and away from the nucleus (Singh, 1996).
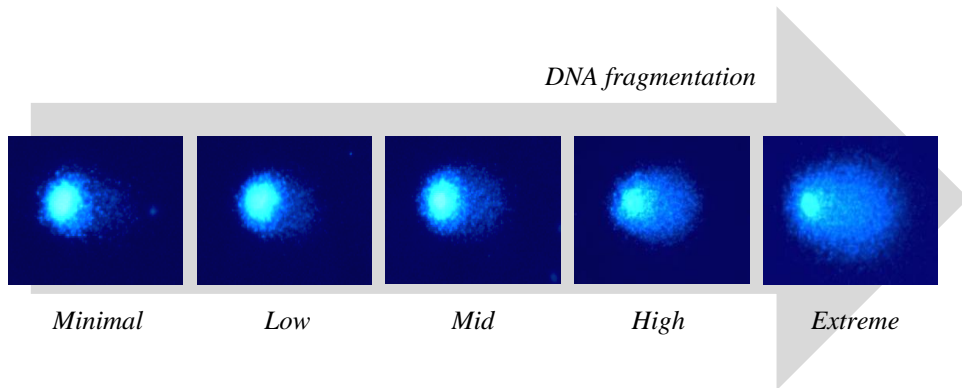

Figure 6: evolution of DNA fragmentation highlighted by the SCGE assay (according to DNA in tail as purposed by Mitchelmore et al., 1998)

Commonly-used parameters of DNA damage are the measures of relative fluorescence intensity of tail (commonly expressed as percentage of DNA in the tail), the tail length, the tail moment (essentially the product of tail length and tail intensity) and the ratio between the length and the diameter of the head of the comet (LDR, length/diameter ratio). Relative tail intensity is the most useful parameter, as it bears a linear relationship to break frequency, is relatively unaffected by threshold settings, and allows discrimination of damage over the widest possible range (from 0 to 100% DNA in tail; Collins, 2004). LDR ratio is a very immediate parameter, since cells exhibiting no damage have a ratio of approximately one (Fairbairn et al., 1995; Rojas et al., 1999). This assay has been widely used for studies in genetic toxicology, for clinical and radiation biology and DNA repair investigations. In recent years, there has been increasing interest regarding its useful application in environmental genotoxicity studies: it has been successfully applied in many aquatic organisms, including many species of fish and aquatic invertebrates, mainly mollusks and crustaceans (Cotelle and Férard, 1999). According to all these reasons, the

comet assay is now considered an early indicator for exposure to a wide variety of genotoxic agents and a sensitive endpoint for detecting DNA damage (Nacci et al., 1996), and therefore, it may be employed as a nonspecific biomarker for the actual genotoxic impact on cells and/or organisms. Several methods were developed to detect the onset of apoptotic cells due to external stressors, as listed by Singh (2000).

The **DNA diffusion assay** is a simple, sensitive, and rapid method for estimating apoptosis in single cells. It was developed by Singh (2000) on human lymphocytes exposed to X rays, and then it was applied in ecotoxicology to several kind of cells, both *in vitro* and *in vivo* (Gichner et al., 2005; Binelli et al., 2009b; Parolini et al., 2009, 2010). The assay follows the procedures developed for the SCGE assay with the exception that, after the cellular lysis, the electrophoretic run is not performed. It involves mixing cells with agarose and making a three-layers microgel on a microscopic slide, then lysing the embedded cells with salt and detergents. Low molecular-weight DNA fragments are allowed to diffuse in the agarose layers in all directions, then precipitated with ethanol and stained with a DNA-binding fluorescent dye (DAPI). The method may be also used to distinguish apoptosis from necrosis, since it was proposed that diffused nuclei with apoptotic and necrotic DNA fragmentation could be distinguished according to their structure from diffused nuclei with genotoxin-induced DNA damage. According to Singh (2000, 2004), apoptotic cells, when tested in the diffusion assay, show a circular gradient of granular DNA with a dense central zone and a lighter, hazy outer zone, giving the overall appearance of a halo due to nucleosome-sized DNA (Figure 7). By contrast, in diffused nuclei due to necrosis the DNA shows a clearly defined outer boundary and a relatively clear appearance.
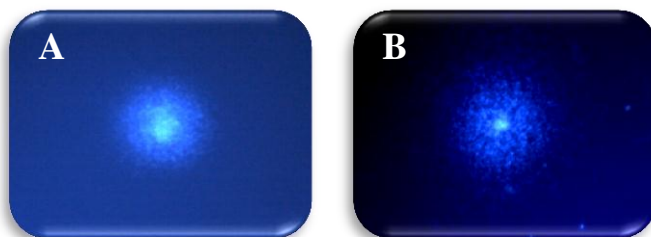

Figure 7: Apoptotic (**A**) and necrotic (**B**) zebra mussel hemocytes according to the guidelines suggested by Gichner et al. (2005)

This sharp outline in necrotic cells may be due to larger sized DNA fragments, which do not diffuse as much as the smaller fragments in apoptotic cells. Nuclei with genotoxin-damaged DNA (but not necrotic or apoptotic) are clearly defined and the nuclei are larger with projections of DNA all around.

The **Neutral Red Retention Assay (NRRA)** is a simple biomarker commonly used to evaluate the destabilization of the lysosome membranes, an indirect parameter of the onset of cellular stress. Lysosomes are membrane-bound organelles in eukaryotic cells. Lysosome enzymes (hydrolases) are capable of degrading biological material and are predominantly sequestered in an inactive form within a thick membrane in order to prevent free-access to cellular constituents. Many chemical, physical and environmental stressors, including pollutants, are known to destabilize lysosome membranes and membrane damage is often proportional to the magnitude of stress (Moore, 1985). Lysosomes are known to play a major role in the sequestration of organic contaminants, including PAHs (Nott et al., 1985). Although the ability to sequester contaminants from sensitive intracellular sites is an advantageous protective mechanism, it also renders the lysosome membrane particularly susceptible to elevated toxicant concentrations, leading to the efflux of hydrolytic enzymes and enhanced autophagy, with a resultant diseased state (Nicholson, 2003). As membrane labialization is generally quantitatively related to the degree of contaminant-induced stress (Moore, 1985) and responsive to a range of inorganic and organic toxicants, it is considered a useful non-specific marker of pollution. Lowe and Pipe (1994) developed an *in vitro* method that was originally set up for fish hepatocytes and subsequently further developed for blood cells of a range of invertebrate species living in water or soil (Weeks and Svendsen, 1996). This method, usually referred to as Neutral Red Retention Time (NRRT), is based on the fact that damaged lysosomes showing membrane destabilization are an indication of cellular critical health status. According to the *in vitro* methodology, the dye is sequestered in the lysosomal compartment when living cells are preloaded with Neutral Red (NR); if the lysosome membranes are damaged, NR leaks out into the cytosol where it can be visualized under the microscope (Figure 8).
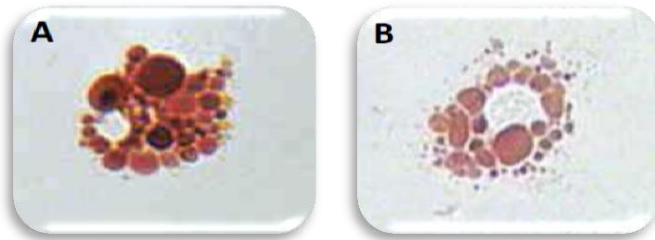
Figure 8: Healthy (**A**) and damaged (**B**) *Mytilus galloprovincialis* hemocytes according to the Neutral Red Retention Assay (from Martínez-Gómez et al., 2005)

The time taken by the dye to leak out into the cytosol is related to the degree of membrane damage. If lysosome membranes are severely damaged, the dye will leak out within few minutes of incubation, whereas healthy lysosomes retain the dye for up to 180 min (Lowe and Pipe, 1994). Reduced lysosome membrane stability has to be considered as an indicator of a general physiological stress, which may also be linked to the increase of oxidative stress.

1.4.4 *in vivo* biomarker battery

In vivo biomarker battery was constituted by the same assays applied in the *in vitro* approach and described above. In addition, it was enlarged by including another test to investigate the genotoxicity induced by xenobiotic, the Micronucleus test (MN test), and the analysis of variations in activity of three antioxidant enzymes, namely catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as that of the detoxifying phase II enzyme glutathione S-transferase (GST).

The **Micronucleus test** (MN test) was originally developed for use on bone marrow and peripheral blood erythrocytes (Heddle, 1973; Schmid, 1977) and then it became widely established in the field of mammalian genetic toxicology. Micronuclei appear when a whole chromosomes or a chromosome fragment fails to migrate with one of the two daughter nuclei formed during mitosis. The first case (chromosome loss) is due to an aneugenic event related to the spindle apparatus, while the second takes place after chromosome breakage. These displaced fragments or chromosomes enter the cytoplasm where they assume the morphology of small micronuclei at the following interphase.

The MN test has the advantage of being applicable to a wide range of different species (plants and animals) without any requirement for a detailed knowledge of the karyotype. The MN test has been widely used for evaluating the genotoxic potential of chemical and physical agents (Maffei et al., 2002; Chung et al., 2002; Ding et al., 2003), the biomonitoring of human populations occupationally exposed to mutagenic agents (Majer et al., 2001; Laffon et al., 2002; Bolognesi et al., 2004), in the search for carcinogenesis-inhibitory compounds (Izzotti et al., 2001; Roy et al., 2003), and it has been also recommended for monitoring in product development and regulatory tests of new drugs (OECD, 2004). The main difference between the comet and the MN assay is the measured endpoint since the comet assay detects DNA strand breakages that can be subsequently repaired by DNA repair systems, while the MN test measures unrepaired DNA lesions (Cotelle and Férard, 1999). In recent years, it has been a useful tool in ecotoxicological studies (Cotelle and Férard, 1999), both in laboratory research and *in situ* ones involving several invertebrate species (Baršiene and Baršyte Lovejoy, 2000; Dolcetti and Venier, 2002), amphibians (Zoll-Moreux and Ferrier, 1999) and fish (Baršiene et al., 2006). The MN test is simple to perform: the cell suspension is fixed on a slide and the nuclei are stained with an opportune stain. In recent years, the use of the fluorescent stain bisbenzimide 33258 (Hoechst) is generally recommended since the results are more sensitive for the quantitative and qualitative analyses of micronuclei. Nuclei (Figure 9) appear very shiny against a dark background, making even the smallest micronuclei visible. After staining, the slides are washed and mounted and they should be coded and blindly scored.
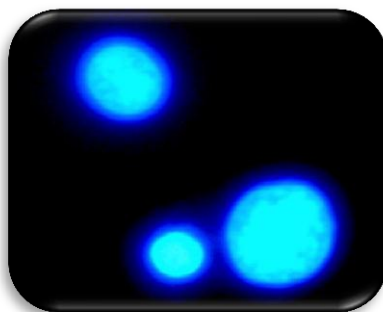

Figure 9: Undamaged (top) and micronucleated nucleus of zebra mussel hemocytes

On the slide, the scoring of micronucleated cell frequencies should be performed, under fluorescent microscope equipped with an immersion objective, at 100× magnification following some well-established criteria. Kirsh-Voelders et al. (2000) suggested that an intracytoplasmatic aggregate having a diameter ranging between 1/3 and 1/16 of that of the main nucleus, located in the same plane of focus, fully separated from the main nucleus and with similar patterns of chromatin should be qualify as a micronucleus (Figure 9). At least 2,000 cells per individual should be scored. However, many species show a very low frequency of spontaneous micronuclei. If weak genotoxicants are tested, the detection of micronucleated cells may be a very rare event, weakening the power of statistical analysis. In this case, a higher number of cells per animal should be scored. The use of an automated scoring device may help increase the number of cells analyzed (Ahmad et al., 2002). It should be remembered that the micronucleus test does not consist of the mere observation of the micronuclei frequencies. Instead, it consists of the study of the variations in these frequencies. At present, the MN test is considered the recommended test for DNA damage detection in ecotoxicological research.

Exposure to some xenobiotics, especially toxic chemical pollutants, may produce an imbalance between endogenous and exogenous ROS and subsequently a decrease of antioxidant defenses. This situation initiates oxidative stress in biological systems, damage to tissues, inflammation, degenerative diseases and aging (Sohal et al., 2002; Finkel and Holbrook, 2000). The interplay between ROS and antioxidant defenses in living aerobic organisms is connected with a series of intracellular antioxidant enzymes, whose roles are to intercept and inactivate reactive radicals. Also, extracellular low molecular-weight antioxidant molecules (such as ascorbate, uric acid, etc.) circulate in biological fluids scavenging free radicals and ROS (Davies, 1995). Living organisms have the ability to synthesize and control specific enzymatic systems which can be used for repair and removal of damaged proteins, lipids, and DNA (Fenech and Ferguson, 2001). Chemical toxic pollutants are important sources of ROS in biological systems. Oxidative stress and oxidative damage to fundamental biomolecules and to antioxidant defenses of organisms is an established field in environmental toxicology and ecotoxicology (Kelly et al., 1998; Regoli et al., 2002a, b). Several molecular biomarkers are used to assess oxidative damage in biomolecules and various aspects of oxidative stress by free radicals in experimental animals. In addition, biomarkers can monitor the status of various antioxidant defense

mechanisms against free radicals. The antioxidant defense system of living organisms can be subdivided into enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic antioxidants, such as glutathione, vitamin E, ascorbate, β-carotene, and urate (de Zwart et al., 1999). The knowledge that oxidative stress and chronic inflammation are related to applications for noninvasive biomarkers of oxidative stress in humans and new pharmacological strategies aimed at supplementing antioxidant defense systems against aging and diseases (Pryor and Godber, 1991). Since the discovery of the importance of free radical damage in the mechanisms of toxicity of many environmental pollutants (xenobiotics) there has been an increased application of biomarkers of oxidative stress in living organisms, especially mammalian systems (Kehrer, 1993; Nordberg and Arner, 2001), but also for plants exposed to air pollution (Scandalios, 1997). Molecular biomarkers of oxidative stress found widespread applications in mechanisms of environmental toxicity and ecotoxicity in aquatic organisms exposed to a variety of chemical pollutants (Livingstone, 2001).

In this study, the enzymatic activity of phase I antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST) were measured in the entire organism according to the observations made by Osman et al. (2007) and Osman and van Noort (2007). They noticed that CAT and GST activities in the whole soft tissue of zebra mussel were much higher than in a single organ. Thus, enzymatic activities were determined by using a spectrophotometer on the cytosolic fraction, as described by Orbea et al. (2002).

Chapter 2 – AIMS and
             RESULT INTERPRETATIONS

In this paragraph, the main results obtained during this three-year research will be reported through the step-by-step description of published articles.

Considering the environmental relevance and the actuality of the PPCP problem, this research was aimed to an in-depth evaluation of the possible adverse effects induced by some therapeutic drugs on a reference biological model, the freshwater bivalve *Dreissena polymorpha*. It is important to highlight the innovation of this study that is the first investigation of sub-lethal effects due to PPCP on freshwater invertebrates. We decided to investigate both the potential cyto-genotoxicity and alterations of the oxidative status of treated specimens induced by a common antimicrobial agent (triclosan; TCS), a widespread antibiotic (trimethoprim; TMP) and three largely used NSAIDs (paracetamol; PCM – diclofenac; DCF – ibuprofen; IBU). These drugs in fact, are largely produced, prescribed, used and diffused in each aquatic ecosystem, and can contribute to pose serious hazard to living biocoenosis.

Our goal was reached by applying a well-tested biomarker battery, according to a stepwise experimental design. The first step of analysis consisted in the application of a particular *in vitro* method that allows the further classification of the chemical toxicity potential and the investigation of the involved mechanism of action.

Exposure consisted of 1 h *in vitro* direct contact between mussel hemocytes and three increasing concentration of each drug, as reported in PAPER I and PAPER II.

PAPER I - Cytotoxic and genotoxic effects of *in vitro* exposures to triclosan and trimethoprim on zebra mussel (*D. polymorpha*) hemocytes

PAPER II - An *in vitro* biomarker approach for the evaluation of non-steroidal anti-inflammatory drugs (NSAIDs) ecotoxicity

Genotoxicity was evaluated by SCGE (Single Cell Gel Electrophoresis) assay and DNA diffusion assay, while the Neutral Red Retention Assay (NRRA) checked cytotoxicity. Each PPCP was able to interact with the treated cellular matrix, leading to significant increases of both genetic and cellular damage. It has allowed the estimation of the involved mechanism of action (MoA) of each drug on zebra mussel hemocytes. The possible rise of oxidative stress, suggested by the destabilization of lysosome membranes, could be considered the main responsible of DNA injuries to treated cells. A widening into this topic

is reported in attached papers. On the basis of obtained data we drawn the first toxicity scale of these drugs for zebra mussel hemocytes, according to which TCS was the most cyto-genotoxic compound, followed by PCM, DCF, IBU and TMP.

It is important to consider that these results were obtained exposing hemocytes to the highest drug concentrations (μM), much higher than those currently measured in the aquatic ecosystems. We decided to neglect the ecological relevance of the *in vitro* works because they were the first screening experiments aimed to the evaluation of the potential toxicity of selected PPCPs on zebra mussel cells and we wanted to ascertain if biomarkers could be applied on *in vitro* bivalve cells. So, according to these considerations, obtained data are only partially usable for a clear environmental risk assessment of PPCPs, because they do not reflect the real situation to which organisms are subjected in ecosystems, but can only furnish a rough idea on the hazard of these chemicals to non-target organisms. Notwithstanding, they surely justify the application of more intensive *in vivo* studies with whole organisms or mesocosms in order to widen the knowledge on this topic.

In our experimental design, *in vitro* approach is essential and absolutely preparatory for the second step, which allows the evaluation of the real effect of pollutants by using *in vivo* assays. By exposing mussels *in vivo*, we can faithfully reproduce both the uptake pathways and the bioavailability of chemicals, allowing to the organism to put in action all its defense mechanisms and metabolism. In order to give a marked ecological relevance to our data and to furnish useful information for the current environmental risk assessment for the selected compounds, we carried out a careful choice of tested concentrations. We decided to test the possible real PPCP toxicity on treated-mussels by exposing them to three different increasing concentrations of each drug, perfectly comparable to those currently revealed in the aquatic ecosystems worldwide.

On the basis of our suggested *in vitro* PPCP scale of toxicity, TCS was the first drug tested by an *in vivo* approach.

PAPER III - *In vivo* experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussel hemocytes

This work confirmed the previous *in vitro* findings, highlighting an extreme toxicity of this antimicrobial agent not only on zebra mussel hemocytes, but also on the whole organism. TCS environmentally relevant concentrations were able to induce a marked increase of

primary DNA damage, as pointed out by all applied SCGE assay endpoints, with values similar to those obtained at the end of a previous exposure to the well-known genotoxic agent benzo(α)pyrene (B(α)P; Binelli et al., 2008). In addition, a significant and drastic increase of apoptotic and micronucleated cells was noticed at each tested concentration. Our data revealed that exposure to 3 nM TCS induced an increase of MN frequency doubled with respect to those revealed in B(α)P experiment at higher treatments. Considering that B(α)P is an assured carcinogenic compound (US EPA, 2005), on the basis of our results TCS can be considered an extreme hazard for zebra mussel and aquatic biocoenosis, being able to enhance the rise of irreversible and dangerous DNA damage. On the basis of obtained data we also supposed a possible TCS MoA, according to with genotoxicity could be induced both by direct interaction DNA-molecule and by the unbalance of oxidative status due to production of reactive oxygen species (ROS).

In order to confirm the latter hypothesis, we decided to evaluate activity alterations of three antioxidant enzymes (superoxide dismutase-SOD; catalase-CAT; glutathione peroxidase-GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST).

PAPER IV - Antioxidant activity in the zebra mussel (*Dreissena polymorpha*) in response to Triclosan exposure

Environmentally relevant TCS concentrations were able to induce a moderate unbalance of treated-specimens oxidative status, as showed by the significant increases of SOD, CAT and GST activity. However, these findings seem to suggest that the rise of genetic damage highlighted in PAPER III was mainly due to the direct interaction between TCS and DNA, with a moderate participation of oxidative stress.

Considering the widespread diffusion of antibiotics in aquatic environment, the second tested drug was trimethoprim.

PAPER V - A multi-biomarker assessment of the impact of the antibacterial trimethoprim on the non-target organism Zebra mussel (*Dreissena polymorpha*)

Notwithstanding this molecule revealed the lower *in vitro* cyto-genotoxicity on zebra mussel hemocytes among the tested drugs, it showed a moderate ability to increase both genetic and cellular damage. A slight increase in DNA damage was registered by apoptosis induction and MN frequency, while significant differences in lysosome membrane stability

from baseline levels were measured at the end of exposures to highest concentrations only. Finally, TMP seemed to have a very low capability to unbalance the oxidative status of treated specimens, although a clear induction of GST activity was noticed. This work highlighted that the only application of *in vitro* method could lead to a wrong evaluation on the hazard of a xenobiotic, suggesting that a tied *in vitro/in vivo* approach should be preferable in risk assessment because it gives more complete and exhaustive report of the pollutant toxicity (Hartmann et al., 2004).

Following our investigation, we tested the potential cyto-genotoxicity of three PPCPs among the most marketed non-steroidal anti-inflammatory drugs (NSAIDs).

PAPER VI - Multi-biomarker approach for the evaluation of cyto-genotoxicity of paracetamol on zebra mussel (*Dreissena polymorpha*)

Environmentally relevant paracetamol (PCM) concentrations did not induce any significant increase of DNA fragmentation, but they were able to trigger the apoptotic pathway and to enhance the formation of micronucleated cells. In addition, the destabilization of lysosome membrane, as well as the unbalance of oxidative status highlighted by alterations in activity of enzymatic system, seemed to suggest that the increase of oxidative stress could be considered the main responsible of measured genetic damage.

PAPER VII - Assessment of the potential cyto–genotoxicity of the nonsteroidal anti-inflammatory drug (NSAID) diclofenac on the zebra mussel (*Dreissena polymorpha*)

This NSAID is commonly considered extremely dangerous for the biocoenosis, since many studies revealed both its acute (Cleuvers, 2003; Ferrari et al., 2003) and chronic toxicity (Triebskorn et al., 2004; Hong et al., 2007) on several different biological models. In addition, it is important to point out that at present DCF is the unique pharmaceutical compound that has produced drastic consequences at population level. It was marked as the responsible of the drastic population decline of three Indian vulture species, namely Indian White-rumped Vulture (*Gyps bengalensis*), Indian Vulture (*Gyps indicus*) and Slender-billed Vulture (*Gyps tenuirostris*), as reported by Oaks et al. (2004). Nonetheless this evidences, strengthened by our *in vitro* data revealing its noteworthy cyto-genotoxicity on zebra mussel hemocytes, environmentally relevant DCF concentrations do not represent a

serious hazard for *D. polymorpha* specimens, since they were not able to induce neither significant cyto-genotoxic effect, nor alterations to the oxidative status of exposed bivalves. In order to conclude the screening investigation of possible NSAIDs environmental toxicity, we have already performed an experiment by exposing zebra mussels to increasing ibuprofen (IBU) concentrations. Obtained data, that are not included in this report, revealed a moderate toxicity of this molecule on treated bivalves, indicating a not negligible IBU hazard on aquatic biocoenosis, differently to that assumed by previous studies (Pascoe et al., 2003; Cleuvers, 2004; Han et al., 2006; Heckmann et al., 2005, 2007; Pounds et al., 2008). Our data suggest that environmentally relevant concentration of different therapeutic drugs could pose low to extreme hazard toward zebra mussel specimens and probably, as consequence, on the aquatic biocoenosis. However, we must not forget that organisms living in aquatic ecosystems are commonly exposed to complex mixtures of pollutants, including PPCPs, which can reveal particular interactions and often induce unexpected effects.

On the basis of this consideration, the third and last step of our experimental design was aimed to a fundamental, but often neglected, ecotoxicological issue, namely the evaluation of potential toxicity of a pollutant mixture. In order to conclude the investigation on NSAIDs ecotoxicity, we decided to expose, by an *in vivo* approach, several zebra mussel specimens to three different mixtures of PCM, DCF and IBU. Usually, the first step of analysis on the toxicity of mixture consists in experiments aimed to the evaluation of its acute toxicity, above all to estimate the toxicological behavior of the chemicals that compound it. Considering that at present neither acute test nor data on the acute toxicity of any PPCP (or mixture) on *D. polymorpha* are available, we decided to carry out a first qualitative evaluation of possible sub-lethal effects induced by the NSAID mixture mentioned above. As previously highlighted, the concentration of each drug in mixture was perfectly comparable to that currently revealed in aquatic ecosystems, in order to give an environmental relevance to our investigation. The lowest concentration reflected the mean level of each NSAID in surface water, while the middle one took into account the mean concentration measured in the effluents of waste water treatment plants worldwide (Kasprzyk-Hordern et al., 2008; Santos et al., 2010). At last, the highest one considered the predicted environmental concentration (PEC) values calculated by many authors for European surface waters (Stuer-Lauridsen et al., 2000; EMEA, 2003; Zuccato et al., 2006;

Carballa et al., 2008; Grung et al., 2008), in order to evaluate the potential toxicity of this mixture in an eventual worst environmental situation. Results are now under preparation, but preliminary data revealed noteworthy sub-lethal effects induced by each administered mixture. In-depth analyses are absolutely necessary in order to complete this first screening evaluation and to explain the toxicological behavior of this mixture in affecting zebra mussel health status.

Simultaneously with these experiments, considering the relevance of *in vitro* methods in screening toxicity evaluation of xenobiotic, primary cultures of zebra mussel hemocytes, gill and digestive gland cells were developed. After the refining of the cell culture procedures, they were applied to assess cytotoxicity of some pharmaceutical compounds.

PAPER VIII – Cytotoxic assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures

Exposure to increasing single drug concentrations (0.001; 0.01; 0.1; 1 and 10 mg/L) of atenolol (ATL), carbamazepine (CBZ), diclofenac (DCF) and gemfibrozil (GEM) showed a significant decrease in cell viability due to CBZ, DCF and GEM exposures according to the Trypan blue exclusion test, while the mitochondrial MTT (3-(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay highlighted a slight to moderate reduction of mitochondrial activity of gill and digestive gland cells. Overall, DCF was the more cytotoxic compound for zebra mussel cells, followed by GEM, CBZ and ATL. Gill cells and hemocytes showed to be the more sensitive targets. Our results lay the groundwork for chronic and *in vivo* toxicity evaluations, which will allow a better definition of the observed toxicity of these molecules in a setting mimicking real environmental exposure. The submitted paper can be consulted within the enclosed CD-ROM.

Chapter 3 – PUBLICATIONS

PAPER I

Binelli Andrea, Cogni Daniele, **Parolini Marco**, Riva Consuelo, Provini Alfredo

Cytotoxic and genotoxic effects of in vitro exposure to Triclosan and Trimethoprim on zebra mussel (*Dreissena polymorpha*) hemocytes

Cytotoxic and genotoxic effects of *in vitro* exposure to Triclosan and Trimethoprim on zebra mussel (*Dreissena polymorpha*) hemocytes

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ABSTRACT

Pharmaceuticals and personal care products (PPCPs) have been detected in several aquatic ecosystems for a number of years, but the potential for biological effects in exposed non-target organisms is only now being reported. In this study the potential cellular damage due to two of the main PPCPs found in aquatic environments was investigated by *in vitro* exposures. Hemolymph samples of the freshwater bivalve *Dreissena polymorpha* were collected and treated with increasing concentrations of the antibacterial agent Triclosan (TCS) and the antibiotic Trimethoprim (TMP). Doses selected for TCS were 0.1, 0.15, 0.2, and 0.3 μM, while 0.2, 1, and 5 μM for TMP exposures, respectively. We evaluated the potential genotoxicity on hemocytes by the SCGE (single cell gel electrophoresis) assay and apoptosis frequency evaluation, while the cytotoxicity was measured by the lysosomal membranes stability test (NRRA, neutral red retention assay). TCS genotoxicity increased in a dose-dependent manner and this pharmaceutical significantly affects hemocyte functionality due to severe DNA injuries at very low doses. In contrast, TMP seems to be less dangerous than TCS for *D. polymorpha* because the cytotoxic and the moderate genotoxic effects noticed were obtained only at very high concentration levels.

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1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a class of emerging environmental pollutants that, upon introduction into aquatic ecosystems, may act on molecules, cells and organs in different organisms through unexpected modes of action (Fent et al., 2006). Several pharmaceuticals widely used in human medicine are excreted unchanged in high quantities and are continuously discharged into aquatic environments (Kummerer, 2004). Notwithstanding that pharmaceuticals are designed to target humans, aquatic organisms exhibiting the same enzyme receptors could also experience those pharmaco-dynamic effects. Moreover, secondary effects not considered important in humans may have major implications for non-mammalian aquatic organisms (Seiler, 2002).

Studies carried out in several Western countries have found more than 80 compounds in water bodies, including analgesics, antibiotics, antiepileptic, β-blockers and lipid regulators (Heberer, 2002). Two of the most used PPCPs worldwide are Triclosan (TCS, 2,4,4′-trichloro-2′-hydroxydiphenyl ether) and Trimethoprim (TMP, 5-[3,4,5-trimethoxy-benzyl]pyrimidine-2,4-diamine) (Fig. 1), whose increasing environmental levels are causing growing concern about their presence in the ecosystems.

TCS is registered as an antibacterial agent, bactericide, disinfectant, and fungicide, and its use began in the 1970s. Use of TCS has risen dramatically in the past few years because it is now present in common products, such as cosmetics, soaps, shampoos, cleaning materials, food packaging materials, oral healthcare products, plastics, shoes and textiles (Jones et al., 2000). TCS is now found throughout the environment, including surface waters, soil and fish tissue as well as in sewage treatment plant influents and effluents (Singer et al., 2002). Although TCS is biodegradable, it has been detected in rivers and lakes at ng/L levels (Balmer et al., 2004). TCS concentrations ranged between 18 and 98 ng/L in Swiss freshwaters (Singer et al., 2002) and between 30 and 90 ng/L in Germany (Wind et al., 2004). In a 1999–2000 study by the U.S. Geological Survey, TCS was found in 57% of the 139 U.S. waterways thought to be susceptible to agriculture or urban activities (Kolpin et al., 2002). Adolfsson-Erici et al. (2000) found three out of five samples of human breast milk contained measurable concentrations of TCS at concentrations up to 30 μg/kg lipid mass. While our current understanding of TCS-induced environmental effects is limited, there is evidence that TCS is acutely and chronically toxic to aquatic organisms (Orvos et al., 2002; Tatarazako et al., 2004). Wilson et al. (2003) have shown that the presence of TCS may influence both the structure and the function of algal communities in stream ecosystems that receive treated wastewater effluent. Recently, Coogan et al. (2007) presented the first report on the algal bioaccumulation potential of TCS. They measured a mean TCS concentration between 100 and 150 ng/g fresh mass in the filamentous
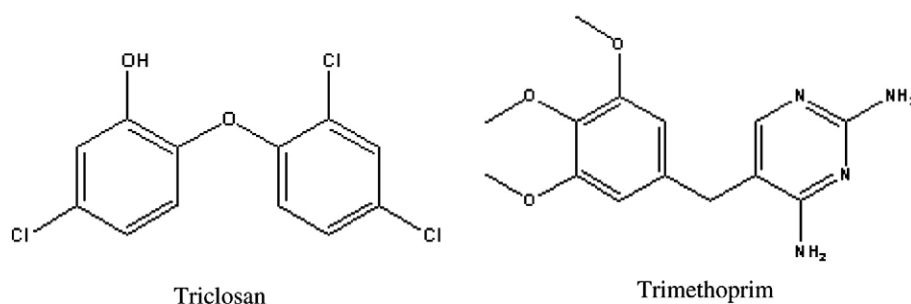
Fig. 1. Structural formulae of Triclosan (A) and Trimethoprim (B).

algae *Cladophora* spp. and a bioaccumulation factor (BAF) in the range of approximately three orders of magnitude.

The TCS antibacterial activity is due to specific inhibition of fatty acid synthesis both in wildlife (Heath et al., 1999) and humans (Liu et al., 2002). Moreover, there is *in vitro* and *in vivo* evidence that TCS can also act in eukaryotic cells through several mechanisms (Canesi et al., 2007).

TMP is a member of a family of synthetic 2,4-diaminopyrimidines with potent microbicidal activity against a wide variety of bacterial species. This antibiotic has been used to successfully treat bacterial infections, particularly common urinary tract infections. TMP interferes with the enzyme dihydrofolate reductase to inhibit the synthesis of tetrahydrofolate, an essential precursor in the synthesis of thymidine. Dihydrofolate reductase inhibition ultimately affects DNA, RNA, and protein synthesis, resulting in stasis or cell death (Baccanari, 1995). Several antibiotics have been discovered in effluents discharging in surface waters (Gagnè et al., 2006), and Clara et al. (2004) recently found 70 ng/L of TMP in several Austrian municipal wastewaters. The literature about the potential genotoxicity of TMP is controversial because positive and negative effects have been reported in the same biological models in both bacterial and mammalian cells (Rasool et al., 1987; Abou-Eisha, 2006).

The aim of this study was to assess the cytotoxicity and genotoxicity of TCS and TMP in short-term *in vitro* experiments carried out with hemocytes from the freshwater bivalve *Dreissena polymorpha*. *In vitro* studies often provide basic information on the nature of tested agents and/or the cellular response because of the relative simplicity of the tested system when compared to complex animal models. These *in vitro* studies should be viewed as a preliminary screening that is part of a structured, tiered approach that will include *in vivo* assays and proteomic approaches to a better understanding of the toxicology of the tested compounds. On the other hand, the working group on genetic toxicology testing from the 4th International workshop on genotoxicity test procedures (IWGT) suggested the use of *in vitro* experiments as initial battery of tests on the strategy for genotoxicity testing to identify chemicals that require follow-up testing (Kirkland et al., 2007).

Although TCS and TMP belong to different PPCP classes, they were chosen for their extensive use worldwide and because they are common representative compounds of their pharmaceutical groups. Moreover, there has been an increasing attention about these two chemicals, shown by the rise of research carried out on their ecotoxicology and mechanisms of toxic action.

Genotoxicity was evaluated by the single cell gel electrophoresis (SCGE) assay and apoptosis frequency evaluation, while the cytotoxic effects were measured by the lysosomal membranes stability test (NRRA or the neutral red retention assay), a classical parameter of cellular stress in bivalves (Lowe et al., 1995).

The selection of *D. polymorpha* as a biological model is mainly due to the fact that bivalves are important members of aquatic ecosystems and are particularly susceptible to environmental stressors. Notwithstanding that marine and freshwater mussels are considered as suitable sentinel-organisms for the entire aquatic biocoenosis, the effects of PPCPs on sessile invertebrates, such as mussels, remain largely unknown at the present time (Gagnè et al., 2006), while many studies have been carried out by mammals and human cell lines. To the best of our knowledge, our study yielded the first data on the genotoxicity and cytotoxicity of PPCPs in this sentinel-organism and one of the first papers for an invertebrate biological model.

2. Materials and methods

TCS and TMP standards were obtained from Sigma-Aldrich (Steinheim, Germany), as well as all other chemicals used for biomarker determination. Dimethylsulfoxide (DMSO) was purchased from VWR International (Milan, Italy).

2.1. Mussel acclimation and maintenance conditions

Several hundred specimens of *D. polymorpha* (Mollusca, Bivalvia) were sampled in Lake Maggiore (Northern Italy) by a scuba diver. Sampling was carried out at 4–5 m of depth, taking care not to remove the mussels from the rocks upon which they were tied to by their byssus. Bivalves were transferred to the laboratory in bags filled with lake water. The rocks were rinsed and introduced into several glass aquaria filled with about 100 L of dechlorinated tap water and maintained at natural photoperiod, constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90% of saturation). Animals were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA) and water was changed every day for at least 1 week to purify the molluscs from the accumulated xenobiotics.

Several specimens with similar shell lengths (about 20 mm) were selected for each *in vitro* test, including control and solvent assays. Mussels were gently cut off from rocks, placed on glass sheets suspended in small glass aquaria (15 L) and maintained for 1 week under the same conditions described above. Only specimens able to re-attach themselves by their byssi on glass sheets were used in the experiments. Mussels were used for the subsequent *in vitro* analyses only when target biomarker assays showed values comparable with controls previously checked.

2.2. Hemocyte preparation and chemical dosing

Two different methods were used to handle hemocytes. For SCGE and apoptosis assays, hemolymph (100 µL) from ten individual mussels was withdrawn from the sinus near the posterior adductor muscle using a hypodermic syringe that contained 200 µL of phosphate-buffered saline (PBS; pH = 7.4). Unlike marine bivalves, no other medium was needed for maintenance of hemocyte suspension. The sample was then held on ice to prevent endogenous damage from occurring during sample preparation and inhibit DNA repair in the unfixed cells. The final hemocyte density was at least 10^4 cells/mL.

The ten cell suspensions were mixed together in a microtube and briefly vortexed. An aliquot of this sample was then added to TCS or TMP (dissolved in DMSO) to obtain a final volume of 1 mL. We tested 0.1, 0.15, 0.2, and 0.3 µM of TCS and 0.2, 1, and 5 µM or TMP, according

to the viability test results. Samples were briefly vortexed and maintained for 60 min in the dark in a temperate room at 20 °C; microtubes were constantly mixed by a horizontally pivoted stirrer. After the exposure, samples were centrifuged at 2000 rpm ($\approx 300 \times g$) at 4 °C for 5 min to stop the effect of contaminants. The supernatant was discharged, and the pellet was re-suspended with 50 μL of PBS.

Only two changes were made to prepare hemocytes for the NRRT assay: centrifugation was carried out at 1500 rpm ($\approx 180 \times g$) to preserve the cellular matrix from mechanical injuries, and the pellet was re-suspended with 300 μL of PBS/EDTA (ethylene-diamine-tetra-acetic acid; 1:1).

2.3. Viability tests and solvent controls

Hemocyte viability was checked by the Trypan blue exclusion method (10 μL of cell suspension was added to 10 μL of 0.4% dye in water) using a Burker chamber and an optical microscope for counting. A pool of five mussels was tested twice for each assay. Solvent controls were carried out with DMSO: several assays were performed with DMSO concentration ranging from 80 ng/L to 1280 ng/L to check the eventual genotoxicity potential of this carrier, always obtaining a cell viability higher than 90%.

2.4. SCGE assay

SCGE assay was performed according to the alkaline (pH > 13) version of the assay developed by Singh et al. (1988), with the subsequent optimization for zebra mussels detailed by Buschini et al. (2003).

Ten 10 μL aliquots of cell suspension mixed with 85 μL of Low Melting Agarose (LMA−0.7%) in PBS (37 °C) were added to ten coated slides (previously dipped in 1% Normal Melting Agarose). Slides were covered by a cover-glass and placed at 4 °C for 40 min until the agarose layer hardened. A third agarose layer was added to the slides in the same way. After agarose solidification, slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH = 10) in a Coplin jar at 4 °C in the dark for at least 1 h.

Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber containing freshly prepared buffer (1 mM Na$_2$EDTA, 300 mM NaOH, pH = 13) and then in an ice–water bath (4 °C). Electrophoresis was then performed at 0.78 V/cm and 300 mA for 10 min. Slides were washed after electrophoresis in a neutralization buffer (0.4 M Tris–HCl, pH = 7.5) and fixed in absolute ethanol. After staining with DAPI (4′,6-diamidino-2-phenylindole) DNA dye (Sigma-Aldrich), a coverslip was placed over the slides. Observations were done under a fluorescence microscope (Leitz DMR, Germany) equipped with an FTC filter. All steps were performed in the dark to minimize additional UV-induced DNA damage. Positive controls were carried out with H$_2$O$_2$ to check the effectiveness of the electrophoresis conditions. All samples were blindly coded and evaluated.

Fifty cells per slide were analyzed using an image analysis system (Comet Score®), for a total of 500 analyzed cells per sample. The ratio between migration length and diameter of the comet head (LDR) was chosen to represent DNA damage data (Bolognesi et al., 2004). This was coupled with the percentage of tail DNA obtained by the Comet Score® and the following DNA damage classes proposed by Mitchelmore et al. (1998): zero or minimal <10%, low damage 10–25%, mid damage 25–50%, high damage 50–75%, and extreme damage >75%. The working group on genetic toxicology testing from the 4th IWGT recently agreed that the percentage of tail DNA is the measure that seems most linearly related to dose and the easiest to understand (Kirkland et al., 2007).

2.5. Apoptosis

The percentage of apoptotic cells was evaluated through the same protocol used for the SCGE assay, as described by Singh (2000). This method is based on the fact that apoptotic cells have numerous alkali–

labile sites (MacManus et al., 2000), which should yield pieces of DNA under exposure to alkaline conditions. These DNA fragments can easily diffuse in the agarose matrix, giving the appearance of a halo with a hazy outline (Singh, 2000).

Briefly, each hemocyte pool was distributed between five different slides processed with the same method described for the SCGE assay. After the hemocytes were lysed, slides were subjected to 5 min of alkaline (pH = 13) DNA unwinding without subsequent electrophoresis. Slides were then washed in neutralization buffer, stained, and fixed in absolute ethanol. Finally, they were labelled with DAPI and observed under fluorescence. Two hundred cells per slide were analyzed for a total of 1000 cells per sample. According to the intense autolysis of genomic DNA, apoptotic cells are characterized by nuclear remnants that resemble pinheads and surrounded by very large DNA halos. Alternatively, necrotic cells are characterized by circular, faint halos and were eliminated from the count.

2.6. Neutral red retention assay

The NRRA method followed the protocol proposed by Lowe and Pipe (1994), whose rationale was that healthy cells could take up and retain larger quantities of the dye (neutral red) than damaged cells.

The neutral red stock solution was made by dissolving 20 mg of dye in 1 mL of DMSO, while the working solution was prepared by diluting 5 μL of stock solution in 2.5 mL of PBS. Microscope slides were coated with 2 μL of polylysine (0.1% in water) with the help of a coverslip. Five slides were used for each sample.

An 80 μL aliquot of hemocyte resuspension was placed carefully on each slide with a hypodermic syringe without the needle to avoid possible cell damage. Slides were suspended on a rack in a light-proof humidity chamber for 20 min, and excess solution was carefully tipped off. Lastly, 40 μL of neutral red working solution was added.

After a 20-min incubation in a humidity chamber, slides were observed under an optical microscope. Slides were examined systematically thereafter at 30 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests were terminated when dye loss was evident in at least 50% of the hemocytes. The mean retention time was then calculated from the five replicates. Between every microscope observation, slides were placed in the humidity chamber.

2.7. Statistical analysis

Data normality and variance homogeneity were verified using the Kolmogorov–Smirnov and Barlett's tests, respectively. We used log-transformed LDR values to normalize the variance.

One-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* test were performed to evaluate eventual significant differences ($p < 0.05$) between controls and treated samples. A Bonferroni *post-hoc* test ($p < 0.05$) was also carried out to check for significant differences among the biomarker end-points measured at the administered doses. All statistical analyses were performed using the software package STATISTICA 6.0.

3. Results

3.1. Hemocyte maintenance conditions

Because hemocytes have a short lifespan in *in vitro* experiments, hemolymph maintained in PBS (1:2) was previously checked at different times (from $t = 0$ min to $t = 180$ min) to control the viability decrease of untreated hemocytes, to select the suitable time of exposure for the following assays. Cell viability was $91 \pm 5\%$ at $t = 0$, while a decrease below than 80% was observed starting from $t = 90$ min. Thus, we opted to perform the exposure assays at $t = 60$ min that guaranteed a cell viability of $83 \pm 3\%$. This is much
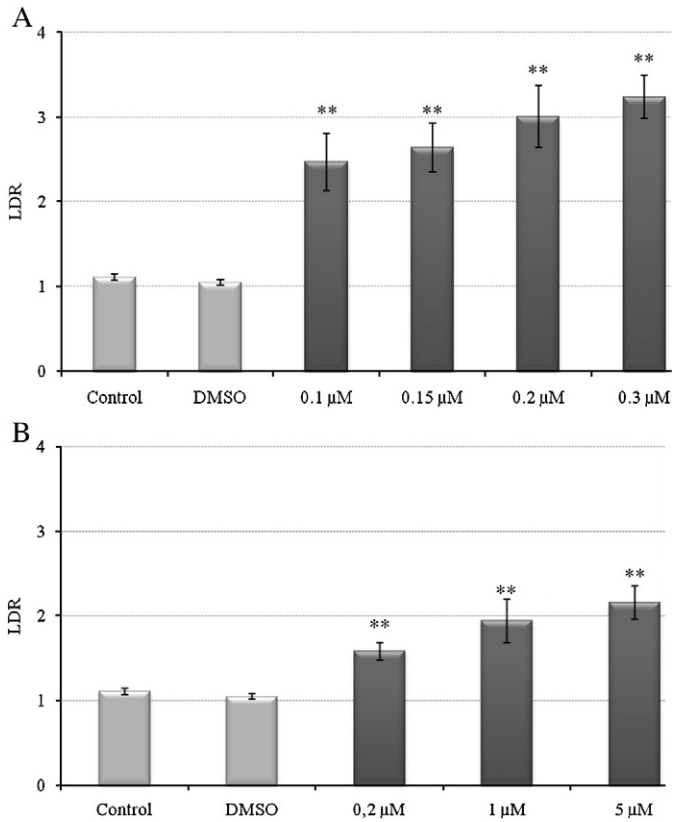
Fig. 2. Length/diameter ratios (LDRs) measured for untreated controls, solvent controls (DMSO = dimethylsulfoxide) and treated samples ($n = 10$). A: Triclosan concentrations; B: Trimethoprim concentrations. Significant values (**$p < 0.01$) refer to the difference between exposed samples and untreated controls. Bars show standard deviation.

higher than the threshold (>70%) for SCGE assay proposed by the expert panel of the IWGTP (Tice et al., 2000).

3.2. SCGE assay results

Baseline levels of length/diameter ratios resulted approximately near one (LDR = 1.1 ± 0.04), as expected for cells not affected by DNA injuries (Fig. 2A, B). Furthermore, no significant differences ($p > 0.05$) were observed between the untreated groups and solvent controls (DMSO).

TCS induced significant ($p < 0.01$) primary DNA damage in the zebra mussel hemocytes at all tested concentrations (Fig. 2A). Very high DNA damage (LDR = 2.5 ± 0.3) was already observed at the lowest tested concentration (0.1 µM), while the highest dose (0.3 µM; LDR = 3.2 ± 0.3) produced a LDR value about 50% higher than the lowest tested dose.

The dose/effect relationship was confirmed by the second end-point used to define primary DNA damage (Table 1); the percentage of hemocytes that fell into the high and extreme DNA damage classes

increased from about 20% at 0.1 µM of TCS to 43% at 0.3 µM, while the majority of hemocyte percentage of control and solvent assays fell into the minimal and low damage classes.

All concentrations tested with TMP exhibited a significant ($p < 0.01$) increase of LDRs up to the highest dose (5 µM), which doubled the baseline levels (Fig. 2B). Although the concentration interval (0.2–5 µM) was much wider than for TCS (0.1–0.3 µM), the difference of DNA injuries due to TMP between the extreme administered doses was about 50%, comparable with that calculated for TCS. Notwithstanding a slight increase of the damaged hemocyte for the two highest classes (Table 1), a greater percentage of cells exposed to TMP fell into minimal and low damage classes (66–76%).

3.3. Apoptosis

Very low baseline levels (3.2%) of apoptotic hemocytes were obtained, and these data are comparable with the data available for this biomarker in *D. polymorpha* (5%; Rocher et al., 2006). No statistical differences were noticed between controls and DMSO-treated hemocytes, while the selected period of exposure to chemicals (60 min) was sufficient to observe DNA fragments generated by apoptosis (Fig. 3A, B).

A concentration of 0.1 µM of TCS was already sufficient to obtain significant apoptotic cells in comparison with baseline values (Fig. 3A). Thus, a threshold over which this chemical begins to induce a significant percentage of apoptosis should be found below this concentration, which corresponds to 30 µg/L of TCS. In addition, we noticed a clear dose/effect relationship, with a linear increase of apoptosis at 0.3 µM of TCS (14.3%) that was approximately five times higher than controls.

Although TMP showed significant differences ($p < 0.01$) from controls, the effect of this chemical was much lower than that of TCS; the highest administered concentration (5 µM) caused only about half of the apoptosis percentage of TCS at 0.3 µM (Fig. 3B). The concentration/effect relationship was also evident for TMP, with an increase up to 75% between the lowest and highest doses.

3.4. Lysosomal membrane stability

Since comparison with other studies carried out with this biomarker in *D. polymorpha* is impossible because of the lack of available data, the baseline level of the dye release can be compared with studies carried out with the genus *Mytilus*, the marine experimental counterpart of the zebra mussel. Although the most frequent NRRT found in several studies was as high as 120 or 180 min in *Mytilus* spp. (Lowe et al., 1995; Fernley et al., 2000; Mamaca et al., 2005), lower NRRT (below 50 min) was observed in a reference site from Greece (Dailianis et al., 2003). The baseline NRRT level (84 ± 13.4 min) measured in zebra mussel hemocytes seems to be much lower than that observed in *Mytilus* sp. The lower retention time measured in zebra mussel can be simply due to inter-specific differences or to a particular characteristic of the collected mussel population, as stated by Dailianis et al. (2003).

Table 1
Percentage of hemocytes in different DNA damage classes (calculated on the basis of DNA percentage in the comet tail) measured in zebra mussels ($n = 10$) exposed to Triclosan and Trimethoprim.

Damage classes	Control	DMSO	Triclosan				Trimethoprim		
			0.1 µM	0.15 µM	0.2 µM	0.3 µM	0.2 µM	1 µM	5 µM
Min	72.2	94.4	24.4	18.8	14.4	9.0	40.8	35.4	30.4
Low	25.4	5.6	23.7	16.6	24.0	10.3	35.4	37.2	35.4
Mid	2.4	0	31.0	34.6	38.8	37.5	20.8	21.8	23
High	0	0	17.3	24.8	18.2	29.8	2.8	4.6	9.4
Extreme	0	0	3.6	5.2	4.6	13.5	0.2	1	1.4

Min = minimal damage < 10% of DNA in the comet tail, low = low damage 10–25%, mid = mid damage 25–50%, high = high damage 50–75% and extreme = extreme damage >75%.
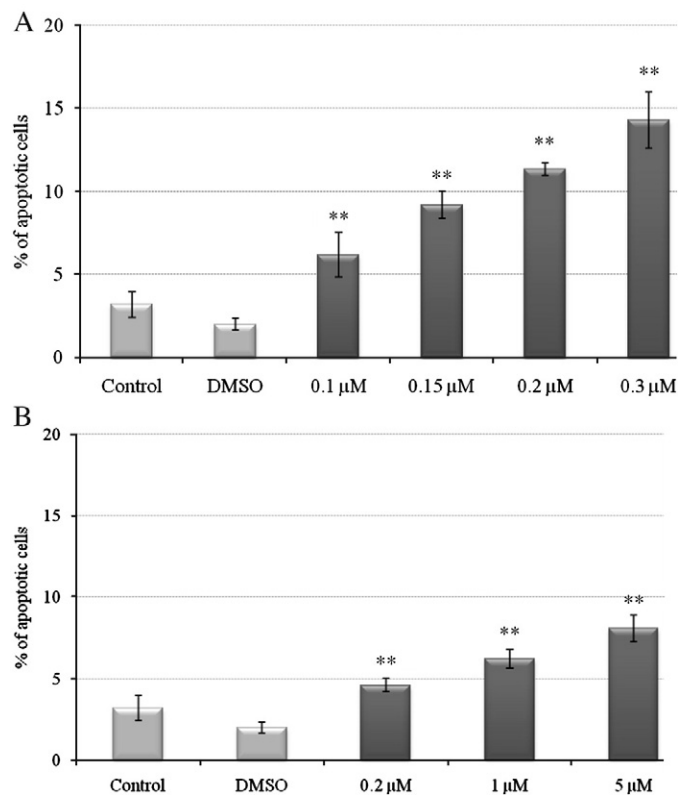
Fig. 3. Percentage of apoptotic cells measured for untreated controls, solvent controls (DMSO = dimethylsulfoxide) and treated samples ($n=5$) carried out with different concentrations of Triclosan (A) and Trimethoprim (B). Significant values (**$p<0.01$) refer to the difference between exposed samples and untreated controls. Bars show standard deviation.
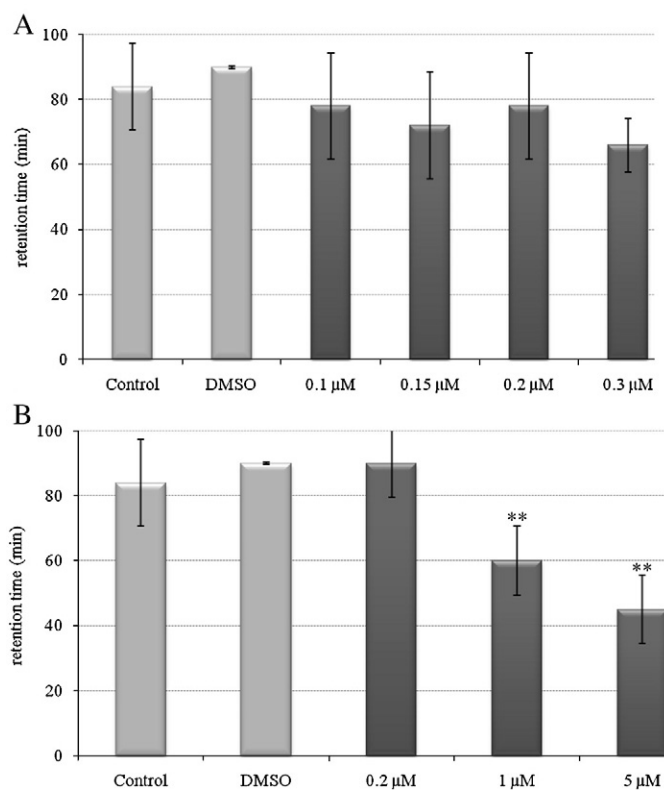

Fig. 4. Neutral red retention time (NRRT) measured for untreated controls, solvent controls (DMSO = dimethylsulfoxide) and treated samples ($n=5$) carried out with Triclosan (A) and Trimethoprim (B). Significant values (**$p<0.01$) refer to the difference between exposed samples and untreated controls. Bars show standard deviation.

No significant differences were obtained between TCS-treated hemocytes and controls (Fig. 4A). The retention time decrease from 0.3 μM TCS was about 22% in comparison with baseline levels and was 16% lower than the lowest administered dose.

In contrast, a clear dose/effect relationship was found for TMP (Fig. 4B), where the two highest doses exhibited significant differences ($p<0.01$) with controls and an NRRT decrease of 29% (1 μM) and 46% (5 μM) from baseline levels, respectively.

4. Discussion

Mussel hemocytes are a useful model for genotoxicity and cytotoxicity assessment because they need only brief manipulation and can be prepared very fast. In addition, hemocytes collected from the hemolymph of mussels should be in the same cell cycle stage because hematopoiesis probably occurs in connective tissue, and they do not divide in the hemolymph (Siu et al., 2004). They are produced by division of blast-like hemocytes and subsequent development of immature, poorly differentiated hemocytes (Hine, 1999). Flow cytometric analysis performed by Mičić et al. (2004) showed that the hemolymph cell cycle kinetics of *Mytilus galloprovincialis* are uniform, although hemolymph contains different hemocyte types, as also demonstrated in *D. polymorpha* (Giamberini et al., 1996).

The main problem encountered to set up the experimental design was the careful selection of tested concentrations because our biomarker battery included both genotoxic and cytotoxic assays and it is well known that DNA damage can be associated with cytotoxicity, depending on tested cellular type (Tice et al., 2000; Kirkland et al., 2007). There was consensus that measures of cytotoxicity need to be evaluated concurrently with each SCGE experiment so that the impact of cytotoxicity on interpretation of Comet assay data can be addressed

(Kirkland et al., 2007). OECD guideline for *in vitro* mammalian genotoxicity test (1997) suggests that, if the maximum concentration is based on cytotoxicity, it should result in approximately 10–20% relative survival cells since both sub-lethal and lethal doses should be investigated. By contrast, a general approach for SCGE assay has been to avoid the testing of doses that decrease viability by more than 30% (Tice et al., 2000). To solve the problem related to these contradictory suggestions, we selected chemical doses that guaranteed hemocyte viability greater than 50% after 60 min of exposure with TCS and TMP (Table 2). Thus, we chose concentrations that enabled the study of sub-lethal effects, while guaranteeing a viability higher or slightly lower than threshold suggested for SCGE assay. On the other hand, the lack of significant cytotoxic effect for all TCS exposure assays (Fig. 4A) opposed to the observed DNA damage (Figs. 2A and 3A) confirmed the kindness of the selected doses because cytotoxicity seemed not interfere with results obtained with SCGE and apoptosis assays.

Table 2

Tested concentrations (μM) of Triclosan and Trimethoprim to check hemocyte viability.

Triclosan		Trimethoprim	
Concentration (μM)	Viability (%)	Concentration (μM)	Viability (%)
0.1	75 ± 6.4	0.1	76 ± 1.5
0.15	64 ± 1.5	1	66 ± 1.5
0.2	51 ± 2.4	3	64 ± 0.5
0.3	52 ± 1.4	5	52 ± 3.0
0.9	47 ± 2.1	7	39 ± 2.0
2.07	35 ± 4.5	10	30 ± 3.0
3.45	28 ± 4.3		
10	27 ± 7.4		

4.1. Cytotoxic and genotoxic effect of TCS

Results obtained with in vitro exposures demonstrated that TCS significantly affects hemocyte functionality due to severe DNA injuries at very low doses, as shown by data from SCGE assays and apoptosis frequency. This is consistent with data from Zuckerbraun et al. (1998) that apoptosis was induced by exposure to 50 μM TCS for 24 h in human gingival cells exposed in vitro.

Because a positive correlation ($R = 0.66$; $p < 0.001$) between LDRs and apoptosis frequency was found, a possible DNA injury sequence can be assumed: an initial stage with an increase of primary DNA damage, shown by SCGE assay, and a subsequent rise of apoptotic cells when damage cannot be repaired. Our results are supported by Steinert (1996), who noticed that an increase in DNA fragmentation was associated with highly apoptotic populations of Mytilus edulis hemocytes. It is widely accepted that apoptosis is due to the activation of endogenous nucleases that cleave nuclear DNA into oligonucleo-somal-sized fragments (Martin et al., 1994).

Despite the limited range (0.1–0.3 μM) of tested TCS concentrations, its genotoxicity, as detected by these two biomarkers, increased in a dose-dependent manner. All LDR values were statistically different (ANOVA, Bonferroni post-hoc test, $p < 0.05$) from each other, except for 0.1 vs. 0.15 μM and 0.2 vs. 0.3 μM, while all apoptosis frequency always showed significant differences ($p < 0.01$). This might be a great environmental problem because small concentration differences in water might show discernable DNA injury differences.

Furthermore, the possible metabolites should be considered as possible interferences in relation to the assessment of TCS toxicity. At least one transformation product, methyl–Triclosan, is relatively stable in the environment and may, therefore, bioaccumulate (Lindstrom et al., 2002; Coogan et al., 2007). Once methylated, TCS lipophilicity increases, facilitating bioaccumulation in fatty tissue and avoiding photodegradation. In a Swiss study (Balmer et al., 2004), the lipid-based concentrations of methyl–Triclosan observed in fish suggested significant bioaccumulation. In our experiments, the TCS stock solution was always maintained at 4 °C in the dark, while the TCS working solutions were freshly prepared to eliminate possible degradation to methyl-TCS. Therefore, due to these precautions and the short exposure time (60 min), the observed effects in hemocytes can be ascribed only to TCS.

TCS toxicity is also highly dependent on its ionized state because molecules are believed to be more likely to cross lipid membranes when in their neutral state (Lipnick, 1995), which is dependent on pH. With a pK_a of 8.1, TCS is predominantly in its neutral form at pH 7.0, and it is predominantly ionized at pH 8.5 (Orvos et al., 2002). Thus, it is absolutely crucial to take pH into consideration during in vitro exposures. In our experiments, TCS was in the more toxic neutral state because PBS at pH 7.4 was utilized.

In contrast, the lysosomal stability assay did not show any significant differences as compared to baseline levels. Although the lack of membrane destabilization seems to indicate the creation of mild cellular stress due to TCS in D. polymorpha, these results should be due only to the low range of tested concentrations. This hypothesis is confirmed by data obtained by Canesi et al. (2007), who showed a significant and concentration-dependent lysosomal membrane destabilization in hemocytes of M. galloprovincialis exposed to 1 μM of TCS, while a dose of 0.1 μM did not cause any significant difference in in vitro assays. Considering the phylogenetic proximity between these bivalves and the similarities among results, the threshold over which TCS is able to significantly increase lysosomal membrane destabilization in these bivalves should be between 0.3 and 1 μM, which corresponds to TCS concentrations of 90 and 300 μg/L, respectively.

In the same article, Canesi et al. (2007) demonstrated that TCS not only affects lysosomal membrane stability, but also exocytosis of lysosomal enzymes both in vitro and in vivo. This means that lysosomal membranes represent a target for the action of this chemical in mussel

hemocytes and supports the hypothesis that TCS action involves interactions with eukaryotic cell membranes (Lygre et al., 2003). In contrast, the lack of significant lysosomal membrane destabilization and the absence of statistical correlations between NRRT and LDRs or apoptosis frequency observed in our study suggest different toxic pathways. The main cytotoxic action mechanism of low TCS concentrations might follow a different pathway, such as the formation of adducts directly on DNA, since TCS was reported to act as a precursor of the 2,8-dichlorodibenzo-p-dioxin (Aranami and Readman, 2007), a well-known DNA-intercalant agent. However, other studies will be necessary to known the real mechanism of TCS administered at low doses.

4.2. Cytotoxic and genotoxic effect of TMP

Unlike TCS, the genotoxic effects of TMP (Figs. 1B and 2B) seem to be due to oxidative stress and the consequent production of ROS because tight negative correlations between LDRs and NRRT ($R = -0.72$; $p < 0.001$) and between apoptosis frequencies and NRRT ($R = -0.84$; $p < 0.001$) were noticed. Moreover, a significant ($p < 0.01$) lysosomal membrane destabilization was obtained at 1 μM and 5 μM TMP (Fig. 4B). Gagnè et al. (2006) showed in the freshwater mussel Elliptio complanata that TMP is one of the most potent inducers of electron transport activity of mitochondria, which is the major ROS source in cells under certain conditions (Jezek and Hlavata, 2005). In addition, oxidative stress occurs when xenobiotics facilitate electron withdrawal by acting as radical scavengers in the mitochondria, leading to hydrogen peroxide formation (Gagnè et al., 2006). Thus, TMP seems also to follow this toxic pathway in D. polymorpha besides the classic mechanism associated with the role of folate in DNA metabolism (Abou-Eisha et al., 1999).

The in vitro cytotoxicity of TMP is much lower than that observed for TCS not only because its lower capacity to decrease hemocyte viability (Table 2) but also because of the lower DNA fragmentation noticed by SCGE assay and the counts of apoptotic hemocytes. Furthermore, tested TMP concentrations (60, 300, and 1500 μg/L) were much higher than environmental levels because TMP was estimated to be present in rivers at 0.21 μg/L (Kim et al., 2007), while Kolpin et al. (2002) detected this chemical in 27.4% of stream water samples at an average of 0.15 μg/L. Unlike TCS, TMP is a very polar compound (log $K_{ow} = 0.91$; Bendz et al., 2005) and is unable to bioaccumulate; consequently, its level in aquatic organisms does not increase much in comparison with the concentration found in aquatic ecosystems. Despite this apparent lower cytotoxicity of TMP as compared to TCS, all three biomarkers measured after TPM exposures showed significant differences from baseline levels. Finally, the relationship between DNA fragmentation and apoptosis formation also seems to be confirmed by TMP treatment, as seen by the tight correlation ($R = 0.75$; $p < 0.001$).

5. Conclusions

Our in vitro results showed a potential danger for aquatic organisms due to TCS exposure, as a high genotoxicity was noticed at low or moderate chemical concentration. Moreover, our data supplied another element in the argument about the genotoxic potential of TMP because they indicated that very high doses of this antimicrobial drug are able to induce both cytotoxic and moderate genotoxic effects in the freshwater zebra mussel.

Since this preliminary screening was carried out with TCS and TMP doses higher than those reported for aquatic ecosystems, these data will be confirmed by in vivo exposures that also consider all possible toxicant bio-transformations and involved metabolic pathways.

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PAPER II

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An *in vitro* biomarker approach for the evaluation of the ecotoxicity of non-steroidal anti-inflammatory drugs (NSAIDs)
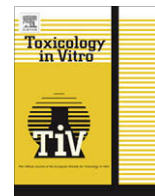
An *in vitro* biomarker approach for the evaluation of the ecotoxicity of non-steroidal anti-inflammatory drugs (NSAIDs)

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently detected pharmaceuticals in aquatic environments. They are the sixth most sold drugs worldwide and are usually found in significant quantities in municipal effluents. The aim of this study was to assess a first screening evaluation of the cytogenotoxicity of three common NSAIDs (diclofenac, ibuprofen and paracetamol) using an *in vitro* biomarker approach on the haemocytes of the freshwater bivalve zebra mussel (*Dreissena polymorpha*). Genotoxicity was evaluated by SCGE (single cell gel electrophoresis) and DNA diffusion assay while cytotoxicity was evaluated by neutral red retention assay (NRRA). The exposure of the haemocytes to increasing concentrations of the three drugs, chosen based on the results of a viability test, revealed high cytogenotoxic potential and allowed the creation of the first toxicity scale for zebra mussel haemocytes (paracetamol < diclofenac < ibuprofen). The present results lay the groundwork for *in vivo* exposures, which will allow for a better definition of the observed cytogenotoxicity of these molecules in a setting miming real environmental exposure.

1. Introduction

Pharmaceutical compounds are an emergent class of contaminants included in the broad category of PPCPs (pharmaceuticals and personal care products). Urban, industrial and hospital wastewater, together with the effluents of aquaculture and zootechnical plants, represent the main pathways of environmental contamination. Many recent studies have revealed the presence of measurable concentrations of around 80–100 of these molecules in the aquatic environment in the high ng L^{-1} to low μg L^{-1} range (Kummerer, 2004; Fent et al., 2006). Among the detected substances, non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most important groups. The sixth most sold drugs worldwide (Langman, 1999), NSAIDs have an estimated annual production of several kilotons (Cleuvers, 2004). NSAIDs such as ibuprofen, naproxen and aspirin are usually found in significant quantities in municipal effluents (Tixier et al., 2003; Ashton et al., 2004). Most NSAIDs act as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes (Gagné et al., 2005). Cyclooxygenase catalyses the formation of prostaglandins and thromboxane from arachidonic acid.

Notwithstanding the presence of pharmaceutical compounds in the environment, due to their continuous use and often abuse and the increasing knowledge about their effects on aquatic flora and fauna, the European Council Directive 2001/83/EC (EC, 2001) only

recently stated that an environmental risk assessment (ERA) might be conducted before authorising the marketing of a medicinal product for human use. Huschek et al. (2004) asserted that, according to EMEA (European Agency for the Evaluation of Medicinal Products), it is fundamental to include in an ERA not only an environmental exposure assessment (Phase I) to estimate the predicted environmental concentration (PEC), but also an evaluation of parameters that indicate toxicological and/or pharmacological effects on biological systems of chemicals, such as genotoxicity, cytotoxicity, neurotoxicity, immunotoxicity or endocrine-disrupting events (Hansen, 2001). At present, pharmaceuticals' acute toxicity is tested on organisms belonging to different levels of biological organisation, such as algae (Yang et al., 2008), cnidarians (Quinn et al., 2008), crustaceans (Haap et al., 2008) mussels (Canesi et al., 2007) and fishes (Choi et al., 2008). These studies, with their focus on short-term effects, play an important role in a tiered approach to environmental risk assessment, but generally they only consider the specific mode of toxic action/potency of pharmaceuticals (CSTEE, 2001). Moreover, Bottoni and Fidente (2005) have underlined that the classical ecotoxicological assays do not seem useful to reach this goal because environmental concentrations of pharmaceuticals are much lower than their effect concentrations for these tests; a more sensitive approach, like the use of biomarkers, was then suggested. The first step can be an *in vitro* screening test using a battery of biomarkers to directly determine the mode of action of a particular pollutant (Gagnaire et al., 2004) and its toxic effect with great precision and reproducibility (Olabarrieta et al., 2001) and also allowing a classification of the

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toxicity potential of the different molecules (Grandi et al., 2006). This preparatory approach is essential for the second step of using *in vivo* assays, which allow for the evaluation of the real effect of the pollutant since the organism can utilise all of its defence mechanisms and provide a more general ecotoxicological screening (Ching et al., 2001). Although both of these approaches have been used separately, their complementarity can give a more complete and exhaustive report of the pollutant's toxicity (Hartmann et al., 2004).

The aim of this study was to assess a first screening evaluation of the cytogenotoxicity of three common NSAIDs, diclofenac (DCF), ibuprofen (IBU) and paracetamol (PCM), using an *in vitro* biomarker approach on the haemocytes of the freshwater bivalve zebra mussel (*Dreissena polymorpha*). Although paracetamol (acetaminophen) does not possess an anti-inflammatory action, it is usually considered an NSAID in toxicology due to its similar mode of action (Misra et al., 1990). The zebra mussel was chosen as the biological model because bivalves play an important role in aquatic ecosystems, and therefore they are particularly susceptible to environmental stressors (Gagnè et al., 2006). It should be borne in mind that bivalves have been shown to possess prostaglandin synthase and COX activity, which they express during gamete maturation and spawning (Osada and Nomura, 1990). Genotoxicity was evaluated by single cell gel electrophoresis (SCGE) assay and apoptosis frequency evaluation; the cytotoxic effects were measured by a lysosomal membranes stability test (NRRA-neutral red retention assay), a classical parameter of cellular stress in bivalves (Lowe et al., 1995). Every assay was carried out on mussel haemocytes because they require only brief manipulation and quick preparation. Moreover, they are directly in contact with contaminants, and their multifunctional roles make them more sensitive than other cell lines (e.g., gills and digestive glands) to internal and environmental factors (Venier et al., 1997), making them a useful cellular matrix to assess the ecotoxicity of potential environmental pollutants.

2. Materials and methods

Standards of paracetamol (*N*-(4-hydroxyphenyl)acetamide; CAS number 103-90-2), diclofenac (2-(2-(2,6-dichlorophenylamino) phenyl)acetic acid; CAS number 15307-86-5) and ibuprofen (2-[4-(2-methylpropyl)phenyl]propanoic acid; CAS number 15687-27-1) were obtained from Sigma–Aldrich (Steinheim, Germany), as well as all other chemicals used for biomarker determination. Dimethylsulfoxide (DMSO; CAS number 67-68-5; purity = 99.5%) was obtained from VWR International (Milan, Italy).

2.1. Mussel acclimation and maintenance conditions

Several hundred specimens of *D. polymorpha*, tied by byssus to the rocks, were collected from Lake Maggiore (Northern Italy) by a scuba diver at a depth of 4–6 m. The mussels were rapidly transferred to the laboratory in bags filled with lake water to reduce environmental stress. The rocks were rinsed and transferred in 100-L glass aquaria filled with tap water, which was maintained in a natural photoperiod at constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90% of saturation). Bivalves were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA), and the water was regularly changed every two days for at least two weeks to help cleanse the bivalves of pollutants bioaccumulated in their soft tissues. Several specimens with similar shell lengths (about 20 mm) were chosen for *in vitro* tests, including controls and solvent. They were gently cut from the rocks and placed on glass sheets suspended in 15-L aquaria filled with tap and dechlorinated

water (1:1 v/v) and maintained at the same conditions described above. Only specimens able to re-form the byssus and reattach themselves to the glass sheet were used in the experiments. Mussels were used for the subsequent *in vitro* analyses only when target biomarker assays showed values comparable with controls previously checked.

2.2. Haemocyte preparation for in vitro method

Exposure consisted of *in vitro* direct contact between haemocytes and selected pollutants as suggested by Gagnaire et al. (2004) and adapted to *D. polymorpha*. Exposure time was extended for 1 h because previous experiments revealed that zebra mussel haemocytes start to die 2 h after withdrawal. Two different methods were employed for drawing and handling mussels' haemocytes. For SCGE and apoptosis assays, haemolymph of ten specimens (about 100 μl) was withdrawn from the sinus near the posterior adductor muscle using a hypodermic syringe filled with 200 μl of phosphate-buffered saline (PBS; pH 7.4). Samples were maintained on ice to avoid endogenous damage during sample preparation and to inhibit DNA repair in the unfixed cells. The final density of haemocytes was constant at 10^4 cells/mL. The ten cell suspensions were put into microtubes and briefly vortexed to homogenise the sample. An aliquot was then added to each microtubes with an appropriate quantity of chemicals to reach a final volume of 1 mL. Samples were briefly mixed with a vortex and then maintained for 60 min in a temperate room at 20 °C in the dark; microtubes were constantly mixed by a horizontally-pivoted stirrer. After exposure, samples were centrifuged at 2000 rpm at 20 °C to stop the effect of contaminants, the supernatant was discharged and the pellet was then re-suspended with 50 μl of phosphate-buffered saline (PBS). Only two changes were made to the protocol of the NRRA: the centrifugation was carried out at 1500 rpm to avoid mechanical damage to the lysosomal membranes, and the pellet was re-suspended with 300 μl of PBS/EDTA (1:1) mixture instead of PBS only.

2.3. Concentration choice

Since zebra mussel haemocytes have a short lifetime in *in vitro* experiments, cell viability was checked in advance to ensure that haemocytes did not die within the entire sample treatment. After a preliminary viability test to evaluate the health status of the specimens, many other tests were carried out with several concentrations of the three molecules to select only chemical concentrations that guaranteed a cell viability higher than 70%, according to recommendation on SCGE assay made by the 4th International Workshop on Genotoxicity Test Procedures (IWGT) (Kirkland et al., 2007). Haemocytes' viability was checked using the Trypan blue exclusion method (10 μl of cell suspension added to 10 μl of 0.4% dye) using a Burker chamber and an optical microscope for the count. A pool of four or five mussels was tested twice for each concentration. We tested three different doses for each chemical in accordance with the results of our viability tests (Table 1).

2.4. SCGE assay

The SCGE assay was basically performed according to the alkaline (pH > 13) version of the assay developed by Singh et al. (1988) with the subsequent optimisation for zebra mussels elaborated by Buschini et al. (2003).

Ten aliquots of 10 μl of cell suspension mixed with 85 μl of low melting agarose (LMA-0.7%) in PBS (37 °C) were spread onto ten coated slides (previously dipped in 1% normal melting agarose), then covered by a coverslip and kept at 4 °C for 5 min until the agarose layer was hardened. A third agarose layer of 85 μl was added

Table 1
Tested concentrations (μM and μg L^{-1}) of diclofenac (DCF), paracetamol (PCM) and ibuprofen (IBU). Numbers written in bold correspond to concentrations chosen for exposure assays.

DCF		PCM		IBU	
μM	(μg L^{-1})	μM	(μg L^{-1})	μM	(μg L^{-1})
0.2	**60**	**0.2**	**30**	**0.2**	**45**
0.5	**156**	**1**	**150**	1	227
0.8	**250**	2	303	**2**	**450**
1	312	**3**	**450**	3	681
2	625	5	757	**4**	**909**
		6.6	1000	5	1136

to the slides in the same way. After agarose solidification, the coverslip was removed, and the slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar at 4 °C in the dark for at least 1 h.

Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber containing freshly prepared buffer (1 mM Na$_2$EDTA, 300 mM NaOH, pH 13) and placed in an ice-water bath (4 °C). Electrophoresis was then performed at 0.78 V/cm and 300 mA for 10 min. After electrophoresis, slides were washed in a neutralisation buffer (0.4 M Tris–HCl, pH 7.5) and fixed in absolute methanol. After staining with DAPI 4′,6-diamidino-2-phenylindole DNA dye (Sigma–Aldrich), coverslips were placed over the slides and observation was carried out using a fluorescence microscope (Leitz DMR, Germany) equipped with a FTC filter. All steps were performed in the dark to minimise additional UV-induced DNA damage. Positive controls were carried out with H$_2$O$_2$ to check the effectiveness of the electrophoresis conditions. All samples were blindly coded and evaluated.

Fifty cells per slide were analysed using an image analysis system (Comet Score®), for a total of 500 analysed cells per sample. The ratio between the migration length and the diameter of the comet head (LDR) was chosen to represent DNA damage (Bolognesi et al., 2004). This was coupled with the percentage of DNA in the tail obtained by the Comet Score® and the following criteria proposed by Mitchelmore et al. (1998): grade of damage zero or minimal <10%, low damage 10–25%, mid damage 25–50%, high damage 50–75% and extreme damage >75%.

2.5. DNA diffusion assay

The evaluation of the frequency of apoptotic cells was carried out by the method described by Singh (2000), basically through the same protocol used for the SCGE assay. This method is based on the fact that apoptotic cells have numerous alkali-labile sites (MacManus et al., 2000), which should yield pieces of DNA once exposed to alkali conditions. These DNA fragments can easily diffuse in the agarose matrix, giving the appearance of a halo with a hazy outline (Singh, 2000). An aliquot of 10 μl was shared among five different slides for each pool of haemocytes and then processed with the same method described above for the SCGE assay. The only methodological difference was that, after the haemocytes' lysing, slides were subjected to 5 min of alkaline DNA unwinding (pH 13) without the subsequent electrophoresis. Then, the slides were washed in the neutralisation buffer and fixed in absolute methanol. Finally, after DAPI dying, slides were observed under a fluorescence microscope. Two hundred cells per slide were counted for a total of one thousand per sample. The endpoint measured was the frequency (%) of apoptotic cells that, according to the intense autolysis of genomic DNA, are characterised by nuclear remnants resembling pinheads surrounded by large DNA halos. These were easily discriminated from necrotic cells, which were

characterised by circular and faint halos that hence were eliminated from the count.

2.6. Neutral red retention assay

The method for the neutral red retention assay followed the protocol proposed by Lowe and Pipe (1994), which overworks the ability of the lysosomal membranes to accumulate a weakly cationic dye (neutral red) and works in accordance with the rationale that healthy cells can retain larger quantities of it than damaged cells. Neutral red stock solution was made by dissolving 20 mg of dye in 1 mL of dimethylsulfoxide (DMSO) while the working solution was prepared fresh every day by diluting 5 μl of stock solution in 2.5 mL of PBS. Using a coverslip, 2 μl of polylysine were spread onto microscope slides. Five slides were prepared for each sample.

An aliquot of 80 μl withdrawn from the haemocytes' suspension was placed onto each slide, being careful to avoid possible mechanical cell damage during hypodermic needle removal. Slides were put in a light-proof humidity chamber for 20 min to allow the haemocytes to stabilise themselves, and then the excess suspension was carefully removed. At last, 40 μl of neutral red working solution was added. After an incubation of 20 min in a humidity chamber, the slides were observed under an optical microscope and again at 15-min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Slides were kept in the humidity chamber between observations. Neutral red retention time was the endpoint measured and corresponded to the time at which at least 50% of the haemocytes exhibited evident dye loss (Lowe et al., 1995). The mean retention time was then calculated from the five replicates.

2.7. Statistical analysis

To ensure that the data conformed to the assumptions of normality and homogeneity of variance, they were tested with the Shapiro-Wilk and Levene's tests, respectively. Data that failed to meet the first assumption were transformed using either a log transformation for the SCGE assay values or an angular transformation (arcsin$\sqrt{P}$) for the % of apoptotic cells. One-way analysis of variance (ANOVA) followed by a Dunnett *post hoc* test was performed to evaluate the eventual significant differences ($p < 0.05$) between controls and treated samples. A Bonferroni *post hoc* test ($p < 0.05$) was also carried out to check the eventual significant differences among the biomarker endpoints measured at the administered doses. All statistical analyses were performed using the software package STATISTICA 7.0.

3. Results

Because haemocytes have a short lifetime in *in vitro* experiments, haemolymph maintained in PBS (1:2) was previously checked at different times (from $t = 0$ min to $t = 180$ min) to control for the decrease in viability of untreated haemocytes to select the suitable length of exposure. Cell viability was 91 ± 5% at $t = 0$, while a decrease below 80% was observed starting at $t = 90$ min. Thus, we opted to perform the exposure assays at $t = 60$ min, which guaranteed a cell viability of 83 ± 3%. Preliminary assays were also carried out to choose the concentrations of DCF, PCM and IBU that allow the study of non-acute toxicity and guaranteed a viability up to 70%.

Baseline levels of all the measured biomarkers are perfectly comparable to those obtained in a previous study made by our research group (Binelli et al., 2009). Finally, no statistical differences between untreated groups and solvent controls were noticed for all end-points.

3.1. SCGE assay results

All of the tested drugs are able to induce significant ($p < 0.01$) primary DNA damage in haemocytes of *D. polymorpha* (Fig. 1). PCM and DCF caused the greatest damage to haemocytes' DNA and also revealed a concentration-related trend for the three tested exposures: DNA fragmentation two times higher than the baseline levels was observed at the lowest tested concentration (0.2 μM), while the highest concentration (3 μM for PCM and 0.8 μM for DCF) caused a further LDR increase of about 65%, reaching a value three times higher than controls (Fig. 1). IBU showed less damage than the other two drugs, reaching an increase of DNA fragmentation 2.5-fold higher than the baseline levels.

The percentage of haemocytes that fell into the high and extreme damage classes grew with increasing concentration for IBU and PCM, ranging from 6% to 23% and from 13% to 27%, respectively (Fig. 2). DCF showed the highest induction of DNA fragmentation as 41% of haemocytes fell in the elevated damage classes beginning from the middle tested dose (0.5 μM).

3.2. DNA diffusion assay results

All of the tested drugs were able to induce irreversible DNA damage, promoting the increase of apoptotic cells (Fig. 3). The lowest
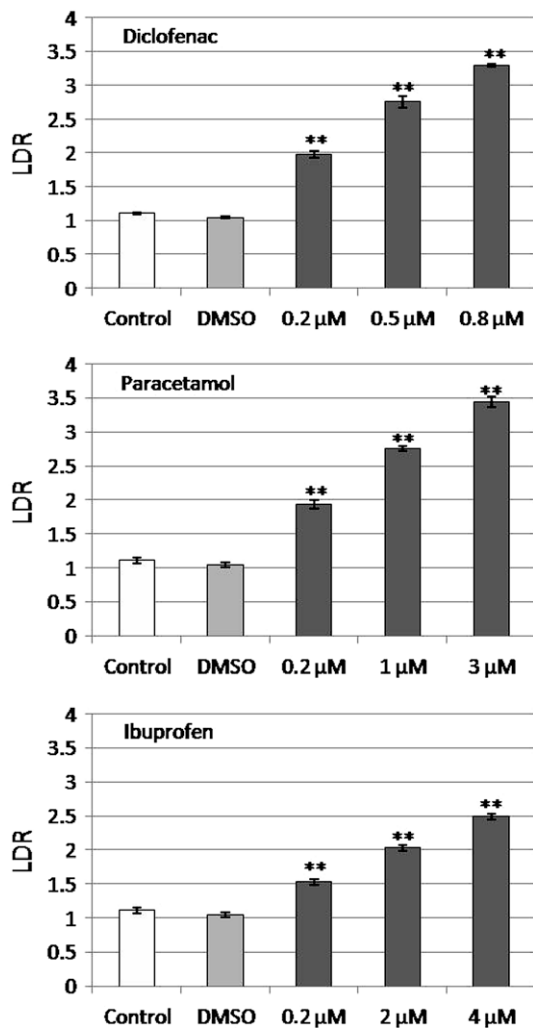
Fig. 1. Means of length/diameter ratios (LDRs) measured during exposure tests. Bars show standard deviation. Significant values (ANOVA, Dunnett *post hoc* test, **$p < 0.01$) are referred to the comparison between treated samples and controls.
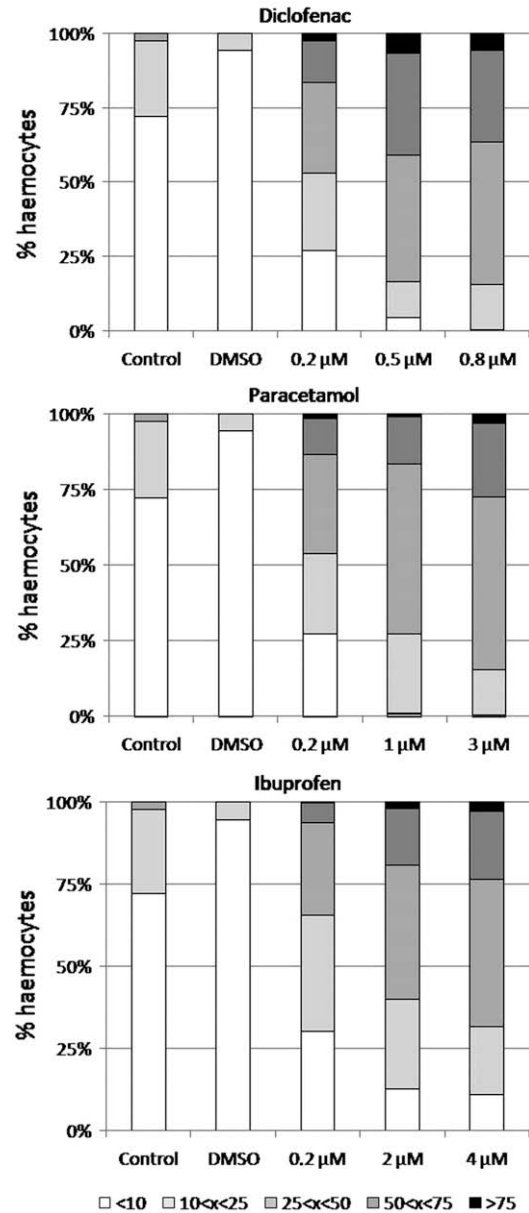
Fig. 2. Distribution of DNA damage (percentage of DNA in the tail): minimal <10; low $10 < x < 25$; mid $25 < x < 50$; high $50 < x < 75$; extreme >75 (Mitchelmore et al., 1998).

concentration of PCM (0.2 μM) was already sufficient to obtain a 2.5-fold increase of apoptosis over baseline levels, while 3 μM caused a further increase of 51%, revealing a clear linear dose-dependent relationship ($r^2 = 0.8973$; $p < 0.01$). This trend was not confirmed by DCF and IBU because these drugs did not induce significant apoptotic phenomena ($p > 0.05$) in zebra mussel haemocytes at 0.2 μM. The middle tested concentration began to promote cell death, showing a percentage of apoptotic cells two times higher than control values. The highest concentration of DCF (0.8 μM) revealed an increase of apoptosis six-fold higher than controls, a value comparable with that obtained with 1 μM of PCM. By contrast, IBU seems to be the less cytotoxic drug since the highest tested dose (4 μM) was able to induce less than 15% of apoptotic cells.

3.3. Neutral red retention assay results

A slight decrease of lysosomal membrane stability was revealed for DCF and IBU (Fig. 4) since statistical differences

Fig. 3. Means of the frequency of apoptotic cells (%) measured during exposure tests. Bars show standard deviation. Significant values (ANOVA, Dunnett *post hoc* test, **$p < 0.01$) are referred to the comparison between treated samples and controls.
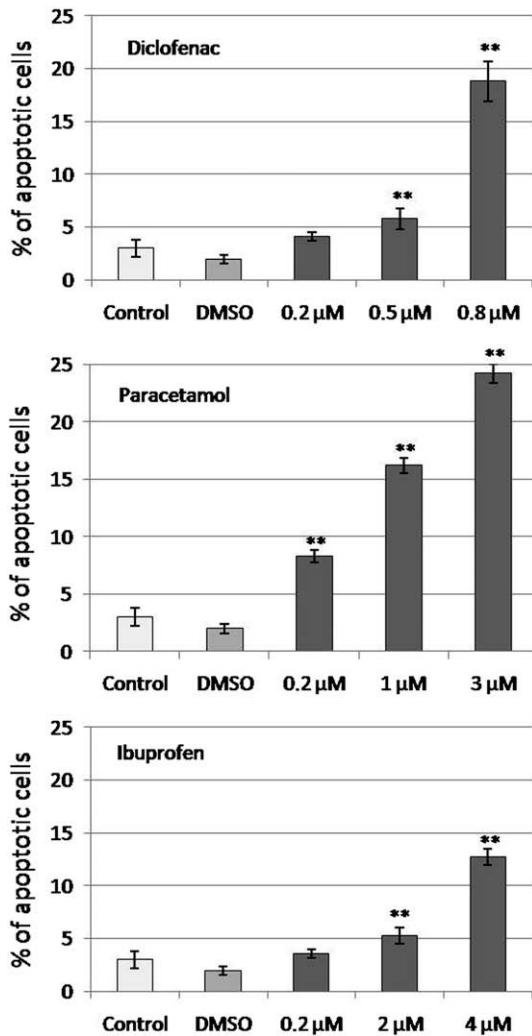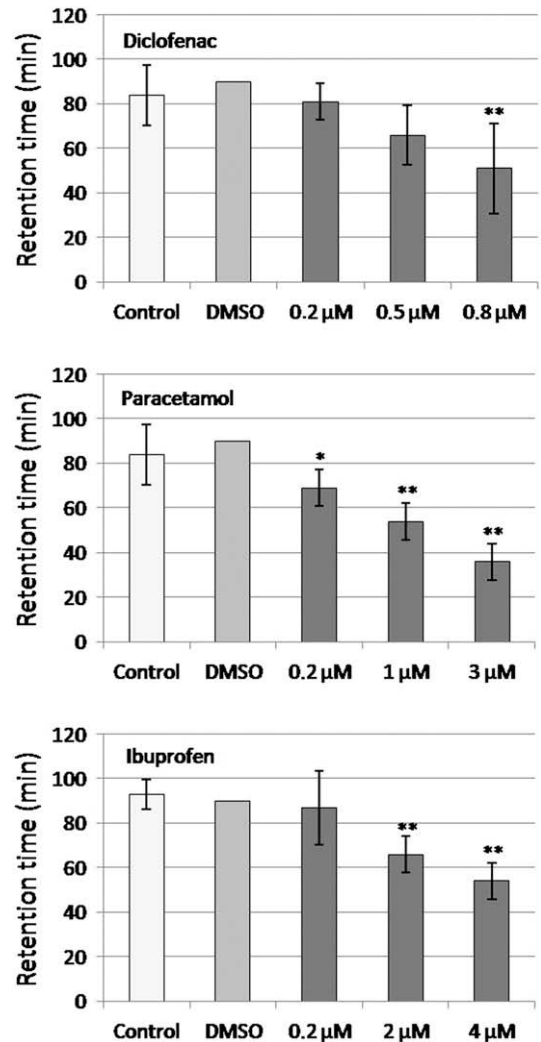

Fig. 4. Means of Neutral red retention times measured during exposure tests. Bars show standard deviation. Significant values (ANOVA, Dunnett *post hoc* test, *$p < 0.05$; **$p < 0.01$) are referred to the comparison between treated samples and controls.

($p < 0.05$) were noticed only at the highest tested dose for the former, while for the latter statistical differences were observed from the middle tested dose with a reduction of retention time of about 40% from baseline levels. On the contrary, PCM revealed a strong decreasing dose/effect relationship ($r^2 = 0.7719$; $p < 0.01$); a significant ($p < 0.05$) retention time (RT) reduction of 18% compared to the control values was already noticed at 0.2 μM, while the middle and high exposure concentrations (1 and 3 μM) showed significant decreases ($p < 0.01$) of 36% and 57%, respectively.

4. Discussion

The main goal of every preliminary screening using the *in vitro* approach should be the investigation of the primary effects of contaminants and the definition of a toxicity scale for the identification of chemicals that require more testing (Kirkland et al., 2007). In order to investigate the potential cyto and genotoxic of three different NSAIDs, several Zebra mussel specimens were exposed to chemical doses much higher than PEC (EMEA, 2003): 10.38 μg L^{-1} for PCM, 5.76 μg L^{-1} for IBU and 1.43 μg L^{-1} for DCF.

4.1. Acute effects

Although the main goal of this study was to known some chronic effects related to these three anti-inflammatory drugs, our data allowed to obtain also information about acute toxicity. Since we tested several concentrations of the selected pharmaceuticals to determine the haemocyte viability, we were also able to calculate the related EC$_{50}$ values, which were 178 μg L^{-1} for DCF, 350 μg L^{-1} for PCM and 1312 μg L^{-1} for IBU, respectively. This preliminary test, which reveals an acute effect (death) on mussel haemocytes, seems to show that IBU possesses the lowest acute toxicity among the tested molecules, reaching EC$_{50}$ values 3.8- and 7-fold higher than PCM and DCF, respectively. This observation is corroborated by EU directive 93/67/EEC, which classifies substances according to their measured effective concentration (CEC, 1996). Applying this classification, IBU is classified as toxic for zebra mussel haemocytes, with an EC$_{50}$ between 1 and 10 mg L^{-1}, while PCM and DCF are very toxic (EC$_{50}$ <0.1–1 mg L^{-1}). However, these values are much higher than environmental concentrations, as also indicated by many studies evaluating the acute toxicity of NSAIDs (Cleuvers, 2004).

The EC$_{50}$ evaluated for Zebra mussel can be not representative for the entire aquatic biocoenosis because the acute toxicity

related to these drugs is highly species-specific. For instance, Cleuvers (2003) indicated that EC_{50} values of the same PPCPs using *Lemna minor* in chronic assays were 5- to 10-fold lower than those obtained using *Daphnia* sp. or an algal test.

4.2. Chronic effects

Considering the very low concentrations of pharmaceuticals in aquatic environments, it could be assumed that chronic effects are generally more likely to occur than acute effects. Results obtained with *in vitro* exposure of cell suspensions demonstrated that each tested drug was able to compromise haemocytes' functionality starting from the lowest dose (0.2 μM), as highlighted by SCGE assay. All LDR values obtained for the three tested concentrations of drugs were statistically different ($p < 0.01$) both from baseline levels and from each other, revealing a clear dose-dependent increase of primary DNA injuries. Comparing LDR values obtained at 0.2 μM, the only dose common to all drugs, IBU induces a DNA fragmentation lower than DCF and PCM according to viability results. However, slight differences were obtained comparing the two different end-points selected to describe SCGE assay results. In fact, while LDR showed similar behaviour for DCF and PCM (Fig. 1), the use of DNA damage classes showed that PCM possesses the greatest capability to induce DNA fragmentation (Fig. 2). Thus, this latter end-point seems to be more sensitive than the simple use of LDR, as also indicated by the 4th IWGT (International workshop on genotoxicity test procedures) that recently proposed the percentage of tail DNA as the measure that seems most linearly related to dose and the easiest to understand (Kirkland et al., 2007).

Although the SCGE assay represents a sensitive and reliable genotoxic test, it can point out only reversible DNA damage that can then be repaired by repairing systems. Thus, results obtained with the apoptosis test should be more representative of the real toxic effects of these drugs. The clear linear dose-effect trend observed by SCGE assay was confirmed only for PCM, which was able to cause a significant ($p < 0.01$) increase of apoptotic cells starting from the lowest exposure dose (Fig. 3). Moreover, comparing the apoptosis percentages measured at the common dose of 0.2 μM, PCM reached an increase of 25% and 50% in comparison with percentages measured for DCF and IBU, respectively.

Notwithstanding the complete lack of scientific literature on the apoptotic effects of NSAIDs in aquatic invertebrates, there is some evidence of their apoptosis induction in several vertebrate cell lines (Gill and Dive, 2000; Zhou et al., 2001; Gómez-Lechón et al., 2003). For many years it was assumed that chemically-induced damage and death occurred by necrosis, but it is now clear that apoptosis could be the major form of chemically-induced cell death and that necrosis is much rarer, occurring only in circumstances of gross cell injury (Raffray and Cohen, 1997; Gill and Dive, 2000). Thus, the evaluation of possible apoptotic effects in invertebrates and the determination of its possible mechanism of action are of fundamental importance. The mitochondrial pathway could explain the induction mechanism observed in zebra mussels. This mechanism involves the mitochondrial permeability transition (MPT), a non-selective inner membrane permeabilisation that is considered a common mechanism that precedes necrotic and apoptotic cell death as revealed in rodent and human hepatocytes exposed *in vitro* to PCM and DCF (Gómez-Lechón et al., 2003; Kon et al., 2007).

Finally, strong positive correlations between LDRs and % of apoptotic cells were found for IBU ($R = 0.92$; $p < 0.01$), DCF ($R = 0.82$; $p < 0.01$) and PCM ($R = 0.99$; $p < 0.01$), indicating that apoptosis is strictly connected with DNA fragmentation. Our results agree with those of Steinert (1996) showing an increase of DNA fragmentation connected to a high frequency of apoptosis in populations of mussel haemocytes. Furthermore, it is widely accepted that apoptosis is due to the activation of endogenous nucleases (Martin et al., 1994), resulting in cleavage of nuclear DNA into oligonucleosomal-sized fragments.

The main mechanism of action of these drugs for the induction of cyto and genotoxicity is probably due to an increase of oxidative stress. In fact, several authors (Masubuchi et al., 2002; Gómez-Lechón et al., 2003) have demonstrated that the mechanism of diclofenac-induced mitochondrial injury seems to involve the generation of reactive oxygen species (ROS) causing oxidative stress to mammal hepatocytes. Also the metabolism of PCM leads to ROS production because this drug is converted to a toxic form, NAPQI (N-acetyl-p-benzo-quinone imine) that is an electrophilic intermediate which is oxidized by cytochrome P450 and converted to a highly reactive and toxic metabolite. In addition, NAPQI can increase the formation of ROS and reactive nitrogen species (RNS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide, and nitro oxide and peroxynitrite, respectively (Yen et al., 2006).

Results obtained by NRRA, that is considered the most reliable of the recommended biomarkers in water quality assessment (UNEP, 1997), could help to confirm this hypothesis since lysosomal membrane destabilisation is a common parameter by which to assess general stress in bivalves (Lowe et al., 1995) and can indicate the induction of oxidative stress. Only PCM was able to destabilise lysosomal membranes starting from 0.2 μM ($p < 0.05$). This could indicate that the production of ROS and the consequent oxidative stress due to PCM could be responsible for the observed DNA damage since tight negative correlations between LDR and NRRT ($R = -0.93$; $p < 0.01$) and between apoptosis frequency and NRRT ($R = -0.91$; $p < 0.01$) were found. Although negative correlations between NRRT and the genotoxicity endpoints were measured both for DCF (NRRT-LDRs, $R = -0.77$; $p < 0.01$ and NRRT-% apoptotic cells, $R = -0.71$; $p < 0.01$) and IBU (NRRT-LDRs, $R = -0.86$; $p < 0.01$ and NRRT-% apoptotic cells, $R = 80.78$; $p < 0.01$), the oxidative stress seems to cause DNA injuries only at the higher tested concentrations (0.8 and 2 μM). In these cases it can be hypothesised that low levels of DCF and IBU can directly produce the observed DNA damage by means of the creation of adducts and/or intercalants without significant ROS production while higher doses are able to create oxidative stress. Several studies (Haap et al., 2008; Atchison et al., 2000; Jones et al., 2003) have pointed out that the cytotoxicity of DCF could also affect protein integrity since the generation of ROS could account for the protein damage. Moreover, another mechanism of action can be derived from the formation of protein adducts, which have been described for DCF in rat liver cells (Hargus et al., 1995; Seitz et al., 1998).

Results obtained with the entire battery of biomarkers seem to show that PCM might be the most cytogenotoxic agent, followed by DCM, while IBU creates the least damage both to DNA and lysosomal membranes. The reason for the greater toxic effect of PCM could actually be due to different bioavailability in comparison to the other two drugs. In fact, PCM has a very low log K_{ow} (0.46) that allows this chemical to enter through the plasmatic membrane of haemocytes more easily than DCM and IBU. Finally, PCM should appear more toxic than expected due to its status as a weak acid (pKa = 9.4) (Lorphensri et al., 2007), which could influence the cell pH.

5. Conclusions

In vitro studies, thanks to their simplicity, reliability and speed, provide basic information on the toxicity of xenobiotics and often assist in hypothesising or testing a probable mechanism of action of pollutants. In addition, they can act as a guide to direct additional research and could be used in environmental risk assessment evaluations. This *in vitro* study, the first directed toward the assessment of cytogenotoxicity of three common pharmaceuticals not only in

D. polymorpha, but also in the whole freshwater bivalve class, revealed a potential danger for aquatic organisms only at high administered doses. PCM exposure caused the greatest DNA damage to mussel haemocytes and was correlated with an elevated destabilisation of lysosomal membranes, a symptom of general stress in bivalves. DCF, despite high genotoxicity noticed at low chemical concentrations, was cytotoxic only at the highest tested doses, while IBU possessed the lowest chronic toxicity. These data allow to draft a first toxicity scale of these molecules (PCM < DCF < IBU) and lay the groundwork for *in vivo* exposures, which will allow better definition of their observed cytogenotoxicity, miming real environmental exposure. In fact, in an environmental situation, many other factors are implicated as well as kinetics of accumulation, exposure duration and defence mechanisms of the organisms (CYP-450, phases I and II enzymatic complexes) that could render less or more toxic the tested molecule.

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PAPER III

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In vivo experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussel hemocytes

In vivo experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussel hemocytes

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ABSTRACT

In this work, we investigated the possible genotoxic and cytotoxic effects of the antibacterial agent Triclosan in hemocytes of the freshwater bivalve Zebra mussel (*Dreissena polymorpha*). For this study, we used several biomarkers for *in vivo* experiments (96 h of exposure) carried out at three possible environmental Triclosan concentrations (1, 2, 3 nM). We used the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and the measure of the apoptotic frequency (Halo assay) to measure the genotoxic potential of Triclosan, and the neutral red retention assay (NRRA) as a measure of lysosomal membrane stability to identify general cellular stress. We observed significant increases in all of the genotoxic biomarkers examined as early as 24 h after initial exposure, as well as a clear destabilization of lysosomal membranes (after 48 h), indicating that this chemical is potentially dangerous for the entire aquatic biocoenosis. A comparison of these *in vivo* data with existing data from *in vitro* experiments allowed us to suggest possible mechanisms of action for Triclosan in this bivalve. Although further studies are needed to confirm the possible modes of action, our study is the first to report on the effects of this widespread antibiotic on freshwater invertebrates.

1. Introduction

Among the emerging class of environmental pollutants of PPCPs (Pharmaceuticals and Personal Care Products), one of the most widely used groups is antibacterial agents, which are used not only for human and veterinary medication, but also for the promotion of growth in livestock and aquaculture species (Daughton and Ternes, 1999). While antibacterial agents at low concentrations are probably not pharmaceutically active in humans, they may still be potential pollutants in aquatic environments (Yang et al., 2008). Triclosan (TCS; 2,4,4′-trichloro-2′-hydroxydiphenyl ether) is one of the main known antibacterial agents and is added to a wide range of consumer products (e.g. toothpastes, soaps, deodorants, textiles, shoes and cosmetics). It has been marketed for over 30 years, and its use has increased over time. The UK Environment Agency (2004) estimated that about 350 tons of this chemical are now used every year in the EU.

TCS is a lipophilic compound (log K_{ow} = 4.8) that has the potential to bioaccumulate and affect non-target organisms. It has been found in four fish species from Sweden, in abundances ranging from 0.24 to 120 mg/kg fresh weight (Adolfsson-Erici et al., 2002), and in the filamentous algae *Cladophora* spp. at 100–150 ng/g fresh weight

(Coogan et al., 2007). Wilson et al. (2003) showed that TCS may influence both the structure and the function of algal communities in stream ecosystems that receive treated wastewater effluent, and there is a huge amount of documentation on the impact of antibacterial residues on microbial processes and the consequential effects on the whole ecosystem (Yang et al., 2008).

TCS has been shown to be biodegradable and photo-unstable, and it continues to breakdown following its release into the aquatic environment. In a laboratory study, Federle and Schwab (2003) reported a mineralization half-life in water of 2.5–3.5 d. In-stream removal half-lives for TCS have been reported to range between 2.1 h (Sabaliunas et al., 2003) and 11.6 h (Morrall et al., 2004), but are likely to be considerably higher in lakes (Singer et al., 2002; Tixier et al., 2002). TCS has been reported to also exist in a methylated form, methyl-TCS (Lindstrom et al., 2002), and can be converted into a dioxin congener (2,8-dichlorodibenzo-*p*-dioxin, DCDD) by photolytic degradation under laboratory conditions (Aranami and Readman, 2007). The extent to which this process takes place in the natural environment, however, is unknown, and therefore it is difficult to assess the environmental significance of this finding (UK Environment Agency, 2004).

Recently, Yang et al. (2008) measured the growth-inhibiting effects of 12 different antibacterial agents and showed that TCS is the most toxic antibacterial compound (NOEC = 200 ng/L) for the freshwater microalga *Pseudokirchneriella subcapitata*. Prior to this study, the lowest reported NOEC was 700 ng/L, which was obtained

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with *Scenedesmus subspicatus* (Orvos et al., 2002). It was on the basis of this value that the most conventional PNEC (predicted no effect concentration) of 70 ng/L for freshwater species was derived. Several studies have found concentrations of TCS in excess of this value in rivers in Germany, England and Switzerland (Singer et al., 2002; Lindstrom et al., 2002; Sabaliunas et al., 2003; Wind et al., 2004): levels ranged from 10 ng/L to 100 ng/L in watercourses that were downstream of sewage treatment plants. Reiss et al. (2002) measured TCS concentrations in US wastewater effluents and found that they ranged from 200 ng/L to 2700 ng/L.

Capdevielle et al. (2007) used the Geography-referenced Regional Exposure Assessment Tool for European Rivers (GREATER) to estimate a PEC (predicted environmental concentration) for the UK that rarely exceeded 200 ng/L of TCS; for North America the PhATE (Pharmaceutical Assessment and Transport Evaluation) model suggested that TCS concentrations could be as high as 850 ng/L without in-stream removal and 250 ng/L with in-stream removal. In the same work, these authors calculated a chronic PNEC of 1550 ng/L by using the species sensitivity distribution (SSD) approach derived from toxicity data of 14 different aquatic species.

The plethora of NOECs and PNECs that have been calculated for aquatic organisms highlights the species-specific effects of TCS and that toxicity studies of different classes of antibacterial agents are urgently required to assess their potential impact on aquatic ecosystems (Yang et al., 2008). Moreover, very few data are available on other sub-lethal effects (genotoxicity, cytotoxicity, impairment of cell signaling, oxidative stress), which can also demonstrate the possible mechanisms of TCS action. This point is particularly true for aquatic invertebrates, which represent more than 90% of the extant species and play important roles in ecosystem function.

The aim of this study was to assess the genotoxicity and cyto-toxicity of TCS *in vivo* with a battery of biomarkers in hemocytes of the freshwater bivalve Zebra mussel (*Dreissena polymorpha*). We used the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and the measure of the apoptotic frequency (Halo assay) to measure the genotoxic potential of TCS; lysosomal membrane stability was also measured by the neutral red retention assay (NRRA) to identify cellular stress. These sensitive and reliable techniques to identify cytotoxicity and genotoxicity potential of different xenobiotics are the most used in ecotoxicology, allowing a direct comparison among different biological models.

This study is the second step of a tiered approach to TCS, which also includes *in vitro* experiments and a proteomic analysis. A previous study (Binelli et al., 2008) presented results from *in vitro* experiments that show that this pharmaceutical significantly affects hemocyte functionality by severely injuring the DNA. While the *in vitro* experiments provide basic information on the nature of the tested agents and/or the cellular response, the *in vivo* experiments allow us to explore the entire effect of toxicant without excluding any biochemical pathway. Moreover, they allow us to evaluate the possible genotoxic and cytotoxic effects of the real bioavailable concentration of the chemical administered in water, mimicking the real world. The overall challenge is thus to integrate individual biomarker observations into a test battery capable of evaluating the total health effect of a particular type of biological model and to suggest a possible mechanism for TCS action. To the best of our knowledge, these data represent the first results on the genotoxicity and cytotoxicity of TCS in this sentinel-organism that links the pelagic and benthic compartments.

2. Materials and methods

The TCS standard (97% purity) and all other chemicals used for biomarker determination were obtained from Sigma–Aldrich

(Steinheim, Germany). Dimethylsulfoxide (DMSO) was purchased from VWR International (Milan, Italy).

2.1. Concentration selection

A major factor to consider when conducting experiments in a laboratory setting is the concentration to select to accurately reflect what occurs in the real world. Since no data existed on genotoxicity and cytotoxicity of TCS for *D. Polymorpha* prior to this work, the chemical concentrations were chosen by following the two PNECs proposed by Orvos et al. (2002) and Capdevielle et al. (2007). We tested 1 nM (290 ng/L), 2 nM (580 ng/L) and 3 nM (870 ng/L) of TCS, which are between the lowest (70 ng/L) and the highest (1550 ng/L) PNECs now available for TCS. The tested concentrations also reflect TCS levels that are expected to exist or have been measured in moderate to highly polluted ecosystems.

2.2. Mussel acclimation and maintenance conditions

Several hundred specimens were sampled (from depth of 3–6 m) from Lake Maggiore (Northern Italy) by a scuba diver and transferred to the laboratory in bags filled with lake water. Mussels were introduced into several glass aquaria filled with about 100 L of dechlorinated tap water and maintained on a natural photoperiod, constant temperature ($20 \pm 1 °C$), pH (7.5) and oxygenation (>90% of saturation). Animals were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA) and water was constantly changed for at least three weeks to purify the molluscs of the accumulated xenobiotics.

Several specimens with similar shell lengths (about 20 mm) were selected for each *in vivo* test, including control and solvent assays. Mussels were placed on glass sheets suspended in small glass aquaria (15 L) and maintained for one week under the same conditions described above. Only specimens that were able to re-attach themselves by their byssi on glass sheets were used in the experiments. Mussels were used for the subsequent *in vivo* experiments only when target biomarkers showed values that were comparable with the previously checked baseline levels.

2.3. Exposure assays

Experiments were performed in semi-static conditions with daily changes of the entire volume of water and the addition of the quantity of chemicals up to the selected concentrations. As carrier of contaminant we used dimethyl sulfoxide (DMSO) that reached a maximum percentage lower than 0.00001% in water (TCS working solution = 10 mg/L).

Since the mineralization half-life of this antibacterial agent is reported to be about 3 d (Federle and Schwab, 2003), a complete change of water and TCS should decrease the transformation of the parent compound into its metabolites.

About 200 specimens were added in each aquarium. Mussels were fed daily with AlgaMac-2000®, which was added 2 h before the water and chemical changes. Temperature, oxygenation and pH were checked daily. Four pools of mussels were collected each day from the aquaria for the SCGE assay, MN test, apoptosis frequencies and NRRA. Cell viability was checked daily by the Trypan blue exclusion method (10 μL of cell suspension added to 10 μL of 0.4% of dye) using the Burker chamber. Viability was always higher than 80%.

2.4. SCGE assay

The SCGE assay was performed using the alkaline (pH > 13) version of the assay developed by Singh et al. (1988), with the sub-

sequent optimization for Zebra mussels detailed by Buschini et al. (2003).

Hemolymph (100 μL) from ten individual mussels was withdrawn from the sinus near the posterior adductor muscle using a hypodermic syringe containing 200 μL of phosphate-buffered saline (PBS; pH 7.4). Ten 10 μL aliquots of cell suspension mixed with 85 μL of Low Melting Agarose (LMA—0.7%) in PBS (37 °C) were added to ten coated slides (previously dipped in 1% Normal Melting Agarose). The slides were covered with cover-glass and placed at 4 °C for 40 min until the agarose layer solidified. A third agarose layer was added to the slides in the same way. After agarose solidification, slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar at 4 °C in the dark for at least one hour.

Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber containing freshly prepared buffer (1 mM Na$_2$EDTA, 300 mM NaOH, pH 13) and then in an ice-water bath (4 °C). Electrophoresis was then performed at 0.78 V/cm and 300 mA for 10 min. Slides were washed after electrophoresis in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) and fixed in absolute ethanol. After staining with DAPI (4′,6-diamidino-2-phenylindole) DNA dye (Sigma–Aldrich), a coverslip was placed over the slides. Imaging was done with a fluorescence microscope (Leitz DMR, Germany) equipped with a FITC filter. All steps were performed in the dark to minimize additional UV-induced DNA damage. Positive controls were carried out with H$_2$O$_2$ to check the effectiveness of the electrophoresis conditions. All samples were blindly coded and always evaluated by only one observer. Each slide was scored following a pre-arranged pathway to reduce the observer's subjectivity. Fifty cells per slide, a total of 500 cells per sample, were analyzed using an image analysis system (Comet Score®). The ratio between the migration length and diameter of the comet head (LDR) was chosen to represent DNA damage. This ratio was coupled to the percentage of tail DNA obtained by the Comet Score®, since the working group on genetic toxicology testing from the 4th IWGT recently agreed that the percentage of tail DNA is the measure that seems most linearly related to dose and the easiest to understand (Kirkland et al., 2007).

2.5. Apoptosis

The percentage of apoptotic cells was evaluated through the same protocol used for the SCGE assay, as described by Singh (2000). Each hemocyte pool was removed and distributed between five different slides, which were processed via the same method described for the SCGE assay. Hemocytes were lysed, and slides were subjected to 5 min of alkaline (pH 13) DNA unwinding without subsequent electrophoresis. Slides were then washed in neutralization buffer, stained and fixed in absolute ethanol. Finally, they were labelled with DAPI and observed under the fluorescence microscope. Two hundred cells per slide were analyzed for a total of 1000 cells per sample. According to the intense autolysis of genomic DNA, apoptotic cells were characterized by nuclear remnants that resemble pinheads and were surrounded by very large DNA halos. Alternatively, necrotic cells were characterized by circular, faint halos and were eliminated from the count.

2.6. Micronucleus test

The MN test was performed according to the method of Pavlica et al. (2000). Hemolymph (100 μL) was obtained from the posterior adductor muscle of 10 mussels with a hypodermic syringe containing 100 μL of PBS and ethylene-diamine-tetra-acetic acid (EDTA, 10 mM) solution (1:1). The cell suspension was placed on a slide and left for 15 min in a humidified chamber at room tempera-

ture to allow the hemocytes to settle. The slides were subsequently fixed in a glutaraldehyde (25% solution) solution (1% in PBS) for 5 min. After rinsing with PBS, the slides were stained with bis-benzimide 33258 (Hoechst; CAS number = 23491-45-4) at a final concentration of 1 mg/mL for 5 min and then washed and mounted in glycerol–McIlvaine buffer (1:1). Slides were kept in the dark at 4 °C prior to examination under the microscope. All samples were coded and blindly evaluated always by only one observer. Each slide was scored following a pre-arranged pathway to reduce the observer's subjectivity. Slides were scored under the fluorescent microscope Leitz DMR equipped with a submerged lens at 100× magnification. Four hundred cells were counted for each slide for a total of 4000 cells/treatment. Only intact and non-overlapping hemocytes were scored. Micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.7. Neutral red retention assay

The NRRA method followed the protocol proposed by Lowe and Pipe (1994) and ICES (International Council for the Exploration of the Sea, 2004), whose rationale was that healthy cells could take up and retain larger quantities of the dye (neutral red) than damaged cells.

The neutral red stock solution was made by dissolving 20 mg of dye in 1 ml of DMSO, while the working solution was prepared by diluting 5 μL of stock solution in 2.5 mL of PBS. Microscope slides were coated with 2 μL of polylysine with the help of a coverslip. Five slides were used for each sample.

Hemolymph was removed using a hypodermic syringe containing 100 μL of PBS/EDTA (1:1). An aliquot of 80 μL of hemocyte resuspension was placed carefully on each slide. Slides were suspended on a rack in a light-proof humidity chamber for 20 min, and excess solution was carefully tipped off. Finally, 40 μL of neutral red working solution were added. After 20-min incubation in the humidity chamber, slides were observed under an optical microscope. Slides were thereafter examined systematically at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests were terminated when the dye loss was evident in at least 50% of the hemocytes (Lowe et al., 1995). The mean retention time was then calculated from the five replicates. Between every microscope observation, slides were returned to the humidity chamber.

2.8. Statistical analyses

Data normality and variance homogeneity were verified using the Kolmogorov-Smirnov and Barlett's tests, respectively. We used log-transformed LDR values to normalize the variance of the Comet test results, while an angular transformation (arcsin $\sqrt{P}$) was used for the MN test data.

A two-way analysis of variance (ANOVA) was performed using biomarker end points as variables and tested concentrations and exposure times as cases. ANOVA was followed by a Bonferroni *post-hoc* test to evaluate the eventual significant differences ($p < 0.05$) between treated samples and related controls (time to time) and among the exposed samples. All statistical analyses were performed using the software package STATISTICA 6.0.

3. Results

3.1. Control and solvent assays

No mortality or significant changes in hemocyte counts were recorded in TCS-treated mussels with respect to controls. Viabil-

ity was checked daily and was always higher than 80%, above the threshold (70%) for the SCGE assay proposed by the expert panel of the IWGTP (International Workshop on Genotoxicity Test Procedures; Tice et al., 2000). No significant differences ($p > 0.05$) were found between control and solvent exposures for any of the tested biomarkers, and no significant temporal differences ($p > 0.05$) were noticed within these two groups during the exposure tests.

3.2. SCGE assay

TCS induced significant ($p < 0.01$) primary genetic damage in the Zebra mussel hemocytes at all of the tested concentrations (Fig. 1a and b). At the end of exposure, 1 nM and 2 nM TCS increased the levels of DNA damage by about two times, while the LDR at 3 nM was more than three times higher than baseline levels (Fig. 1a). Although the primary DNA damage seemed to follow the same trend in the two lowest tested concentrations, a clear dose/effect relationship ($F = 736.6$; $p < 0.01$) was obtained for all the doses, as well as a significant ($F = 511.4$; $p < 0.01$) time/effect correlation.

The same temporal trend was also obtained with the second measured end point, which showed a significant increase ($p < 0.01$) of DNA fragments in the comet tails in all the experiments (Fig. 1b). A percentage of tail DNA ranging from 18% to 31% of fragmentation was already obtained after 24 h of exposure, indicating that TCS has a fast genotoxic effect on Zebra mussel hemocytes; fragmentation increased to 31–40% by the end of the experiment.
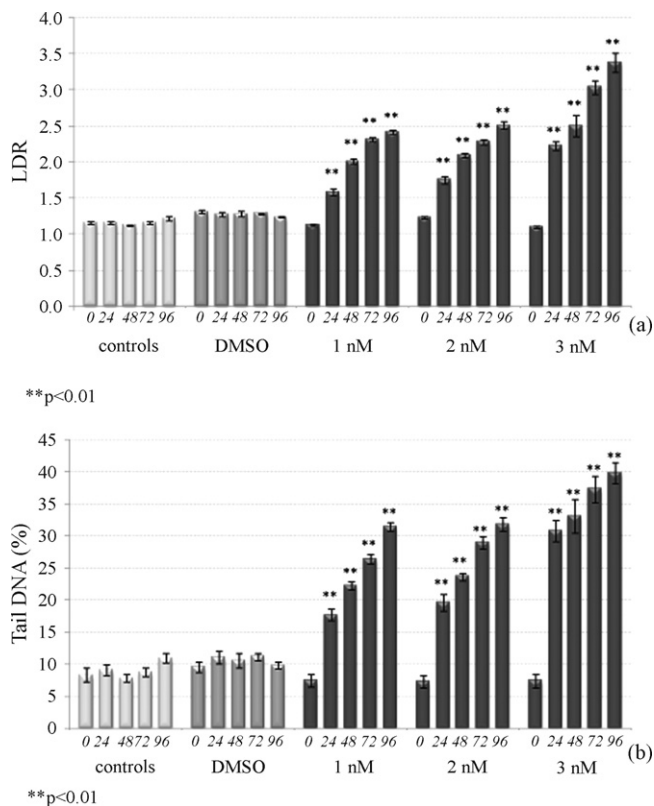

Fig. 1. Temporal trend (h) of the means of LDRs ± SEM (a) and of the % tail DNA (b) calculated for Zebra mussel hemocytes by the Comet-Score® for controls, solvents and treated samples with TCS. Significant values (two-way ANOVA, Bonferroni *post-hoc* test, $p < 0.05$) refer to the comparison between treated samples and controls at the same time.
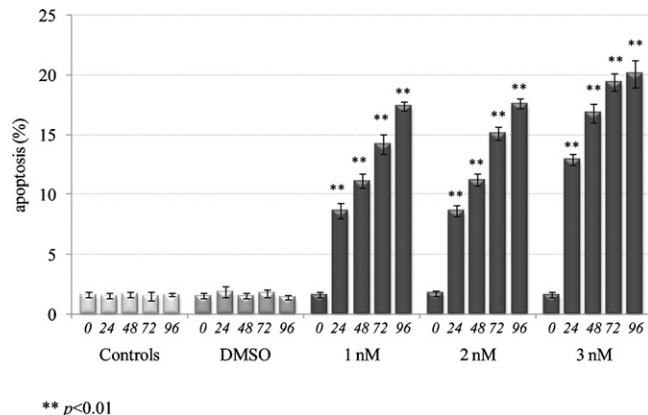
Fig. 2. Temporal trend (h) of the means of apoptosis frequency ± SEM calculated for Zebra mussel hemocytes for controls, solvents and treated samples with TCS. Significant values (two-way ANOVA, Bonferroni *post-hoc* test, $p < 0.05$) were obtained for the comparison between treated samples and controls at the same time.

3.3. Apoptosis

We observed concentration-dependent ($F = 566.1$; $p < 0.01$) and time-dependent ($F = 346.3$; $p < 0.01$) increases in the percentage of apoptotic hemocytes that were significantly different ($p < 0.01$) from the controls in all of the experiments (Fig. 2). A concentration of 1 nM TCS (290 ng/L) was able to produce an apoptotic frequency that was about six times higher than the baseline level. The highest percentage of apoptosis ($20.1 \pm 2.6\%$), about 12 times that of the controls, was obtained with 3 nM TCS.

3.4. MN assay

Significant ($p < 0.01$) irreversible genetic damage was found in all of the experiments (Fig. 3), with a concentration-dependent ($F = 141.2$; $p < 0.01$) and time-dependent increasing trend ($F = 56.8$; $p < 0.01$). TCS was able to quickly interfere with the DNA, as indicated by a MN% of more than twice the baseline levels after 24 h and between 20 and 40‰ at the end of the exposure. Moreover, the genotoxic potential of TCS seems to act also at low concentrations in Zebra mussel specimens, as shown by the significant increase in MN frequency that was observed with 1 nM of TCS.
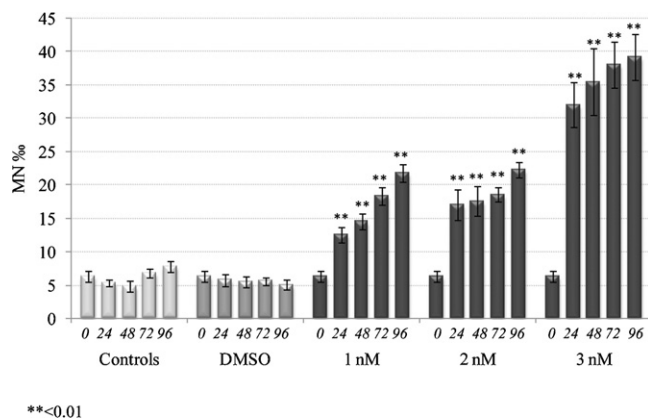
Fig. 3. Temporal trend (h) of the means of micronuclei frequency ± SEM calculated for Zebra mussel hemocytes for controls, solvents and treated samples with TCS. Significant values (two-way ANOVA, Bonferroni *post-hoc* test, $p < 0.05$) were obtained in the comparison between treated samples and controls at the same time.

Fig. 4. Temporal trend (h) of the means of retention times ± SEM of the Neutral Red dye calculated for Zebra mussel hemocytes for controls, solvents and treated samples with TCS. Significant values (two-way ANOVA, Bonferroni *post-hoc* test, $p < 0.05$) refer to the comparison between treated samples and controls at the same time.
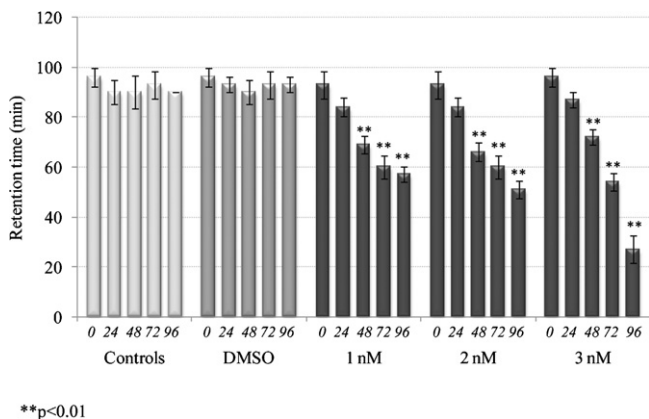
3.5. Lysosomal membrane stability

Lysosomal membrane stability is considered to be the most reliable of the recommended biomarkers for water quality assessment (UNEP, 1997). The retention time (RT) of the Neutral Red dye observed in control assays was about 90 min (Fig. 4), which is comparable with that found in previous studies carried out with *D. polymorpha* (Binelli et al., 2008) but lower than those measured in the genus *Mytilus* (Lowe et al., 1995; Fernley et al., 2000; Mamaca et al., 2005).

TCS was able to produce a destabilization of the lysosomal membrane in a dose-dependent ($F = 32.6$; $p < 0.01$) and time-dependent manner ($F = 49.5$; $p < 0.01$). The general cellular stress measured with this assay did not appear until after 48 h, since no significant membrane destabilization was noticed in the first 24 h. No statistical differences were measured between 1 nM and 2 nM values, while the highest tested dose (3 nM) was significantly ($p < 0.05$) different from the other two concentrations at $t = 96$ h.

The dye decrease that was measured at the end of the exposure time ranged between 37% and 70% of the baseline levels, demonstrating that this antibacterial agent was clearly able to induce cellular stress.

4. Discussion

Because PPCPs are found in the effluents of wastewater treatment plants, an understanding of potential effects of these chemicals on aquatic organisms is important for proper environmental monitoring and management. The acute and chronic ecotoxicological tests, which are often not sensitive enough and not able to detect adverse effects of pharmaceuticals, cover only a small set of laboratory organisms and more specific tests are needed (Fent et al., 2006). A tiered, structured approach based on biomarkers might be a complementary tool to gain in-depth knowledge of the potential environmental risk of PPCPs. The *in vitro* experiments should be used as preliminary screening, both for determining the most toxic chemicals that require follow-up testing and for gaining basic knowledge of the nature of the tested agents and/or the cellular response (Fent et al., 2006; Kirkland et al., 2007). Moreover, the use of only one cell type in *in vitro* toxicological tests can minimize the individual variability that is often seen in biological responses (Zuccato et al., 2006). Using the *in vivo* approach, carried out under laboratory conditions, allows us to gain toxicological information for the selected compound in the absence

of complicating environmental factors. The main challenge is the selection of contaminant levels and exposure route (e.g. injection or addition of chemical to water). Several studies have been carried out using very high concentrations of PPCPs, far higher than their real environmental levels. On one hand, using this level can reveal possible effects that the tested chemical can exert on the selected biological model; on the other hand, these experiments may not accurately reflect the real risk for the aquatic biocoenosis. The final step of this tiered approach is the testing of PPCP mixtures, which are only rarely studied, and field studies to confirm laboratory findings.

We have carried out the first two steps of this tiered approach. In a separate paper, we report on the *in vitro* experiments (Binelli et al., 2008) that we have conducted with TCS under identical conditions for mussel maintenance, hemocyte withdrawal and sample treatment as in the present *in vivo* assays. The only differences were related to the time of exposure to TCS (1 h) and to the centrifugation to stop the effect of contaminant. We demonstrate that TCS has a clear genotoxic potential when administered at moderate concentrations (0.1, 0.15, 0.2, 0.3 μM) but does not induce a destabilization of the lysosomal membrane stability, a classical parameter of cellular stress in bivalve (Lowe et al., 1995). This result was also previously reported by Canesi et al. (2007), who noticed no significant differences in the stability of the lysosomal membrane in hemocytes of *Mytilus galloprovincialis* in *in vitro* experiments using 0.1 μM of TCS. In contrast, clear damage was observed at TCS concentrations of 1 μM and above. Thus, these assays, which were performed with two different biological models, show that *in vitro*, only high concentrations of TCS are able to interfere with the lysosomal membranes of bivalves. Moreover, our *in vitro* results, combined with the observation that *in vivo* there is significant destabilization of lysosomal membranes (Fig. 4), seem to indicate that the effect of TCS on mussel lysosomes is probably caused by a dramatic increase in extra-cellular oxidative stress that is produced through metabolic pathways, a process that is impossible to predict from *in vitro* assays. On the other hand, good correlation has been reported between the impairment of antioxidant activity, the neutral red retention time and changes in DNA integrity in different organisms (Regoli et al., 2002, 2004; Camus et al., 2002; Mamaca et al., 2005). Other authors (Kirchin et al., 1992; Winston et al., 1991, 1996) have suggested that reactive free radicals, such as reactive oxygen species (ROS) and xenobiotic derivatives, contribute to the damaging effects on the lysosomal membranes and that the intralysosomal environment is already a site of oxyradical production (Winston et al., 1991; Livingstone, 2001).

The genotoxicity of TCS in Zebra mussel hemocytes has previously been demonstrated with other *in vivo* biomarkers (Comet and MN assays, apoptosis), which were significantly different from baseline levels as early as 24 h after initial exposure. Since the first significant lysosomal membrane destabilization was not observed until 48 h of exposure, it is likely that other complementary toxic pathways exist, in addition to the production of oxidative stress as a consequence of membrane interference.

The results that we obtained from both the *in vitro* and *in vivo* experiments allow us to suggest a possible mechanism of action for TCS in *D. polymorpha* (Fig. 5). Unfortunately, our data can be compared only with results from studies of different biological models, because no other data on Zebra mussels exposed to PPCPs exist.

The increase of oxidative stress is likely to be the main toxic pathway, since the NRRA carried out *in vivo* indirectly confirmed the production of ROS. TCS also stimulates the activity of glutathione transferase (GST), which conjugates glutathione to both endogenous and exogenous substrates, indicating that this compound may represent a substrate for phase II enzymes of blue mussel (Canesi et al., 2007). Also, Gagné et al. (2006) found that there were changes
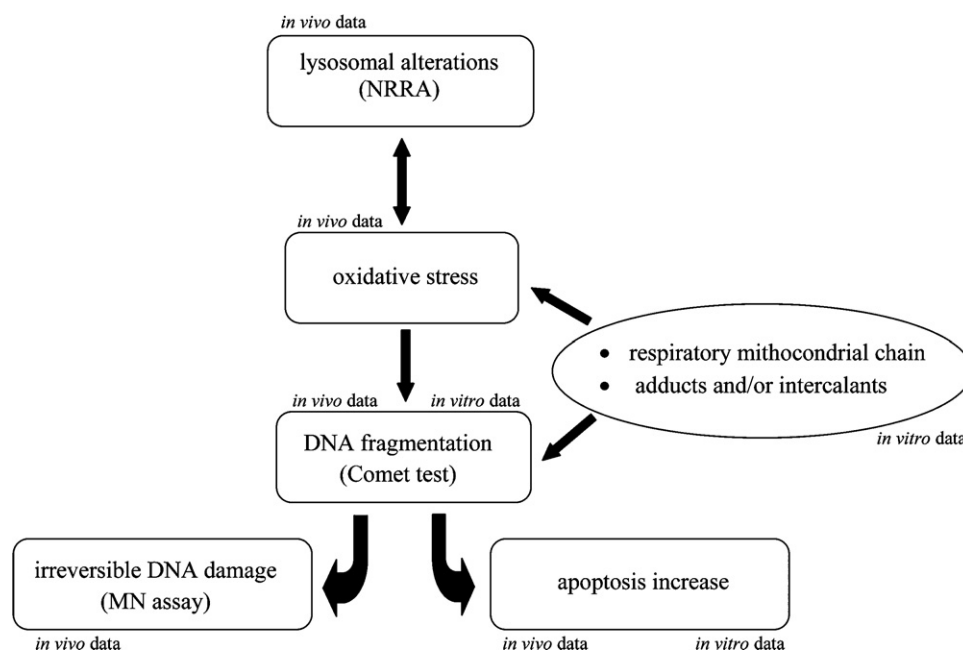
Fig. 5. Possible mechanisms of action of Triclosan in hemocytes of Zebra mussel.

in the activities of antioxidant enzymes and levels of lipid peroxidation in tissues of the freshwater bivalve *Elliptio complanata* upon exposure to municipal effluents containing PPCPs. The increase of ROS and the consequent oxidative stress could produce an increase in DNA fragmentation, as indicated by the Comet test (Fig. 1 a and b). This effect has already been noted with moderate levels of TCS, corresponding to 290 ng/L. Although the time-dependent increase in the amount of genetic damage can be partially overcome by the defense mechanisms of the mussel, the LDRs that were observed at the end of the exposure time are comparable with those found in previous experiments performed with benzo(α)pyrene (Binelli et al., 2008).

The dramatic increase in DNA fragmentation that was observed in the SCGE assay make also irreversible genetic damage, as indicated by the positive correlation ($R = 0.57$; $p < 0.05$) between LDRs and micronucleus millesimal frequency (MN‰). Also the MN‰ of TCS is similar to that of another known genotoxic compound, B(α)P: the MN frequency (39‰) obtained with 3 nM TCS was more than double the MN percentage (15‰) obtained with 7.9 nM of B(α)P after 96 h of exposure (Binelli et al., 2008).

Damaged hemocytes can choose to follow the programmed cell death (PCD) pathway if DNA injuries dramatically increase and its defense systems are not able to repair the genetic damage. The frequency of apoptosis was positively correlated to both LDRs ($R = 0.84$; $p < 0.05$) and MN frequencies ($R = 0.57$; $p < 0.05$) and inversely correlated with NRRT ($R = -0.73$; $p < 0.05$). This last correlation is particularly interesting because it suggests a possible relationship between the lysosomal membrane destabilization and PCD. On the other hand, lysosomes in digestive gland epithelial cells of different species of marine mussels that are exposed to contaminants have characteristic pathological alterations that include swelling of the digestive cell lysosomes, increased fragility of the lysosomal membrane, excessive build-up of unsaturated neutral lipid in the lysosomal compartment and accumulation of lipofuscin (Lowe, 1988; Moore, 1990). These changes are accompanied by atrophy of the digestive epithelium, which could involve augmented autophagic processes, although there is also evidence for cell deletion analogous to apoptosis in mammals (Lowe, 1988). PCD can be divided into apoptosis (PCD Type I) and autophagic cell

death (PCD Type II); it appears to be a phylogenetically ancient phenomenon and occurs in both physiological and disease states (Bursch, 2001; Zhao et al., 2001). Lysosomal changes are involved in both types of cell death, and they should not be considered mutually exclusive processes (Bursch, 2001). Thus, changes in lysosomes can predispose the cells to deletion by programmed cell death or can simply represent a symptom of the beginning of the chain-reaction that leads to PCD, including increases in DNA fragmentation and oxidative stress.

Our *in vitro* data suggest that there are other complementary mechanisms of action (Fig. 5), since several genotoxic effects of TCS were noticed (Binelli et al., in press) in the absence of the extracellular antioxidant pathways. The first mechanism is a possible increase of intracellular oxidative stress, which could arise from a pathological increase in the production of oxyradicals (mainly H_2O_2, which is produced in mitochondria by dismutation of $O_2^{\bullet-}$) through the cellular mitochondrial chain enzymatic complexes (Brunk and Terman, 2002; Kudin et al., 2004; Moore et al., 2006). Since no statistical differences between exposed and untreated hemocytes were noticed in the neutral red retention assay *in vitro* (Binelli et al., 2008), it appears that low concentrations may only weakly increase oxidative stress to levels that are not able to significantly destabilize lysosomal membranes. Alternatively, TCS may act as a DNA adduct and/or DNA intercalant to directly exert genotoxic effects. Additionally, TCS has been reported to act as a precursor of the 2,8-dichlorodibenzo-*p*-dioxin (Aranami and Readman, 2007), which is a well-known DNA-intercalating agent.

5. Conclusions

In conclusion, our results seem to indicate that there are different complementary mechanisms of action to explain the genotoxicity of TCS in Zebra mussel, connected both to oxidative stress and/or to a direct effect on DNA. Other, in-depth studies, such as the evaluation of antioxidant enzymes, the possible interference with hormone metabolism and the role played by its metabolites, will be necessary to fully understand the cytotoxic effect of TCS on this sentinel-organism. Moreover, our data demonstrate the potential genotoxicity and cytotoxicity of environmental levels of TCS in

Zebra mussel specimens and point out a possible danger for the entire aquatic community.

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PAPER IV

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Antioxidant activity in the Zebra mussel (*Dreissena polymorpha*) in response to Triclosan exposure

Antioxidant Activity in the Zebra Mussel (*Dreissena polymorpha*) in Response to Triclosan Exposure

Andrea Binelli · Marco Parolini ·
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Abstract The biocide triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy)phenol) is commonly used in several personal care products, textiles, and children's toys. Because the removal of TCS by wastewater treatment plants is incomplete, its environmental fate is to be discharged into freshwater ecosystems, where its ecological impact is largely unknown. The aim of this study was to determine the effect of TCS on the antioxidant enzymatic chain of the freshwater mollusk zebra mussel (*Dreissena polymorpha*). We measured the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST) in zebra mussel specimens exposed to 1 nM, 2 nM, and 3 nM TCS in vivo. The mussels were exposed for 96 h, and the enzyme activities were measured every 24 h. We measured clear activation of GST alone at all three dose levels, which shows a poor induction of the antioxidant enzymatic chain by TCS. CAT and SOD were activated only at 3 nM, while GPx values overlapped the baseline levels.

Keywords Biomarkers · Pharmaceuticals · Risk assessment · Enzymes · Mussels

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1 Introduction

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy)phenol) is a widely used antibacterial agent, the applications of which range from many consumer products (soaps, toothpastes, clothes, deodorants, and cosmetics) to textiles and children's toys. Approximately 1,500 tons of TCS are produced annually worldwide, of which 350 tons/year are utilized in Europe (Singer et al. 2002). Despite the entry of TCS into wastewater treatment plants (WWTPs) at low (μg l^{-1}) concentrations, it is incompletely removed, resulting in final effluent concentrations in the range of several hundreds of nanograms per liter (Capdevielle et al. 2007; Gomez et al. 2007). Thus, TCS has been reported as one of the most frequently detected pharmaceuticals and personal care products (PPCPs) in surface waters (Kolpin et al. 2002). Moreover, reactions with free chlorine in WWTPs and photolysis of the parent compound may increase the production of several dangerous by-products, as recently found by Buth et al. (2009). These authors demonstrated the photochemical conversion of three chlorinated TCS derivatives to three polychlorodibenzo-*p*-dioxins that possess higher toxicity than 2,8-dichlorodibenzo-*p*-dioxin, the sole TCS photoproduct previously known.

The high lipophilicity of this antibacterial agent (log K_{ow}=4.8) (Coogan et al. 2007) represents another environmental problem, as TCS possesses the potential to bioaccumulate and to produce many deleterious effects on aquatic nontarget organisms. Adolfsson-Erici

et al. ([2002]) found TCS in four fish species from Sweden (0.24–120 mg kg^{-1} fresh weight), and Coogan et al. ([2007]) recently measured concentrations of TCS in the filamentous algae *Cladophora* spp., ranging between 100 ng g^{-1} and 150 ng g^{-1} fresh weight. The study of Coogan and La Point ([2008]) on TCS bioaccumulation using the caged aquatic snail *Helisoma trivolvis* showed rapid bioaccumulation up to 58.7 ppb in snail tissue.

Several researchers have shown many adverse effects due to TCS exposure in different organisms. Recently, Oliveira et al. ([2009]) demonstrated deleterious effects on zebrafish (*Danio rerio*), both as adults and during the early stages, including embryotoxicity, hatching delay, and biomarker alterations. There is also much evidence demonstrating endocrine disruption resulting from TCS exposure: Veldhoen et al. ([2006]) showed that TCS acts as an endocrine disruptor in *Rana catesbeiana*, inducing early metamorphosis in a naturally premetamorphic stage of the tadpole. Other studies also indicate endocrine disruptor effects of TCS in Japanese medaka (*Oryzias latipes*), where it demonstrated to be androgenic (Foran et al. [2000]) or estrogenic (Ishibashi et al. [2004]).

In a previous study, our research group demonstrated high cytogenotoxic effects of this pollutant on the zebra mussel (*Dreissena polymorpha*) when they are exposed to environmental concentrations of TCS (Binelli et al. [2009a]). In particular, TCS was able to produce a significant increase of DNA fragmentation after only 24 h of exposure, followed by a rise of micronucleated and apoptotic cells at levels even higher than those observed after exposure to benzo(α) pyrene (Binelli et al. [2008]). We also noticed a significant ($p<.05$) destabilization of the lysosomal membranes, even when starting from 48 h of exposure. These data and comparisons with results obtained previously regarding in vitro exposure of the hemocytes of this freshwater mussel to TCS (Binelli et al. [2009b]) drove us to hypothesize different complementary mechanisms of action for TCS in *D. polymorpha* in which the direct effect on DNA and oxidative stress are connected (Binelli et al. [2009a]).

The aim of this study was to verify the role played by TCS in the increase of oxidative stress by measuring the activity of some antioxidant enzymes. In particular, we determined the activities of superoxide dismutase (SOD), catalase (CAT),

and glutathione peroxidase (GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST), which is strongly tied to the defense chain that is activated against reactive oxygen species (ROS). We exposed several zebra mussel specimens to identical concentrations of TCS (1 nM, 2 nM, and 3 nM) using the same maintenance conditions as followed in our previous experiments. We evaluated the enzyme activities everyday over a total exposure time of 96 h. This study represents the first assessment of the potential effect of TCS on *D. polymorpha* antioxidant defense mechanisms and is also the first evaluation of the role played by oxidative stress on the DNA damage observed in this biological model, which represents a very useful biological model and a key species between the pelagic and benthic compartments.

2 Materials and Methods

The TCS standard (CAS number 9012-63-9, 97% purity) and other chemicals used for biomarker determination were obtained from Sigma-Aldrich (Steinheim, Germany). Dimethylsulfoxide (DMSO) was purchased from VWR International (Milan, Italy).

2.1 Mussel Acclimation and Maintenance Conditions

Several hundred specimens were sampled (3–6 m of depth) from Lake Maggiore (Northern Italy) by a scuba diver and transferred to the laboratory in bags filled with lake water. The mussels were rinsed under running tap water and introduced into several glass aquaria filled with approximately 100 L of dechlorinated tap water and maintained on a natural photoperiod, at constant temperature (20±1°C), pH (7.5) and oxygenation (>90% of saturation). The mussels were fed daily with an algae replacement-substitute enrichment medium (AlgaMac-2000®; Bio-Marine Inc., Hawthorne, CA), and the water was changed every 2 days for at least 3 weeks to purify the mollusks of any accumulated xenobiotics.

Several specimens of similar shell length (about 20 mm) were selected for each in vivo test, including the control and solvent assays. Mussels were placed on glass sheets suspended in small glass aquaria (15 L) and maintained for 1 week under the same

conditions as described above. Only specimens able to reattach themselves through their byssi to the glass sheets were used in the experiments. Mussels were used for the subsequent in vivo experiments only when target biomarkers were at comparable levels with previously checked baseline.

2.2 Exposure Assays

Experiments were performed in semistatic conditions with daily changes of the entire volume of water and with chemicals added to the selected concentrations. We selected the same doses used in the previous study that was carried out to evaluate the in vivo cytogeno-toxicity of TCS (Binelli et al. 2009a): 1 nM (290 ng/L), 2 nM (580 ng/L), and 3 nM (870 ng/L) of TCS. These values ranged between the lowest (70 ng/L) and the highest (1,550 ng/L) predicted no-effect concentrations (PNECs) currently available for TCS (Orvos et al. 2002; Capdevielle et al. 2007).

As the contaminant carrier, we used DMSO in water at a maximum percentage of less than 0.009% (TCS working solution=10 mg l^{-1}).

Fifty mussels were added to each aquarium and fed daily with AlgaMac-2000®, which was added 2 h before the water and chemical changes. The temperature, oxygenation, and pH were checked daily. A pool of six to eight mussels was collected each day from each aquarium, snap frozen in liquid nitrogen, and stored at −80°C for subsequent enzymatic assays.

The enzymatic activities were determined spectro-photometrically as described by Orbea et al. (2002) using the entire organism as the small size of the mussels (average length=20 mm) did not allow the use of different tissues due to the small quantity of enzymes contained. Measurements were carried out in triplicate using the cytosolic fraction extracted from a pool of six to eight entire mussels (≈1-g fresh weight) homogenized in 100 nM phosphate buffer (100 mM KCl and 1 mM EDTA, pH 7.4) using a Potter homogenizer. We also added three specific protease inhibitors (1:10): dithiothreitol (DTT, 100 mM), phe-nanthroline (Phe, 10 mM), and trypsin inhibitor (TI, 10 mg/ml). The homogenate was centrifuged at 2,500 rpm (500×g) for 15 min at 4°C. The superna-tant was then transferred into clean tubes and centrifuged again at 12,000 rpm (2,000×g) for 30 min at 4°C. Finally, the supernatant was ultra-centrifuged at 45,000 rpm (100,000×g) for 90 min at

4°C. The cytosolic fraction was held on ice and immediately processed for protein determination and enzymatic activity assay.

CAT activity was determined in the cytosolic fractions by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 50 mM potassium phosphate buffer, pH 7.

SOD activity was determined in the cytosolic fraction as the inhibition of the rate of cytochrome c reduction (observed at 550 nm) by superoxide anion generated from the xanthine oxidase/hypoxanthine reaction. The final concentrations of the reagents were as follows: potassium phosphate buffer (50 mM, pH 7.8), hypoxanthine (0.05 mM), xanthine oxidase (0.008 mU/ml), and cytochrome C (0.01 mM). The activity is given in SOD units (1 SOD unit=50% inhibition of the xanthine oxidase reaction).

GPx activity was measured in the cytosolic fraction by monitoring the consumption of NADPH at 340 nm during the formation of reduced glutathione by gluta-thione reductase. The reaction medium consisted of the following: 0.2 mM H_2O_2 substrate in 50 mM potassi-um phosphate buffer, pH 7, containing additional glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U/ml), and NADPH (0.12 mM).

GST activity was measured by adding reduced glutathione (1 mM) and 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM) in phosphate buffer (80 mM, pH 7.4) to the cytosolic fraction. The resulting reaction was monitored for 1 min at 340 nm.

The total protein content of all samples was measured using the method of Bradford (1976) and bovine serum albumin as the standard.

2.3 Statistical Analyses

Data normality and variance homogeneity were verified using the Shapiro-Wilk test and Levene's test, respectively. To identify dose/effect and time/effect relationships, we performed a two-way analysis of variance (ANOVA) using time and the concentra-tion of TCS as variables and biomarker end points as cases. The ANOVA analysis was followed by Bonferroni post hoc tests to evaluate the eventual significant differences ($p<.05$) between the treated samples and the related controls (comparing time with time) and among the exposures.

Pearson's correlation test was carried out to compare the antioxidant enzyme activities and cyto-

genotoxic biomarkers (micronucleus test, apoptosis determination, the Comet test and destabilization of the lysosomal membranes) in the three exposure assays to investigate possible correlations between various biological responses. All statistical analyses were performed using the STATISTICA 7.0 software package.

3 Results

We tested the solvent (DMSO) for possible effects on the selected enzymes by using the highest concentration reached in water (0.009%) as an additional control. This assay showed that DMSO did not produce significant ($p > .05$) changes in enzyme activity, and no significant ($p > .05$) temporal differences were noticed within the control and solvent groups during the exposure tests. All control data from these enzymatic assays agreed with those obtained in previous studies (Osman and Van Noort 2007; Faria et al. 2009) using *D. polymorpha*. Moreover, no significant ($p > .05$) differences were noticed among the controls for the entire period of exposure, showing the validity of the selected maintenance conditions.

The activity of the phase II detoxifying enzyme, GST, showed a very similar temporal behavior for the three different concentrations tested, as we noticed a clear increase of activity after just 24 h of exposure (Fig. 1). The maximum significant ($p < .01$) induction of GST was obtained at $t = 48$ h, at which time it had been increased by 53% (1 nM), 44% (2 nM), and 72% (3 nM) in comparison with the starting values. Moreover, these enzymatic activities were about

47% higher than the respective controls. In contrast, after this rise, the enzyme activity fell to values comparable to baseline levels, with the exception of the 3 nM test samples that remained significantly different from the controls.

CAT and SOD followed the same trends, as demonstrated by the lack of any observable effect from exposure to 1 nM and 2 nM TCS (Figs. 2 and 3). The only significant ($p < .05$) inductions of these two antioxidant enzymes were noticed at the highest dose after $t = 48$ h and $t = 72$ h, with a trend similar to GST. In particular, the highest values measured for CAT and SOD were 40% and 60% higher, respectively, than those observed at $t = 0$ h. Finally, GPx showed no significant ($p > .05$) induction due to TCS exposure, although a sporadic but significant ($p < .05$) inhibition was registered after $t = 72$ h at 2 nM TCS (Fig. 4). Notwithstanding these results, we found significant ($p < .05$) dose/effect and time/effect relationships for all the tested enzymes (Table 1).

4 Discussion

We tested these enzymes as they form the best-known defense chain against increasing levels of ROS and consequently oxidative stress, as shown in Fig. 5. Some xenobiotics can be biotransformed by cytochrome P450 (CYP 450) into quinone and semiquinone radicals that produce oxygen radicals ($O_2^-{}^\circ$) through the redox cycle or by CYP 450 directly. Although the production of quinones from TCS by mussels was not still investigated, Yu et al. (2006) demonstrated that the photocatalytic oxidation of this antibacterial agent produced both

Fig. 1 Temporal trend of glutathione transferase (mean±standard error of the mean [SEM]) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *$p < .05$, **$p < .01$
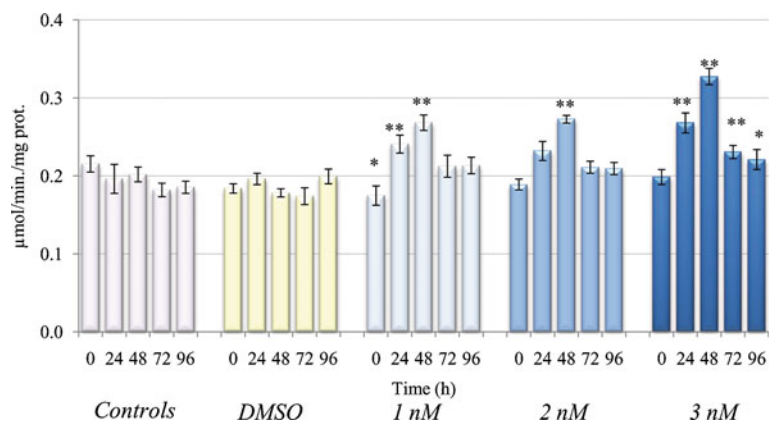
Fig. 2 Temporal trend of CAT (mean±SEM) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *p<.05, **p<.01
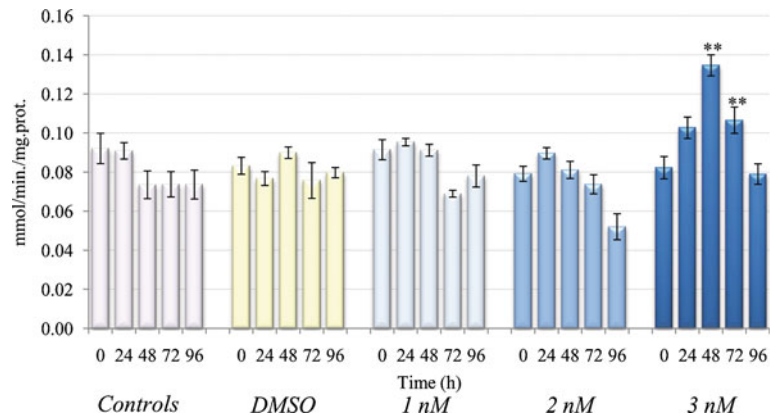
quinone (2-chloro-5-(2,4-dichlorophenoxy)-benzo-quinone) and hydroquinone (2-chloro-5-(2,4-dichlor-ophenoxy) benzene-1,4-diol) of TCS. Moreover, the same authors estimated that the maximum concentration of hydroquinone was 50% less than that of quinone. The capability of TCS to be transformed into quinones was also observed by Zhang and Huang (2003).

Another degradative pathway that can be followed by TCS is due to the role of GSTs (Fig. 5) that are members of an isoenzyme family that catalyze the conjugation of several xenobiotics to glutathione (GSH). The action of CYP 450 and GST normally produce free radical intermediates that can be then transformed into oxygen and hydrogen peroxide by SOD. Hydrogen peroxide, a powerful and potentially harmful oxidizing agent, is then metabolized into H_2O and O_2 by CAT. In addition, GPx is able to catalyze the decomposition of hydrogen peroxide, completing the defense chain against ROS. Unfortunately, if this defense mechanism is saturated or inhibited, hydro-

gen peroxide can be transformed to OH° in the presence of Fe^{2+} by the Fenton reaction. OH° is the most toxic free radical intermediate, as it is able to create several DNA injuries and can damage proteins and lipids.

The use of D. polymorpha as a biological model to investigate the role played by TCS in the increase of oxidative stress is particularly interesting as this mussel possesses a particular enzyme (DT-diapho-rase) that is able to protect itself against quinone toxicity though the transformation of semiquinones into hydroquinones. Recently, Osman et al. (2004) proposed that the in vivo formation of ROS by quinone metabolism was suppressed in D. polymor-pha. Thus, the possible increase of oxidative stress due to TCS can be also due to the possible saturation of DT-diaphorase.

This research completed the data that had been previously obtained both in vitro and in vivo on zebra mussel specimens exposed to TCS demonstrating clear and significant cytogenotoxicity (Binelli et al.

Fig. 3 Temporal trend of SOD (mean±SEM) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *p<.05, **p<.01

Fig. 4 Temporal trend of GPx (mean±SEM) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *$p < .05$, **$p < .01$
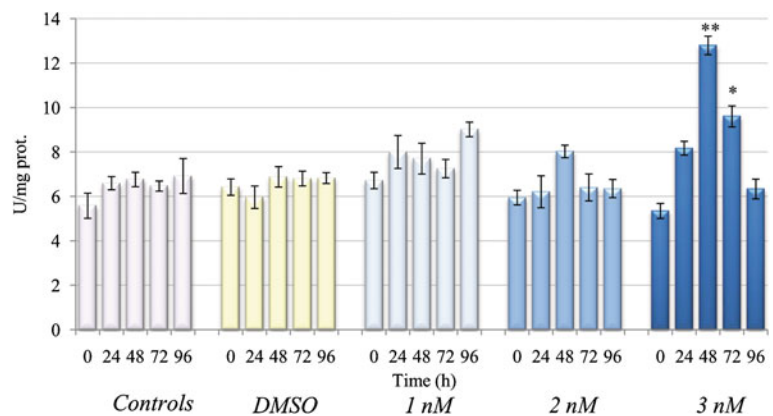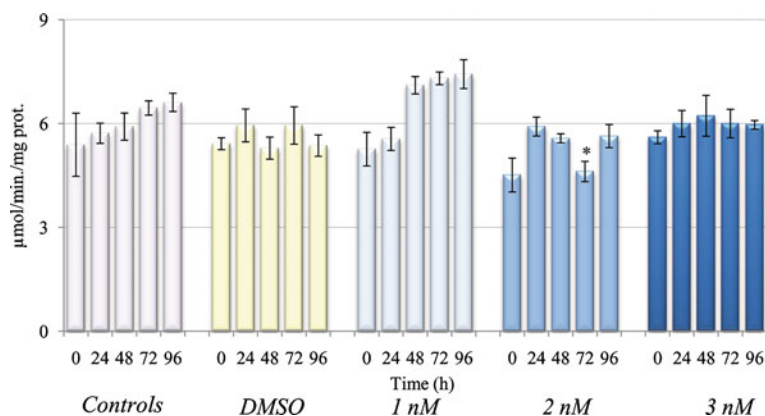

2009a, b). In proposing a possible mechanism of action of this contaminant, we supposed both a direct effect on DNA and an increase of oxidative stress that can indirectly produce several types of damage on the informational macromolecules.

Overall, triclosan did not produce an evident or significant change of the enzymatic activities of CAT (Fig. 2), SOD (Fig. 3), or GPx (Fig. 4) in zebra mussels, except at the highest TCS dose tested (3 nM). The only enzyme significantly different from controls also at the lowest administered dose was GST (Fig. 1). Moreover, we observed an intrinsic variability in enzyme activities that has been also confirmed by several authors who found absolutely inhomogeneous behavior for antioxidant enzymes (Regoli et al. 2003; Osman et al. 2007; Xiao et al. 2007). However, we can suggest possible explanations for their particular behavior with the help of cytogenotoxic results previously obtained (Binelli et al. 2009a). For instance, the parabolic slope observed for GST, CAT, and SOD at 3 nM highlights a strong response of the antioxidant defense mechanism until $t = 48$ h. In light of the heavy cytogenotoxic effects noticed in vivo using the same TCS dose (Binelli et al. 2009a), the dramatic decrease

of enzyme activity observed at the later exposure times might be due to a decrease of the entire metabolism. This could be a sign of effects occurring through the entire organism, not only at the cellular level.

A very similar behavior of the antioxidant enzymes to that measured in the present research was found by Cheung et al. (2002) in the marine bivalve *Perna viridis* exposed to polychlorinated biphenyls (PCBs): no increase in activity was observed for CAT and SOD, but a significant correlation ($p < .01$) was obtained between GST and PCB levels measured in mussel soft tissues. In addition, Oliveira et al. (2009) found that GST, as well as cholinesterase and lactate dehydrogenase, had the most sensitive end point of the biomarker analyzed when they evaluated the acute and chronic effects of TCS on zebrafish (*D. rerio*) early-life stages and adults. The highest response found in our study just for this detoxifying enzyme highlighted two different consequences: First, TCS can also be clearly considered as a substrate for the phase II enzymes in *D. polymorpha*, as previously indicated by Canesi et al. (2007). Second, the lack of activation of GPx can be due simply to the significant increase of GST activity, as these two enzymes are competitors for the same substrate, glutathione (Fig. 5). Although hydrogen peroxide can also be degraded by CAT, the failure of antioxidant defense arising from the blocking of GPx activity and poor CAT activation can lead to an overproduction of OH°, with a consequent increase of oxidative stress (at least at the highest tested concentration).

To evaluate the role played by the production of ROS in the evident DNA damage obtained previously in *D. polymorpha*, we performed a Pearson's correlation test between the cytogenotoxic data and enzymatic activi-

Table 1 Time/effect and dose/effect relationships for the four tested antioxidant enzymes (two-way ANOVA, Bonferroni post-hoc test, $p < .05$)

	CAT	GPx	SOD	GST
Time/effect	$F = 18.5$	$F = 6.73$	$F = 13.36$	$F = 60.1$
	$p < .01$	$p < .01$	$p < .01$	$p < .01$
Dose/effect	$F = 27.57$	$F = 10.77$	$F = 15.25$	$F = 76.35$
	$p < .01$	$p < .01$	$p < .01$	$p < .01$

Fig. 5 Scheme of the anti-oxidant enzymatic chain and the relative production of ROS. The measured enzymes are indicated in *green*, while the oxygen free radicals and the cytogeno-toxic damage in *red*
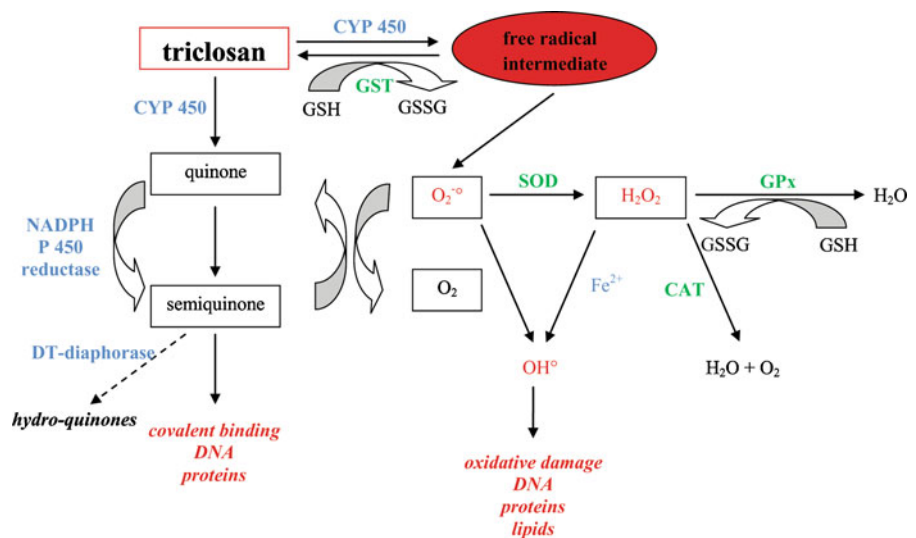
ties (Table 2). Unfortunately, this statistical approach also did not give a definitive response to the role played by oxidative stress; although significant ($p < .05$) correlations were obtained between the antioxidant enzymatic activities and the cytogenotoxic end points at concentrations of 1 nM and 3 nM, no correlations were found when using the intermediate concentration (CAT excluded). Moreover, whereas the activity of CAT was positively correlated with apoptosis and the formation of micronuclei at 3 nM, it was negatively correlated with the same end points at 1 nM, confirming the great variability of these enzymatic responses.

As our study showed a significant effect/dose relationship (Table 1) of TCS on antioxidant enzymes, we suggest that its mechanism of action in *D. polymorpha* can be due to cooperative action between the direct damage on DNA and cellular metabolism, and the role played by the antioxidant enzymes. Based on the significant increase of GST, CAT, and SOD at 3 nM, we can infer that the antioxidant protective effect is activated by TCS only at high doses, whereas the direct effect on DNA by the parental compound and/or metabolites is the main followed mechanism of action at low doses.

However, *D. polymorpha* possesses DT-diaphorase, which is able to protect the mollusk from the superoxide anion (Fig. 5). The lack of SOD activation at low doses may simply indicate the very low production rate of this radical, which is then not consequently transformed into H_2O_2, the selective substrate of CAT and GPx. Moreover, GST (the only enzyme activated at all three tested concentrations) may be sufficient to protect

mussels through the formation of other free radical intermediates that cannot activate the antioxidant enzymes. However, when the concentration of TCS rises, the double defense mechanism composed by DT-diaphorase and GST may not be sufficient to counter the production of $O_2^{-\circ}$, as indicated by the activation of the complete antioxidant enzymatic battery. In other words, our data seem to show a very active protective mechanism in *D. polymorpha* against the formation of oxidative stress that should be investigated more extensively. We can also consider that a longer exposure time, as happens in the environment, might produce variations of the antioxidant enzyme activities also at lower concentrations.

Bearing in mind the capability of several environmental pollutants to inhibit the activity of some enzymatic complexes, another possible explanation for the toxicity of TCS can arise from potential blocking of the antioxidant enzymes. In this case, the increase of ROS should produce a consequent increase in the oxidative stress that may be the primary agent responsible for the observed cytogenotoxic damage. However, this alternative hypothesis is less probable, as we noticed a clear activation of CAT and SOD at the highest administered dose, denying inhibition of the antioxidant enzymatic chain by TCS.

5 Conclusions

The results obtained by measuring the levels of several antioxidant enzymes do not completely explain the

Table 2 Pearson's correlation coefficients obtained by using all the cytogenotoxic endpoints monitored and the enzyme activities measured at 1 nM, 2 nM, and 3 nM ($*p<.05$; $**p<.01$)

	LDR	% DNA	APO	MN	NRRT	CAT	GPX	SOD
1 nM								
% DNA	0.94**							
APO	0.95**	0.96**						
MN	0.81**	0.82**	0.83**					
NRRT	-0.90**	-0.87**	-0.88**	-0.69**				
CAT	-0.64**	-0.58**	-0.58**	-0.42**	0.61**			
GPX	0.81**	0.71**	0.75**	0.64**	-0.75**	-0.50**		
SOD	0.36*	0.35*	0.42**	0.26	-0.37**	0.05	0.25	
GST	0.30*	0.34*	0.34*	0.28	-0.21	0.21	0.31*	0.15
2 nM								
% DNA	0.95**							
APO	0.95**	0.94**						
MN	0.65**	0.69**	0.71**					
NRRT	-0.92**	-0.88**	-0.92**	-0.67**				
CAT	-0.57**	-0.52**	-0.57**	-0.25	0.56**			
GPX	0.26	0.25	0.22	0.28	-0.20	0.02		
SOD	0.23	0.13	0.15	0.09	-0.26	0.00	0.21	
GST	0.26	0.20	0.20	0.24	-0.22	0.32*	0.48**	0.70**
3 nM								
% DNA	0.92**							
APO	0.91**	0.89**						
MN	0.64**	0.75**	0.74**					
NRRT	-0.83**	-0.69**	-0.79**	-0.55**				
CAT	0.09	0.25	0.29*	0.28*	0.15			
GPX	0.24	0.34*	0.35*	0.30*	-0.13	0.48**		
SOD	0.29*	0.42**	0.47**	0.40**	0.01	0.79**	0.51**	
GST	0.15	0.34*	0.34*	0.33*	0.12	0.86**	0.54**	0.80**

LDR length/diameter ratio, *% DNA* percentage of tail DNA, *APO* frequency of apoptotic cells, *MN* frequency of micronuclei, *NRRT* neutral red retention time. Correlations among the enzyme activities and cytogenotoxic end points are indicated in *rectangles*

mechanism of action of triclosan in *D. polymorpha* because only the phase II detoxifying enzyme GST was clearly and significantly induced. In contrast, CAT and SOD were activated only at the highest administered dose, and GPx remained at levels similar to the baseline. Thus, the cytogenotoxicity of TCS previously revealed on zebra mussels even at low doses appears to be mainly due to a direct effect of the parental compound and/or its several metabolites (methyl-triclosan and polychlorodibenzo-*p*-dioxins) rather than an increase of ROS and consequent oxidative stress. The role played by DT-diaphorase to protect this mollusk against the production of ROS that is also induced by TCS should be investigated in the future.

The entire set of data obtained by our research group on the ecotoxicity of TCS shows very dangerous effects on *D. polymorpha*. Consequently, our results indicate a potential danger for the entire aquatic biocoenosis. We hope that the increasing number of studies on the effects of TCS on different biological models can cast light on its possible environmental risk.

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PAPER V

Binelli Andrea, **Parolini Marco**, Cogni Daniele, Pedriali Alessandra, Provini Alfredo

A multi-biomarker assessment of the impact of the antibacterial trimethoprim on the non-target organism Zebra mussel (*Dreissena polymorpha*)

A multi-biomarker assessment of the impact of the antibacterial trimethoprim on the non-target organism Zebra mussel (*Dreissena polymorpha*)

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ABSTRACT

A battery of eight biomarkers was applied in the freshwater mussel *Dreissena polymorpha* to evaluate potential sub-lethal effects of the antimicrobial trimethoprim (TMP, 5-[3,4,5-trimethoxybenzyl]pyrimidine-2,4-diamine). Mussels were exposed for 96 h to increasing concentrations (1, 3, 10 nM) of TMP in *in vivo* experiments. We determined the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test), the apoptotic frequency (Halo assay) and the lysosomal membrane stability (Neutral Red Retention Assay) in mussel hemocytes. Moreover, to reveal whether the oxidative status was altered, measurements of the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the phase II detoxifying enzyme glutathione S-transferase (GST) were performed using the cytosolic fraction extracted from a pool of entire mussels. The biomarker battery pointed out only a moderate cyto- and genotoxicity on Zebra mussel hemocytes since only a slight increase in DNA damage was registered by apoptosis induction and MN frequency, while significant differences of lysosomal membrane stability from baseline levels were measured at 3 and 10 nM at the end of exposures only. Finally, TMP seems to have a very low induction capability or even an inhibitory effect on the activities of antioxidant enzymes, but a clear significant induction on GST.

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1. Introduction

Pharmaceuticals and Personal Care Products (PPCPs) are developed to achieve specific biological effects related to human and animal health care, as well as to livestock farming and aquaculture growth promotion. Although pharmaceuticals are primarily designed to target humans, there are concerns about their potential impacts on non-target organisms because of their physicochemical and biological properties (Seiler, 2002). More than 100 pharmaceutical compounds, covering various therapeutic classes, have been reported in sewage, surface water, groundwater and even drinking water worldwide (Heberer, 2002; Choi et al., 2008). Antibiotics are considered to have a high priority for environmental risk assessment because of their extensive use in humans and animals. Moreover, these drugs can potentially cause damage to the ecosystem by affecting key species and by promoting the development and spread of resistant genes in the environment (Costanzo et al., 2005). Sanderson et al. (2004) suggested a qualitative assessment of risk that ranks pharmaceuticals according to the probability and potential severity of their environmental impact and health effects: antibiotics > sex hormones > cardiovascular > antineoplastics. The use of antibiotics is also constantly

increasing, as indicated by recent research carried out by the European Surveillance of Antimicrobial Consumption (ESAC, 2007). This previous study showed that countries in the south and east of Europe have the highest consumption, whereas consumption is much lower in Northern and Western Europe. In particular, the antibiotic consumption in 2006 ranged between 27.8 and 32.4 defined daily doses (DDD) per 1000 inhabitants per day in France and Greece, while in the Netherlands and Russian Federation the consumption was lower (9.6–14.1 DDD/1000 inh./day). Italy remains one of the European countries with the highest use of antimicrobials and it is ranked fourth in the EU, with a consumption of 26.7 DDD/1000 inh./day (ESAC, 2007).

Trimethoprim (TMP, 5-[3,4,5-trimethoxybenzyl]pyrimidine-2,4-diamine) is a widely used antimicrobial drug and one member of the family of synthetic 2,4-diaminopyrimidines. TMP has potent microbicidal activity against a wide variety of bacterial species (Lampert and O' Grady, 1992), and forms metal complexes with useful antimalarial properties (Ajibade and Kolawole, 2008). TMP inhibits the enzyme dihydrofolate reductase to block the synthesis of tetrahydrofolate, an essential precursor in the synthesis of thymidine. Loss of this nucleoside ultimately impacts DNA, RNA and protein synthesis, resulting in stasis or cell death (Baccanari, 1995). The acute effects of TMP were investigated in some non-target organisms by Halling-Sørensen et al. (2000). This group found an $EC_{50} = 110$ mg/L in the green freshwater microalga *Pseudokirchneriella subcapitata*, very similar to that observed in the cyanobacterium *Microcystis*

aeruginosa ($EC_{50} = 112$ mg/L) and crustacean *Daphnia magna* ($_{48h}EC_{50} = 123$ mg/L). However, these values are several orders of magnitude higher than environmental concentrations found both in wastewaters and freshwaters. Chang et al. (2008) measured levels of TMP from 0.09 to 0.34 ng/L in four Japanese rivers, while this chemical was detected at a concentration up to 546 ng/L in the South Korean Han River (Choi et al., 2008). With regard to European contamination, Kasprzyk-Hordern et al. (2008) measured TMP concentrations up to 183 ng/L in the River Ely (South Wales, UK). Additionally, average concentrations ranging from 12 to 27 ng/L were found in five different sampling sites of the Seine (France; Tamtam et al., 2008). These values are much lower than the two available PNECs (Predicted No Effect Concentrations) of 180 µg/L (Halling-Sørensen et al., 2000) and 16 µg/L (Grung et al., 2008), calculated by using either acute toxicity results or a combination of classical acute and chronic toxicity data, respectively. By contrast, very few data are available on other sub-lethal effects (e.g., genotoxicity, cytotoxicity, impairment of cell signaling, oxidative stress) that could result from the biochemical activity of TMP. In particular, there is considerable controversy over the potential cytotoxicity and genotoxicity of TMP. This is largely due to conflicting reports from the scientific literature and from the manufacturers that describe both positive and negative effects within the same bacterial and mammalian cell models (Abou-Eisha, 2006).

The purpose of this study was to measure several biomarker end-points to examine eventual sub-lethal effects of TMP in the freshwater bivalve Zebra mussel (*Dreissena polymorpha*), a useful sentinel organism for the evaluation of possible danger due to synthetic chemicals on aquatic ecosystems. We measured eight individual biomarker responses, and integrated them into a battery capable of detecting the net effect of this chemical on Zebra mussel specimens exposed to three different concentrations of TMP. We used the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and the measure of apoptotic frequency (Halo assay) to investigate the genotoxicity potential of TMP, while the lysosomal membrane stability, as measured by the Neutral Red Retention Assay (NRRA), was selected for the identification of generic cellular stress. Moreover, we also determined the response of antioxidant enzymes by measuring the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST).

2. Materials and methods

TMP standard (98% purity) was obtained from Sigma-Aldrich (Steinheim, Germany), as well as all other chemicals used for biomarker determination. Dimethylsulfoxide (DMSO) was purchased from VWR International (Milan, Italy).

2.1. Dose selection

Doses to be tested under laboratory conditions must be carefully selected in order to give information with maximum utility in the real world. Since the aim of this study was to investigate particular sub-lethal effects, and bearing in mind that no data on TMP toxicity was available for *D. polymorpha*, chemical doses were chosen according to both the concentrations found in freshwaters worldwide and the PEC (Predicted Environmental Concentration) calculated according to EMEA (European Medicines Evaluation Agency) guidelines. We selected 1 nM (0.29 µg/L), 3 nM (0.87 µg/L) and 10 nM (2.9 µg/L) concentrations of TMP. The first of these doses is absolutely comparable with most of the measured environmental concentrations (MECs). The middle concentration is slightly higher than the maximum value found in literature to date (Choi et al., 2008), while 10 nM is comparable to the EMEA PEC (2.4 µg/L; Grung et al., 2008).

2.2. Mussel acclimation and maintenance conditions

Several hundred specimens were sampled in Lake Maggiore (Northern Italy) by a scuba diver and transferred to the laboratory in bags filled with lake water. The mussels were then introduced into several glass aquaria filled with about 100 L of dechlorinated tap water, and maintained at natural photoperiod, constant temperature (20 ± 1 °C), pH (7.5), and oxygenation (>90% of saturation). Animals were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA) and water was constantly changed for at least three weeks to purify the mollusks from accumulated xenobiotics. Then, several specimens with a similar shell length (about 20 mm) were selected for each *in vivo* test, including control and solvent assays. Mussels were placed on glass sheets suspended in small glass aquaria (15 L) and maintained for one week under the conditions described above. Only specimens that were able to re-attach themselves by their byssi on glass sheets were used in the experiments. Mussels were used for the subsequent *in vivo* experiments only when target biomarkers showed values comparable to previously checked baseline levels.

2.3. Exposure assays

Experiments were performed under semi-static conditions, with daily changes of the entire volume of water and the addition of chemicals up to the selected concentrations. Since TMP is a chemical very resistant to biodegradation (Halling-Sørensen et al., 2000), the administered dose was assumed to be constant in solution over each 24 h period.

Mussels were fed daily with AlgaMac-2000®, which was added 2 h before each water and chemical change. Temperature, oxygenation and pH were checked daily. Several mussels were collected each day from the aquaria to be processed and used for biomarker analyses. We used hemocytes to measure cyto- and genotoxic biomarkers, while the enzymatic activity was measured by using entire organisms. Hemocyte viability was checked daily by the Trypan blue exclusion method (10 µL of cell suspension added to 10 µL of 0.4% dye) using a Burker chamber.

2.4. Micronucleus test

The MN test was performed according to the method of Pavlica et al. (2000). Hemolymph (100 µL) was obtained from the posterior adductor muscle of 10 mussels with a hypodermic syringe containing 100 µL of PBS and ethylenediaminetetraacetic acid (EDTA, 10 mM) solution (1:1). The cell suspension was placed on a slide and left for 15 min in a humidified chamber at room temperature to allow the hemocytes to settle. The slides were subsequently fixed with glutaraldehyde (25% solution, diluted to 1% in PBS) for 5 min. After rinsing with PBS, the slides were stained with bisbenzimide 33258 (Hoechst; CAS number 23491-45-4) at a concentration of 1 mg/mL for 5 min, prior to being washed and mounted in glycerol–McIlvaine buffer (1:1). Slides were kept in the dark at 4 °C prior to examination under the microscope. All samples were coded and blind evaluated. Slides were scored under a Leitz DMR fluorescent microscope equipped with a submerged lens at 100× magnification. Four hundred cells were counted for each slide, giving a total of 4000 cells/treatment. Only intact and non-overlapping hemocytes were scored. Micronuclei were identified by the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.5. SCGE assay

The SCGE assay was performed under alkaline conditions (pH > 13) as described by Singh et al. (1988), with the subsequent optimization for Zebra mussels detailed by Buschini et al. (2003). Hemolymph

(100 μL) from ten individual mussels was withdrawn from the sinus near the posterior adductor muscle using a hypodermic syringe containing 200 μL of phosphate-buffered saline (PBS; pH = 7.4). Ten 10-μL aliquots of cell suspension were mixed with 85 μL of Low Melting Agarose (LMA-0.7%) in PBS (37 °C), and added to ten coated slides (previously dipped in 1% Normal Melting Agarose). Slides were covered by a cover-glass and placed at 4 °C for 40 min until the agarose layer hardened. A third agarose layer was added to the slides in the same way. After agarose solidification, slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH = 10) in a Coplin jar at 4 °C in the dark for at least 1 h.

Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber containing freshly prepared buffer (1 mM Na_2EDTA, 300 mM NaOH, pH = 13) and then in an ice-water bath (4 °C). Electrophoresis was then performed at 0.78 V/cm and 300 mA for 10 min. Slides were washed after electrophoresis in a neutralization buffer (0.4 M Tris–HCl, pH = 7.5) and fixed in absolute ethanol. After staining with DAPI (4′,6-diamidino-2-phenylindole) DNA dye (Sigma-Aldrich), a coverslip was placed over the slides. Observations were done under a fluorescence microscope (Leitz DMR, Germany) equipped with a FTC filter. All steps were performed in the dark to minimize additional UV-induced DNA damage. Positive controls were carried out with H_2O_2 to check the effectiveness of the electrophoresis conditions. All samples were blindly coded and evaluated.

In total, 50 cells per slide were analyzed using an image analysis system (Comet Score®), for a total of 500 analyzed cells per sample. The ratio between migration length and diameter of the comet head (LDR) was chosen to represent DNA damage data. This was coupled with the percentage of tail DNA obtained by the Comet Score® assay, since the working group on genetic toxicology testing from the 4th IWGT (International Workshop on Genotoxicity Test Procedures) recently agreed that the percentage of tail DNA is the measure most linearly related to dose and the easiest to understand (Kirkland et al., 2007).

2.6. Halo assay

The percentage of apoptotic cells was evaluated through the same protocol used for the SCGE assay, as described by Singh (2000). Each hemocyte pool was withdrawn and distributed between five different slides that were processed using the method described above for the SCGE assay. Hemocytes were lysed, and the slides were subjected to 5 min of alkaline (pH = 13) DNA unwinding without subsequent electrophoresis. Slides were then washed in neutralization buffer, stained, and fixed in absolute ethanol. Finally, they were labeled with DAPI and observed under fluorescence. Two hundred cells per slide were analyzed for a total of 1000 cells per sample. Necrotic cells were eliminated from the count.

2.7. Neutral Red Retention Assay

The NRRA method followed the protocol proposed by Lowe and Pipe (1994) and ICES (International Council for the Exploration of the Sea, 2004). The neutral red stock solution was made by dissolving 20 mg of dye in 1 mL of DMSO, while the working solution was prepared by diluting 5 μL of stock solution in 2.5 mL of PBS. Microscope slides were coated with 2 μL of polylysine with the help of a coverslip. Five slides were used for each sample. Hemolymph was withdrawn using a hypodermic syringe that contained 100 μL of PBS/EDTA solution (1:1), as described above. An aliquot of 80 μL of hemocyte resuspension was placed carefully on each slide. Slides were suspended on a rack in a light-proof humidity chamber for 20 min, and excess solution was carefully tipped off. Lastly, 40 μL of neutral red working solution was added. After a 20-min incubation in the humidity chamber, slides were observed under an optical microscope. Slides were examined systematically thereafter at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests were terminated when dye loss was evident in at least 50% of the hemocytes. The mean retention time was then calculated from the five replicates.

2.8. Enzymatic activities

Unlike cytotoxic and genotoxic biomarkers, the enzymatic activities were measured in the entire organism following observations made by Osman et al. (2007) and Osman and van Noort (2007) that CAT and GST activities in the whole soft tissues were much higher than in single gills. Enzymatic activities were determined spectrophotometrically as described by Orbea et al. (2002). Measurements were carried out in triplicate, using the cytosolic fraction extracted from a pool of 6–8 entire mussels (≈1 g fresh weight) homogenized in 100 nM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) using a Potter homogenizer. We also added specific protease inhibitors (1:10): dithiothreitol (DTT, 100 mM), phenanthroline (Phe, 10 mM) and trypsin inhibitor (TI, 10 mg/mL). The homogenate was centrifuged at 500 g for 15 min at 4 °C. The supernatant was then transferred into clean tubes and centrifuged again at 2000 g for 30 min at 4 °C. Finally, the supernatant was ultra-centrifuged at 100,000 g for 90 min at 4 °C. The cytosolic fraction was held in ice and immediately processed for protein determination and enzymatic activity. CAT activity was determined in the cytosolic fractions by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 80 mM potassium phosphate buffer, pH 7.

SOD activity was determined in the cytosolic fraction by measuring the degree of inhibition of cytochrome c reduction at 550 nm by superoxide anion generated by the xanthine oxidase/hypoxanthine reaction. The concentrations of the reagents were as follows: potassium phosphate buffer (50 mM, pH 7.8), hypoxanthine (50 μM), xanthine oxidase (1.87 mU/mL) and cytochrome c (10 μM). The activity is given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction).

GPx activity was measured in the cytosolic fraction by monitoring the consumption of NADPH at 340 nm during the formation of reduced glutathione by glutathione reductase. The reaction medium consisted of the following: 0.2 mM H_2O_2 substrate in 100 mM potassium phosphate buffer, pH 7, containing additional glutathione (2 mM), sodium azide (0.5 mM), glutathione reductase (2 U/mL) and NADPH (120 μM).

GST activity was measured by adding reduced glutathione (20 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) in phosphate buffer (pH 7.4) to the cytosolic fraction. The resulting reaction was monitored for 1 min at 340 nm.

The total protein content of all samples was measured according to the method of Bradford (1976) using bovine serum albumin as a standard.

2.9. Statistical analyses

Data normality and variance homogeneity were verified using the Kolmogorov–Smirnov and Barlett tests, respectively. We used log-transformed LDR values to normalize the variance of the Comet test results, and an angular transformation (arcsin $\sqrt{P}$) for the MN test data. To identify dose/effect and time/effect relationships a two-way analysis of variance (ANOVA) was performed using time and TMP concentrations as variables, while biomarker end-points as cases. ANOVA was followed by Bonferroni *post-hoc* test to evaluate eventual significant differences ($p < 0.05$) between treated samples and related controls (time to time) and among exposures.

The Pearson's correlation test was carried out on all measured variables in the three exposure assays to investigate possible correlations between various biological responses. All statistical analyses were performed using the software package STATISTICA 7.0.

3. Results

No mortality or changes in hemocyte counts were recorded in TMP-treated mussels with respect to their controls. Moreover, hemocyte viability was checked daily and was always higher than 80%. We also tested the solvent (DMSO) for possible cytotoxic effects by using the highest concentration reached in water (0.03%) as an additional control. This assay showed that DMSO did not produce any significant ($p > 0.05$) DNA damage or changes in enzyme activity, and no significant ($p > 0.05$) temporal differences were noticed within the control and solvent groups during the entire exposure tests. All control data from the cyto- and genotoxic assays agreed with those obtained in previous studies carried out by our research group on this biological model (Riva et al., 2007; Binelli et al., 2008; Binelli et al., 2009a,b). Also, baseline levels obtained for enzymatic activities are similar to those found by Osman and van Noort (2007) and Faria et al. (2009).

3.1. MN, SCGE, HALO and NRRT assay results

The two-way ANOVA showed for MN assay an overall significant ($p < 0.05$) difference not only between each treatment and controls, but also among all the three treatments. Moreover, we noticed significant overall dose/effect (two-way ANOVA; $p < 0.01$) and time/effect (two-way ANOVA; $p < 0.01$) relationships. Fig. 1 shows the temporal trend of the five assays carried out for the evaluation of micronucleated hemocytes and the statistical differences obtained by the comparison of treated samples with the correspondent controls (two-way ANOVA, Bonferroni post-hoc test, $p < 0.05$).

By contrast, we obtained a significant (two-way ANOVA; $p < 0.01$) difference only between the overall temporal trend of LDRs at 10 nM and controls (Fig. 2a,b) and no significant dose/effect and time/effect relationship were noticed. Moreover, results obtained using LDRs showed a homogeneity higher than data obtained from averaging the percentage of DNA found in the tail of the comets.

The measured apoptotic cell frequencies showed clear dose/effect (two-way ANOVA; $p < 0.01$) and time/effect (two-way ANOVA; $p < 0.01$) relationships for all the tested concentrations (Fig. 3). In addition, all the treatments and controls resulted significantly (two-way ANOVA; $p < 0.05$) different by each other, excluding 1 nM vs 3 nM. Although the level of programmed cell death was only two/three times higher than the control level (1.5%) in the 1 nM and 3 nM cases, it increased to 13% at a dose of 10 nM TMP.

Although TMP (Fig. 4) is able to destabilize lysosomal membranes in an overall dose-dependent (two-way ANOVA; $p < 0.01$) and time-dependent (two-way ANOVA; $p < 0.01$) manner, only the temporal
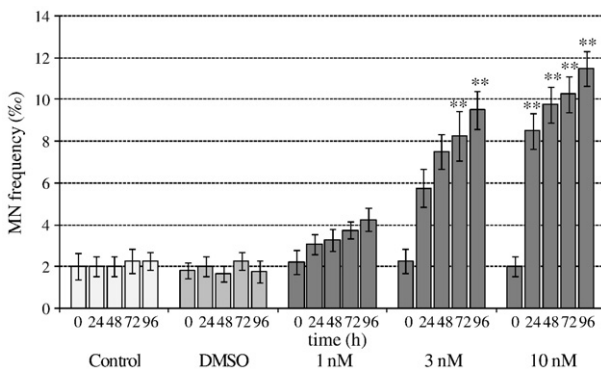

Fig. 2. Results (mean values ± SEM; standard error of the mean) of the SCGE assay expressed by the length/diameter ratio (a) and the mean percentage of tail DNA (b). Measurements employed Zebra mussel hemolymph samples ($n = 30$) exposed to three different TMP doses. Significant differences (two-way ANOVA, Bonferroni post-hoc test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

trend at 10 nM was significantly (two-way ANOVA; $p < 0.05$) different both to controls and 1 nM assay.

3.2. CAT, SOD, GPx, GST activity

No homogeneous pattern was found because several samples showed an induction of enzymatic activity, while others exhibited a clear decrease in comparison with baseline levels (Fig. 5). We noticed a significant (two-way ANOVA; $p < 0.05$) induction of activity at 3 nM for CAT, GPx and GST enzymes, but basically TMP seems to have an inhibitory effect. The two-way ANOVA confirms this effect because the activity of all the enzymes at 3 nM was statistically ($p < 0.05$) different from the other two administered doses (SOD excluded).

Fig. 1. Frequency of micronucleated hemocytes (mean values ± SEM) observed in D. polymorpha specimens ($n = 30$) exposed to three concentrations of TMP. Significant differences (two-way ANOVA, Bonferroni post-hoc test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

Fig. 3. Percentages of apoptotic hemocytes (mean values ± SEM) measured by the Halo assay in Zebra mussel specimens ($n = 15$) exposed to 1 nM, 3 nM and 10 nM TMP. Significant differences (two-way ANOVA, Bonferroni post-hoc test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).
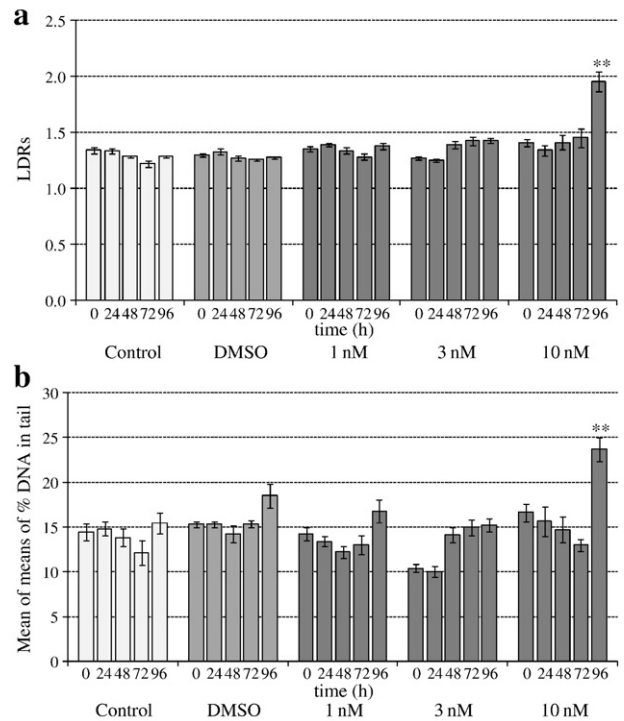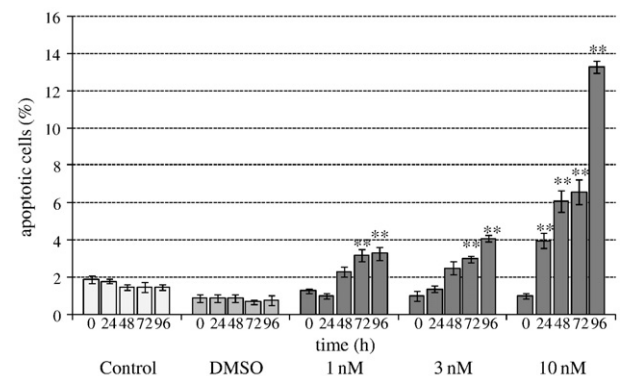
Fig. 4. Mean retention time (RT) of neutral red loss (mean values ± SEM) from lysosomes in hemocytes of Zebra mussels ($n = 15$) exposed to increasing concentrations of TMP. Significant differences (two-way ANOVA, Bonferroni *post-hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).
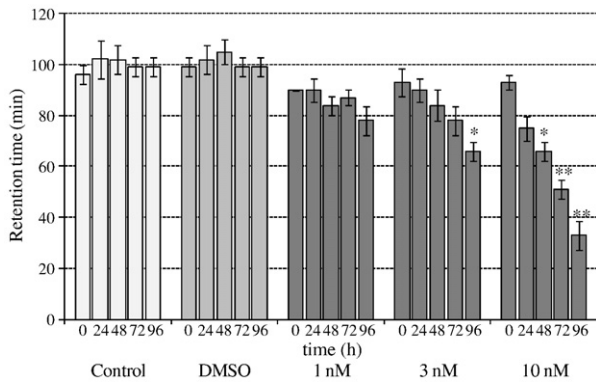
GST activity exhibited a significant (two-way ANOVA, $p < 0.01$) rise of temporal trend between 1 nM and 3 nM, although activity clearly decreased (two-way ANOVA, $p < 0.01$) at a dose of 10 nM.

The temporal trends of GPx for all the administered doses were significantly (two-way ANOVA, $p < 0.01$) different from controls, but with an induction in enzyme activity observed only at 3 nM. A similar trend was also found for CAT activity, while no significant variations from controls were observed for SOD, excepted at 1 nM (two-way ANOVA; $p < 0.01$).

4. Discussion

The freshwater bivalve *D. polymorpha* has been used in recent years not only as a sentinel organism for the identification of contamination due to persistent organic pollutants (POPs), heavy metals and PPCPs, but also as a key species to determine the biological risk associated with several chemicals present in freshwater ecosystems. In this context, our work, through laboratory exposures, sought to characterize those dose-dependent changes induced by the antibiotic trimethoprim in this freshwater mussel by means of a biomarker battery. Additionally, some effort has been made to address

potential roles that specific oxyradicals might play in perturbing oxidative status and triggering cytotoxic and genotoxic damage.

A previous study was carried out to determine the cytotoxicity of TMP towards Zebra mussel hemocytes exposed *in vitro* (Binelli et al., 2009b), the only study examining the impact of TMP in this sentinel organism until now. This research pointed out that TMP has a moderate tendency to induce DNA damage, since the observed significant increases in LDR values and apoptosis induction were obtained at administered doses (60, 300, and 1500 µg/L) much higher than environmental levels. Moreover, we hypothesized that TMP possibly increases oxidative stress; this was indicated indirectly by the significant decrease of lysosomal membrane stability (NRRA) that resulted also correlated to LDR and apoptosis.

The *in vivo* results, carried out at environmental doses, confirm the TMP-induced effects observed in *in vitro* experiments. A significant increase in DNA damage was registered by two of the examined genotoxic biomarkers (apoptosis induction and MN frequency). On the contrary, a slight increase in DNA damage was detected at 10 nM by alkaline SCGE assay, which highlights DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA–DNA/DNA protein cross-linking and SSB associated with incomplete excision repair sites (Tice et al., 2000). The lack of correlation between this biomarker, which registers primary repairable genetic damage, and those that detect fixed DNA damage seems to indicate that TMP is able to interfere on DNA by means of great genetic injuries, such as the formation of double-strand breaks (DSB), translocations or production of adducts/intercalants to DNA. Zebra mussels seem able to repair minor DNA damage made by this chemical administered at environmental doses, while hemocytes enter apoptosis or produce micronuclei only when broad damage is evident. Thus, the significant production of micronuclei observed at 3 nM and 10 nM (Fig. 1) should indicate that the main mechanism of TMP cytotoxicity involves an aneuploidogenic pathway, instead of clastogenic effects or that the latter are produced by very large genetic injuries. The dramatic increase in apoptotic cells, found also at the lowest tested dose (Fig. 3), can confirm this hypothesis since hemocytes chose programmed cell death without transitioning through a previous DNA fragmentation, as contrarily indicated by other studies with different pollutants (Martin et al., 1994; Steinert, 1996; Binelli et al., 2009a).

Dose/effect relationships similar to those observed for apoptosis and MN frequency could not be extracted from the antioxidant

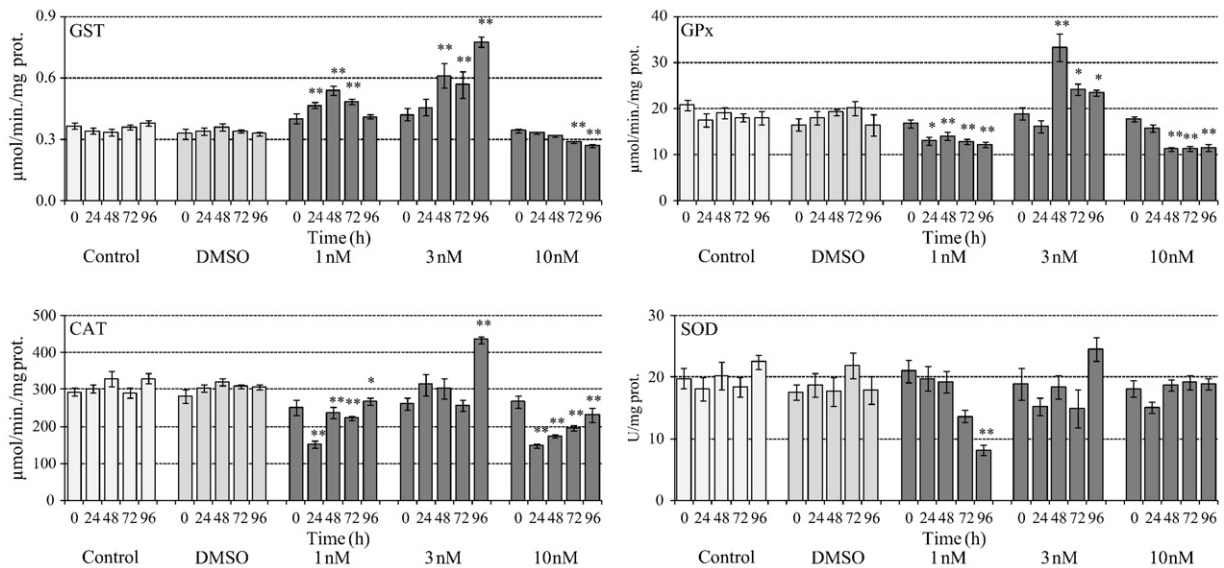**Fig. 5.** Effects of increasing doses of TMP on the activity of glutathione S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) measured in the whole organism of Zebra mussels (pool of 6–8 specimens). Significant differences (two-way ANOVA, Bonferroni *post-hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

enzyme data, due to complex variations in behavior between data sets. On the other hand, antioxidants are part of a very complex homeostatic system, and the redox status is buffered by several interactions. During metabolic processes, a small proportion (2–3%) of free-radicals may escape from the antioxidant defense, causing oxidative damage (Valavanidis et al., 2006) that can be increased by exposure to several pollutants. Scientific literature provides several examples of elevated variability of antioxidant responses with transient or limited changes reported for different species, tissues and contaminants (Regoli et al., 2003; Osman et al., 2007; Xiao et al., 2007). This should be true also for aquatic organisms, even if the regulation of their antioxidant systems in relation to pollutants is not well understood (Livingstone, 2001).

CAT, GPx and GST activities exhibited a significant rise at 3 nM of TMP, while we measured significant decreases in these antioxidant activities at 1 nM and 10 nM (Fig. 5). Interestingly, several other authors have reported this particular behavior. For example, Regoli et al. (2003) revealed a general inhibition of CAT in eels (*Anguilla anguilla*) exposed to benzo[α]pyrene (B[α]P), 2,3,7′,8′-tetrachlorodibenzo p-dioxin (TCDD), β-naphthoflavone (BNF) and Arochlor 1254, while GPx was not significantly affected by Arochlor 1254 and BNF. This is in line with the observations made by Osman et al. (2007), who reported a decline of CAT in *D. polymorpha* specimens exposed to different sediment extracts contaminated by several POPs.

Taken together, the general decrease in CAT and GPx activity observed at 10 nM, along with the lack of an observable SOD response, could indicate that sub-lethal damage from TMP at higher doses compromised the ability of this mussel to respond in an adaptive manner (Regoli et al., 2003). On the other hand, the decreased enzyme activity observed at 1 nM could be a kind of hormetic response arising from TMP's reduction of the bacterial charge present in the mussel soft tissues. In fact, more than 30 species of indigenous bacteria have been isolated from the Zebra mussel, including members of *Aeromonas* and *Pseudomonas* that are potentially opportunistic pathogens to animals and humans (Gu and Mitchell, 2002). Thus, TMP administered at low doses might reduce the basal inflammation produced by bacteria and consequently the baseline levels of antioxidant enzymes.

Since many PPCPs require metabolic oxidation to be efficiently eliminated, their long-term exposure could lead to oxidative stress. The activation of the phase II detoxifying enzyme GST found also at very low TMP concentrations seems to confirm the possible increase of oxidative stress due to the excretion mechanism. On the other hand, this very polar chemical (log $K_{ow} = 0.91$; Kim et al., 2007) can be easily glutathione-conjugated by GST, probably in a direct way or subsequently through weakly active phase I biotransformation enzymes. In fact, fish and bivalves appear to lack the CYP2C sub-type of CYP-450, one of the main isoenzymes for drug metabolism (Miners and Birkett, 1998; Stresser et al., 2000).

Correlation analysis was performed on all measured biomarkers for all three administered doses to point out eventual relationships between genotoxicity and oxidative stress (Table 1). The genotoxicity revealed after exposure to 3 nM TMP seems to be due primarily to oxidative stress, because significant ($p < 0.05$) correlations were obtained between genotoxic end-points and several antioxidant enzymes. Another confirmation was made by the negative significant ($p < 0.05$) relationship between lysosomal membrane stability and all the genotoxic biomarkers and antioxidants (GPx excluded). In fact, good correlations were reported between impairment of antioxidant capability, neutral red retention times, and changes in DNA integrity in different organisms (Camus et al., 2002; Regoli et al., 2002; Mamaca et al., 2005). Other authors (Kirchin et al., 1992; Winston et al., 1996) suggested that reactive free-radicals, such as ROS and xenobiotic derivatives, contribute to the damage of lysosomal membranes and that the intralysosomal environment is already a site of oxyradical production.

Also the rise of genotoxicity at 10 nM (Figs. 1 and 3) suggests that TMP toxicity might be due to the observed inhibition of antioxidant activities as a consequence of increased oxidative stress, as also indicated by several authors in some biological models exposed to different chemicals (Regoli et al., 1998; Hinson et al., 2004; Yen et al., 2006; Osman et al., 2007). Furthermore, another possible mechanism

Table 1
Pearson's correlation obtained by using all measured end-points (pool of 18–24 specimens) at 1 nM (A), 3 nM (B) and 10 nM (C).

	LDR	% DNA	APO	MN	NRRT	CAT	GPx	SOD
A								
% DNA	0.238							
APO	0.074	0.109						
MN	−0.087	0.129	**0.351***					
NRRT	−0.146	−0.144	**−0.393****	−0.130				
CAT	0.104	0.232	**0.478****	0.094	**−0.435****			
GPx	−0.076	0.016	**−0.479****	−0.214	0.179	**0.281***		
SOD	−0.164	−0.187	**−0.716****	−0.238	**0.429****	**−0.351***	**0.538****	
GST	−0.144	**−0.360***	0.102	0.042	−0.046	−0.163	−0.087	0.186
B								
% DNA	**0.879****							
APO	**0.603****	**0.632****						
MN	0.260	**0.322***	**0.656****					
NRRT	**−0.484****	**−0.511****	**−0.731****	**−0.517****				
CAT	0.246	0.229	**0.605****	**0.351***	**−0.533****			
GPx	**0.398****	**0.451****	**0.406****	**0.296***	−0.126	0.078		
SOD	0.186	0.170	**0.439****	0.119	**−0.294***	**0.674****	0.210	
GST	**0.533****	**0.611****	**0.792****	**0.481****	**−0.639****	**0.729****	**0.503****	**0.521****
C								
% DNA	**0.819****							
APO	**0.647****	**0.462****						
MN	**0.309***	0.148	**0.655****					
NRRT	**−0.564****	**−0.386***	**−0.920****	**−0.670****				
CAT	0.269	0.276	−0.017	**−0.415****	0.0103			
GPx	**−0.336***	−0.091	**−0.687****	**−0.678****	**0.749****	0.271		
SOD	**0.344***	−0.002	**0.281***	0.072	**−0.280***	**0.324***	**−0.384****	
GST	**−0.554****	**−0.319***	**−0.889****	**−0.608****	**0.895****	−0.040	**0.727****	**−0.295***

LDR = length/diameter ratio; % DNA = percentage of tail DNA; APO = frequency of apoptotic cells; MN = frequency of micronuclei; NRRT = Neutral red retention time; CAT = catalase; GPx = glutathione peroxidase; SOD = superoxide dismutase; GST = glutathione S-transferase. Significant correlations (*$p < 0.05$; **$p < 0.01$) are indicated in bold.

of action can stem directly from TMP's well-known suppression of DNA synthesis. TMP acts by interfering with the action of dihydrofolate reductase, thereby inhibiting synthesis of tetrahydrofolic acid. Tetrahydrofolic acid, in turn, is an essential precursor in the synthesis of the thymidine monophosphate (dTMP) and its downstream product, thymidine triphosphate, is one of the four nucleoside triphosphates that are used in DNA synthesis. Moreover, tetrahydrofolic acid is a key precursor for the purine bases of DNA and RNA, and also for methionine. This large-scale disruption of pathways critical to nucleic acid synthesis might create severe DNA damage, produced either directly or by the wrong action of DNA repair mechanisms. Damage of this type may not register within the alkaline SCGE assay (Fig. 2), but might be able to significantly increase both micronuclei formation (Fig. 1) and apoptosis (Fig. 3).

Finally, the significant ($p < 0.05$) correlation between GST activity and all the genotoxic end-points observed at 3 nM corroborates the hypothesis that GST plays a role in the increase of oxidative stress responsible of DNA damage through the glutathione conjugation with TMP.

Data obtained in this study shed some light on the controversial results found in scientific literature about the cyto- and genotoxicity of TMP, because its effect on DNA and its capacity to modify the scavenging of radicals by antioxidant enzymes strictly depend on its administered dose and exposure time. Moreover, the right evaluation of possible adverse effects on the selected biological model is also dependent on the selected biochemical end-points.

5. Conclusions

This work confirmed results obtained from our previous *in vitro* study of TMP exposure, namely that this antibiotic possesses cytotoxic and genotoxic potential against the non-target organism *D. polymorpha*. In fact, this research highlighted that TMP is able to inflict DNA damage on Zebra mussel hemocytes at existing environmental doses, bearing in mind that apoptosis and micronucleus induction increased markedly at 10 nM, a concentration comparable with the present PEC.

TMP's mechanism of action seems mainly due to the increase of oxidative stress, even if with some differences related to the administered dose. A strong relationship between genotoxic effects and the induction of antioxidants was noticed only at 3 nM. On the contrary, the absence of this correlation at a higher concentration suggests that TMP might also induce DNA damage by means of its inhibition of dihydrofolate reductase or by a heavy increase of oxidative stress that produces a reduction of the antioxidant defenses.

Our study also demonstrated that the use of a biomarker battery is absolutely crucial to identify the total toxic effects due to an environmental pollutant, like TMP, because of the imprecise selection of measured biochemical responses could drive to a wrong environmental risk assessment.

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PAPER VI

Parolini Marco, Binelli Andrea, Cogni Daniele, Provini Alfredo

Multi-biomarker approach for the evaluation of the cyto-genotoxicity of paracetamol on the zebra mussel (*Dreissena polymorpha*)

Multi-biomarker approach for the evaluation of the cyto-genotoxicity of paracetamol on the zebra mussel (*Dreissena polymorpha*)

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ABSTRACT

Paracetamol (PCM; N-(4-hydroxyphenyl)acetamide) is a widely used analgesic and antipyretic agent that is utilized in human medicine. Its use is so widespread that it is constantly being introduced into global water bodies where it reaches concentrations up to several $\mu g \, L^{-1}$. A battery of eight biomarkers was applied in the freshwater bivalve *Dreissena polymorpha* in order to evaluate its potential sub-lethal effect. Mussels were exposed for 96 h to increasing environmental concentrations (1, 5, 10 nM) of PCM. Cyto-genotoxicity was determined in mussel hemocytes by the lysosomal membrane stability (Neutral Red Retention Assay), the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and assessments of the apoptotic frequency (DNA diffusion assay). Moreover, in order to evaluate the probable alterations to the mussels' oxidative status, measurements of the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the detoxifying enzyme glutathione S-transferase (GST) were performed using the cytosolic fraction extracted from a pool of entire mussels. The biomarker battery demonstrated moderate cyto-genotoxicity in zebra mussel hemocytes since no primary DNA fragmentation was measured by the SCGE assay and only a slight increase in fixed DNA damage was registered by apoptotic and MN frequencies. Significant destabilization of the lysosomal membrane from baseline levels was evident at 5 and 10 nM at the end of the exposures, as was a high induction capacity of the activities of CAT and GST.

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1. Introduction

Pharmaceutical compounds are an emerging class of environmental pollutants that are extensively and increasingly being used in human and veterinary medicine (Fent et al., 2006). Due to their continuous production, consumption and often abuse, many studies have shown worldwide measurable concentrations of about 100 of these drugs in the aquatic environment in the high $ng \, L^{-1}$ to low $\mu g \, L^{-1}$ range (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999; Kümmerer, 2001, 2004; Heberer, 2002). Since pharmaceuticals have physico-chemical characteristics that are similar to those of harmful xenobiotics (Sanderson et al., 2004), they could be potentially dangerous to aquatic organisms (Fent et al., 2006) that are particularly important non-target species, as they are exposed to contaminants over their whole life-span. At present, only the acute toxicity of a few pharmaceuticals has been tested on organisms belonging to different trophic levels (Canesi et al., 2007; Choi et al., 2008; Haap et al., 2008; Quinn et al., 2008; Yang et al., 2008), but these data are not suitable for an accurate risk assessment since chronic effects are much more probable (Fent, 2003; Crane et al., 2006). However, the environmental levels of

some pharmaceuticals are lower than the acute effect concentrations measured by these assays (Ferrari et al., 2004; Bottoni and Fidente, 2005). Currently, studies on chronic toxicity in aquatic organisms are increasing to encompass a variety of different aquatic species (Huggett et al., 2002; Pascoe et al., 2003; Quinn et al., 2008), although these data are completely lacking for many pharmaceuticals (Carlsson et al., 2006).

Among the many classes of pharmaceuticals, the non-steroidal-anti-inflammatory drugs (NSAIDs) are one of the most important groups. NSAIDs inhibit the synthesis and release of prostaglandins acting as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and the cyclooxygenase-2 (COX-2) isoenzymes (Gagné et al., 2005). With an annual production of several kilotons (Cleuvers, 2004), NSAIDs are the sixth most sold drugs worldwide (Langman, 1999). Additionally, since some of these pharmaceuticals can be purchased without medical prescription, their consumption could be even higher. Due to the continuous and increasing application, as well as their pharmacokinetic properties (half-life, urinary and fecal excretion, metabolism, etc.), NSAIDs can reach detectable concentrations both in sewage and in surface water (Cleuvers, 2004). Many authors have reported levels of these drugs exceeding $1 \, \mu g \, L^{-1}$ in wastewaters and in the effluents of sewage treatment plants (STP), while lower concentrations have been found in surface

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waters (Stumpf et al., 1996; Ternes et al., 1998; Farré et al., 2001; Heberer, 2002; Metcalfe et al., 2003).

Although the analgesic and antipyretic agent paracetamol (PCM; N-(4-hydroxyphenyl)acetamide) does not possess a real anti-inflammatory action, it is usually considered an NSAID in toxicology due to its very similar mode of action (Misra et al., 1990). Since it is considered a safe drug at therapeutic doses, it can be purchased as an over-the-counter preparation in most countries, and it is currently the most widely used drug worldwide (An et al., 2009). Due to the huge production and quantity of use, it is reported as one of the most frequently detected pharmaceuticals to be found in surface waters, wastewaters and drinking water. In a survey of 139 US streams, Kolpin et al. (2002) detected PCM in 24% of tested samples at a median concentration of 0.11 $\mu g\,L^{-1}$, with a maximum detection level up to 10 $\mu g\,L^{-1}$, which is in perfect agreement with the PEC (Predicted Environmental Concentration) calculated by Kim et al. (2007) for Korean waters (16.5 $\mu g\,L^{-1}$). In worldwide surface waters, the median concentration of this compound is 0.055 ± 0.051 $\mu g\,L^{-1}$ (Bound and Voulvoulis, 2006; Gros et al., 2006), while in raw wastewaters was detected at a higher median concentration of 48 ± 75 $\mu g\,L^{-1}$ (Gros et al., 2006; Han et al., 2006).

Due to its widespread presence in aquatic ecosystems, PCM is one of the possible dangerous compounds for the entire aquatic biocoenosis (Crane et al., 2006; Schulte-Oehlmann et al., 2007). Although very few studies have been carried out to evaluate its environmental risk, Henschel et al. (1997) classified this drug as harmful to aquatic organisms on the basis of some ecotoxicological tests in different biological models including bacteria, algae, Daphnia spp., and fish embryos. According to this assumption, on the basis of the Predicted No Effect Concentration (PNEC) value of 9.2 $\mu g\,L^{-1}$, Kim et al. (2007) calculated a PCM hazard quotient that correspond to 1.8, suggesting potential adverse ecological consequences. In order to increase the knowledge about its possible environmental effects, the purpose of this study was to assess the cytogenotoxicity of PCM on the freshwater bivalve zebra mussel (Dreissena polymorpha) by a multi-biomarker approach. This mussel was chosen as a biological model because mollusks plays an important role in freshwater ecosystems and are particularly susceptible to environmental stressors (Gagné et al., 2006). Additionally, previous studies have revealed that D. polymorpha is an useful and sensible organism capable of highlighting sub-lethal effects when exposed to synthetic chemicals, like persistent organic pollutants (Riva et al., 2007; Binelli et al., 2008b) and pharmaceuticals (Binelli et al., 2008a, 2009). We measured the end-points of eight individual biomarkers, whose integrated response can be helpful for detecting the sub-lethal effects caused by PCM on zebra mussel specimens exposed to three different environmental concentrations of this drug. Its genotoxic potential was evaluated by the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and the measure of apoptotic frequency (DNA Diffusion assay), while the cytotoxicity was measured by a lysosomal membrane stability test (NRRA – Neutral Red Retention Assay), a classical parameter of generic cellular stress in bivalves (Lowe et al., 1995). Additionally, we also measured the activity of three antioxidant phase I enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST) to study in-depth the toxicity of PCM and to investigate its possible mechanism of action.

2. Materials and methods

Standard PCM (CAS number 103-90-2) was obtained from Sigma–Aldrich (Steinheim, Germany), as well as all other chemicals used for biomarker determination. Dimethylsulfoxide (DMSO;

CAS number 67-68-5; purity = 99.5%) was obtained from VWR International (Milan, Italy).

2.1. Mussel acclimation and maintenance conditions

Several hundred specimens of D. polymorpha tied by the byssus to the rocks were collected by a scuba diver at a depth of 4–6 m in Lake Lugano (Northern Italy), which is considered a reference site due to its low xenobiotic pollution (Binelli et al., 2005). The mussels were rapidly transferred in laboratory in bags filled with lake water, the rocks were rinsed and introduced into 100-L glass aquaria filled with tap water, which was maintained at a natural photoperiod, constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90% of saturation). Bivalves were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA), and the water was changed regularly every two days for at least two weeks to gradually purify the mollusks of the pollutants that had accumulated in their soft tissues. Several specimens (n = 300 for each aquarium) with the same shell length (about 20 mm) were chosen for in vivo tests, including a control assay. They were gently cut from the rocks and placed on glass sheets suspended in 15-L aquaria filled with tap and de-chlorinated water (1:1 v/v) and maintained at the same conditions described above. Only specimens able to re-form their byssus and reattach themselves to the glass sheet were used in the experiments. Mussels were used for the subsequent in vivo exposures only when target biomarkers showed values comparable with baseline levels previously checked.

2.2. Concentration choice

Doses to be tested under laboratory conditions must be carefully selected in order to give information with maximum utility in the real world. Since the purpose of this study was to investigate particular sub-lethal effects, and bearing in mind that no data on the toxicity of PCM was available for D. polymorpha, drug doses were chosen according to the concentration currently found in freshwaters worldwide. We selected 1 nM (0.154 $\mu g\,L^{-1}$), 5 nM (0.75 $\mu g\,L^{-1}$) and 10 nM (1.51 $\mu g\,L^{-1}$) concentrations of PCM. The first and second doses are comparable with most of the measured environmental concentrations (MECs; Bound and Voulvoulis, 2006; Grujić et al., 2009). The highest one is comparable with the maximum level revealed currently in aquatic ecosystems (1.95 $\mu g\,L^{-1}$; Kolpin et al., 2004), but much lower than the PEC value calculated by Kim et al. (2007) for Korean water (10 $\mu g\,L^{-1}$) and by Stuer-Lauridsen et al. (2000) for the European Union (65.4 $\mu g\,L^{-1}$).

2.3. Exposure assays

Exposure assays were conducted in semi-static conditions. The entire water volume was changed daily and PCM was added up to the selected concentration. Given the hydrophilic nature of PCM (log K_{ow} = 0.46; Lorphensri et al., 2007), a PCM working solution (10 $mg\,L^{-1}$) was prepared by using deionized water. Exact volumes of this working solution were added daily to each aquarium, until the desired exposure concentrations were reached. The complete water and chemical changes should guarantee a constant solution concentration of PCM over each 24-h period and prevent losses of contaminant as well as the transformation of the parental compound into its metabolites (Binelli et al., 2009). Mussels were fed daily with AlgaMac-2000®, which was added 2 h before each water and chemical change. Temperature, oxygenation and pH were checked daily. Several specimens (n = 33) were collected each day from the control and exposure aquaria to measure cyto- and genotoxic biomarkers in the hemocytes, whose viability was

checked by the Trypan blue exclusion method. The entire soft tissue of the other 25 specimens was frozen in liquid nitrogen and maintained at −80 °C until the enzymatic activity was measured.

2.4. SCGE assay

The SCGE assay was basically performed according to the alkaline (pH > 13) version of the assay developed by Singh et al. (1988), with the subsequent optimizations for the zebra mussel detailed by Buschini et al. (2003). A total of 100 μL of hemolymph from ten specimens was withdrawn from the sinus near to the posterior adductor muscle with a hypodermic syringe containing 200 μL of phosphate-buffered saline (PBS; pH 7.4). Ten aliquots of 10 μL of cell suspension mixed with 85 μL of low-melting agarose (LMA-0.7%) in PBS (37 °C) were spread onto ten coated slides (previously dipped in 1% normal-melting agarose; NMA), then covered by a coverslip and kept at 4 °C for 40 min until the agarose layer was hardened. A third layer of 85 μL was added to the slides in the same way. After agarose solidification, the coverslip was removed and slides were placed in a lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) in a Coplin jar at 4 °C in the dark for 1 h. Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber filled with freshly prepared buffer (1 mM Na$_2$EDTA, 300 mM NaOH, pH 13) and then in an ice-water bath (4 °C). Electrophoresis was then performed at 0.78 V cm^{-1} (25 V) and 300 mA for 10 min. After the electrophoresis, slides were washed with a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 5 min and fixed in absolute ethanol for other 5 min. After staining with DAPI (4′,6-diamidino-2-phenylindole) DNA dye (Sigma–Aldrich), a coverslip was placed over the slides. The observations were carried out under a fluorescence microscope (Leitz DMR, Germany) equipped with a FITC filter. All steps were performed in the dark in order to minimize additional UV-induced DNA damage. Positive controls were carried out exposing hemocytes to H$_2$O$_2$ to check the effectiveness of the electrophoresis conditions. All samples were blindly coded and evaluated; 50 cells for each slide were analyzed using an image analysis system (Comet Score®) for a total of 500 analyzed hemocytes per sample. Two DNA damage end-points were evaluated: the ratio between migration length and comet head diameter (LDR) and the percentage of tail DNA. The first one was chosen to represent DNA damage data, the second since the working group on genetic toxicology testing from the 4th IWGT (International Workshop on Genotoxicity Test Procedures) recently agreed that the percentage of tail DNA is the measure most linearly related to dose and the easiest to understand (Kirkland et al., 2007).

2.5. DNA diffusion assay

The evaluation of the frequency of apoptotic cells was carried out by the method described by Singh (2000), based on the same protocol used for the SCGE assay. An aliquot of 10 μL was shared among five different slides for each sample and then processed with the same method described above for the SCGE assay. The only methodological difference was that, after lysing the hemocytes, slides were subjected to 5 min of alkaline DNA unwinding (pH 13) without the subsequent electrophoresis. Slides were then washed in the neutralization buffer and fixed in absolute ethanol. Finally, after DAPI dying, they were observed under a fluorescence microscope. Two hundred cells per slide were analyzed for a total of 1000 cells per sample. Necrotic cells were eliminated from the count.

2.6. Micronucleus test

The MN test was performed according to the method developed by Pavlica et al. (2000). Hemolymph (100 μL) was withdrawn by the sinus near the posterior adductor muscle with a syringe filled with 100 μL of PBS and ethylenediaminetetraacetic acid (EDTA, 10 mM) solution (1:1 v/v). The cell suspension was spread on a slide and left for 15 min in a humidity chamber at room temperature to allow the hemocytes to settle. The hemocytes were then fixed with glutaraldehyde (25% solution, diluted to 1% in PBS) for 5 min. After rinsing the excess fixative with PBS, the slides were stained with bisbenzimide 33258 (Hoechst; CAS number 23491-45-4) at a concentration of 1 mg mL^{-1} for 5 min, prior to being washed and mounted in glycerol-McIlvane buffer (1:1 v/v). Slides were kept in the dark at 4 °C until examination under a Leitz DCM fluorescence microscope that was equipped with a submerged lens at 100× magnification. All samples were coded and evaluated by a blinded observer. Four hundred cells were counted for each slide, for a total of 4000 cells per sample. Only intact and non-overlapping hemocyte nuclei were scored. Micronuclei were identified by the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN‰) was calculated.

2.7. Neutral Red Retention Assay (NRRA)

The NRRA method followed the protocol proposed by Lowe and Pipe (1994) and International Council for the Exploration of the Sea (ICES, 2004). The Neutral Red stock solution was prepared by dissolving 20 mg of dye in 1 mL of dimethylsulfoxide (DMSO), while the working solution was made by diluting 5 μL of stock solution in 2.5 mL of PBS. Microscope slides were previously coated with 2 μL of polylysine with the help of a coverslip. Five slides were used for each sample. Hemolymph was withdrawn using a hypodermic syringe containing 100 μL of PBS/EDTA solution (1:1 v/v), as described above. The entire withdrawal was spread carefully on each slide. Slides were suspended on a rack in a light-proof humidity chamber for 20 min, and excess solution was carefully tipped off. Lastly, 40 μL of Neutral Red working solution was added. After 20 min of incubation in the humidity chamber, slides were observed under an optical microscope. Slides were examined systematically thereafter at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests were terminated when dye loss was evident in at least 50% of the hemocytes. The mean retention time (NRRT) was then calculated from the five replicates.

2.8. Enzymatic activity

The enzymatic activities were measured in the entire organism according to the observations made by Osman et al. (2007) and Osman and van Noort (2007) that CAT and GST activities in the whole soft tissue were much higher than in a single gill. Enzymatic activities were determined spectrophometrically as described by Orbea et al. (2002). Measurements were carried out in triplicate using the cytosolic fraction extracted from a pool of 6–8 entire mussels ($\approx$1 g fresh weight) homogenized in 100 nM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) using a Potter homogenizer. Specific protease inhibitors (1:10) were also added to the buffer: dithiothreitol (DTT, 100 mM), phenanthroline (Phe, 10 mM) and trypsin inhibitor (Try, 10 mg mL^{-1}). The homogenate was centrifuged at 500g for 15 min at 4 °C. The supernatant was subsequently transferred into clean tubes and centrifuged again at 2000g for 30 min at 4 °C. Finally, the supernatant was ultra-centrifuged at 100 000g for 90 min at 4 °C. The cytosolic fraction was held in ice and immediately processed for the determination of protein and enzymatic activities. The total protein content of all samples was
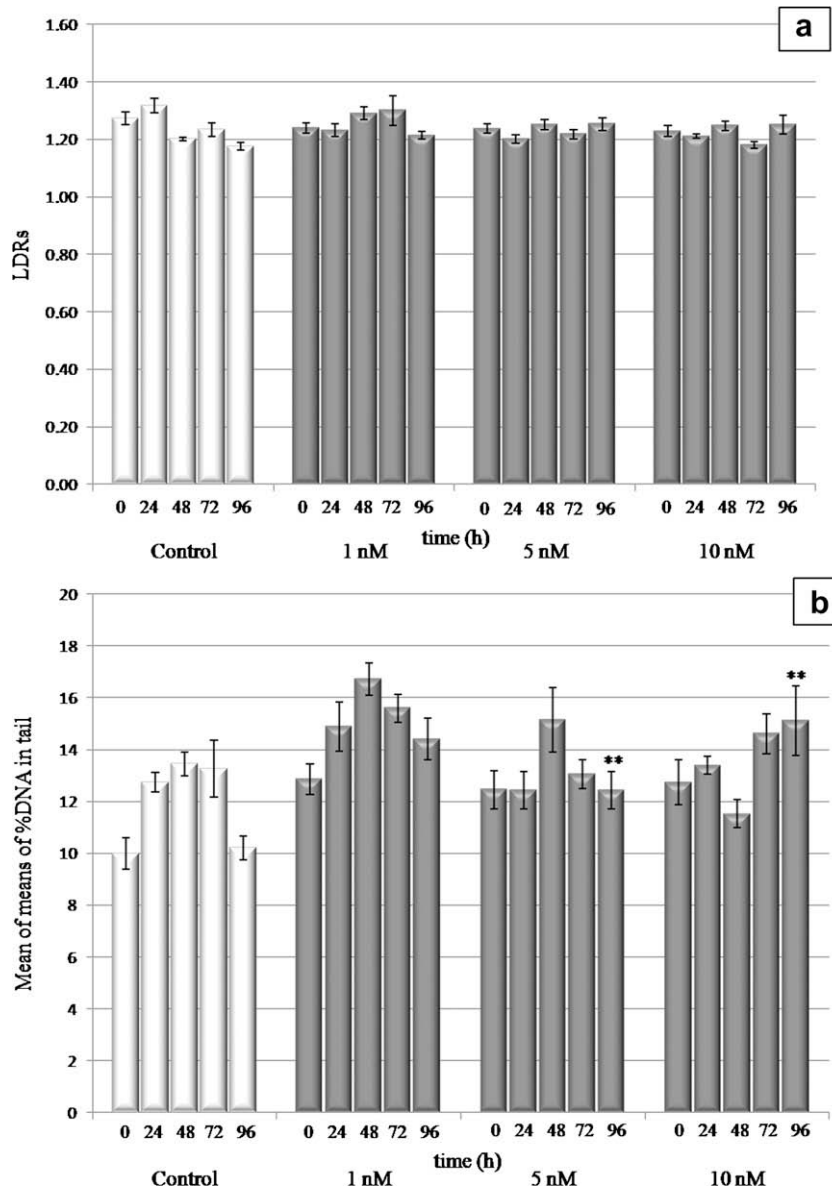
Fig. 1. Mean values (±SEM) of the SCGE assay expressed by the length/diameter ratio (LDR; a) and the mean of the mean percentages of DNA in tail (b). Significant differences (two-way ANOVA, Bonferroni *post hoc* test, **$p < 0.01$) are referred to the comparison between treated mussels and the correspondent control (time to time).

determined according to the Bradford method (1976) using bovine serum albumin (BSA) as the standard. The activity of each enzyme (CAT, SOD, GPx and GST) was measured in the cytosolic fraction. CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 80 mM potassium phosphate buffer (pH 7). SOD activity was determined by measuring the degree of inhibition of cytochrome c reduction at 550 nm by superoxide anion generated by the xanthine oxidase/hypoxanthine reaction. The concentrations of the reagents used during these reactions were as follows: potassium phosphate buffer (50 mM, pH 7.8), hypoxanthine (50 μM), xanthine oxidase (1.87 mU mL^{-1}) and cytochrome c (10 μM). The activity is given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction).

GPx activity was measured by monitoring the consumption of NADPH at 340 nm during the formation of reduced glutathione by glutathione reductase. The reaction medium consisted of the following: 0.2 mM H_2O_2 substrate in 100 mM potassium phosphate buffer (pH 7), containing additional glutathione (2 mM), so-

dium azide (NaN_3; 0.5 mM), glutathione reductase (2 U mL^{-1}) and NADPH (120 μM). GST activity was measured by adding reduced glutathione (20 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) in phosphate buffer (pH 7.4) to the cytosolic fraction. The resulting reaction was monitored for 1 min at 340 nm.

2.9. Statistical analysis

Data normality and homoscedasticity were verified using the Shapiro–Wilk and Levene's tests, respectively. To identify dose/effect and time/effect relationships a two-way analysis of variance (ANOVA) was performed using time and PCM concentrations as variables, while biomarker end-points served as cases. The ANOVA was followed by a Bonferroni *post hoc* test to evaluate eventual significant differences ($p < 0.05$) between treated samples and related controls (time to time) as well as among exposures. The Pearson's correlation test was carried out on all measured variables in the three exposure assays to investigate possible correlations between

various biological responses. All statistical analyses were performed using the STATISTICA 7.0 software package.

3. Results

3.1. Baseline levels

No mortality or changes in hemocyte viability were recorded in the control aquarium. In comparison to the corresponding control value, however, significant viability differences ($p < 0.01$) were recorded in bivalves exposed to the higher PCM dose, beginning from 72 h of exposure (data not showed). The viability of the hemocytes was always higher than 78%, according to recommendations made by the 4th International Workshop on Genotoxicity Test Procedures (IWGTP), which suggested a viability >70% for the overall genotoxicity assays (Kirkland et al., 2007). All control data from the tested cyto- and genotoxic biomarkers agreed those obtained by our research group in previous studies (Riva et al., 2007; Binelli et al., 2008a,b; Binelli et al., 2009). Moreover, also the baseline levels obtained for enzymatic activities were similar to those obtained in zebra mussel specimens by Osman and van Noort (2007), Binelli et al. (2009) and Faria et al. (2009).

3.2. Cyto-genotoxicity assay results

PCM did not induce primary genetic damage in zebra mussel hemocytes at each tested concentration, since no significant differences ($p > 0.05$) were noticed about the LDR values between controls and PCM-treated specimens at each exposure time and dose (Fig. 1A). Neither time-dependent (two-way ANOVA, Bonferroni *post hoc* test; $F = 0.79$; $p > 0.05$) nor dose-dependent ($F = 1.86$; $p > 0.05$) relationships were noticed for the LDR end-point. By contrast, considering the mean of the percentage of DNA in the tail, both time/effect ($F = 9.148$; $p < 0.01$) and dose/effect ($F = 9.166$; $p < 0.01$) relationships were evident (Fig. 1B). Moreover, we observed an overall significant difference ($p < 0.01$) between each PCM treatment and control. In addition, at the end of the exposure, 5 nM and 10 nM PCM increased significantly ($p < 0.01$) the levels of the percentage of DNA in the tail.

According to the tail DNA data, the MN assay (Fig. 2) showed an overall significant ($p < 0.05$) difference not only between each treatment and the controls, but also between 10 nM and the other two tested doses. Moreover, we noticed significant overall dose/effect ($F = 20.48$; $p < 0.01$) and time/effect ($F = 19.32$; $p < 0.01$) relationships. At the end of the exposure at 5 nM and 10 nM, the data indicated a significant ($p < 0.01$) frequency of micronuclei about 3.7 and 5 times higher than baseline levels, respectively. By contrast, the measured apoptotic cell frequencies (Fig. 3) showed neither a time/effect ($F = 1.82$; $p > 0.05$) nor a dose/effect ($F = 1.6$; $p > 0.05$) relationship and no differences ($p > 0.05$) were observed between each treatment and the controls or among each PCM dose. The only significant difference ($p < 0.05$) in the apoptotic frequency was noticed at the end of exposure at 10 nM, with about a fourfold increase of apoptosis compared to the correspondent baseline level.

Finally, data obtained by the NRRA (Fig. 4) showed that PCM was able to induce a significant destabilization of lysosomal membranes in an overall dose-dependent ($F = 33.43$; $p < 0.01$) and time-dependent ($F = 23.29$; $p < 0.01$) manner. The statistical approach highlighted an overall significant difference ($p < 0.05$) not only between each treatment and baseline levels, but also among each other. A decreasing temporal trend was recorded at each dose and a significant ($p < 0.01$) increase of cellular stress in bivalves was noticed at the highest dose after only 48 h of exposure.

3.3. Enzymatic activity results

CAT, GPx and GST showed a very similar trend with a clear and significant ($p < 0.05$) induction of their enzymatic activity starting from 5 nM, while only SOD exhibited a more complex enzymatic trend (Fig. 5). Notwithstanding, we notice a clear dose/effect (two-way ANOVA, $p < 0.01$) and time/effect ($p < 0.01$) relationship for each single enzyme. The CAT activity reached values about two-fold higher than the correspondent controls at the end of the exposure at the higher dose. Moreover, the higher treatment differed significantly ($p < 0.01$) from the others. The GPx, like CAT, showed a significant ($p < 0.01$) increase of 63% compared to controls after 96 h at 5 nM and after 24 h at 10 nM. Each dose differed significantly from controls ($p < 0.01$) and 5 nM and 10 nM as well as from the lowest dose ($p < 0.01$). Each single PCM dose induced significant ($p < 0.05$) changes in the GST activity that exhibited an increasing trend similar to CAT and GPx at 5 nM and 10 nM, with significant differences ($p < 0.01$) compared to the correspondent controls after 72 h of exposure at each concentration. By contrast, SOD was characterized by a drastic increase ($p < 0.01$) in activity after only 24 h at 1 nM, followed by a return to baseline levels. The middle dose showed a similar behavior, but data were not significantly different from controls. PCM showed a slight, but non-significant increasing trend at 10 nM. Notwithstanding this high variability of responses, each dose trend differed significantly from the controls ($p < 0.05$), and the lowest concentration differed ($p < 0.01$) also from 5 nM and 10 nM.

4. Discussion

The first approach to characterize the possible adverse effects due to a specific chemical could be to utilize *in vitro* assays that provide basic information concerning the toxicity of xenobiotics and often help elucidate the probable mechanism of action of pollutants. In previous *in vitro* screening studies on zebra mussel hemocytes, we pointed out the high capability of PCM to induce both cytotoxic and genotoxic damage (Parolini et al., 2009). Results obtained by this previous research showed that PCM has a high tendency to induce DNA damage, as highlighted by the significant increase in LDR values and apoptotic cell frequency. Moreover, data obtained by NRRA revealed that PCM was able to significantly reduce the lysosomal membrane stability. These results, obtained at administered doses (30, 150 and 450 $\mu g\ L^{-1}$) much higher than environmental levels, revealed the possible adverse effects that PCM can cause to *D. polymorpha* but may not completely reflect the real risk for the organism and consequently for the aquatic biocoenosis. The *in vivo* assays yield additional toxicological information for the selected compound and help to thoroughly complete and analyze the topic of the environmental risk assessment. *In vivo* results gave quite different responses than *in vitro* experiments, confirming that a tiered approach can give a more exhaustive picture of the pollutants' toxicity (Hartmann et al., 2004). While the *in vitro* assays showed clear primary DNA damage highlighted by the Comet Test, PCM tested *in vivo* at environmental concentrations was not able to produce significant DNA fragmentation, except for two values obtained by the use of the percentage of DNA in the tail, considered by the IWGTP as the measure that is most linearly related to dose (Kirkland et al., 2007). The lack of correlation ($p > 0.05$) between the Comet Test end-points, which evaluate primary repairable genetic damage, and the other biomarkers of genotoxicity (DNA diffusion assay and MN test) that detect fixed DNA damage, seems to indicate that the PCM genotoxicity appeared in *D. polymorpha* through great genetic injuries, such as double strand breaks (dsb), translocations, and inversions, without a previous increase in DNA fragmentation. Repairing systems are
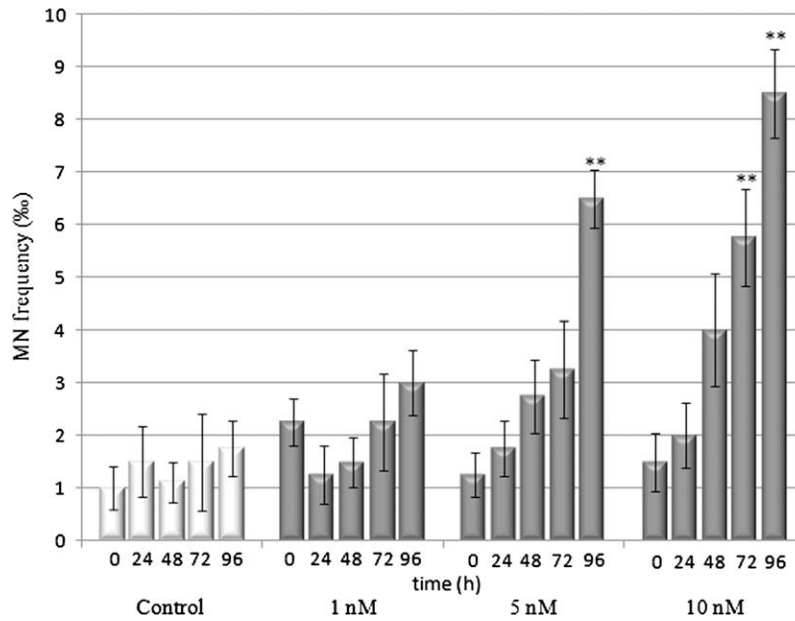
Fig. 2. Frequency of micronucleated hemocytes (mean‰ ± SEM) measured in zebra mussel specimens. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, [**]$p < 0.01$) are referred to the comparison between treated mussels and the correspondent control (time to time).
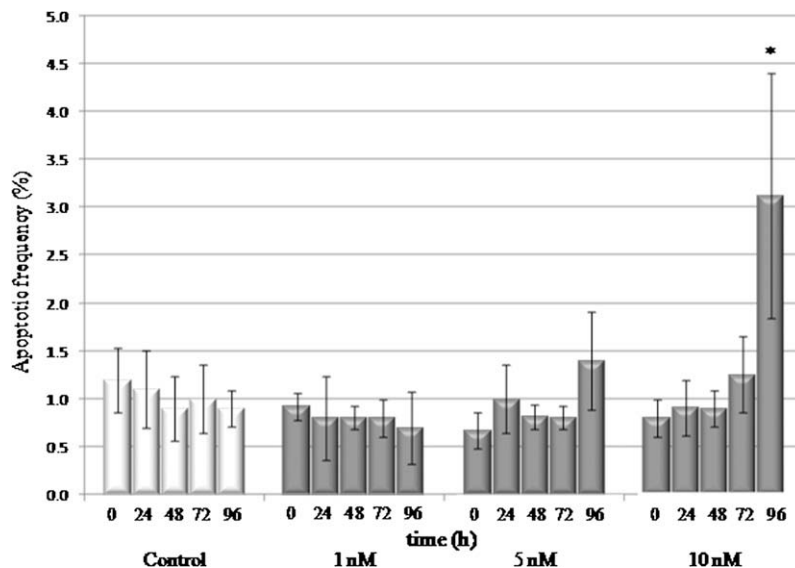

Fig. 3. Percentages of apoptotic hemocytes (mean values ± SEM) measured by the DNA diffusion assay in zebra mussel specimens. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, [*]$p < 0.05$) are referred to the comparison between treated mussels and the correspondent control (time to time).

capable of repairing minor DNA damage caused by PCM, while hemocytes activate the apoptotic processes or produce micronuclei only when broad genetic damage is evident. As suggested by Binelli et al. (2009) for the exposure of zebra mussel specimens to the antibacterial trimethoprim, the significant increase of micronuclei ($p < 0.01$) observed at the end of the exposure at 5 nM and 10 nM (Fig. 2) should indicate that one of the mechanism of PCM cytotoxicity involves the aneuploidogen pathway, instead of clastogenic effects or that the latter are produced by broad genetic damage. Additionally, the significant ($p < 0.05$) increase of apoptotic cells (Fig. 3) measured at the end of exposure at 10 nM, the strong correlation with micronuclei frequency noticed already at 5 nM and the lack of correlation with SCGE end-points can confirm the hypothesis that hemocytes chose a programmed cell death only

in presence of great DNA injuries, without transitioning through the DNA fragmentation.

Since the genotoxicity potential of PCM was registered only at the end of exposure (Figs. 2 and 3), we can suppose that this hydrophilic compound can be readily eliminated by the detoxification system through feces at least at the beginning of exposure. Afterward, when the levels of PCM increase in tissues, broad genetic damage appears at the highest doses and cells can choose either to follow the programmed death or the micronuclei production. However, this hypothesis does not explain the possible mechanism of action by which PCM damages macromolecules. Many authors (Hazai et al., 2002) have suggested that the metabolism of PCM leads to ROS production since the parental molecule is biotransformed in the toxic NAPQI (N-acetyl-*p*-benzoquinoneimine), which
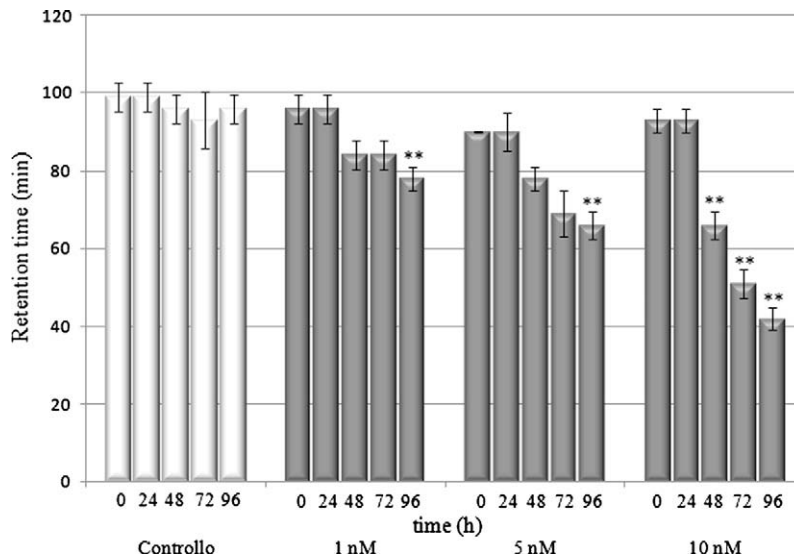
Fig. 4. Neutral Red Retention Time (mean NRRT values ± SEM) measured in zebra mussel specimens. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, **$p < 0.01$) referred to the comparison between treated mussels and the correspondent control (time to time).
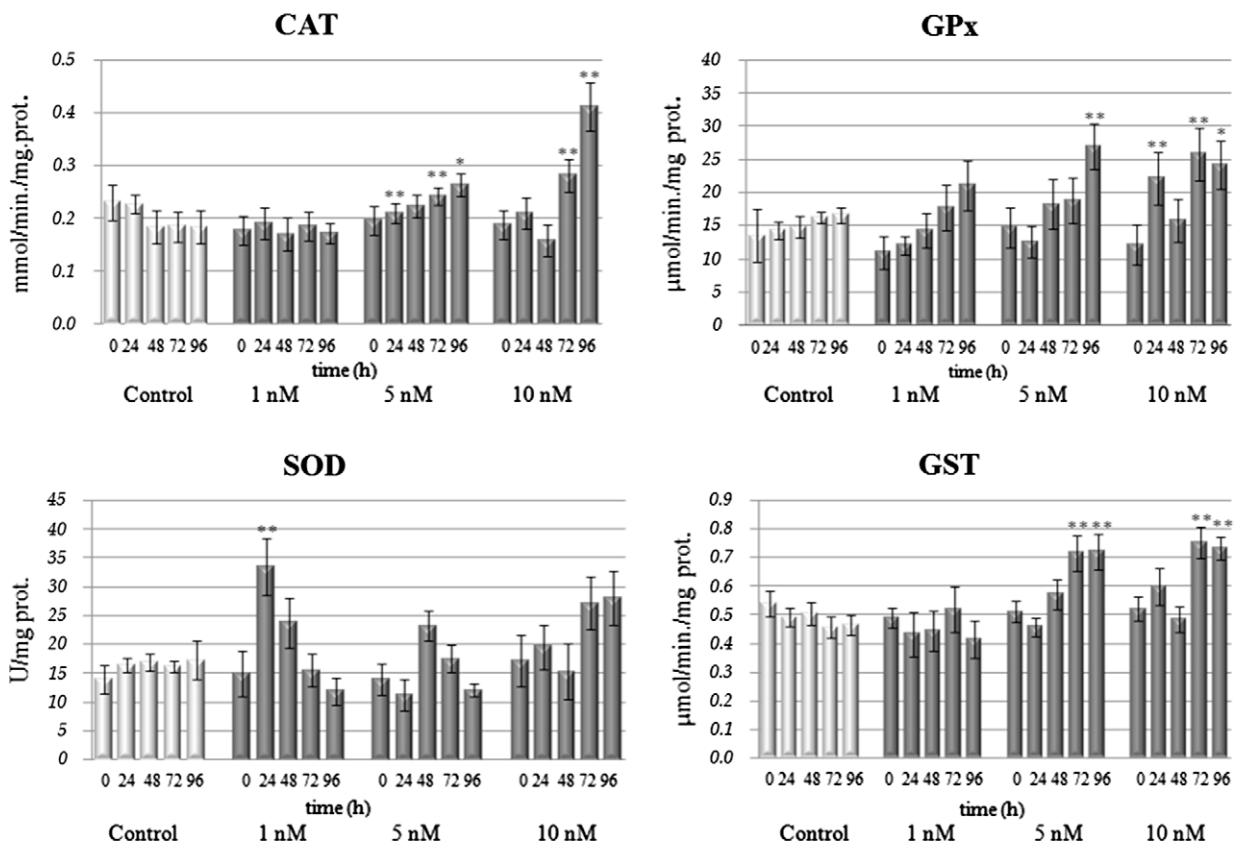
Fig. 5. Effects of increasing doses of PCM on the activity of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione S-transferase (GST) measured in the body of zebra mussels. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, *$p < 0.05$, **$p < 0.01$) are referred to the comparison between treated mussels and the correspondent control (time to time).

besides being a reactive metabolite that interacts with proteins and nucleic acids (Huber et al., 2009), it is an electrophilic intermediate and can increase the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide anion, hydroxyl radical, hydrogen peroxide, nitro oxide and peroxynitrite (Yen et al., 2006). Furthermore, results obtained measuring

the bivalve enzymatic activity explain the onset of genetic damage with the increase in oxidative stress through the production of ROS.

SOD activity in *D. polymorpha* exposed to PCM showed a very particular response since it significantly ($p < 0.01$) increased only at the lowest concentration after 24 h of exposure (Fig. 5). The lack

of a significant SOD response at 5 nM and 10 nM could indicate that sub-lethal damage caused by PCM at higher doses compromised the ability of the zebra mussel to respond in an adaptive manner (Regoli et al., 2003). On the other hand, the antioxidant chain reaction is not blocked by the lack of SOD induction because of several other cellular enzymes, such as those contained in peroxisomes, are able to generate H_2O_2 (Khessiba et al., 2005) that represents the selective substrate for the subsequent antioxidant enzymes. The significant induction of CAT and GPx obtained in our study was also observed in several previous studies carried out in mussels exposed to different xenobiotics. A significant increase of CAT was observed in the digestive gland of a ribbed mussel (*Geukensia demissa*) exposed to paraquat (Wenning et al., 1988), in *M. edulis* exposed to menadione (Livingstone et al., 1990) and in a green-lipped mussel (*Perna viridis*) exposed to polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides (OCs) (Richardson et al., 2008). Furthermore, Pan et al. (2006) have found a similar GPx increasing trend in the *Chlamys ferrari* hemolymph exposed to benzo(α)pyrene, benzo(k)fluoranthene and their mixture. The significant increase ($p < 0.01$) of GST activity was already found by many authors (Gagné et al., 2004; Canesi et al., 2007; Binelli et al., 2009) both in the entire body and in the hepatic glands of some mussel species exposed to different xenobiotics, and it seems to confirm the possible increase in oxidative stress due to an excretion mechanism. On the other hand, PCM is a very polar chemical (log K_{ow} = 0.46; Lorphensri et al., 2007) and can be easily conjugated to glutathione by GST through weakly active phase I biotransformation enzymes. This pathway was verified in mammals where high PCM concentrations are oxidized by enzymes of the CYP 450 mixed-function oxidase (MFO) system to NAPQI (Corcoran et al., 1980; Bowles et al., 2005), which quickly reacts with glutathione, resulting in the formation of a glutathione conjugate mediated by GST (Huber et al., 2009). On the basis of these findings we can hypothesize a possible mechanism of action of PCM in the zebra mussel (Fig. 6): PCM is oxidized by CYP 450 to the highly reactive NAPQI that can directly interact with proteins and nucleic acids. In addition, this electrophilic intermediate can increase the formation of ROS and reactive nitrogen species (RNS) that can react and create broad injuries to DNA. Finally, GST detoxifies NAPQI, catalyzing its conjugation with glutathione. To verify this suggested mechanism of action it will be necessary to investigate the formation of NAPQI and/or its glutathione-conjugated metabolite. Also the significant decrease of the NRRT (Fig. 4), which is a common parameter for the assessment of general stress in bivalves and an indicator of the induction of oxidative stress (Lowe et al., 1995), along with the significant negative correlation with apoptosis and micronuclei end-points obtained at 5 nM
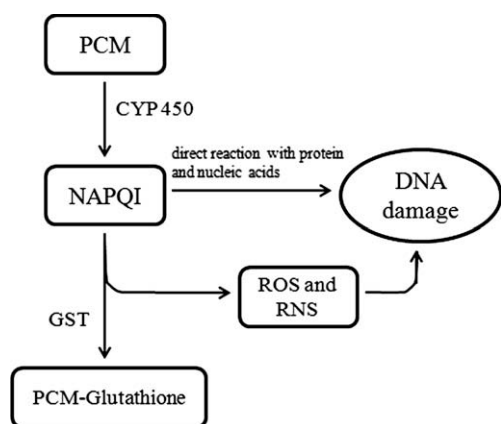

Fig. 6. Supposed mechanism of action of PCM in zebra mussel.

Table 1
Pearson's correlation matrix obtained by using all measured end-points (pool of 18–24 specimens) at 1 nM, 5 nM and 10 nM. Significant correlations (*$p < 0.05$) are indicated in bold.

	LDR	%DNA	MN	Apo	NRRT	CAT	SOD	GPx
1 nM								
%DNA	**0.79**							
MN	−0.03	0.01						
Apo	−0.07	−0.04	0.02					
NRRT	−0.08	−0.01	−0.19	0.09				
CAT	**−0.32**	−0.21	−0.05	0.16	0.22			
SOD	0.06	**0.35**	−0.26	−0.17	**0.29**	0.17		
GPx	−0.09	−0.24	0.27	−0.24	**−0.65**	0.06	**−0.40**	
GST	−0.09	−0.05	−0.03	0.18	0.24	**0.35**	−0.09	0.13
5 nM								
%DNA	**0.78**							
MN	0.16	0.27						
Apo	0.05	0.17	**0.33**					
NRRT	−0.08	−0.21	**−0.50**	−0.23				
CAT	**0.39**	**0.37**	0.21	0.21	−0.25			
SOD	0.04	0.17	−0.18	**−0.32**	−0.10	0.02		
GPx	0.26	**0.32**	**0.60**	**0.34**	**−0.72**	**0.55**	0.01	
GST	0.18	0.26	**0.55**	0.26	**−0.77**	0.21	0.08	**0.79**
10 nM								
%DNA	**0.81**							
MN	0.06	0.12						
Apo	−0.04	0.10	**0.45**					
NRRT	0.00	−0.11	**−0.69**	**−0.51**				
CAT	0.04	0.16	**0.62**	**−0.69**	**−0.70**			
SOD	0.05	0.14	**0.49**	**0.50**	**−0.60**	**0.75**		
GPx	−0.19	−0.19	**0.36**	0.28	**−0.54**	**0.59**	**0.70**	
GST	−0.15	0.01	**0.49**	**0.43**	**−0.64**	**0.78**	**0.85**	**0.85**

LDR = length/diameter ratio; % DNA = percentage of tail DNA; Apo = frequency of apoptotic cells; MN = frequency of micronuclei; NRRT = Neutral Red Retention Time; CAT = catalase; GPx = glutathione peroxidase; SOD = superoxide dismutase; GST = glutathione S-transferase.

and 10 nM confirm that the oxidative stress could be the main pathway for the formation of DNA damage.

The correlation analysis (Table 1) performed on all measured biomarkers could be an useful tool to support the proposed mechanisms of action. The genotoxicity revealed mainly for the highest administered dose is most likely due simply to the increase in oxidative stress, since overall significant ($p < 0.05$) correlations were found between genotoxic end-points and all the antioxidant enzymes. Moreover, another confirmation was made by the significant negative ($p < 0.05$) correlation between NRRT, genotoxic biomarkers and antioxidants, which was in accordance with work by many authors who reported good correlations between lysosomal membrane destabilization, impairment of the antioxidant defense system and DNA injuries (Regoli et al., 2002; Mamaca et al., 2005; Binelli et al., 2009). In addition, other authors have suggested that reactive free-radicals contribute to the destabilization of lysosomal membranes and that the intralysosomal environment is already an oxyradical production site (Kirchin et al., 1992; Winston et al., 1996). Finally, the significant correlation ($p < 0.05$) between GST and phase I enzymes confirms the activation of the entire enzymatic defense chain of the zebra mussel. Moreover, it supports the thesis that GST play a fundamental role in the protection against PCM, but at the same time that it is also the responsible for the insurgence of oxidative stress, through the PCM conjugation with glutathione.

5. Conclusions

Since this work is only partially in accordance with results obtained from our *in vitro* study on the evaluation of cyto-genotoxicity of PCM, it confirms that the tiered approach can give a more exhaustive and detailed report of the pollutant toxicity. The new

data revealed the capacity of this drug to induce moderate genotoxicity in bivalves exposed to environmental concentrations, but they do not show any primary DNA damage, unlike the *in vitro* results. This genetic damage was probably due both to the increase in oxidative stress and/or to a direct interaction between its metabolite NAPQI with DNA. These data confirm the hypothesis that PCM could be a problematic compound for aquatic organisms, although the current environmental concentrations can seemingly cause only low and moderate adverse effects. On the other hand, the significant DNA injuries found at the end of exposure can show a possible delay of PCM genotoxic action that can be confirmed by longer assays. Nonetheless, considering the increasing production and use of this drug, its environmental presence and hazardous impact on aquatic organisms could quickly increase.

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PAPER VII

Parolini Marco, Binelli Andrea, Provini Alfredo

Assessment of the potential cyto–genotoxicity of the nonsteroidal anti-inflammatory drug (NSAID) diclofenac on the Zebra mussel (*Dreissena polymorpha*)

Assessment of the Potential Cyto–Genotoxicity of the Nonsteroidal Anti-Inflammatory Drug (NSAID) Diclofenac on the Zebra Mussel (*Dreissena polymorpha*)

Marco Parolini · Andrea Binelli · Alfredo Provini

Abstract A battery of eight biomarkers was used on the freshwater bivalve *Dreissena polymorpha* in order to evaluate potential sub-lethal effects of the nonsteroidal anti-inflammatory drug diclofenac (DCF; 2-[(2,6-dichlorophenyl)amino]phenylacetic acid). By an in vivo approach, mussels were exposed for 96 h to increasing concentrations (0.3, 1, and 2 nM) of DCF perfectly comparable with current surface water levels. We determined the single cell gel electrophoresis assay, the apoptotic frequency (DNA Diffusion assay), the micronucleus test (MN test), and the lysosomal membrane stability (Neutral Red Retention Assay) in mussel hemocytes. Moreover, the activity of catalase, superoxide dismutase, glutathione peroxidase, and the phase II detoxifying enzyme glutathione *S*-transferase was measured in the cytosolic fraction extracted from a pool of entire bivalves to reveal possible alterations of the oxidative status of exposed specimens. The biomarker battery pointed out a negligible cyto- and genotoxicity on zebra mussel hemocytes since only a slight decrease of lysosomal membrane stability from baseline levels was measured at the end of exposures at the highest concentration (2 nM). In addition, environmental concentrations of DCF seem to have a negligible effect on the activities of antioxidant and detoxifying enzymes.

Keywords Diclofenac · Cyto-genotoxicity · Biomarker · *Dreissena polymorpha*

1 Introduction

In recent years, many investigations have been reported the current widespread occurrence of pharmaceutical and personal care products (PPCPs) in the environment, notably in the aquatic compartment (Roberts and Thomas 2006; Gómez et al. 2007). According to these evidences, the issue of the potential adverse effects of these molecules on the aquatic biocoenosis has begun to receive an increasing interest within the scientific community. Many tons of pharmacologically active substances are commonly used in human and veterinary medicine, and following their proper therapeutic use, they are excreted, either unchanged or metabolized, via urine and feces directly into surface waters or into sewers. However, since sewage treatment plants (STPs) are not able to completely eliminate most of these compounds, they end in surface water tough. Here, since pharmaceuticals were designed to affect a biological target Halling-Sørensen et al. (1998), they may pose a potential environmental threat to aquatic non-target organisms, even at low concentrations.

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Among the revealed compounds, the nonsteroidal anti-inflammatory drugs (NSAIDs) are a group of agents that share similar pharmacologic properties and are widely used for control of pain and inflammation. Being the sixth most sold drugs worldwide (Langman 1999), NSAIDs have an estimated annual production of several kilotons (Cleuvers 2004). Diclofenac (DCF; 2-[(2,6-dichlorophenyl)amino]phenylacetic acid) is a well-known phenylacetic acid NSAID, commonly used to reduce inflammation and pain associated with arthritis and other conditions, such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis (Todd and Sorkin 1988). As all NSAIDs, DCF inhibit the cyclo-oxygenases COX-1 and COX-2 (Laneuville et al. 1994; Gierse et al. 1995), which catalyze the formation of prostaglandins and thromboxane from arachidonic acid. Because of its huge worldwide selling, often over-the-counter and large prescription volume, DCF has been identified as one of the main pharmaceuticals present in the aquatic ecosystems. Dermal application results the main emission pathway of the active compound (Heberer and Feldmann 2005) due to its relative small absorption on skin (5–10%) while the remaining is released into water by washing (Letzel et al. 2009). For this reason, DCF is recognized as a very important pollutant due to its low removal rate during the wastewater treatment process. It is frequently detected at low microgram-per-liter range in STP effluents of Europe and North and South America (Deng et al. 2003; Koutsouba et al. 2003; Ashton et al. 2004; Lindqvist et al. 2005; Roberts and Thomas 2006; Gómez et al. 2007). Accordingly, DCF is commonly found also in surface waters in concentrations covering a wide range from few nanograms per liter up to the micrograms per liter (Metcalfe et al. 2003; Ashton et al. 2004; Lindqvist et al. 2005; Gros et al. 2006). Nonetheless, these findings, its environmental fate, and above all its ecotoxicological effects on non-target organism are only poorly understood. Although the recent decline of three Indian gyps vulture populations (Indian White-rumped Vulture, *Gyps bengalensis*; Indian Vulture, *Gyps indicus*; and Slender-billed Vulture, *Gyps tenuirostris*) has been related to diclofenac-induced renal failures (Oaks et al. 2004; Taggart et al. 2007), at present still few investigations report the adverse effects of DCF on aquatic organisms. Some studies were carried out on *Vibrio fisheri* (Zhang et al. 2008a), several algal

species, *Daphnia magna* (Cleuvers 2003; Ferrari et al. 2003), and *Danio rerio* (Dietrich and Prietz 1999) which demonstrated that this drug has a low acute toxicity as short-term EC_{50}, with tested concentrations much higher than those currently present in the surface waters. However, continuous release of DCF may lead to chronic exposure of aquatic organisms and consequently lower effect concentrations. Triebskorn et al. (2004) highlighted that 1 µg/L DCF was able to cause harmful effects on liver, kidney, and gills of rainbow trout, while Hong et al. (2007) showed that the expression of biomarker genes was related to cellular toxicity, genotoxicity, and estrogenic effects in Japanese medaka fish (*Oryzias latipes*) exposed to low DCF concentration. Notwithstanding these evidences, at present, no study was carried out on invertebrate species to study the possible DCF sublethal effects.

In order to increase the knowledge about this topic, the purpose of this study was to assess the DCF potential cyto–genotoxicity on the freshwater bivalve zebra mussel (*Dreissena polymorpha*) by a multibiomarker approach. This mollusk was chosen as a biological model because it plays an important role in freshwater ecosystems, and it is particularly susceptible to environmental stressors (Gagné et al. 2006). Additionally, previous studies have shown that *D. polymorpha* is a useful and sensible organism to point out sublethal effects when exposed to synthetic chemicals, like persistent organic pollutants (Binelli et al. 2008a; Riva et al. 2007) and pharmaceuticals (Binelli et al. 2008a, 2009; Parolini et al. 2010), also at lowest concentrations. We measured the end-points of eight individual biomarkers, whose integrated response can be helpful for detecting the sublethal effects caused by DCF on zebra mussel specimens. Bivalves were exposed to three different DCF concentrations, similar to those currently found in surface waters worldwide. DCF genotoxic potential was evaluated by the single-cell gel electrophoresis (SCGE) assay, the measure of apoptotic frequency (DNA Diffusion assay), and the micronucleus test (MN test). Cytotoxicity was measured by the Neutral Red Retention Assay (NRRA) that evaluates the lysosomal membrane stability, a classical parameter of generic cellular stress in bivalves (Lowe et al. 1995). Additionally, we also measured the activity of three antioxidant phase I enzymes, catalase (CAT), superoxide dismutase (SOD), and glutathione perox-

idase (GPx), as well as the phase II detoxifying enzyme glutathione S-transferase (GST) in order to study in-depth the possible redox status unbalance of the exposed mussels.

2 Materials and Methods

Standard DCF (CAS number 15307-86-5) was obtained from Sigma–Aldrich (Steinheim, Germany), as well as all other chemicals used for biomarker determination. Dimethyl sulfoxide (DMSO; CAS number 67-68-5; purity=99.5%) was obtained from VWR International (Milan, Italy).

2.1 Mussel Acclimation and Maintenance Conditions

Several hundred specimens of $D.$ $polymorpha$ tied by their byssi to the rocks were collected on September 2008 by a scuba diver at a depth of 4–6 m in Lake Lugano (Northern Italy), which is considered a reference site due to its low xenobiotic pollution (Binelli et al. 2005). Mussels were quickly transferred in laboratory in bags filled with lake water. Rocks were rinsed under running tap water and introduced into 100-L glass holding aquaria filled with tap and lake water (75:25v/v) in order to avoid a drastic chemist water change and to guarantee a food supply to mussels for the first 24 h of acclimation. Specimens were maintained at a natural photoperiod, constant temperature (20±1°C), pH (7.5), and oxygenation (>90% of saturation). Bivalves were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA), and water was regularly changed for at least 2 weeks to gradually purify the mollusks by the possible pollutants previously accumulated in their soft tissues. Several specimens (∼2,000) with the same shell length (∼20 mm) were chosen for in vivo tests, including control and solvent assay. They were gently cut from the rocks and placed on glass sheets suspended in 15-L aquaria filled with 10 L of tap water and maintained at the same conditions described above. Three hundred specimens were put in each aquarium. Only specimens that were able to reform their byssi and reattach themselves to the glass sheet were used in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method, while biomarker baseline levels were checked weekly. Mussels were exposed to DCF only when target biomarkers levels were comparable with baseline levels obtained in previous studies.

2.2 Concentration Choice

In order to give an ecological value to our research and to provide information with maximum utility in the real world, tested doses were carefully checked. Since the purpose of this study was to investigate particular sublethal effects and bearing in mind that no in vivo data on the toxicity of DCF were available for $D.$ $polymorpha$, drug doses were chosen according to the current concentration found in freshwaters worldwide. We selected 0.3 nM (95 ng/L), 1 nM (318 ng/L), and 2 nM (637 ng/L) DCF concentrations that correspond to the lowest, mean, and highest concentrations measured in surface waters and reported by Zhang et al. (2008).

2.3 Exposure Assays

Exposure assays were conducted in semi-static conditions and were protracted for 96 h. Previous studies demonstrated that this period of time is enough to highlight sublethal effects on PPCP-treated zebra mussel specimens, also exposed to low and environmentally relevant concentrations (Binelli et al. 2008a, 2009; Parolini et al. 2010). Control, DMSO, and exposure aquaria were processed at the same time. The entire water volume (10 L) was changed daily, and DCF was added up to the selected concentrations. Given the lipophilic nature of DCF (log K_{ow}=4.4; Cleuvers 2004) and its low water solubility (23.73 mg/L at 25°C; Research Corporation 2006), we used dimethyl sulfoxide (DMSO) as carrier solvent. So, a DCF working solution (10 mg/L) was prepared by using DMSO. In order to exclude possible adverse cyto–genotoxic effects towards bivalve specimens due to the carrier solvent we prepared a solvent control aquarium, in which a maximum percentage of 0.006% DMSO in water was added daily. Exact volumes of working solution were added daily to each exposure aquarium, until the desired concentrations were reached. The complete water and chemical change were carried out daily, and they should guarantee a constant solution concentration of DCF over each 24-h period and prevent losses of contaminant as well as the transformation of the

parental compound into its metabolites. Bearing in mind that *D. polymorpha* seems to accumulate the pollutants mainly from water (Binelli and Provini 2003), specimens were fed daily 2 h before each water and chemical change in order to avoid the adherence of the chemical to food particles and to prevent the reduction of DCF bioavailability. Temperature (20 ± 1°C), pH (7.5), and oxygenation (>90% of saturation) were checked daily. Several specimens ($n=33$) were collected every 24 h for 5 days (96 h) from the control, solvent, and exposure aquaria in order to measure cyto–genotoxicity in the hemocytes. The entire soft tissue of other 20 specimens was frozen in liquid nitrogen and kept at –80°C until the enzymatic activity was measured.

2.4 SCGE Assay

The SCGE assay was basically performed according to the alkaline (pH>13) version developed by Singh et al. (1988), with the subsequent optimizations for the zebra mussel detailed by Buschini et al. (2003). Hemolymph (100 μl) from ten specimens was withdrawn from the sinus near to the posterior adductor muscle with a hypodermic syringe containing 200 μl of phosphate-buffered saline (PBS; pH 7.4). Ten aliquots of 10 μl of cell suspension were embedded in two layers of low-melting agarose (LMA 0.7%) in PBS (37°C). They were spread onto ten coated slides (previously dipped in 1% normal-melting agarose; NMA) and kept at 4°C in the dark. After agarose solidification, slides were placed in a lysis solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 8 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) at 4°C in the dark for 1 h. Alkaline DNA unwinding was carried out for 5 min in a gel electrophoresis chamber filled with freshly prepared buffer (1 mM Na$_2$EDTA, 300 mM NaOH, pH 13) and kept in an ice-water bath (4°C). Electrophoresis was then performed at 0.78 V/cm (25 V) and 300 mA for 10 min. After electrophoresis, slides were washed with a neutralization buffer (0.4 M Tris–HCl, pH 7.5), fixed in absolute ethanol and stained with DAPI (4′,6-diamidino-2-phenylindole) DNA dye (Sigma–Aldrich). Slides were then observed under a fluorescence microscope (Leitz DMR, Germany) equipped with a FITC filter. All steps were performed in the dark in order to minimize additional UV-induced DNA damage. Positive controls were carried out

exposing hemocytes to H$_2$O$_2$ to check the effectiveness of electrophoresis conditions. All samples were blindly coded and evaluated; 50 cells for each slide were analyzed using an image analysis system (Comet Score®) for a total of 500 analyzed hemocytes per sample. Two common DNA damage endpoints were evaluated: the ratio between migration length and comet head diameter (LDR) and the percentage of tail DNA.

2.5 DNA Diffusion Assay

The evaluation of apoptotic cell frequency was carried out by the method described by Singh (2000), based on the same protocol used for the SCGE assay. A hemolymph aliquot of 10 μl was shared among five different slides for each sample and then processed with the same method described above for the SCGE assay. After lysis step, slides were subjected to 5 min of alkaline DNA unwinding (pH 13) without the subsequent electrophoresis. Slides were washed in the neutralization buffer, fixed in absolute ethanol, and after DAPI dying, they were observed under a fluorescence microscope. Two hundred cells per slide were analyzed for a total of 1,000 cells per sample. Necrotic cells were excluded from the count.

2.6 MN test

The MN test was performed according to the method developed by Pavlica et al. (2000). Hemolymph (100 μl) from 10 zebra mussel specimens was spread on 10 slide and left for 15 min in a moisture chamber at room temperature to allow the hemocytes settling. Slides were fixed with glutaraldehyde (25% solution, diluted to 1% in PBS) and stained with bisbenzimide 33258 (1 mg/mL; Hoechst, CAS number 23491-45-4), prior to being washed and mounted in glycerol-McIlvane buffer (1:1 v/v). Slides were kept in the dark at 4°C until examination under a Leitz DCM fluorescence microscope equipped with a submerged lens at ×100 magnification. All samples were coded and blindly evaluated. Four hundred cells were counted for each slide, for a total of 4,000 cells per sample. Only intact and non-overlapping hemocyte nuclei were scored. Micronuclei were identified by the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency (MN ‰) was calculated.

2.7 NRRA

The NRRA method followed the protocol proposed by Lowe and Pipe (1994) and International Council for the Exploration of the Sea (2004). The Neutral Red stock solution was prepared by dissolving 20 mg of dye in 1 mL of DMSO, while the working solution was made by diluting 5 μL of stock solution in 2.5 mL of PBS. Hemolymph (100 μL) was carefully spread onto polylysine-coated slides. Forty microliters of Neutral Red working solution was added to cell suspension and after 20 min of incubation, slides were observed under an optical microscope. Slides were examined systematically thereafter at 15-min intervals in order to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests were terminated when dye loss was evident in at least 50% of the hemocytes. Five slides were used for each sample. The mean retention time (NRRT) was then calculated from the five replicates.

2.8 Enzymatic Activity

The enzymatic activities were measured in the entire organism according to Osman et al. (2007) and Osman and van Noort (2007) and were determined spectrophometrically as described by Orbea et al. (2002). Measurements were carried out in triplicate using the cytosolic fraction extracted from a pool of 6–8 entire mussels (≈1 g fresh weight) homogenized in 100 nM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) containing specific protease inhibitors (1:10): dithiothreitol (100 mM), phenanthroline (10 mM), and trypsin inhibitor (Try, 10 mg/mL). The homogenate was centrifuged at $500 \times g$ for 15 min at 4°C. The supernatant was subsequently centrifuged at $2,000 \times g$ for 30 min at 4°C, and finally, it was ultra-centrifuged at $100,000 \times g$ for 90 min at 4°C. The cytosolic fraction was kept in ice and immediately processed for the determination of protein and enzymatic activities. The total protein content of each sample was determined according to the Bradford method (1976), using bovine serum albumin as standard. The activity of each enzyme (CAT, SOD, GPx, and GST) was measured in the cytosolic fraction. CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 80 mM potassium phosphate buffer (pH 7). SOD activity was determined by measuring

the degree of inhibition of cytochrome c (10 μM) reduction at 550 nm by superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 μM) reaction. The activity is given in SOD units (1 SOD unit=50% inhibition of the xanthine oxidase reaction).

GPx activity was measured by monitoring the consumption of NADPH at 340 nm, by using 0.2 mM H_2O_2 substrate in 100 mM potassium phosphate buffer (pH 7), containing additional glutathione (2 mM), sodium azide (NaN_3; 0.5 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). GST activity was measured by adding reduced glutathione (20 mM) and 1-chloro-2,4 dinitrobenzene in phosphate buffer (pH 7.4) to the cytosolic fraction. The resulting reaction was monitored for 1 min at 340 nm.

2.9 Statistical Analysis

Data normality and homoscedasticity were verified using the Shapiro–Wilk and Levene's tests, respectively. To identify dose/effect and time/effect relationships, a two-way analysis of variance (ANOVA) was performed using time and DCF concentrations as variables, while biomarker end-points served as cases. Just in case of significant values, ANOVA was followed by a Bonferroni post hoc test to evaluate eventual significant differences ($p < 0.05$) between treated samples and related controls (time to time), as well as among exposures. All statistical analyses were performed using the STATISTICA 7.0 software package.

3 Results

3.1 Baseline Levels

In Table 1 were reported the viability values of control and DCF-treated zebra mussel specimens. No mortality or decreasing hemocyte viability was recorded in the control aquarium, with values higher than 93%. The hemocyte's daily viability from DCF-treated specimens was always higher than 89% and did not show any significant ($p > 0.05$) difference in comparison with the corresponding control values. The hemocyte viability agreed with the recommendations made by the 4th International Workshop on Genotoxicity Test Procedures (IWGTP) that suggested a viability >70% for the

Table 1 Results of hemocyte viability (mean % values ± standard deviation)

	Control	DMSO	0.3 nM	1 nM	2 nM
Hemocyte viability (%)					
$t=0$	94.91 (±0.38)	94.76 (±0.16)	89.85 (±3.04)	89.96 (±0.96)	91.97 (±4.64)
$t=24$	93.69 (±1.15)	90.36 (±4.03)	94.50 (±0.50)	97.04 (±2.24)	93.38 (±0.88)
$t=48$	93.10 (±1.15)	93.79 (±0.68)	90.97 (±2.06)	93.60 (±2.08)	93.90 (±2.55)
$t=72$	94.89 (±0.47)	94.01 (±1.11)	96.03 (±3.00)	97.00 (±1.00)	93.86 (±1.87)
$t=96$	92.62 (±1.89)	94.87 (±1.60)	94.86 (±4.45)	91.90 (±5.14)	91.54 (±3.21)

Measurements employed in triplicate on zebra mussel hemolymph samples exposed to three different DCF concentrations. No significant differences (two-way ANOVA, $p>0.05$) were pointed out by the comparison between treated mussels and the correspondent controls (time to time)

overall genotoxicity assays (Kirkland et al. 2007). We also tested the solvent (DMSO) as an additional control to exclude its possible cyto–genotoxic effects by using the highest concentration reached in water (0.006%). This assay showed that DMSO did not produce any significant ($p>0.05$) change in hemocytes viability, DNA damage, or changes in enzyme activity. No significant ($p>0.05$) temporal differences were noticed within the control and solvent group during the entire exposure tests. All control data from each cyto- and genotoxic assays agreed with those obtained in previous studies carried out by our research group on this biological model (Binelli et al. 2008a, b, 2009). In addition, baseline levels obtained for enzymatic activities were similar to those found by Osman and van Noort (2007), Faria et al. (2009), and Binelli et al. (2009).

3.2 Cyto–Genotoxicity Biomarker Results

Time-dependent (two-way ANOVA, Bonferroni post hoc test; $F=2.945$; $p<0.05$) and dose-dependent ($F=2.465$; $p<0.05$) relationships were noticed for the LDR endpoint although DCF did not induce any primary genetic damage in zebra mussel hemocytes. No significant differences ($p>0.05$) were noticed for LDR values between controls and DCF-treated specimens at each exposure time and concentration (Fig. 1a). Accordingly, no significant differences ($p>0.05$) were revealed also considering the mean of % tail DNA (Fig. 1b), even if both time/effect ($F=9.228$; $p<0.01$) and dose/effect ($F=3.721$; $p<0.01$) relationships were obtained.

No significant ($p>0.05$) induction of apoptosis was observed between DCF-treated mussels and controls; nonetheless, a weak increase of the apoptotic cell

frequency at 2 nM was measured (Fig. 2a). According to SCGE and DNA diffusion assays, MN test did not reveal a significant ($p>0.05$) increase of micronucleated cell frequency at each tested concentration (Fig. 2b). Finally, NRRA (Fig. 3) showed a significant dose-dependent ($F=3.80$; $p<0.05$) and time-dependent ($F=3.67$; $p<0.05$) reduction of lysosomal membranes stability. A slight decreasing trend was evident at 1 nM, but a significant ($p<0.05$) increase of cellular stress in DCF-treated bivalves was noticed only at the end of the exposure at 2 nM, with values 26% lower than the corresponding control.

3.3 Enzyme Activity Results

In Fig. 4 were reported clockwise the results of CAT, SOD, GPx, and GST enzymatic activity. The CAT activity (Fig. 4) did not show any significant differences ($p>0.05$) between DCF-treated and corresponding control specimens, at each exposure time and concentration. SOD activity was not influenced by the exposure to environmental DCF concentrations, showing homogeneous values during the assays that did not differ significantly ($p>0.05$) from the baseline levels.

GPx activity was characterized by both time- ($F=8.010$; $p<0.01$) and dose-dependent ($F=14.294$; $p<0.01$) relationships and showed different trends according to the tested concentration. A progressive GPx activity decreasing trend was registered at 1 nM and a significant ($p<0.01$) inhibition measured after 72 h of exposure with respect to the correspondent control, while at 2 nM, the enzyme activity was perfectly comparable with those measured in the control specimens with the exception for the significant ($p<0.01$) inhibition measured after 24 h.

Fig. 1 Results (mean values ± SEM; standard error of the mean) of the SCGE assay expressed by the length/diameter ratio (**a**) and the mean percentage of tail DNA (**b**). Measurements employed the zebra mussel hemolymph samples (*n*=10) exposed to three different DCF concentrations. No significant differences (two-way ANOVA, *p*>0.05) were pointed out by to the comparison between treated mussels and the correspondent control (time to time)
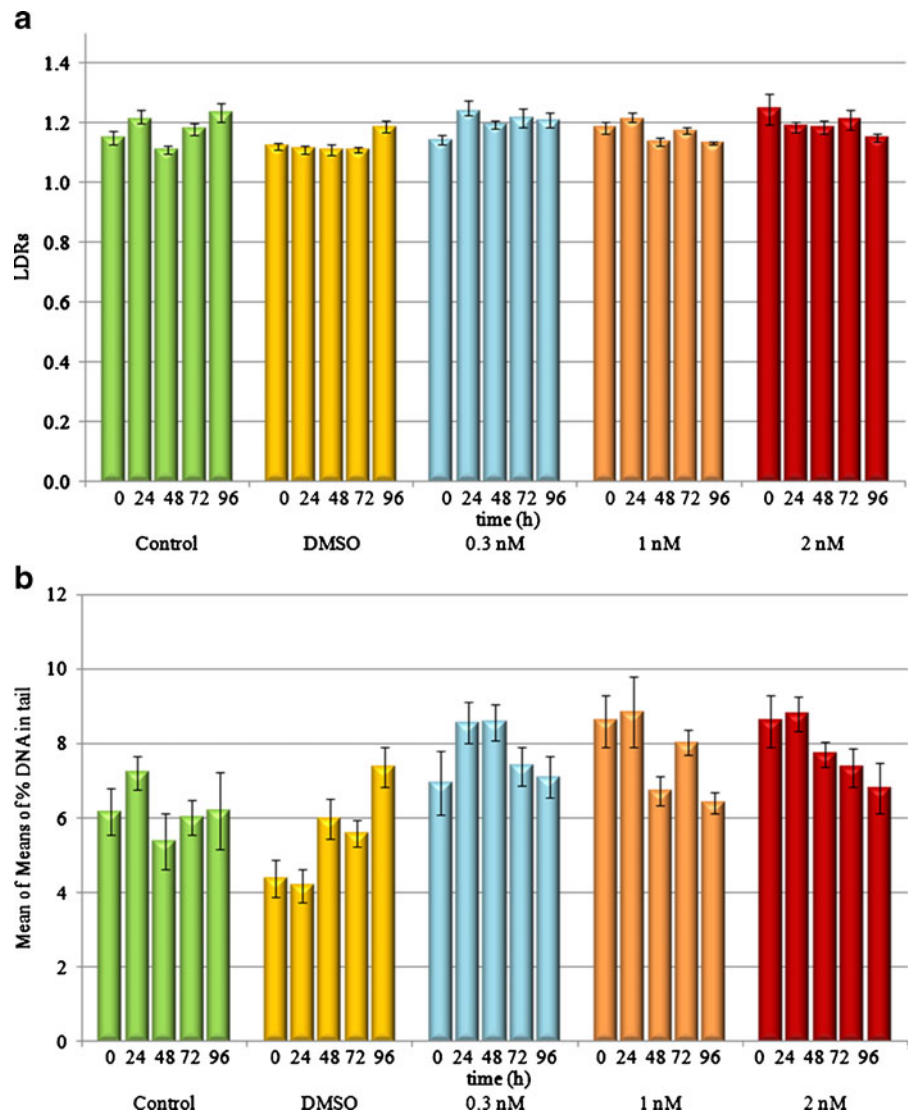
Finally, GST activity showed both time–effect (*F*= 17.171; *p*<0.01) and dose–effect (*F*=15.902; *p*<0.01) relationship exhibiting a parabolic trend at each tested concentration, with the only significant (*p*<0.01) increase of enzyme activity measured after 48 and 72 h at the lowest (0.3 nM) and highest (2 nM) concentration, respectively.

4 Discussion

In vivo biomarker assays are suitable tools to investigate the real ecotoxicological profile of xenobiotics since they are very useful for addressing further environmental risk assessment. On the other

hand, they accurately reproduce the environmental situation, miming both the real accumulation pathways and the effective bioavailability of pollutants. Moreover, they allow the exposed specimens to put into practice all their defense mechanisms, unlike in vitro assays.

In vivo exposures showed that DCF environmentally relevant concentrations were not able to produce any significant DNA fragmentation on zebra mussel hemocytes even after 96 h exposure, as highlighted by the lack of significant increases (*p*>0.05) of LDR values in comparison to baseline levels (Fig. 1a). In addition, the analysis of the mean percentage of tail DNA (Fig. 1b), considered by the IWGTP as the measure that is most linearly related to dose (Kirkland

Fig. 2 Percentages of apoptotic hemocytes (mean values ± SEM) measured by the DNA Diffusion assay ($n=5$) (**a**) and frequency of micronucleated hemocytes (mean values ± SEM) observed in *D. polymorpha* specimens ($n=10$) (**b**) exposed to increasing DCF concentrations. No significant differences (two-way ANOVA, $p>0.05$) were highlighted in both assays by the comparison between treated mussels and the correspondent control (time to time)
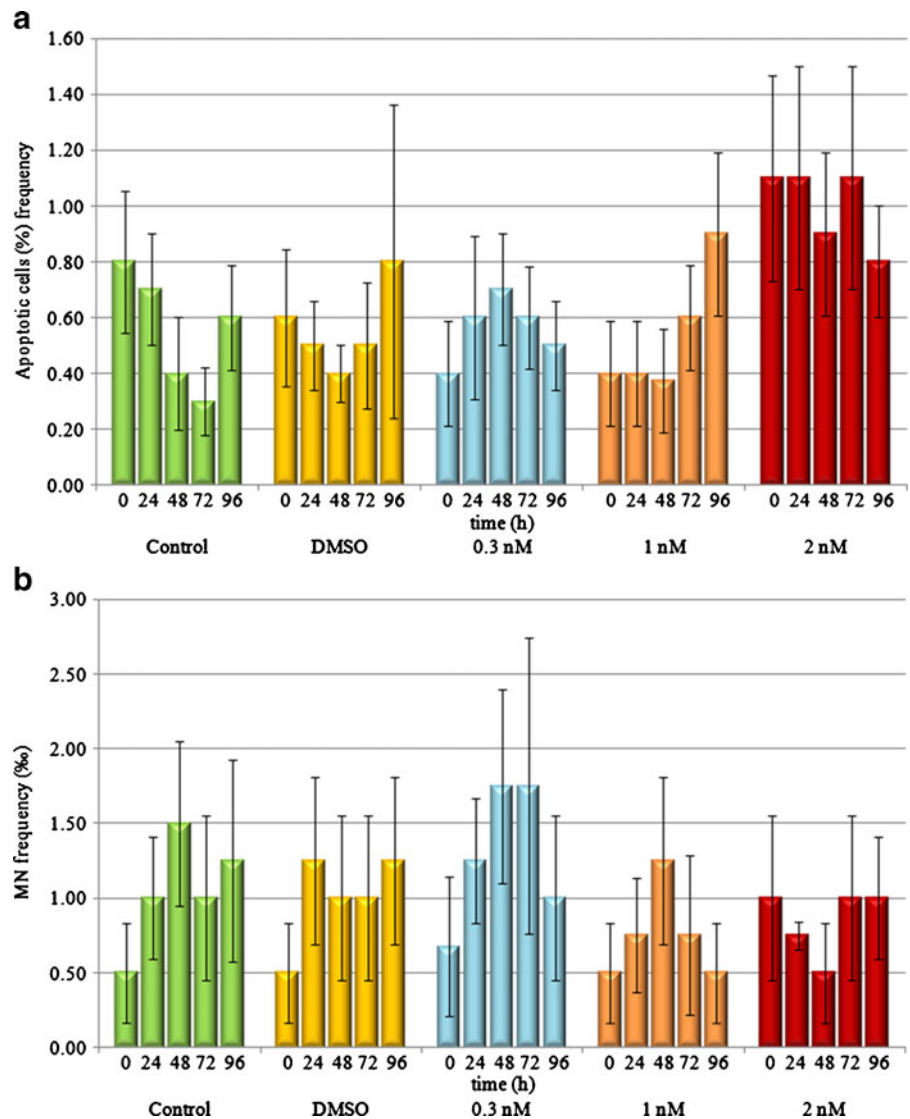

et al. 2007), seems to confirm the inability of tested concentrations to induce significant ($p>0.05$) DNA single-strand breaks to zebra mussel hemocytes. According to SCGE endpoints, DNA Diffusion assay and MN test also confirmed that tested DCF concentrations do not seem genotoxic to zebra mussel hemocytes because they were not able to produce fixed DNA injuries, as pointed out by the lack of significant ($p>0.05$) increase of apoptotic (Fig. 2a) and micronucleated cells (Fig. 2b). Our findings disagree with results obtained by Hong et al. (2007) that showed the induction of the p53 gene expression after only 4-day exposure to 1 μg/L DCF in *O. latipes* specimens. The p53 gene is a critical biomarker for analyzing the carcinogenicity and DNA damage due

to environmental toxicants since its product plays a key role in cell cycle arrest, apoptosis, and the DNA repair pathway (Hong et al. 2007). Moreover, Triebskorn et al. (2004) showed that even environmentally relevant concentrations of DCF (1 μg/L) lead to cellular reactions in the liver, kidney and gills of rainbow trout (*Oncorhynchus mykiss*), which decreases the renal and gill functionality.

Nonetheless, the evidences mentioned above and bearing in mind that previous in vivo studies demonstrated the high sensitivity of *D. polymorpha* to pharmaceuticals (Parolini et al. 2010), our data revealed that DCF does not seem to be genotoxic towards *D. polymorpha* and its hazard seem to be negligible, at least for this aquatic species.

Fig. 3 Mean of NRRT of neutral red loss (mean values ± SEM) from lysosomes in hemocytes of zebra mussels (*n*=5) exposed to increasing concentrations of DCF. Significant differences (two-way ANOVA, Bonferroni post hoc test, *p*<0.05) were referred to the comparison between treated mussels and the correspondent control (time to time)
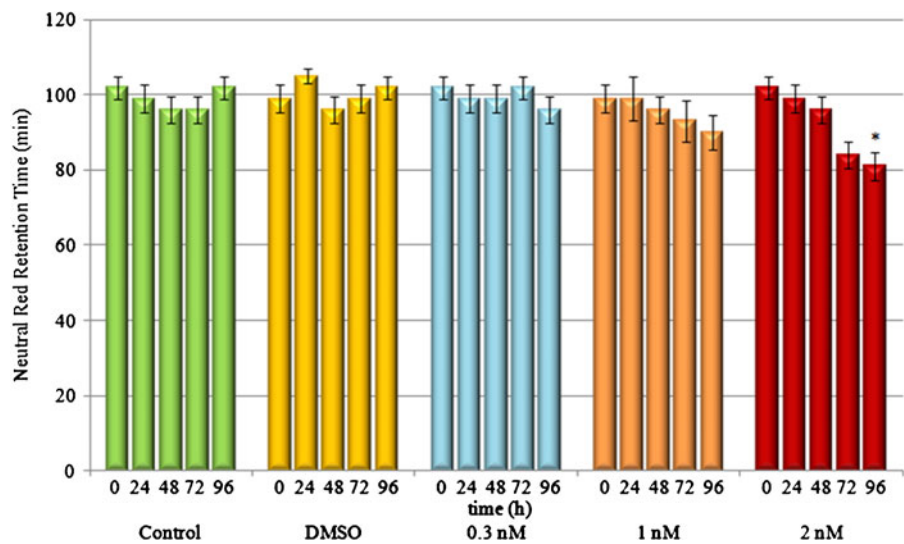
The apparent discrepancy between our in vivo genotoxicity findings and previous studies can be explained bearing in mind that the latter researches were carried out on vertebrate species that could be more sensitive to DCF with respect to invertebrates. Different metabolic pathways or different genes could be involved in the rise of genotoxic effects between vertebrates and invertebrates. According to genotoxicity results, DCF environmental concentrations were not able to produce a cellular stress in the exposed specimens, since the analysis of the retention time showed a slight but significant decrease (*p*<0.05) of the lysosomal membrane stability only at the end of the exposure at 2 nM (Fig. 3). Notwithstanding many authors (Kirchin et al. 1992; Winston et al. 1996; Binelli et al. 2008a) have reported good correlations between lysosomal membrane destabilization and the impairment of the antioxidant defense system (Regoli et al. 2002; Mamaca et al. 2005; Binelli et al. 2009), and others have suggested that reactive free radicals contribute to the destabilization of lysosomal membranes (Kirchin et al. 1992; Winston et al. 1996), this relationship seems not to be confirmed in this study. The activity of antioxidant (CAT, SOD, and GPx) and detoxifying (GST) enzymes (Fig. 4) resulted extremely changeable, showing floating trends at each tested concentration and time, showing that low DCF concentrations are not able to induce even a meaningful oxidative stress to zebra mussel specimens. This huge variability could be explained bearing in mind that antioxidants are part of a very complex

homeostatic system and the redox status is buffered by several interactions. Scientific literature provides several examples of elevated variability of antioxidant responses with transient or limited changes reported for different species, tissues and contaminants (Regoli et al. 2003; Osman et al. 2007; Xiao et al. 2007; Binelli et al. 2009).

Nonetheless, environmental DCF concentrations were not able to induce cytotoxic effects on zebra mussel-treated specimens, many studies carried out on different biological models (Cantoni et al. 2003; Boelsterli 2003; Tang 2003; Yan et al. 2005) are in agreement to affirm that exposure to higher DCF concentrations (ranging between few μg/L up to mg/L) can decrease the effectiveness of defense system towards reactive oxygen species (ROS). In addition, Lauer et al. (2009) showed that very high (10 to 500 μM) DCF concentrations induce alteration on the CYP-450 expression, decreasing the excretion ability of the organism and forming some more toxic metabolites and a very reactive quinone intermediate able to bind to proteins and nucleic acids (Evans et al. 2004). These metabolites may further produce oxidative stress by either generating ROS via redox cycling or depletion of the oxidative stress protective tripeptide glutathione (Boelsterli 2003; Tuschl et al. 2008). This evidence could represent a possible explanation on the very different toxicological behavior noticed in vertebrates and the lack of cyto–genotoxic effects found in zebra mussel. In fact, although invertebrates have several isoforms of the CYP-450, the activity of
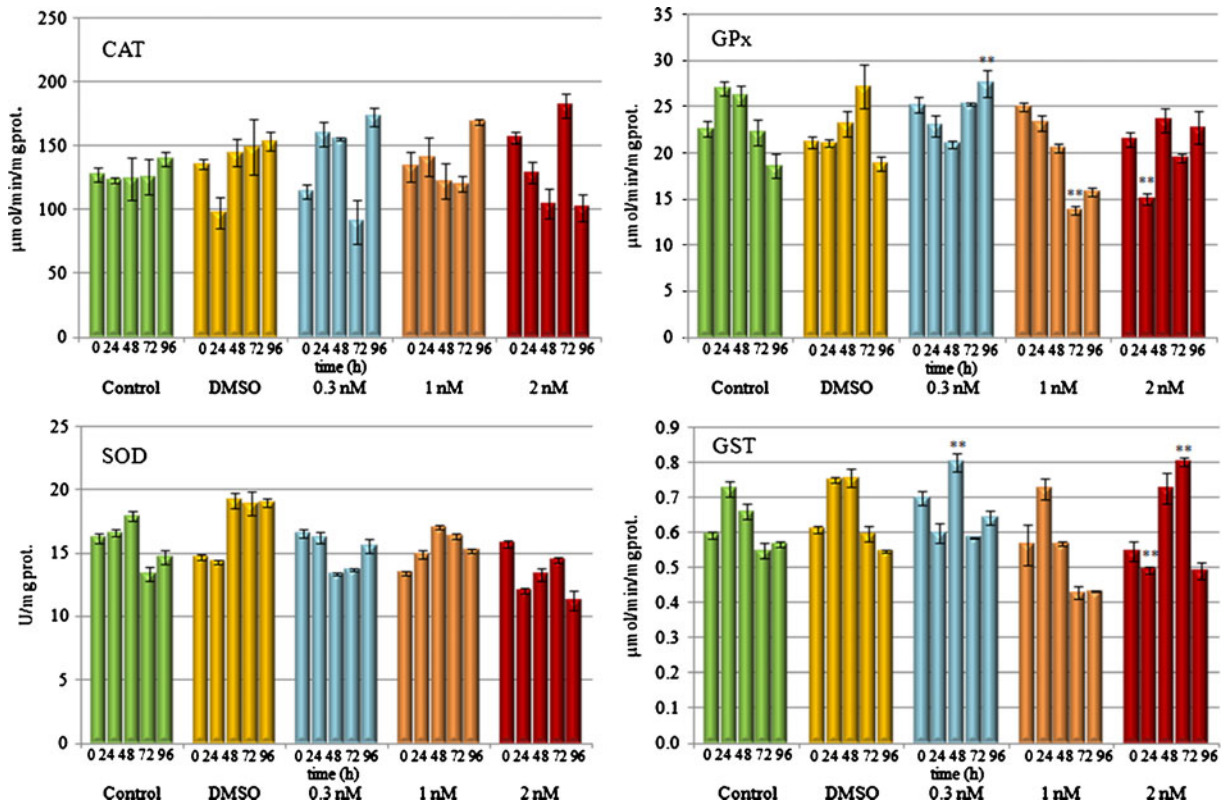
Fig. 4 Effects of increasing doses of DCF on the activity (mean ± SEM) of CAT, GPx, SOD, and GST measured in the whole soft tissues of zebra mussels (pool of 6–8 specimens). Significant differences (two-way ANOVA, Bonferroni post hoc test, $p<0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time)

this detoxifying enzymatic system is less active than that of vertebrates Mitchelmore et al. (1990), and it cannot produce the toxic metabolites that are identified as responsible of the DCF toxicity (Lauer et al. 2009). Effectively, recently, Osman et al. (2004) demonstrated that *D. polymorpha* owns the cytosolic flavo-enzyme DT-diaphorase (NAD(P)H:quinone oxidoreductase) that mediates the two-electron reduction of quinones into hydroquinones which are more stable and less likely to undergo auto-oxidation. Thus, DT-diaphorase is able to protect zebra mussel against quinone toxicity, as also suggested by these authors which proposed that in vivo ROS formation by quinone metabolism was even suppressed in *D. polymorpha*. This skill could rend this bivalve more resistant towards DCF than aquatic vertebrates, which on the contrary seem to be more sensitive even if exposed to environmental concentrations. Obviously, these hypotheses should be further confirmed by proteomic or genomic techniques or by using different invertebrate biological models.

5 Conclusions

This work highlights that current DCF environmental concentrations do not seem able to produce DNA damage and unbalance the redox homeostasis of *D. polymorpha*. The lack of adverse effect pointed out during the 96-h exposure does not allow to estimate the possible mechanism of action of this drug on zebra mussel, notwithstanding in scientific literature that it is well documented in other biological models. Our data enlarge the knowledge about the DCF ecotoxicity, indicating that current environmental concentrations do not pose a relevant hazard for zebra mussel and aquatic invertebrates. However, with respect to the great amount of evidences about DCF cyto–genotoxicity reported by previous studies on other biological models, further in-depth analyses should be necessary in order to confirm these findings and to estimate the toxicity threshold of this NSAID for invertebrates, since an increase of its environmental levels, anything but unlikely due to its great

consumption, can lead to a dangerous increase of DCF riskiness towards the aquatic biota.

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PAPER VIII

Parolini Marco, Quinn Brian, Binelli Andrea, Provini Alfredo

Cytotoxic assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures

This paper can be consulted within the enclosed CD-ROM

Chapter 4 – CONCLUSIONS and REMARKS

According to literature evidences, pharmaceutical and personal care products may be considered new widespread and hazardous environmental pollutants. Several studies investigated their potential danger on different biological models, but often these researches pointed out only few aspects of their toxic potential. Our work enlarged the knowledge on this issue, investigating the adverse effects induced by some drugs on a freshwater non-target model organism by a multi-biomarker battery, focusing on cyto-genotoxicity. In order to reach our goal, a stepwise approach based on tied *in vitro/in vivo* biomarker battery was used. This integrated experimental design was chosen because it can give a more complete and exhaustive report on the toxicity of new pollutants. On the basis of obtained findings, we can suggest that our particular *in vitro* method, consisting in a direct contact between hemocytes and toxicant, owns all the features to be considered an excellent screening tool, because it is rapid, reliable, inexpensive, responsive and reproducible. Moreover, considering that mussel hemocytes require only brief manipulation, quick preparation and are very sensitive to xenobiotics, their use in ecotoxicological studies is strictly recommended, as also shown by our data.

Exposure to high concentrations of each selected drugs induced noteworthy increases of both cellular and genetic damage on *D. polymorpha* hemocytes, indicating a potential hazard of these PPCPs on this bivalve species. These data allow drafting a first toxicity scale of selected molecules and laying the groundwork for further *in vivo* exposures, which could allow better definition of the observed cyto-genotoxicity. The second step of this work consisted in the *in vivo* approach that considers many other factors implicated in the xenobiotic toxicity, such as kinetics of accumulation, exposure duration and defense mechanisms of the organisms (e.g. CYP-450, phase I and II enzymatic systems), which could render less or more toxic the tested compound.

Our *in vivo* findings highlighted the potential toxicity of some PPCP towards zebra mussel, since environmentally relevant concentrations of some selected drugs were able to induce significant cyto-genotoxic effects and alterations in the oxidative status on treated specimens. These data gave us also the opportunity to suggest possible mechanisms of action involved in the rise of induced sub-lethal effects. Since biomarker data are not sufficient to obtain an exhaustive response on drug mechanism of toxicity in zebra mussel, other in-depth analyses should be performed. This goal will be reached in further researches by applying more innovative, introspective, but often pioneering in ecotoxicology,

techniques. Among these, the so-called "omics techniques" represent a powerful approach to study both molecular and cellular processes, as well as the adaptation and the response of specimens exposed to environmental pollutants. Proteomic will be used to evaluate the modifications of protein pattern in a specific biological model in order to investigate the involved mechanism of action of each drug. By correlating tested drug and changed proteins, it will be possible to obtain a drug fingerprint for the selected biological model and formulate hypotheses on the mechanism leading the rise of adverse effects.

Finally, our data confirmed the usefulness of zebra mussel in works aimed to the evaluation of toxicity induced by new potentially hazardous pollutants, such as pharmaceutical and personal care products. In addition, we suggest the use of a multi-biomarker approach in ecotoxicological studies aimed to the evaluation of sub-lethal induced effects. By measuring only one end-point in fact, the response of the cell/organism to the pollutants could not be indicative of its real toxicity. On the contrary, by using a suite of responses, we can obtain an accurate overview on the toxic potential and mechanism of action of tested xenobiotic. In order to enlarge the latter issue, we think that the application of powerful techniques, such as proteomic, should be absolutely a priority, considering that biomarker data are not enough to formulate definitive hypotheses. Finally, according to the increasing production, selling volumes, use and abuse of therapeutic compounds, the increase of their concentration in water is surely expected, with the consequent enhancement of risk toward the biocoenosis. In order to investigate effects caused by this possible and worrisome future scenario, and confirm the real PPCP environmental risk toward the aquatic biocoenosis, further experiments should be absolutely necessary to verify chronic effects induced by higher concentrations, as well as by long-term exposures, considering that organisms are exposed to these pollutants for their whole life.

Chapter 5 – REFERENCES

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Appendix – LIST OF OTHER PUBLICATIONS

All these papers can be consulted within the enclosed CD-ROM.

PAPER IX - Sarkar, S.K., Binelli, A., Riva, C., **Parolini, M.**, Chatterjee, M., Bhattacharya, A. K., 2008. Organochlorine Pesticide Residues in Sediment Cores of Sunderban Wetland, Northeastern Part of Bay of Bengal, India, and their Ecotoxicological Significance. Archives of Environmental Contamination and Toxicology 55, 358-371.

PAPER X - Binelli, A., Sarkar, S., Chatterjee, M., Riva, C., **Parolini, M.**, Bhattacharya, B., Bhattacharya, A.K., Satpathy, K. K., 2009. Congener profiles of polychlorinated biphenyls (PCBs) in core sediments of Sunderban mangrove wetland (N.E. India) and their ecotoxicological significance. Environmental Monitoring and Assessment 153, 221–234.

PAPER XI - Binelli, A., Sarkar, S., Chatterjee, M., Riva, C., **Parolini, M.**, Bhattacharya, B., Bhattacharya, A.K., Satpathy, K. K., 2008. A comparison of sediment quality guidelines for toxicity assessment in the Sunderban wetlands (Bay of Bengal, India). Chemosphere 73, 1129-1137.

PAPER XII - Tremolada, P., **Parolini, M.**, Binelli, A., Ballabio, C., Comolli, R., Provini, A., 2009. Preferential retention of POPs in the North aspect of mountains. Environmental Pollution 157, 3298-3307.

PAPER XIII - Tremolada, P., **Parolini, M.**, Binelli, A., Ballabio, C., Comolli, R., Provini, A., 2009. Seasonal changes and temperature-dependent accumulation of polycyclic aromatic hydrocarbons in high-altitude soils. Science of the Total Environment 407, 4269-4277.

PAPER XIV - Binelli, A., Cogni, D., **Parolini, M.**, Provini, A., 2010. Multi-biomarker approach to investigate the state of contamination of the R. Lambro/R. Po confluence (Italy). Chemosphere 79 (5), 518-528.

PAPER XV - Riva, C., Binelli, A., **Parolini, M.**, Provini, A., 2010. The case of pollution of Lake Maggiore: a twelve years research with the bioindicator mussel *Dreissena polymorpha*. Water Air and Soil Pollution 210, 75-86.

PAPER XVI - Matozzo, V., Binelli, A., **Parolini, M.**, Locatello, L., Marin, M.G., 2010. Biomonitoring of environmental pollution in the Lagoon of Venice using the clam *Ruditapes philippinarum* as sentinel species: biomarker responses and contamination levels. Journal of Environmental Monitoring 12, 776-786.

PAPER XVII - Tremolada, P., Comolli, R., **Parolini, M.**, Moia, F., Binelli, A. One year cycle of DDT concentrations in high-altitude soils. Water Air and Soil Pollution (in press). DOI 10.1007/s11270-010-0596-5.

PAPER XVIII - **Parolini, M.**, Binelli, A., Matozzo, V., Marin, M.G., 2010. Persistent organic pollutants in sediments from the Lagoon of Venice - a possible hazard for sediment-dwelling organisms. Journal of Soils and Sediments 10, 1362-1379.

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