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CYTO-GENOTOXIC EFFECTS AND PROTEIN ALTERATIONS INDUCED BY SOME PHARMACEUTICAL COMPOUNDS AND ILLICIT DRUGS ON NON-TARGET ORGANISMS

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Abstract

In the last decades the aquatic environment has been continuously loaded by several new classes of pollutants and a lot of monitoring studies have been conducted worldwide in order to study their widespread environmental distribution. Among them, pharmaceuticals and personal care products (PPCPs) as well as illicit drugs received particular attention from scientific community. Indeed, their huge consumption and indiscriminate use led their environmental levels in the aquatic environments at not negligible and sometimes also alarming concentrations, which could compromise the health of the biocoenosis.

Notwithstanding their presence in the environment is now a well-established issue, for most of these chemicals their possible adverse effects toward aquatic biocoenosis still remain unknown. Taking into account that these emerging environmental pollutants have physic-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. However, especially for what concern illicit drugs, there is a lack of ecotoxicological studies about their sub-lethal effects on non-target organisms or, when they are available, they are inadequate since they consider only the possible acute effects. Actually, these substances are present in environment at concentrations that difficultly could give acute effects, while chronic ones and sub-lethal effects are more probable.

In order to partially fill this gap, our research group applied a step-wise approach to evaluate possible adverse effects of these molecules on the freshwater bivalve *Dreissena polymorpha* (zebra mussel), trying also to understand their possible mechanism of toxic action. This mussel was chosen as biological model because the invertebrates constitute more than the 90% of living species. Moreover, they play an important role in freshwater ecosystems and are particularly susceptible to environmental stressors. Additionally, previous studies have revealed that *D. polymorpha* is an useful and sensible organism able to highlight sub-lethal effects when exposed to synthetic chemicals.

We applied a suite of biomarkers to first investigate the cyto-genotoxic effects of some PPCPs: trimethoprim (TMP), triclosan (TCS), paracetamol (PCM), diclofenac (DCF) and ibuprofen (IBU). Our results highlighted that triclosan showed highest cyto-genotoxicity and thanks to the application of the biomarker response index we draw a scale of toxicity of these PPCPs, integrating data obtained from different biomarkers end-points: TCS>TMP>IBU>DCF>PCM. Moreover, the analysis of zebra mussels antioxidant enzymes activity gave us the possibility to infer a plausible mechanism of action of TCS, that probably

exert its toxicity both by the induction of oxidative stress but especially through a direct action on DNA, since it can act as a DNA adduct and/or DNA intercalant.

Our results confirmed that these kinds of molecules could have a negative impact on the aquatic ecosystem, and their adverse effects on non-target organisms should not be underestimated. A similar approach has been employed also to test, for the first time, cyto-genotoxicity of the emerging pollutants cocaine (CO) and benzoylecgonine (BE). Our preliminary results, obtained by the analysis of crucial biomarkers end-points to test cyto-genotoxicity of two CO environmental concentrations, suggested a hazard of this molecule toward *Dreissena polymorpha* and probably also toward the entire aquatic biocoenosis.

Taking into account these findings and since CO is rapidly metabolized into BE once assumed, we then decided to test BE effects on zebra mussel after a long-term exposure, considering also that BE's environmental concentrations are often higher than those of the parental compound. The analysis of biomarkers end-points pointed out a clear cyto-genotoxicity of this new aquatic pollutant on *Dreissena polymorpha* at environmental concentrations, highlighting its action as oxidative stressor. To better understand BE mechanism of action, we then applied both classic proteomic and redox proteomic approach that confirmed the role of BE to induce oxidative stress in zebra mussel and also to compromise the energetic metabolism of our biological model.

This research represents the first effort to invetsigate the ecotoxicity of this new class of environmental pollutants, and we hope that it could be the starting point to a more in-depth study on the potential environmental risk for this kind of contaminants, especially considering the occurrence of measurable concentrations of several other illicit drugs in freshwaters and their possible high biological activity.

Chapter 1 *– STATE of ART*

1.1 PPCPs AS ENVIRONMENTAL POLLUTANTS

The occurrence of PPCPs (Pharmaceutical and Personal Care Products) in the aquatic environment has been receiving increasing attention in recent years, since their pollution is now recognized as an environmental concern in many industrialized countries. This has led to the creation of an extensive area of research, including among others: their chemical identification and quantification; elucidation of transformation pathways when present in wastewater treatment plants (WWTPs) or in environmental matrices; assessment of their potential biological effects and development and application of advanced treatment processes for their removal.

Although PPCPs are thought to be harmful to public health as potential bioactive chemicals in the environment also at trace level concentrations (ng/L to mg/L; Kümmerer, 2009a), these substances are still considered as emerging pollutants in waterbodies because they still remain unregulated or are currently undergoing a regularization process (Esplugas et al., 2007).

Their occurrence is reported worldwide in a range of aquatic environments, such as lakes, rivers, freshwater catchments, estuaries, reservoirs and marine waters. Nevertheless, due to their large number (ranging in an order of thousands), only few of these compounds are toxicologically evaluated.

The worldwide consumption of pharmaceuticals has increased significantly since the 1950s as a direct result of a combination of factors including population growth, the fast development of medical science, ageing of the population, and practitioners' prescription habits (OECD, 2009). 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, around 3,000 drugs were available in the United States in 2010 (Bruce et al., 2010) and about 4,900 active ingredients are currently authorized by the Therapeutic Goods Administration for use in Australia (TGA, 2011). Moreover, a large number of these molecules are used also in veterinary applications. The pharmaceutical consumption pattern for different countries is not identical and some drugs may be forbidden or replaced by new related ones. In England, Germany and Australia, the amounts for the most frequently used drugs are 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). 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. 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. After administration, PPCPs can be transformed in the human body into more polar and soluble forms as metabolites or as conjugates of glucuronic and sulphuric acid (Heberer, 2002a; Nikolaou et al., 2007). Parental compounds and their metabolites are readily excreted with urine and faeces and enter into urban wastewater treatment plants (WWTPs).

In this way, these chemicals are continuously introduced in the aquatic environment, where they are detected at trace concentrations and become pseudo-persistent.

In fact, most urban wastewaters are contaminated by medicinal compounds (Jones et al., 2005) and several reviews have reported the occurrence and fate of pharmaceuticals in the environment, where they are ubiquitous in soil and water (Daughton and Ternes, 1999; Khetan and Collins, 2007; Kümmerer, 2009a; Pauwels and Verstraete, 2006; Rahman et al., 2009). This pollution arises from emission from production sites, direct disposal of overplus drugs in households, excretion from urine or faeces after drug administration to humans and animals and water treatments in fish farms. Agriculture and pharmaceutical industries (Kümmerer, 2009a) and hospitals are often pointed out as major contributors to pharmaceutical residues in influents of municipal STPs (Hawkshead, 2008; Ternes et al., 2006) (Fig. 1).

Veterinary pharmaceuticals, for example, contaminate soil directly via manure and surface and ground waters by runoff from fields (Khetan and Collins, 2007).

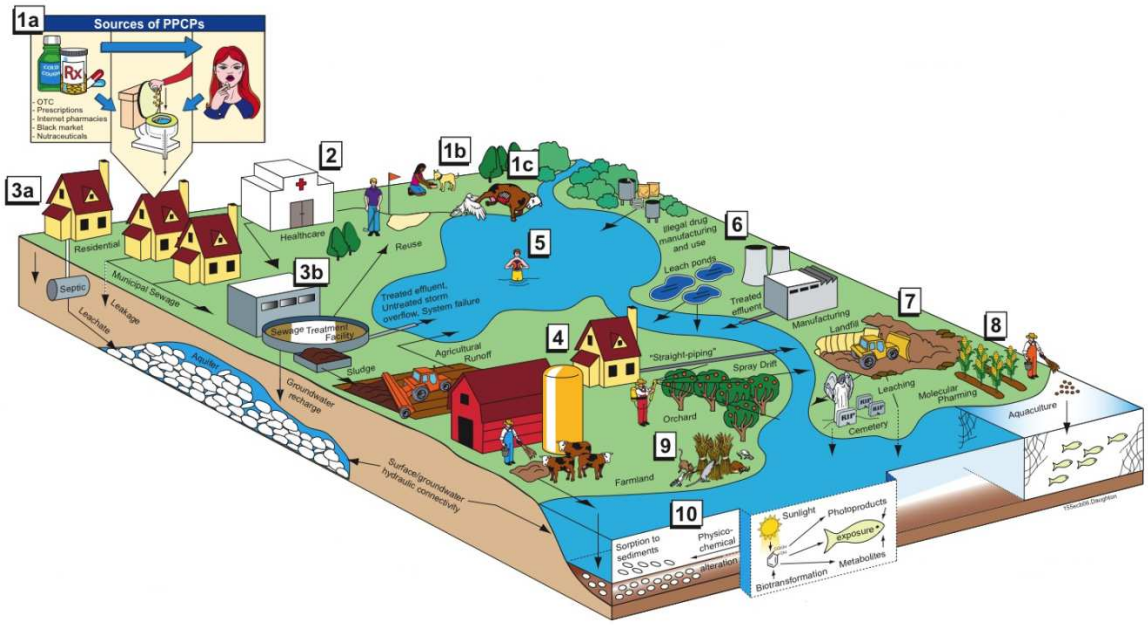


Figure 1: Origins and fate of PPCPs in the Environment (modified from U.S. Environmental Protection Agency Office of Research and Development, National Exposure Research Laborator, Environmental Sciences Division, Environmental Chemistry Branch). 1) usage by individuals (1a) and pets (1b); 2) release of treated/intreated hospital wastes to domestic sewage systems; 3) Release to private septic/leach fields (3a), wastewater treatment plants (3b) and overflow of untreated sewage from storm events (3b); 4) farming and breeding; 5) direct release to open waters via washing/bathing/swimming; 6) manufacturing; 7) landfills; 8) aquaculture and molecular pharming; 9) release of drugs that serve double duty as pest control agents; 10) ultimate environmental transport/fate.

Pharmaceuticals persist in the environment mainly because of its incomplete elimination in wastewater treatment plants (WWTPs), remaining between 60% and 90% of them after the action of biodegradation, deconjugation, sorption and photodegradation steps. Some of these compounds are eliminated by chemical or biological processes while others are degraded during sewage treatment processes or removed from the water phase by adsorption onto solid phase (e.g. sludge) (Jones et al., 2005). Data recently reported show that some pharmaceuticals are accumulated in sewage sludge. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent.

The STP effluents then contain bio-recalcitrant un-metabolized and metabolized pharmaceutical residues that are released in the receiving surface waters (Klavarioti et al., 2009).

Moreover, sewage sludge from WWTPs can be considered a secondary PPCPs contamination source, since after its application in agricultural fields, contamination of soils, runoff into surface water but also drainage may occur (Fig. 1).

The existence of drugs in environmental waters was first reported in the 1970s by Tabak and Brunch (1970), Norpoth et al. (1973), and Garrison et al. (1976), and the first studies reporting the existence of drugs in wastewater go back in the 1980s (Fielding et al., 1981; Richardson and Bowron, 1985).

Although the input sources are well-known, the behaviour and fate of pharmaceuticals and their metabolites in the aquatic environment is not well known. Considering these issues, an increasing number of studies were aimed to the quali-quantitative evaluation of PPCPs in aquatic environments.

According to Runnalls et al. (2010), approximately 150 pharmaceutical compounds have been detected in sources including wastewater, surface water, groundwater and more recently drinking water and they are thought to be harmful to public health as potential bioactive chemicals in the environment (Kümmerer, 2009a).

The accurate quantification of pharmaceuticals, especially in environmental samples can be an analytical challenge, because of the complexity of the matrix and their low levels of occurrence. Thanks to the improvements of chemical analysis methods, many studies have confirmed the existence of pharmaceuticals in aquatic matrices, sediments, soils, and sludge.

Many studies reported measurable concentrations, ranging between ng/L to low μg/L, of a wide range of about 80-100 pharmaceutical compounds belonging to different therapeutic classes, and some of their metabolites in in sewage, rivers, creeks, seawater, groundwater and even drinking water from many European and US countries (Halling-Sorensen et al., 1998; Ternes, 1998; Kolpin et al., 2002; Boyd et al., 2003; Kolpin et al., 2004; Ashton et al., 2004; Gross et al., 2004). The most frequently detected classes of pharmaceuticals in environment are non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, antibiotics, lipid regulators, steroids and related hormones, beta-blockers, and cancer therapeutics (Nikolaou et al., 2007). While among personal care products, disinfectants (e.g. triclosan) and fragrances are the most commonly detected compounds in surface water throughout the world (reviewed by Brausch and Rand 2011).

Of environmental concern, it is not necessarily a high production volume of a certain pharmaceutical, but the environmental persistence and critical biological activity (e.g. high toxicity, high potency for effects on biological key functions such as reproduction). Pharmaceuticals having environmental relevance share the following properties: often, but not always, high production volume combined with environmental persistence and biological activity, mainly after long-term exposure. This affects the water quality and may constitute a potential risk for the ecosystems and the human and animal welfare (Klavarioti et al., 2009).

PPCPs released into the environment may impose toxicity potentially on any level of the biological hierarchy (Kümmerer, 2009a), since many non-target organisms possess human- and animal-alike metabolic pathways, similar receptors 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.

Despite this, there is a gap in legislation regarding the environmental contamination of pharmaceuticals. This probably arises because available data is insufficient to quantify a precise contamination profile. Abundant conclusive studies concerning chronic toxicity are also lacking so that it becomes impossible to infer the risks of long-term exposure of pharmaceuticals and their metabolites on fauna and flora. 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. The European Union Directive 92/18/EEC introduced for the first time, the requirement for an environmental risk assessment, as a prerequisite to obtain marketing authorization for veterinary pharmaceuticals. For this purpose, the European Agency for the Evaluation of Medicinal Products (EMEA) published a "Note for Guidance" (1998) where guidelines to assess the environmental risk of veterinary medicinal products are established. The European Commission extended its concerns to pharmaceuticals for human use by publishing the Directive 2001/83/EC which was subsequently amended by the Directive 2004/27/EC. These directives established that marketing authorization for new medical products for human use should be accompanied by an environmental risk assessment (ERA), whose guidelines were set out by EMEA (2006). Similarly, the US Food and Drug Administration (FDA) require ERA to be performed for human and veterinary medicines on the effects on aquatic and terrestrial organisms before a product can be marketed. However, an environmental assessment is required only if the estimated environmental concentration of the pharmaceutical at the point of the entry is above 1 μg/L (FDA-CDER, 1998), which corresponds to about 40 tons 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. Actually many non-target organisms (which possess human- and animal-alike metabolic pathways, similar receptors or biomolecules) are therefore inadvertently exposed to active substances released into the environment (Daughton and Ternes, 1999; Fent et al., 2006). It is also 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. In particular, aaquatic organisms are important targets, since they are exposed to several of these drugs for their whole life (Fent et al., 2006).

An increasing number of researches are carrying out in order to evaluate the potential dangerousness of these new environmental pollutants on the biocoenosis, but for pharmaceuticals risk assessment, studies on acute effects in organisms belonging to different trophic levels (i.e. algae, zooplankton and other invertebrates and fish) predominate relatively to chronic ones (Yang et al., 2008; Quinn et al., 2008; Haap et al., 2008; Canesi et al., 2007; Choi et al., 2008).

Moreover, scientific community has mainly concerned their attention only on few therapeutic classes, such as NSAIDs, blood lipid lowering agents, antibiotics and sex hormones.

Recently diclofenac, 17alpha-ethinylestradiol and 17beta-estradiol have been classified as priority substances (PS) in the field of water policy, since they were recognized as chemicals presenting a significant risk to or via the aquatic environment at EU level (Commission proposal on priority substances – COM(2011)876)). Other pharmaceuticals (45%) displayed a wide variability of acute toxicity values that makes the classification difficult. In addition, according to other findings, several 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). Indeed, the effects observed in these studies occur at much higher concentrations than the relevant environmental ones. Actually, acute toxicity data is only valuable when accidental discharge of the drugs occurs, since the environmental concentrations usually reported for these compounds are low, typically in a factor of one thousand. Moreover, life-cycle analyses are not reported and toxicity to benthic and soil organisms has very rarely been evaluated. Although the risk of acute toxic effects in the environment with the current use of pharmaceuticals is unlikely, bioaccumulation and chronic toxicity tests are still scarce (Daughton and Ternes, 1999; Fent et al., 2006) probably due to the complex experimental work involved. Sub-lethal effects cannot be excluded because of lack of chronic ecotoxicity data (Khetan and Collins, 2007). For instance, it is accepted that some pharmaceuticals may cause long-term, irreversible changes to the micro-organisms genome, even at low contents, which therefore increases their resistance to them (Esplugas et al., 2007; Klavarioti et al.,

2009). Furthermore, these pollutants often occur as complex mixtures, giving rise to 'drug cocktails' whose toxicity has been seldom predicted, as in the case of beta-blockers (Cleuvers, 2005). On the other hand, some pharmaceuticals have also been classified as endocrine disrupting compounds (EDCs) since they cause harmful effects on the human endocrine system (Esplugas et al., 2007; Klavarioti et al., 2009; Rahman et al., 2009). Currently, the best knowledge on chronic effects related to pharmaceutical compounds exists only 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) and neuroactive compounds (Ferrari et al., 2003; Pascoe et al., 2003; Thaker, 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 for the in-depth study of the environmental hazard of pharmaceutical compounds (Bottoni and Fidente, 2005).

1.1.1 TRICLOSAN

Triclosan (TCS) or 2,4,4′- trichloro-2′-hydroxy-diphenyl ether is one of the main known antibacterial ingredient used since 1968 as antiseptic, disinfectant, and preservative in clinical settings. It is present in various consumer products including cosmetics, household cleaning products and toys and it has also been incorporated on the surface of medical devices, plastic materials, textiles, and kitchen utensils. The UK Environment Agency (2004) estimated that about 350 tons of this chemical are used every year in the EU and in 2010 it was removed from the UE list of additives for use in plastic use food-contact materials (Commission decision 2010/169/EU).

The incomplete removal of TCS wastewater treatment (Bester 2005; Halden and Paull 2005; Klein et al. 2010) may pose a threat to non-target organisms by way of effluent. TCS is considered an ubiquitous pollutant, since its presence has been worldwide reported in

wastewater, rivers, lakes, sediments, surface water, aquatic organisms (fish) as well as in human milk (McAvoy et al., 2002; Aguera et al., 2003; Dayan, 2007; Ying et al., 2007; Chu and Metcalfe 2007; Kasprzyk-Hodern et al., 2008; Chalew and Halden 2009; Reiss et al. 2009; Kim et al., 2009; Dougherty et al., 2010).

An USGS study monitoring 95 compounds in surface water throughout the United States, found TCS to be one of the most frequently detected compounds with surface water concentrations as high as 2.3 µg/L (Kolpin et al., 2002). For all published studies conducted to-date, TCS has been detected in 56.8% of surface water samples with a median concentration of about 50 ng/L (reviewed by Brausch and Rand, 2011), whereas concentrations in WWTP effluents were considerably higher up to 650 ng/L (Lindstrom et al., 2002) and 1600 ng/L (Halden and Paull, 2005). In Europe, the presence of TCS in surface water was investigated in Germany, Italy, Greece, Slovenia, Spain, Romania, Switzerland, and the UK, showing concentrations up to 285 ng/L. It has also been reported in biosoils at µg/g levels or 1000-fold greater than those in aqueous concentrations (Xia et al., 2010).

TCS is a lipophilic compound (log K_{ow} = 4.8) that has the potential to bioaccumulate and affect non-target organisms. In spite of this and its presence in the environment, to date there is limited data available on the toxicity of TCS for non-target aquatic organisms (Yang et al., 2008; Oliveira et al., 2009; Nassef et al., 2010; Palenske et al., 2010).

TCS accumulation has been described for freshwater aquatic organisms mainly in lower trophic-level organisms such algae, crustacean and fish: 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 the 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 and it could be present also in its methyl derivative methyl triclosan (M-TCS), a more stable and lipophilic compound to the parental compound (Lindstrom et al., 2002). M-TCS 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-pdioxins 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).

Toxicity and ecotoxicity studies showed a variety of non-target organisms (bacteria, plants, fish, birds, protozoa, and mammals) sensitive to TCS exposure (Capdevielle et al. 2008; Chalew and Halden 2009; Lyndall et al. 2010; Fuchsman et al. 2010). The lowest observed effect concentration (LOEC) values for the phytoplankton taxa ranged between 100 and 1200 ng/L, whereas no effect concentration (NOEC) ranged from 500 to 8300 ng/L (reviewed by Bedoux et al., 2012). Ricart et al. (2010) showed that environmental concentrations of TCS caused an increase of bacterial mortality with a NOEC of 210 ng/L, while for the cyanobacteria *Anabaena flosaquae* the NOEC was 800 ng/L (Orvos et al., 2002). For what concern fish taxon LOEC and NOEC ranged between 31.6 and 313 µg/L and from 15 and 162 µg/L, respectively (reviewed by Bedoux et al., 2012). Subsequently, PNEC (Predicted No Effect Concentration) values for freshwater species were derived: 70 ng/L on the basis of NOEC of 700 ng/L for the algae *Scenedesmus subspicatus* (Orvos et al., 2002) and 1550 ng/L by using the species sensitivity distribution (SSD) approach derived from toxicity data on 14 different aquatic species of North America (Pharmaceutical Assessment and Transport Evaluation). If we considered environmental levels mentioned above, it is evident that TCS concentrations often exceed these values. 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 few works are available on chronic toxicity or on other sub-lethal effects (genotoxicity, cytotoxicity, impairment of cell signaling, oxidative stress), which can also demonstrate the TCS possible mechanisms of action (MoA).

1.1.2 TRIMETHOPRIM

Trimethoprim (TMP; 5-[3,4,5-trimethoxybenzyl]pyrimidine-2,4-diamine), the earliest antibacterial diaminopyrimidine introduced for clinical use, is still widely used as sulphonamide potentiator both in human and veterinary medicine. It 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).

The environmental load of TMP is substantial and as a consequence of its mobility in soil and slow biodegradation (Boxall et al., 2003; Chemwatch, 2009).

TMP has been shown to withstand sewage water treatment, with almost 100% of the environmental load transferred to the final effluent (Lindberg et al., 2005). In fact, TMP has been recently detected in various aquatic systems usually at concentration ranged from 0.34 ng/L to 546 ng/L wordwilde (Chang et al., 2008; Choi et al., 2008; Kasprzyk- Hordern et al., 2008; Tamtam et al., 2008; Kümmerer, 2009b; Santos et al., 2010) and occasionally at concentrations up to 2 µg/L (Le and Munekage, 2004).

Because of their extensive use in humans and animals and their pharmacology activities, TMP and antibiotics in general are considered molecules having high priority in environmental risk assessment and it is reasonable that they can 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).

For this reason, there are a lot of studies concerning TMP acute effects on aquatic model organisms and generally they are not elicited by environmentally realistic concentrations (Kim et al., 2007; Halling-Sørensen et al., 2000). For example, PNEC values available of 180 µg/L (Halling-Sørensen et al., 2000) and 16 µg/L (Grung et al., 2008), are much higher than environmental TMP levels.

However, few data are available on other sub-lethal effects coupled with considerable controversy about its potential cytotoxicity and genotoxicity. Only recently Binelli et al. (2009) have shown that TMP is able to inflict DNA damage on zebra mussel hemocytes at 2.9 mg/L concentration. But conflicts about its effects still remain, since literature describe both positive and negative effects within the same bacterial and mammalian cell models (Abou-Eisha, 2006).

1.1.3 NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely used pharmaceuticals prescribed to reduce inflammation and manage pain in primary care. In particular, **diclofenac** (**DCF**; 2-[(2,6-dichlorophenyl)amino] phenylacetic acid) and **ibuprofen** (**IBU**; ((+/-)-2-(p-isobutylphenyl) propionic acid with R and S isomers) are two of

the commonly used NAIDs to treat arthritis, rheumatic disorders, pain and fever (Todd and Sorkin, 1988; Hayashi et al., 2008).

With an annual production of several kilotons (Cleuvers, 2004), NSAIDs are the sixth most sold drugs worldwide (Langman, 1999). For example, ibuprofen 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).

Additionally, since some of these pharmaceuticals can be purchased without medical prescription, their consumption could be even higher. The discharge of these therapeutic agents from production facilities, hospitals and private household effluent, as well as improper disposal of unused drugs, pose a burden on the environment (Christensen, 1998). NSAIDs have been detected worldwide both in sewage and in surface waters (Ternes et al., 1998; Farré et al., 2001; Metcalfe et al., 2003; Cleuvers, 2004; Koutsouba et al., 2003; Löffler and Ternes, 2003; Carballa et al., 2004; Tauxe-Wuersch et al., 2005; Yamamoto et al., 2009). Stumpf et al. (1999) were the first to report the presence of diclofenac in the aquatic environment. Ibuprofen and diclofenac were even detected in drinking water in Germany at low ng/l concentrations (Heberer, 2002b). A monitoring study carried out in Canada showed that the NSAIDs naproxen, ibuprofen and diclofenac were amongst the most frequently detected in WWTP effluents at maximum concentrations of 1.2, 0.77 and 0.75 μg/l respectively (Lishman et al., 2006). In the UK an increasing number of studies have reported the presence of NSAIDs in WWTP effluents and rivers (Thomas and Hilton, 2004; Jones et al., 2007; Kasprzyk-Hordern et al., 2008). Hilton et al. (2003) observed that diclofenac was the most frequently detected in WWTP effluents.

Diclofenac, ibuprofen, naproxen, paracetamol and some of their metabolites are often found in surface water in up to μg/L range (Calamari et al., 2003; Ashton et al., 2004; Clara et al., 2004; Weigel et al., 2004a, 2004b; Fent et al., 2006).

The function of NSAIDs in humans is basically to inhibit the enzymes that catalyze the biosynthesis of prostaglandin - which is partially responsible for causing pain and inflammation - 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). Prostaglandins are also present in organisms such as fish, amphibians, birds, corals, sponges, and marine algae, where they carryout various functions, including defense mechanisms. Moreover, cyclooxygenase enzymes are conserved in many vertebrates and invertebrates, thus NSAIDs may affect prostanoids biosynthesis and cause adverse effects in non-target organisms. Another specific effect for ibuprofen was growth inhibition of certain

gram-positive bacteria when exposed to low concentrations of the this drug in the environment (Halling-Sorensen et al., 1998).

Considering this, several studies investigated the environmental risk assessments of these drugs by using classic acute and chronic tests. Farré et al. (2001) analysed the acute toxicity of NSAIDs on the luminescence of bacteria *Vibrio fischeri* and reported a 50% reduction in bioluminescence (EC_{50}= 30 min) of 13.5 mg/L for diclofenac, 19.2 mg/L for ibuprofen and ketoprofen and 35 mg/l for naproxen. Jones et al. (2002) and Carlsson et al. (2006) have compiled the acute toxic effects of pharmaceuticals including diclofenac, naproxen, ibuprofen and ketoprofen. They concluded that diclofenac and ibuprofen are potentially dangerous to the environment. Of all the NSAIDs diclofenac showed the highest acute toxicity to aquatic species with lowest observed effect concentration often below 100 mg/L (LOEC) (Fent et al., 2006). Nonetheless these kinds of studies demonstrated acute effects on aquatic organisms especially for what concern diclofenac and ibuprofen, they tested concentrations much higher than those currently present in the surface waters (Dietrich and Prietz, 1999; Cleuvers, 2003; Ferrari et al., 2003; Halling-Sørensen et al., 1998; Cleuvers, 2003, 2004; Heckmann et al., 2005; Han et al., 2006). Moreover fewer researches were carried out to investigate chronic effect, especially in aquatic invertebrates. For example studies conducted on different biological models, such as *Hydra vulgaris* (Pascoe et al., 2003), *Lemna minor* and *Synechocystis sp.* (Pomati et al., 2004), indicated that acetylsalicylic acid, paracetamol and IBU probably has very little impact on aquatic biota.

An earlier study reported the toxicity of diclofenac in three Gyps vulture species with catastrophic declines in the populations in Pakistan and India (Oaks et al., 2004). Since then extensive research has been carried out on chronic toxicity of environmentally relevant concentrations of diclofenac especially in fish. It is a general consensus that chronic exposure to diclofenac can impair renal functions in fish (Triebskorn et al., 2004; Schwaiger et al., 2004; Hoeger et al., 2005).

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. But, especially for what concern DCF, there is a complete lack of studies carried out on invertebrate species.

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 (Han 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.

Since their continuous release into aquatic environment, these molecules (DCF, IBU, PCM as firsts) may lead to chronic exposure of aquatic organisms and consequently lower effect concentrations. Notwithstanding these evidences, at present, very few studies were carried out to study their possible sub-lethal effects.

1.2 DRUGS OF ABUSE AS NEW ENVIRONMENTAL CONTAMINANTS

Illicit drug use and trafficking are international issues that have negative impacts across the social and economic spectrum, from the public health of individuals to the large-scale stability of national borders. Statistics show that around a third of European citizens have tried an illicit drug, while overdose claims the life of at least one citizen every hour (European Monitoring Centre for Drugs and Drug Addiction, 2010). Ever-changing patterns in illicit drug production, demand and supply need a program of frequent monitoring.

In particular, the recent United Nations Office on Drugs and Crime (UNOC) report has estimated that between 149 and 272 million people (3.3–6.1% of the population aged 15–64)

used illicit substances at least once in 2009 (UNODC, 2011). Among these, cannabis was the most used (125–203 million people), followed by amphetamine-group substances, cocaine and opiates.

This problematic has become, especially in recent years, an important social-economic and clinical issue. Following this evidence, the scientific community started to give ever more space to the investigation of these substances in environment. Although stable or downward trends for most of the drugs mentioned above have been estimated in major regions of consumption (UNODC, 2011), their estimated global production (7,700 tons for opium, 845 tons for cocaine, 2,200-9,900 tons for cannabis in 2008) is comparable to that of widely used pharmaceutical compounds (Santos et al., 2010). Nonetheless these alarming evidences, up through the 90s the emerging studies on pharmaceutical compounds in environment inexplicably excluded from consideration the contributions by the so-called illicit drugs. This is probably due to the ambiguity in what exactly defines an illicit drug; in fact, even if several of them are illegal, many others are medical pharmaceuticals having valuable therapeutic use, such as morphine and oxycodone.

As observed for pharmaceuticals, the main source of environmental contamination by illicit drugs is the human consumption. In fact, after the ingestion of a “drug dose”, active parent compounds (unchanged parent drugs) and/or metabolites are excreted in consumers’ urine, entering urban wastewater and reaching sewage treatment plants (STPs) that are able to remove this kind of contaminants only partially.

Indeed, for what concern the whole drugs spectrum the removal rates are rather low, ranging between 45 and 94%, but when there is an increase of water concentrations, it falls down till 25 % (for MDMA, methylenedioxymethamphetamine) and 35 % (for BE, benzoylecgonine), especially after high usage periods (Bijlsma et al., 2009). This can lead to their introduction into surface (rivers, lakes, sea) or drinking waters (Boleda et al., 2009; Huerta-Fontela et al., 2008a,b; Kasprzyk-Hordern et al., 2009; Postigo et al., 2010; Zuccato et al., 2008a).

The first studies that included illicit drugs into the concerns surrounding pharmaceuticals in environment were conducted only in 1999 and in 2001 (Daughton and Ternes, 1999; Daughton, 2001). But only in 2005, thanks to a study by the Mario Negri institute on the presence of cocaine in the River Po (Zuccato et al., 2005), illicit drugs have been identified as a novel class of environmental contaminants. Since that moment, an increasing number of monitoring surveys have showed the occurrence of these chemicals, as well as of their corresponding metabolites, in both STPs effluents and surface waters worldwide, pointed out

drugs that have been detected in environmental media are generally those most widely used worldwide.

Zuccato et al. (2008b) reported the finding of cocaine (CO) and its metabolite BE, opiates (morphine, codeine), and methadone in Italian main rivers (Po, Lambro, Olona, Arno) in the range from 0.5 to 183 ng/L. Ecstasy (3,4-methylenedioxy-N-methylamphetamine; MDMA), methamphetamine (MA), the cannabinoid 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC – COOH) and the other metabolites of cocaine (norbenzoylecgonine, norcocaine, cocaethylene) were less abundant (0.1–8 ng/L). In the same study also concentrations in River Thames were detected, with levels varying from 2 ng/L for methamphetamine to 42 ng/L for BE.

Subsequently a study conducted in the Ebro River basin (Postigo et al., 2010) highlighted that the most abundant compounds present in effluent sewage waters of different treatment plants were BE, ephedrine (EPH) and morphine (MOR) with median concentrations of 115, 92, and 46 ng/L, respectively. Median concentrations in treated sewage water were between 2 (for MDMA, MOR, and THC-COOH) and 23 (for CO) times lower than median concentrations in raw sewage water. Instead in surface waters the most abundant and ubiquitous compounds were BE, its precursor CO, and EPH that were present in all investigated surface water samples at levels up to 346, 59, and 145 ng/L, respectively.

In general, the levels of detection observed in this study and the contamination pattern of illicit drugs and metabolites in surface river waters are comparable to those reported in similar monitoring studies in Europe by other authors (Bijlsma et al., 2009; Boleda et al., 2009; Huerta-Fontela et al., 2008a; Kasprzyk-Hordern et al., 2009; Van Nuijs et al., 2009a; Van Nuijs et al., 2009b).

MA and MDMA were also found in a small WWTP in Kentuky (USA, Loganthan et al., 2009) with concentrations varied widely from non-detects to 300 ng/L.

Kasprzyk-Hordern in 2010 described the contamination pattern of illicit drugs worldwide. From this analysis CO and its main metabolite BE resulted as most abundant illicit drugs presents in the aquatic environment. They have been quantified in surface water at concentrations up to about 120 ng/L and 200 ng/L respectively, with maximum levels in WWTPs where BE reached concentrations of 10 mg/L and 3 mg/L in wastewater influent and effluent respectively.

Amphetamines have been also frequently found in rivers across Europe at levels reaching 50 ng/L, while the most abused Δ-9-tetrahydrocannabinol (THC), an active constituent of cannabis, its major metabolites, THC-COOH and 11-hydroxy-THC (OH-THC) have also been quantified in rivers and/or wastewater at low ng/L.

Even if concentrations founded in environment are relatively low or moderate, risks for the aquatic biocoenosis and consequently for human health cannot be excluded since CO, AM and MDMA have proved potent pharmacological activities in mammalian systems, and their presence as complex mixtures in surface waters — together with residues of many therapeutic drugs — may lead to unforeseen pharmacological interactions causing toxic effects to aquatic organisms (Brecher and Consumers Union of United States 1972; Rehm et al. 2006).

Illicit drugs share several features with therapeutic drugs that make them potentially dangerous not only for humans (through a direct usage) but also for aquatic ecosystems. Indeed they are ubiquitous, polar and non- volatile chemicals and so persistent in the aquatic compartment. Biological effects from psychotropic substances can in fact occur even at low environmental concentrations, as recently reported for mixtures of therapeutic pharmaceuticals (Pomati et al., 2006). Moreover, just to their continuous intake, also less persistent compounds can affect the behavior of exposed organisms (Guler and Ford 2010) and accumule in fish tissues (Schultz et al. 2010, 2011). Notwithstanding these aspects of ecological relevance, today there is a lack of information about their possible adverse effects to non-target organisms.

1.2.1 COCAINE AND BENZOYLECGONINE

The 2011 report of the UNODC reveals that despite a slight decrease in production and consumption in the last few years, cocaine remains, after opiates, the second most problematic drug of abuse in the world, and the main addiction problem in North America, affecting over 2% of the adult population (15–64 years) in the USA. In Europe, cocaine ranks second in most abused illicit drugs, right after cannabis, with an estimated prevalence of cocaine use between 0.8 and 0.9% of the population aged 15–64, corresponding to 4.3–4.8 million people who used cocaine at least once in 2009 (UNODC 2011).

Cocaine is an alkaloid (Tab. 1), a neurotoxin that protects the coca plant (*Erythroxylem* spp.) from herbivory by critically disrupting insect motor control (Nathanson et al., 1993).

In both mammals (Kelley and Berridge, 2002; Wise, 2004) and insects (Roeder, 2005; Wolf and Heberlein, 2003), cocaine operates by blocking biogenic amine reuptake transporters (Corey et al., 1994; Gallant et al., 2003), thereby disrupting biogenic amine signalling (Bainton et al., 2000; McClung and Hirsh, 1999; Nathanson et al., 1993) and preventing reuptake and recycling of released dopamine (Fig. 2a). In this way, cocaine increase extra-neuronal dopamine, which results in stimulation of brain reward circuits.

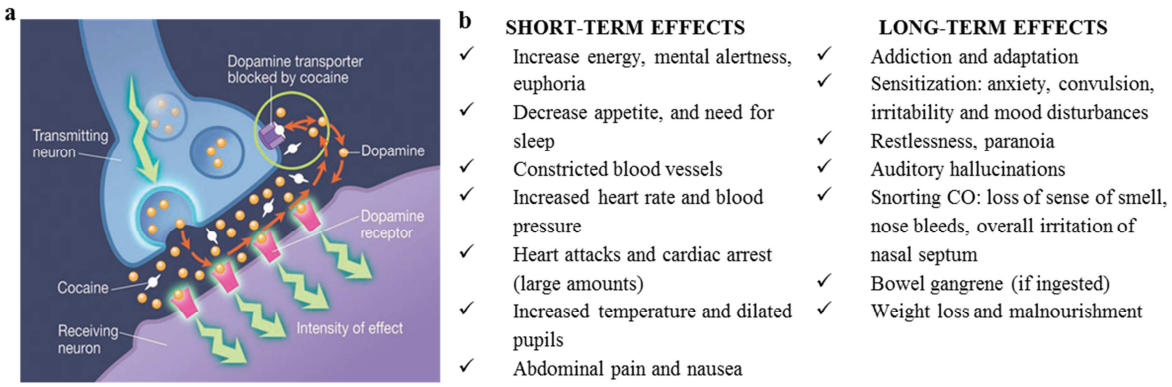


Figure 2: Cocaine's mechanism of toxic action in mammals (a) and its short- and long-term effects (b) in humans.

In mammals, the biogenic amine systems disrupted by cocaine [principally dopamine (DA)] modulate both motor control and reward processing (Cenci, 2007; Uhl et al., 2002; Wise and Rompre, 1989; Wise, 2004) and it is probably through this diverse pharmacology that cocaine produces such a wide spectrum of effects at central nervous system and cardiovascular system level (Fig. 2b) (Schindler et al., 1995; Cunha-Oliveira et al., 2006; Goldstein et al., 2009). In humans, cocaine is also highly toxic at medium to high doses but highly rewarding and reinforcing at low doses and potentially addictive (Kelley and Berridge, 2002; Sullivan et al., 2008).

There are also many evidences about the toxicity of CO at cellular level. CO induced apoptosis in an *in vitro* study on cortical neurons of fetal mice (Nassogne et al., 1997), but was found only to be a weak clastogen (Yu et al., 1999) and not to be mutagenic or carcinogenic (Salvadori et al., 1998) always in rodents. In the latter two studies, the possible mechanism by which CO exerts its cytotoxic effects seems to be the oxidative damage by reactive oxygen species. CO is known to generate ROS only when it is metabolized (Kanel et al., 1990).

The induction of apoptosis by CO has been demonstrated also in coronary artery cells (He et al., 2000), rat myocardial cells (Xiao et al., 2000) and rats testes (Li et al., 2003).

The cytotoxic effects of CO have also been reported in model organisms used to investigate mechanisms of drug action in humans such as in the protozoan *Tetrahymena pyriformis*. Indeed, Stefanidou et al. (2002) observed a stimulation of the mitotic process in this protozoan, which due to the subsequent stimulation of the DNA synthesis indicates a mitogenic effect of cocaine products. Anderson-Brown et al. (1990) instead demonstrated that CO is able inhibits DNA synthesis in the adult and developing rat brain.

Literature is instead lacking of studies inherent its adverse effects on non-target organisms. Only information about modification of the behaviour of some biological models is available. Carter et al. (2006) analysed the effects of repeated cocaine on learning, memory and reinstatement in the pond snail, *Lymnaea stagnalis*, demonstrating that CO was able to modify the interaction between the original memory trace and active inhibition of this trace through extinction training. Subsequently Barron and co-authors (2009) highlighted CO stimulation of pathways for reward assessment and processing demonstrating this chemical affected bee's dance behaviour by modulation of brain reward system. The first knowledge about accumulation of CO in a non-target organism tissues was carried out only in 2012: after 1 month of exposure to a nominal concentration of 20 ng/L, CO was found in different tissues of the treated Silver eels (*Anguilla Anguilla*) at concentrations up to 30.5 pg/g in brain and, at low concentrations, in almost all tissues of post-exposure recovery eels (Capaldo et al., 2012).
In humans, CO it is almost completely metabolized within two hours of administration and only a small percentage (1-9%; Baselt, 2004) is excreted in urine as the unchanged drug (Fig. 3). The main *in vivo* metabolism of cocaine in humans is its hydrolysis to BE mainly by liver carboxylesterase-1 (hCE-1). Cocaine may also be hydrolyzed to ecgonine methyl ester (EME) by liver carboxylesterase- 2 (hCE-2) and pseudocholinesterases (PChE). The parental compound is indeed excreted as BE for the 45% of the administrated dose and as EME for the 40%. Only a minor part (1-3%) is N-demethylated by CYP3A in humans to norcocaine (NCOC) (Goldstein et al., 2009). Anhydroecgonine methyl ester (AEME) and cocaethylene (CE) are two other metabolites known to potentiate cocaine-induced toxicity and are formed when the drug is used whether in the free base form ('crack) (Erzouki et al. 1995) or concomitantly with alcohol (Dean et al. 1992; Harris et al. 2003) (Fig. 3).

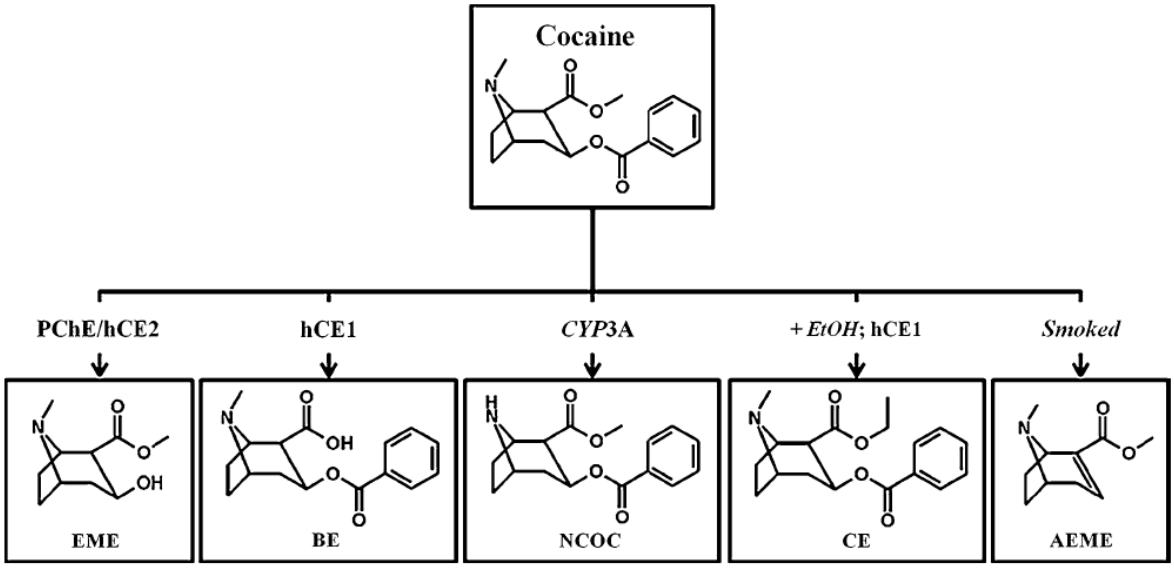
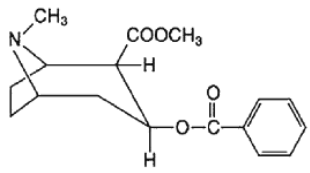
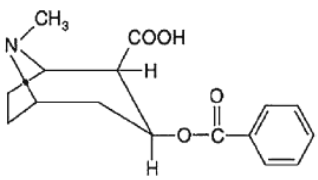


Figure 3: Schematic representation of major metabolic pathways of cocaine in humans, and enzymes involved (Valente et al., 2012).

Due to its rapid metabolism, cocaine has a short elimination half-life of about 1 hour and urine assays for cocaine use typically measure the cocaine metabolite BE, which has an elimination half-life of 6 hours (Tab. 1). Urine testing for BE is typically reported to give positive results for 1 to 2 days after recent cocaine use. BE can be detected for up to 5 days in casual users. In chronic users, urinary detection is possible for as long as 3 weeks (Roldan, 2008).

Table 1: Structures and physico-chemical data of the selected drugs of abuse

Structure	Compound	Empirical formula	MW (kDa)	pKa	*Log Kow*	Half-life (hours)
	CO	$C_{17}H_{21}NO_4$	303.15	8.6	3.08	1
	BE	$C_{16}H_{19}NO_4$	289.13	3.2 (pKa_1) 10.1 (pKa_2)	2.72	5-6

Benzoylecgonine is the corresponding carboxylacid of the methyl ester of CO (Tab. 1) and it has been demonstrated to have vasoconstrictive properties; however, it does not appear to cross the blood–brain barrier readily (Goldstein et al., 2009).

Since its longer half-life, it is now established that BE might be responsible for some of the delayed cerebrovascular events previously attributed to CO. For instance Brogan et al. (1992) associated the recurrent vasoconstriction of human coronary arterial (after 90 minutes of CO administration) to a temporal correspondence with an increasing blood concentration of CO's main metabolites, BE and EME. Madden et al. (1995) found that cat's middle cerebral arteries contracted more vigorously to BE than to CO itself. Indeed, BE-induced decrease in diameter was 2.2 times greater than the decrease due to CO. Moreover they showed that CO and BE exert vasoconstrictor effects through different mechanisms: CO appear to act primarily through adrenergic nerves and receptors coupled with activation of intracellular calcium stores; BE's action appear to depend extensively on the influx of extracellular calcium. The latter has been shown to have a central role in the modulation of both apoptotic and necrotic cell death (Waring, 2005) and many investigation have shown that it is essential for production of reactive oxygen species (ROS), since elevation of intracellular calcium level is responsible for activation of ROS-generating enzymes (the Krebs cycle) and formation of free radicals by the mitochondria respiratory chain (Gordeeva et al., 2003). Nikolettos et al. (2012) showed also that BE increase myometrium contractility in rat through an adrenergic mechanism.

Despite this in-depth knowledge of CO and BE's adverse effects in humans and vertebrates, no data are available on their acute or chronic effects on non-target organisms. Although cocaine, psychotropic substances and smart drugs (amphetamines, pills) are often thought innocuous and are widely consumed in our society, these chemicals are potential environmental toxicants due to their pharmaceutical activity (Castiglioni et al., 2006).

Research about the presence of these biologically active compounds in the environment is vital in order to improve knowledge on the occurrence, fate and exposure of these compounds and their potential impact on aquatic and human life.

For instance, Cecinato and co-authors (2009) highlighted that CO resulted as the most important illicit substance occurring in the atmosphere, where its concentrations varied widely from 13 pg/m^3 to 2800 pg/m^3 (Cognard et al., 2005). Since in humans CO is largely excreted in urine as metabolites, the BE levels in aquatic environment are higher than those of parental compound with concentrations up to the µg/L range (Castiglioni et al., 2006, 2011; Gheorghe et al., 2008; van Nuijs et al., 2009b; Postigo et al., 2010) (Tab. 2).

Table 2: Most relevant concentrations of selected illicit drugs in European surface waters.

	WWTPs influents	WWTPs effluent	Rivers	Reference
Cocaine	465 ng/L	/	44 ng/L	reviewed in *Zuccato and Castiglioni, 2009 (Italy)*
	753 ng/L	/	115 ng/L	*Van Nuijs et al., 2009a (Belgium)*
	820 ng/L	540 ng/L	/	*Bijlsman et al., 2009 (Spain)*
	961 ng/L	31.1 ng/L	59.2	*Postigo et al., 2010 (Spain)*
	109 ng/L	65.2	14	*Baker et al., 2011 (UK)*
Benzoylecgonine	1468 ng/L	/	183 ng/L	reviewed in *Zuccato and Castiglioni, 2009 (Italy)*
	2 258 ng/L	/	520 ng/L	*Van Nuijs et al., 2009a (Belgium)*
	10 500 ng/L	6 790 ng/L	/	*Bijlsman et al., 2009 (Spain)*
	2 790 ng/L	510 ng/L	346 ng/L	*Postigo et al., 2010 (Spain)*
	368.3 ng/L	293.3 ng/L	52.5 ng/L	*Baker et al., 2011 (UK)*

In addition, a recent study conducted on influents of wastewaters in Belgium (Van Nuijs et al., 2012) demonstrated that these molecules are pseudo-persistent in aquatic environment. BE showed concentration stability for 28 h, with a slight increase (0.2% per h), while CO and EME showed decreasing concentrations (40% and 20% after 12 h, respectively). Authors explained these findings by a partial degradation of CO to BE.

Notwithstanding these important environmental aspects and nonetheless the increasing knowledge on the occurrence of CO and BE in the aquatic environment, today the ecotoxicological investigation about their impact on aquatic biocoenosis is absolutely inadequate.

1.3 ROLE OF BIOMARKERS IN DRUGS ENVIRONMENTAL RISK ASSESSMENT

Considering the lack of information on the potential sub-lethal effects induced by PPCPs and illicit drugs 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.

In the early phase of environmental monitoring, the most common and simply approach was to measure physical and chemical variables with the occasional implementation of biological variables (Lam & Gray, 2003).

Indeed, the simplest way to assess the pollution status of a specific ecosystem is to carry out chemical analysis of water, soil, biological extracts and other environmental samples. However, given the large number, complexity and sometimes low toxicity threshold of environmental chemicals, chemical analysis alone may not offer meaningful assessment of the pollution status of the studied ecosystem. Moreover, chemical analysis *per se* offers little insight to the environmental fate or biological threat posed by pollutants.

With the realization that some environmental pollutants, such as herbicides and insecticides, also produced deleterious effects, attention moved away from contaminant monitoring to measuring biological effects.

It is now well established that contaminants can cause changes at all levels of biological organization (Fig. 4). In some cases research still focused only on the measurement of chemicals in organisms – that have the great benefit of quantifying that fraction which is bioavailable and thereby has potential to cause harmful effects – or measuring changes only at the population level, by which only an indirect inference can be made with regard to the cause of the population decline (Simpson and Norris, 2000).

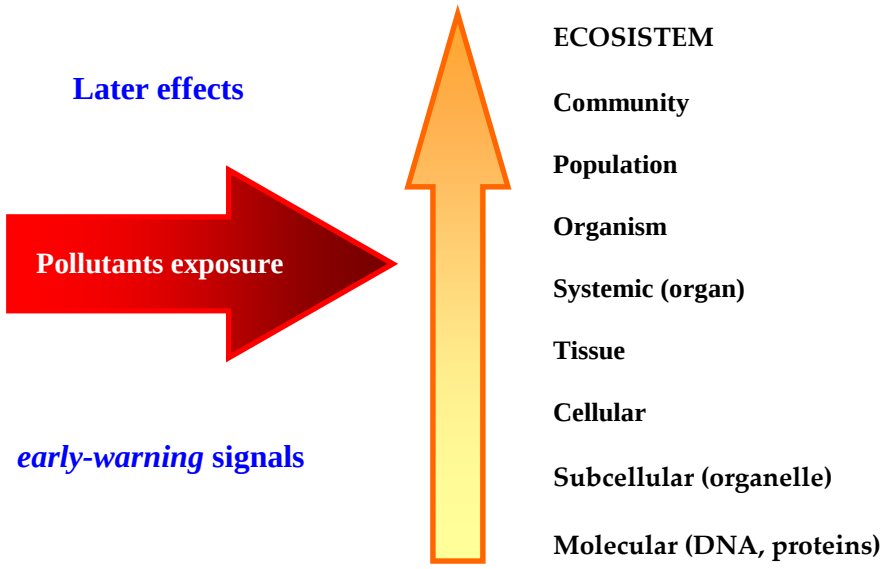


Figure 4: Schematic representation of the sequential order of responses to pollutant stress within a biological system.

It is now recognized that if the focus of contaminant studies in streams or rivers is only on community change, subtle or chronic biological effects that may result in irreversible long-term changes could be occurring in apparently healthy ecosystems but would not be initially detected (Maher et al., 1999).

Although the population, community and ecosystem are important levels for the monitoring of toxic effects, the primary effect of xenobiotics was first revealed at the sub-cellular level by impaired biological function (as biochemical and molecular variations, enzymatic activity modifications, DNA alterations) (Fig. 4). Its rapid evaluation can allow the activation of some procedures for the pollutant impact reduction before the damage reaches the higher hierarchical levels.

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 PPCPs and illicit drugs (Bottoni and Fidente, 2005). In contrast to the simple measurement of contaminants bioaccumulation 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). However, the identification of the eventual effects on the community due to contaminant exposure is generally more complex both because targets of pollutants can be highly species-specific and because chemical mixtures with additives, synergic or opposite effects are normally found in the environment.

Biomarkers were originally defined as any biochemical, histological, or physiological alterations or manifestations of environmental stress (NRC, 1987). They have been classified as biomarkers of exposure to a toxicant, biomarkers of effects of exposure, or biomarkers of susceptibility to the effects of exposure (Peakall and Shugart, 1993). More recently, this definition has been challenged by several authors (Adams, 1990; McCarty and Munkittrick, 1996; Engel and Vaughan, 1996) and the term biomarker is now more commonly used in a more restrictive sense, namely biochemical sub-lethal changes resulting from individual exposure to xenobiotics.

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; McCarthy et al., 1990) (Fig. 5).

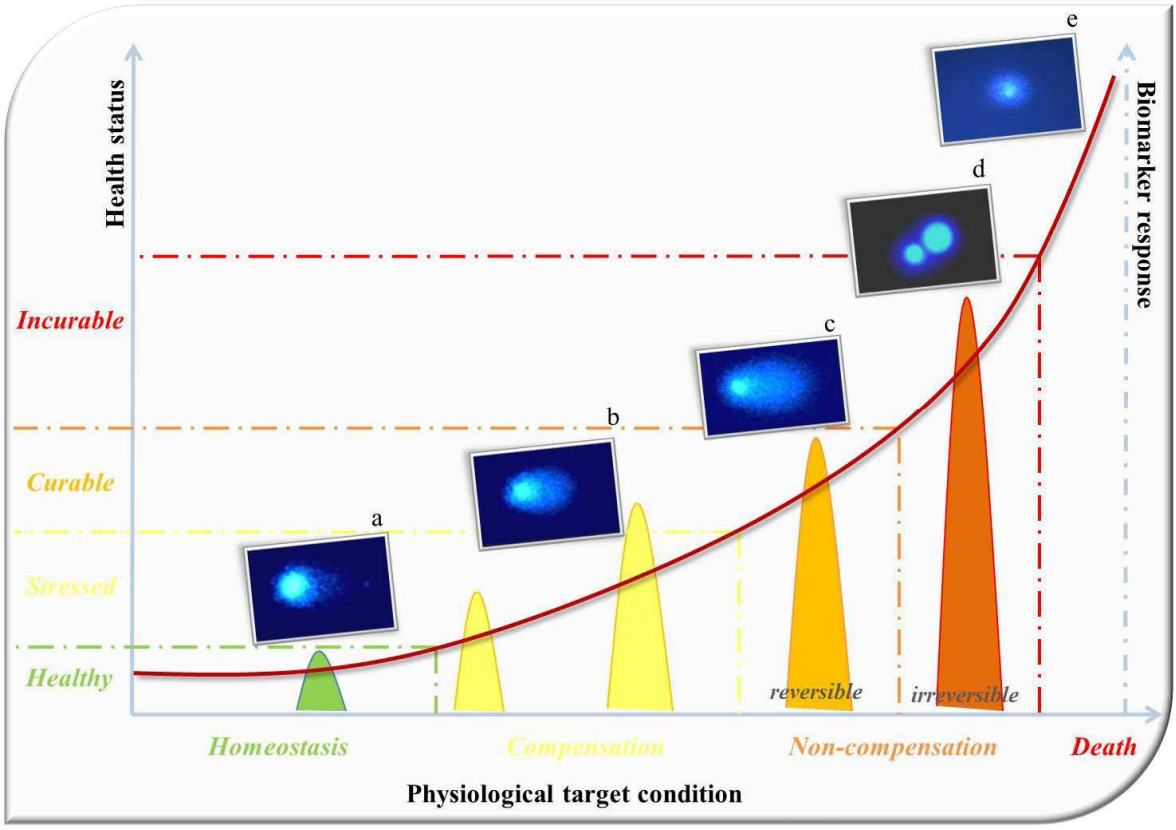


Figure 5: Relationship between exposure to pollutant, health status and biomarker responses. The exponential magenta line shows the progression of the health status of an individual in relationship with increase of exposure time and pollutant concentration. Green broken line represents the threshold 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).

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 non-destructive 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).

The advantages of applying biomarkers, often to complement traditional chemical analysis in environmental monitoring programs, are considerable (McCarthy and Shugart, 1990; Depledge, 1994; Wells et al., 2001; Handy et al., 2003):

- ✓ Biomarker responses may indicate the presence of a biologically available contaminant, rather than a biological inert form of contamination.
- ✓ Using a suite of biomarkers may reveal the presence of contaminants that were not suspected initially.
- ✓ Biomarkers responses often persist long after a transient exposure to a contaminant that has then degraded and is no longer detectable. Thus, biomarkers may detect intermittent pollution events that routine chemical monitoring may miss.
- ✓ Biomarker analyses are, in many cases, much easier to perform and are considerably less expensive than a wide range of chemical analyses.

Sometimes there is variability in biomarker responses that may be attributed to abiotic (temperature, salinity, dissolved oxygen, etc.) or biotic factors (genotype, phenotypic plasticity, tolerance, age, sex, body size, etc.). However, it is a widely held misconception that these sources of variability render the biomarker responses insensitive compared to traditional chemical monitoring techniques. Indeed, there are several options for minimizing variability, such as the use of a suite of biomarkers, since it is very important to measure several biomarkers at the same time in the same biological model. Moreover, if there is evidence that an abiotic/biotic factor can modulate the response during an exposition to a toxicant under controlled laboratory conditions suitable exposures to that factor should be done. In addition, connection should be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. At the end, careful selection of a "reference sites" from which take a control response obtained from depurated organisms maintained under laboratory conditions it may be appropriate. The combination of these approaches could reduce the variability to such an extent that long term monitoring using biomarkers could be a realistic option for regulatory authorities (Handy et al., 2003).

Biomarkers may also 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. Notwithstanding, they have seldom been used for decision making in regulatory ecotoxicology, but they could play a crucial role in assessing risks of some drugs. 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., 2003; Crane et al., 2006).

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 the 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 ROLE OF PROTEOMICS IN ECOTOXICOLOGY

Risk assessment of pollution levels can be achieved by analysing the routes by which pollutants enter ecosystems and, then, accumulate along trophic networks (Newman and Clements, 2008). However, this mode of analysis assumes that the biological effects of chemicals of interest are established. Actually, the biological effects of emerging pollutants are often poorly understood. The application of systems biology approaches has been proposed as an ideal tool to assess effects of pollutants at the cellular level because this approach could provide a more comprehensive risk assessment of the cellular effects than do the current, generally more narrowly focused methods (Calzolai et al., 2007; Lemos et al., 2010; Lopez-Barea and Gomez-Ariza, 2006; Waters and Fostel, 2004). There is now increasing awareness that no single biomarker will serve to indicate the full effect of environmental pollutants (Galloway et al., 2004a, 2004b), but it is absolutely necessary to use multiple biomarkers, to investigate effects in different organs within a sentinel species (Depledge and Galloway, 2005). Moreover, analogous to diagnosis in human medicine, it is recognized that most pollutant effects depend on the determination of suites of responses, rather than any pollutant-specific or disease specific response.

There is also an increasing interest in understanding the cellular mechanisms of organism-level responses to changes in the physical and chemical environment, including changes in temperature, osmolality, oxygen concentration and pollutants, as well as the mechanisms of developmental pathways, infections, and symbioses.

At this regard a new trend in ecotoxicology and biomedical research is the application of so-called “omics” technologies. These are methods that have the potential to monitor complete classes of cellular molecules such as messenger RNAs, proteins and intermediary metabolites in a single analysis in organisms exposed to different types of chemical stressors (Ankley et al., 2006), compared to traditional analyses that rely on only one endpoint. By allowing simultaneous analysis of thousands of genes, proteins, and metabolites, these new global technologies have enabled a wider approach to biological questions, since toxicity generally involves not only changes in a single gene but rather a cascade of gene interactions (Nuwaysir et al., 1999; Aardema and MacGregor, 2002). Some authors recommended 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). These methodologies have been already widely used in human medicines and in toxicology studies. In particular proteomics is a well-established area of research in molecular medicine because the evaluation of changes in protein expression patterns can provide information on pathogenic signalling pathways and the identification of human disease markers (Petricoin and Liotta, 2003).

In the last decade, 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 also in the field of ecotoxicology (Miracle et al., 2003; Viant et al., 2003; Snape et al., 2004).

These studies have demonstrated an effective methodology for characterising the modes of action and the mechanisms of toxicity for pollutants with a high potential for identifying novel biomarkers (Dowling and Sheehan, 2006; López-Barea and Gómez-Ariza, 2006; Monsinjon and Knigge, 2007).

Snape et al. (2004) proposed the term “ecotoxicogenomics” to describe the integration of genomics (transcriptomics, proteomics, and metabolomics) into ecotoxicology, and defined it as “the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures.” The authors emphasized the need for ecotoxicology to move toward a more holistic approach, which integrates high-throughput “omics” technologies. Identification of endpoints and responses from such an approach could potentially improve risk assessment through a clearer insight into mechanisms of actions gained by an increased level of information obtained at the molecular level. Improved knowledge regarding cellular control and defence mechanisms will allow a more robust extrapolation between model species and target species (MacGregor, 2003), as well as reducing uncertainties involved in predicting threshold levels of various types of toxicity.

It has also been suggested that genetic variation is the major cause for variation in susceptibility to disease and toxicant exposure variants (Aardema and MacGregor, 2002; Ashton et al., 2002; Botstein and Risch, 2003), indicating that a certain set of genes or proteins could be used to discover sensitive species and (sub)populations.

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, to date, few ecotoxicological studies have utilized "omics" technologies. Transcriptomics, e.g., microarray studies, have led the way in this effort, but several proteomics studies have now established the feasibility of a proteomic approach (Lemos et al., 2010).

Among "omics" technologies, **environmental proteomics** or ecotoxicoproteomics - the study of changes in the abundance of proteins and their post-translational modifications - has become a powerful tool for generating hypotheses regarding how the environment affects the biology of non-target organisms. With its rapidly expanding analytical tools, it provides a means to study the changes occurring at the level of the proteome - the entire protein pool - in response to both the external environment and ontogenetic events in animals, plants, and bacteria.

The approach has obvious applications to ecotoxicology since it has the potential both to identify previously unknown protein biomarkers and to gain insights into toxicity mechanisms. Since the proteome is a dynamic quantity, it holds out the promise of detecting subtle changes in sentinel species as they adapt to altered surroundings.

Notwithstanding the improvement of proteomics methodologies both for electrophoresis procedures and mass spectrometry analyses, the protein identification in the field of ecotoxicology is often challenging because of the limited information contained in the available databases, especially for non-model organisms, whose genomes have not been fully sequenced (Tomanek, 2011). Conversely, more genome data are available for bacteria, fungi and plants, which are not as extensively used in ecotoxicology. Although this drawback, proteomics has much to offer even in species poorly represented in sequence databases. There is significant scope for greater application of *de novo* sequencing approaches in LC separations and in spot identification in 2-DE, thus circumventing current limitations of sequence data. Several research groups have pioneered the proteomics approach to ecotoxicology and it has the potential to become the assessment method of choice for emerging pollutants, even if the field is still in an early stage of development since it has to overcome several shortcomings. First of all, some of the proteomics studies are not fully quantitative; in addition, sample sizes are often low and provide little statistical power to detect a small but relevant change in protein abundance. Protein identification has been limited, since, as mentioned above, protein identification relies on available sequence

information. Then the success of proteomics projects focusing on non-model organisms is largely dependent on these resources. Actually, many studies have been limited by a paucity of genomic information and thus identified only a few significant (or changing) proteins, but with more EST (translated expressed sequence tag) libraries available, it is possible to identify more than 50% of the proteins from such sentinel non-model species as *Mytilus*. Finally, acute and long-term exposures as well as exposures to a range of pollutant concentrations are needed to detect possible nonlinear low-dose responses (Calabrese et al. 2007). Despite these current shortcomings, proteomics provides a powerful approach for ecotoxicologists in order to assess the biological effects of emerging pollutants and to investigate their MoA. It also will allow greater understanding of the consequences and fate of pollutants in biological systems ranging from organelles to cells, tissues, individual animals and communities. Unfortunately, none of these approaches has been used for detecting or assessing human/veterinary pharmaceutical or illicit drugs effects, although their use in this field is absolutely recommendable.

1.4.1 REDOX PROTEOMICS

Proteomics applied to assess the biological effects of pollutants in non-target organisms is beginning to reveal some of the systemic changes that occur on the cellular level (Tomanek, 2011). First, a qualitative description of cellular changes shows that the systemic changes occurring during exposure are pollutant-specific. Second, proteins common to many pollutant-stress responses include oxidative stress proteins, cytoskeletal proteins, chaperones, proteases, and proteins involved in the detoxification of xenobiotics. Together, these changes suggest that the production of reactive oxygen species (ROS) leads to the denaturation of proteins as well as wide-ranging modifications of cytoskeletal elements. In this scenario, post-translational modifications (PTMs) present a novel frontier to assess the biological effects of pollutants. Although the types of PTM number in the hundreds (Walsh, 2006), only changing patterns of carbonylation, glutathionylation, thiol-modifications, and ubiquitination have been studied in response to pollutants (Sheehan, 2006). The former three are modifications caused by the increased production of ROS and can occur due to a change in the oxidative environment of the cell.

Radicals and non-radical oxidants can be generated by a wide variety of different processes in biological systems (e.g. by-products of mitochondrial electron transport and during inflammation), but they can also occur as a response to a wide range of exogenous agents (e.g. UV, pollutants).When this production overcomes the cellular defence mechanisms, oxidative

stress occurs (Dalle-Donne et al., 2005). Most highly reactive oxidants, including many radicals, react with virtually all biological molecules, including DNA, RNA, cholesterol, lipids, carbohydrates, proteins and antioxidants. Since proteins are the most abundant non aqueous component of the cell, they are major targets of ROS (they adsorb ~ 68% of ROS) and numerous post-translational, reversible or irreversible modifications have been characterized, which may lead to a change in the structure and/or function of the oxidized protein (Davies, 2005).

Redox proteomics is an increasingly emerging branch of proteomics aimed at identifying and quantifying redox-based changes within the proteome both in redox signalling and under oxidative stress conditions (Butterfield et al., 2012; Sultana and Butterfield, 2011).

In the last few years, combined proteomics, mass spectrometry (MS) and affinity chemistry-based methodologies have contributed in a significant way to provide a better understanding of protein oxidative modifications occurring in various biological specimens under different physiological and pathological conditions (e.g., Butterfield et al., 2012; Sultana and Butterfield 2011). Oxidative stress can cause change in levels of specific proteins detectable by protein staining and image analysis (Patton, 2002). Similarly, redox-based processes altering the *pI* or *Mr* of proteins (e.g., charge isomerization, protein backbone cleavage, and crosslinking) are detected as altered 2D SDS–PAGE spots. In practice, a surprisingly small number of changes to the absolute amounts of individual proteins is usually observed. Notwithstanding this, redox-based modification of proteins in certain proteins is often quite extensive.

Most researches have focused especially on perspectives of ageing (Levine and Stadtman, 2001), cell signalling (Bigelow and Squier, 2005), and the aetiology of human disease (Halliwell and Gutteridge, 1989) with also the aim to identify a novel panel of biomarkers to better understand the molecular mechanisms of a disease together with identification of specific targets of oxidative damage. Moreover redox proteomics will play a pivotal role also in the search for new therapeutic targets and their validation, and in the design and testing of new drugs against those pathologies related to altered redox homeostasis (Butterfield and Dalle-Donne, 2012).

ROS can modify and inactivate proteins in a wide variety of ways. Sulphur-containing molecules are notoriously susceptible to oxidation. Cysteine thiols (-SH) can be irreversibly oxidised to sulphinic (-SO2H) and cysteic (-SO3H) acids or reversibly oxidised to sulphenic acid (-SOH), thiyl radicals (-S.) or nitrosothiols (-SNO). Methionines can be oxidised to sulphoxides and sulphones. Amino acid side-chains can be irreversibly converted to

aldehyde/ketone groups collectively called protein carbonyls (Ghezzi and Bonetto, 2003; Levine and Stadtman, 2001). These result in partially unfolded and inactivated proteins which may be rapidly cleared from cells. Other reversible redox lesions of proteins include glutathionylation - which can protect cysteine residues from oxidation (Schafer and Buettner, 2001) - and formation of methionine sulphoxide, an indicator of cell ageing (O'Sullivan et al., 2005). Thus, given that a great number of pollutants are known to increase production of ROS, PTMs caused by ROS and subsequent changes in levels of protein degradation have the potential to be sensitive global markers of pollutant stress.

In the field of ecotoxicology the redox proteomics approach is only recently recognize as a powerful tool to better understand the molecular mechanisms of toxicity (which can differ with identity of pro-oxidant). For example, blue mussels (*M. edulis*) from polluted sites in Ireland showed greater levels of carbonylated proteins but few changes in protein abundance in gill and digestive tissues in comparison to control sites (McDonagh et al. 2005). Subsequent exposure of mussels to H_2O_2 followed by 2-DE showed that oxidative stress increases levels of carbonylated proteins (McDonagh et al. 2005). Carbonylation was also used as a biomarker to discover that p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) causes oxidative stress in the clam *Ruditapes decussatus* (Dowling et al. 2006).

There is evidence that a number of abundant proteins in *Mytilus* are targets of ROS (McDonagh & Sheehan 2007). Specifically, actin, PDI, and other chaperones (heat shock protein gp96 and calreticulin) have been shown to form intra-molecular disulphide bonds in response to exposure to the pro-oxidant menadione, possibly sequestering ROS before they can cause uncontrollable damage to other proteins, lipids, or ribonucleotides.

Redox proteomics was also used to study the effects of GNP (gold nanoparticles) on protein thiols of *Mytilus edulis* (Tedesco et al., 2010) and Chora et al. (2010) showed that NP exposure generated ROS in gill and digestive gland of *R. decussatus* that significantly altered the proteome. Results also highlighted the advantage of using redox proteomics in the assessment of protein ubiquitination and carbonylation, which may be markers of oxidative stress in *R. decussatus*.

Because of the range of irreversible and reversible modifications possible in proteins, redox proteomics offer a rout to identifying new protein targets for ROS toxicity with insights to likely mechanisms.

1.5 MUSSELS AS BIOLOGICAL MODEL IN ECOTOXICOLOGY

Since the aquatic environment is often the ultimate ecological compartment of anthropogenic pollutants, 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.

Because of their interactionswith water and aquatic sediments, bivalves are amongst the most popular sentinel species used in ecotoxicology, so that the term “Mussel Watch” was coined. Since the mid-1970s, scientists of several countries have used bivalve-filter feeding molluscs to monitor several contaminants, from heavy metals to pesticides and other organic pollutants, in coastal marine waters (Farrington et al., 1987). The 1990s saw a rapid increase in use of mussels as tools for measuring POP (Persistent Organic Pollutants) loads in the environment. This is because of their wide geographical and taxonomic distribution, their abundance, ease of identification, sedentary habit and the fact that they are mainly filter-feeders (Bayne, 1978). Bivalves are often quite tolerant to chemical pollution and survive by adapting to changing pollution status. They can bioaccumulate pollutants, bacteria and viruses from their surrounding environment, which has made them especially useful in ecotoxicology (Depledge and Galloway, 2005; Peakall, 1994). 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 sentinel-organisms, 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 molluscs, 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 favours 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) and pharmaceuticals (Binelli et al., 2009; Parolini et al., 2009, 2010).

1.5.1 ZEBRA MUSSEL (*Dreissena polymorpha*)

Dreissena polymorpha (Pallas, 1771) is a sessile bivalve, forming dense colonies on various hard substrates in lentic and lotic freshwaters and it is able to survive also in estuarine slightly brackish waters (it tolerates salinity up to about 5 g/L). It has brownish-yellowish shells with dark and light coloured zigzag banding which confers to it the common name "zebra mussel" (Fig. 6).

Native to the drainage basins of the Black, Caspian and Aral Seas, it was found across Europe before the last glacial period (Starobogatov and Andreeva, 1994).

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 during the 19th century occupied most of inner water systems of western and central Europe rapidly (Kinzelbach, 1992).

Regnum: Animalia
Phylum: Mollusca
Classis: Bivalvia
Subclassis: Heterodonta
Ordo: Veneroida
Superfamilia: Dreissenoidea
Familia: Dreissenidae
Genus: *Dreissena*
Species: *D. polymorpha* (Pallas, 1771)

Figure 6: Adult individuals of zebra mussel (*Dreissena polymorpha*) and their systematic classification.

In the 1920's it appeared in Sweden, in the 1960's it was found in alpine and subalpine European lakes (Pollux et al., 2003) and reached Italy in 1969 (Lake Garda, Giusti and Oppi, 1972), where subsequently 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 now considered as a typical representative of Italian malacofauna. It arrived in Ireland around 1993 and was first recorded in the Ebro River in 2001 (Delivering Alien Invasive Species Inventories for Europe, DAISIE, 2006). In 1988 it first appeared in Lake St. Clair and rapidly spread throughout the Great Lakes of North America (Hebert et al., 1989). Further range expansions are expected in temperate latitudes of the Northern Hemisphere and to South America, South Africa, Australia and New Zealand.

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 obligated 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, usually with a ratio 1:1. Fertilisation takes place externally and synchronise spawning occurs once they overcome 8 mm and is influenced by water temperature and trophic conditions (Binelli et al., 2001). A mature female may produce one million eggs per year. Spawning begins at 12-15 ºC it can be profuse at 18-20 ºC; and may take place over a period of 3-5 months (Fig. 7) (Sprung, 1987, 1989; Borcherding and De Ruyter van Staveninck, 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.

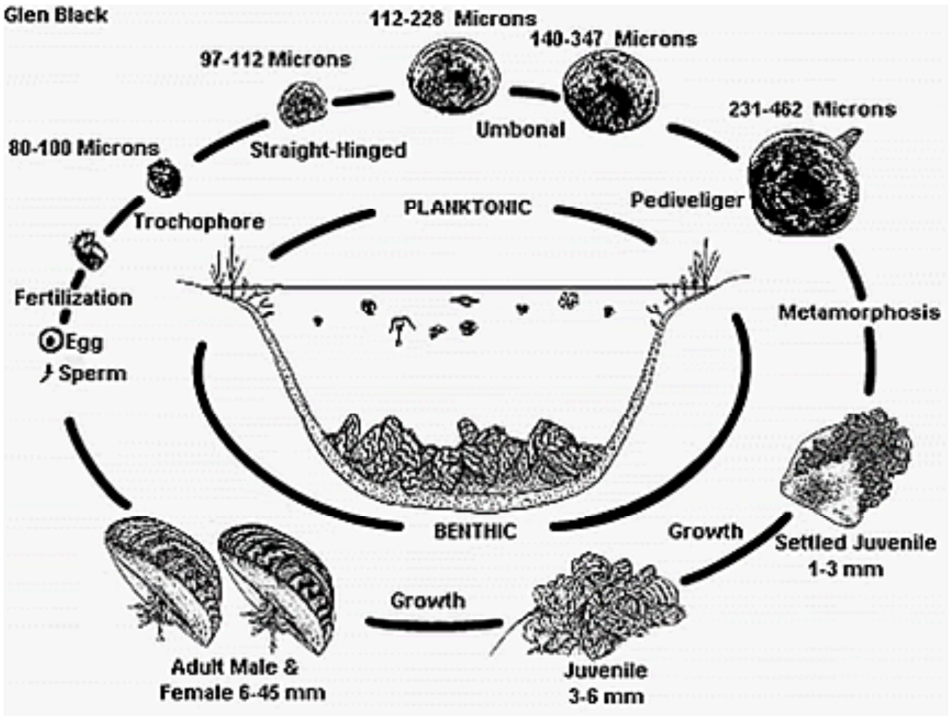


Figure 7: Zebra mussel (*Dreissena polymorpha*) life cycle. Its annual cycle can be divided in three periods: a pre-reproductive period (autumn-May) when the maturation of gametes occurs, a reproduction period, and a short post reproductive period.

Zebra mussels are unusual among freshwater molluscs in that they have planktonic larval stages (trochophore, veliconcha, pediveliger) that more closely resemble marine than freshwater bivalves (Neumann and Jenner, 1992). Dispersal occurs mainly during this pelagic state, when they are transported by currents. Secondary it can occur by the drifting of post-larvae and young adults using byssal and/or mucous threads. 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 other molluscs and crustaceans, also interfering with their movements (Lyakhnovich et al., 1994). In this way it competes for space and food with native mussels and other filter-feeding organisms. 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). It is evident how it can have multiple economic impacts, including: fisheries (interference with fishing gear, prey for commercial fish, alteration of fish communities), aquaculture (fouling of cages), water abstractions (clogging of water intake pipes), aquatic transport (fouling of ship hulls and navigational constructions). Invasion of the zebra mussels to the North America is causing annual multimillion losses to the economy. 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 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), since its filtration activity can lead to phytoplankton consumption resulting in increased water clarity.

This mussel possesses 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.

1.5.2 BIOMARKERS APPLIED ON ZEBRA MUSSEL SPECIMENS

Since mussels have acquired a global importance as sentinel organisms in aquatic environments, a growing number of studies is now available on pollutants effects on many biomarkers in both field-sampled animals and in those exposed to specific chemicals under controlled conditions (Peakall, 1994; Cajaraville et al., 2000; Livingstone et al., 2000; Oehlmann et al., 2002; Roméo et al., 2003; Smolders et al., 2003; Petrović et al., 2004; Depledge and Galloway, 2005).

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, 2009b,c; 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 evaluation of xenobiotic effects on organisms could be done *in vitro* or *in vivo*. 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). Instead, the latter approach has the advantage to allow a more general ecotoxicological screening (Ching et al., 2001), since it consider the whole organisms or mesocosms (Blaauboe, 2008; Gura, 2008) that mimes the natural environmental situation. In this way, 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 their defence mechanisms.

1.5.2a *in vivo* BIOMARKER BATTERY

We applied the Single Cell Gel Electrophoresis (SCGE) assay, the DNA diffusion assay and micronucleus test as biomarker of genetic damage on zebra mussel haemocytes exposed by an *in vivo* approach. 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). In addition, the increase of oxidative stress was investigated through 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 Stransferase (GST). Instead, to detect consequences of oxidative stress the protein carbonyl content (PCC) and the oxidation of polyunsatured fatty acids (LPO) were measured.

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).

Östling and Johanson (1984) were the first to develop a micro-gel electrophoresis technique for detecting DNA damage at the level of the single mammalian cell under neutral conditions. The neutral conditions used greatly limited the general utility of the assay. Singh et al. (1988) and Olive et al. (1990) independently modified the assay by developing alkaline versions (pH 13 and pH 12.3, respectively). 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 crosslinks, 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). Since the introduction of the alkaline (pH>13) Comet assay in 1988, the breadth of applications and the number of investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include its fairly simple methodology, sensitivity for detecting low levels of DNA damage,

requirement for small numbers of cells, flexibility, low costs, ease of application; the ability to conduct studies using relatively small amounts of a test substance; and rapid production of data. During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring to DNA repair processes to genetic toxicology and "ecogenotoxicology" (Tice et al., 2000; Collins, 2004; Knopper and McNamee, 2008). It has been successfully applied in many aquatic organisms, including many species of fish and aquatic invertebrates, mainly molluscs and crustaceans (Cotelle and Férard, 1999).

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 viewed using a fluorescent microscope. The distance and/or amount of DNA migration (Fig. 8) 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).

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).

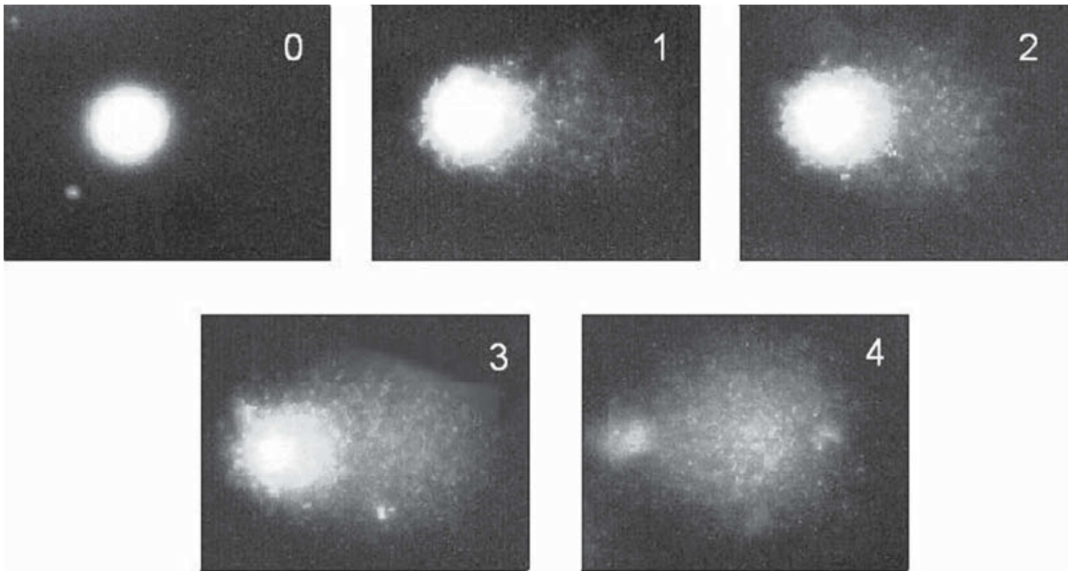


Figure 8: Images of comets, stained with DAPI and evolution of DNA fragmentation highlighted by the SCGE assay (according to DNA in tail as purposed by Mitchelmore et al., 1998): 0) Minimal; 1) Low; 2) Mid; 3) High; 4) Extreme (Apoptosis).

A highly significant contribution of the SCGE assay to genetic toxicology is in its application to *in vivo* studies. As only a small numbers of cells are required for analysis, literally any tissue or organ is amenable to investigation. In conducting *in vivo* Comet studies, care should be taken to avoid conditions that would lead to positive results that do not reflect genotoxicity but may arise from DNA damage (i.e., DSB) associated with apoptosis or necrosis. Apoptotic or necrotic cells exhibit very small or non-existent heads and very large diffuse tails (Fig. 8(4)). These cells are commonly referred to as tear-drops, ghost cells, or hedgehog cells. Such cells can be produced after exposure to cytotoxic agents and/or non-genotoxic agents and should be excluded from analysis (Knopper and McNamee, 2008).

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 kinds of cells (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 (Fig. 9). By contrast, in diffused nuclei due to necrosis the DNA shows a clearly defined outer boundary and a relatively clear appearance. 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.

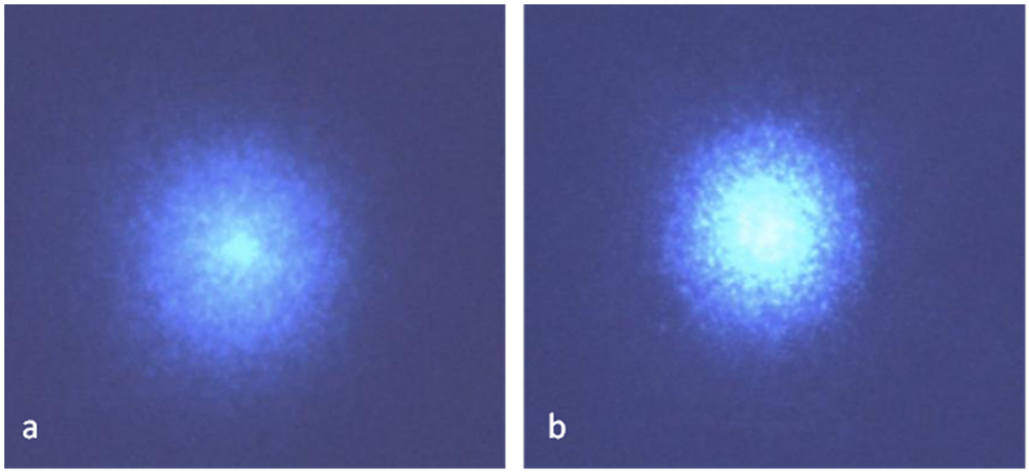


Figure 9: Apoptotic (a) and necrotic (b) zebra mussel hemocytes according to the guidelines suggested by Gichner et al. (2005).

The **Micronucleus test** (MN test) is a cytogenetic test commonly used in various biological systems for monitoring environmental genotoxicity. It 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.

The method consists of scoring cells with one or several cytoplasmic nuclei of reduced size associated with the main cellular nucleus. 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 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.

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 (Fig. 10). At least 2,000 cells per individual should be scored.

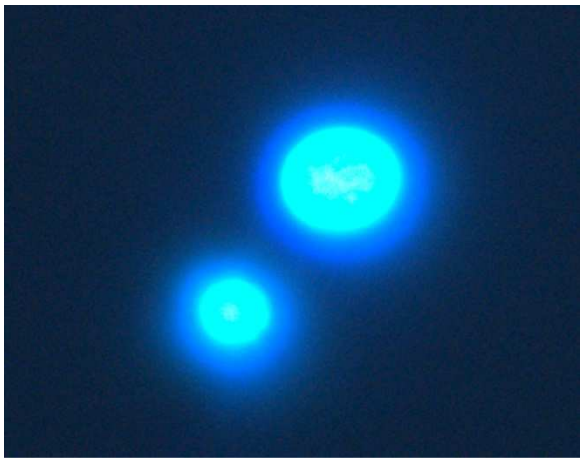


Figure 10: Micronucleated nucleus of zebra mussel hemocytes.

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 analysed (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.

The **Neutral Red Retention Assay (NRRA)** is a simple biomarker commonly used to evaluate the destabilization of the lysosome membranes.

Lysosomes are subcellular organelles bounded by a semipermeable lipoprotein membrane containing a battery of about 60 hydrolytic enzymes. Acting optimally at an acid pH, lysosomes are collectively capable of degrading all classes of macromolecules of indogenous (intracellular) and exogenous (extracellular) origin. Lysosomes are in all probability ubiquitous in the cells of eukaryotic organisms, with the notable exception of mammalian red blood cells.

Lysosomes are involved in a diverse range of cellular activities including digestion, reproduction, membrane repair, developmental processes, programmed cell death (apoptosis), protein turnover and the immune response. In addition to cellular molecules and pathogens, lysosomes accumulate a wide range of toxic compounds including PCBs, PAHs and metals which are damaging to cells (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).

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). For this reason lysosomal damage, expressed as an increase in the permeability of the lipoprotein membrane, is well established as a useful non-specific biomarker of contaminant induced stress (Moore, 1990; Lowe and Pipe, 1994).

Lowe and Pipe (1994) developed a 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. The dye is sequestered in the lysosomal compartment when living cells are preloaded with Neutral Red (NR) (Fig. 11); it is a lipophilic dye and as such will freely permeate the cell membrane. Within cells the compound becomes trapped by protonation in the lysosomes and accumulated in these organelles, where it can be visualized microscopically. The degree of trapping of this lysosome-tropic marker depends on the pH of the lysosome as well as the efficiency of its membrane associated proton pump (Segien, 1983). If the lysosome membranes are damaged, NR leaks out into the cytosol where it can be visualized under the microscope (Fig. 11).

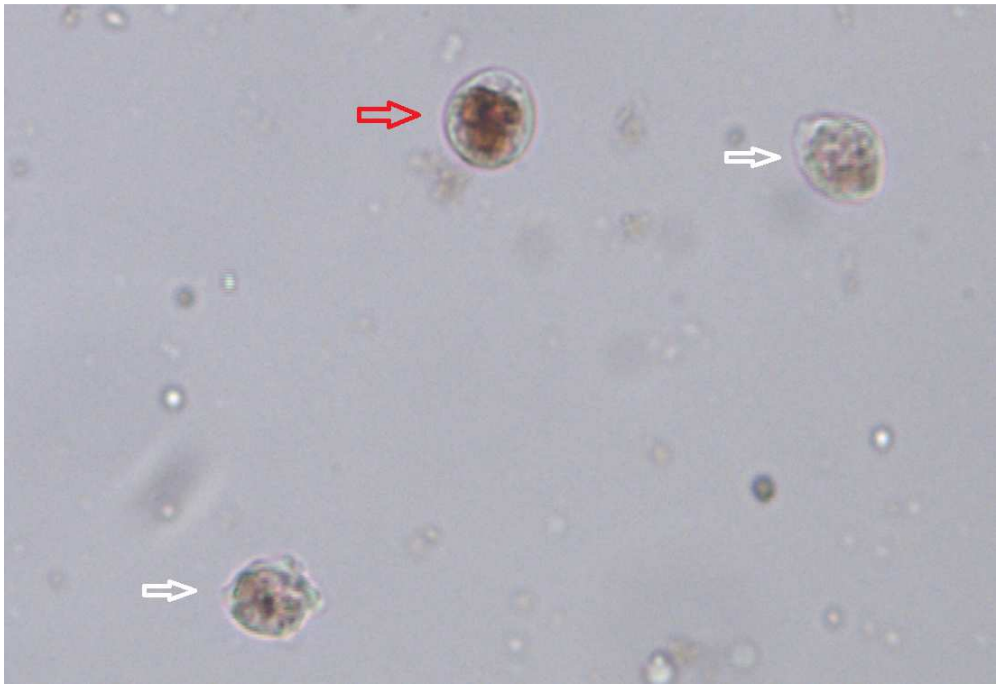


Figure 11: Healthy (red arrow) and damaged (white arrows) zebra mussel hemocytes according to the Neutral Red Retention Assay.

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 (Winston et al., 1996).

Many environmental pollutants exert their toxic effects by altering the redox status of the cell. Molecular oxygen (O_2) has two unpaired electrons in its outer shell and this paramagnetic property makes O_2 essentially unreactive with organic molecules unless it is first activated (Cadenas, 1989). This is achieved in a series of univalent reaction resulting in the successively formation of ROS (Fig. 12).

ROS are relevant to ecotoxicology purposes for at least three reasons. Firstly, they are continuously produced in natural contexts (O'Sullivan et al., 2005); secondly, many environmental pollutants can stimulate generation of ROS (Lopez-Barea et al., 2006); thirdly, many ROS feature in stress-response pathways as part of signal transduction (Schrek and Baeurle, 1991). Because most organisms are exposed to ROS either as a result of external or internal chemical events, cells have evolved very elaborate defences to protect key components from oxidative damage (Fig. 12). Exposure to some xenobiotics, especially toxic chemical pollutants, may produce an imbalance between endogenous and exogenous ROS and subsequently a decrease of antioxidant defences. 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 defences 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).

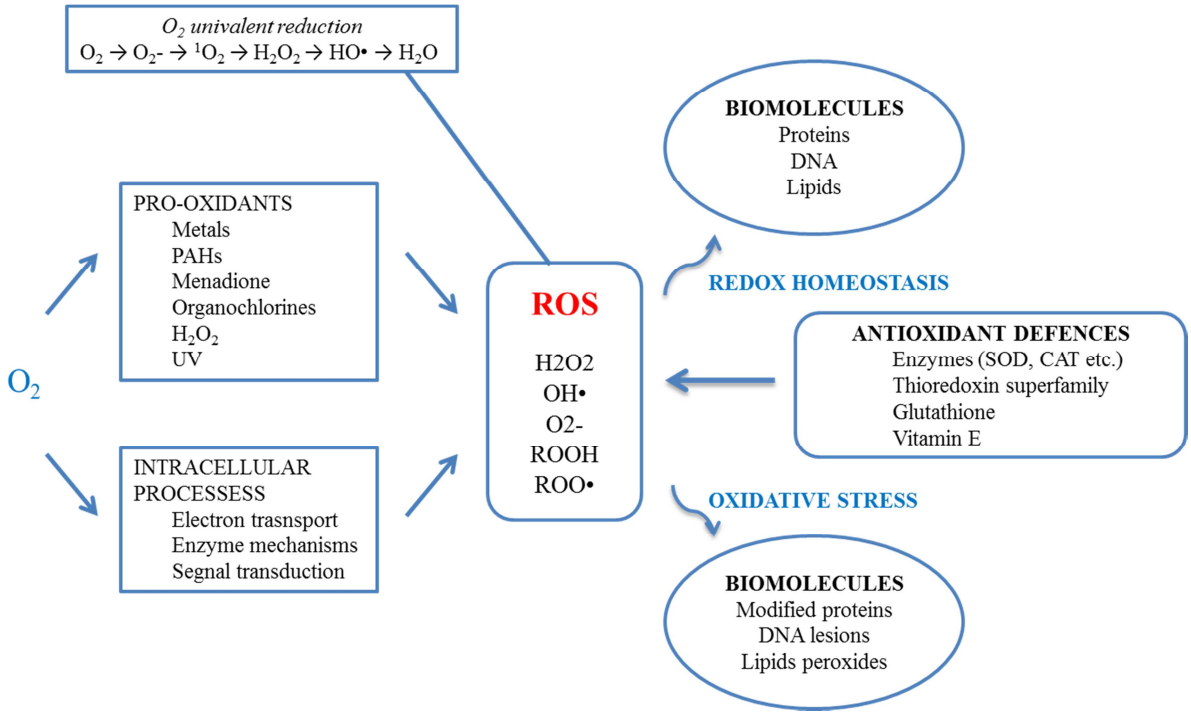


Figure 12: OS arises from ROS derived by univalent reduction of O_2, when antioxidant defences are overcome. They can arise from endogenous or external sources (PAHs – polyaromatic hydrocarbons).

Biomarkers can monitor the status of various antioxidant defence mechanisms against free radicals. The **antioxidant defence 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 non-invasive biomarkers of oxidative stress in humans and new pharmacological strategies aimed at supplementing antioxidant defence 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 our 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).

If the oxidative stress arises, a lot of consequences are possible, such as lipid peroxidation (LPO) and the oxidation of protein residues with the consequently increase of protein carbonyls. Oxidation of polyunsaturated fatty acids is a very important consequence of oxidative stress; it is the oxidative deterioration of lipids containing a number of C-C double bonds (Rice-Evans and Burdon, 1993). Membrane poly-unsaturated fatty acids are particularly susceptible to lipid peroxidation and since membranes form the bases of cellular organelles, its damage is highly detrimental to the functioning of the cell and its survival. Moreover, a large number of toxic by-products are formed during LPO and these have effects at a site away from area of their generation. Hence they behave as toxic "second messengers" and can modulate, at very low and non-toxic concentrations, several cell functions, including signal transduction, gene expression, cell proliferation and more generally the response of target cells (Raha and Robinson, 2000). LPO was measured by the **quantification of thiobarbituric acid-reactive substances (TBARS)** according to Ohkawa (1979). Samples were analyse in triplicate (n=3) from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 50 mM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) containing 1 mM DTT and 1 mM PMSF using a Potter homogenizer. The amount of TBARS formed was measured by reading absorbance at 535 nm (extinction coefficient of $1.56*10^5$ M/cm) and results were expressed as nmol TBARS formed/g fresh weight.

An important part of cellular damage caused by ROS is the oxidation of amino acid residues on proteins, forming protein carbonyls. In addition, carbohydrate and lipid derivatives can react with proteins to form adducts that can be analysed. **Protein carbonyl content (PCC)** is the most widely used marker of oxidative modification of proteins (Mecocci et al., 1998; Chevion et al., 2000). Oxidation products and carbonyl derivatives of proteins may result from oxidative modifications of amino acids and side chains, reactive oxygen-mediated peptide cleavage and from reactions with lipid and carbohydrate products (Labieniec et al., 2004). Hence, the presence of these irreversible modifications may indicate that the proteins have been subjected to oxidative free radical damage (De Zwart et al., 1999). As for LPO, PCC was measured in triplicate from a pool of three whole mussels in the same homogenization buffer and for its quantification the reaction with 2,4-dinitrophenylhydrazine (DNPH) was employed according to Mecocci et al. (1998). The carbonyl content was

calculated from the absorbance measurement at 370 nm with the use of molar absorption coefficient of 22,000 mol/cm and expressed as nmol/mg protein.

These tests are well established oxidative stress indices, widely used both in human investigations on aging and diseases (Chevion et al., 2000; Zafrilla et al., 2006; Chakraborty et al., 2009), toxicology (Döger et al., 2011), animal reproduction studies (Domìnguez-Rebolledo et al., 2010) and ecotoxicology (Livingstone, 2001; Verlecar et al., 2008; Faria et al., 2010).

1.5.3 PROTEOMICS APPLIED ON ZEBRA MUSSEL SPECIMENS

Although still in a pioneering way, proteomics has, in the last decade, been applied to bivalves to support the toxicological studies. Data have been gathered from a broad range of hazardous chemicals (e.g. metals, polyaromatic hydrocarbons, diallylphtalate, polybrominated diphenyl ethers) (Knigge et al., 2004; Dowling et al., 2006; Chora et al., 2009) and also natural toxins, from both laboratorial and field work (Nzoughet et al., 2008; Nzoughet et al., 2009; Martins et al., 2009; Puerto et al., 2011).

Pollutant chemicals can react with proteins in a variety of ways including formation of adducts (e.g. benzo[a]pyrene; Sugihara and James, 2003), alteration of phosphorylation status (e.g. phorbol esters and akadoic acid; Forman et al., 1998), alteration of thiols (e.g. reactive oxygen species, ROS; Dalle-Donne et al., 2005) and conversion of side chains to aldehyde or ketone groups (ROS; Dalle-Donne et al., 2005). These interactions may alter the p*I* or lead to a change in the level of specific proteins, all of which could be detectable with **proteomics approaches**.

To detect these changes in the field of ecotoxicology the most frequently methods used have been two-dimensional gel electrophoresis (2-DE) of proteins and mass spectrometry (MS). Since the original development of 2-DE (O'Farrell, 1975), several technical innovations have contributed to improving the robustness and reliability of this technique (Lilley et al., 2002). Of key importance was the development of IPGs, which abolished cathodic drift and improved reproducibility (Celis and Gromov, 1999), development of sensitive staining methods (Rabilloud, 1992), availability of powerful image analysis systems (Westergren-Thorssin et al., 2001) and improved MS and bioinformatics methods for spot identification (Stults and Arnott, 2005; Chalkley et al., 2005). Multiple analyses are possible on 2-DE gels giving both abundance and chemical information (Chevallet et al., 1997; Patton, 2002). This can allow comparison of expression levels as well as reporting alterations in structural proteins, and key proteins of the oxidative stress defence mechanisms, metabolism of

xenobiotics, cell signaling, protein stabilization, energy metabolism and metabolism of lipids, and amino acids (Riva et al., 2011; Leung et al., 2011; Thompson et al., 2011). However, the lack of genomic sequences from bivalves is still preventing large-scale utilization of MS-based proteomics in this area. Indeed, spot quantification and identification remain major stumbling blocks in many species used in ecotoxicology. However, affinity methods for selection of sub-proteomes (Lee and Lee, 2004) and advanced MS approaches can simplify this problem, and can also be considered to better describe the protein post-translational modifications (PTMs) induced by the action of hazardous chemicals (Campos et al., 2012).

The first approach in aquatic toxicology to proteomic analysis was conducted by Shephard and Bradley (2000) and Shephard et al. (2000) on the bivalve *Mytilus edulis* exposed to copper, polychlorinated biphenyls (PCBs) and salinity stress. They used the overall pattern of protein spots in 2-DE as a protein expression signature (PES) that could be used to reveal effects in animals exposed to a variety of pollutants and although these studies were performed in laboratory conditions gave a starting point in the use of proteomics in other aquatic species and in field experiments. Subsequently, other bivalves such as *Mytilus galloprovincialis* (Lopez et al., 2001) and *Perna viridis* (Leung et al., 2011), clams *Ruditapes decussatus* (Dowling et al., 2006; Chora et al., 2008; Chora et al., 2009), *Chamelea gallina* (Rodriguez-Ortega et al., 2003), the freshwater mussel *Unio pictorum* (Marie et a., 2010), and rock oyster *Saccostrea glomerata* (Thompson et al., 2011) were utilized in proteomic studies, employing 2-DE, PES and MS (peptide mass fingerprint and tandem MS).

Unfortunately the scientific literature is completely lacking about proteomics studies on zebra mussel and although *D. polymorpha* can be used as an extremely flexible environmental monitor, there are very few data regarding the evaluation of possible changes in protein patterns due to exposure to environmental contaminants. Since this bivalve is comparable to *Mytilus* sp. (Giambérini and Cajaraville, 2005), which has been used for several proteomic studies (Apraiz and Cristobal, 2006; Magi et al., 2008), in this study we applied proteomics tools on zebra mussel specimens previously used with its marine counterpart.

Tissue selection for comparative proteomic analyses is a crucial point. In this study, differences in protein expression levels were studied in mussel gills because they are the first uptake site for many toxicants in the aquatic environment and are known to be affected by exposure to pollutants (Gómez-Mendikute et al., 2005). Moreover the gills of *D. polymorpha* are independent organs that can be easily dissected to offer a pure tissue sample (Quinn et al., 2009).

Methods were partially modified from Colombo et al. (2009). Briefly we obtained cytosolic soluble fraction from zebra mussel gills after their homogenization in a hypertonic buffer; proteins were quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA) and precipitated. Then 2-DE was performed. After appropriate staining, gel images were obtained and analysed with image analysis software to reveal varied spots (proteins) between treatments. Spots of interest were subsequently isolated from gels and submit to in-gel trypsin digestion to allow mass spectrometry identification. In this study we used a MALDI-TOF/TOF (Matrix-assisted Laser Desorption Ionization - Time of Flight) mass spectrometer obtaining a peptide mass fingerprint plus MS/MS data (Fig. 13).

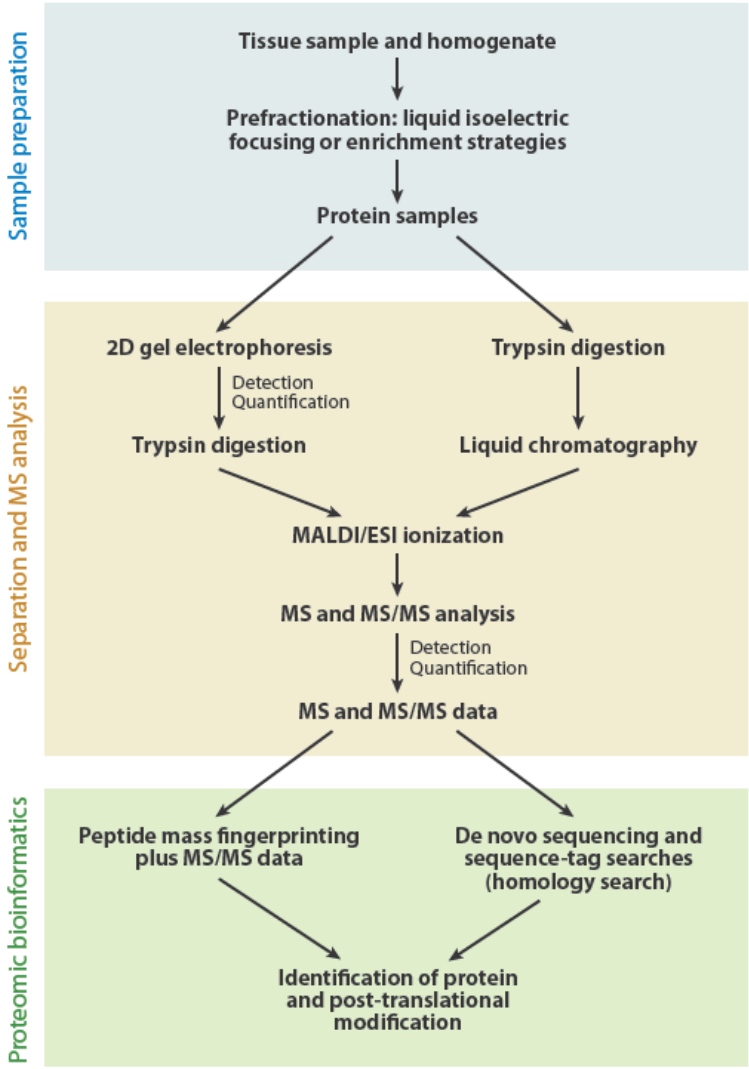


Figure 13: Overview of different proteomic work flows (Tomanek, 2011). Abbreviations: 2D (two-dimensional) gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

A complementary 2-DE based proteomic approach to PES and/or spot identification may be offered by probing changes in the chemical status rather than the abundance of individual proteins. An attractive set of targets is presented by post-translational modifications arising from OS such as carbonylation, glutathionylation and effects on protein thiols pattern (Levine and Stadtaman, 2001; Dalle-Donne et al., 2005). Redox-based processes altering the p*I* or M_r of proteins (e.g., charge isomerization, protein backbone cleavage, and crosslinking) are detected as altered 2D SDS-PAGE spots (Sheehan, 2006).

OS can cause change in levels of specific proteins detectable by protein staining and image analysis (Patton, 2002) and can cause up- and down-regulation of key proteins (Regoli et al., 2002), such as those involved in energy metabolism, in redox regulation and production of reducing equivalents such as NADPH (Kültz, 2005). In response to cellular stress, molecular chaperones that stabilize denaturing proteins during cellular stress, proteases that regulate protein turnover, and proteins that sense and repair DNA and RNA damage and that are involved in fatty acid metabolism are also activated (Kültz, 2005; Petrak et al., 2008; Wang et al., 2009). Because of the high number of reversible and irreversible modifications possible in proteins, **redox proteomics** offers a route to detecting these effects and providing new molecular markers of the presence of pollutants in the environment with insights to likely mechanisms of action of xenobiotics.

One of the first works in which the proteomic approach was applied on bivalves has presented a combination of gel electrophoresis and western blotting for the detection of carbonylation and glutathionylation of proteins in *M. edulis* (McDonagh et al., 2005) sampled from a polluted and reference site in Cork Harbour, Ireland. The same research group assessed for the first time disulphide bonds (S-S) of proteins in *M. edulis* exposed to hydrogen peroxide (H_2O_2) to elicit oxidative stress (McDonagh et al., 2006).

Afterwards the study of some PTMs have been used in other bivalve species such as *R. decussatus* (Dowling et al., 2006; Chora et al., 2008) or in *M. edulis* (Tedesco et al., 2008) to understand the effects of emerging pollutants such as cadmium, nonylphenol and nano-particles, under laboratory conditions. These studies demonstrated that the toxicity associated to these compounds may derive from an excessive increase of protein PTMs with subsequent protein inactivation (Chora et al., 2009; Apeti et al., 2010; Tedesco et al., 2008), so that in bivalves, carbonylation and ubiquitination of proteins assessed through redox proteomics have recently been applied as biomarkers of OS (Dowling et al., 2006; Prevodnik et al., 2007; McDonagh and Sheehan, 2008). Modern proteomic technologies evaluating PTMs in subproteomes are slowly increasing in the environmental research (Nesatyy and Suter, 2008),

but current methods applied in medical research, involving the isolation/enrichment of oxidative stress modified proteins/peptides, combined with extensive LC-MS/MS analysis may contribute to the identification of modified proteins and their residues. Such methods will increase our understanding of the role of protein PTMs in the toxicity induced by environmental contaminants (Madian and Regnier, 2010).

Notwithstanding this, today no redox proteomic approach has been applied on *Dreissena polymorpha* specimens. Thus we investigated, for the first time, the OS effects on zebra mussel gill's proteome as a consequence of exposure to illicit drugs, adapting methods previously used on *M. edulis* (Tedesco et al., 2010). Briefly we obtained cytosolic soluble fraction from zebra mussel gills pools and then incubated it with 5 – Iodoacetamidofluorescein (IAF; Baty et al., 2002) and Fluorescein - 5 – thiosemicarbazide (FTSC; Ahn et al., 1987) to label protein thiol groups and carbonyls respectively. Different aliquots of protein extracts were resolved using 1-DE to determine the overall quantity of thiol-containing proteins and carbonylation (equal amounts of protein were loaded in 12 wells - 3 replicates for each treatment - and repeated at least 3 times) and then 2D-SDS PAGE (four biological replicates for each treatments), in order to evaluate changes in protein expression profiles to see what kind of proteins is involved in the toxicity process.

As described above, we excised spots of interest from 2-DE gels and, after tryptic digestion, analyzed them using MALDI-TOF/TOF and a liquid chromatography-tandem mass spectrometer that allow *de novo* sequencing analysis increasing the chances of *D. polymorpha* proteins recognition in databases.

Chapter 2 *– AIMS, DEVELOPEMENT OF THE EXPERIMENTAL DESIGN and MAIN RESULTS*

The ecotoxicology should follow a multidisciplinary approach, combing the science of chemistry, toxicology, pharmacology, epidemiology and ecology with an understanding of the sources, fates and effects of chemicals in the environment.

Moreover, in the last few years, the need for an in-depth knowledge of the response of organisms to the most frequent and persistent pollutants and a deep characterization of the molecular markers and mechanisms involved in the toxic processes has become of priority importance. The large scale analyses of proteins, genes and other biomolecules, provided by OMICs approaches can contribute to this search. In this sense, the present thesis aims to integrate the classic approach of ecotoxicology, especially regarding the employment of cyto-genotoxic biomarkers, with the more holistic OMICs techniques, in particular with the environmental proteomic approach to analyse the proteome of organisms and to identify variations in the proteins induced by xenobiotics.

In particular this thesis is comprised of a collection of articles published, in course of publication or submitted to several international scientific journals that aimed to characterize the effects and mechanisms of action of different classes of new environmental pollutants, PPCPs and illicit drugs, on the freshwater bivalve *D. polymorpha*. Each article is fully reported in Chapter 3.

The list of the articles grouped on the basis of the main topic is presented below.

Few years ago, our research group focused its attention on a new class of environmental contaminants: PPCPs. We previously analysed the effects on zebra mussel of different chemicals attributable to this category, such as the antibiotic TMP (*in vivo* effects; Binelli et al., 2009a) and some NSAIDs both in *in vitro* (Parolini et al., 2009) and *in vivo* studies carried out both as single molecules (PCM, Parolini et al., 2010; DCF, Parolini et al., 2011a; IBU, Parolini et al., 2011b) and as mixture (Parolini et al., 2012).

Among personal care products the most in depth characterized chemical by our research group was TCS that showed a clear and significant cyto-genotoxicity both *in vitro* and *in vivo* studies (Binelli et al., 2009b; Binelli et al., 2009c). In order to complete the analysis of its effects and to confirm its mechanism of action, we verified its role in the increase of oxidative stress by measuring the activity of some antioxidant enzymes (SOD, CAT, GPx) and GST at three different doses (1 nM, 2 nM, 3 nM corresponding to 290, 580 and 870 ng/L respectively):

I. ANTIOXIDANT ACTIVITY IN THE ZEBRA MUSSEL (*Dreissena polymorpha*) IN RESPONSE TO TRICLOSAN EXPOSURE.

We measured enzymes activities every 24 h during a 96 h exposure's period. The only enzyme significantly different from controls also at the lowest administered dose was GST, while CAT and SOD were activated only at the highest dose tested and GPx values overlapped the baseline levels. Our results did not completely explain the MoA of triclosan, since we previously noticed a high induction of DNA damage also at the lowest doses (Binelli et al., 2009b; Binelli et al., 2009c). These effects are probably due to a direct action of TCS that induce oxidative stress only at higher doses. Moreover, *D. polymorpha* defense mechanisms, as 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. Considering the whole set of data collected by our research group, it is evident that this chemical represents a potential danger for the aquatic biocoenosis, since showed very dangerous effects on zebra mussel.

Subsequently, we compared the cyto-genotoxic effects induced by the exposure to a common environmentally relevant concentration (1 nM) of TCS, TMP, DCF, IBU and PCM to draw an appropriate toxicity scale and to identify the therapeutic that should need more in-depth investigations. By using all the measured end-points we calculated a biomarker response index (BRI) that allows to rank and compare the hazard of tested PPCPs toward the zebra mussel, minimizing the variation of responses, as in-depth shown in the second published article:

II. APPLICATION OF A BIOMARKER RESPONSE INDEX FOR RANKING THE TOXICITY OF FIVE PHARMACEUTICAL AND PERSONAL CARE PRODUCTS (PPCPs) TO THE BIVALVE *Dreissena polymorpha.*

The scale of toxicity was as follows: TCS>TMP>IBU>DCF>PCM, confirming that TCS is the most dangerous pharmaceutical among those tested. These results suggest a further in-depth investigations for an accurate environmental risk assessment for these chemicals in order to protect the entire aquatic community.

We then turned our attention on the new relevant class of environmental contaminants: illicit drugs. CO and its main metabolite BE are among the most abundant illicit drugs in the freshwater ecosystems and the study following is part of a broader project designed to evaluate the sub-lethal effects of not just CO but also its primary metabolites, BE and EME.

Since no data are available on their acute or chronic effects on the aquatic community and in particular on *Dreissena polymorpha*, we first evaluate possible cyto-genotoxicity of the parental compound in zebra mussel through a battery of biomarkers (Comet test, MN test,

DNA diffusion assay and NRRA) after a 96h of exposure under laboratory conditions. We exposed mussels to three environmentally relevant CO concentrations of 40 ng/L (0.13 nM), 220 ng/L (0.73 nM) and 10 µg/L (32.96 nM) also to give a marked ecological relevance to our research and to mimic true environmental conditions especially for what concern the European contest. In detail, the first dose corresponded to the highest CO concentration found in Italian freshwater (Zuccato et al., 2008b) while the second dose is the European average of the STP levels (Kasprzyk-Hordern et al., 2008; 2009; van Nuijs et al., 2009a). Finally, we calculated the Predicted Environmental Concentration (PEC) as highest dose from data available on River Po basin (Northern Italy) and following the EMEA (European Medicines Evaluation Agency) guidelines (EMEA, 2006).

The choice of experimental conditions was given from the fact that several other chemicals have produced cyto-genotoxic damage within the same period of exposure (Riva et al., 2007b; Binelli et al., 2008, 2009a,c; Parolini et al., 2010):

III. ILLICIT DRUGS AS NEW ENVIRONMENTAL POLLUTANTS: CYTO-GENOTOXIC EFFECTS OF COCAINE ON THE BIOLOGICAL MODEL *Dreissena polymorpha*.

Our preliminary results highlighted the capability of CO to induce a clear DNA damage in *D. polymorpha*, confirming a previous study that demonstrated CO genotoxicity in mammalian models (Alvarenga et al., 2011). CO was also able to induce destabilization of lysosomal membranes, suggesting also a possible induction of oxidative stress as a consequence of CO biotransformation.

This work is an example of a preliminary research with the aim to identify the possible cyto-genotoxic effects of an emerging pollutant at environmental concentrations, which in this case has highlighted a possible danger of illicit drugs for freshwater biocoenosis. After this early screening, it is critical to carry out studies to evaluate the late effects of long-term exposure, bearing in mind that in aquatic ecosystem animals are exposed to xenobiotics over their whole life-cycle.

In this contest, since in humans CO is rapidly metabolized into BE and since the aquatic levels of BE are much higher than those of parental compound, we performed an *in vivo* long-term exposure (14 days) of zebra mussel specimens to this metabolite at the two environmental concentrations of 0.5 µg/L (1.7 nM) and 1 µg/L (3.4 nM) of BE. We also

enriched the battery of biomarkers with antioxidant enzymes and LPO and PCC assays, in order to in-depth investigate its cytotoxicity and possible induction of oxidative stress.
Thus, end-points of ten different biomarkers (three of genotoxicity, one of cytotoxicity and six as oxidative stress indices) were measured with the aim to detect both sub-lethal BE effects and also to suppose its possible mechanism of action in *D. polymorpha*:

IV. SUB-LETHAL EFFECTS CAUSED BY THE COCAINE METABOLITE BENZOYLECGONINE TO THE FRESHWATER MUSSEL *Dreissena polymorpha.*

This is the first in-depth ecotoxicological study on the possible adverse effects due to this psychotropic substance. Results obtained pointed out that 14 days exposure to environmentally relevant BE concentrations were able to induce remarkable relevant toxic effects to different levels of biological organization in zebra mussel specimens. Indeed, we noticed a significant ($p<0.05$) destabilization of lysosomal membranes with a contemporary alteration of defense enzyme activities at both exposure concentrations. BE was also able to infer primary and fixed DNA damage at the highest dose tested. These findings led us to infer a probable involvement of oxidative stress in BE toxicity, confirmed by the increase of both LPO and PCC levels especially for what concern the highest concentration.
This pioneering research underlined how illicit drugs effects on aquatic biocoenosis must not be underestimated. Indeed, it could be possible that in environment, where other chemicals are present, additive and synergic effects arise resulting in possible higher toxicity of these molecules.
To confirm our hypothesis about BE mechanism of action we needed a more in-depth investigation using more powerful techniques. With the introduction of proteomic approach in our experimental design we partially achieved this objective. As well in depth described in previous sections, the application of proteomics to the field of ecotoxicology have been demonstrated an effective methodology for characterizing the modes of action and the mechanisms of toxicity for pollutants.
However, until now, there is a completely lack of proteomics studies applied on *D. polymorpha*. Since that, before applying these methodologies on zebra mussel in order to investigate MoA of PPCPs and illicit drugs, we proceeded to verify the suitability of a proteomic approach in this useful bio-indicator species. We then assessed changes in protein expression profiles in the gills of zebra mussel specimens after an *in vivo* exposure to the

chemical model benzo(α)pyrene (B[α]P). Considering this has been the first proteomic study on this biological model, we investigated also the role of gender in proteome response and also the possible dose-dependent relationships. We then analyzed separately male and female mussels exposed for 7 days to two different concentrations of B[α]P. We measured also B[α]P bioaccumulation in mussel soft tissues to ensure a real exposure to the selected chemical:

V. A PROTEOMIC STUDY USING ZEBRA MUSSELS (*D. polymorpha*) EXPOSED TO BENZO(α)PYRENE: THE ROLE OF GENDER AND EXPOSURE CONCENTRATIONS.

Through 2-DE electrophoresis tools we evaluated overall changes in expression profiles for 28 proteins in exposed mussels. No protein varied in common between concentrations, while a marked gender difference in altered protein levels emerged after exposure to B[α]P.
Spots of interest were manually excised from 2-DE gels and analyzed by MALDITOF/ TOF mass spectrometry. The most significant proteins that were identified as altered were related to oxidative stress, signal transduction, cellular structure and metabolism. This preliminary study indicated the feasibility of a proteomic approach in zebra mussel and provided a starting point for the further application of proteomics to this ecotoxicological model, confirming the need to increase the number of proteomic studies in invertebrates in order to increase their representation in databases and the successful identification of their most relevant proteins.
After verifying this point, in order to conclude the characterization of BE as new aquatic pollutant, we carried out other in-depth study about its MoA through a classic proteomic approach to see what kind of proteins were involved in its toxicity mechanisms. Thus, *D. polymorpha* specimens were subjected to a 14-day exposure of two different concentrations (0.5 µg/L and 1 µg/L) of BE. Changes in protein expression profiles were investigated in gill cytosolic fractions from control/exposed mussels using 2-DE electrophoresis. We evaluated an overall change in expression profiles for 18 proteins in exposed mussels, 7 and 11 of which were, respectively, over- and under-expressed. The comparative analysis of protein data sets showed 4 proteins that varied commonly and with the same trend in expression between the two different BE concentrations. Putative identification of differentially expressed proteins was performed with RP-HPLC coupled to ESI-TOF-MS and a *de novo* sequencing approach. The wide range of proteins affected suggested that BE has marked effects on various biological processes. Furthermore the role played by BE in inducing oxidative stress was further examined also in gills by measuring PCC and LPO, confirming an oxidative action exerted by BE (Riva et al., 2012).

Moreover, since BE showed a potential as oxidative stressor, we applied also redox proteomics approach with the aim to detect redox modifications against *D. polymorpha* gill proteins and to investigate what kind of proteins and so what metabolic pathways were involved. Exposures were performed *in vivo* for a period of 14 days and modifications on protein thiols and carbonyl groups in mussel gills were evaluated. We maintained the same experimental conditions and same concentrations (0.5 and 1 µg/L) pervious described, in order to even evaluate the possible dose-dependent changes in the proteome. These results are shown in the next article:

VI. A REDOX PROTEOMIC INVESTIGATION OF OXIDATIVE STRESS CAUSED BY BENZOYLECGONINE IN THE FRESHWATER BIVALVE *Dreissena polymorpha.*

One dimensional electrophoresis did not reveal a reduction in protein thiols content, while underlined a significant increase of protein carbonylation at both doses tested. We then performed protein profiling using 2-DE with subsequent MALDI-TOF and TOF/TOF with LIFT technique and LTQ-Orbitrap (Linear ion trap combined with Orbitrap mass spectrometer) yielding *de novo* protein sequences suitable for database searching. We identified a heat shock protein and the cytochrome c, that is considered a conventional biomarker of protein oxidation. These results confirmed the presence of oxidative stress caused by BE. Moreover, we supposed also the adaptation of mussel gill tissue to prevent huge damages as consequence of the presence of high oxidative stress. This could be done through glutathionylation of abundant proteins, as showed by the decrease of carbonylation of three different forms of tubulin. At the end, oxidative modifications were detected also in proteins involved in the glucose metabolism: this is of interest, since up- or down-regulation of metabolic enzymes are often related to stress status after exposure to chemical contaminants or OS. Our findings confirmed a significant cellular stress under the BE exposure regime used here, that could compromise also the energetic metabolism of our biological model.

At the end, this study illustrated the potential of proteomic techniques to uncover differences in protein redox status in *D. polymorpha*.

Chapter 3 – *ARTICLES*

PAPER I

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

ANTIOXIDANT ACTIVITY IN THE ZEBRA MUSSEL (*Dreissena polymorpha*) IN RESPONSE TO TRICLOSAN EXPOSURE.

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

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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 cytogenotoxicity 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 spectrophotometrically 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), phenanthroline (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 supernatant 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 ultracentrifuged 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 glutathione reductase. The reaction medium consisted of the following: 0.2 mM H_2O_2 substrate in 50 mM potassium 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 concentration 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^{-}°) 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

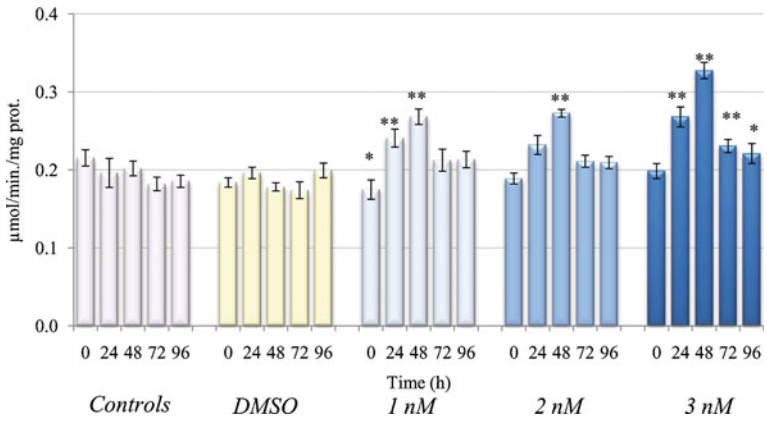

Fig. 1 Temporal trend of glutathione transferase (mean±standard error of the mean [SEM]) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *$p<.05$, **$p<.01$

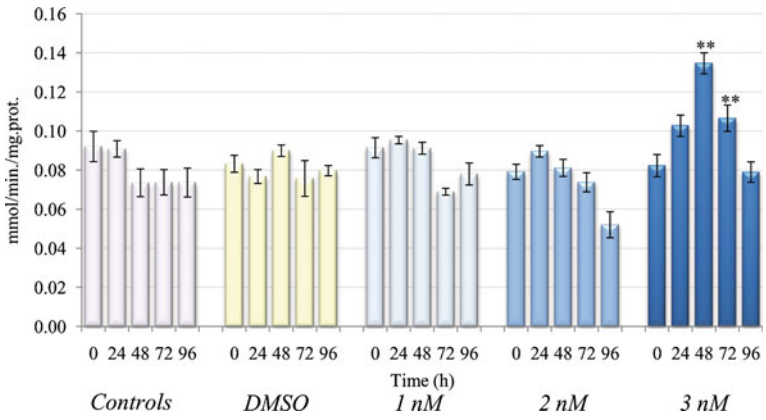

Fig. 2 Temporal trend of CAT (mean±SEM) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *$p<.05$, **$p<.01$

quinone (2-chloro-5-(2,4-dichlorophenoxy)-benzoquinone) and hydroquinone (2-chloro-5-(2,4-dichlorophenoxy) 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, hydrogen 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-diaphorase) 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. polymorpha*. 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.

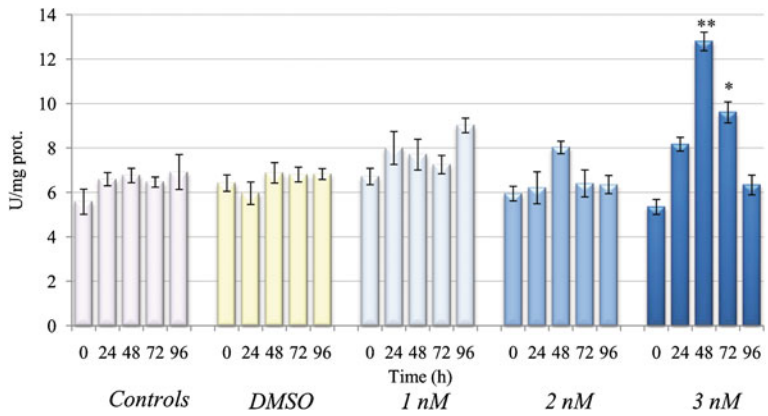

Fig. 3 Temporal trend of SOD (mean±SEM) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *$p<.05$, **$p<.01$

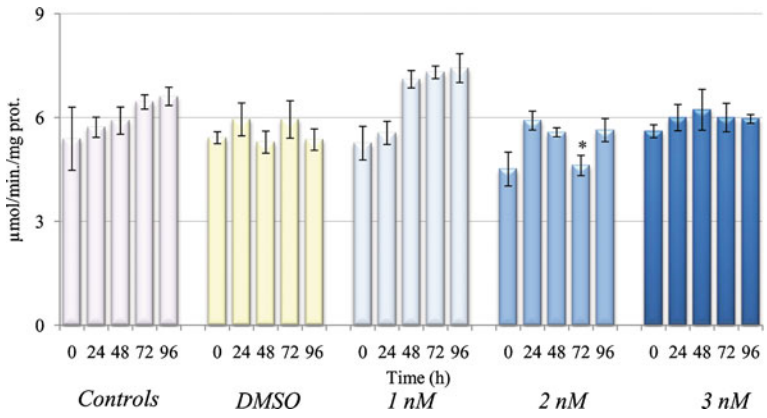

Fig. 4 Temporal trend of GPx (mean±SEM) caused by triclosan administered at 1 nM, 2 nM, and 3 nM compared to relative controls and solvent controls. ANOVA, Bonferroni post-hoc test, *$p<.05$, **$p<.01$

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.

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	$F=27.57$	$F=10.77$	$F=15.25$	$F=76.35$
	$p<.01$	$p<.01$	$p<.01$	$p<.01$

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-

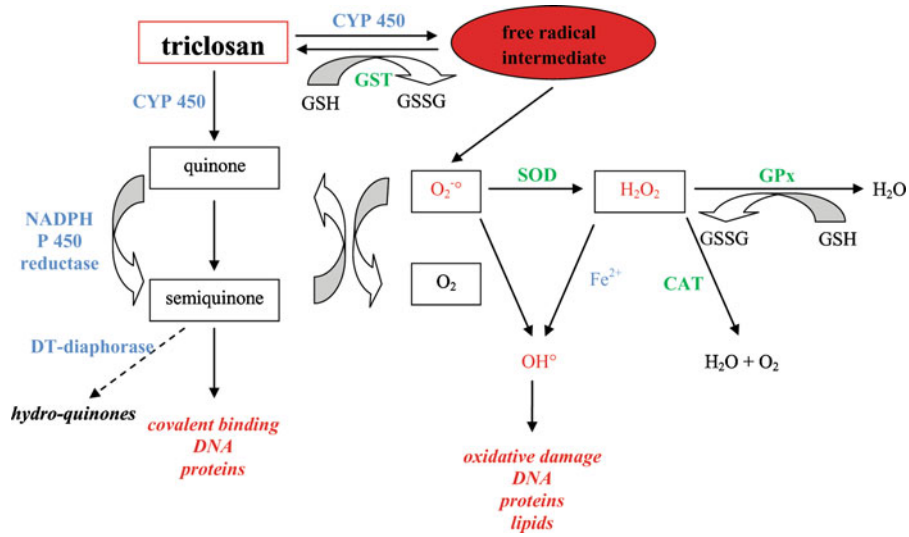

Fig. 5 Scheme of the antioxidant enzymatic chain and the relative production of ROS. The measured enzymes are indicated in *green*, while the oxygen free radicals and the cytogenotoxic damage in *red*

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 II

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APPLICATION OF A BIOMARKER RESPONSE INDEX FOR RANKING THE TOXICITY OF FIVE PHARMACEUTICAL AND PERSONAL CARE PRODUCTS (PPCPs) TO THE BIVALVE *Dreissena polymorpha*.

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Application of a Biomarker Response Index for Ranking the Toxicity of Five Pharmaceutical and Personal Care Products (PPCPs) to the Bivalve *Dreissena polymorpha*

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Abstract Pharmaceuticals and personal care products (PPCPs) have been detected in several aquatic ecosystems during the last two decades, but their potential for biological effects to nontarget organisms is only now being studied. The aim of this study was to compare and rank the cyto-genetic effects induced by 96-hour exposure to an environmental concentration (1 nM) of triclosan (TCS), trimethoprim (TMP), diclofenac (DCF), ibuprofen (IBU), and paracetamol (PCM) on the freshwater bivalve *Dreissena polymorpha* by integrating biological responses of eight biomarker into a simple biomarker response index (BRI). The application of the BRI decreased the wide biomarker variability and enabled toxicity ranking of the tested PPCPs as follow: TCS > TMP > IBU > DCF = PCM. This approach allowed us to draw an accurate PPCP scale of toxicity of the most dangerous drug and to address further in-depth investigations.

During the last two decades, pharmaceutical and personal care products (PPCPs) have begun to be considered as new environmental pollutants due to the increasing knowledge on their widespread occurrence in water systems and their potential hazard toward aquatic organisms. In fact, measurable levels of hundreds of PPCPs were found in the μg/L range in sewage-treatment plant effluents and in the ng/L range in surface waters (Santos et al. 2010). Because drugs have an intended biological activity and are often extremely persistent and bioavailable, they are potentially dangerous to nontarget organisms, even at low concentrations (Fent et al. 2006). Recent studies have pointed out that PPCP-induced acute effects occur only at concentrations in the mg/L range (Fent et al. 2006; Haap et al. 2008; Quinn et al. 2008). However, because current PPCP environmental levels are lower than the threshold effect level for the classical ecotoxicological acute assays (Ferrari et al. 2003) and because aquatic organisms are exposed to low concentrations during their entire life span, chronic effects are much more probable (Crane et al. 2006). Studies on chronic toxicity induced by PPCPs are increasing in a variety of biological models, even if these data are lacking for several compounds (Carlsson et al. 2006) and species. To enlarge the amount of knowledge on this topic, our recent investigations highlighted notable sublethal effects induced by short-term exposures to five common PPCPs: triclosan (TCS; Binelli et al. 2009a), trimethoprim (TMP; Binelli et al. 2009b), paracetamol (PCM; Parolini et al. 2010), diclofenac (DCF; Parolini et al. 2011a), and ibuprofen (IBU; Parolini et al. 2011b) on the freshwater bivalve *Dreissena polymorpha*. TCS is an antibacterial agent used in several health care products, whereas TMP is an antibiotic with potent microbicidal activity against a wide variety of bacterial species. DCF, IBU, and PCM have been reported as three of the most frequently used nonsteroidal anti-inflammatory drugs (NSAIDs) worldwide. Investigations were focused on these specific therapeutics because they are extensively used, represent their pharmaceutical groups, and are among the most detected PPCPs in the aquatic environment. In fact, NSAIDs and antibiotics are the most relevant therapeutic classes in terms of environmental contamination because they represent, respectively, 16 and 15 % of drugs detected in

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monitoring surveys worldwide (Santos et al. 2010). Moreover, their notable and increasing environmental levels are causing growing concern about their possible hazard toward the aquatic ecosystems. Even if data from the studies mentioned previously were useful to identify the adverse effects caused by the tested PPCPs, simple analysis of the changes in different physiological parameters generally prevent us from obtaining a correct evaluation of overall changes in the health status of organisms induced by pollutant exposure (Sforzini et al. 2011) and to accurately rank the toxicity of different molecules because of the wide variability in biomarker responses. Thus, the application of approaches integrating responses from biomarker analyses into a synthetic index can allow an easier interpretation of complex ecotoxicological data (Moore et al. 2004; Sforzini et al. 2011). For instance, an algorithm to integrate and explain biomarker data from the bivalve *Mytilus* spp. was recently developed, and it was proven to be a reliable and easy-to-use tool to rank the levels of pollutant-induced stress in mussels (Dagnino et al. 2007; Franzellitti et al. 2010). In addition, Hagger et al. (2008) applied an integrated biomarker response index (BRI) to evaluate the health status of *M. edulis* from 10 United Kingdom estuaries and to assess the influence of seasonality on biomarker responses in the same species (Hagger et al. 2010). However, until now these approaches were exclusively used in field studies to discriminate the pollution of different areas (Hagger et al. 2008, 2010) and/or in laboratory experiments to investigate dose-dependent changes in organism health status after chemical exposure (Sforzini et al. 2011). Therefore, the aim of this conclusive study was (1) to investigate in-depth the toxicity of a realistic concentration (1 nM) of TCS, TMP, DCF, IBU, and PCM—which previous studies have largely omitted—toward zebra mussel specimens; and (2) to integrate the wide biomarker data set obtained from 1 nM PPCP exposures into a BRI to draw an appropriate scale of toxicity and to identify the PCPP(s) that requires more in-depth investigation.

Materials and Methods

Because mussel sampling, acclimation, and laboratory maintenance conditions were described in detail elsewhere (Binelli et al. 2009a, b; Parolini et al. 2010, 2011a, b), they are only briefly explained here. Zebra mussel specimens were sampled in the Lake Lugano, transferred to the laboratory in bags filled with lake water, and kept in depuration under controlled laboratory conditions. Exposure assays were performed under semistatic conditions for 96 h. To give remarkable ecological relevance to our research and to compare the effects of each single drug, bivalves were exposed to a concentration (1 nM) similar for each PCPP, which reflected the mean of the concentrations measured in surface waters worldwide. In detail, we exposed zebra mussels to 290 ng/L TCS, 290 ng/L TMP, 318 ng/L DCF, 200 ng/L IBU, and 154 ng/L PCM, respectively. Exposure procedures and biomarker methods have been thoroughly described in the studies previously mentioned and are briefly reported in Supplementary Materials.

BRI

We calculated a BRI in accordance with previous approaches by Hagger et al. (2008and 2010) with slight modification. The changes in individual biomarker values over a stress gradient yield characteristic trends, such as increasing, decreasing, or bell-shaped response profiles (Hagger et al. 2010). For instance, genotoxic biomarkers usually follow an increasing trend and the NRRA a decreasing one, whereas enzymatic activity shows a bell-shaped curve. Hence, this knowledge on biomarker trends, combined with baseline data, was used to calculate the percentage alteration level (AL) as the % deviation of treated sample mean values from that of the control. Because for each tested biomarker, no significant differences ($p > 0.05$) were noted among levels from specimens in control aquaria during 96-hour tests and the corresponding levels at the beginning ($t = 0$ h) of each single exposure, we considered the $t = 0$ h value as the control for each biomarker. We calculated the % AL for each biomarker and time of exposure. Then a range of biomarker responses was divided into four categories, to which was assigned a specific score. Similar to Dagnino et al. (2007), biomarker responses that showed small differences (±20 %) with respect to the corresponding control, although statistically significant, were not considered of biological relevance and were assigned a score of 1. Deviation from control ranging from ±20 % to ±50 % were assigned a score of 2 because these changes can indicate the first physiological responses of the organism, whereas large differences from baseline levels (±50–100 %), showing notable alterations induced by stressors, were assigned a score of 3. Finally, deviations that largely overcame the corresponding recorded baseline levels (> ±100 %) were scored with 4. Because the enzyme activity usually follows a bell-shape trend and its decrease after the achievement of the maximum expression means a situation of high stress, when an enzyme follow a clear bell-shape trend, we decided to calculate the alteration of enzyme activity with respect to the highest obtained value. Then we scored the AL in the same way described previously. In addition, each biomarker was also weighted according to its level of biological organization: According to Hagger et al. (2010), the cellular biomarkers

included in our suite (single-cell gel electrophoresis assay, micronucleus [MN] test, DNA diffusion assay, and NRRA) were weighted as 2, whereas molecular assays [activities of super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione s-transferase (GST)] were weighted as 1 because it is assumed that an alteration at the cellular level will have a greater impact on the health of the organisms than changes at the molecular level. The final BRI value after 96 h of exposure for each tested PPCP was then calculated using the following equation:

$$\mathrm{BRI} = \sum((\mathrm{biomarker_n score\ at\ t} = 0 + \ldots.. + \mathrm{biomarker_n score\ at\ t} = 96) * \mathrm{biomarker_n weighting}) / \sum \mathrm{biomarker_n\ weighting}),$$

where $_n$ is the measured biomarker. The BRI was then used to discriminate the PPCP that could most powerfully alter the health status of zebra mussel and to accurately rank the PPCPs' toxic potential in an accurate scale of toxicity.

Statistical Analysis

Data normality and homoscedasticity were verified using Shapiro–Wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA) was performed to investigate possible time-effect relationships using time as a variable, whereas biomarker end points served as cases. ANOVA was performed between treated samples and related control and followed by Bonferroni post hoc test to evaluate significant differences ($p < 0.05$) among the adverse effects induced by each single PPCP during the

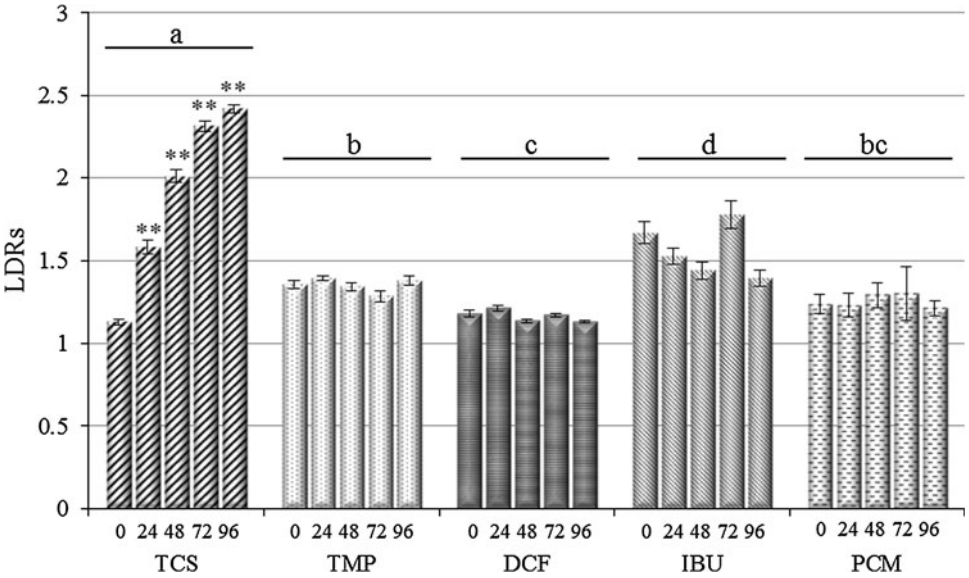

Fig. 1 Primary DNA damage expressed by the length/diameter ratio (LDR, mean values ± SEM). Measurements employed the zebra mussel hemolymph samples ($n = 10$) exposed to 1 nM of selected drugs. Significant differences (two-way ANOVA, Bonferroni post hoc test; $^{**}p < 0.01$) were noted on comparison between treated mussels and the correspondent control. Different *letters* above histograms indicate significant differences among PPCP treatments ($p < 0.01$)

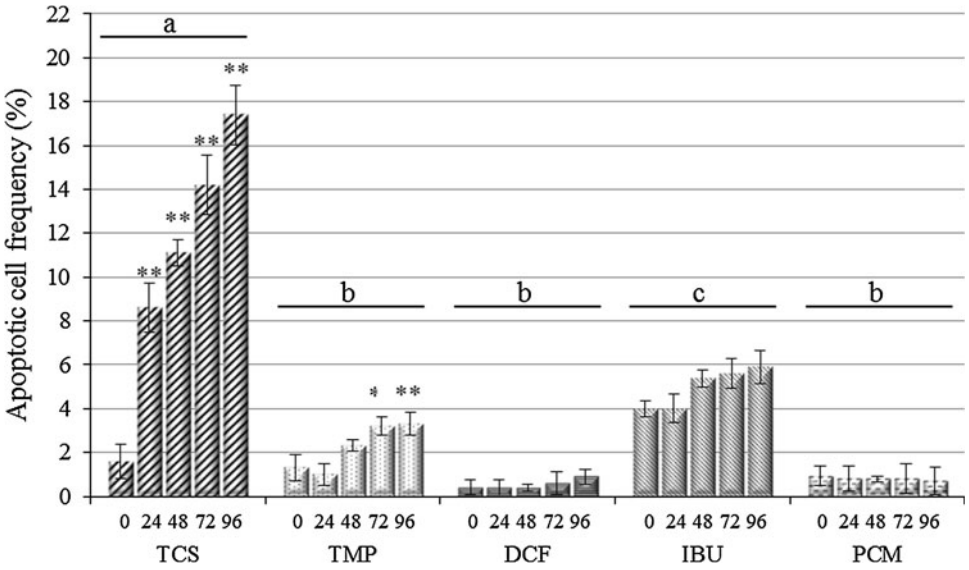

Fig. 2 Percentages of apoptotic hemocytes (mean values ± SEM) measured by the DNA diffusion assay ($n = 5$) in *D. polymorpha* specimens exposed to 1 nM of selected drugs. Significant differences (ANOVA, Bonferroni post hoc test; $^{**}p < 0.01$) were noted on comparison between treated mussels and the correspondent control. Different *letters* above the histograms indicate significant differences among PPCP treatments ($p < 0.01$)

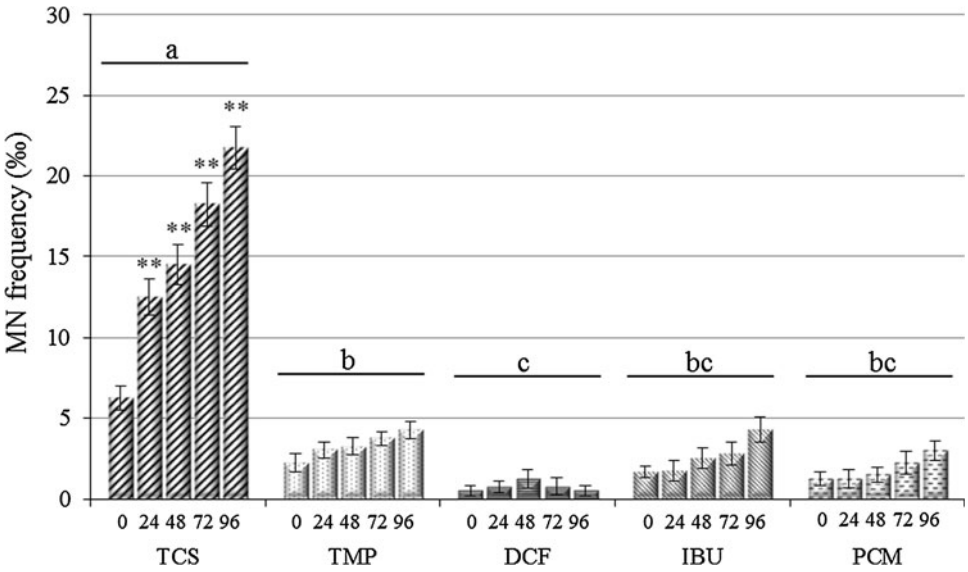

Fig. 3 Frequency of micronucleated hemocytes (mean values ± SEM) measured by the MN test ($n = 10$) in *D. polymorpha* specimens exposed to 1 nM of selected drugs. Significant differences (two-way ANOVA, Bonferroni post hoc test; $^{**}p < 0.01$) were pointed out on comparison between treated mussels and the correspondent control. Different *letters* above the histograms indicate significant differences among PPCP treatments ($p < 0.01$)

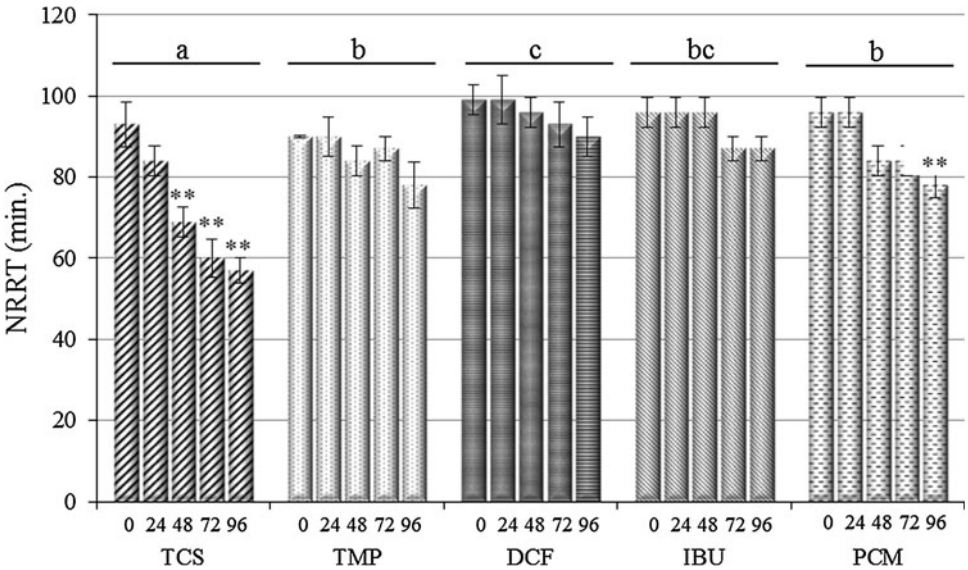

Fig. 4 Mean NRRT of neutral red loss (mean values ± SEM) from lysosomes in hemocytes of zebra mussels ($n = 5$) exposed to 1 nM of selected drugs. Significant differences (ANOVA, Bonferroni post hoc test; $^{**}p < 0.01$) were noted on comparison between treated mussels and the correspondent control. Different letters above the histograms indicate significant differences among PPCP treatments ($p < 0.01$)

exposures. In addition, factorial ANOVA, followed by Bonferroni post hoc test, was performed to identify significant differences ($p < 0.05$) among PPCP-induced effects for each measured biomarker. All statistical analyses were performed using STATISTICA 7.0 software (Statsoft Inc., USA).

Results

Control values of DNA fragmentation (LDR from the SCGE assay; Fig. 1), apoptosis (DNA diffusion assay; Fig. 2) MN test (Fig. 3), and NRRA (Fig. 4) measured for each PPCP were perfectly comparable among each other, with the exception of MN values obtained in the TCS exposure, which could reflect a different physiological status of bivalves due to a different sampling period. However, all baseline levels were similar to those measured in previous studies (Binelli et al. 2008a, b) and were included in the physiological range of this bivalve species. Baseline levels of enzymatic activity in mussels exposed to PPCPs were comparable among each other, with the exception of those measured in bivalves exposed to TCS (Table 1). No significant differences ($p > 0.05$) were noticed among levels from specimens in control aquaria during 96-hour tests and the corresponding levels at the beginning ($t = 0$ h) of each single exposure. TCS was able to induce marked primary lesions to hemocyte DNA already after only 24 h of exposure ($p < 0.01$) and followed a significant time-dependent relationship ($F = 292.17$; $p < 0.01$). The other therapeutics did not cause any significant ($p > 0.05$) increase of DNA primary damage

Table 1 SOD, CAT, GPx, and GST activity (mean values ± SEM) measured in pools of zebra mussel soft tissue ($n = 3$) after exposure to 1 nM of each single PPCP

PPCP	Time (h)	SOD U/mg protein	CAT mM/min/mg protein	GPx μM/min/mg protein	GST μM/min/mg protein
TCS[a]	$t = 0$	6.720 ± 0.370	0.092 ± 0.005	5.262 ± 0.484	0.175 ± 0.012
	$t = 24$	8.002 ± 0.741	0.095 ± 0.001	5.559 ± 0.373	**0.241 ± 0.011****
	$t = 48$	7.703 ± 0.693	0.091 ± 0.003	**7.109 ± 0.250****	**0.268 ± 0.009****
	$t = 72$	7.256 ± 0.412	**0.069 ± 0.002****	**7.310 ± 0.182****	**0.212 ± 0.014***
	$t = 96$	9.019 ± 0.328	0.078 ± 0.015	**7.431 ± 0.417****	**0.213 ± 0.010***
TMP[b]	$t = 0$	21.050 ± 1.270	0.252 ± 0.014	16.816 ± 0.650	0.401 ± 0.017
	$t = 24$	19.826 ± 1.394	**0.152 ± 0.006****	**13.054 ± 0.653****	**0.467 ± 0.010***
	$t = 48$	19.315 ± 1.199	0.238 ± 0.011	**14.127 ± 0.584***	**0.540 ± 0.016****
	$t = 72$	**13.680 ± 0.705***	0.223 ± 0.004	**12.907 ± 0.366****	**0.486 ± 0.009****
	$t = 96$	**8.235 ± 0.553****	0.269 ± 0.007	**12.275 ± 0.428****	0.411 ± 0.007
IBU[b]	$t = 0$	13.264 ± 0.554	0.070 ± 0.003	14.733 ± 0.275	0.208 ± 0.004
	$t = 24$	14.130 ± 1.297	0.060 ± 0.011	**38.337 ± 0790****	0.250 ± 0.010
	$t = 48$	17.046 ± 2.247	0.088 ± 0.006	**34.729 ± 2.362****	**0.359 ± 0.030****
	$t = 72$	13.922 ± 0.564	0.064 ± 0.008	**23.634 ± 0.640***	0.239 ± 0.003
	$t = 96$	**22.758 ± 2.022****	**0.098 ± 0.010****	**33.073 ± 1.453****	**0.316 ± 0.011****
DCF[b]	$t = 0$	13.432 ± 1.559	0.134 ± 0.011	25.004 ± 0.401	0.565 ± 0.057
	$t = 24$	14.941 ± 1.079	0.141 ± 0.014	23.276 ± 0.867	**0.725 ± 0.030****
	$t = 48$	17.058 ± 1.081	0.122 ± 0.013	**20.595 ± 0.444****	0.569 ± 0.005
	$t = 72$	16.358 ± 0.740	0.120 ± 0.006	23.817 ± 0.499	**0.429 ± 0.001***
	$t = 96$	15.199 ± 1.020	0.169 ± 0.002	**15.739 ± 0.470****	**0.433 ± 0.003***
PCM[c]	$t = 0$	14.852 ± 1.615	0.177 ± 0.010	10.944 ± 1.001	0.484 ± 0.014
	$t = 24$	**33.498 ± 1.991****	0.192 ± 0.012	12.025 ± 0.552	0.432 ± 0.031
	$t = 48$	23.780 ± 1.763	0.170 ± 0.012	14.232 ± 1.024	0.444 ± 0.028
	$t = 72$	15.529 ± 1.182	0.185 ± 0.010	**17.737 ± 1.419****	0.518 ± 0.031
	$t = 96$	11.958 ± 0.944	0.173 ± 0.007	**21.079 ± 1.525****	0.415 ± 0.027

Different letters after drug names indicate significant differences ($p < 0.01$) among PPCP treatments

Significant differences (ANOVA, Bonferroni post hoc test; *$p < 0.05$; **$p < 0.01$) were noted on comparison between treated mussels and the correspondent control

(Fig. 1). Similarly, TCS induced a significant time-dependent ($F = 105.90$; $p < 0.01$) increase of apoptotic cell frequency, with 96-hour values being sixfold greater than the corresponding control (Fig. 2). Significant time-dependent ($F = 18.02$; $p < 0.01$) increases of apoptotic frequency were also noticed after 72 h of exposure to 1 nM TMP, whereas the selected concentration of each NSAID did not trigger the apoptotic process. A significant ($p < 0.01$) increase of micronuclei frequency was noticed also after TCS exposure, with values doubled with respect to the control as early as 24 h of exposure (Fig. 3). Even if a slight time-dependent trend of icronuclei frequency was found during TMP ($F = 3.96$; $p < 0.05$), IBU ($F = 2.69$; $p < 0.05$), and PCM ($F = 2.76$; $p < 0.05$) exposures, no significant ($p < 0.05$) increase with respect to the control was found. Data obtained by the NRRA (Fig. 4) highlighted a progressive time-dependent ($F = 15.86$; $p < 0.05$) decrease of the stability of lysosome membranes after TCS treatment, showing at $t = 96$ h a membrane destabilization 39 % greater than at $t = 0$ h. At the end of exposure to PCM, a significant ($p < 0.01$) time-dependant decrease of neutral red retention time (NRRT) was found ($F = 6.18$; $p < 0.01$). In contrast, although significant time-dependencies were noticed for TMP ($F = 2.76$; $p < 0.05$) and IBU ($F = 2.68$; $p < 0.05$), no significant NRRT decrease ($p > 0.05$) in treated bivalves compared with the control was measured during the exposure period. Table 1 lists the results of the enzyme activities measured in zebra mussel soft tissues during exposures to the considered PPCPs. Slight imbalances of SOD and CAT activity were found after exposure to all of the tested therapeutics. TMP induced a significant time-dependent ($F = 13.95$; $p < 0.05$) decrease of SOD activity, with values being 2.5-fold lower than those at $t = 0$ h. In contrast, IBU significantly ($p < 0.01$) increased enzyme activity at the end of the exposure, according to a significant

time-dependent relationship ($F = 6.15$; $p < 0.05$). Significant time-dependent increases of GPx levels were noticed in TCS ($F = 10.46$; $p < 0.05$), IBU ($F = 29.91$; $p < 0.05$), and PCM ($F = 12.83$; $p < 0.05$) exposures, with values being ≤2.7-fold greater than in control mussels measured at $t = 24$ h subjected to IBU treatment. In contrast, a significant time-dependent decrease was induced by TMP ($F = 10.03$; $p < 0.05$) and DCF ($F = 41.24$; $p < 0.05$). Last, a significant ($p < 0.01$) floating alteration of GST was noticed after exposures to TCS, TMP, IBU, and DCF, whereas PCM did not affect the activity of this detoxifying enzyme.

Discussion

The discussion of biomarker data obtained by our previous studies mainly focused on results from the highest exposure concentrations because they caused the most remarkable effects to treated-mussels (Binelli et al. 2009a, b; Parolini et al. 2010, 2011a, b), thus allowing us to suppose the drug mechanism of action in bivalves. However, reconsidering our findings, we can also state that the effects induced by concentrations of TCS, TMP, PCM, DCF, and IBU similar to those currently found in surface water worldwide cannot be neglected, thus confirming the hazard of tested drugs as well as the sensitivity of biomarker techniques and their usefulness in pharmaceutical-risk assessment. Slight variations in cellular and genetic end points were caused by 96-hour exposure to 1 nM TMP, DCF, IBU, and PCM, suggesting low risk for zebra mussels. In contrast, more alarming adverse effects were induced by TCS because significant ($p < 0.01$) increases of cyto-genetic damage, as well as relevant imbalances of enzymatic activities, were noticed after only 24 h of exposure. These findings pointed to TCS as the most toxic among tested PPCPs. Significant differences (factorial ANOVA, Bonferroni post hoc test; $p < 0.05$) between adverse effects induced by TCS and all of the other drugs, with the exception of SOD and CAT, were found for all of the measured end points. Moreover, by considering the whole biomarker response, the toxicity of this antibacterial was significantly different ($p < 0.01$) from that of other therapeutics. Notwithstanding, it is not easy to accurately rank the toxicity of the other PPCPs by simple interpretation of biomarker results. Overall, by considering the entire suite of investigated end points, TMP toxicity was significantly ($p < 0.01$) different from that of DCF and IBU, whereas it was similar to that of PCM. It is also interesting to note that within the same therapeutic class (NSAIDs), responses to treatments were notably different. With the exception of the length/diameter ratio (LDR) parameter (Fig. 1), the other end points showed different changes from basal levels, as well as different trends, depending on the administered drug. For instance, IBU treatment induced significant increases in MN and apoptotic frequencies (Fig. 3), whereas DCF and PCM did not cause fixed genetic damage. Similarly, IBU significantly increased the activity of all of the antioxidant enzymes and GST, whereas significant ($p < 0.01$) decreases of GPx and GST were induced by DCF and variations in SOD and GPx by PCM (Table 1). Notwithstanding, even if a wide variability in the NSAID-induced responses was found among single biomarkers, no significant ($p > 0.05$) differences in their overall toxicity were noticed. When considering only biomarker data, IBU seems to be the second most toxic molecule, followed by TMP, whose toxicity seems to be similar to that of PCM. However, although it is widely known that the assessment of several biomarkers is the best approach to the understanding of adverse effects and mechanism of action of pollutants on organism (Viarengo et al. 2007; Sforzini et al. 2011), the simple examination of the simultaneous changes of dissimilar biological parameters is not enough to rank the hazard of different pollutants because of the remarkable variability in biomarker responses. The application of procedures able to integrate the biomarker responses within a simple synthetic index could help to minimize variation of responses (Hagger et al. 2006), to draw an accurate scale of toxicity of PPCPs, and to obtain useful information for environmental risk assessment. Our specific BRI computed on measured end points in zebra mussel specimens under our experimental conditions (Fig. 5a) efficiently decreased the large degree of variability of responses, confirmed TCS as the compound that most affects bivalve health status, and allowed a more accurate hazard ranking of the other tested drugs to zebra mussel as follows: TMP > IBU > DCF = PCM. It is interesting to note that the BRI approach overturned the PPCP scale of toxicity based on simple biomarker interpretation and statistical analysis, thus identifying that TMP affects mussel health status more than IBU. Moreover, the BRI-based ranking highlighted differences in the hazard of tested NSAIDs, however, whilst statistical analysis, in fact, did not show any difference both in single end points and whole toxicity among NSAIDs, the BRI highlighted that IBU toxicity can be considered greater than that of DCF and PCM. The highest toxicity of TCS with respect to the other PPCPs could be related to its lipophilic feature (log $K_{ow} = 4.76$; Kim et al. 2009) and agreed-on comparative results on the toxicity of different PPCPs (Yang et al. 2008; Kim et al. 2009). However, excepting TCS, our BRI-based scale of toxicity partially disputed the theory according to which the toxicity of a molecule is directly correlated with its log K_{ow}. Even if TMP is a hydrophilic chemical (log $K_{ow} = 0.91$; Bendz et al. 2005), in fact, it was the second most toxic molecule among the tested ones, showing a greater toxicity

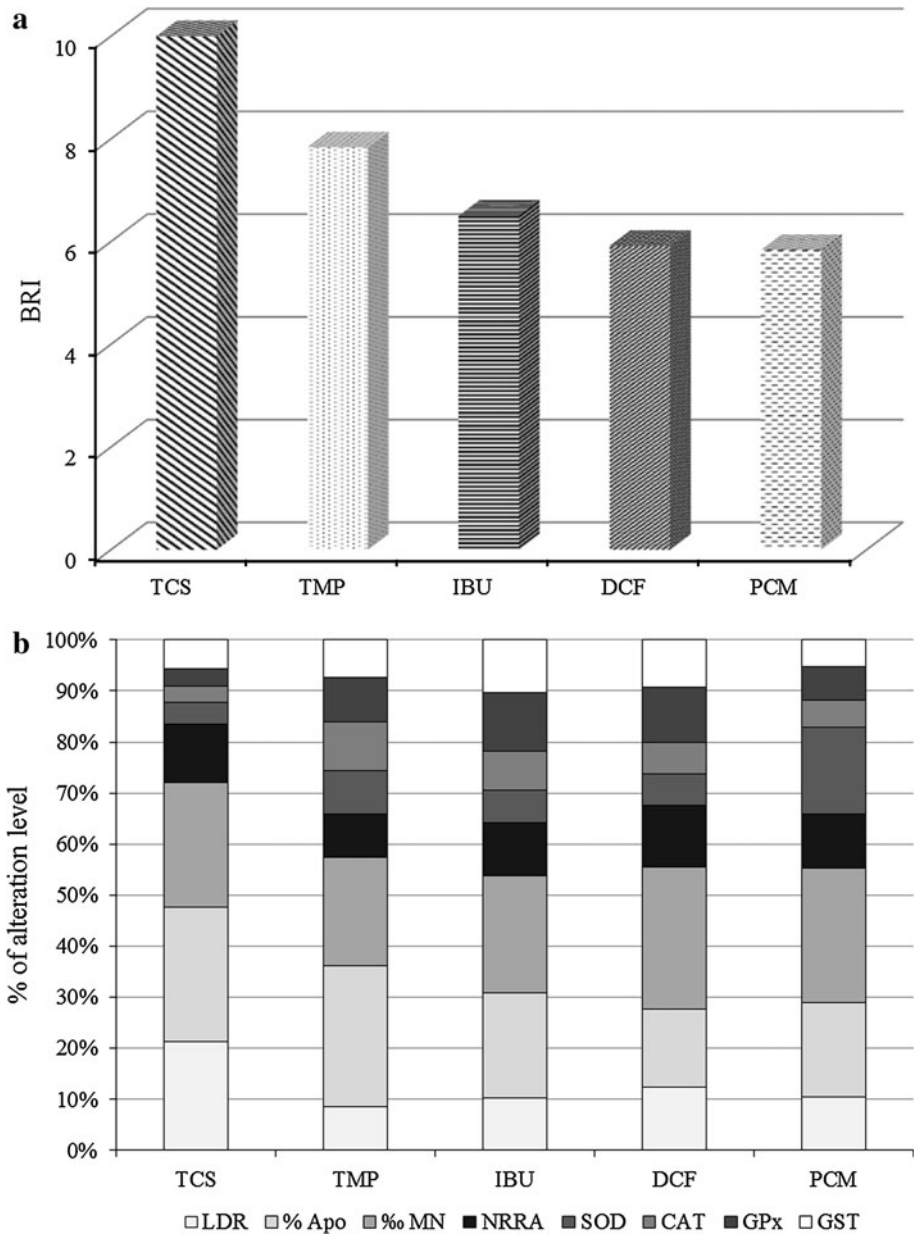

Fig. 5 **a** BRI value calculated for exposure to 1 nM of each tested PPCP. **b** Percentage AL level indicates the contribution of each single biomarker to the total BRI

with respect to IBU (log K_{ow} = 3.97; Kim et al. 2009) and, above all, to DCF (log K_{ow} = 4.4; Cleuvers 2004). DCF's low toxicity was absolutely unexpected because several evidences of its marked hazard toward aquatic vertebrates are available in the scientific literature (Triebskorn et al. 2004; Hong et al. 2007) such that it was recently included in the new revised list of priority substances by the European Commission (2012). The toxicity of tested NSAIDs to zebra mussel also disputed findings by Cleuvers (2004), who found that the acute toxicity of DCF was 1.5- and fivefold greater than IBU in *D. magna* and *D. subspicatus*, respectively. In addition, our BRI-based NSAIDs scale of toxicity was also in reverse order with respect to the in vitro scale based on data from four biomarkers applied to zebra mussel hemocytes (Parolini et al. 2009). This opposite toxic behavior could be due to differences in experimental approaches because the in vitro bioavailability of drugs could be different from that in vivo, and the sensitivity of single cells could be different from that of the entire organism. In vivo exposures, in fact, accurately mimic the real environmental situation by also taking in consideration the potential effects of metabolic byproducts, which are often more toxic than parental compounds. Hence, it is important to bear in mind that the BRI-based scale of toxicity was much more accurate and trustworthy than the previous in vitro scale because it was built on in vivo data reflecting the real health status of the organism. Moreover, the BRI considered the responses of a suite

of eight different assays, weighting each of them according to their level of biological targets. When the set of biomarkers is relatively large, e.g., six to eight, the weight of one factor is markedly decreased compared with cases in which three to four biomarkers are used because the number of biomarkers included in the calculation plays an important role affecting the ''relative weight'' of each biomarker in the final index value (Broeg and Lehtonen 2006). For instance, by calculating the BRI only on genotoxic data obtained in the present study, the NSAIDs toxicity scale becomes similar to the in vitro one: PCM $\approx$ IBU $>$ DCF. In addition, by applying a specific weight to each biomarker according to its level of biological target, we avoided undervaluing or overvaluing the contribution of a specific adverse effect and, subsequently, the real hazard of a compound. By considering the non-weighted alteration from the baseline level for each single biomarker, we determined that the main contribution to TCS toxicity was genetic damage (64 % of the AL), whereas cytotoxic damage was lower (36 %). In contrast, other PPCPs mainly caused cellular damage to zebra mussel because NRRA and enzyme activity alterations accounted for 54–60 % of total AL, whereas genetic damage was lower (40–46 %). By including the weight of biomarkers into the BRI calculation, we completely reconsidered our data (Fig. 5b). In fact, TCS-induced DNA damage accounted for the 73 % of the total BRI, whereas for the other PPCPs, genetic damage was much more important, accounting for 51–65 % of the BRI, with the contribution of cytotoxic damage being only 41–46 % of the BRI.

Conclusion

The integration of biomarker data into a simple BRI confirmed TCS as the most problematic tested PPCP for zebra mussel and allowed an accurate classification of the toxicity of other drugs, thus limiting the response variability of applied biomarkers. However, even if our BRI scale of toxicity gave useful information on the toxicity of selected PPCPs, it could be improved by including other biomarker data. In fact, by evaluating other kind of effects at different levels of biological organization, such as physiological alterations, the information on PPCP toxicity should be strengthened and the toxicity ranking even more accurate. Last, it is important to bear in mind that indices such as the BRI are oversimplifications of a complex exposure scenario and also of the multiple physiological responses in the target organisms. Therefore, the results obtained by using integrated indices should be taken as (1) useful tools to formulate an exhaustive and preliminary risk assessment of PPCPs as well as (2) further actions in the attempt to investigate the hazard of dangerous molecules as well as to be used as basis for environmental management. In conclusion, based on these considerations, broader information on PPCPs ecotoxicity must be a priority in environmental-risk assessment. This is particularly true for TCS, whose induced sublethal effects, mechanism of action for aquatic organisms, and effects on population dynamics, must be investigated to clarify its real ecological hazard for the aquatic ecosystems.

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PAPER III

Binelli Andrea, **Pedriali Alessandra,** Riva Consuelo, Parolini Marco

ILLICIT DRUGS AS NEW ENVIRONMENTAL POLLUTANTS: CYTO-GENOTOXIC EFFECTS OF COCAINE ON THE BIOLOGICAL MODEL *Dreissena polymorpha*.

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Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha*

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ABSTRACT

The increase in global consumption of illicit drugs has produced not only social and medical problems but also a potential new environmental danger. Indeed, it has been established that drugs consumed by humans end up in surface waters, after being carried through the sewage system. Although many studies to measure concentrations of several drugs of abuse in freshwater worldwide have been conducted, no data have been available to evaluate their potentially harmful effects on non-target organisms until now. The present study represents the first attempt to investigate the cyto-genotoxic effects of cocaine, one of the primary drugs consumed in Western Countries, in the biological model *Dreissena polymorpha* by the use of a biomarker battery. We performed the following tests on Zebra mussel hemocytes: the single cell gel electrophoresis (SCGE) assay, the apoptosis frequency evaluation and the micronucleus assay (MN test) for the evaluation of genotoxicity and the lysosomal membranes stability test (neutral red retention assay; NRRA) to identify the cocaine cytotoxicity. We exposed the molluscs for 96 h to three different nominal concentrations in water (40 ng L^{-1}; 220 ng L^{-1}; and 10 μg L^{-1}).

Cocaine caused significant ($p < 0.05$) primary DNA damage in this short-term experiment, but it also caused a clear increase in micronucleated cells and a marked rise in apoptosis, which was evident in samples from even the lowest environmental cocaine concentration. Because cocaine decreased the stability of lysosomal membranes, we also highlighted its cytotoxicity and the possible implications of oxidative stress for the observed genotoxic effects.

1. Introduction

In the last decades, the scientific community has focused on new types of environmental contaminants, such as pharmaceuticals and personal care products (PPCPs) and drugs of abuse due to their possible dangerous effects on non-target organisms. In particular, the use and abuse of illicit drugs has increased dramatically within Western Counties in particular, matching the consumption levels of pharmaceuticals used for therapeutic purposes (Santos et al., 2009). The recent World Drug Report (UNODC, 2010) estimates that between 155 and 250 million people (3.5–5.7% of the population aged 15–64) used illicit substances at least once in 2008. Globally, cannabis users comprise the largest number of illicit drug users (129–190 million people). Amphetamine-group substances rank as the second most commonly used drugs, followed by cocaine and opiates. In Europe, cocaine remains the second most used illicit drug after cannabis. The annual prevalence of cocaine use in Europe ranges from 0.8% to 0.9% of the population aged 15–64, or approximately 4.5–5 million people who reportedly used cocaine in 2008/2009 (UNODC, 2010).

Humans excrete illicit and therapeutic drugs and their metabolites, and these compounds end up in sewage treatment plants (STPs) where they are often partially removed (Zuccato et al., 2008; Domènech et al., 2009). Several studies have demonstrated the presence of drugs of abuse not only in STPs worldwide (Zuccato et al., 2000; Postigo et al., 2010) but also in the receiving surface waters, which may have implications for humans and wildlife (Pomati et al., 2006). Cocaine concentrations in European surface waters range from <LOQ (limit of quantification) to hundreds of ng L^{-1} (Castiglioni et al., 2006; Gheorghe et al., 2008; Van Nuijs et al., 2009; Kasprzyk-Hordern, 2010). Zuccato and co-workers (2005) showed that kilograms of cocaine residue travel down the River Po, the main river in Italy, on a daily basis. Cocaine, morphine, ecstasy and methamphetamines possess very powerful pharmacological effects, and their presence in surface waters could produce unforeseeable interactions with other drugs and pharmaceuticals (Zuccato et al., 2008).

Although some researches have focused on the development of reliable methodologies to measure illicit drug concentrations in aquatic environments, no data are available on the acute or chronic effects of these drugs on the aquatic community.

We evaluated possible cyto-genotoxic effects of cocaine (benzoylmethylecgonine) exposure in the mollusk Zebra mussel (*Dreissena polymorpha*), which is a widespread aquatic organism

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in Europe and North America. We maintained mussels in a controlled laboratory setting and exposed them to three different cocaine concentrations (40 ng L^{-1}; 220 ng L^{-1}; 10 μg L^{-1}) for 96 h to investigate the short-term effects of the drug. Notably, several other chemicals have produced cyto-genotoxic damage within the same period of exposure (Riva et al., 2007; Binelli et al., 2008a,b, 2009; Parolini et al., 2010).

This study is the first of a wider body of research designed to evaluate the role of not just cocaine but also its primary metabolites, benzoylecgonine and ecgonine methyl ester, as new environmental contaminants. We chose to test cocaine first because not only it is the parent compound, but it has also been reported to have cytogenotoxic effects in model organisms that are used to investigate mechanisms of drug action in humans. Stefanidou et al. (2002) observed a stimulation of the mitotic process in the protozoan *Tetrahymena pyriformis*, which due to the subsequent stimulation of the DNA synthesis indicates a mitogenic effect of cocaine products. Cocaine also inhibits DNA synthesis throughout the entire adult rat brain and in the developing rat brain (Anderson-Brown et al., 1990). Furthermore, cocaine induces apoptosis in the cortical neurons of fetal mice (Nassogne et al., 1997).

We investigated the potential cyto-genotoxicity of cocaine on mussel hemocytes using a biomarker battery. We performed the single cell gel electrophoresis (SCGE) assay, the apoptosis frequency evaluation and the micronucleus assay (MN test) for the genotoxicity evaluation. The lysosomal membrane stability test (neutral red retention assay; NRRA) was used to identify cocaine cytotoxicity in Zebra mussels. To the best of our knowledge, our work represents the first study to evaluate the possible damage produced by a specific drug of abuse in a freshwater biota.

2. Materials and methods

The cocaine standard (purity >99%) was purchased from Alltech Applied Science (State College, United States) while the other reagents used for biomarker measures were purchased from Sigma–Aldrich (Steinheim, Germany). We diluted the acetone stock solution (1 g L^{-1}) to 10 mg L^{-1} in bi-distilled water (work solution), which was then used to obtain the desired cocaine concentration in experimental aquaria.

2.1. Dose selection

Because our study represents the first experiment designed to evaluate the potential dangers of cocaine as an environmental contaminant to aquatic organisms, no data are available regarding cocaine toxicity threshold. We decided to investigate the cyto-genotoxicity of this illicit drug administered at environmental concentrations that mimic true environmental conditions throughout world. Thus, we selected three different doses based on biomonitoring studies recently conducted in Italy and Europe: 40 ng L^{-1} (0.13 nM), 220 ng L^{-1} (0.73 nM) and 10 μg L^{-1} (32.96 nM). The first dose is based on the highest cocaine concentration found in Italian freshwater (Zuccato et al., 2008) while the second dose is the European average of the STP levels (Kasprzyk-Hordern et al., 2008; 2009; van Nuijs et al., 2009). Finally, we calculated the Predicted Environmental Concentration (PEC) as highest dose from data available on River Po basin (Northern Italy) and following the EMEA (European Medicines Evaluation Agency) guidelines (EMEA, 2006). We evaluated a theoretical PEC of about 10 μg L^{-1}.

2.2. Mussel acclimation and maintenance conditions

Several hundred specimens were sampled in the reference site of Lake Lugano (Northern Italy–Switzerland) in which no cocaine (<LOQ) has been found (Castiglioni et al., 2006). Mussels were quickly transferred to the laboratory in bags filled with lake water and introduced into glass aquaria filled with approximately 100 L of dechlorinated tap water. Specimens were maintained on a typical photoperiod (16 h of light and 8 h of dark) with a daylight lamp (3000 lux), 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 2 weeks to purify the molluscs from the possible accumulation of xenobiotics.

Two hundreds fifty specimens with similar shell lengths (approximately 20 mm) were selected for each *in vivo* test, including control assays. Mussels were placed on glass sheets suspended in small glass aquaria (15 L) and maintained for 1 week under the same conditions described above. Only specimens that were able to re-attach themselves to the glass sheets by their byssi were used in the experiments. Mussels were used for the subsequent *in vivo* experiments only when their target biomarkers were comparable to previously established baseline levels.

2.3. Exposure assays

The single previously published paper on cocaine degradation (Postigo et al., 2011) reported that a slight transformation (less than 5%) was observed in distilled water after 22 h of keeping the solution in the dark at room temperature. Moreover, the same authors conducted photolysis experiments in which natural solar photolysis only achieved approximately 22% photo-transformation of cocaine after 60 h of sunlight exposure. Because our experiments were performed in semi-static conditions with daily changes of the entire volume of water and the addition of chemicals in selected doses, the cocaine concentrations in water should have been self-maintained at levels similar to nominal doses.

Mussels were fed daily with AlgaMac-2000®, which was added 2 h before 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, DNA diffusion assay and NRRA, respectively. We evaluated biomarker responses in the Zebra mussel hemocytes because these evaluations require only brief manipulations and very fast preparations. Moreover, hemocytes are quickly recruited during defense and immune reactions and their multifunctional roles render them more sensitive to internal and environmental factors than other cells (Venier et al., 1997).

2.4. Cell treatment and viability

Mussel valves were gently opened using a scalpel, and hemolymph was aspirated from the sinus near the posterior adductor muscle with a hypodermic syringe containing different volumes of phosphate-buffered saline (PBS). The volume of hemolymph recovered per mussel was approximately 100 μL, with a final cell density of approximately 10^7 cells mL^{-1}. Cell viability was checked using the Trypan blue exclusion method (10 μL of cell suspension added to 10 μL of 0.4% dye) immediately after withdrawal or treatment using a Burker chamber. Tests were only performed on cells showing a viability of $\geqslant$80%, according to the *in vivo* Comet test as in Tice et al. (2000).

2.5. Biomarker analyses

A detailed description of all the methodologies conducted for the chosen biomarkers can be found in Binelli et al. (2009). Thus, we report only a brief description of the procedures that were followed. We made one single slide for every mussel from each

aquarium (control and exposure tanks) in order to have several replicates: 10 different replicates for SCGE and MN assays, five replicates for NRRA and apoptosis assay.

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

The percentage of apoptotic cells was evaluated using the same protocol as in the SCGE assay described by Singh (2000). Two hundred cells per slide were analyzed for a total of 1000 cells per sample. In addition, we separately counted the percentage of necrotic cells characterized by circular, faint halos rather than nuclear remnants that resemble pinheads and are surrounded by the very large DNA halos typical of apoptotic cells.

The MN test was performed according to the method of Pavlica et al. (2000). Four hundred cells were counted per slide for a total of 4000 cells treatment^{-1}. Only intact and non-overlapping hemocytes were scored. Micronuclei were identified using the criteria proposed by Kirsch-Volders et al. (2000), calculating the MN frequency (MN‰). The NRRA method was performed according to the protocol proposed by Lowe and Pipe (1994), whose rationale was that healthy cells can take up and retain larger quantities of dye (neutral red) than damaged cells. Lysosomal membrane stability is considered the most reliable of the recommended biomarkers for water quality assessment (UNEP, 1997). Slides were examined systematically at 15 min intervals to determine at what time point 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 dye retention time was then calculated from the five replicates.

2.6. Statistical analyses

Data normality and variance homogeneity were verified using the Shapiro–Wilk and Levene's tests, respectively. We log-transformed LDR values to normalize the variance of 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 testing concentration and exposure times as cases. The ANOVA was followed by a Bonferroni *post hoc* test to evaluate significant differences ($p < 0.05$; $p < 0.01$) between treated samples and controls (time to time) across exposure levels. All statistical analyses were performed using the software package STATISTICA 7.0.

3. Results

No mortality or significant changes in hemocyte counts were recorded in cocaine-treated mussels with respect to controls. Background measures of each biomarker were comparable to those obtained in previous research on Zebra mussels (Pavlica et al., 2001; Bolognesi et al., 2004; Rocher et al., 2006; Riva et al., 2007; Binelli et al., 2008a,b; Parolini et al., 2010). We observed slight primary damage to the DNA of Zebra mussel hemocytes exposed to cocaine for a short period of time (Fig. 1). A clear dose/effect relationship (two-way ANOVA; $F = 14.37$; d.f. = 199; $p < 0.01$) and a significant ($F = 3.4$; d.f. = 199; $p < 0.05$) time/effect correlation were obtained for LDR although we found significant differences ($p < 0.01$) compared with controls only at the end of the low- and high-dose exposures. This primary damage produced by cocaine also caused chromosomal aberrations, as shown by the significant

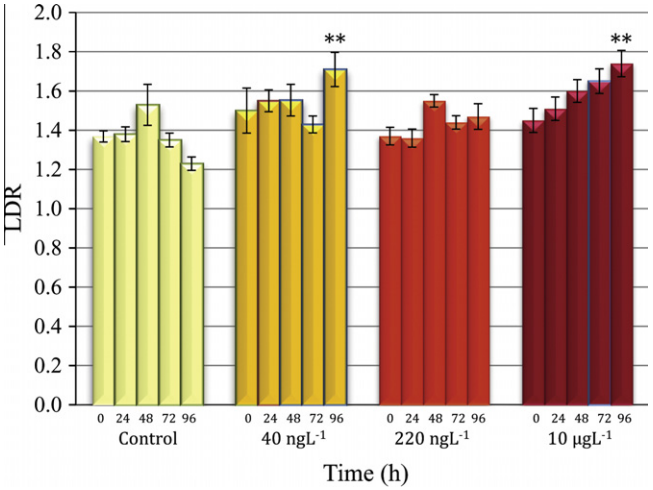

Fig. 1. Results (mean values ± SEM; standard error of the mean) of the SCGE assay expressed by the length/diameter ratio (LDR). Measurements employed Zebra mussel haemolymph samples ($n = 30$) exposed to three different cocaine doses. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

increase ($p < 0.05$) of micronuclei obtained in the highest tested dose after only 72 h of exposure (Fig. 2). Moreover, we found dose/effect ($F = 6.19$; d.f. = 199; $p < 0.01$) and time/effect ($F = 14.23$; d.f. = 199; $p < 0.01$) dependencies for MN frequency.

The largest irreversible effect produced by cocaine exposure was manifested in an increase in the number of apoptotic cells (Fig. 3). We found significant differences ($p < 0.05$) compared with background levels as early as 24 h post-exposure to cocaine in both the intermediate (220 ng L^{-1}) and highest (10 µg L^{-1}) administered concentrations. Approximately 12% of hemocytes were lost to programmed cell death after 72 h of exposure for the calculated TEC, five times higher than controls. The ability of cocaine to produce apoptosis was confirmed by the dose/effect ($F = 98.34$; d.f. = 99; $p < 0.01$) and time/effect ($F = 10.45$; d.f. = 99; $p < 0.01$) relationships. To better evaluate the health status of Zebra mussel specimens, we also measured the percentage of necrotic cells; although no statistical differences were obtained, we noticed an

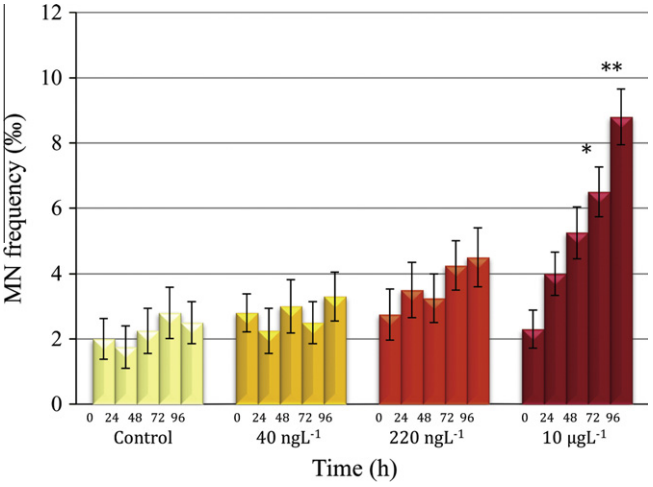

Fig. 2. Frequency of micronucleated haemocytes (mean values ± SEM) observed in *D. polymorpha* specimens ($n = 30$) exposed to cocaine. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

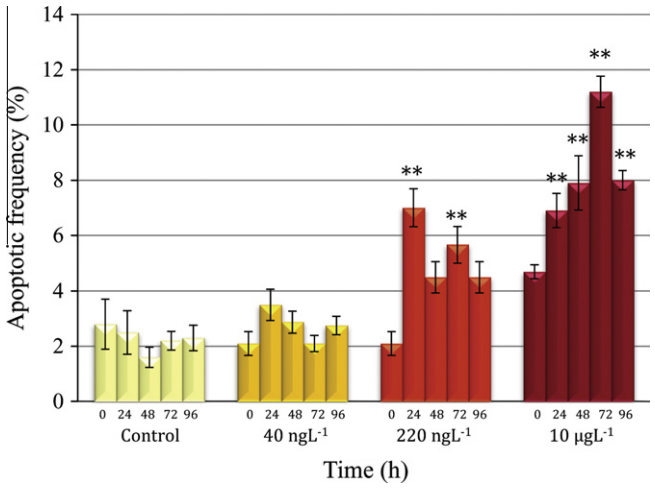

Fig. 3. Percentages of apoptotic haemocytes (mean values ± SEM) measured by the Halo assay in Zebra mussel specimens ($n = 15$) exposed to cocaine. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

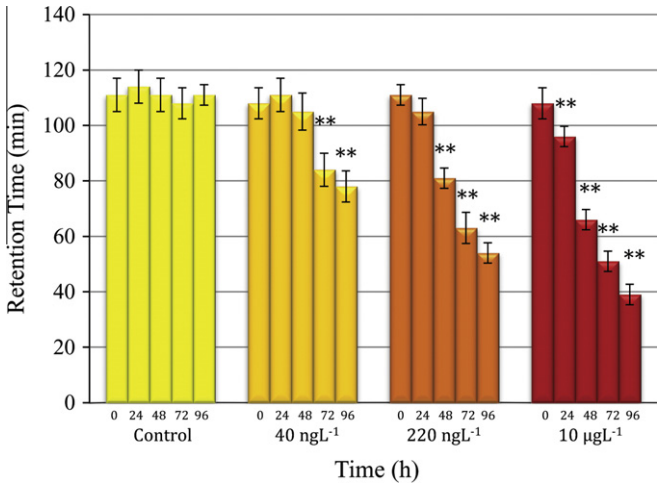

Fig. 5. Mean retention time (RT) of neutral red loss (mean values ± SEM) from *lysosomes in* haemocytes of Zebra mussels ($n = 15$) exposed to cocaine. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

increase in necrosis mainly at the end of the exposure period in the 10 µg L^{-1} group, which was approximately double the level observed 24 h earlier (Fig. 4).

Cocaine is also able to produce significant ($p < 0.05$) cytotoxic effects, as demonstrated by the NRRA results (Fig. 5). This biomarker was the only outcome that showed a dramatic decrease in lysosomal membrane stability ($p < 0.01$) for the lowest tested dose. It is interesting to note that the increase in cocaine concentration corresponded to an earlier decrease in dye retention time: 72 h for 40 ng L^{-1}, 48 h for 220 ng L^{-1} and only 24 h for 10 µg L^{-1}. At the end of exposure, the decreases in dye retention time (RT) reached nearly 30% of the background levels for the lowest dose, 50% for the intermediate dose and 67% for the highest dose.

4. Discussion

Cocaine is generally studied in the context of its effects on human health and in particular as a possible cause of neurological and cardiac damage. For this purpose, thousands of studies are available on mammalian biological models of cocaine exposure in doses similar to those measured in humans. However, these studies are not useful for highlighting the possible dangerous effects of cocaine on aquatic biocoenosis or protecting non-target organisms because the tested doses are much higher than environmental concentrations.

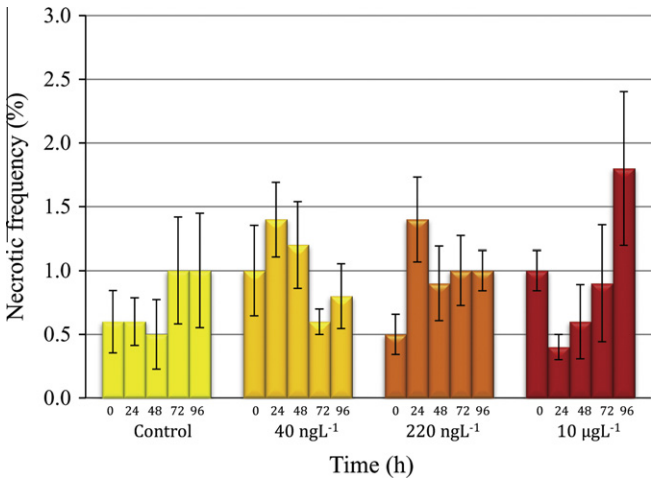

Fig. 4. Percentages of necrotic haemocytes (mean values ± SEM) measured in Zebra mussel specimens ($n = 15$) exposed to cocaine. Significant differences (two-way ANOVA, Bonferroni *post hoc* test, $p < 0.05$) were referred to the comparison between treated mussels and the correspondent control (time to time).

Unfortunately, to date no data have been available on the ecological effects of cocaine. For this reason, the results of our first screening study on the role of illicit drugs as new environmental pollutants cannot be compared with other ecotoxicological data.

The capability of cocaine to increase DNA fragmentation in Zebra mussel hemocytes as soon as 96 h after the onset of exposure (Fig. 1) highlights its possible genotoxicity on aquatic biocoenosis as previously demonstrated in mammalian models. In a recent study, Alvarenga co-workers (2011) showed clear primary DNA damage in mice 1 h after exposure to cocaine but at levels (1.7–7 mg kg^{-1} body weight) much higher than both typical therapeutic doses (0.28 and 0.57 mg kg^{-1} body weight; Goldstein et al., 2009) and those tested in the current paper.

The genotoxic action of cocaine was most evident in the micronucleus assay and specifically in the apoptosis frequency evaluation (Figs. 2 and 3). Indeed, cocaine produced significant structural or numerical chromosome aberrations due to short-term exposure to environmental concentrations and an increasing trend towards hemocytes entering programmed cell death rather than the mitotic divisions that would generate serious DNA damage in daughter cells. We noticed a slight but non-significant decrease in apoptosis at $t = 96$ h for the highest concentration, which could be due to the decreasing health of the entire organism. Indeed, we observed an increase in the number of necrotic cells at the end of exposure to 10 µg L^{-1} (Fig. 4) although it was non-significant, which could potentially suggest the onset of acute effects of cocaine on hemocytes. However, Cuhna-Oliveira and co-workers (2006) have demonstrated that the behavioral changes induced in rat neurons by cocaine are based on the induction of cell death by apoptosis or necrosis.

There is a clear discrepancy between the low levels of DNA fragmentation as measured by the Comet assay (Fig. 1) and biomarkers that measure fixed genetic damage (Figs. 2 and 3); this was especially true for apoptotic frequency. A possible explanation is that the discrepancy is due to the capability of Zebra mussels to repair low-levels of DNA damage, while major injuries (double strand breaks, translocations, DNA adducts or intercalation), that are able

Table 1
Pearson's correlation obtained by using the cytogenotoxic end-points measured for the three tested cocaine concentrations. Significant correlation are indicated in bold.

		LDR	MN frequency	Apoptosis
40 ng L^{-1}	MN frequency	0.17		
	% of apoptosis	0.27	0.07	
	NRRT	−0.08	−0.02	**0.37****
220 ng L^{-1}	MN frequency	0.15		
	% of apoptosis	0.05	0.17	
	NRRT	**−0.29***	−0.27	−0.18
10 μg L^{-1}	MN frequency	**0.34***		
	% of apoptosis	**0.35***	**0.41****	
	NRRT	**−0.52***	**−0.69****	**−0.68****

MN frequency = frequency of micronucleated cells.
% of apoptosis = percentage of apoptotic cells.
NRRT = Neutral red retention time.
* $p < 0.05$.
** $p < 0.01$.

to induce apoptosis and necrosis, can be directly produced by cocaine. Indeed, this drug directly induces the activity of different types of caspases, namely types −2, −3 and −9, which play important roles in apoptosis, necrosis and inflammation (Cunha-Oliveira et al., 2006).

Alternatively, the direct genotoxic action of cocaine could be due to alterations in proteins involved in the complete segregation of the genome. Several protein components, such as mitotic spindle proteins, the protein complex that regulates the separation of sister chromatids during cell division (cohesins) and the proteins of phase G2/M complex, can produce aneuploidy if damaged. This suggestion is supported by several studies conducted in the protozoon *T. pyriformis* (Stefanidou et al., 2011), mouse oocytes (Combelles et al., 2000) and human fetuses (Meyer and Zhang, 2009), in which aneuploidy effects of cocaine were demonstrated.

Both of the hypotheses described above could produce genomic conditions that are not compatible with cellular life, and consequently, both conditions could activate the mechanism for programmed cell death.

The results from our NRRA assay, which was conducted to evaluate the cytotoxicity of cocaine, show clear destabilization of lysosomal membranes for even the lowest administered dose (Fig. 5). Because reactive free-radicals, such as reactive oxygen species (ROS), contribute to the damaging effects on the lysosomal membranes and the intra-lysosomal environment is already a site of oxyradical production (Livingstone, 2001), another possible mechanism of action of cocaine is that it increases oxidative stress. Moreover, strong correlations have been reported between impairment of antioxidant capability, neutral red retention time and changes in DNA integrity in different organisms (Regoli et al., 2004; Mamaca et al., 2005). The cyto-genotoxicity observed in our study could be due to an increase in ROS as a consequence of cocaine biotransformation into N-hydroxy norcocaine and norcocaine nitroxide by Cyp-450 activity (Díez-Fernández et al., 1999). An increase in oxidative stress can produce both direct DNA damage and alterations to protein complexes involved in metabolism of genetic material, inducing apoptotic processes and micronuclei formation. Indeed, the capability of ROS to induce micronuclei as a consequence of cocaine metabolism has been demonstrated by Yu and colleagues (1999) in the ovarian cells of hamsters (*Cricetulus grisou*). In addition, cocaine exposure has been shown to cause an increase in apoptosis in rat hepatocytes, an effect that was correlated with an increase in oxidative stress (Díez-Fernández et al., 1999).

The correlation analyses performed among the different biomarker end-points seems to confirm the possible role of ROS and oxidative stress in genetic damage as a result of cocaine exposure (Table 1). We found a highly significant ($p < 0.01$) correlation among NRRT, apoptotic cell frequency and MN induction beyond significant relationships ($p < 0.05$) for genotoxicity biomarkers. It appears that these different mechanisms of action that are already confirmed in other biological models may also be verified for the Zebra mussel.

5. Conclusion

The results obtained in this study show clear cyto-genotoxic effects of cocaine in a common non-target organism, highlighting a possible danger of illicit drugs for freshwater biocoenosis. Our findings are novel in the field of drug research because until this point, only ecological data related to environmental concentrations and how these contaminants are eliminated by STPs have been available. This research is the first of several investigations we will conduct on the role of illicit drugs as new aquatic pollutants, because a great deal of research needs to be done to achieve a complete view of the ecological implications of cocaine discharge. For instance, it will be crucial to perform in-depth studies on the mechanisms of action of these molecules, because they could be different from those already established in mammalian biological models. Another important area for study will certainly be to evaluate the effects of not only the parent compounds but also the metabolites and combinations of metabolites and their possible impact on metabolic pathways. Our work highlights the effects of cocaine in a short-time experiment ($t = 96$ h) because our goal was exclusively to first identify the possible cyto-genotoxic effects of environmental cocaine concentrations, but it will absolutely critical to carry out studies to evaluate the late effects of long-term exposures. In this context, our research group is performing long-term experiments with cocaine and its main metabolite (benzoylecgonine).

We hope that these encouraging results on the effects of cocaine on non-target organisms will open up new opportunities to investigate the potential environmental risks of these types of contaminants, as has already been done for many pharmaceuticals.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.10.056.

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PAPER IV

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SUB-LETHAL EFFECTS CAUSED BY THE COCAINE METABOLITE BENZOYLECGONINE TO THE FRESHWATER MUSSEL *Dreissena polymorpha*.

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Sub-lethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*

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HIGHLIGHTS

- We investigated sub-lethal effects induced by benzoylecgonine (BE) to *D. polymorpha*.
- Realistic BE concentrations caused notable adverse effects in treated bivalves.
- Significant imbalances of oxidative status was induced by both the treatments.
- Oxidative stress caused remarkable adverse effects to cellular macromolecules.

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ABSTRACT

Illicit drugs have been recognized as emerging environmental pollutants that could represent a potential risk for aquatic communities. Even if many studies have shown the occurrence of several drugs of abuse and their metabolites in freshwaters in the High ng/L to Low μg/L range worldwide, no information on their potentially harmful effects on non-target organisms is available. The aim of this study was to investigate sub-lethal effects induced by the main metabolite of cocaine, the benzoylecgonine (BE), on the freshwater bivalve *Dreissena polymorpha*. Mussels were exposed under semi-static conditions for 14 days to two environmentally relevant BE concentrations (0.5 μg/L and 1 μg/L) and induced adverse effects were evaluated through the application of a suite of ten different biomarkers. We applied on bivalve hemocytes the single cell gel electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test) to investigate DNA injuries, while the neutral red retention assay (NRRA) was used to assess BE cytotoxicity. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities, as well as the lipid peroxidation (LPO) and protein carbonyl content (PCC), were measured as oxidative stress indices in zebra mussel homogenates. Significant decrease in lysosomal membrane stability and imbalances of defense enzyme activities were found at both exposure concentrations, suggesting the involvement of oxidative stress in BE toxicity. Significant increases in LPO and PCC, as well as in primary (DNA strand breaks) and fixed DNA damage (apoptotic and micronucleated cell frequency), were found at the highest BE treatment, confirming that adverse effects to macromolecules were due to the increase of BE-induced oxidative stress.

1. Introduction

The recent World Drug Report has estimated that between 149 and 272 million people (3.3–6.1% of the population aged 15–64) used illicit substances at least once in 2009 (UNODC, 2011). Among these, cannabis was the most used (125–203 million people), followed by amphetamine-group substances, cocaine and opiates. Even if stable or downward trends in use and abuse of the most of drugs mentioned above have been estimated in major regions of consumption (UNODC, 2011), their consumption levels match those of common pharmaceuticals used for therapeutic purposes (Santos et al., 2012). Despite these alarming evidences, up through the 90s, the emerging studies on pharmaceutical compounds in environment (PiE) inexplicably excluded from consideration the contributions by the so-called illicit drugs. This is probably due to the ambiguity in what exactly defines an illicit drug; in fact, even if several of them are illegal, many others are licit medical pharmaceuticals having valuable therapeutic use, such as morphine and oxycodone. Therefore, they must be considered within the PiE category. This is particularly true considering that these molecules share several features with pharmaceutical compounds: even if they belong to structurally diverse group of chemicals, they have extremely high potential for biological effects in humans and non-target organisms, and their pathway to enter the aquatic environment should

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not differ from that of medical and veterinary therapeutics. After the drug dose consumption, in fact, active parent compounds or metabolites are excreted in consumers' urine and feces, as well as in hair and sweat, enter urban wastewater and reach sewage treatment plants (STPs; Castiglioni et al., 2011a,b). Since these substances are often only partially removed by the STPs (Zuccato et al., 2008; Domènech et al., 2009) they pass into surface waters, contributing to the environmental contamination. Notwithstanding, not until the 1999 and 2001 was the scope of concerns surrounding pharmaceuticals in environment to include illicit drugs (Daughton and Ternes, 1999; Daughton, 2001). Only recently, drugs of abuse have been identified as a group of emerging pollutants, attracting the interest of analytical and environmental chemistry (Richardson, 2009; Zuccato and Castiglioni, 2009). An increasing number of monitoring surveys have showed the occurrence of cocaine (CO), amphetamines (AMP), Δ^9-tetrahydrocannabinol (THC), ecstasy (3,4-methylenedioxy-N-methylamphetamine; MDMA), opiates (heroin, morphine and codeine), as well as of their corresponding metabolites, in both STP effluents and surface waters worldwide in ng/L concentrations (Zuccato et al., 2005, 2008; Postigo et al., 2010). Although current environmental levels are quite low, risks for both the human health and the aquatic community cannot be excluded. Psychotropic substances, in fact, have high pharmacological activities, and their presence as complex mixtures in surface waters, together with residues of many other therapeutics, may lead to unforeseeable pharmacological interactions causing toxic effects to aquatic organisms. In spite of the increasing knowledge on the occurrence of illicit drug residues in the aquatic environment, information on their possible adverse effects to non-target organisms is absolutely inadequate. The first effort to fill this gap was made by Binelli and coauthors (2012), who investigated the potential sub-lethal effect induced by environmentally relevant concentration of cocaine towards the bivalve *Dreissena polymorpha*, pointing out its remarkable cyto-genotoxicity. However, since in humans cocaine is largely excreted in urine as metabolites (45% benzoylecgonine and 40% ecgonine methyl ester of the administered dose) and only a small percentage as the unchanged drug (1–9% of the administered dose; Baselt, 2004), in aquatic environment the levels of the benzoylecgonine (BE), are higher than those of the parental compound (Castiglioni et al., 2006, 2011a,b; Gheorghe et al., 2008; van Nuijs et al., 2009; Postigo et al., 2010). Notwithstanding, the knowledge on BE potential toxic effects to non-target organism is completely lacking. For these reasons, our research was aimed to evaluate sub-lethal effects induced by the benzoylecgonine towards a classical freshwater biological model, the zebra mussel *D. polymorpha*. Thanks to its widespread diffusion, peculiar physiological characteristics and high sensitivity to several environmental pollutants, including pharmaceutical and personal care products (PPCPs; Binelli et al., 2009a,b; Parolini et al., 2010, 2011; Parolini and Binelli, 2011; Parolini and Binelli, 2012), this bivalve species is widely used in ecotoxicology. We exposed mussels to two environmentally relevant BE concentrations (0.5 and 1 μg/L) for 14 days, investigating the potential BE toxicity by using an *in vivo* multi-biomarker approach. The end-points of ten different biomarkers were measured and their integrated response was used both in detecting sub-lethal BE-induced effects and in supposing its possible mechanism of action in zebra mussel specimens. In detail, BE cytotoxicity was evaluated on hemocytes by the neutral red retention assay (NRRA), a simple indicator of general cellular stress in bivalves (Lowe et al., 1995). The activity of three antioxidant phase I enzymes, namely catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the phase II detoxifying enzyme glutathione S-transferase (GST), the lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured as oxidative stress indices in bivalve homogenates. In addition, primary (DNA strand breaks) and fixed (apoptotic and micronucleated cell frequency) genetic damage were investigated on zebra mussel hemocytes by the single cell gel electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test), respectively.

2. Materials and methods

The benzoylecgonine (BE) standard (CAS number 519-09-5; purity > 99%) was purchased from Alltech-Applied Science (State College, PA, USA), while all the reagents used for biomarker analyses were purchased from Sigma-Aldrich (Steinheim, Germany). BE stock solution (1 g/L in methanol) was diluted to 10 mg/L in bi-distilled water (working solution), which was then used to obtain the desired BE concentration in experimental aquaria.

2.1. Exposure concentration selection

Since this study represents the first experiment aimed to evaluate the potential hazard of BE towards non-target aquatic organisms, no data regarding its toxicity threshold are currently available in scientific literature. So, to give a marked ecological relevance to our research, we decided to expose mussels to two environmentally relevant BE concentrations, mimicking real conditions to which specimens might be subjected during their whole life span. Thus, according to the most recent European monitoring studies, we exposed mussel to 0.5 μg/L (1.7 nM, Low) and 1 μg/L (3.4 nM, High) of BE. The first treatment reflected the highest BE concentration found in surface waters (van Nuijs et al., 2009) while the second one was similar to the mean BE concentration measured in the influents of STPs (Castiglioni et al., 2006, 2011a,b; Postigo et al., 2010).

2.2. Experimental design

Zebra mussel specimens were collected in April 2011 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 chemical (Binelli et al., 2005) and drug pollution (Zuccato et al., 2008). Mussels were gently cut off from the rocks, quickly transferred to the laboratory in bags filled with lake water and placed in 15 L glass-holding aquaria filled with tap and lake water (50:50 v/v) to avoid a drastic change in the chemical composition of the water and to guarantee a food supply for the mussels during the first 24 h of acclimation. Bivalves were maintained in aquaria filled with 10 L of tap and deionized water (50:50 v/v), previously de-chlorinated by aeration, under a natural photoperiod with constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (> 90% of saturation). The bivalves were fed daily with lyophilized algae (*Spirulina* spp.) and water was regularly renewed every two days for 2 weeks to depurate the mollusks of any possible pollutants that had previously accumulated in their soft tissues. Only specimens that were able to re-form 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, whereas biomarker baseline levels were checked weekly. Mussels were exposed to BE concentrations only when target biomarker levels were comparable with baseline ones obtained in previous studies (Parolini et al., 2010, 2011; Parolini and Binelli, 2011). 200 specimens per aquarium having similar shell length (20 ± 2 mm) were exposed under semi-static conditions for 14 days. Control and exposure aquaria were processed at the same time. The whole water volume (10 L) was renewed on a daily basis and exact volumes of working solution (10 mg/L) were added daily to the exposure aquaria until reaching the chosen concentrations. The complete water and chemical renewal should guarantee a constant BE concentration over a 24-h period and prevent losses of contaminants, as well as the degradation of parental compound, since BE degradation in surface water occurs only after 26 h (van Nuijs et al., 2012). Moreover, we evaluated the BE concentration in both working solution and exposure aquaria at each time of biomarker analysis by HCT Ultra (Bruker, Germany). Water samples were spiked with 1 μg/L of benzoylecgonine-D_3 (BE-D_3) as internal recovery standard before purification and concentration by SPE (HLB 1 cc, Waters). BE quantification was made by a calibration curve (0.1–10 μg/L; R = 0.99) and internal standard recovery was > 90%. Working

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solution concentration was confirmed as 10 mg/L. No BE residues were found in control aquarium (<0.01 μg/L), while the mean BE concentrations measured during the exposure were very close to the nominal ones and were 0.44 ± 0.08 μg/L for the Low treatment and 0.85 ± 0.23 μg/L for the High one, respectively. Specimens were fed 2 h before the daily change of water and BE solution to avoid its adherence to algae and to prevent the reduction of its bioavailability to bivalves. Several specimens (n = 30) were collected at t = 0 and after 4, 7, 11 and 14 days from control and exposure aquaria to evaluate both baseline levels and BE-induced sub-lethal effects, respectively. Bivalve hemolymph was withdrawn and cyto-genetic biomarkers were evaluated on hemocytes. After the withdrawal, the zebra mussel soft tissues were immediately frozen in liquid nitrogen and stored at −80 °C until the analysis of LPO and PCC. Lastly, the soft tissues of other 15 specimens were frozen in liquid nitrogen and stored at −80 °C until the enzymatic activity was measured.

2.3. NRRA, enzyme activity and oxidative stress biomarkers

The NRRA method was applied on mussel hemocytes following the protocol proposed by Lowe and Pipe (1994). 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 finished when dye loss was evident in at least 50% of the hemocytes. The mean retention time was calculated from five replicates (n = 5). The activity of SOD, CAT, GPx, and GST was measured in triplicate (n = 3) in the cytosolic fraction extracted from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 100 mM 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). The homogenate was centrifuged at 15.000 *g* for 1 h at 4 °C. The sample was held 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 (1976) method using bovine serum albumin as a standard. Enzymatic activities were determined spectrophotometrically as described by Orbea et al. (2002). Briefly, the CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 67 mM potassium phosphate buffer (pH 7). The SOD activity was determined by measuring the degree of inhibition of cytochrome c (10 μM) reduction at 550 nm by the 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). The GPx activity was measured by monitoring the consumption of NADPH at 340 nm using 0.2 mM H_2O_2 substrate in 50 mM potassium phosphate buffer (pH 7) containing additional glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). Lastly, the GST activity was measured by adding reduced glutathione (1 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. Lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured in triplicate (n = 3) from a pool of three whole mussels (≈0.3 g fresh weight) homogenized in 50 mM phosphate buffer (pH 7.4; KCl 100 mM, EDTA 1 mM) containing 1 mM DTT and 1 mM PMSF using a Potter homogenizer. LPO level was assayed by the determination of thiobarbituric acid-reactive substances (TBARS) according to Ohkawa (1979). The absorbance was read at 532 nm after removal of any fluctuated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of 1.56 10^5 M/cm and expressed as nmol TBARS formed/g fresh weight. For carbonyl quantification the reaction with 2,4-dinitrophenylhydrazine (DNPH) was employed according to Mecocci et al. (1998). The carbonyl content was calculated from the absorbance measurement at 370 nm with the use of molar absorption coefficient of 22 000 mol/cm and expressed as nmol/(mg protein).

2.4. Genotoxicity biomarkers

Since methods and procedures of cyto-genotoxicity biomarkers applied in this study were well-described in detail by Parolini et al. (2010), only a brief description of the followed techniques was reported here. Genetic biomarker was performed on zebra mussel hemocytes. The alkaline (pH > 13) SCGE assay was performed following the method adapted for the zebra mussel by Buschini et al. (2003). Fifty cells per slide were analyzed using an image analysis system (Comet Score®), for a total of 500 analyzed cells per specimen (n = 10). Two DNA damage end-points were evaluated: the ratio between migration length and comet head diameter (LDR) and the percentage of DNA in tail. The apoptotic cell frequency was evaluated through the protocol described by Singh (2000). Two hundred cells per slide were analyzed for a total of 1000 cells per sample (n = 5). The MN test was performed according to the method of Pavlica et al. (2000). Four hundred cells were counted per each slide (n = 10) for a total of 4000 cells/treatment. Micronuclei were identified by the criteria proposed by Kirsch-Volders et al. (2000), and the MN frequency was calculated (MN‰).

2.5. 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 BE concentrations as variables, while biomarker end-points served as cases. The ANOVA was followed by a Fischer's LSD *post-hoc* test to evaluate significant differences ($^*p<0.05$; $^{**}p<0.01$) 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 exposure assays to investigate possible correlations between the different biological responses. All statistical analyses were performed using the STATISTICA 7.0 software package.

3. Results

3.1. Baseline levels of applied biomarkers

Very low mortality was observed in the control (2%) and exposure aquaria (2.5%) during the 14-days experiments. The average hemocyte viability of bivalves from control, low and high exposure tanks was 78 ± 4%, 79 ± 8% and 82 ± 7%, respectively, agreeing the recommendations made by the 4th International Workshop on Genotoxicity Test Procedures (IWGTP), which requires a viability > 70% to perform the SCGE and genotoxicity assays (Kirkland et al., 2007). Baseline levels of cyto-genetic biomarker and enzyme activity were perfectly comparable with those obtained in previous laboratory studies (Binelli et al., 2009 a,b; Parolini et al., 2010, 2011; Parolini and Binelli, 2011; Parolini and Binelli, 2012) and fell within the physiological range of this bivalve species. No information on PCC baseline levels in zebra mussel was available, however they were in the same order of magnitude with respect to those measured in the bivalve *Unio tumidus* (Labieniec and Gabryelak, 2004). Lastly, LPO baseline levels were in accordance with those found by Quinn et al. (2011) in zebra mussel visceral mass homogenate.

3.2. NRRA and oxidative stress biomarker results

A decreasing NRRT trend was recorded at both tested concentration (Fig. 1), following significant time- ($F = 17.73$; $p<0.01$) and dose-dependent ($F = 48.90$; $p<0.01$) relationships. A significant ($p<0.01$) increase of cellular stress in treated bivalves was observed as early as 11 days at the Low treatment, while it was found ($p<0.01$) already after 4 days of exposure to High, reaching values 3.5-fold lower than the corresponding control at the end of the test. The trends of the

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antioxidant (SOD, CAT and GPx) and detoxifying enzymes (GST) are reported in Fig. 2. All enzyme activities depended on exposure concentration, since an increasing trend was noticed at Low treatment, while a decreasing one was observed at High. In detail, SOD followed significant time- ($F=2.82$; $p<0.05$) and concentration-dependent ($F=93.69$; $p<0.01$) relationships. At Low, it showed a significant ($p<0.01$) increase of activity as early as 4 days of exposure, with values 62% higher than the corresponding control, reaching values 2.4-fold higher than starting levels at $t=14$ days. On opposite, significant ($p<0.01$) decrease in its activity was found at High treatment, with values 2-fold higher than controls at the end of the test. A significant time-dependent ($F=4.25$; $p<0.01$) increase of CAT was found at Low, showing an activity more than halved with respect to baseline levels. The High treatment induced a significantly different ($F=64.19$; $p<0.01$) response in CAT activity with respect to Low; significant ($p<0.01$) inhibition of CAT was found as early as 11 days of exposure, showing values 80% lower than controls at $t=14$ days. GPx activity followed an overlapping trend than CAT for both treatments, showing overall time ($F=5.25$; $p<0.01$) and concentration ($F=42.98$; $p<0.01$) dependencies. The GST activity showed a concentration-dependent relationship ($F=26.47$; $p<0.01$) even if no time dependency was found ($F=1.67$; $p>0.05$). A significant ($p<0.01$) increase of GST activity was found at the end of Low exposure with values 32% higher than controls. In contrast, an early significant ($p<0.01$) decrease of GST was noticed at High treatment, showing values halved than the corresponding control already after 4 days of exposure. 14 days exposure resulted in significantly time- ($F=2.95$; $p<0.01$) and concentration-dependent ($F=9.84$; $p<0.01$) increased LPO levels (Fig. 3a). At High, significant ($p<0.01$) increase of lipid peroxidation was found already after 4 days of exposure, reaching $t=14$ days values 45% higher than controls. Protein carbonyl content followed overall time ($F=8.82$; $p<0.01$) and concentration ($F=79.04$; $p<0.01$) dependencies, showing notable significant ($p<0.01$) increase of protein carbonylation at the end of the treatment, with values 2-fold higher than the corresponding control (Fig. 3b).

3.3. Genetic biomarker results

According to time ($F=27.89$; $p<0.01$) and concentration ($F=34.22$; $p<0.01$) dependencies, significant increases of LDR values were found as early as 7 days of exposure at both Low and High treatment, showing values 52% and 60% higher than the corresponding control at the last test day, respectively (Fig. 4a). Similarly, the mean percentage of DNA in the comet tail (Fig. 4b) was doubled compared to controls at the end of exposure to both the BE concentrations, following significant time- ($F=28.36$; $p<0.01$) and concentration ($F=26.30$; $p<0.01$) relationships. Significant ($p<0.01$) time- ($F=6.50$; $p<0.01$) and concentration- ($F=4.58$; $p<0.05$) increase of apoptotic cell frequency (Fig. 5a) was found after 11 and 14 days of exposure to High, showing values 2.5-fold higher than those measured at $t=0$ days. Lastly, the frequency of micronucleated cells followed both time ($F=16.79$; $p<0.01$) and concentration ($F=45.38$; $p<0.01$) dependencies, pointing out significant ($p<0.01$) increase of micronuclei as early as 4 days of exposure to High, reaching values 3-fold higher with respect to corresponding control at the end of the experiment (Fig. 5b).

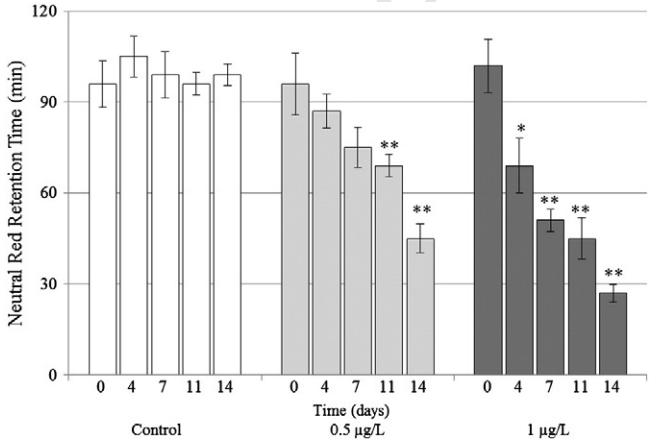

Fig. 1. Mean of neutral red retention time (NRRT) of neutral red loss (mean ± SEM) from lysosomes in the hemocytes of zebra mussels ($n=5$) exposed to BE concentrations. Significant differences (two-way ANOVA, Fischer's LSD *post-hoc* test, $^{*}p<0.05$; $^{**}p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

4. Discussion

Results from the present study pointed out that 14 days exposure to environmentally relevant BE concentrations were able to induce remarkable sub-lethal effects to different levels of biological organization in zebra mussel specimens. At sub-cellular level, the first detectable symptom of the onset of toxicity is often associated with the lysosomes (Regoli et al., 1998), which have an important role in the cellular allocation of xenobiotics and, simultaneously, are preferential targets of these toxic molecules (Lowe et al., 1992). NRRA results showed that both the BE treatments significantly ($p<0.01$) affected the lysosome membrane stability of mussel hemocytes. The significant ($p<0.01$) decrease in NRRT suggested a situation of general cellular stress in bivalves, which was likely linked to the induction of oxidative stress (Lowe et al., 1995). The destabilization of lysosomes in mussels, in fact, is affected by the production of oxyradicals generated by the exposure to contaminants, both internally and externally of the lysosome membrane (Regoli et al., 1998). Indeed, different alterations to these organelles (including damage to their membranes) have been related to the increase of peroxidative processes (Winston et al., 1996), which are common pathways of toxicity induced by several environmental pollutants and are associated with the increase of reactive oxygen species (ROS). This can happen either by the straightforward activation of processes that lead to their synthesis or indirectly acting on antioxidant enzymes (including SOD, CAT, and so forth) and scavengers, decreasing cell defenses. Many studies have shown significant correlation between lysosome membrane destabilization and the impairment of antioxidant enzymes in aquatic organisms (Regoli et al., 2002), including zebra mussel exposed to pharmaceutical and personal care products (Binelli et al., 2009 a,b; Parolini et al., 2010). Since enzymatic antioxidant defense system is based on"cascade" reactions of SOD, CAT, and GPx, changes in their activities provide information on organism responses to pro-oxidant chemicals (Viarengo et al., 2007). According to previous studies, we found significant ($p<0.05$) correlations between the NRRA and antioxidants (Table 1), indicating that both BE exposures can imbalance zebra mussel enzymatic defense against ROS. It is interesting to note that while we found a negative correlation between NRRA and enzymes at Low exposure, these end-points were positively correlated at High administered dose, suggesting a dose-dependent antioxidant response to BE treatments. The increased activity of all the antioxidants indirectly suggested that BE promotes free radicals production. In fact, at Low, an early significant ($p<0.05$) increase of SOD activity (Fig. 2) was measured, indicating the activation of this enzyme to counterbalance the production of the superoxide anion. SOD is the first enzyme involved in the reactions against oxyradicals catalyzing the dismutation of two superoxide anions (O_2^- and OH^-) into molecular oxygen and hydrogen peroxide (H_2O_2). The significant ($p<0.01$) enhancements of CAT and GPx activities resulting from the early activation of SOD, pointed out their complementary role in metabolizing hydrogen peroxide (Box et al., 2007) into H_2O and O_2, completing the defense chain against ROS. In addition, the enhancement in GST activity confirmed the activation of the natural antioxidant defense system due to BE exposure and the induction of detoxification

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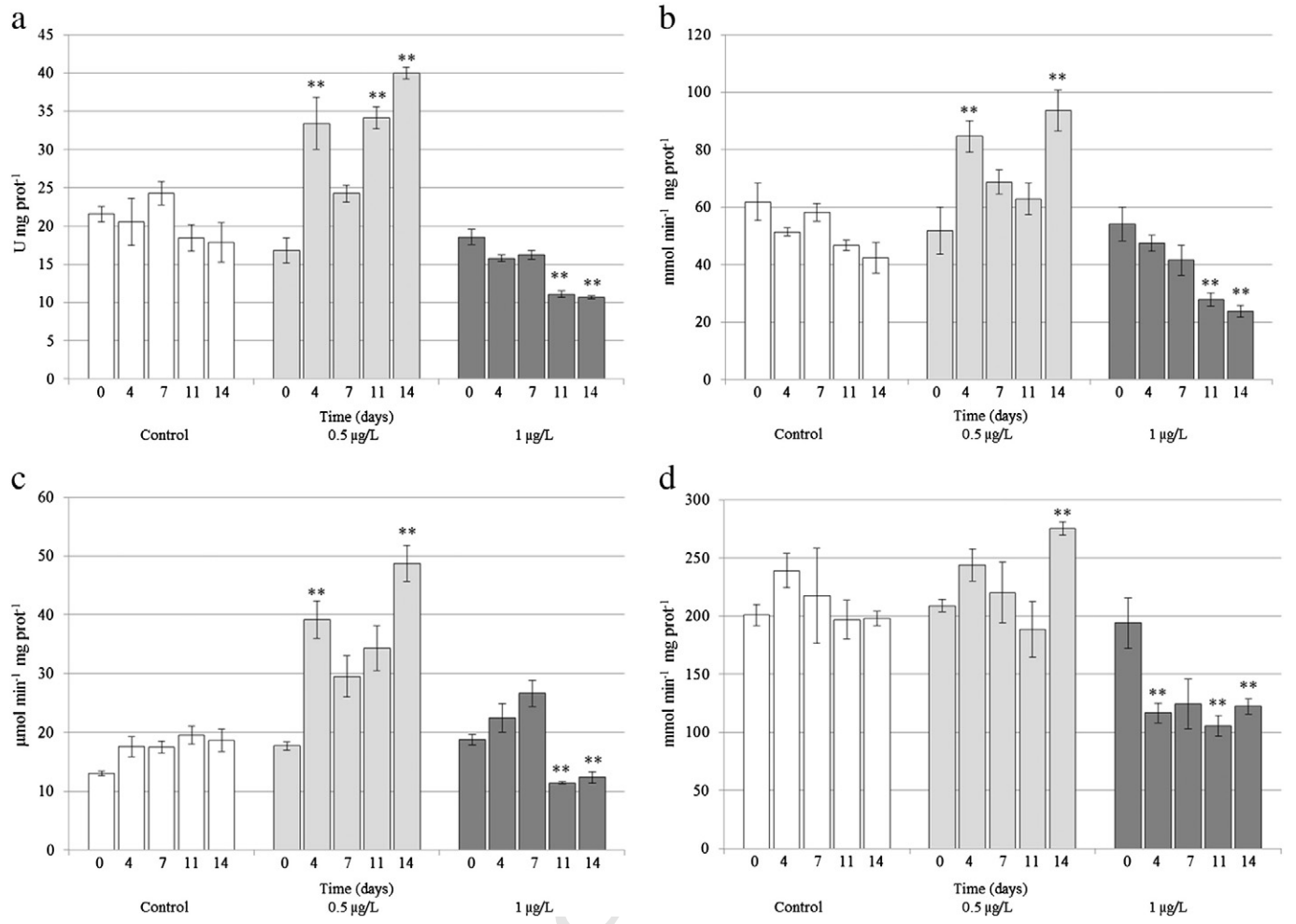

Fig. 2. Effects of BE treatments on the activity (mean ± SEM) of superoxide dismutase (SOD; a), catalase (CAT; b), glutathione peroxidase (GPx; c), and glutathione S-transferase (GST; d), measured in the whole soft tissue of zebra mussels (n = 3; pool of 3 specimens). Significant differences (two-way ANOVA, Fischer's LSD *post-hoc* test, $^{*}p<0.05$; $^{**}p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

process, as opposed to the pro-oxidation forces that are mediated by this enzyme (Elia et al., 2007).

The significant correlation ($p<0.05$) among SOD, CAT, GPx and GST activity found at Low treatment (Table 1), confirmed the activation of the whole *D. polymorpha* enzymatic defense chain against BE-induced ROS, whose compensatory capacity might efficiently counteract their harmful action and prevent the raise of oxidative stress. In fact, even if during metabolic processes a small proportion of free radicals may escape from the protective shield of antioxidants, we found just a slight time-dependent increase in both primary (Fig. 3a and b) and fixed genetic damage (Fig. 4a and b), as well as in lipid peroxidation and protein carbonylation (Fig. 5a and b, respectively). In contrast, results from High BE treatment pointed out an opposite antioxidant response of zebra mussel specimens with respect to Low. No significant ($p>0.05$) variation in enzyme activities were found until t = 7 days of exposure, but significant ($p<0.01$) time-dependent decrease of SOD, CAT and GPx, as well as of GST, compared to controls were found after 11 days (Fig. 2), suggesting a drastic impairment of antioxidant defenses. On the other hand, similar results were observed in *Perna viridis* gills and hepatopancreas after exposure to benzo(α)pyrene and Aroclor 1254, where antioxidant enzymes were unable to counteract intracellular ROS overproduction (Cheung et al., 2004). Our findings were also in accordance with several studies indicated that levels of antioxidant enzymes may increase when bivalves are exposed to Low concentrations of chemical, or during short-term exposures, but may decrease, or even be inhibited, at higher dosages, or with prolonged exposure (Valavanidis et al., 2006; Wang et al., 2011). Hence, the inhibition of antioxidants keeps the organism from counteracting the toxicity of ROS due to High BE exposure, leading to an oxidative stress situation. The lysosomal membrane stability results (Fig. 1), following the development of the pollutant-induced stress, can help to correctly interpret the physiological changes in antioxidant enzymatic activity (Viarengo et al., 2007). The time-dependent decrease of NRRT pointed out an extreme cellular stress in High-treated bivalves confirming that bivalves suffered a drastic oxidative stress situation, which might cause oxidative damage to cellular macromolecules, such as lipids of membranes, proteins, and DNA (Livingstone, 2001). High BE exposure induced significant ($p<0.01$) increase in lipid peroxidation (Fig. 3a), an indirect indicator of oxidative stress (Cheung et al., 2002), as early as 4 days of exposure, confirming that bivalves suffered a remarkable oxidative stress situation. Similarly, significant ($p<0.01$) increases of carbonylated proteins (Fig. 3b) were found at High administered concentration. Oxidation products and carbonyl derivatives of proteins may result from oxidative modifications of amino acids and side chains, reactive oxygen-mediated peptide cleavage and from reactions with lipid and carbohydrate products (Labieniec and Gabryelak, 2004). Hence, the presence of these irreversible modifications may indicate that proteins have been subjected to oxidative free radical damage (De Zwart et al., 1998). In fact, previous redox proteomics analyses showed significant increase in protein carbonylation in zebra mussel gills after 14 days exposure to the same concentration of BE, as well as oxidative modifications in different classes of proteins, such as those of the cytoskeleton, energetic metabolism and

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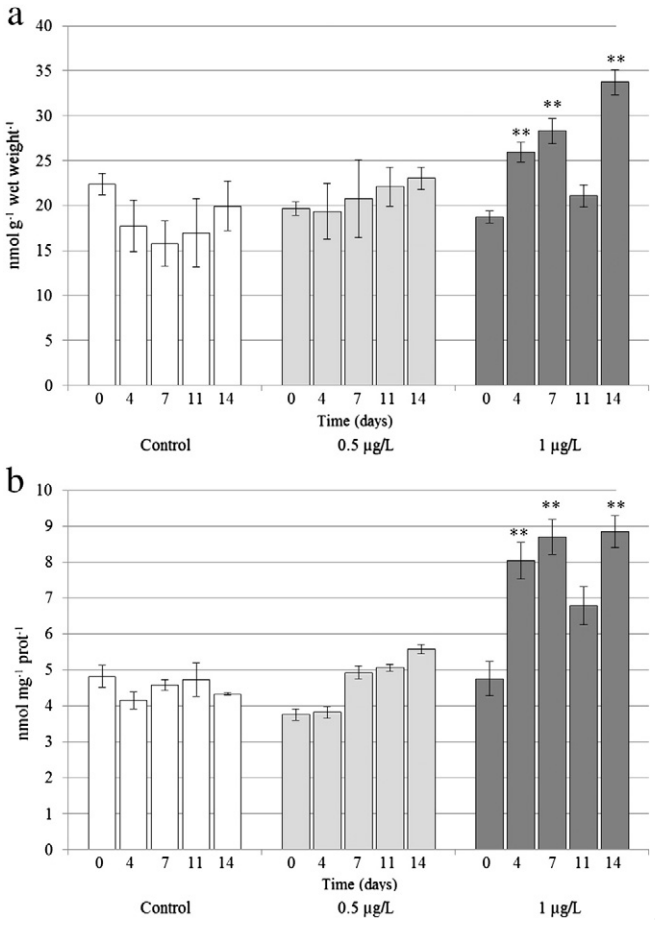

Fig. 3. Measure (mean ± SEM) of lipid peroxidation (a) and protein carbonylation (b) in zebra mussels (n = 3; pool of 3 specimens) specimens treated with two BE concentrations. Significant differences (two-way ANOVA, Fischer's LSD *post-hoc* test, $^{*}p<0.05$; $^{**}p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

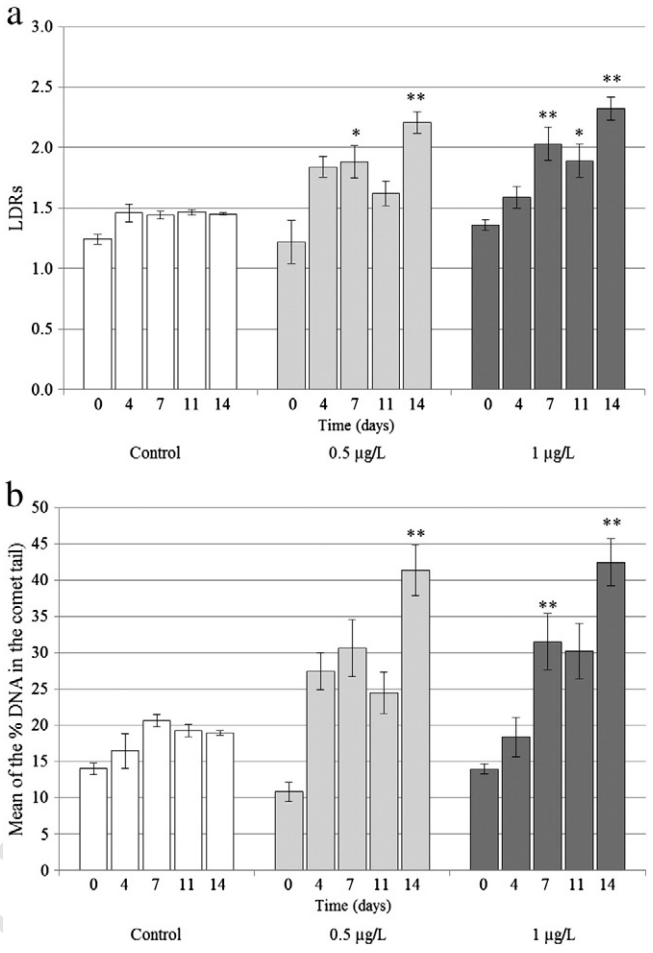

Fig. 4. Results (mean ± SEM; standard error of the mean) of the SCGE assay expressed by the length/diameter ratio (a) and the mean percentage of tail DNA (b). Measurements were carried out on zebra mussel hemolymph samples (n = 10) exposed to BE concentrations. Significant differences (two-way ANOVA, Fischer's LSD *post-hoc* test, $^{*}p<0.05$; $^{**}p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

Q3

stress response (Pedriali et al., 2012). These results support our biomarker data and confirm the involvement of oxidative stress in BE toxicity to zebra mussel. Lastly, DNA is the other target macromolecule involved in cellular oxidative injury. Genetic damage can be traced to direct reaction between DNA and free radical species, which might lead to several alterations including damaged bases, inter- and intra-strand crosslinks and single strand breaks. Many studies have highlighted that the increase of pollutant-induced ROS could cause remarkable changes in the integrity of DNA in different organisms (Regoli et al., 2002; Mamaca et al., 2005), including zebra mussel (Binelli et al., 2009a,b; Parolini et al., 2010; Parolini and Binelli, 2012). On the other hand, covalent binding to certain breakdown products of lipid hydroperoxides, such as MDA, can result in DNA strand breaks and crosslinks (Cheung et al., 2002), while it is well known that carbonyl compounds are toxic due to their carcinogenic properties (Labieniec and Gabryelak, 2004). The analysis of both the SCGE end-points pointed out significant ($p<0.01$) time-dependent increase of DNA fragmentation as early as 7 days of exposures (Fig. 4a and b). The strict negative correlation ($p<0.05$) between DNA fragmentation end-points and all the enzyme activities suggested that ROS could directly cause DNA fragmentation. However, the significant ($p<0.05$) positive correlations among LPO, PCC and SCGE end-points (LDRs and % of DNA in tail) can also suggest the possible involvement of lipid peroxidation and protein carbonyls byproducts in the raise of primary genetic damage. The noteworthy increase in DNA primary lesions can subsequently generate irreversible fixed genetic damage. It has already been suggested that DNA strand breaks are the main contributor to micronuclei induction (Van Goethem et al., 1997) and the primary apoptosis inducing factor in zebra mussels (Binelli et al., 2009 a,b; Parolini et al., 2010). Our data confirmed this relationship, since significant ($p<0.01$) increases of both apoptotic (Fig. 5a) and micronucleated (Fig. 5b) cells were noticed at High BE concentration. Moreover, the overall significant ($p<0.05$) correlation between all the genetic end-points (Table 1) confirmed the strict link between primary and fixed genetic damage. Similar relationships were also found in zebra mussel specimens exposed for 96-h to High cocaine concentration (10 µg/L; Binelli et al., 2012). Unfortunately, current results on BE are not comparable with those from the preliminary study on CO, since they were obtained by a short-term experiment testing concentrations different with respect to those here selected, suggesting the necessity of further comparative studies.

5. Conclusions

The results obtained in this study showed that benzoylecgonine exposures induced remarkable adverse effects to a common non-target organism at environmental concentrations, highlighting its possible hazard to freshwater communities. 14 days experiments showed that treatments to realistic environmental concentrations induced notable imbalances of enzyme defense chain, leading to an oxidative stress situation in specimens exposed to High dose. Consequently, BE induced marked damage to different macromolecules, such as lipids of biological membranes, proteins and DNA. However, this BE mechanism of action

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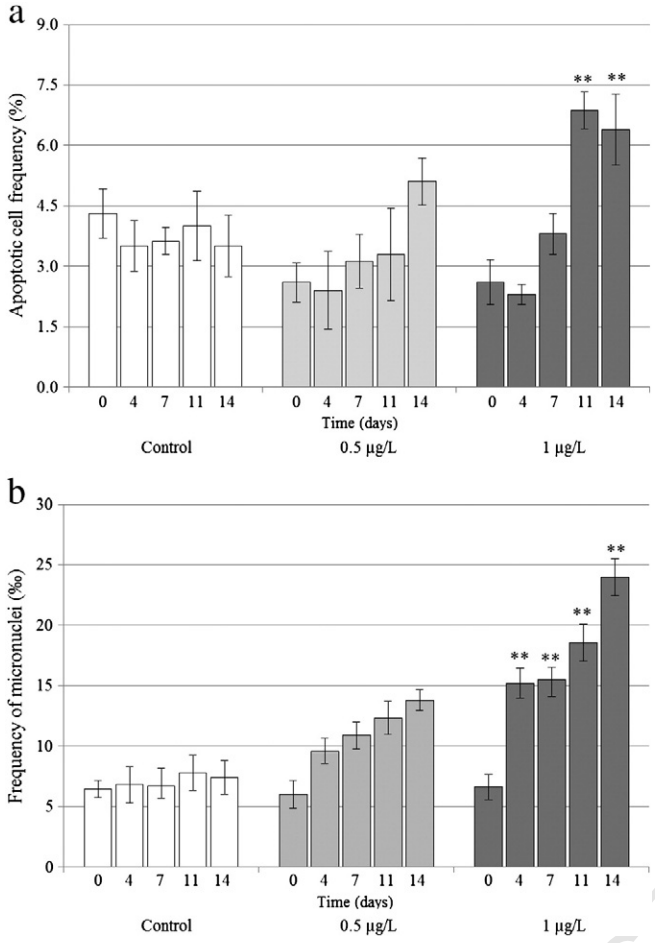

Fig. 5. Percentages of apoptotic hemocytes (n = 5; mean ± SEM) measured by the DNA diffusion assay (a) and frequency of micronucleated hemocytes (b) (n = 10; mean ± SEM) observed in *D. polymorpha* specimens exposed to BE treatments. Significant differences (two-way ANOVA, Fischer's LSD *post-hoc* test, $*p<0.05$; $**p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

Q4

in zebra mussel based on biomarker results should need to be confirmed by using powerful techniques, such as the so-called "omic techniques". Our findings must not be absolutely underestimated since in the real environmental situation bivalves are exposed to BE for their whole life-span, resulting in possible higher toxicity. This is particularly true considering the continuous use of cocaine worldwide, which could lead to a continuous input of its main metabolite in the aquatic system and hazardous impact on non-target aquatic organisms. Based on these considerations, in-depth investigations on BE toxicity should be a priority in environmental risk assessment, in order to enhance knowledge on its possible sub-lethal effects, the mechanism of action for aquatic organisms and effects on population dynamics, which would clarify its true ecological hazard for the aquatic communities. Moreover, considering the notable hazard of CO and BE to zebra mussel, the occurrence of measurable concentrations of several other illicit drugs in freshwaters and their possible high biological activity, further investigations on their induced adverse effects towards non-target organism must be performed.

6. Uncited reference

Q15

Huerta-Fontela et al., 2007

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Table 1

Q5 Pearson's correlation matrix obtained by using all measured biomarker end-points at Low and High BE treatment. Significant correlations ($p<0.05$) are indicated in bold.

	LDR	%DNA	MN	Apo	NRRT	CAT	GPx	GST	SOD	LPO
Low										
%DNA	**0.95**									
MN	0.23	0.22								
Apo	0.21	0.26	0.26							
NRRT	**−0.60**	**−0.62**	**−0.40**	**−0.56**						
CAT	**0.58**	**0.53**	**0.33**	**0.33**	**−0.58**					
GPx	**0.59**	**0.58**	**0.39**	**0.45**	**−0.53**	**0.59**				
GST	**0.38**	**0.33**	0.12	**0.38**	−0.16	**0.45**	**0.43**			
SOD	**0.55**	**0.48**	**0.50**	**0.45**	**−0.64**	**0.78**	**0.75**	**0.41**		
LPO	0.24	**0.30**	0.01	0.04	−0.19	−0.12	0.02	−0.08	−0.03	
PCC	**0.45**	**0.47**	**−0.74**	**0.40**	**−0.74**	**0.37**	**0.35**	0.16	**0.53**	0.09
High										
%DNA	**0.94**									
MN	**0.46**	**0.52**								
Apo	**0.52**	**0.56**	**0.53**							
NRRT	**−0.75**	**−0.75**	**−0.68**	**−0.70**						
CAT	**−0.58**	**−0.60**	**−0.61**	**−0.85**	**0.81**					
GPx	−0.19	−0.27	**−0.43**	**−0.58**	**0.39**	**0.44**				
GST	**−0.48**	**−0.46**	**−0.38**	**−0.44**	**0.61**	**0.62**	−0.03			
SOD	**−0.51**	**−0.55**	**−0.62**	**−0.75**	**0.68**	**0.73**	**0.50**	**0.52**		
LPO	**0.51**	**0.52**	**0.49**	0.20	**−0.56**	**−0.46**	0.06	**−0.38**	**−0.34**	
PCC	**0.55**	**0.49**	**0.45**	0.13	**−0.58**	**−0.35**	0.20	**−0.49**	**−0.39**	**0.57**

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PAPER V

Riva Consuelo, Binelli Andrea, Rusconi Francesco, Colombo Graziano, **Pedriali Alessandra,** Zippel Renata, Provini Alfredo

A PROTEOMIC STUDY USING ZEBRA MUSSELS (*D. polymorpha*) EXPOSED TO BENZO(α)PYRENE: THE ROLE OF GENDER AND EXPOSURE CONCENTRATIONS.

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A proteomic study using zebra mussels (*D. polymorpha*) exposed to benzo(α)pyrene: The role of gender and exposure concentrations

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ABSTRACT

It has recently been established that the use of proteomics can be a useful tool in the field of ecotoxicology. Despite the fact that the mussel *Dreissena polymorpha* is a valuable bioindicator for freshwater ecosystems, the application of a proteomic approach with this organism has not been deeply investigated. To this end, several zebra mussel specimens were subjected to a 7-day exposure of two different concentrations (0.1 and 2 μg L^{-1}) of the model pollutant benzo[α]pyrene (B[α]P). Changes in protein expression profiles were investigated in gill cytosolic fractions from control/exposed male and female mussels using 2-DE electrophoresis. B[α]P bioaccumulation in mussel soft tissue was also assessed to validate exposure to the selected chemical. We evaluated overall changes in expression profiles for 28 proteins in exposed mussels, 16 and 12 of which were, respectively, over- and under-expressed. Surprisingly, the comparative analysis of protein data sets showed no proteins that varied commonly between the two different B[α]P concentrations. Spots of interest were manually excised and analysed by MALDI-TOF/TOF mass spectrometry. The most significant proteins that were identified as altered were related to oxidative stress, signal transduction, cellular structure and metabolism. This preliminary study indicates the feasibility of a proteomic approach with the freshwater mussel *D. polymorpha* and provides a starting point for similar investigations. Our results confirm the need to increase the number of invertebrate proteomic studies in order to increase the following: their representation in databases and the successful identification of their most relevant proteins. Finally, additional studies investigating the role of gender and protein modulation are warranted.

1. Introduction

Proteomics is a well-established area of research in molecular medicine because the evaluation of changes in protein expression patterns can provide information on pathogenic signalling pathways and the identification of human disease markers (Petricoin and Liotta, 2003). In the past decade, the application of proteomics to the field of ecotoxicology (ecotoxicoproteomics or environmental proteomics) has begun to develop (Monsinjon and Knigge, 2007). These studies have demonstrated an effective methodology for characterising the modes of action and the mechanisms of toxicity for pollutants with a high potential for identifying novel biomarkers (Dowling and Sheehan, 2006; López-Barea and Gómez-Ariza, 2006; Monsinjon and Knigge, 2007).

Because aquatic environments are often the ultimate ecological compartment for anthropogenic pollutants, their organisms in particular have received growing interest in the application of proteomic methodologies to ecotoxicology. While vertebrates with well-annotated genomes, such as *Danio rerio* or *Xenopus laevis*, have been successfully used for identifying proteins affected by a toxicant or stressor (De Wit et al., 2008; De Souza et al., 2009; Huang et al., 2010; Serrano et al., 2010), the situation is more problematic in the case of organisms for which only limited genomic information is available, as is the case for some invertebrates. This limitation can be partially overcome by cross-species protein identifications using databases from other sequenced organisms. Several reports demonstrate that this approach can successfully identify some classes of proteins, especially the most ubiquitous and/or highly conserved proteins (López et al., 2002), even if the amount of successfully identified proteins is less than that usually obtained with fully sequenced organisms (Monsinjon and Knigge, 2007).

Despite the apparent drawbacks in applying a proteomic approach to organisms with genomes that are not fully sequenced, an increase in awareness of the enormous advantages of this tech-

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nique has encouraged investigators to divert more resources and time to exploring the effects of toxicants or physical stressors at the protein level of several ecologically relevant species (Lemos et al., 2010). However, there are still some issues to resolve. For instance, a frequently underestimated aspect is the role of sex on protein profile of a selected biological model. Even if the effect is not clear at the phenotypic level, different genders may present different sensitivities to toxicants (Lemos et al., 2010). Moreover, with the exception of a few isolated reports (Hanisch et al., 2010), there is a lack of knowledge on the evaluation of the proteomic response in organisms exposed to different toxicant concentrations. In fact, most published ecotoxiproteomic studies are confined to one tested concentration (Gündel et al., 2010).

One of the most valuable invertebrate models in freshwater environments is the bivalve zebra mussel (*Dreissena polymorpha*), which has been extensively used for biomonitoring metals and persistent organic pollutants (Camusso et al., 2001; Binelli et al., 2004; Riva et al., 2008; Voets et al., 2009) and for the evaluation of several biomarkers (Osman et al., 2007; Riva et al., 2007; Contardo-Jara and Wiegand, 2008; Binelli et al., 2009; Parolini et al., 2010). Although *D. polymorpha* can be used as an extremely flexible environmental monitor, there is a paucity of data regarding the evaluation of possible changes in protein patterns due to exposure to environmental contaminants.

The purpose of this study was to verify the suitability of a proteomic approach in this useful bio-indicator species of freshwater environments. This aim was realised by assessing changes in protein expression profiles in the gills of zebra mussel specimens after an *in vivo* exposure to the model PAH (polycyclic aromatic hydrocarbon) benzo(α)pyrene (B[α]P). In order to provide a more in-depth evaluation of this approach, we decided to investigate the role of gender in proteome response by separately analysing male and female mussels. Moreover, we chose to test the effect of two different concentrations of B[α]P to evaluate possible proteome dose-dependent relationships. We identified proteins the level of which changed in response to B[α]P exposure by MALDI-TOF/TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry). B[α]P bioaccumulation in mussel soft tissues was also assessed to ensure a real exposure to the selected chemical. This study is the first preliminary analysis of proteomic modulation in *D. polymorpha* gills exposed to a toxicant and provides a starting point for the further application of proteomics to this potential ecotoxicological model.

2. Materials and methods

2.1. Exposures

About 100 sexually mature *D. polymorpha* specimens were collected (3–6 m depth) in April 2009 from a pristine site (Lake Lugano, Northern Italy) by a scuba diver and immediately transferred to the laboratory in bags filled with lake water. Only mussels with similar shell lengths (20 ± 2 mm) were selected for experiments. Twenty specimens were placed on glass sheets suspended in small glass aquaria (3 L) filled with dechlorinated tap water and acclimatised for one week at a natural photoperiod and constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (90% saturation). Animals were fed daily with an algae replacement substitute-enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA), and water was changed daily (100% renewal). Only specimens that were able to attach themselves to the glass sheets were used in the experiments.

Exposures were performed for 7 days under semi-static conditions (Binelli et al., 2008) with daily changes of the entire volume of water and the addition of B[α]P (Dr. Ehrenstorfer, 99.5% purity) dissolved in DMSO (dimethyl sulfoxide) to yield the final test concentrations (0.1 and 2.0 μg L^{-1}). A control group exposed solely to DMSO was tested in parallel, and the final solvent concentration in each treatment was the same (0.001%). DMSO was chosen as a suitable vehicle for dissolving B[α]P as its use has previously been reported (Venier et al., 1997; Hoarau et al., 2006; Binelli et al., 2008) and because it is recognised as compatible in ecotoxicological assessments (De la Torre et al., 1995). In addition, we used a DMSO concentration well below the 0.01% concentration recommended by Organization for Economic Cooperation and Development (OECD, 2000).

Mussels were fed daily for 1 h before changing water and adding contaminant to allow the intake of B[α]P only by water. After a 7-day exposure, the gills of 4 males and 4 females from each experimental condition were removed, frozen in liquid nitrogen, and stored at −80 °C until analysis. Tissue selection for comparative proteomic analyses is a crucial point. In this study, differences in protein expression levels were studied in mussel gills because they are the first uptake site for many toxicants in the aquatic environment and are known to be affected by exposure to pollutants (Gómez-Mendikute et al., 2005). In *D. polymorpha*, the gills are independent organs that can be easily dissected to offer a pure tissue sample (Quinn et al., 2009). A gonad smear for each mussel was examined by light microscopy for gender determinations. The remainder of the mussel soft tissue was stored at −20 °C for chemical analyses to evaluate B[α]P bioaccumulation.

2.2. Sample preparation

Gills from individual mussels were homogenised in 800 μL of ice-cold buffer A [20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), 10 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate ($Na_4P_2O_7$), 10 mM β-glycerophosphate, supplemented with complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)] and centrifuged at 800 × *g* for 10 min (4 °C). The collected supernatant was then centrifuged at 100,000 × *g* for 1 h (4 °C). The resulting supernatant was used for two-dimensional electrophoresis (2-DE) and represents the cytosolic soluble fraction. Proteins were quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA). Before 2-DE, each sample, which contained 300 μg of protein, was precipitated using a chloroform/methanol mixture and resolubilised in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM DTT, 1% IPG buffer 3–10 NL and 1.2% DeStreak solution (GE Healthcare, UK).

2.3. Two-dimensional electrophoresis (2-DE)

The first dimension was carried out with an 18 cm pH 3–10 non-linear gradient IPG strip (GE Healthcare). Solubilised samples were used to rehydrate IPG strips before isoelectrofocusing. Strips were rehydrated for 1 h (at 20 °C) without current and then for 12 h at 30 V in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM DTT and 1% IPG buffer 3–10 NL (GE Healthcare). Strips were focused at 20 °C for a total of 70,000 V h at a maximum of 8000 V using the Ettan IPGphor II system. Focused IPG strips were reduced (1% DTT) and alkalised (2.5% iodoacetamide) in equilibration buffer (6 M urea, 2% SDS, 30% glycerol and 50 mM Tris/HCl, pH 8.8.) just before loading onto a 12.5% 24-cm, 1-mm thick acrylamide gel. The strips were overlaid with 1% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and run in sets of four in an Ettan DALTsix electrophoresis unit (GE Healthcare) at 25 °C with a pre-run step at 2 W/gel for 1 h and a run step at 15 W/gel until the blue dye front had run off the bottom of the gels. The gels were silver

stained using the MS-compatible ProteoSilver™ Plus Kit, (Sigma) according to the manufacturer's instructions.

2.4. Image analysis

After silver staining, gel images were obtained using an ImageScanner II and analysed with ImageMaster 2D Platinum software (GE Healthcare). Each experimental group (DMSO control, low and high B[α]P concentration) consisted of 8 gels from mussel gills (four from 4 males and four from 4 females for a total of 24 gels). B[α]P effects were investigated in male and female controls compared to exposed males and females, respectively. In order to detect any eventual substantial difference between control genders, an intersex analysis set was evaluated (4 DMSO control males *versus* 4 DMSO control females).

Spot detection was performed automatically based on the following parameters: (i) minimal area > 5 pixels, (ii) smooth factor > 2.0 and (iii) saliency > 1.0. Manual editing was then performed to remove streaks, speckles and artefacts. Relative spot volume (%V = 100 × V single spot/V all spots, where V is the integrated OD over the spot area) was used for quantitative analyses in order to reduce experimental errors due to protein loading and staining. Because silver staining has a dynamic range of 1–2 orders of magnitude (Miller et al., 2006), the weakest and the strongest spots evaluated in this study fell within this range. Finally, we chose a master gel to match to spots from the other gels. Gel alignments were carried out based on landmark annotations. Because a good correlation (matching >70%) was obtained between the pairs of gels, no manual editing was necessary to correct for either false positives or false negatives. Only spots that were appropriately matched among all replicates from each experimental group were included in further analyses. Spots corresponding to differentially expressed proteins were statistically evaluated in terms of mean relative volume by the Student's *t*-test for unpaired samples after normality (Shapiro–Wilks' *W*-test) and homogeneity of variances (Levene's test) testing. The significance level was defined as $p < 0.05$. In order to avoid eventual false significant results, a minimum of a 50% change cut-off relative to the control was used as a further criterion for differential expression (Schlags et al., 2005).

2.5. In-gel trypsin digestion

Protein spots were manually excised from silver-stained gels with a razor blade, chopped into 1 mm^3 pieces and collected into LoBind tubes (Eppendorf, Germany). Gel pieces were destained with silver de-staining solution (ProteoSilver™ Plus Kit, Sigma–Aldrich, USA), washed with 100 μL of 50% (vol/vol) acetonitrile in ammonium bicarbonate (50 mM, pH 7.4), dehydrated in 100 μL of acetonitrile for 5 min and completely dried in a speed-vac (Thermo Savant, Savant Instruments, USA) after solvent removal. Digestion was performed for 2 h at 37 °C with sequencing grade modified trypsin diluted in ProteaseMAX™ surfactant (Promega, USA), which improves recovery of longer peptides and provides an increased sequence coverage. Digested samples were centrifuged at 16,000 × *g* for 10 s, and the digestion reaction with extracted peptides was transferred into a new tube. Trifluoroacetic acid was added to a final concentration of 0.5% to inactivate trypsin. Finally, samples were reduced (≈5 μL) by speed-vac and immediately analysed.

2.6. Protein identification

Two microliters of a tryptic-digested sample was mixed with 2 μL of saturated α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid and spotted onto a MALDI target plate (1 μL). Mass spectra were acquired using an Ultraflex III Bruker MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) operating in reflectron mode with 20-kV accelerating voltage and 23-kV reflecting voltage. MALDI mass spectra were calibrated using the Peptide Calibration Standard (700–4500 Da) from Bruker Daltonics. Data acquisition and data processing were performed with flexControl and flexAnalyses software (Bruker Daltonics) using a proprietary "Top Hat" base-line tool along with the "SNAP" peak detection algorithm, which was set to a signal-to-noise ratio of 6, maximal number of peaks as 100 and quality factor threshold of 50. The obtained peptide mass list was used for PMF (peptide mass fingerprint) database searching with the MASCOT search engine (http://www.matrixscience.com) in NCBInr/SwissProt databases. The main search parameters were the following: Metazoa (species); NCBInr/SwissProt (databases); 1.2 Da (mass tolerance); $[M+H]^+$ and monoisotopic; carbamidomethylation (fixed modification); oxidation at methionine (variable modification) and up to one allowed missed cleavage site. Results with confidence interval % (C.I. %) values greater than 95% based on MASCOT MOWSE were considered a positive identification.

2.7. B[α]P chemical analyses

About 1 g of lyophilised mussel tissue was analysed accordingly to the protocol described in detail in Riva et al. (2010). Two microliters of a final sample was injected into a Trace GC 2000 gas chromatograph (Thermo Electron, Austin TX, USA) coupled with a Polaris Q Ion Trap mass spectrometer with an AS 2000 autosampler (Thermo Electron). Instrument conditions for B[α]P were as follows: PTV injector in split/splitless mode; He carrier gas (constant flow rate, 1.1 mL min^{-1}); RTX-5MS (30 m, 0.25 mm ID, Restek Chromatography, USA); transfer line temperature 280 °C; ion source temperature 260 °C; He damping gas flow 0.3 mL min^{-1}; and EI-SIM (selective ion monitoring) mode with EI 70 eV. Excalibur 1.3.1 software was used for data processing. Multi-level calibration curves were created for quantification, and good linearity ($R^2 > 0.98$) was achieved for tested intervals, which included all the concentration ranges found in the samples. Analyte identification was based on the retention time, ion chromatograms and intensity ratios of the ions monitored. The B[α]P limit of quantification (LOQ, defined as three times the noise level) corresponded to 0.2 ng g^{-1} lipid weight.

3. Results

Zebra mussels were exposed to B[α]P concentrations of 0.1 and 2 μg L^{-1} for 7 days, and bioaccumulation analyses were performed at the end of the exposure period. Mussel behaviour was judged to be normal by checking visible siphons pumping in and out and responding to mechanical stimuli (rapid siphon retraction and shell valve closure). No dead mussels were recorded for the entire duration of the experiment.

3.1. Chemical analyses

After exposure, B[α]P levels in mussel soft tissues increased in a concentration-dependent manner ranging from 48 (for the lowest concentration) to 242 ng g^{-1} lipids (for the highest concentration), which corresponded to 7 and 32 ng g^{-1} dry weight, respectively. The B[α]P contents were also measured in DMSO exposed mussels, and the value was below the LOQ value.

3.2. Protein expression patterns of B[α]P exposure

A representative two-dimensional gel image of cytosolic soluble proteins from zebra mussel gill tissue is reported in Fig. 1. Two-dimensional electrophoresis of extracted proteins resulted in a high separation and resolution with respect to molecular weight (MW)

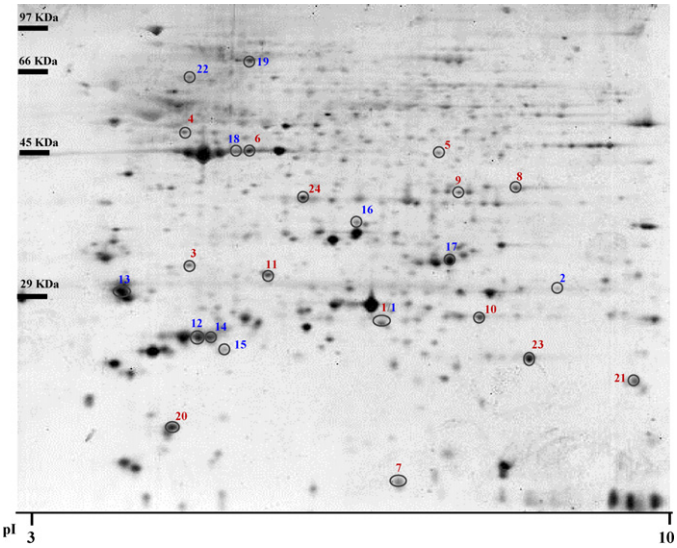

Fig. 1. Representative gel image of proteins from *D. polymorpha* gills. Three hundred micrograms of protein was separated on non-linear wide-range immobilized pH gradients (pH 3–10, NL/18 cm) in the first dimension and 12% SDS-PAGE for the second dimension. Proteins were stained with ProteoSilver Stain MALDI compatible (SIGMA). Spots significantly up-/down-regulated are numbered in red/blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

and isoelectric point (pI). On average, about 450 protein spots per gel were visualised and detected using silver staining and ImageMaster Analysis Software. Most of the proteins were concentrated on a pI ranging from 5 to 8. In terms of MW, most of the detected proteins were between 20 and 66 kDa.

The proteome comparisons between the control and exposed mussels were made analysing males and females separately. On the whole, we found a total of 28 differentially expressed proteins ($p < 0.05$, Fig. 2). The comparison between the control and exposed group (Fig. 2B and C) indicated that mussels exposed to the lower B[α]P concentration showed a higher tendency towards down-regulation (i.e., 71% of the total differently expressed proteins). Inversely, mussels exposed to the higher B[α]P concentration showed an up-regulation (i.e., 91% of the total differently expressed proteins). Fourteen spots (4 in male and 10 in female specimens) were differentially expressed in mussel gills after exposure to 0.1 $\mu g\,L^{-1}$ B[α]P (Fig. 2B). In males, 3 spots showed 1.6- to 2.5-fold increase, while only 1 was down-regulated (a 1.8-fold decrease). In females, proteins were almost exclusively down-regulated. We detected 9 spots with 1.6- to 3.5-fold decreases, and only one was increased by 2.6 fold. Only one spot (spot no. 4) varied commonly and with the same over-expression trend between the genders. Exposure to the higher B[α]P concentration revealed 11 differentially regulated proteins, 5 in males and 6 in females, respectively (Fig. 2C). Males exhibited 5 differently expressed proteins, all up-regulated (1.7- to 3.3-fold increase). Females also showed a strong over-expression pattern. Five proteins were up-regulated (2.6- to 4.5-fold increase), and only one showed a 3.2-fold decrease in expression. Only one over-expressed protein (spot no. 9) varied commonly between genders. Surprisingly, no proteins were found to vary commonly between the two B[α]P experimental doses.

The intersex analyses between gills from control male and female specimens (Fig. 2A) revealed that only 3 protein spots were differentially expressed with 2 over- (1.6- to 2.3-fold increase) and 1 under-expressed (1.8-fold decrease). Two of these proteins were also regulated after the 0.1 $\mu g\,L^{-1}$ B[α]P exposure. Spot no. 1 was over-expressed in the intersex analyses (2.3-fold increase) and found to be down-regulated in exposed females (1.6-fold decrease). On the contrary, spot no. 2 was under-expressed (1.8-fold decrease) and also down-regulated in exposed males (1.8-fold decrease).

3.3. Protein identifications

Proteins that showed significant differences were selected for MALDI-TOF/TOF identification. We excised 19 of the 24 differentially expressed spots. The other 5 spots (nos. 9, 13, 15, 16, 22) were too low in abundance and/or too close to other spots to be isolated and excised with certainty. A total of 7 proteins were positively identified using MALDI-TOF/TOF mass spectrometry. Because *D. polymorpha* is not a model organism, most of the identified protein sequences were absent from databases, and protein identification was achieved by homology. Spot identities, Mr (kDa), pI value, the predicted biological role, and protein coverage are presented in Table 1. The remaining 12 proteins were not identified. This was mainly because their abundances were too low or because their database scores were not sufficiently high to yield unambiguous matches.

4. Discussion

D. polymorpha is a valuable freshwater bioindicator species and is considered the freshwater counterpart of *Mytilus* (Giambérini and Cajaraville, 2005), which has been used for several proteomic studies (López et al., 2001; Apraiz and Cristobal, 2006; Magi et al., 2008). In our study, we evaluated the suitability of proteomics in the zebra mussel for the first time. The experimental design was set up to investigate changes in protein expression profiles in mussel gills and to evaluate the role of different concentrations of the chemical tested. Moreover, the modulation in protein expression was investigated by analysing males and females separately to detect possible gender influences on protein expression, which is an aspect often disregarded in ecotoxicoproteomics.

One of the main problems in experimental design in ecotoxicological investigations is the careful selection of the concentrations of the chemical. In our study, *D. polymorpha* specimens were exposed to B[α]P concentrations that are considered environmentally relevant (Irwin et al., 1997). In addition, the chosen values were below the estimated lethal toxicity concentration (LC_{50}) determined for freshwater organisms (5 $\mu g\,L^{-1}$ for 48 h or a longer exposure; Neff, 2002). The choice of the tested concentrations was also made on the basis of results obtained in our previous study, which was carried out on zebra mussels haemocytes and highlighted a significant genotoxic effect of B[α]P within a range of 0.1–10 $\mu g\,L^{-1}$ (Binelli et al., 2008). The determination of the chemical bioaccumulation in the organism during the exposure assay is also a crucial point. This guarantees that the effects evaluated are due to the effective accumulation of the toxicant and allows a rigorous investigation of the concentration–effect relationship. B[α]P shows a rapid accumulation in bivalve molluscs after only 7 days, and the levels reached at the end of the exposure are comparable to those detected in mussels from moderate or heavily polluted environments (Gaspare et al., 2009; Valavanidis et al., 2008). Thus, the observed changes in the zebra mussel proteome can also be obtained in natural aquatic ecosystems and mimic the real world. None of the B[α]P exposure assays caused mortality in mussels over the entire exposure time, which indicated that the protein expression changes in our experiments responded only to the toxic properties of the chemical and not to an acute biological damage by the compound.

On the whole, our study indicated that B[α]P can significantly modulate protein expression profiles in gills. Detailed descriptions on the role of gender, the effects of different B[α]P concentrations in

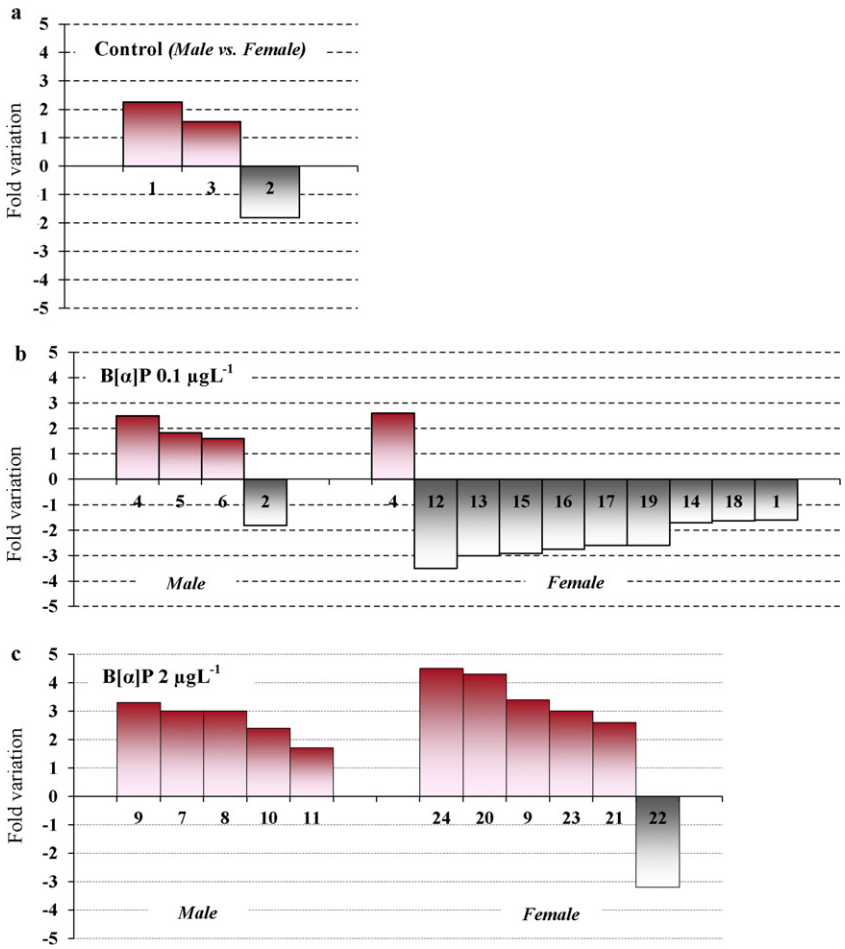

Fig. 2. Sets of protein spots differentially expressed in *D. polymorpha* gills. Graphs are organised to show the results from intersex analyses of control mussel, male *versus* female (a), mussels exposed to 0.1 μg L^{-1} B[α]P *versus* DMSO control mussels (b) and mussels exposed to 2 μg L^{-1} B[α]P *versus* DMSO control mussels (c). Data are grouped on the *x-axis* with the results relative to the male on the left side and to the female on the right side. The *y-axis* represents the fold change (in terms of relative spot volume, % V) of the protein spots, where a *positive value* indicates an increase in abundance ( ) and a *negative value* indicates a decrease in abundance ( ).

the proteome response and an overview of the identified proteins are reported below.

4.1. Gender differences in proteome response after B[α]P exposure

While clinical proteomic investigations using test organisms tend to rely on gender-controlled experiments (Amelina and Cristobal, 2009; Metskas et al., 2010), there is a lack of information regarding the role of gender in proteome response in the ecotoxiproteomic field. The examination of differences between exposed male and female mussels and respective controls revealed a complex response pattern (Fig. 2). Mussel gill tissue was expected to not respond to a toxin in a sex-specific manner (Monsinjon et al., 2006). Notwithstanding, a marked gender difference in altered protein levels emerged after exposure to B[α]P. In fact, the proteome pattern was completely different. The exception was only two proteins, spot nos. 4 and 9, which varied commonly in male and female mussels at the lower and the higher B[α]P concentrations (Fig. 2B and C), respectively.

A marked gender difference in response to PFOA (perfluorooctanoic acid) has been well-described from the comparison of male and female protein profiles in the rare minnow (*Gobiocypris rarus*) (Wei et al., 2008). When they reported specific, and in some cases, opposed proteomic responses in male and female zebrafish (*D. rerio*) exposed to a mixture of BFRs (brominated flame retardants), Kling et al. (2008) confirmed the importance of considering gender in ecotoxicoproteomics to accurately determine a contaminant-exposure effect.

Our observations are also supported by other mussel studies that demonstrate substantial gender influence in response to B[α]P exposure. For example, in B[α]P metabolic research using microsomes from *Mytilus edulis* digestive glands, the highest B[α]P hydroxylase activity was reported in a female rather than a male (Livingstone and Farrar, 1984). In order to investigate gender-specific differences in gene transcription, Brown et al. (2006) exposed *M. edulis* to B[α]P and found that >50% of transcripts were more responsive in females rather than males. Because the differences between male and female protein profiles were significant after exposure to B[α]P, our results indicated the need to consider *D. polymorpha* gender in proteomic-based investigations.

In addition, the results of the intersex analyses between male and female control mussels (Fig. 2A) showed that only 3 proteins (spot nos. 1, 3 and 2) were differently expressed between genders. It is important to highlight that if differences exist between control male and female organisms, these should be considered in a proteomic evaluation after a toxin exposure.

4.2. Effects of different B[α]P concentrations on proteome response

In a cell-response study to toxicant exposure, dose-related responses should be considered because different doses, which

Table 1
Identified proteins differentially expressed in *D. polymorpha* gills cytosolic fraction after B[α]P exposure.

Spot no.[a]	AC no.[b]	Homologous protein (organism)	Biological role/process	Theoretical/experimental		MASCOT score[c]	Sequence coverage (%)[d]	Gender/exposureconcentration[e]	Fold change[f] (↑/↓)
				Mr (kDa)	pI				
1	Q8BK84	Dual specificity phosphatase DUPD1 (*M. musculus*)	Cell signalling pathways	24.2/26	6.05/6.12	93	34	Sex specific Female (L)	2.3 ↑
									1.6 ↓
2	A4IGD2	N-acetyltransferase 8-like protein (*D. rerio*)	Metabolic process	32.3/30	8.65/8.52	86	33	Sex specific Male (L)	1.8 ↓
									1.8 ↓
4	P60712	Actin, cytoplasmic 1 (*B. taurus*)	Cytoskeletal	41.7/43	5.29/5.11	117	43	Male (L)	2.5 ↑
5	Q22067	Probableaspartateaminotransferase, cytoplasmic (*C. elegans*)	Metabolic process	45.5/45	6.93/6.62	80	17	Male (L)	1.8 ↑
6	O17320	Actin (*C. gigas*)	Cytoskeletal	41.8/45	5.30/5.53	101	41	Male (L)	1.6 ↑
8	P81431	Alcoholdehydrogenase class-3 (*O. vulgaris*)	Cell redox homeostasis	40.4/40	7.83/7.81	76	31	Male (H)	3 ↑
12	Q5ZJF4	Peroxiredoxin-6 (*G. gallus*)	Cell redox homeostasis	25.0/24	5.72/5.20	81	49	Female (L)	3.5 ↓

[a] Spot number on 2-DE map (Fig. 2).
[b] Accession number in SwissProt database.
[c] MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com).
[d] Percentage of aminoacid sequence coverage of matched peptidesin the identified proteins.
[e] Gender and exposure condition (L, low = B[α]P 0.1 μg L^{-1}; H, high = B[α]P 2 μg L^{-1}).
[f] Fold change increase (↑) or decrease (↓) (in terms of relative spot volume, %V).

belong to different toxic ranges, may actually induce dose-dependent or completely different responses (Shen et al., 2006). Moreover, the identification of altered protein expressions that are induced by only one stressor condition may not be sufficient to identify the toxicity pathways or the mode of action of a chemical. Ultimately, new potential biomarkers may not be established. Surprisingly, the comparative analysis results of our proteome data sets showed no proteins that varied commonly between the two different B[α]P concentrations. Even though there was a 20-fold difference in nominal B[α]P concentrations tested, the gap in B[α]P levels measured in mussel soft tissues after 7 days of exposure was only 4.5- to 5-fold. We were surprised to find no common protein responses in so small of a concentration range.

On the other hand, protein patterns for a cellular response can change dramatically over time and will not be fixed during later events (Shen et al., 2007). In fact, changes produced by external factors, such as a toxicant administration, can cause unpredicted perturbations in the contents of individual proteins, and the responses of individual proteins may not be linear when a cell is exposed to various concentrations of a chemical. Thus, it can be very difficult to establish a reliable dose-response relationship for a proteome (Randić and Estrada, 2005).

Even if not deeply investigated, especially in ecotoxiproteomic research, the available literature regarding dose-dependent protein responses is not in agreement. For example, in a *M. edulis* proteomic study, investigators performed a Cu^{2+} dose response investigation (20–40–60–80 ppb for 24 h) and found 11 proteins consistently altered across the range of concentrations tested (Shepard and Bradley, 2000). A proteomic analysis performed on developing tadpoles (*X. laevis*) exposed to 0.1 and 1 ppm Aroclor 1254 showed only six commonly up-regulated proteins between both experimental conditions with a total of 60 differentially expressed proteins (Gillardin et al., 2009).

Our results are also consistent with a proteomic analysis of human amniotic epithelial FL cells exposed to three different concentrations of *anti*-benzo(α)pyrene-7,8 dihydrodiol-9,10-epoxide (BPDE) (Shen et al., 2007) in which there was no common protein with an altered expression in all three tested exposures.

On the other hand, our results could also reflect the influence of natural variation of individuals within a sample group and be due to genetic or developmental differences. Organism response to chemical stresses is a complex reaction that depends on the type of chemical and is influenced by many internal and external factors (Pyza et al., 1997). Moreover, when animals are moved from their natural environment to a test environment in the laboratory, they are always subjected to new stress factors that are difficult to identify and control (Prevodnik et al., 2007).

High variability for the expression of stress-responsive proteins in single individuals (Pyza et al., 1997; Wheelock et al., 1999) and among individual mussels used to test the effects of environmental contaminants on protein expression (Knigge et al., 2004; Prevodnik et al., 2007) has been described. These observations and our results indicate that proteomic profile changes cannot be considered simple amplifications of low-concentration responses in the case of high concentration exposures. Without question, a further exposure of zebra mussel specimens to a broad range of B[α]P concentrations could help us to more deeply investigate this topic. In addition, the use of a large number of replicate samples or the use of pooled samples would be necessary to reduce the influence of an eventual interindividual variation.

4.3. Description of the identified proteins

The PMF approach enabled us to identify 7 of the 24 differentially expressed proteins. To identify the proteins of interest, we encountered two main problems: limitation of protein amount and the absence of *D. polymorpha* sequences in protein/gene databases. The former is a limiting factor in protein identification after in-gel digestion because an insufficient amount of peptide generates low signal intensities, and protein identification is very difficult (Verhoeckx et al., 2005). The second problem is related to the poor representation of genomic information for several organisms, e.g., bivalves, within the available databases. This problem has already been addressed in previous reports (López et al., 2002; Monsinjon and Knigge, 2007). Because the determination of complete peptide sequences and cross-species identification via databases has recently improved the identification-based proteomics approach within the field of ecotoxicology, one possible solution would be *de novo* sequencing (Monsinjon and Knigge, 2007). On the other hand, this approach is time-consuming, laborious and expensive for non-model species that are totally absent from current sequence databases (Vioque-Fernández et al., 2009). Moreover, in our specific case, the use of *de novo* sequencing could have been difficult because it requires a high number of ions for MS analyses and, therefore, a higher amount of protein from a spot sample (Martínez-Fernández et al., 2008), which was our first limitation.

The proteins identified by PMF (Table 1) and the possible role played by B[α]P in their modulation provides some interesting points for discussion. At the lower B[α]P concentration, two over-expressed proteins (spot nos. 4 and 6) were identified as actin isoforms in males. Conventional actin is one of the principal components of the eukaryotic cytoskeleton, and it has a central role in cellular processes ranging from cell motility to intracellular transport and cell organisation (Goodson and Hawse, 2002). Actin is expressed in different isoforms that are generally obtained in eukaryotes by alternative splicing of duplicated and highly conserved genes. Actin proteins usually contain 375 or 376 amino acids with high sequence similarities and small differences in molecular weights between isoforms (42–43 kDa), which are best separated according to their respective isoelectric points (Jonsson et al., 2006). In mussels, B[α]P metabolism is known to lead to the generation of quinones and reactive oxygen species (ROS) (Mitchelmore et al., 1998). There is strong evidence for the role of ROS in inducing a number of cytoskeletal rearrangements (Dalle-Donne et al., 2001; Huot et al., 1997) and specifically triggering an increase in actin levels (Clarkson et al., 2002). Actin is over-expressed after moderate to severe pancreatic injury (Zhong and Omary, 2004). The authors suggested that actin over-expression may have a compensatory or protective role in the cell. Because oxidative stress can interfere with actin polymerisation and stress fiber formation, an increase in its levels may provide a reserve of "normal" actin. As oxidative stress is a prominent feature of B[α]P toxicity in mussels, it may ultimately generate complex cytoskeletal rearrangements in gill cells.

Two proteins associated with cell redox homeostasis were also identified. They were peroxiredoxin-6 (Prdx6), which was highly down-regulated (3.5-fold decrease) in females exposed to the lower B[α]P concentration, and an alcohol dehydrogenase class III (ADH3), which was strongly increased (3-fold) in males after exposure to the higher B[α]P concentration. Peroxiredoxin (Prdx) family proteins are ubiquitously found in prokaryotic and eukaryotic species (Leyens et al., 2003; Wood et al., 2003) and prevent cellular radical damage as antioxidant scavengers. Prdx6, in particular, is a 1-cysteine peroxiredoxin involved in antioxidant processes with the additional ability to reduce phospholipid hydroperoxides (Fisher et al., 1999) and repair membrane damage caused by oxidative stress (Chowdhury et al., 2009). Prdx6 gene induction enables an organism to control the cellular increase of ROS levels and, thereby, avoid cellular damage (David et al., 2007). On the other hand, a decrease in Prdx6 expression has been described in various experimental models that are characterised by an increase in cellular oxidative stress (Brixius et al., 2007; Pak et al., 2006). Our results may indicate an impairment of mussel gills to withstand oxidative-induced damage exerted by B[α]P metabolism.

ADH3 has widespread occurrence in both prokaryotic and eukaryotic organisms and constitutes a key enzyme in the detoxification of endogenous and exogenous formaldehyde. However, it is also important in nitric oxide (NO) homeostasis (Jensen et al., 1998; Haqqani et al., 2003). Dutta et al. (2010) used different cell lines to demonstrate that B[α]P administration resulted in an elevation of ROS. This depressed antioxidant protein levels, enhanced expression of inducible nitric oxide synthase and increased production of cellular NO. Thus, we can assume that the marked increase in ADH3 levels may be a response to the elevated levels of NO due to B[α]P exposure.

Another over-expressed protein in males exposed to 0.1 $\mu g\,L^{-1}$ B[α]P was identified as aspartate aminotransferase (AAT). This protein is involved in metabolic process and plays a key role in the regulation of carbon and nitrogen flux in amino acid metabolism (Boutet et al., 2005). In aquatic organisms, transaminases are affected by exposure to hydrocarbons (Narvia and Rantamäki, 1997), and an AAT activity has been found in all mussel tissues (Bishop et al., 1983). In fish, altered aminotransferase activities are caused by the presence of B[α]P (Oikari and Jimenez, 1992). A dietary B[α]P administration causes a significant increase in AAT activity in rockfish serum (Kim et al., 2008).

A decrease in dual specific phosphatase (DUSP) abundance was observed in female mussels exposed to 0.1 $\mu g\,L^{-1}$ B[α]P. DUSPs are a subclass of protein tyrosine phosphatases uniquely able to hydrolyse the phosphate ester bond on both a tyrosine and threonine or serine residue on the same protein. They have a central role in the complex regulation of cell-signalling pathways for the mitogen-activated protein kinase (MAPK) signalling pathways and for cell cycle progression (Camps et al., 2000). MAPK cascades are central to fundamental signalling networks that respond to extracellular events by influencing cell growth, differentiation, apoptosis and stress responses. In carcinomas, DUSPD1 mRNA is expressed at relatively high levels during the early stages of tumour development, but mRNA expression visibly decreases in the later stages of tumour progression (Rauhala et al., 2005). This observation is remarkably evident in advanced hepatocellular carcinomas, which often have down-regulated DUSPD1 protein levels (Calvisi et al., 2008). The decrease in DUSP expression observed in *D. polymorpha* may be the consequence of a situation of heavy stress induced by B[α]P at the lower tested concentration.

Finally, we noted a decreasing N-acetyltransferase (NAT) profile in male mussels exposed to the lower B[α]P concentration. Proteins that belong to the NAT family are highly conserved (Butcher et al., 2002) and have been extensively studied for their role in phase II xenobiotic metabolism. NAT-dependent acetylation is considered one of the major biotransformation pathways for various drugs and environmental xenobiotics (Hein et al., 2000). Digestive glands and gills are the principal mussel detoxification organs. They have phase I detoxification enzymes concentrated in the former and phase II in the latter (Fitzpatrick et al., 1995). Because enzymatic activity is inhibited by *in vivo* exposure to hydrogen peroxide or nitric oxide derivatives, an intracellular redox condition is able to modulate the human NAT-1 isoform (Atmane et al., 2003). Thus, an impairment in NAT detoxification activity may expose mussel gill cells to harmful effects.

DUSP and NAT are also two of the three proteins differently expressed between male and female control specimens (Fig. 2A). As gender-specific basal level differences may contribute to the

responses observed in the present study, data interpretation with regards to these proteins should be carefully checked.

5. Conclusions

Although a proteomics approach in environmental toxicology is extremely promising, progress is slow for those species with no proteomic/genomic information. The limited or non-existent sequence data for ecotoxicologically relevant organisms, the need for bioinformatics tools and the cost of the technology are currently delaying the growth of ecotoxiproteomics and making the interpretation of the results difficult. Protein identification is clearly a prerequisite for unravelling the underlying mechanisms of toxicology, which is a prevalent application of proteomic science. The present report shows not only the utility of proteomic techniques to study differences in protein expression in the freshwater mussel *D. polymorpha*, but also that the interpretation of data sets by this approach should be carefully checked. For instance, the role of gender on protein modulation, the responses generated by different toxicant concentrations, how B[α]P induces the observed changes in the identified proteins and how protein perturbation affects the associated cellular processes in mussel gills need to be deeply investigated. Finally, our preliminary results confirm the need to increase the number of proteomic studies in invertebrates in order to increase their representation in databases and the successful identification of their most relevant proteins.

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PAPER VI

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A REDOX PROTEOMIC INVESTIGATION OF OXIDATIVE STRESS CAUSED BY BENZOYLECGONINE IN THE FRESHWATER BIVALVE *Dreissena polymorpha*.

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A redox proteomic investigation of oxidative stress caused by benzoylecgonine in the freshwater bivalve *Dreissena polymorpha*

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Drugs of abuse and their human metabolites have been recently recognized as emerging environmental contaminants. Notwithstanding the fact that these kinds of compounds share some features with pharmaceuticals, their ecotoxicology has not yet been extensively investigated, although some of their characteristics may potentially threaten aquatic ecosystems. One of the most abundant drugs found in rivers and wastewaters is benzoylecgonine (BE), the main metabolite of cocaine. We applied a redox proteomics approach to evaluate changes in the proteome of *Dreissena polymorpha* exposed to two different concentrations of BE (0.5 and 1 μg/l). Exposures were performed *in vivo* for a period of 14 days and the effect of oxidative stress on protein thiol and carbonyl groups in mussel gills were evaluated. One-dimensional electrophoresis did not reveal a reduction in protein thiol content but showed a significant increase of protein carbonylation at both doses tested. Then, protein profiling using two-dimensional gel electrophoresis was performed with subsequent matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) and TOF/TOF with LIFT technique and linear ion trap combined with orbitrap mass spectrometer (LTQ-Orbitrap). This yielded *de novo* protein sequences suitable for database searching. These preliminary results and protein identifications obtained suggest that BE causes oxidative stress. Oxidative modifications were detected in differing classes of proteins such as those of the cytoskeleton, energetic metabolism and stress response.

Supporting information may be found in the online version of this article.

Keywords: benzoylecgonine; *Dreissena polymorpha*; redox proteomics; protein thiol; protein carbonyl

Introduction

Drugs of abuse and their metabolites have been recently recognized as an important emerging category of environmental contaminants and these compounds have become pseudo-persistent in the aquatic environment due to their widespread production and consumption.[1] The estimated global production of major illicit drugs is comparable to that of legitimate pharmaceuticals.[2,3] As with pharmaceuticals, the main source of contamination is *via* human consumption followed by continuous release into wastewaters. Since sewage treatment plants (STPs) are able only partially to remove this class of contaminant, several illicit drugs and their metabolites can accumulate within the aquatic environment, in which they can produce negative effects on the biocoenosis. Notwithstanding this, the ecotoxicology of drugs of abuse and their metabolites has not yet been extensively investigated and monitoring studies have been conducted only in recent years in some geographical areas. Some of these studies showed that benzoylecgonine (BE) is one of the most abundant of this drug category found in rivers and wastewaters.[4,5] BE is the corresponding carboxylic acid resulting from hydrolysis of the methyl ester of cocaine (CO), catalyzed by carboxylesterases in liver, and subsequently excreted in urine. Indeed, it has been found at concentrations up to 10 μg/l and 3 μg/l in wastewater influents and effluents, respectively,[6] whilst in surface waters it can reach concentrations of 520 ng/l. [7]

Since drugs and their metabolites are continuously released into the aquatic environment after human consumption, we chose the freshwater bivalve zebra mussel (*Dreissena polymorpha*) as a biological model to investigate ecotoxicological effects of BE by analysis of biomarkers and application of redox proteomics to improve knowledge of BE's mechanism of action (MOA). *D. polymorpha* is a popular sentinel organism because of its wide geographical distribution (present in large parts of European and American water bodies and sporadically present in the Far East and Africa), sensitivity to environmental pollutants and fast bioaccumulation due to a high filtration rate.[8–11] One of the main effects of environmental pollutants (e.g. PAHs, metals, pharmaceuticals) on non-target organisms is an increase in oxidative stress (OS) [12,13] due to over-production of reactive oxygen species (ROS). If excess ROS overcome the cell's antioxidant defenses, they can interact with important cell components causing structural changes resulting in toxicity. Proteins absorb ~70% of ROS in OS and redox

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lesions in proteins have the potential to complicate the proteome both by altering levels of individual proteins and by changing their covalent structure with consequences for protein function and turnover.[14] Redox proteomics aims to detect and analyze redox-based changes within the proteome both in sub-stress scenarios such as redox signalling and in OS. Changes in redox potential can covalently modify amino acid side chains by carbonylation, glutathionylation,[15] or oxidation of cysteine –SH groups (e.g. to form disulfides, mixed disulfides or sulfenic [SOH]/sulfinic/[SO_2H]/sulfonic acids [SO_3H]) (Figure 1a).[16] Sulfur-containing residues (cysteine and methionine) are especially susceptible to oxidation which can have functional significance and lead to increased turnover of damaged proteins. Other residues (e.g. lysine, arginine, proline) can be oxidatively converted to reactive aldehyde or ketone groups (carbonyls) causing inactivation, crosslinking or breakdown of protein (Figure 1b).[17] These modifications are readily detectable in 2DE by labelling modified proteins with specific reagents, such as 5′-iodoacetamide fluorescein (IAF), a popular fluorescent dye for labelling protein thiols[18] and fluorescein-5′-thiosemicarbazide (FTSC) for protein carbonyl groups (Figure 1).[19]

Proteomics enables comparison of dynamic responses to stress by simultaneously comparing hundreds to thousands of proteins yielding an unbiased and integrative overview of changes in protein abundance.[20] It takes advantage of high-throughput separative and analytical techniques for detection of change in level/status of specific proteins. Proteomics has the potential to make a valuable contribution to environmental monitoring and risk assessment,[21] but it is only in recent years that ecotoxicoproteomics has been used to probe effects of environmental contaminants.[22,23] Despite this promise, genomes of popular sentinel organisms are often only poorly characterized and lack of sequence information impedes protein identification.[24] Our research group previously made the first attempt to verify the suitability of the proteomic approach in *D. polymorpha* and this confirmed its feasibility providing a starting point for similar investigations.[25]

The present work aims, for the first time, to investigate sub-lethal effects of BE on *D. polymorpha* using proteomics-based detection of redox modifications to gill proteins. We report here one-dimensional electrophoresis (1DE) analysis of general OS effects revealing quantitative carbonylation of proteins. These were further investigated by protein profiling using two-dimensional gel electrophoresis (2DE) with subsequent matrix assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) with LIFT technique.[26] Peptide mass fingerprinting (PMF) was used to screen a protein sequence database and, in some cases, this provided sufficient information for protein identification. Tandem mass spectrometry (MS/MS) fragment ion analysis of selected peptides can be used for improved identification. Although MALDI-TOF/TOF is a very suitable tool for analysis of peptides and proteins thanks to its high sensitivity, fast data acquisition, ease of use, and robust instrumentation,[27] identification of proteins in non-standard model organisms remains problematic.[24,25] The database search fails if the sequence of the protein is not available or when unexpected modifications and amino acid substitutions are present in real samples. Recently, this limitation has been overcome to some extent using a *de novo* sequencing strategy,[28] in which partial or complete amino-acid sequences are obtained using either manual or automated *de novo* peptide sequence analysis. This approach has been successfully applied in recent studies with incomplete or non-sequenced organisms in order to identify their proteins.[29–31] For all these reasons we decided to analyze spots of interest by linear ion trap combined with orbitrap mass spectrometer (LTQ-Orbitrap) yielding *de novo* protein sequences suitable for database searching, in order to increase the possibility of identification. A particular advantage offered by redox proteomics is that, while the abundance and activity of specific proteins may not necessarily have altered on proteomic comparison, clear evidence of OS-induced structural effects on protein structure can be adduced allowing identification of potential redox target proteins. This is a relatively novel approach in aquatic toxicology.

Materials and methods

BE (CAS number 519-09-5; purity >99%) was purchased from Alltech Applied Science (State College, PA, USA), while the other reagents used for biomarker measures were from Sigma-Aldrich (Steinheim, Germany). We diluted the methanol stock solution (1 g/l) to 10 mg/l in twice-distilled water (working solution) that was used to reach the selected concentration in exposure aquaria.

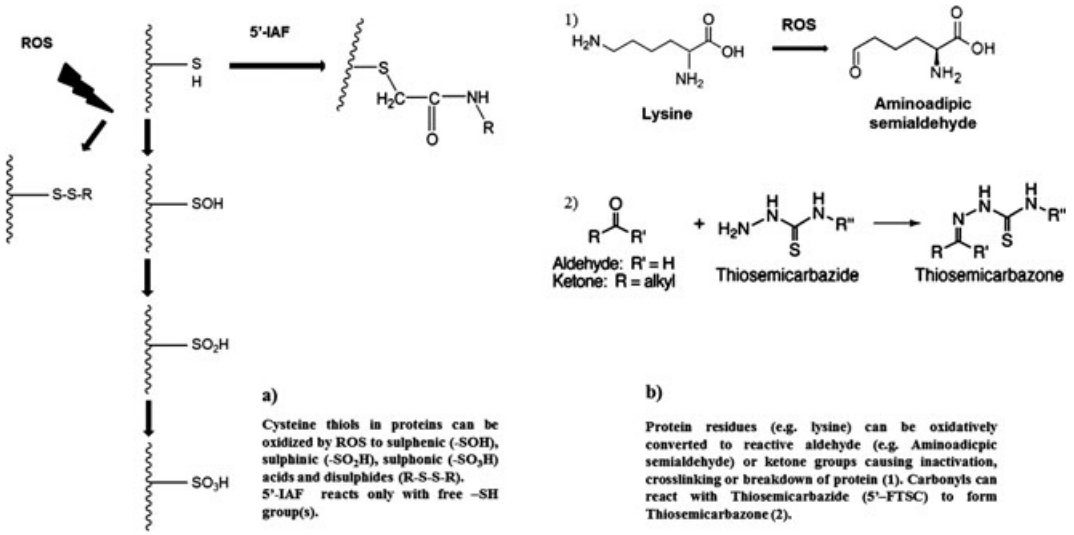

Figure 1. Oxidative modifications of amino acid side chains: oxidation of cysteine –SH groups (a) and carbonylation (b) and their specific labeling.

Dose selection

As no data about the toxicity of BE on *D. polymorpha* are available, drug doses according to the concentration-range reported in freshwaters were chosen: 0.5 μg/l (1.73 nM), and 1 μg/l (3.46 nM). The first was based on the highest BE concentration reported in European freshwater,[7] while the second was the highest concentration found in wastewater treatment plants (WWTPs). [32]

Exposures

D. polymorpha specimens were collected (3–6 m depth) in February 2011 from a reference site (GPS Coordinates: 45°52'0" N - 08°52'0"; Lake Lugano, Northern Italy) by a scuba diver and immediately transferred to the laboratory in bags filled with lake water. Colonized rocks were rinsed and introduced into 100-l glass aquaria filled with tap water, which was maintained at a natural photoperiod and constant temperature (20 ± 1 °C), pH (7.5) and oxygenation (>90% of saturation). The bivalves were fed daily with an algae replacement-substitute-enrichment medium (AlgaMac-2000, Bio-Marine Inc., Hawthorne, CA, USA), and water was changed regularly every two days for one week. After this period, several hundred animals with similar shell lengths (20 ± 2 mm) were gently cut from the rocks, placed on a glass sheet and maintained in 12-l aquaria filled with tap and de-chlorinated water (1:1 *v/v*) under identical conditions to those described above for two weeks to completely purify the molluscs from bio-accumulated pollutants. Only specimens able to re-form their byssus and re-attach themselves to the glass sheet were used for subsequent *in vivo* experiments.

A control and two exposure aquaria (one for lowest BE dose tested and the other for the highest one) were processed simultaneously. Exposures were carried out for 14 days and performed in semi-static conditions[33] with daily changes of the entire volume of water and the addition of the contaminant to yield the final test concentrations. This kind of exposure conditions should guarantee a constant BE concentration over a 24-h period. Indeed, a recent study[34] demonstrated that BE degradation does not occur in surface water after 35 h. Moreover, mussels were fed daily two hours before changing water to allow the intake of BE only by water. After 14 days, gills of 20 animals for each experimental condition were removed, frozen in liquid nitrogen and stored at -80 °C until required. In *D. polymorpha*, the gills are independent organs that can be easily dissected to offer a pure tissue sample.[35]

Sample preparation

Gills from individual mussels were pooled (4 pools of five animals from each experimental condition) and homogenized by RW20 digital laboratory stirrer in 3 volumes of buffer to weight (10 mM Tris/HCl, pH 7.2, 500 mM sucrose, 1 mM EDTA, 0.15 M KCl, 1 mM PMSF). The homogenate was centrifuged at 20,000×*g* at 4 °C for 1 h, and the supernatant was collected. Protein concentrations were determined by the method of Bradford (1976)[36] with bovine serum albumin (BSA) as a standard and fractions stored at -80 °C.

Labelling of proteins

Thiol groups in protein extracts were labelled by adding IAF in dimethyl sulfoxide to a final concentration of 200 μM and incubating at room temperature for 2 h in the dark. IAF is a thiol-specific reagent which reacts only with free thiols but not with oxidized variants such as sulfenic/sulfinic/sulfonic acids, disulfides or nitrosothiols (Figure 1a).[18] The fluorescein moiety of IAF provides a ready means for detecting proteins containing free thiols in electrophoretic separations. For fluorometric determination of protein carbonyl groups in oxidized proteins on polyacrylamide gels, carbonyls in protein extracts were labelled with FTSC to a final concentration of 200 μM and treated identically as for IAF. Before electrophoresis, proteins were precipitated using trichloroacetic acid (TCA) and acetone for samples labelled with IAF and TCA for those labelled with FTSC.

Electrophoresis procedures

After precipitation, samples (120 μg protein) were re-solubilized in a buffer containing 0.5 M Tris-HCl, pH 6.8, 0.26% glycerol, 0.2% sodium dodecyl sulphate (SDS; 10%) and 0.02% bromophenol blue (0.5%). Proteins were then resolved by 1DE on 12% SDS polyacrylamide (SDS PAGE) gels,[37] using an Atto AE-6450 mini PAGE system at 90 V for 1 h and 150 V until the electrophoresis was complete. Gels were scanned in a Typhoon 9400 scanner (GE Healthcare, Little Chalfont, Buckinghamshire, UK; Ex_{max} 490–495 nm; Em_{max} 515–520 nm) and were subsequently stained with Coomassie G250. Equal amounts of protein were loaded in 12 wells (3 technical replicates for each control and BE pool).

2DE analysis was performed on gill protein extracts for control and BE-treated pools (200 μg protein) incubated with IAF or FTSC respectively, and precipitated as described above. Four biological replicates were obtained from four gill pools for each treatment. After protein labelling, gill protein extracts were re-suspended in rehydration buffer containing 5 M urea, 2 M thiourea, 2% CHAPS, 4% ampholyte (Pharmalyte 3-10, Amersham-Pharmacia Biotech, Little Chalfont, Bucks., UK), 1% Destreak reagent (Amersham-Pharmacia Biotech) and trace amounts of bromophenol blue. A final volume of 125 μl was loaded on 7-cm pH 3 to 10 nonlinear immobilized pH gradient (IPG) strips (BioRad, Hercules, CA, USA) and rehydrated overnight for at least 15 h in the dark. Proteins were focused in a Protean isoelectric focusing (IEF) Cell (BioRad) with linear voltage increases: 250 V for 15 min: 4000 V for 2 h; then up to 20 000 Vh. After focusing, strips were equilibrated for 20 min in equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% dithiothreitol and then for 20 min in equilibration buffer containing 2.5% iodoacetamide. Equilibrated strips were embedded in molten agarose (0.5%) containing a trace of bromophenol blue atop 12% SDS PAGE gels and were electrophoresed at 4 °C at 90 V for the first 30 minutes and then at 150 V until the dye front reached the end of the gel. 2DE gels were scanned (Typhoon 9400 scanner) as described above to reveal thiol-containing proteins labelled with IAF and ketones labelled with FTSC. Gels were then visualized by Coomassie G250 to reveal total protein.

Quantification of proteins

For each 1DE gel, all bands detected by the Typhoon 9400 scanner were subsequently analyzed by Quantity One image analysis software (BioRad, Hercules, CA, USA) measuring the total intensity for each lane (Figures 2a and 2b). They were quantified as arbitrary units (A.U.). All 1DE gels stained with Coomassie blue G250 were scanned in a GS-800 calibrated densitometer (BioRad

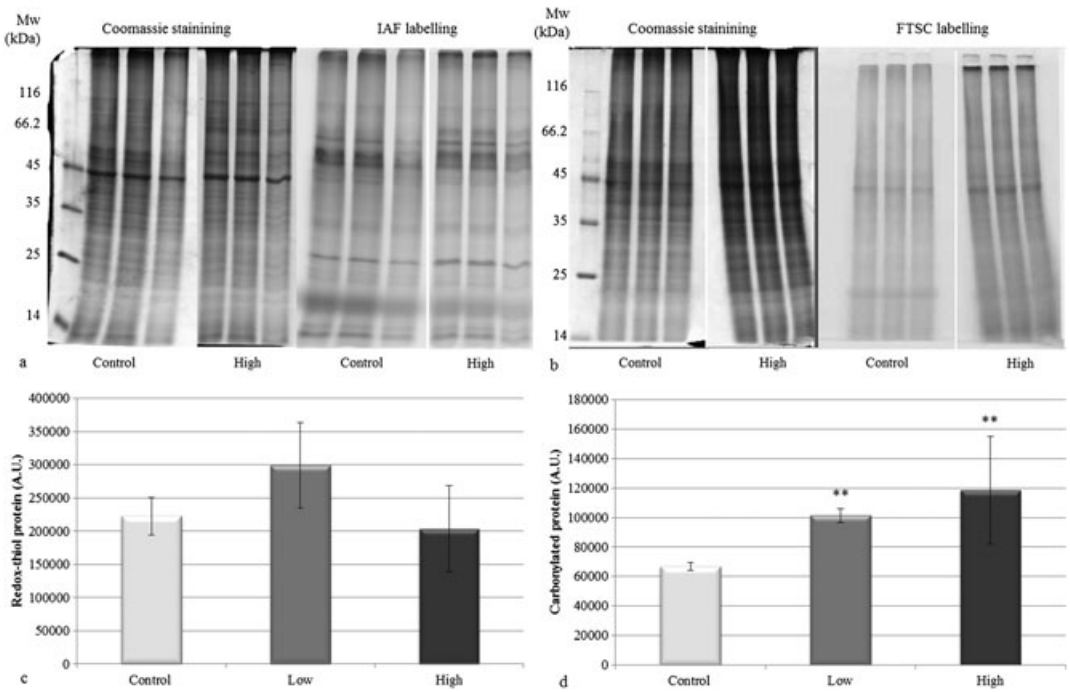

Figure 2. (a), (b) Representative images of 1DE gels stained with Coomassie G-250 (scanned with GS-800 calibrated densitometer) and with IAF/FTSC labeling (scanned with Typhoon 9400 scanner). Total optical densities for each lane obtained from FTSC and IAF staining were normalized with those from Coomassie G-250 staining of the same gel. (c) Levels of proteins containing thiol groups in gills of *D. polymorpha* exposed to BE. (d) Levels of proteins containing carbonyls in gills of *D. polymorpha* exposed to BE. Values shown are averages of total band optical density ± S.D. of each lane of one dimensional gel. Analyses were performed in triplicate (ANOVA, Dunnett *post-hoc* test; **$p<0.01$).

Hercules, CA, USA) and the optical density from each lane was measured by Quantity One image analysis software as described above (Figures 2a and 2b). Total optical densities for each lane were normalized with those from Coomassie staining from the same lane in each gel. Three replicates (technical replicates) from four different extracts (biological replicates) for each treatment were performed in 1DE. Values (means ± SD) were compared by one-way analysis of variance (ANOVA). The application of *Shapiro-Wilk* and *Levene's* tests assured the normal distribution ($p > 0.05$) and the homogeneity ($p > 0.05$) of data analyzed. The Dunnett *post-hoc* test was performed to discriminate between groups of means.

2DE image analysis and in-gel trypsin digestion

Scanned 2DE gel images (a total of 12 gel images, 4 gel images for each treatment) were analyzed using BioRad PDQuest software, version 7.3.1. During the analysis the fixed co-ordinates per gel were kept as 10x10, by identifying the smallest, faintest and largest spots with background normalization. Protein spots were automatically detected and the results were manually verified and edited where needed. Gels were matched using all-to-all spot matching, avoiding introduction of bias caused by the use of a reference gel. Matching was iteratively evaluated and parameters refined to optimize match quality. Integrated intensities were measured for each spot, background corrected, and then normalized with *Total Quantity in Valid Spots* method (the raw quantity of each spot in a member gel is divided by the total quantity of all the spots in that gel that have been included in the Master). This removed systematic gel intensity differences originating from variations in staining, scanning time and protein loading by mathematically minimizing the median expression difference between matched spots.

After 2DE, proteins were visualized with colloidal Coomassie Brilliant Blue G-250. To correct for possible inter-gel protein loading differences, the *Total Quantity in Valid Spots* for each fluorescent gel was normalized to the *Total Quantity in Valid Spots* in the corresponding Coomassie blue-stained gel. Normalized values from control group were compared with normalized values from BE treatment groups using a one-tailed paired Student's t-test. Spots of interest were then manually excised with a razor blade, chopped into 1 mm^2 pieces and collected into LoBind tubes (Eppendorf, Germany). Gel pieces were de-stained in 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) at 37 °C for 30 min, washed with 200 µl of 50% (vol/vol) acetonitrile in ammonium bicarbonate (50 mM, pH 7.4), dehydrated in 200 µl of acetonitrile for 30 s and completely dried in a speed-vac (Thermo Savant, Savant Instruments, Pittsburgh, PA, USA) after solvent removal.

Digestion was performed for 2 h at 37 °C with sequencing grade modified trypsin diluted in ProteaseMAX™ surfactant (Promega, Madison, WI, USA), which improves recovery of longer peptides and provides increased sequence coverage, as previously described.[25] Digests were centrifuged at 16 000×*g* for 10 s, and the digestion reaction with extracted peptides was transferred into a new tube. Trifluoroacetic acid was added to a final concentration of 0.5% to inactivate trypsin. Finally, samples were reduced (≈5 µl) by speed-vac and immediately analyzed.

Protein identification

Tryptic-digested samples (2 µl) were mixed with 2 µl of saturated α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid and spotted onto a MALDI target plate (1 µl). Mass spectra were acquired using an Ultraflex III Bruker MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany)

operating in reflectron mode with 20-kV accelerating and 23-kV reflecting voltages. MALDI mass spectra were calibrated using the Peptide Calibration Standard (700–4500 Da) from Bruker Daltonics. Data acquisition and processing were performed with flexControl and flexAnalyses software (Bruker Daltonics, Bremen, Germany) using a proprietary 'Top Hat' base-line tool along with the 'SNAP' peak detection algorithm, which was set to a signal-to-noise ratio of 6, maximal number of peaks as 100 and quality factor threshold of 50. The peptide mass list obtained was used for PMF database searching with the MASCOT search engine (http://www.matrixscience.com) in NCBInr/Swiss- Prot databases. The main search parameters were as follows: Metazoa (species); NCBInr/SwissProt (databases); 1.2 Da (mass tolerance); $[M+H]^+$ and monoisotopic; carbamidomethylation (fixed modification); oxidation at methionine (variable modification) and up to one allowed missed cleavage site. Results with confidence interval % (C.I. %) values greater than 95% based on MASCOT MOWSE were considered a positive identification.

Samples, which were analyzed firstly by PMF from MALDI-TOF without obtaining a positive match, were additionally analyzed using LIFT-TOF/TOF MS/MS from the same target. At least one precursor ion (the most intense one) per sample was chosen for MS/MS analysis. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of jointly-migrating parent and fragment ions in a timed ion gate, ions were lifted by 19 kV to high potential energy in the LIFT cell.

After further acceleration of the fragment ions in the second ion source, their masses could be simultaneously analyzed in the reflector with high sensitivity. As for PMF, LIFT spectra were interpreted with the Mascot software (Matrix Science Ltd, London, UK). Database searches, through Mascot were performed via BioTools 2.2 software (Bruker Daltonics, Bremen, Germany). MS/MS tolerance of 0.5 Da and one missing cleavage site for MS/MS search were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as a criterion for correct identification.[38,39]

Protein in-gel digestion and identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Protein in-gel digestion was performed according to Marsoni *et al.*,[40] with some modifications. Briefly, excised 2DE gel fragments were incubated three times in 200 µl of HPLC-grade H_2O and 200 µl of 50% acetonitrile (ACN) (both steps for 15 min at room temperature). Gel pieces were then dehydrated in 70 µl of 100% ACN for 5 min, hydrated in 70 µl of 100 mM ammonium bicarbonate (NH_4HCO_3) for 5 min, washed in 70 µl of 50 mM NH_4HCO_3/50% ACN for 15 min and dried in a vacuum centrifuge. For protein digestion, 30 µl of trypsin solution [Promega; 20 ng/µl in 50 mM NH_4HCO_3, pH 8] were added to each sample and incubated for 45 min at 4 °C. The supernatants were replaced with 30 µl of 50 mM NH_4HCO_3 and samples were finally incubated for 16 h at 37 °C. Gel pieces were sonicated for 15 min in a cool water bath, centrifuged at 12,000×g for 15 min and the resulting peptide mixture was acidified by addition of 1 µl TFA per sample. After collecting supernatants, tryptic fragments were extracted from gels as described by Marsoni *et al.*,[40] redissolved in 40 µl of 0.1% formic acid and stored at −80 °C. Tryptic peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Ultimate 3000 (Dionex) HPLC coupled with a high resolution LTQ-Orbitrap spectrometer (Thermo). Chromatography separations were conducted on a XBridge C18 column (300 µm I.D. × 100 mm length and 3.5 µm particle size, Waters), using a linear gradient from 5 to 90% acetonitrile, containing 0.1% formic acid with a flow of 4 µl/min, including the regeneration step. A typical separation lasted 70 min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of 250–2000 m/z followed by full MS/MS scan for the most intense ion from the MS scan). Raw data files were converted to mzXML/dta formats and processed through the Global Proteome Machine (GPM) software (www.thegpm.org). MS/MS spectra were searched against all databases by this tool. Moreover, we performed *de novo* sequencing from mass spectra data using the Pepnovo algorithm. Resulting peptides were investigated by the sequence similarity search algorithm MS BLAST (http://genetics.bwh.harvard.edu/msblast/index.html). As indicated by Habermann *et al.*,[41] significance of hits was evaluated according to the MS BLAST scoring scheme: for every reported hit, the score of the top-ranked HSP was compared with the corresponding threshold score for a single-matched HSP from the MS BLAST scoring table, depending on the number of queried peptides.

Results and discussion

Protein thiols and carbonyls

1DE revealed that BE did not cause extensive oxidation of thiol-containing proteins (Figure 2c). This may be because cysteine is the second least-abundant residue in proteins[42] and therefore may not be quantitatively modified by ROS induced by BE. OS may also cause conformational changes that render some protein thiols more reactive toward cationic groups and changing their susceptibility to alkylation, either by increasing their exposure to the matrix or by decreasing their *pKa* values.[43] In addition, transient OS may trigger protective mechanisms such as protein S-glutathionylation and formation of disulphides to protect thiols against irreversible oxidation.[44,45] Our findings reflect those of previous work on *Mytilus edulis*, which showed that gold nano-particles and menadione caused significant decrease of total protein thiols in digestive gland, but not in gills or mantles.[23] By contrast, after 14 days exposure, a significant ($p < 0.01$) increase in carbonyl groups at both doses was observed (Figure 2d) with a clear dose/effect relationship ($F = 9.23$; $p < 0.01$). Carbonylation is a quantitative protein modification in response to OS which is often triggered by hydroxyl radicals. It is an irreversible modification and is a widely used general marker for oxidative damage in proteins.[46,47] FTSC labelling has previously been used in the detection of protein carbonyls and FTSC-labelled proteins can be detected without Western blotting.[48,49] Considering these results, it is plausible that xenobiotics entering animals via gills may cause transient OS resulting in protein glutathionylation and long-term chronic exposure to pollutants. This, in turn, can result in higher levels of irreversible modifications such as carbonylation. These general effects were investigated in more depth by 2DE.

Protein oxidation patterns in 2DE separations

Representative 2DE images of cytosolic soluble proteomes from zebra mussel gill tissue are shown in Figure 3. Approximately

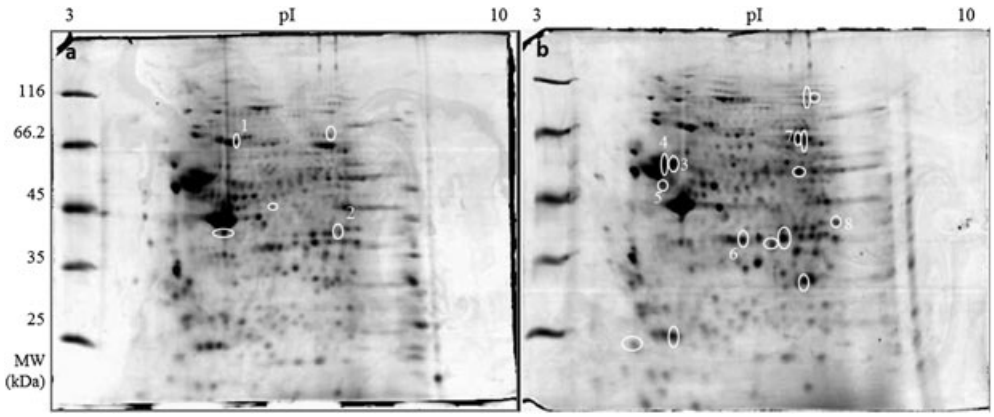

Figure 3. Protein expression profiles (PEPs) of mussels exposed to BE. Proteins were separated by 2-D SDSPAGE and stained with colloidal Coomassie Brilliant Blue G-250. Figures show spots of interest in representative gels belong from IAF (a) and FTSC (b) treated samples. Numbers correspond to those in Table 1 (spots identified). Additional fluorescence gels images are reported in Supplemental material (Fig. A and Fig. B).

350 fluorescently-labelled protein spots per gel were visualized and detected using the Typhoon scanner and these were analyzed by PDQuest. Comparison of control and exposed mussels highlighted a total of 11 (low dose) and 13 (high dose) differentially expressed thiol-containing protein spots (fold > 1.5; $p < 0.05$), respectively. Matching based on spot volume revealed 8 and 11 thiol-containing protein spots with higher volumes in exposed mussels to the lowest and highest dose, respectively, than controls ($p < 0.05$), of which 5 spots were most significant ($p < 0.01$). These results seemed to confirm those obtained from 1DE, since BE did not cause stronger effect on thiol-containing proteins of exposed samples compared to controls. Comparison of carbonyl-labelled patterns revealed a higher number of differentially expressed spots ($p < 0.05$): 23 (low dose) and 35 (high dose). Moreover, 4 spots disappeared in treatment samples. At the lowest dose tested, matching revealed 11 spots with higher volumes in treated mussels than controls ($p < 0.05$), of which 7 were most significant ($p < 0.01$). Instead, 12 spots showed lower volumes in treatment samples than controls ($p < 0.05$), of which 4 were most significant ($p < 0.01$). At the highest dose tested matching revealed 32 spots with lower volumes in treatments than controls ($p < 0.05$), 9 of which were most significant ($p < 0.01$). Only 3 spots had higher volumes in exposed mussels compared to controls. The high number of varying spots, especially at the highest dose tested, demonstrated a strong effect of BE on carbonyl groups, suggesting a role for BE in the alteration of redox status in *D. polymorpha* gills and confirming the results obtained with 1DE. As mentioned in the section 1, identification of altered protein expressions that are induced by only one stressor may not be sufficient to identify the toxicity pathways or the MOA of a chemical. For this purpose, we analyzed the pattern of variation at two different doses and comparison of IAF labelling highlighted only one spot varying in common between treatments, while, with FTSC labelling, 9 spots varied in common between treatments, of which one showed increased intensity while the others decreased. Always for FTSC, among the four spots which disappeared in treatment samples, two were in common between concentrations. Despite the high number of spots varied for each treatment a limited number of spots varied in common and a higher number of spots varied at the highest dose tested. This uncoordinated response to different concentrations of the same compound is not surprising and could be explained by the principle that the severity, or the probability of effect, may be related to the dose or exposure level.[50] Actually in the toxicoproteomics field, investigations of toxicant exposure dose-dependent changes have been reported.[51,52] Despite this, in a previous study on *D. polymorpha* we found no proteins varying in common between two different benzo[α]pyrene concentrations.[25] In this regard we can consider that changes produced by external factors, such as toxicant administration, can cause unpredicted perturbations in the levels of individual proteins and responses of individual proteins may not be linear when a cell is exposed to various concentrations of a chemical. It can therefore be very difficult to establish a reliable dose-response relationship for a proteome.[53] We could hypothesize that this kind of response is strictly dependent on the selected contaminant, the doses tested and the biological model used. It should be clearly noted that only spots carrying a fluorescent label are detected in the present study and interpretation must therefore focus on availability of carbonyl or thiol group, respectively, for each fluorescent label. This is a novel means of detecting redox-mediated structural effects on target proteins. However, no inferences can be drawn regarding either the abundance or activity of particular proteins as would be usual in expression proteomics studies.[51]

Protein identification

Among differentially labelled protein spots we selected only the most intense and well-defined (20 protein spots in total) for subsequent MS analysis (Figure 3). Some spots were either too low in abundance and/or too close to each other to be isolated and excised with certainty. In particular, we focused our attention on 7 spots (1 belonging to the IAF group and 6 to the FTSC group) that varied in common between concentrations. These were analyzed by LC-MS/MS, to try to obtain more information on the MOA of BE. Among those 7 spots 3 proteins were successfully identified (Table 1c/d). Other 5 proteins, of 13 analyzed, were positively identified using MALDI-TOF/TOF (Table 1a/b). In conclusion, from of the total number of 20 spots analyzed, 12 proteins were not identified successfully, most likely due to low protein abundance producing insufficient tryptic peptides for MS, or the database searching scores being too low to yield unambiguous matches.

As shown in Table 1, we identified hsc70 which showed oxidation of thiols, and a cytochrome c that was subject to an increase

Table 1. Identified proteins differentially expressed in *D. polymorpha* gills cytosolic fraction after BE exposure by using Mascot, GPM and MS BLAST

a) Identification performed with PMF and MASCOT

Spot[a]	AC n° [b]	Homologous protein (*Organism*)	Biological role/process	Theoretical/experimental Mw (kDa)	Theoretical/experimental pI	MASCOT Score[c] (%)	No. matched/submitted peptide	Variation (fold > 1.5; $p < 0.05$)[d]
1 IAF	Q9U639	Heat shock 70 kDa protein cognate 4 (*Manduca sexta*)	ATP binding/response to stress	71/66	5.3/5.4	60 (19%)	11/26	↓
4 FTSC	P41383	Tubulin alpha-2/alpha-4chain (*Patella vulgata*)	Cytoskeletal	50/55	4.9/4.9	181 (39%)	15/18	↓
5 FTSC	P30883	Tubulin beta-4 chain (*Xenopus laevis*)	Cytoskeletal	50/50	4.8/4.9	184 (39%)	20/27	↓

b) Identification performed with LIFT-TOF/TOF MS/MS and MASCOT

Spot[a]	AC n° [b]	Homologous protein (*Organism*)	Biological role/process	Theoretical/experimental Mw (kDa)	Theoretical/experimental pI	MASCOT Score[e] (%)	Observed/calculated mass of selected peptide (peptide sequence)	Variation (fold > 1.5; $p < 0.05$)[d]
2 IAF	P04970	Glyceraldehyde-3-phosphate dehydrogenase 1 (*Caenorhabditis elegans*)	Oxidoreductase activity/glycolysis	36/40	7.6/6.9	71 (4%)	1765.8/1764.8 Da (K.LVSWYDNEGYSNR)	↑
8 FTSC	P54216	Fructose-bisphosphate aldolase (*Caenorhabditis elegnans*)	Catalyticactivity/ glycolisis	39/40	6.3/7	57 (3%)	1325.8/1324.7 Da (K.KPWALTFSYGR)	↓

c) Identification performed using MS/MS data and the GPM

Spot[a]	AC n° [b]	Homologous protein (*Organism*)	Biological role/process	Theoretical/experimental Mw (kDa)	Theoretical/experimental pI	log(e)[f]	Peptides	Variation (fold > 1.5; $p < 0.05$)[d]
3 FTSC	Q5H7U8	Tubulin (*Crassostrea gigas*)	Cytoskeletal	50/50	4.9/5	-84.0	TIGGGDDSFN TFFSETGAGK AVFVDLEPTV VDEVR QLFHPEQLVT GKEDAANNYA R EIVDLVLDR IHFPLATYAP VISAEK DVNAAIATIK TIQFVDWCPT GFK LIGQIVSSIT ASLR VGINYQPPTV VPGGDLAK VPGGDLAKVQ R	↓

(Continues)

Table 1. *(Continued)*

Spot[a]	AC n° [b]	Homologous protein (*Organism*)	Biological role/process	Mw (kDa)	p*I*	MASCOT Score[c] (%)	No. matched/submitted peptide	Variation (fold > 1.5; $p < 0.05$)[d]
6FTSC	Q7Y6Q1	Cytochrome c subunit I (*Ruditapes philippinarum*)	Oxidoreductase activity/transport	28/40	7.1/5.8	-7.1	GTMYFMFSIW SGLMGTGLS MLEDGQLYNL VVTAHGLVMI FFLVMPMMIG GFGNWLIPLM LK MILLLGSTYV DGGAGTGWTI YPPLSSIGYH SGR	↑
d) Identification performed using *de novo* sequencing with MS BLAST				Theoretical/experimental				
Spot[a]	AC n° [b]	Homologous protein (*Organism*)	Biological role/process	Mw (kDa)	p*I*	HSPs Score[g] (%)	Alignement	Variation (fold > 1.5; $p < 0.05$)[d]
7 FTSC	Q0KHB7	Phosphoenolpyruvate carboxykinase [GTP] (*Crassostrea gigas*)	Nucleotide binding/ gluconeogenesis	71/66	6.5/6.6	108 (55%)	Query: 1 BXXXXSFGSGYGGNSLLGKBXXXXXXA 27 + SFGSGYGGNSLLGK+ A Sbjct: 235 RREIVSFGSGYGGNSLLGKKCFALRIA 261	↓

[a]Spot number corresponding to spots in Figure 3.
[b]Accession number in SwissProt database.
[c]MASCOT score (Matrix Science, London, UK; http//www.matrixscience.com) obtained from PMF analysis, (%) sequence coverage.
[d]Fold change increase (↑) or decrease (↓) (in terms of relative spot volume, %V).
[e]MASCOT score (Matrix Science, London, UK; http//www.matrixscience.com) obtained from LIFT-TOF/TOF MS/MS, (%) sequence coverage.
[f]the base-10 log of the expectation that any particular protein assignment was made at random (E-value).
[g]Score of the top-ranked HSP compared with threshold score for a single-aligned HSP determined for each size of the query. (%) identities (# of amino acid residues identified in MS/# of amino acids in database protein sequence) × 100.

in carbonylation. These results are very interesting since both of these proteins are involved in the oxidative stress process.[54,55] Furthermore, spot 6 (identified as cytochrome c) was one of the spots that varied in common between doses. In a recent study the carbonyl contents of control and oxidized horse (*Equus caballus*) cytochrome c (HCC) was evaluated as a conventional biomarker of protein oxidation after *in vitro* cell exposure to UV/H_2O_2.[56] With extended reaction time (oxidation dose), these workers observed a significant increase in protein carbonyl content. Considering these findings and, bearing in mind the 1-DE results, it is likely that BE acts as a pro-oxidant in *D. polymorpha*. Since no data are available on the MOA of BE in invertebrates, we can only compare our data to results obtained in vertebrates. In particular, several studies carried out in humans and mammals have already demonstrated how negative effects of BE are greater than those caused by cocaine itself[57] and a temporal association between recurrent cocaine-induced coronary vasoconstriction and elevated blood concentrations of the major cocaine metabolites has been demonstrated.[58] BE exerts vasoconstriction by acting on the influx of extracellular calcium. Abnormal variations of intracellular calcium concentration are related to increased ROS levels, activation of antioxidant defense mechanisms and trigger irreversible cellular events leading to apoptosis.[59] Moreover, Goldstein *et al*.[60] suggested that the BE half-life (about 5–6 h) – much longer than that of cocaine (0.7–1.5 h) – can be related to long-term BE toxicity.

Three different forms of tubulin (one of which varied in common between doses) were identified. These showed a decrease of carbonylation on BE treatment. This situation could be due to glutathionylation, which may be an adaptation of mussel gill tissue to allow animals to survive in the presence of high oxidative stress. Previous studies conducted in rats[61] and humans[62] showed highly-abundant proteins such as cytoskeletal proteins were glutathionylated during diamide stress thus preventing irreversible oxidation. Furthermore, McDonagh *et al*.[63] have demonstrated that, in mussels, some low-abundance proteins are heavily carbonylated while some abundant proteins are not carbonylated at all. In the same study they observed that gill in mussels is the main site of protein glutathionylation and carbonylation as a result of oxidative stress and that this tissue can also be affected in animals experimentally exposed to high levels of the pro-oxidant H_2O_2. To verify our hypothesis it would be interesting to investigate glutathionylation levels also in *D. polymorpha* gills after exposure to BE.

Variation of three enzymes involved in glycolytic and/or gluconeogenic pathways was also observed (Table 1). This is of interest since up- or down-regulation of metabolic enzymes are often related to stress status after exposure to chemical contaminants or OS. Thus, the alteration of redox status could further confirm the ability of BE to trigger OS in *D. polymorpha* with implications for energy metabolism. Gillardin *et al*.[52] found an overexpression of such enzymes in *Xenopus laevis* after exposure to polychlorinated biphenyl mixture Aroclor 1254. This result could be due to an increased requirement for both energy and protein synthesis/degradation pathways.[64] Shi *et al*.[65] demonstrated that increased cellular ROS level benefits human cancer cells through up-regulation of glycolysis. Finally, the analysis of protein expression profiles about the quantitative variation of spots after exposure to BE, could confirm our hypothesis, since it could be possible that BE up-regulates the glycolytic/gluconeogenic pathways. Key antioxidant systems such as thioredoxin, glutaredoxin, glutathione and glutathione-ascorbate cycle depend heavily on $NADPH^+$ rather than $NADH^+$ for reducing equivalents. On exposure to pro-oxidants, cells need to shift rapidly from pathways producing $NADH^+$ to others – such as the pentose phosphate pathway – that produce $NADPH^+$. In this regard, it is interesting to note that glyceraldehyde 3-phosphate dehydrogenase is a well-known redox sensor in cells, as well as being a glycolytic enzyme.[66]

Conclusions

This is the first study using fluorescence-based detection of redox modification of *D. polymorpha* proteins and it demonstrates sublethal effects of BE on this aquatic organism. Notwithstanding the use of powerful tools such as LC-MS/MS and *de novo* sequencing, the poor representation of this organism in sequence databases makes extensive interpretation of proteomic results problematic. However, the present report illustrates the potential of proteomic techniques to uncover differences in protein redox status in *D. polymorpha*. We detected a clear increase of protein carbonylation after exposure to BE suggesting the presence of OS especially at the highest dose tested. BE leads to modification of some proteins involved in glucose metabolism and some which are cytoskeletal components of the cell. Moreover, identification of a heat shock protein and cytocrome c suggests significant cellular stress under the BE exposure regime used here. In future work it will be important to test differing doses of contaminants to understand better how BE induces the changes observed in the identified proteins and how these changes may affect associated cellular processes in this mussel.

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Chapter 4 – *GENERAL CONCLUSIONS and REMARKS*

Even if scientific literature highlighted that the PPCPs widespread distribution is a well-established environmental issue, their toxic potential is only partially investigated, since only few aspects of their toxicity have been pointed out. Our research group, in the past few years, tried to give a more comprehensive view about this topic. In order to reach this goal, a stepwise *in vivo* approach using a suite of biomarkers has been used, with an increasing attention toward the comprehension of the mechanisms of toxic action.

On the basis of results obtained in the present work we were able to conclude that among PPCPs tested, TCS showed highest cyto-genotoxicity on *D. polymorpha*, and after verifying its antioxidant enzyme activities in response to TCS exposure, we inferred that there are different complementary mechanisms of action to explain the genotoxicity of this personal care product in zebra mussel, connected both to oxidative stress and/or to a direct effect on DNA. Moreover, data collected from the analysis of different biomarkers end-points and their subsequently integration through the biomarker response index allowed us to draft a toxicity scale of selected molecules (TCS>TMP>IBU>DCF>PCM), highlighting that adverse effects on aquatic biocoenosis of some PPCPs at environmental concentrations should not be underestimated.

A similar approach has been employed also to test cyto-genotoxicity of the emerging pollutants cocaine and benzoylecgonine. This research is the first regarding the effects on non-target organisms of this kind of substances as new environmental contaminants. Illicit drugs were recognized as emerging pollutants only in recent few years, since their presence in the aquatic environment has been demonstrated worldwide at concentrations similar to those of PPCPs products. Our findings pointed out a clear cyto-genotoxic effect of CO on *Dreissena polymorpha* after a short-time exposure, since our first goal was to identify the possible cyto-genotoxic effects of environmental cocaine concentrations. A more in-depth study on late effects of BE (the main metabolite of CO) showed remarkable adverse effects at environmental concentrations, highlighting its possible hazard to freshwater communities. Also in this case, we focused our attention on the mechanism of toxic action of these molecules especially for what concern BE, since it showed higher cyto-genotoxicity than the parental compound. The proteomic approach gave us the possibility to confirm the BE mechanism of action inferred from biomarkers results: BE exerts its toxic action on zebra mussel through the increasing of oxidative stress, compromising also the energetic metabolism. CO and BE constitute a considerable hazard to *Dreissena polymorpha* and possible adverse effects on the entire aquatic biocoenosis could not be underestimated.

We hope that this first ecotoxicological investigation could be the starting point to a more in-depth study on the potential environmental risk for this kind of contaminants, especially considering the occurrence of measurable concentrations of several other illicit drugs in freshwaters and their possible high biological activity. Moreover, given their continuous input in aquatic ecosystem, studies about late effects of illicit drugs must be conducted, even at higher concentrations than environmental ones, to better understand their mechanisms of action. Furthermore, considering that aquatic organisms live closely with all these substances for the throughout their life-cycle, it should be interesting also to investigate the effects of a mixture of illicit drugs and pharmaceuticals, since addictive and synergic effects are plausible. Our research highlighted moreover the suitability of a battery of biomarkers in order to obtain a more comprehensive view of adverse effects caused by a pollutant at different biological levels and the *in vivo* approach is of crucial importance to take into account the mechanisms of defense of the biological model considered, in order to infer the mechanisms of toxicity. However, our findings confirmed also that suggested mechanism of action needs to be confirmed by using more powerful techniques, such as proteomics, especially when an adequate information on the contaminant ecotoxicity is lacking. In the present study this approach has allowed us to obtain a more accurate overview on the real toxic potential and MoA of new environmental pollutants.

Finally, *Dreissena polymorpha* has proved once again to be a very useful biological model for ecotoxicological studies, but to improve our knowledge about this organism, more proteomics and genomics studies are necessary, in order to enlarge data available in databases.

Chapter 5 – *REFERENCES*

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Appendix *– OTHER PUBLICATION*

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Variation of Antioxidant Activity in *Dreissena polymorpha* Specimens Exposed to 2,2′,4,4′,5,6′-Hexa BDE (BDE-154)

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Abstract We evaluated the imbalance of the oxidative status in zebra mussel (*Dreissena polymorpha*) specimens exposed for 96 h to environmentally relevant concentrations (0.1, 0.5, and 1 μg/L) of the 2,2′,4,4′,5,6′-hexa BDE (BDE-154). The activities of three antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and the phase II detoxifying enzyme glutathione S-transferase (GST), were measured in the cytosolic fraction from a pool of zebra mussels. Significant variations in the activity of each single enzyme were noticed at each treatment, indicating that exposure to BDE-154 was able to impair the oxidative status of treated bivalves through the increase of reactive oxygen species. In detail, SOD and GPx were significantly induced, while CAT and GST were depressed with respect to the baseline levels. These data have confirmed that the raise of oxidative stress is the main cause of the BDE-154-induced genetic damage observed in a previous study on the zebra mussel.

Keywords 2,2′,4,4′,5,6′-Hexa BDE (BDE-154) · Antioxidant enzymes · Zebra mussel

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1 Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of halogenated organic compounds that are widely used as flame retardants in electronic equipment, plastics, textiles, and building materials. PBDEs potentially contain 209 different congeners, varying in both number and position of bromination, but only few of them are included in the commercial PBDE formulations, named penta-, octa-, and deca-BDE, respectively. Since the 1970s, these chemicals have been extensively used in several industrial applications. However, recently, due to growing environmental and human health concerns, penta- and octa-formulations were banned in European Union in 2004 (Restriction of Hazardous Substances Directive; EU 2002/95/CE), while in USA, their manufacturing was voluntary ended. Lastly, also the use of deca-BDE in the EU has been banned since 1 July 2008 (European Court of Justice 2008). Notwithstanding, recent restrictions have not eliminated PBDE releases from products currently in-service or new manufactured products (La Guardia et al. 2006). Since PBDEs are polymer additives and are not chemically bound to materials, they are known to leach into the surrounding environment as a result of manufacturing, usage, and disposal of PBDE-containing products (de Wit 2002), so they have become ubiquitous contaminants in the environment. In recent years, several researches have been focused on PBDEs because of growing concerns about their occurrence, bioaccumulative potentials in wildlife and human tissues (de Wit 2002; McDonald 2002), and

continuously rising concentrations with a doubling time of about 5 years in human blood, milk, and tissues during the past 30 years (Hites 2004). To date, investigations have reported over than 40 specific PBDEs, ranging from mono- to deca-BDEs in dissimilar environmental matrices. Among these, in contrast to the production capacity, the less brominated BDEs, in particular the main components of the penta-BDE formulation, namely tetra- (BDE-47), penta- (BDE-99, −100) and hexa-BDE (BDE-153, −154), are the predominant congeners detected in biological samples (Wollenberger et al. 2005). Nonetheless their widespread distribution and their high bioavailability observed across several species, including fish and mussels (Darnerud 2003), current knowledge on potential toxic effects induced by PBDEs is limited. Some studies investigated the toxicity of such BDE congeners and/or their hydroxylated metabolites on classical mammalian models, pointing out a wide range of effects, such as alteration in phase I biotransformation, morphological changes in hepatic and thyroid size and histology, interference with thyroid hormone homeostasis, developmental neurotoxicity, and immunological alterations (Darnerud 2003). In aquatic organisms, such as fish, PBDEs seem to have a low acute toxicity, but they were able to inhibit liver enzyme activity and to affect phase I and phase II biotransformation, produce fatty livers, alter blood levels of glucose and hematocrit, and reduce spawning success (Holm et al. 1993; Tjarnlund et al. 1998). Unfortunately, to date, toxicity information on invertebrates is limited to few studies regarding selected congeners, which have revealed BDE-induced adverse effects on the development, reproduction, and growth rate in algae, crustaceans (Breitholtz and Wollenberger 2003; Kallqvist et al. 2006), and shrimps (Wollenberger et al. 2005; Key et al. 2008). In addition, a recent study by Parolini and Binelli (2012) have highlighted as two low-brominated congeners, namely BDE-100 and BDE-154, were able to induce cyto-genotoxic responses in zebra mussel specimens, suggesting that the raise of genetic damage could be due to the onset of oxidative stress. At present, the information on the potentiality of BDE congeners to increase the oxidative stress and produce DNA damage is limited to BDE-47 and BDE-209 (He et al. 2008; Jin et al. 2010; Yan et al. 2011), but any information can confirm this relationship for other compounds. So, the aim of the present study was to assess the potential capability of BDE-154 to imbalance the oxidative status of *Dreissena polymorpha*-treated specimens and confirm its genotoxic mechanism on this bivalve species. Zebra mussels were exposed to three BDE-154 environmentally relevant concentrations (0.1, 0.5, and 1 μg/L), and the activities of three antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and the phase II detoxifying enzyme glutathione S-transferase (GST), were measured in the cytosolic fraction extracted from a pool of bivalves. Simultaneously, the assessment of levels of each single congener in zebra mussel soft tissues was performed, allowing an investigation of the concentration–effect relationship.

2 Materials and Methods

2.1 Reagent and Standards

Standard of 2,2′,4,4′,5,6′-hexa BDE (BDE-154; CAS number 207122-15-4) used in exposure assays was purchased from AccuStandard (New Haven, CT, USA). All reagents and chemicals used for biomarker determination were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents used in chemical analyses were pesticide grade. Florisil (100–200 mesh) was obtained from Fluka (Steinheim, Germany), while silica gel for column chromatography (70–230 mesh) was supplied by Sigma-Aldrich (Steinheim, Germany). BDE–commonly occurring congeners mixture used for quali-quantitative determination of investigated congeners and $^{13}C_{12}$-labeled mixture used as internal surrogate standard (PDE-MXB; composed by $^{13}C_{12}$-labeled BDE-28, 154, and 183) were purchased from AccuStandard (New Haven, CT, USA).

2.2 Mussel Acclimation and Maintenance Conditions

Several hundred zebra mussel specimens 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 chemical pollution (Binelli et al. 2005), and quickly transferred to laboratory in bags filled with lake water. After rinsing under running tap water, rocks were immersed into 100-L glass holding aquaria filled with tap and lake water (75:25 *v*/*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. Then, bivalves were maintained in tap water, dechlorinated by aeration, at natural photoperiod, constant

temperature (20±1°C), pH (7.5), and oxygenation (>90% of saturation) and fed daily with an algae replacement–substitute–enrichment medium (AlgaMac-2000®, Bio-Marine Inc., Hawthorne, USA). Water was regularly changed for 14 days to gradually purify mollusks of 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. They were placed on glass sheets suspended in 15-L aquaria filled with 10 L of dechlorinated tap water and maintained at the same conditions described above. Three hundred specimens were put in each aquarium. Only specimens that were able to re-form their byssi and reattach themselves to the glass sheet were used in the experiments. No mortality was noticed during 96 h experiments, and mussel viability was checked daily by the Trypan blue exclusion method. Bivalves were exposed to BDE-154 only when target biomarker levels were comparable with baseline ones obtained in previous studies (Binelli et al. 2009; Parolini et al. 2010).

2.3 Exposure Assays

In order to give a marked ecological relevance to our research, BDE exposure concentrations were as similar as possible to those measured in surface water. We selected 0.1, 0.5, and 1 μg/L of BDE-154 as experimental concentrations. Ninety-six-hour exposure assays were conducted in semi-static conditions since previous studies demonstrated that this period of time is enough to highlight sublethal effects on POP-treated zebra mussel specimens, including PBDEs, also exposed to low and environmentally relevant concentrations (Riva et al. 2007; Binelli et al. 2008a). Control, solvent control (0.1% of acetone; ACE), and exposure aquaria were processed at the same time. A working solution (10 mg/L) for each congener was prepared by diluting the BDE standard in acetone. Exact volumes of working solution were added to each exposure aquarium after the complete water change (10 L), until the desired concentrations were reached. The complete water and chemical change were carried out on a daily basis. This procedure should guarantee a constant concentration of BDE-154 over each 24-h period and prevent losses of contaminant, as well as the transformation of the parental compound into its metabolites. 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 chemical bioavailability. Temperature (20±1°C), pH (7.5), and oxygenation (>90% of saturation) were checked daily. Every 24 h, the entire soft tissue of 20 specimens from each aquarium, including control and solvent control, was immediately frozen in liquid nitrogen and stored at −80°C until the enzymatic activity was measured. Simultaneously, the soft tissues of other 33 specimens, used for a parallel investigation on BDE-154 cyto-genotoxicity, were pooled, frozen, and stored at −20°C until chemical analyses were performed. A detailed description of PBDE chemical analysis was reported elsewhere by Binelli et al. (2008b).

2.4 Enzyme Activities

A detailed description of the sample preparation procedures has been reported by Parolini et al. (2010). The activity of each enzyme (SOD, CAT, GPx, and GST) was measured in triplicate in the cytosolic fraction extracted from a pool of six to eight whole mussels (≈1 g fresh weight). The total protein content of each sample was determined according to the Bradford method (1976) using bovine serum albumin as a standard. Enzymatic activities were determined spectrophotometrically as described by Orbea et al. (2002). Briefly, The CAT activity was determined by measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 substrate in 67 mM potassium phosphate buffer (pH 7). The SOD activity was determined by measuring the degree of inhibition of cytochrome c (10 μM) reduction at 550 nm by the 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). The GPx activity was measured by monitoring the consumption of NADPH at 340 nm using 0.2 mM H_2O_2 substrate in 50 mM potassium phosphate buffer (pH 7) containing additional glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL), and NADPH (120 μM). Lastly, the GST activity was measured by adding reduced glutathione (1 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.

2.5 Statistical Analysis

Data normality and homoscedasticity were verified using the Shapiro–Wilk and Levene's tests, respectively. Two-way analysis of variance (ANOVA) was performed to

investigate possible time–effect and dose–effect relationships using time and BDE-154 concentrations as variables, whereas the biomarker endpoints served as cases. ANOVA was followed by the Bonferroni post hoc test to evaluate 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 performed on all of the measured variables in the three exposure assays to investigate possible correlations between investigated biological responses. All of the statistical analyses were performed using the STATISTICA 7.0 software package.

3 Results

3.1 BDE-154 Bioaccumulation Results

In Fig. 1, the concentration (log nanograms per gram lipid weight) of BDE-154 bioaccumulated during the exposures was reported. Baseline levels of BDE-154 were measured in the soft tissue of control and solvent control specimens and on average were 9.29±4.10 ng/g lipid weight. Values measured at the beginning of the exposure ($t=0$ h) were perfectly comparable with controls for each single BDE congener. Already after 48 h of exposure, concentrations of BDE-154 in zebra mussel soft tissues were on average 178-fold higher than those measured at $t=0$ h, while at the end of the tests, levels were on average 1,074-fold higher than background ones.

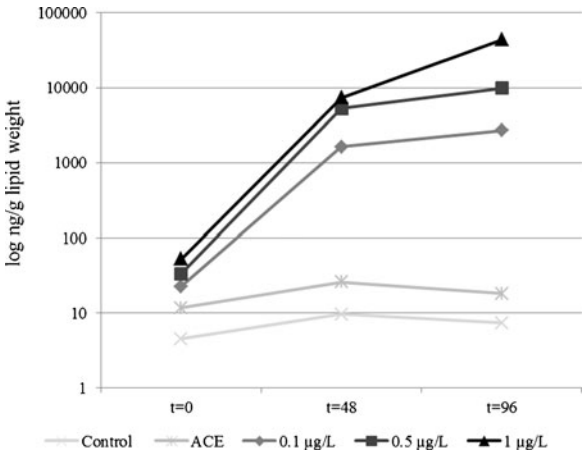

Fig. 1 Levels (log nanograms per gram lipid weight) of BDE-154 measured in the soft tissues of zebra mussel specimens ($n=1$, pool of 30 specimens) at the beginning ($t=0$) and after 48 and 96 h of exposure to the three selected treatments

3.2 Enzyme Activity Results

Baseline levels of each investigated enzyme activity were perfectly comparable with those obtained in previous studies (Binelli et al. 2009; Parolini et al. 2010, 2011a, b) and fell within the physiological range of this bivalve species. Overall, the statistical analysis showed significant ($p<0.05$) differences between each BDE-154 treatment and controls, as well as among treatments, for SOD, GPx, and GST. In addition, significant ($p<0.05$) differences in SOD, GPx, and GST activity were noticed between each exposure time ($t=24$; $t=48$; $t=72$, and $t=96$) and the beginning of the exposure ($t=0$). In detail, a significant increase of SOD activity (Fig. 2) was noticed already after only 24 h of exposure at each treatment, following a significant time-dependent ($F=140.93$; $p<0.01$) and concentration-dependent ($F=176.91$; $p<0.01$) relationship. At the end of each exposure, SOD activity was threefold higher than the correspondent baseline levels.

A progressive activation of GPx (Fig. 3) was observed in each experiment according to significant time- ($F=24.49$; $p<0.01$) and concentration-dependent ($F=27.21$, $p<0.01$) relationships. At 0.1 μg/L, GPx showed an increasing trend until 48 h of exposure, with values increased by 60% with respect to corresponding control, followed by a return to baseline levels at the end of the test. A significant ($p<0.01$) induction of GPx activity was noted after 48 h at 0.5 μg/L and after 24 h of exposure at 1 μg/L, showing at $t=96$ h values increased by 44% and 40% than the corresponding controls, respectively. The CAT activity (Fig. 4) followed a time-dependent relationship ($F=3.23$; $p<0.05$). A decreasing activity trend was found at each concentration (excluding to 0.5 μg/L from 72 up to 96 h), even if a significant ($p<0.05$) reduction of CAT was measured only after 96 h of exposure at 1 μg/L, showing values decreased by 33% with respect to the corresponding control. Lastly, the GST activity (Fig. 5) was characterized by both time-($F=79.08$; $p<0.01$) and concentration-dependent ($F=154.11$; $p<0.01$) relationships. At each treatment, the GST followed a significant ($p<0.01$) decrease of its activity already after only 24 h of exposure, showing values more than halved in comparison with the corresponding controls.

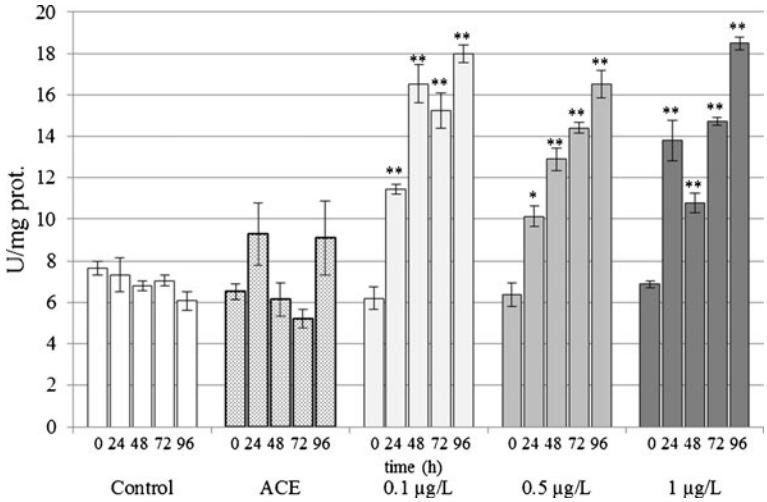

Fig. 2 Variations induced by BDE-154 treatments on the activity (mean ± SEM) of superoxide dismutase in the soft tissue of zebra mussels (n=3; pool of six to eight specimens). Significant differences (two-way ANOVA, Bonferroni post hoc test, *p<0.05; **p<0.01) were referred to the comparison between treated mussels and the corresponding control (time to time)

4 Discussion

Such researches have pointed out that the toxicity of brominated flame retardants, including PBDEs, may be the result of oxidative stress induced by the overproduction of reactive oxygen species (ROS) (Fonnum et al. 2006; Tseng et al. 2008). Currently, no data are available regarding the capability of BDE-154 to induce the imbalance of the oxidative status in the zebra mussel, even if Hakk et al. (2009) showed that oxidation and oxidative debromination are the favored metabolic pathways for BDE-154 in mammals. In a previous study, Parolini and Binelli (2012) investigated the sublethal effects (cyto-genotoxicity) induced by environmentally relevant concentrations of BDE-154 on zebra mussel specimens. The application of the neutral red retention assay (NRRA) highlighted that this congener was notably cytotoxic towards treated bivalves and it caused both primary and fixed genetic damage. Authors suggested that DNA injuries could be due to an impairment of the oxidative status of treated mussels, since the generic cellular stress pointed out by NRRA can be considered an indirect indicator of oxidative stress (Lowe et al. 1995). The destabilization of lysosomes in mussels, in fact, is affected by the production of oxyradicals generated by the exposure to contaminants, both internally and externally of the lysosome membrane (Regoli et al. 1998). Indeed, different alterations to these organelles (including damage to their membranes) have been related to the increase of peroxidative

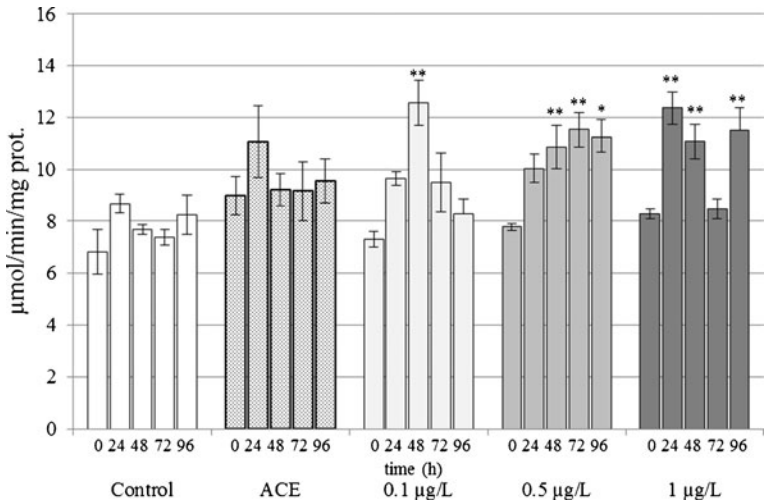

Fig. 3 Variations induced by BDE-154 treatments on the activity (mean ± SEM) of glutathione peroxidase in the soft tissue of zebra mussels (n=3; pool of six to eight specimens). Significant differences (two-way ANOVA, Bonferroni post hoc test, *p<0.05; **p<0.01) were referred to the comparison between treated mussels and the corresponding control (time to time)

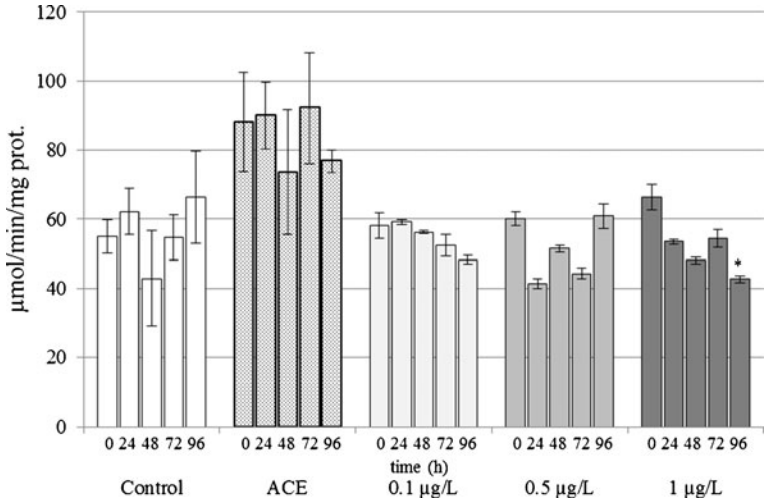

Fig. 4 Variations induced by BDE-154 treatments on the activity (mean ± SEM) of catalase in the soft tissue of zebra mussels (n=3; pool of six to eight specimens). Significant differences (two-way ANOVA, Bonferroni post hoc test, *p<0.05) were referred to the comparison between treated mussels and the corresponding control (time to time)

processes (Winston et al. 1996), which are common pathways of toxicity induced by several environmental pollutants and are associated with ROS. Chemical analyses pointed out a notable bioaccumulation of BDE-154 in zebra mussel soft tissues at each tested concentration (Fig. 1). After the exposure, bivalves put in action their mechanisms of defense, including P450 isoforms and dissimilar enzymes, in order to detoxify the administered chemical. During phase I enzyme-mediated reactions, the parent xenobiotic can be metabolized to form unstable radical metabolites which have the potential to attack oxygen molecules, giving rise to dangerous oxyradicals and thus causing oxidative stress (Kappus 1985). Biological systems have developed during their evolution adequate enzymatic (and nonenzymatic) antioxidant mechanisms to protect their cellular components from ROS and oxidative damage. Enzymatic antioxidant defense system is based on "cascade" reactions of SOD, CAT, and GPx and the correlation between BDE body burden concentrations and all the enzymatic responses (Table 1) suggested the activation of the whole defense chain against BDE-154-induced ROS. SOD is the first defense enzyme involved in the reactions against oxyradicals (Kappus 1986) and it catalyzes the dismutation of two superoxide anions (O_2^- and OH) to molecular oxygen and hydrogen peroxide (Fridovich 1998). Its action is of primary importance to prevent lipid peroxidation induced by the superoxide anion and its increasing activity was showed in previous studies on mollusks exposed to different contaminants (Cheung et

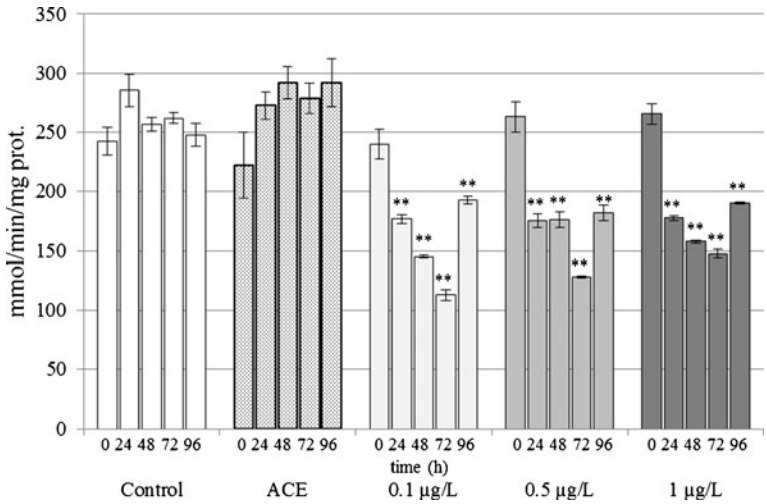

Fig. 5 Variations induced by BDE-154 treatments on the activity (mean ± SEM) of phase II glutathione S-transferase in the soft tissue of zebra mussels (n=3; pool of six to eight specimens). Significant differences (two-way ANOVA, Bonferroni post hoc test, *p<0.05; **p<0.01) were referred to the comparison between treated mussels and the corresponding control (time to time)

Table 1 Pearson's correlation matrix obtained by using all of the enzymatic biomarker endpoints and the measured BDE-154 body burden concentrations under the three selected treatments (n=18)

	SOD	CAT	GPx	GST
0.1 μg/L				
CAT	**−0.53**			
GPx	**0.50**	0.14		
GST	**−0.60**	0.23	**−0.50**	
[BDE-154]	**0.96**	**−0.89**	0.29	**−0.60**
0.5 μg/L				
CAT	0.02			
GPx	**0.86**	−0.26		
GST	**−0.62**	**0.68**	**−0.73**	
[BDE-154]	**0.99**	0.07	**0.92**	**−0.84**
1 μg/L				
CAT	**−0.72**			
GPx	**0.42**	**−0.55**		
GST	**−0.56**	**0.62**	**−0.33**	
[BDE-154]	**0.96**	**−0.71**	**0.64**	**−0.31**

Significant correlations (p<0.05) are indicated in bold

al. 2004; Richardson et al. 2008). Exposure to BDE-154 was able to significantly (p<0.01) activate the SOD at each tested concentration (Fig. 2), indicating an increased production of the superoxide anion (O_2^-) which is subsequently transformed to H_2O_2, initiating the induction of other antioxidant enzymes (Kappus 1986). Hydrogen peroxide, a powerful and potentially harmful oxidizing agent, is then metabolized into H_2O and O_2 by CAT and GPx, completing the defense chain against ROS. The significant (p<0.01) increase of the GPx activity (Fig. 3) observed at 0.5 and 1 μg/L BDE-154 can be considered an indirect confirmation of the early SOD activation (Maria and Bebianno 2010). Moreover, it suggested that mussels activated enzymes to counteract the overproduction of the superoxide anion, as confirmed by the significant (p<0.05) correlations among SOD, GPx, and CAT, above all at the highest concentrations (Table 1). However, even if CAT plays a complementary role to GPx in the elimination of hydrogen peroxide (Box et al. 2007), it did not show any significant variation of activity, with the exception of a significant inhibition at the end of the exposure at 1 μg/L treatment. It could suggest a weak involvement of CAT in removal of BDE-154-induced hydroxyl radical and/or a selective inhibition mediated by this compound at high dosage (Wang et al. 2011). The discrepancy of response between CAT and GPx activity was already found both in vertebrates (Jifa et al. 2006) and invertebrates, including bivalves (Cheung et al. 2004) and may indicate a dissimilar capability of response to oxidative stress. It has been demonstrated that CAT is active at rather high H_2O_2 concentrations, playing a minor role in its catabolism at low production, but when the rate of H_2O_2 is enhanced, its importance increases (Vidal-Liñán et al. 2011). GPx probably acts maintaining normal cell functions, whereas CAT acquires importance in the response to outstanding oxidative stress (Vidal-Liñán et al. 2011). According to this hypothesis, our data seem to indicate that BDE-154 environmentally relevant concentrations can enhance the oxyradicals production in zebra mussel specimens but the organism try to counteract this dangerous situation in order to prevent the onset of high oxidative stress. Alternatively, as suggested by previous studies (Guyton et al. 1996; Cheung et al. 2004), it is conceivable that substrate competition between GPx and CAT might be the cause of the inhibition of CAT during the exposures, as GPx was induced at the same time. A similar response towards pro-oxidant forces was noticed in the bivalve *Perna viridis* treated with Aroclor 1254, showing a significant activation of SOD and GPx, but a depression of CAT (Cheung et al. 2004). Lastly, even if GST is involved in the phase II of metabolism process and plays an important role in the conjugation of electrophilic compounds with glutathione necessary for the detoxification of xenobiotics, it has been also suggested that it

may be a complement of the antioxidant defenses in mussels (Vidal-Liñán et al. 2011). Such studies showed that the increases of GST activity after exposure to different halogenated compounds in mussels are positively correlated with tissue concentrations (Cheung et al. 2002). However, laboratory studies showed depression of GST activity of mussel cytosol when exposed to different xenobiotics (Akcha et al. 2000; Cheung et al. 2004). Even if a significant correlation between GST and BDE-154 tissue levels was found, our data pointed out a significant reduction of GST activity at each treatment, already after only 24 h of exposure. The lack of a GST activity increase could be associated with a GR inhibition, which leads to lack of reduced glutathione (GSH) recycling in the cells. Considering that GSH is used as cofactor by GST and GPx, the inhibition of the former could be due to the completion for the same substrate, as suggested by Binelli et al. (2011). Moreover, GST depression could be related to the transcription of GST-pi gene; the failure of GST synthesis limited the protein availability in the cytosol (Hoarau et al. 2006). This particular response can be due to an inhibitory effect caused by high concentration of contaminant in mussel tissues or, alternatively, an adaptive response in which mussels may have abandoned the GST detoxification pathway in favor of a different one (Cheung et al. 2004). Even if the investigated antioxidant parameters did not response in a clear increasing manner with respect to BDE-154 body burden concentrations, the significant correlation among all of them, particularly at the highest concentration (Table 1), confirmed the activation of the whole enzymatic defense chain and, indirectly that genetic damage induced by BDE-154 might be caused by the raise of oxidative stress due to an overproduction of ROS. Many studies have highlighted that the increase of ROS can produce remarkable changes in the integrity of DNA in different organisms (Regoli et al. 2002; Mamaca et al. 2005). ROS in fact can interact with DNA by the formation of adducts, alkali-labile sites, and single- or double-stranded breaks, causing the reduction of its integrity. These suggestions were confirmed by the correlation analysis between antioxidant activities and the endpoints of the single cell gel electrophoresis assay showed by Parolini et al. (2011a). At 1 μg/L treatment, the most responsive one, a significant correlation was found between SOD (r= 0.51), GPx (r=0.35), CAT (r=−0.38), and the LDR values, as well as between SOD (r=0.52), GPx (r= 0.39), CAT (r=−0.48), and the mean of the percent of DNA in the comet tail. These relationships confirmed that primary DNA damage found by authors was induced by an overproduction of ROS that the antioxidant defense system cannot counteract. In conclusion, our data confirmed that BDE-154 was able to imbalance the oxidative status of zebra mussel-treated specimens, leading to an overproduction of ROS and to the subsequent raise of oxidative stress, when the balance between the pro-oxidant mechanisms and antioxidant defenses is overcome.

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