UNIVERSITA' DEGLI STUDI DI MILANO
SCUOLA DI DOTTORATO IN TERRA, AMBIENTE E BIODIVERSITA'
DIPARTIMENTO DI BIOSCIENZE
DOTTORATO DI RICERCA IN BIOLOGIA ANIMALE
CICLO XXVII

TESI DI DOTTORATO DI RICERCA

PHARMACEUTICALS AND ILLICIT DRUGS AS NEW ENVIRONMENTAL CONTAMINANTS: ECOTOXICOLOGICAL EFFECTS AND NEW DEPURATION METHODOLOGIES

BIO/07

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ABSTRACT

Pharmaceuticals and personal care products (PPCPs) and illicit drugs are now widely considered emerging aquatic contaminants because of their high usage worldwide and their consequent presence in surface water with concentrations ranging from ng/L to µg/L. In fact, once hired, these substances are eliminated by urine and faeces in their unaltered form or as metabolites and poured in sewage system. However, the traditional wastewater treatment plants (WWTPs) are not able to eliminate these compounds from wastewaters, consequently spilled placed in the aquatic environment.

Thus, this research project suggests a natural process based on the natural bivalve filtration as a complementary method to traditional wastewater treatment, seeking thereby to remove the abovementioned contaminants from the aqueous matrix. In detail, we chose the invasive freshwater mussel *Dreissena polymorpha* to study this possible alternative depuration method. For this purpose, we built a pilot-plant within the Milano-Nosedo WWTP in which twenty removal Plexiglas® panels were placed. Moreover, we placed about 40,000 *D. polymorpha* specimens on these substrates appointed to the wastewater filtration. During the experiment we tested the abatement of 13 pharmaceutical compounds (atenolol, carbamazepine, ciprofloxacin, clarithromycin, dehydro-erythromycin, diclofenac, furosemide, ibuprofen, hydrochlorothiazide, ketoprofen, naproxen, paracetamol and ofloxacin), 4 illicit drugs (cocaine, benzoylecgonine, methamphetamine and methadone) and 7 heavy metals (Al, Cr, Cu, Fe, Mn, Ni and Pb) after *D. polymorpha* exposition.

Because of the well-known presence of PPCPs and illicit drugs in the environment, the second part of this project was devoted to the assessment of their ecotoxicological effects. We decided to evaluate the potential chronic toxicity of these chemicals on the same model organism used in the bio-filtration process since *D. polymorpha* is particularly representative of the aquatic ecosystem and, at the same time, sensitive to environmental changes.

The illicit drugs and PPCPs assayed at environmental concentrations in this study were the following: morphine, 3,4-methylenedioxymethamphetamine (MDMA), tonalide and galaxolide. Furthermore, because in the environment such substances form complex mixtures, we also tested the toxicity of an illicit drugs' mixture composed by cocaine, benzoylecgonine, morphine, MDMA and amphetamine administered at environmental concentrations.

The chronic toxicity of these substances was investigated by a biomarker battery: cytotoxicity was evaluated by the Neutral Red Retention Assay (NRRA) while, we assessed the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) for monitoring the raise of oxidative stress, the phase II detoxifying enzyme glutathione-S-transferase (GST), as well as the levels of lipid peroxidation (LPO) and protein carbonylation

content (PCC) in order to highlight eventual oxidative damage. The potential injury at the genetic level induced by PPCPs and illicit drugs was assessed on *D. polymorpha* haemocytes by Comet Test, Micronucleus Test (MN test), percentage of apoptotic frequencies (DNA Diffusion Assay) and DNA fragmentation (DNA Precipitation Assay). Finally, the filtration rate of *D. polymorpha* has been tested as a physiological biomarker.

Data obtained on the *D. polymorpha* removal ability due to the bio-filtration process are very encouraged. The bivalve activity has shown a great capacity in the concentration reduction of various PPCPs, illicit drugs and mainly heavy metals. The ecological importance of these results is high, taking into account the ecotoxicological effects of these substances on the aquatic communities.

The main results for the evaluation of ecotoxicological effects of some emerging environmental contaminants highlighted that morphine and MDMA seem to induce a significant increase of oxidative stress in the bivalve, but not followed to genetic fixed damage; these results were partially confirmed by the exposure to the illicit drugs' mixture that showed a significant increase in antioxidant enzyme activity and in protein carbonylation content.

Finally, the exposure to the musks galaxolide and tonalide caused a significant increase in the DNA fragmentation percentage on bivalve haemocytes.

Therefore, the results obtained during this research project contributed to point out the importance of new wastewater depuration methods allowing the removal of emerging contaminants whose ecotoxicological effects must not be underestimated, as they may ultimately affect the higher biological levels. Moreover, the use of an invasive species such as *D. polymorpha* in the depuration context may also have interesting implications for the economic sector and initiate an exploitation processes of alien species, that, being difficult to remove, constitute a serious problem for ecosystems.

Despite the good results obtained, further studies are necessary to clarify both the bio-filtration treatment and toxic effects of illicit drugs and PPCPs on aquatic communities.

Chapter 1

STATE OF ART

1 PHARMACEUTICALS AND ILLICIT DRUGS AS AQUATIC EMERGING CONTAMINANTS

Before the industrial age, urban wastewater contained almost exclusively biodegradable substances, as nitrogen and phosphorus compounds, as well as the organic matter. However, the chemical industry advent at the end of '800 has created a severe problem, due to the disposal of numerous synthetic substances, used in agricultural, civil or industrial context. The main problem related to these synthesis compounds (POPs, Persistent Organic Pollutants), is connected to their high environmental persistence (Wania and Mackay, 1996; Warren *et al*., 2003). The POP degradation is a very slow process and the resulting transformation products are very stable and often more toxic than the parental molecules; in addition, POPs are frequently characterized by low water solubility and high lipophilicity, which allows them to accumulate in the organisms and along the food web (Jones and de Voogt, 1999). For these reasons, in the last decades, the chemical impact assessment on the organisms has focused almost exclusively on traditional priority pollutants, such as pesticides. These substances, however, are only part of the much broader set of xenobiotics that is possible to identify in the environment. Another group of biologically active molecules that only recently has been receiving attention as aquatic emerging pollutant includes pharmaceutical and personal care products (PPCPs) and illicit drugs. In fact, after their assumption or use, these molecules are excreted in their unaltered form or as metabolites with urine, faeces and sweat, and are conveyed into the sewer system (Ternes, 1998; Zuccato *et al*., 2000; Heberer, 2002; Castiglioni *et al*., 2006).

The traditional wastewater treatment plants (WWTPs), being designed to remove organic matter and nutrient from wastewater, leave the concentration of the above mentioned contaminants unaltered; these pollutants are quite persistent and can be found in concentration higher than 80% in the WWTP effluents, potentially causing important adverse effects for aquatic communities (Ternes *et al*., 1998).

The problems associated with the presence of PPCPs and drugs of abuse in wastewater are nowadays extremely relevant; to better understand this phenomenon, however, it is more convenient to separately analyze the extremely different origin and nature of the two considered pollutant groups.

1.1 *Pharmaceuticals and personal care products (PPCPs)*

The use of PPCPs in human and veterinary medical field is facing a growing expansion (Fent *et al*., 2006). It is estimated that in the European Union (EU) about 3,000 different pharmaceutical substances are normally used only in human therapy (Fent *et al*., 2006).

The massive use of pharmaceutical compounds has therefore encouraged the scientific community to carry out quali-quantitative studies for monitoring their presence in surface waters. Early research in this direction began in the 70s, when clofibric acid traces were found in the treated United States (USA) wastewater (Garrison *et al*., 1976). Further studies, thanks to the analytical instrumentation improvement, have confirmed the presence of pharmaceuticals such as ibuprofen, propranolol and their metabolites in surface water and sewage effluents at concentrations ranging from ng/L to µg/L (Halling-Sørensen *et al*., 1998; Ternes, 1998; Kolpin *et al*., 2002; Boyd *et al*., 2003; Calamari *et al*., 2003; Ashton *et al*., 2004; Gross *et al*., 2004; Wiegel *et al*., 2004 a, b).

Among the mostly abundant pharmaceuticals found in the environment (Figure 1) there are the non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, diclofenac, naproxen, ketoprofen and paracetamol, together with antibiotics, lipid regulators (statins and fibrates), estrogen, antiepileptic and β-blockers (Santos *et al*., 2010). The overt presence of these substances in the environment is a clear signal of pollution due to human activities: in this scenario, the antiepileptic carbamazepine, being extremely persistent, has been proposed as an anthropogenic marker (Clara *et al*., 2004).

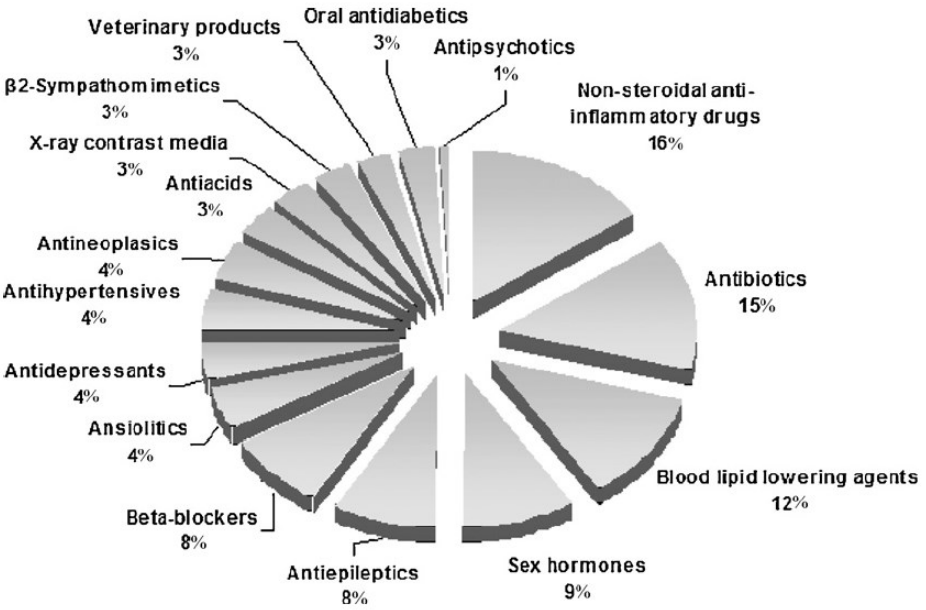

Figure 1: Percentages of pharmaceuticals detected in the environment (Santos *et al*., 2010).

Despite the pharmaceutical compounds is the predominant part of the PPCP group, it is necessary to include in this category other compounds present in the environment with non-negligible concentrations, such as synthetic musks, used as fragrance in the cosmetic industry. Synthetic

musks can be divided into three groups, based on their chemical structures: nitromusks, polycyclic musks and macrocyclic musks. Such substances, unlike pharmaceuticals, do not undergo metabolic processes (Ternes *et al*., 2004), since their use is mainly external, and are transferred unchanged in wastewater. Galaxolide and tonalide are the most widely used polycyclic musks (Gatermann *et al*., 2002; Kuhlich *et al*., 2011) in the cosmetic industry, and their concentrations in the WWTP effluents has been demonstrated to achieve levels in the µg/L order (Zeng *et al*., 2007; Shek *et al*., 2008; Guo *et al*., 2010).

However, PPCP pollution do not only interested the sewage effluents and surface waters. Due to the close interconnection of the various environmental compartments (Figure 2), in fact, even drinking water (Putschew *et al*, 2000; Zuccato *et al*, 2000; Stackelberg *et al*, 2004) and groundwater (Ternes *et al*., 2001) have been shown to contain PPCPs with concentrations in the ng/L order. In this regard, studies conducted by Heberer and Stan (1997) and by Reddersen and co-workers (2002) have shown the presence of some pharmaceuticals such as phenazone, propiphenazone and clofibric acid in Berlin drinking water. It should be considered that PPCPs with high volatility and polarity are persistent in the aqueous matrix (Daughton and Ternes 1999; Crane *et al*., 2006) and can be degraded by various chemical-physical processes such as photolysis, regulated by additional factors as the solar radiation intensity and the presence of photosensitizer elements (Boreen *et al*., 2003; Bartels and Tümpling 2007). At the same time, the non-detection of a specific compound in water does not imply that it has been completely removed but its degradation may have led to analytically undetectable metabolite (Daughton and Ternes 1999; Zwiener *et al*., 2001; Heberer 2002).

Despite the overt presence of these substances in freshwaters, there is currently a lack in the legislation aimed at protect and safeguard the ecosystems by the PPCP presence. Indeed, although the concentration of such compounds in inland waters is low (Hartig *et al*., 1999; Kasprzyk-Hordern *et al*., 2007, 2009), they may cause dysfunction in non-target aquatic organisms (Lintelmann *et al*., 2003).

Only in the lasts years, the National institutions published a detailed reference guide on how pharmaceuticals should be evaluated according to their environmental impacts. The prerequisite for pharmaceutical product registration is represented by the ecotoxicity tests (established in 1995 in accordance with the European Directive 92/18 EEC and EMEA, 1998 for veterinary pharmaceutical products). The European Commission has issued a guideline (Directive 2001/83/EC), indicating that an authorization for a medicinal product for human use must be accompanied by an environmental risk assessment (ERA). At the same time, the Food and Drug Administration (FDA) has published a guide for the evaluation of pharmaceuticals for human use; according to this guide, pharmaceutical companies are required to provide a product environmental assessment when the

predicted concentration in the aquatic ecosystem is ≥ 1 mg/L (FDA-CDER, 1998), which corresponds to about 40 t of pharmaceutical released in the environment. For veterinary pharmaceuticals, the ERA is required by the FDA in the United States since 1980 (Boxall *et al*., 2003). These guidelines concern only the pharmaceutical products, while for illicit drugs the situation is extremely ambiguous, taking into account that the same definition of "drug of abuse" turns out to be somewhat controversial.

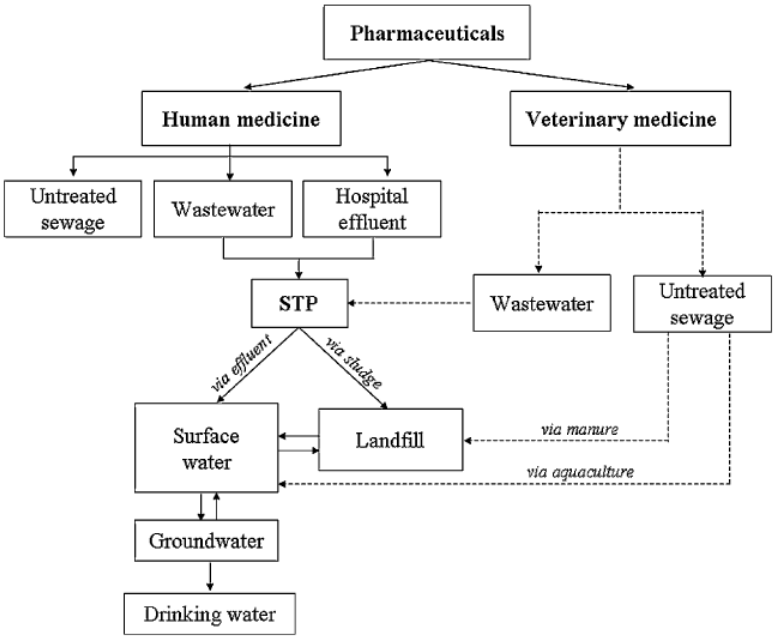

Figure 2: Summary of the PPCP sources and fate (adapted from Kümmerer 2001; Santos *et al*., 2010).

1.2 *Illicit drugs*

While for the pharmaceutical compounds numerous studies about their distribution in the water compartment are present in the scientific literature, the amount of data available on the illicit drugs is insufficient. This is probably due to the ambiguity regarding what exactly defines an "illicit" substance, because of the erroneous idea that illicit drugs are necessarily illegal. Instead, some of them are also used in the medical field; the most evident examples are the opioids such as morphine, codeine, methadone and oxycodone, which are analgesics used in the pain treatment.

It is possible to define a "drug of abuse" as a natural, semi-synthetic or synthetic substance that once assumed induces a pharmacological effect on the organism and can cause physical and/or psychological tolerance or dependence. In addition, a drug can be defined “illegal” when its use is outside of the medical context and his production and detention are not allowed by national or

international laws (Hall *et al*., 2008). In this category are also included the drugs used legally in the medical field but produced, acquired or consumed illegally.

According to effects induced on target organisms, drugs of abuse can be divided into three categories: stimulating the central nervous system (CNS) activity, such as cocaine and amphetamines, depressants the CNS activity, such as morphine and heroin, and psychedelics as cannabis and hallucinogens.

According to the latest World Drug Report (UNODC, 2014), the four classes of drugs of abuse most commonly used worldwide (Figure 3) are represented by opioids and opiates (as morphine and heroin), cocaine, cannabinoids (hashish and marijuana), amphetamine and amphetamine-type stimulants (ATSs, as methamphetamine and ecstasy). The illegal use of these substances varies greatly among countries and several social factors, but the consumption is in any case high, comparable to that of common pharmaceuticals (Santos *et al*., 2010).

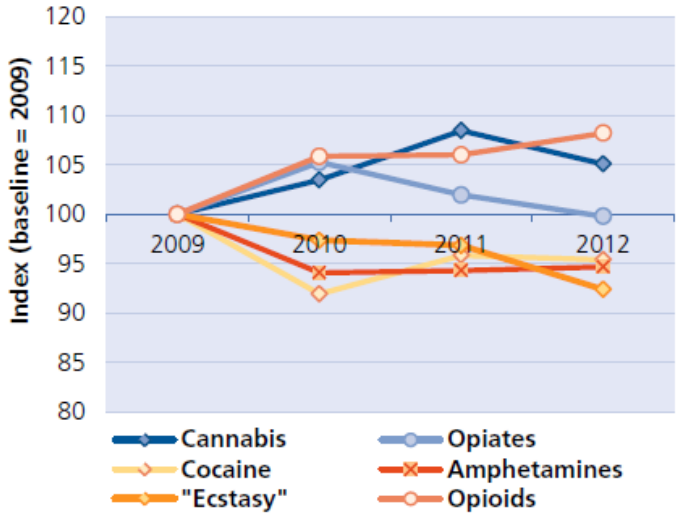

Figure 3: Trends in the use of the most common illicit drugs from 2009 to 2012 (UNODC, 2014).

The UNODC report has estimated that, in 2012, 243 million people corresponding to 5.2% of the world population with an age between 15 and 64 years has used at least once a drug of abuse (UNODC, 2014). However, it is believed that these results underestimate the real situation, since the data have been obtained only by indirect methods including police reports, illicit drug seizures, deaths related to their consumption, addicts statements and data provided by aid agency. Because of this reason, in order to improve the knowledge about the consumption of illicit drugs, a new experimental method based on the concentration measurement of these substances found in WWTP effluents, called Sewage Epidemiology Approach, has been developed (Daughton, 2001).

The illicit drugs, as PPCPs, are mostly characterized by high polarity, which makes them poorly bioaccumulative, even if most of these substances have a very high environmental persistence, which allows their detection in ecosystems for a long period of time after their release (Pal *et al*., 2013). In the last decade a growing number of studies have shown the presence of various illicit drugs and their metabolites in waste and surface waters worldwide in concentrations ranging from tens to hundreds of ng/L (Zuccato *et al*., 2008; Bartelt-Hunt *et al*., 2009; Loganathan *et al*., 2009; Mari *et al*., 2009; Berset *et al*., 2010; Pal *et al*., 2013). Therefore, these substances, being designed to carry out their action at very low concentrations, could cause significant deleterious effects on non-target organisms (Pal *et al*., 2013).

The active components of illicit drugs, as well as those of PPCPs, are very complex molecules with different physico-chemical and biological properties. Generally, the characteristics of a single compound cannot be considered as an indicator of the entire chemical class, because a chemical structure can be not accompanied by an identical action mechanism. This feature makes the removal of these substances from the wastewater by conventional WWTPs extremely difficult.

2 WASTEWATER TREATMENT

Conventional systems are not suitable for the emerging contaminant removal, and this lead to the contamination of the WWTP water bodies' outlet. This limitation should be an incentive for the scientific community to give particular attention to alternative treatments that improve the efficiency of traditional systems.

2.1 *Conventional treatment systems*

The conventional WWTPs are carried out through different treatments in order to remove compounds with different chemical-physical characteristics. The wastewater treatments can be classified as follow (Figure 4):

1) pre-treatment (screening, grit and oil removal);
2) primary treatment (primary sedimentation);
3) secondary treatments (biological oxidation and secondary sedimentation);
4) tertiary treatment (nitrogen and phosphorus removal);
5) disinfection.

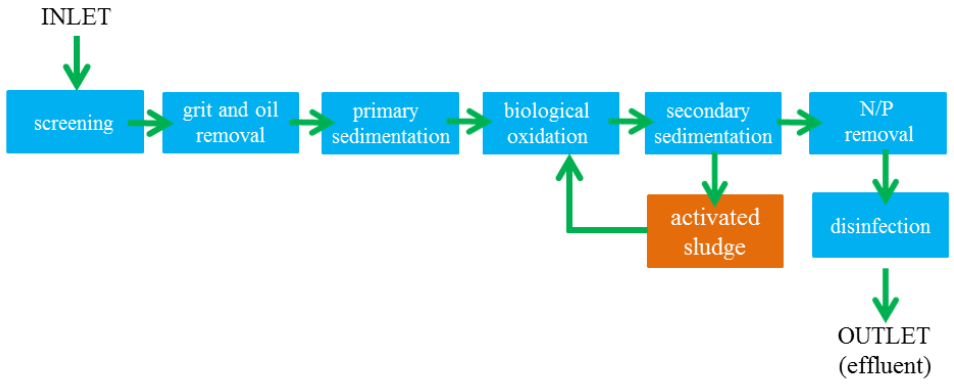

Figure 4: Summary of the depuration processes in a traditional WWTPs.

The preliminary treatments are used to remove from incoming WWTP wastewater materials that have size or characteristics potentially deleterious for subsequent purification steps; there are included in this category a screening processes, the grit and the oil removal. After this treatment, the primary process consists in the separation of heavier materials from the water by sedimentation, which will constitute the so-called primary sludge. The primary treatment affects both the component already present in sedimentable form in the wastewater incoming at the WWTP, both

the suspended materials rendered sedimentable after a coagulation-flocculation stage. The process of primary sedimentation is often combined with the secondary, or biological, treatment. This step can lead to a 25-30% of removal of the BOD (Biochemical Oxygen Demand) allowing an abatement of the biodegradable organic substances, which represent the ideal substrate for bacterial growth. Through this process the organic matter is converted, under aerobic conditions, in secondary sludge, easily removable from the aqueous matrix. These biological processes, based on the bacterial biomass oxidant activity, are divided into suspended-growth and attached growth systems (fixed-film). In the first case, the bacterial mass remains in suspension in the aqueous matrix due to aeration systems while, in the second process, the bacterial colonies form a biofilm on appropriate supports in contact with the wastewater.

Instead, the tertiary treatments allows the nitrogen and phosphorus elimination. Nitrogen is removed mostly through biological treatments: biochemical reactions involved in this process are nitrification and denitrification by *Nitrosomonas* and *Nitrobacter*. On the contrary, phosphorus is removed by chemical precipitation, introducing iron or aluminum salts in the treatment tank, which determine the formation of insoluble phosphates separable by decantation.

These phases are followed by disinfection treatments conducted through chemical/physical procedures (as peracetic acid, ozonation or ultraviolet radiation) aimed to eliminating pathogenic bacteria present in the WWTP effluents. The treated waters are then put in the effluent.

However, despite the complex integration of different treatments within the WWTPs, many pollutants are poorly removed, such as heavy metals, pharmaceuticals, illicit drug and their metabolites. For this reason, in recent years the scientific community has been looking for alternative or experimental treatments that complete and support the traditional depuration process.

For example, chemical/physical systems as precipitation/neutralization, ion exchange, membrane separation, reverse osmosis, electrodialysis and activated carbon adsorption are used in the heavy metals removal (Matheickal *et al*., 1987; Atkinson *et al*., 1998; Ahluwalia and Goyal, 2007). Since these techniques have high operating costs, the scientific community has focused his attention on the development of new systems.

2.2 *Alternative natural treatments*

The treatment cycle described above is characteristic of a traditional WWTP, capable of dealing with a high inhabitant equivalent (the amount of biodegradable organic substances, derived from a civil user, conveyed into the sewer in a period of one day). To treat smaller inhabitant equivalents, the alternative natural treatments can be used as simpler and economic systems. In this regard, the Italian Legislative Decree 152/99, and subsequent updates, drives to alternative systems for all settlements with an equivalent population ranged between 50 and 2,000 equivalent inhabitants.

Among the natural alternative treatments, constructed wetlands (Figure 5) and lagooning are the most representative.

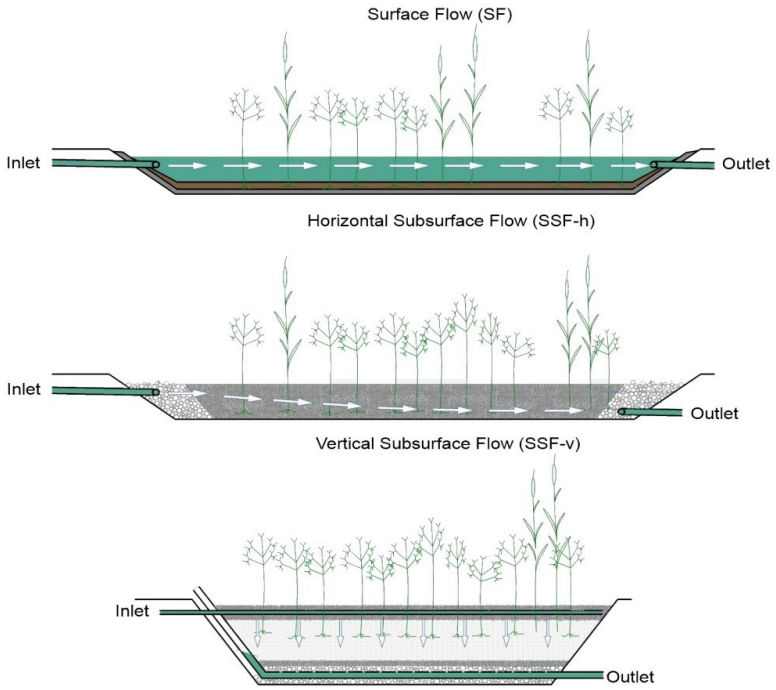

Figure 5: Diagram of the three types of constructed wetland (modified from: http://www.akzero.org/).

The first attempt on the use of chemical/physical and biological processes typical of natural wetlands in controlled systems date back to the mid-50s (Seidel, 1955). After the first trials, twenty years of subsequent research has allowed to create the first constructed wetland full-scale, built in Germany in 1977 (Kickuth, 1977). Afterwards, in the early 80s, constructed wetlands have been used for wastewater treatment in Denmark and, later, in Austria and Switzerland. These systems are artificially propagated in moist environments, where the wastewaters are conveyed.

Constructed wetland systems are characterized by the presence of typical wetland plants (e.g. *Phragmites australis*, *Eichhornia crassipes* and *Lemna spp.*), rooted in the substrate or floating on the water surface which, together with adherent microorganisms, are suitable for several pollutant removal. Therefore this method, exploiting the natural phenomena of bioaccumulation, adsorption and biotransformation, is particularly efficient in the organic matter and nutrient removal, as well as in the heavy metal reduction (Certini and Scalenghe 1999; Dhote and Dixit, 2009), while few

studies were performed on the abatement of PPCPs (Matamoros and Bayona, 2006; Matamoros *et al*., 2007).

These alternative method can be classified into three groups, according to the effluent flow pathway; in the systems with surface flow (SF; Figure 5), wastewater is constantly above the substrate on which the aquatic plants are fixed. Conversely, in subsurface flow systems (SSF), the water level always remains below the surface layer of the plant substrate.

According to the pathway imposed to the wastewater, constructed wetlands are called SSF-h when the flow occurs horizontally, or SSF-v when the flow is vertical. Both SSF systems are suitable to treat wastewater with low concentrations of particulate matter, because of the obstacle to the flow due to the substrate. On the other hand, SSF have a high pollutant removal efficiency *per* unit area, due to the greater substrate porosity.

The lagooning, instead, is a purification system in which the effluent remains for a long time in artificial basins where oxidation and fermentation processes take place through microorganisms, in a similar manner to what occurs in natural water bodies. Since lagooning requires large surfaces, its use is not common in Italy: it has been applied mainly in the seasonal food field (Vismara *et al*., 2001) or in warm climates, where the environmental conditions are favorable for year-round (Von Sperling, 1996).

2.3 *Experimental natural treatments: a reinterpretation of the bio-filtration old concept*

The technologies described above are commonly used in civil water purification. However, both traditional and alternative natural systems have some limitations, as inability in the removal of some pollutants. For these reasons methods as biosorption, algal exopolysaccharide exploitation and bio-filtration are in the testing phase.

The term biosorption is define as the passive pollutants uptake from an aqueous solution by a dead or a non-growing microbial biomass (Beveridge and Doyle, 1989; Volesky, 1990; Aksu 2005).

The use of dead microorganisms provides undoubted benefits: it can treat toxic wastewater, is easy and inexpensive regarding both the manufacture and the storage, has no nutrient need and can be cleaned and reused for several cycles. However, there are difficulties related to the saturation and recovery of the contaminated microbial biomass (Ahluwalia and Goyal, 2007).

Exopolysaccharides are a class of substance produced by a large variety of organisms, such as bacteria and fungi; these compounds are responsible for the microbial flocculation in the wastewater biological treatment, leading to the formation of activated sludge. Even some microalgae are capable to produce exopolysaccharides, especially in stressful situations (Avnimelech *et al*., 1982). Some studies have shown how this compound, produced from

microalgae of the genera *Chlamydomonas*, *Phacus*, *Euglena* and *Chlorella,* are able to improve the activated sludge flocculation in the biological oxidation tanks and, moreover, significantly increase the nitrogen removal rate (Shipin *et al*., 1998, 1999).

Finally, the bio-filtration process refers to the pollutant removal by the use of a synthetic filter where a bacterial biofilm lives, as occurs in the biological treatment with biodisk. However, there are not experimental evidence about the efficiency of the above mentioned experimental methods in the removal of some emerging contaminants. Therefore, this research project aims to reinterpret, in an innovative key, the term "bio-filtration", through the development of a new purification system for emerging contaminant removal based on the physiological filtration activity of an invasive freshwater bivalve: *Dreissena polymorpha*.

2.3.1 *D. polymorpha use in the wastewater depuration context*

D. polymorpha (Pallas 1771; Figure 6) is a mollusk belonging to the family Dressenidae, present in both lotic and lentic waters. The occurrence of this species in estuaries of the central Paratethys dates back 10-11 million years ago and then spread in rivers, estuaries and coastal waters of the Pontic-Caspian Region (Gollasch and Leppäkoski, 1999). Subsequently, the flourishing development of inland waterway transport has led to the diffusion of this bivalve throughout Central and Northern Europe (Annoni *et al*., 1978). The multiple passive distribution modes, such as the accession to the transport ship keel or to the timber transported by the Baltic Sea, have allowed *D. polymorpha* to colonize areas far from its original areal.

Figure 6: *D. polymorpha* specimens.

In Italy, the first findings of *D. polymorpha* dates back to the seventies, followed by subsequent studies that show its presence in most inland waters of this country (Giusti and Oppi, 1973; Bodon *et al*., 2005; Cianfanelli *et al*., 2007).

D. polymorpha has some physiological and ecological characteristics that may be useful in depuration processes: 1) high filtration rate, in the range of 5 to 400 mL/bivalve/h (Ackerman, 1999; Baldwin *et al*., 2002); 2) high population density (up to 700,000 individuals/m^2; Pathy, 1994); 3) indirect bioaccumulation of many lipophilic contaminants in the soft tissues. In this context, it is also important to considered an additional physiological aspect of *D. polymorpha*: its filtering activity optimally occurs in temperature ranging between 10 and 20 °C (Noordhuis, 1992; McMahon, 1996), that is the average temperature of the civil WWTP effluents. This would make the bio-filtration treatment effective also during winter period.

Previous studies regarding the use of *D. polymorpha* in the environmental management, such as eutrophication control (Reeders and Bij de Vaate, 1990) and nutrient elimination from aqueous matrix (Piesik, 1983), support the aims of this research. However, this work appears to be extremely innovative because for the first time, according to our knowledge, the bivalve filtration has been exploited in a WWTP to remove emerging contaminants. For this purpose, we built a pilot-plant (Figure 7B) at the Milano-Nosedo WWTP (North Italy) in which several tests were carried out to assess *D. polymorpha* adaptation to wastewater and its filtration capacity in the removal of some recalcitrant pollutants.

The pilot-plant used in the study is made from a stainless steel tank with a volume of 1000 L where twenty removable Plexiglas® panels were included (Figure 7A). Approximately 40,000 *D. polymorpha* specimens, collected by scuba divers in Lake Lugano and Lake Maggiore, were allowed to adhere by their byssus to the panels. In addition, a recycling tank, with a capacity of 200 L, was installed. Since *D. polymorpha* is a dangerous fouling agent (Arpita *et al*., 2013), all discharges from the pilot-plant were fitted with grids; furthermore, the bio-filtration system was placed before both the sand filters and the disinfection treatment with peracetic acid, in order to kill any adults or larvae eventually escaped from the pilot-plant, as suggested by Elliot and co-workers (2005, 2008). In this context, the use of invasive alien species, as *D. polymorpha*, could have interesting economic implications, related to the exploitation of the same non-native species now present in aquatic ecosystem and difficult to eliminate.

We monitored some pharmaceuticals (atenolol, carbamazepine, ciprofloxacin, clarithromycin, dehydro-erythromycin, diclofenac, furosemide, ibuprofen, hydrochlorothiazide, ketoprofen, naproxen, paracetamol, and ofloxacin), illicit drugs (cocaine, benzoylecgonine, methamphetamine and methadone) and heavy metals (Al, Cr, Cu, Fe, Mn, Ni, Pb) present in the wastewater (Table 1),

pumped directly from the WWTP canal into the pilot-plant, during the first 4 h and after one day of bivalve exposure (see chapters 2 and 3). In this regard it is important to take into consideration that PPCPs and drugs of abuse, once placed in water bodies downstream of the WWTPs, could give rise to serious ecotoxicological effects on aquatic communities (Fent *et al*., 2006).

Therefore, the second part of my PhD project was deputy to the assessment of sub-lethal effects of some PPCPs and illicit drugs on *D. polymorpha*; in particular, the attention has been focused on the effects of illicit drugs and personal care products, because few studies have ever investigated the ecotoxicity of these molecules.

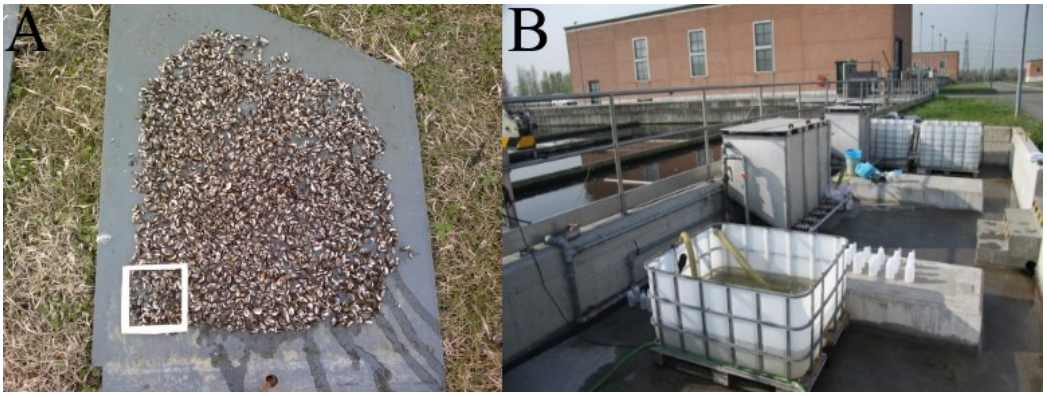

Figure 7: A) Plexiglas® panel with bivalves deputy to the wastewater bio-filtration. B) Pilot-plant structure at the Milano-Nosedo WWTP; in the foreground the recycling tank, while, in the background, the steel tank with bivalves inside.

Group	Compound	Effluent Concentration (ng/L)	Action/Medical Use
Pharmaceuticals			
NSAIDs	diclofenac ibuprofen ketoprofen naproxen paracetamol	0.3-2,400[*] 1.41-6,718.3[*] 8-351[*] 12.0-7,962.1[*] 1.8-4,300[*]	Analgesic, antipyretic and anti-inflammatory effects. Action on the arachidonic acid metabolism through the cyclooxygenase inhibition (COX-1 and COX-2).
antibiotics	clarithromycin dehydro-erythromycin	12-232[*] 361-811[*]	Bacterial infection treatment. Action on specific microbial structure.
antiepileptic/ antidepressants	carbamazepine	<5-2,100[*]	Neuronal activity decrease through the slowdown in the sodium channel recovery; increase of γ-aminobutyric acid (GABA) inhibitory effects; interference with both the inositol cycle and glycogen synthase kinase (GSK).
diuretics	furosemide hydrochlorothiazide	585[**] 439.1[**]	Promote liquid elimination and reduce simultaneously blood pressing in the arteries acting at the nephron level.
β - blocker	atenolol	40-1,168[*]	Control of hypertension, angina pectoralis, myocardial infarction, arrhythmia and sympathomimetic function through the inhibition of β-adrenergic receptors.
Illicit Drugs			
cocaine	cocaine benzoylecgonine (cocaine metabolite)	< 0.99-540 [***] < 0.92- 3,425[***]	Euphoria induction by blocking the dopamine recapture and, consequently, dopaminergic neurotransmission enhancement.
amphetamine-type stimulants (ATSs)	metamphetamine	1.05-350[***]	Psychostimulant by norepinephrine and dopamine release from nerve endings.
opioids	methadone	3.4-87[***]	Analgesic also used in replacement therapy to reduce the opiate dependence. Opioids bind to endorphin receptors, activating the inhibitory functions.

Table 1: Pharmaceuticals and illicit drugs considered in the *D. polymorpha* removal tests with respective concentrations detected in WWTP effluents ([**]Zuccato *et al*., 2005; [*]Santos *et al*., 2010; [***]Pal *et al*., 2013) and their action mechanism on target organisms.

3 ECOTOXICOLOGICAL EFFECTS OF EMERGING CONTAMINANTS

Pharmaceutical compounds and illicit drugs are designed to act on specific molecular and biochemical processes in the target organisms. For this reason, once released into the environment, they may have potential toxicological and/or pharmacological effects on organisms having the same or similar target structures (Fent *et al*., 2006). In this context, it is important considering that several mechanism of action are not completely understood for both pharmaceutical compounds and illicit drugs yet, and ecotoxicological studies are therefore difficult to carry out, also because few data are available about the toxicity of these substances on aquatic and terrestrial communities.

However, some classical assays to test the acute toxicity of different pharmaceutical compounds have been performed on algae (Yang *et al*., 2008), crustaceans (Haap *et al*., 2008), mussels (Canesi *et al*., 2007) and fishes (Choi *et al*., 2008), detecting acute effects below 100 mg/L in 17% of cases (Fent *et al*., 2006). The acute toxicity induction at high concentrations is unlikely to occur in the environment; however, the widespread use of these substances and their constant input into wastewater result in a continuous exposure of the aquatic communities, which could therefore manifest sub-lethal effects even at low toxic concentrations. Similarly, acute toxicity studies conducted on some synthetic musks have highlighted effect at concentrations higher than those found in the environment (Gooding *et al*., 2006).

The more exhaustive information on chronic toxicity associated with these substances regard almost exclusively the pharmaceutical compounds, while few research were conducted on illicit drugs and synthetic musks. A pivotal study in the chronic toxicity scenario has been carried out on the active ingredient of the contraceptive pill 17α-ethinylestradiol (EE2), which leads the vitellogenin induction at environmental concentrations in different fish species (Lange *et al.*, 2001; Brian *et al*., 2005; Fenske *et al*., 2005; Parrot and Blunt, 2005).

Regarding illicit drugs, the first study concerning the assessment of sub-lethal effect was conducted on cocaine where the dopaminergic signaling transmission has been investigated at retina and brain level on *Danio rerio*, that manifest a behavioral disorders and decreased vision after the exposure (Darland and Dowling, 2001). Subsequently, Gagné and co-workers (2010) evaluated the neurochemical effects of morphine on freshwater mollusk *Elliptio complanata*; after injections of morphine increasing doses (0.7; 0.35 and 0.75 g/g fresh weight), the changes in the certain neurotransmitters concentrations, such as dopamine, serotonin, glutamate and the γ-aminobutyric acid (GABA), as well as the dependent activity of acetylcholinesterase (AChE), dopamine and serotonin were monitored. Morphine injections caused a reduction in the serotonin and AChE levels, accompanied by increase of dopamine and GABA concentrations. A more recent study conducted by Binelli and co-workers (2012) showed how a 96 h exposure to three different cocaine

concentrations (40 ng/L, 220 ng/L and 10 µg/L) on the bivalve *D. polymorpha* have been able to alter the lysosomal membrane stability and induce considerable primary and fixed genetic damage on the bivalves. At the same time, subsequent research conducted on the main cocaine metabolite, benzoylecgonine, showed a decrease of the lysosomal membrane stability, accompanied by a significant increase in oxidative stress and genetic damage on *D. polymorpha* haemocytes (Parolini *et al*., 2013). A similar result has been obtained by exposing the bivalves for 14 days to the cannabis active principle Δ-9-tetrahydrocannabinol (THC; Parolini *et al*., 2014).

These findings clearly suggest how the PPCP and illicit drug presence in the environment, even at very low concentrations, may pose a hazard to the aquatic community and confirm the necessity of further research for a proper risk management and the ecosystem preservation. Therefore, the biomarker use on a biological model appears to be of great interest to understand the environmental risk of these contaminants.

3.1 *Chronic toxicity evaluation: a biomarker approach*

The exposure assessment and contaminant effect on the non-target organisms remains one of the major issues to be addressed in the environmental management. The classic ecotoxicological assay carried out by mollusks, algae, bacteria and fishes, are not particularly effective for the determination of chronic toxicity of illicit drugs and certain PPCPs, since their environmental levels are lower than the effect concentrations of these chemicals. For this reason, the ecotoxicology looks towards the development of most suitable methods based on the study of the so-called "early adverse effects", caused by contaminants and measurable at different levels of biological organization. In this sense, biomarkers have proved to be of great utility. They are defined as any change in biochemical, cellular or physiological level, measurable in a body as a response to exposure to one or more pollutant compounds (Huggett *et al*., 1992; Depledge and Fossi, 1994). The first biological effect of a contaminant occurs at biochemical and molecular level, such as modification of the enzymatic activity or DNA alterations, and only afterwards, according to a cascade mechanism, can be found also at higher levels of the biological organization.

However, while the effects shown at subcellular level are then reflected in individual damage, it is not easy to understand if the same changes can have an impact at the ecosystem level. Thus, the prediction of contaminant effects on a whole ecosystem based on the simple observation of a single biomarker is a speculative approach that needs a deeper investigation. For this reason, the use of a biomarker battery that investigates the biological parameters at different levels of hierarchical organization is an excellent approach for formulating hypotheses based on the toxicity and the mechanism of action (MOA) of the tested molecules.

Currently, the law does not provide the biomarker use in the ERA context, because the relationship between damage at the subcellular and at the higher levels of biological organization has not been clarified yet. In the early future, more detailed studies are necessary in this direction, in order to clarify this relationship and to identify the point at which an organism passes from the homeostasis to a compensatory or irreversibility state (Fossi, 1998).

3.1.1 *Biomarker classification*

The biomarker batteries used in the present study can be grouped into four distinct categories according to the type of damage obtained: biomarkers of genetic damage, cytotoxicity, oxidative stress and physiological effects, which will be briefly described in the following paragraphs.

3.1.1.1 *Biomarkers of genetic damage*

A genotoxic agent alters the structure, the informational content, the DNA segregation or temporarily inhibits its replication. In the present project, the following biomarkers of genetic damage has been applied:

1) **Single Cell Gel Electrophoresis (SCGE) Assay or Comet Assay**: it is an electrophoretic technique used to measure a primary damage (repairable) to the DNA of single cells (Tice *et al*., 2000). This technique allows the identification of a wide range of agents that induce single or double strand breaks, alkali-labile sites and to promote the crosslink formation (DNA-protein, DNA-DNA intra-and inter-strand; Albertini *et al*., 2000; Singh, 2000). The name of this assay derives from the fact that during the electrophoretic cycle, the DNA damaged strands are attracted by the anode, thus forming the characteristic "comets". Cells with more consistent damage show an increased rate of migration towards the anode and then a more pronounced comet.
2) **DNA Precipitation Assay:** as for the comet assay, this biomarker highlights primary damage on the single and double DNA helix. The cells are lysed with an anionic detergent and then treated with potassium chloride (KCl); this allows the precipitation of proteins, intact DNA and phosphates, while the damaged DNA remains in suspension and is quantified using fluorochromes.
3) **DNA Diffusion Assay**: this technique highlights the DNA fragmentation at the single cell level and discriminates apoptotic from necrotic cells. In the apoptotic cell, a series of alterations lead to the production of DNA fragments of different length, multiples of approximately 185-200 bp. Degraded chromatin granules migrate towards the nucleus

periphery, while discrete fragments of nuclear material following subsequently the plasma membrane, where they are delimited by evaginations of the membrane itself. These fragments are therefore detached from the rest of the cell taking with it part of the cytoplasm and nuclear material and giving rise to the so-called apoptotic bodies that are phagocytized by neighboring cells. The apoptotic cell, therefore, appears as a circular gradient of DNA around a central denser zone while the peripheral zone has poorly defined edges.

4) **Micronucleus test**: the term "micronucleus" means a small mass of intracytoplasmic chromatin, used as an indirect index of chromosomal aberration (Castello and Silvestri, 1999). The frequency of the micronucleus is, therefore, a reliable assessment of the chromosomal damage. During the mitotic anaphase some alterations can occur, such as chromosomal fragments that lack the centromere, that do not properly segregate or whole chromosomes that retard the migration along the mitotic spindle. During the telophase, the nuclear membrane is reconstructed also around the fragments and chromosomes that did not previously migrate (Migliore and Simi, 1998). In the interphase cells, intracytoplasmic particles are originated, that have round or oval form clearly distinct from the nucleus. The micronucleus frequency can be considered as an indicator of the presence of genotoxic substances, which are not removed properly by the body defense mechanisms (Mersch *et al*., 1996).

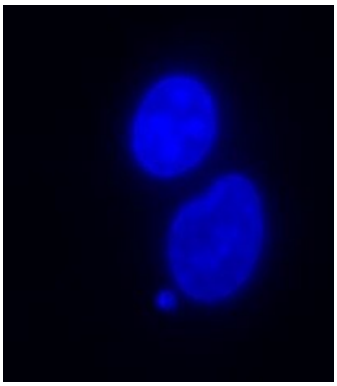

Figure 8: Cells with micronucleus highlighted with Hoechst fluorescent dye (https://www.uni-due.de/).

3.1.1.2 *Biomarkers of citotoxicity*

This biomarker category is used to evaluate the contaminant toxicity based on the changes at subcellular level, in particular at the lysosome level; these vesicular organelles originate from the Golgi apparatus and contain several lytic enzymes. Lysosomes have the ability to accumulate different metals and toxic organic contaminants (Moore, 1990); their role exposes them to a particular vulnerability situation towards certain xenobiotics that can lead to damage of the

lysosome itself. The lysosomal membrane stability is usually measured by means of the **Neutral Red Retention Assay (NRRA)**. The cells are stained with Neutral Red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride), a lipophilic dye that diffuses through the membrane and penetrates into lysosomes. Its concentration in the lysosomes, therefore, is superior to that in cytoplasm. If the lysosomes are intact, the dye can be retain for a long time; on the contrary, if their membranes are damaged, the Neutral Red comes out and spreads into the cytoplasm pointing out the cytotoxic effects of the tested substance.

3.1.1.3 *Biomarkers of oxidative stress*

Cells can tolerate the oxidative stress derived from the natural metabolism, thanks to the action of efficient antioxidant defense systems. In contrast, a higher stress can cause a considerable alteration of cellular metabolism that lead to cell transformation or death. The main causes of oxidative damage are the Reactive Oxygen Species (ROS), molecules continuously produced to basal levels by the body due to the oxidizable biomolecule metabolism (Gutteridge, 1994). However, their levels can significantly increase under special conditions, such as exposure of the organism to radiation or toxic agents and/or pollutants. The most important ROS are the superoxide radical ($\bullet O_2^-$), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2). The most relevant consequences arising from the excessive ROS presence are the DNA structural alteration, the inhibition or activation of several signal transduction pathways, the intercellular communication block and the apoptotic process. The damage extent is the result of the imbalance between the ROS production and their removal by antioxidant defenses. These protection systems are represented by a series of enzymes that catalyze the reactions that transform ROS into non-reactive species or a less hazardous and their extent can be used as a biomarker of oxidative stress (Van der Oost *et al*., 2003).

Among the antioxidant enzymes, the ones investigated in this project are:

1) **Catalase (CAT)**: is an inducible enzyme that metabolizes H_2O_2 to molecular oxygen and water. It is a hemoprotein widespread among aerobic organisms and is mainly localized in peroxisomes.
2) **Superoxide Dismutase (SOD)**: this metalloenzyme consists of two homodimers localized in peroxisomes. SOD catalyzes the dismutation reaction of the $\bullet O_2^-$ to oxygen and H_2O_2.
3) **Glutathione Peroxidase (GPx)**: is an important antioxidant defense, particularly against the H_2O_2 formation. GPx is a soluble protein of 85 kDa, formed by four subunits containing a selenocysteine that constitutes the enzyme catalytic center. GPx is also able to catalyze the

reduction of H_2O_2 to form alcohols (Manduzio *et al*., 2004): in this case the glutathione (GSH; a peptide) acts as an electron donor and is oxidized (GSSG). However, through the action of glutathione reductase (GR) and in the presence of NADPH, this peptide is regenerated.

In addition to the antioxidant defenses, the detoxifying enzymes are activated to increase the xenobiotics hydrophilicity, promoting their excretion. Among these, the **Glutathione S-Transferase (GST)** is an inducible enzyme that catalyzes the assemblage of the GSH in an electrophilic substrate. It has been recently suggested to use the measurement of the cytosolic GST levels as a pollution indicator.

ROS can also cause oxidative damage to proteins and lipids, resulting in damage of cellular functions. The evaluation of **Protein Carbonilation Content (PCC)** and **Lipid Peroxidation (LPO)** are the main indicators used in the ecotoxicology to assess the oxidation of proteins and lipids by the Fenton/Haber-Weiss reaction.

3.1.1.4 *Physiological biomarkers*

An organism can also respond to a contaminant exposure with modifications at higher hierarchical levels, such as at a physiological level. A xenobiotic compound may in fact interfere with the organism metabolism and affect one or more life processes. Among these, the **Filtration Rate** assessment is the most widely used physiological biomarker in ecotoxicological studies that utilize filtering organisms as *D. polymorpha*. This parameter is influenced by changes in both biotic and abiotic factors such as age, size, life cycle period, temperature, food and exposure to contaminants (Morton, 1971; Walz, 1978; Dorgelo and Smeenk, 1988; Sprung and Rose, 1988; Reeders and Bij de Vaate, 1990). The decrease in the filtration rate is evaluated using measurements of the removal of Neutral Red dye added in the exposure tank at different times (Palais *et al*., 2012).

3.2 *D. polymorpha as biological model in ecotoxicology*

D. polymorpha, besides its use in the improvement of water quality (Richter, 1986; Antsulevich, 1994; Elliot *et al*., 2008; Schernewski *et al*., 2012; McLaughlan and Aldridge 2013), has been used for many years as a biological indicator in biomonitoring operations, thanks to its peculiar eco-physiological characteristics, such as sessility, ubiquitous presence, adaptability, high filtration rate and relatively long life cycle; furthermore, this bivalve is an excellent bioaccumulator of many xenobiotics such as Polychlorinated Biphenyls (PCBs; Provini *et al*., 1997; Binelli *et al*., 2004), Chlorinated Pesticides (Binelli and Provini, 2003a,b), Polycyclic Aromatic Hydrocarbons (PAHs;

Fisher *et al*., 1993) and heavy metals (Camusso *et al*., 2001). Moreover, in the last years, *D. polymorpha* has been chosen as model species in studies designed to evaluate the toxicity of PPCPs (Binelli *et al*., 2009a,b,c; Parolini *et al*., 2010; 2011a,b) and illicit drugs (Binelli *et al*., 2012; Parolini and Binelli, 2013; Parolini *et al*., 2013; Parolini *et al*., 2014). For all these reasons, *D. polymorpha* can now be considered the freshwater counterpart of *Mytilus spp.*, and has been proposed as a good biological indicator of inland waters (see chapter 4).

Therefore, in this research, the sub-lethal effects of some illicit drugs and PPCPs (synthetic musks; Table 2) detected in surface water with non-negligible concentrations were evaluated, by means of a biomarker battery conducted on *D. polymorpha* (see chapters 5, 6, 7 and 8). In this context, it is important to consider that the chronic toxicity assessment of these molecules (Table 2) is part of a larger project aimed at the characterization of the sub-lethal effects of PPCPs and drugs of abuse detected in surface waters. The concentration of the tested substances refers to their environmental levels, giving an ecological relevance to the study.

Group	Compound	Surface Water Concentration (ng/L)	Tested Concentration (ng/L)
Illicit Drugs			
opiates	morphine	1.74-89[*]	50 and 500
amphetamine-type stimulants (ATSs)	3,4-methylenedioxymethamphetamine (MDMA) or ecstasy	0.2-14.1[*]	50 and 500
illicit drug mixture	cocaine benzoylecgonine (cocaine metabolite) morphine amphetamine MDMA	0.5-44[*] 1.6-316[*] 1.74-89[*] 1-309[*] 0.2-14.1[*]	50 300 100 300 50
Personal Care Products			
synthetic musks	galaxolide tonalide	<0.05-12,500[**] <0.25-6,800[**]	100 and 500 20 and 80

Table 2: Compounds assayed in the ecotoxicological analysis on *D. polymorpha* with relative tested and detected concentrations ([**]Esche, 2004; [**]Villa *et al*., 2012; [*]Pal *et al*., 2013).

4 PUBLICATION LIST

Chapter 2:

*Paper 1: Andrea Binelli, **Stefano Magni**, Carlo Soave, Francesca Marazzi, Ettore Zuccato, Sara Castiglioni, Marco Parolini, Valeria Mezzanotte (2014). The biofiltration process by the bivalve D. polymorpha for the removal of some pharmaceuticals and drugs of abuse from civil wastewaters. Ecological Engineering 71, 710-721.*

In this work, we tested the filtering efficiency of the bivalve *D. polymorpha* in the removal of some drugs of abuse and pharmaceutical compounds from wastewater in a civil WWTP (Milano-Nosedo WWTP). The research focused specifically on the abovementioned contaminants, as their abatement by traditional treatment systems is problematic. This research has been pivotal for the use of the bivalve bio-filtration process in an experimental system, taking into account that *D. polymorpha* is a highly invasive species and its potential exploitation could have interesting implications for economic and ecological fields.

Contribution: In this research I made the management of the pilot-plant, containing the bivalves deputies to the wastewater bio-filtration, at the Milano-Nosedo WWTP. Moreover, I carried out all the emerging contaminant removal tests.

Chapter 3:

*Paper 2: **Stefano Magni**, Marco Parolini, Carlo Soave, Francesca Marazzi, Valeria Mezzanotte, Andrea Binelli (2014). Removal of heavy metals from wastewater using zebra mussel bio-filtration process. Journal of Environmental Chemical Engineering. Under review.*

This work is closely connected with the previous one; in this context, the filtering efficiency of the bivalve *D. polymorpha* in the removal of heavy metals from wastewater has been tested. These contaminants, although not considered as emerging pollutants, are still poorly removed by traditional depuration systems causing adverse effects on aquatic organisms.

Contribution: In this research I made the management of the pilot-plant at the Milano-Nosedo WWTP and I carried out all heavy metal removal tests from wastewater.

Chapter 4:

*Paper 3: Andrea Binelli, Camilla Della Torre, **Stefano Magni**, Marco Parolini (2015). Does zebra mussel (Dreissena polymorpha) represent the freshwater counterpart of Mytilus in ecotoxicological studies? A critical review. Environmental Pollution 196, 386-403.*

D. polymorpha, besides being used in some studies as a bio-filtering agent to improve the water quality, has been used for many years as model organism in ecotoxicological studies. In this review, we presented all the studies conducted on this bivalve for the ecotoxicological effect and risk assessment, from the biomonitoring studies to its application in the 'omics' techniques. Therefore, the aim of this article is to propose the bivalve *D. polymorpha* as the *Mytilus spp.* freshwater counterpart as a biological model for inland waters.
Contribution: In this review I have collected, from scientific literature, the freshwater biomonitoring studies conducted using *D. polymorpha* as model organism.

Chapter 5:
*Paper 4: **Stefano Magni**, Marco Parolini, Andrea Binelli (2014). Sublethal effects induced by morphine to the freshwater biological model Dreissena polymorpha. Environmental Toxicology DOI: 10.1002/tox.2202.*

This research is part of a larger project aimed at assessing the sub-lethal effects of illicit drugs. In this study, we evaluated the morphine chronic toxicity on the bivalve *D. polymorpha* during 14 exposure days applying biomarkers of oxidative stress, biomarkers of genotoxicity and physiological biomarkers. Two morphine concentrations have been tested: an environmental concentration (measured in surface waters) of 0.05 µg/L and a higher concentration of 0.5 µg/L that allowed identifying a toxicity scale of the tested molecules by response integration in an appropriate index (Biomarker Response Index).
Contribution: In this work I have conducted the bivalve exposure tests and I have carried out all the considered biomarkers.

Chapter 6:
*Paper 5: Marco Parolini, **Stefano Magni**, Andrea Binelli (2014). Environmental concentrations of 3,4-methylenedioxymethamphetamine (MDMA)-induced cellular stress and modulated antioxidant enzyme activity in the zebra mussel. Environmental Science and Pollution Research 21, 11099-11106.*

In this paper, we evaluated the MDMA (ecstasy) chronic toxicity on the bivalve *D. polymorpha* during 14 exposure days applying a biomarker battery. Two concentrations of MDMA have been tested: an environmental concentration of 0.05 µg/L and a higher concentration of 0.5 µg/L that,

having already been tested in previous studies in our laboratory on other illegal drugs, has allowed us to identify a toxicity scale among the same tested molecules.
Contribution: In this work I have conducted the bivalve exposure tests and I have carried out all the considered biomarkers.

Chapter 7:
*Paper 6: Marco Parolini, **Stefano Magni**, Sara Castiglioni, Ettore Zuccato, Andrea Binelli (2014). Realistic mixture of illicit drugs impaired the oxidative status of the zebra mussel (Dreissena polymorpha). Chemosphere. Under review.*

We evaluated the sub-lethal effects of an illicit drug mixture on the bivalve *D. polymorpha* during 14 exposure days applying a biomarker battery. This study has a high ecological valence, since the drugs of abuse released into the surface waters form complex mixtures that may cause additive or synergistic toxic effects on non-target organisms. For this reason, the tested mixture was performed with illicit drugs at environmental concentration: cocaine (50 ng/L), benzoylecgonine (300 ng/L), amphetamine (300 ng/L) morphine (100 ng/L) and MDMA (50 ng/L).
Contribution: In this work I have conducted the bivalve exposure tests and I have carried out all the biomarkers.

Chapter 8:
*Paper 7: Marco Parolini, **Stefano Magni**, Irene Traversi, Sara Villa, Antonio Finizio, Andrea Binelli (2014). Environmentally relevant concentrations of galaxolide (HHCB) and tonalide (AHTN) induced oxidative and genetic damage in Dreissena polymorpha. Journal of Hazardous Materials. Accepted.*

This study is part of a larger project aimed at assessing the sub-lethal effects of PPCPs. In this study we evaluated the chronic toxicity of two synthetic musks (galaxolide and tonalide, two personal care products widely used in the cosmetic industry) on the bivalve *D. polymorpha* during 21 exposure days applying a suite of biomarkers. Two concentrations for each considered musks have been tested: 100 ng/L and 500 ng/L of galaxolide and 20 ng/L and 80 ng/L for tonalide.
Contribution: In this work I have measured all the considered biomarkers.

Chapter 2

PAPER 1

The biofiltration process by the bivalve *D. polymorpha* for the removal of some pharmaceuticals and drugs of abuse from civil wastewaters

(Ecological Engineering 71, 710-721)

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The biofiltration process by the bivalve *D. polymorpha* for the removal of some pharmaceuticals and drugs of abuse from civil wastewaters

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ABSTRACT

This study shows the evaluation of the possible use of the freshwater bivalve *Dreissena polymorpha* for the removal of some recalcitrant contaminants, namely many pharmaceuticals and drugs of abuse that are not sufficiently removed from civil wastewaters. This mollusk has an enormous filtering capability and is highly resistant to natural and anthropogenic stresses and to a significant bioaccumulation of lipophilic contaminants. All these characteristics may be particularly useful for the removal of compounds not easily eliminated by conventional wastewater treatment processes. To verify this hypothesis an experimental study was conducted at the pilot scale using a pilot plant installed in the largest wastewater treatment plant of Milan (Milano-Nosedo, Italy). First, we presented results obtained in several preliminary tests in order to evaluate the capability of zebra mussel specimens to survive in different wastewater mixtures, its filtering capacity and the possible influence of bio- and photo-degradation in the abatement of the molecules of interest. Finally, data obtained in the final tests demonstrated a capacity of this filter-feeder to reduce the concentrations of several pharmaceuticals and drugs of abuse higher than that obtained by the simple natural sedimentation, suggesting a possible implementation of the bio-filtration process in wastewater management.

1. Introduction

Today, the main challenge in the wastewater treatment of Western countries is the abatement of several environmental pollutants recalcitrant to removal, such as pharmaceuticals and personal care products (PPCPs), drugs of abuse and related metabolites. These compounds have been detected in sewage flows, surface and groundwater with levels generally ranging from traces to pbb values (Fatta-Kassinos et al., 2011). Despite it is known that these chemicals are to be associated with adverse effects in aquatic organisms at environmental relevant concentrations, it is now well accepted that conventional WWTPs are not designed to quantitatively remove micropollutants, because they are basically designed for the elimination of macropollutants (suspended solids, organic matter and nutrients). Thus, in the last decade, intense efforts have been made to improve the performances of WWTPs in respect to micropollutants' removal by introducing new steps designed to remove such contaminants more efficiently (Batt et al., 2007; Clara et al., 2005a; Nakada et al., 2007). Thus, a plethora of several different new and pioneering methods have been developed and tested as final wastewater treatment to remove these pollutants, such as activated carbon adsorption (Yu et al., 2008), ozonation and advanced oxidation processes (Kim et al., 2008), membrane filtration (Snyder et al., 2007), reverse osmosis (Lee et al., 2012; Dolar et al., 2012) and bio-filtration (Reungoat et al., 2011). Unfortunately, each of them possesses several drawbacks and/or most of them are able to eliminate only specific contaminants. In fact, the current consensus on treatment in the research community is that no single technology can completely remove pharmaceuticals because of their very particular physical–chemical characteristics and that integration of removal technology may prove essential to handling of today's mixtures of compounds in wastewater (Fatta-Kassinos et al., 2011). Moreover, their efficiency of removal is significantly affected by several factors: the physicochemical properties of pharmaceuticals, the treatment processes employed, the age of the activated sludge, the hydraulic retention time (HRT) and

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0925-8574/

environmental parameters such as temperature and light intensity (Clara et al., 2005b; Andreozzi et al., 2003).

In this context, we tried for the first time worldwide a natural, not expensive and energy-free treatment for the abatement of some recalcitrant environmental pollutants from wastewaters exploiting the physiological characteristic of the freshwater bivalve zebra mussel (*Dreissena polymorpha*), which is well known for its huge filtering capacity. This filter feeder can filter a wide range of suspended particles of greater than 0.7 μm from the water (Sprung and Rose, 1988), whose a percentage is assimilated (typically in the range of 15–40 μm; Ten Winkel and Davids, 1982) and the rest deposited to the benthic zone as faeces and pseudofaeces. Furthermore, zebra mussel has an enormous filtering capacity, ranging from 5 to 400 mL/bivalve/h (Ackerman, 1999; Baldwin et al., 2002) and the capability to reach a high population density, with more than 700,000 specimens/m^2 (Pathy, 1994). Its possible use in the wastewater treatments is also promoted by the great resistance to physical and chemical changes (e.g. temperature, pH, hardness and salinity) and the ability to attach itself to hard substrates by the byssus. McLaughlan and Aldridge (2013) consider the freshwater filter feeders as "keystone organisms" or "ecosystem engineers" because they are characterized by a system that processes large volumes of water in order to trap and concentrate food from their surrounding. On the other hand, there are several evidences in the use of zebra mussel for environmental management as biofilter. Piesik (1983) examined almost thirty years ago the possible application of *D. polymorpha* in eutrophication control in a canal of Poland, concluding that these mussels succeeded in removing dissolved nutrients from the water. Richter (1986) gave preliminary results of a study in Lake Tjeukemeer (The Netherlands), which suggested zebra mussels could be effective tool for reducing algal density in Dutch lakes. Antsulevich (1994) proposed the construction of artificial reefs in the Neva Bay (Finland), which could be periodically removed and cleaned from uncrusting zebra mussels to improve the water quality. More recently, Elliott et al. (2008) tested the influence of different ecological factors, such as the density of the mollusks, the current speed and the amount of food, on the zebra mussel filtration capacity. These authors also suggested that zebra mussels could be used as an on-site industrial bio-filter for water treatment. Finally, Schernewski et al., 2012 considered the opportunities of using zebra mussel cultivation in the Szczecin Lagoon (Germany) in order to improve water quality. Although these evidences based on the capability of zebra mussel to be exploited in the environmental management, no data are available until now on the possible use of this filter feeder as complementary treatment in WWTPs. Thus, this study is a novelty in the wastewater management overview and could represent a possible starting point to take advantage on the natural characteristics of an aquatic organism that can be enforced in the prevailing engineering and chemical approaches. Our idea started from the consideration that many environmental chemicals are bound to suspended particulate matter (SPM) in different ways, depending not only on lipophilic properties, such as persistent organic pollutants (POPs), but also on specific other characteristics (cation exchange, complexation and hydrogen bonding), typical of some relatively polar or ionic chemical classes, such as pharmaceuticals and drugs of abuse (Lahti and Oikari, 2011; Baker and Kasprzyk-Hordern, 2011; Petrie et al., 2013; Darwano et al., 2014). Thus, we hypothesize that the very high filtration rate due to hundreds of thousands of zebra mussel specimens can decrease the concentration of several pollutants in wastewater by the increase of SPM deposition on the bottom of the plant. Moreover, the high filtration rate allows a fast intake of environmental pollutants in the mussel soft tissues, increasing their elimination from the wastewater. To verify this hypothesis, we carried out this research at the pilot scale at the largest WWTP of Milan (Milano-Nosedo; Northern Italy) by the construction of a pilot-plant that was implemented with other facilities during the entire study. In detail, we investigated the possible use of zebra mussel to decrease the concentrations of 13 pharmaceuticals (atenolol, carbamazepine, ciprofloxacin, clarithromycin, dehydroerythromycin, diclofenac, furosemide, ibuprofen, hydrochlorothiazide, ketoprofen, naproxen, ofloxacin, paracetamol) and 4 drugs of abuse (cocaine, benzoylecgonine, methamphetamines and methadone) still present in the outflow of the largest WWTP of Milan. Moreover, we showed also the results obtained in the needful preliminary tests carried out to verify the capacity of this mussel to live in different wastewater mixtures, the better conditions to maintain higher the filtration rate and data from tests conducted to evaluate the percentage of decrease of the chemical oxygen demand (COD), chosen as starting marker of the filtration capability of the zebra mussel specimens.

2. Materials and methods

2.1. The pilot-plant

Fig. 1 shows the pilot-plant and other equipment used for this project. In detail, the pilot-plant was a stainless steel tank with a volume of 1000 L (l = 154 cm, h = 102 cm, w = 80.5 cm) with 20 removable Plexiglas® panels (size of 70 × 40 cm; Fig. 2) to which the zebra mussel specimens attached themselves. The particular arrangement of panels forced the wastewater into a zigzag pathway to increase its residence time (Fig. 2). More than 2000 zebra mussel specimens were attached to each panel, for a total of approximately 40,000 individuals in the pilot-plant. The tank had a hopper bottom with five outflow valves to allow the collection and elimination of the sediments, faeces and pseudo-faeces (Fig. 1). A submerged pump collected the treated effluent directly from the canal between the sedimentation tanks and the sand filters of the WWTP. This position also guaranteed the lack of the possible accidental release of *D. polymorpha* specimens into the WWTP outflow because the sand filters and the following disinfection process (by peracetic acid) should stop and kill any leaked organism. Moreover, we added several plastic grids with a narrow mesh to all valves and outputs from the pilot-plant to prevent any mussel outflow. In the canal from which the effluent was pumped to the pilot-plant, the temperature ranged between 14 and 24 °C, the optimal survival temperature for zebra mussels (McMahon, 1996). We also added a 200 L re-circulation tank (Fig. 1) to feed the plant by re-circulation to investigate various hydraulic retention times (30, 60, 90, 120, 15, 180, 210, 240 min). The re-circulation pump allowed a maximum waste flow of 3500 L/h. Finally, two "attachment tanks", in which the zebra mussels can attach themselves to panels by the byssus, completed the entire facility, in addition to a "nursery" (Fig. 1) in which the panels with the attached mussels can be stored before their placement into the pilot-plant. The pilot-plant and other facilities were completely cleaned cyclically by tap water and before each test to guarantee the best conditions and the elimination of the possible "memory effect" from the previous assay.

The mussels were collected by scuba divers at different times from Lake Maggiore and Lake Lugano, located at the Italy-Switzerland border. We gently detached the mussels from the submerged substrates (rocks, branches, anthropic wastes) and rapidly transferred them to the WWTP of Milano-Nosedo by refrigerated bags to decrease any stress factors.

First, we conducted the following preliminary tests: (1) evaluation of the time and method required for the mussels' adhesion to the panels; (2) adaptation of the individuals to the fed effluent; (3) control of the possible concentration decrease of pharmaceuticals

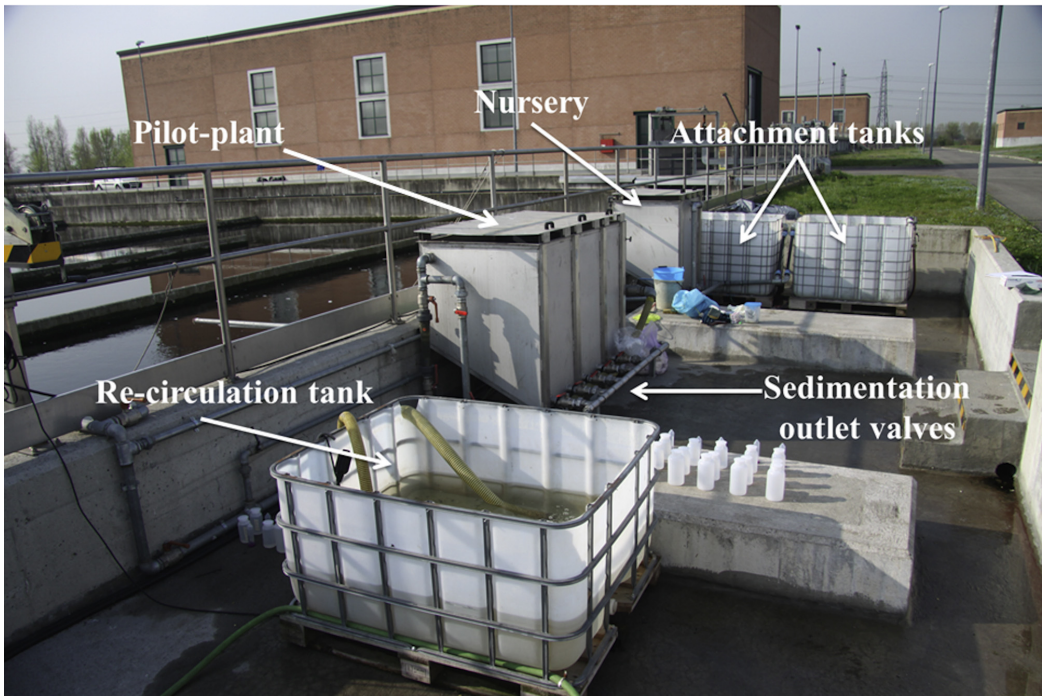

Fig. 1. Structure of the pilot-plant and all the facilities.

and drugs of abuse due to photo- and bio-degradation and (4) evaluation of the removal efficiency of COD by the zebra mussels. Only after the positive check of these conditions, we moved to the final assays to evaluate the possible abatement of some pharmaceuticals and illicit drugs in the final WWTP outflow.

2.2. Analytical characterization of the fed effluent

As mentioned above, the bio-filtration was first fed on the secondary effluents (after biological treatment and secondary settling). As the effluent had very low COD, suspended solid and fecal coliform concentrations, tests were performed on different inflow/outflow mixtures to evaluate the performance of the bio-filtration for different pollutant concentrations and provide the mussels with the needed feed. The mean starting chemical characteristics and the *Escherichia coli* counts in the used mixtures are reported in Table 1.

2.3. Adaptation of mussels to the effluent

Once adherent to the Plexiglas® panels, the *D. polymorpha* specimens were laid inside the pilot-plant and placed in contact with the wastewater, which was pumped into the tank directly from the post-sedimentation canal. The idea of using *D. polymorpha* in the tertiary treatments assumed that the mussels could live in the effluents, which have physico-chemical properties different from those of the freshwater. Thus, some tests were conducted to control the good health conditions of the animals. We fed the mussels twice a week with *Spirulina* spp. to provide the needed food as the effluent had very low organic matter content ($BOD_5 \approx 3$ mg/L). We monitored the mussel health status by measuring the total content of proteins and lipids in their soft tissues. To evaluate these physiological parameters, many bivalves (five mussels for proteins and thirty for lipids' determination) were gently cut off from the panels at different times by the excision of the byssus and frozen at −20 °C until the analyses. Unfortunately, the mussel soft tissue obtained at each time point was sufficient to perform the measurement of proteins in triplicate (about 1.2 g fresh weight for each pool) but not for the lipid determination (about 7 g fresh weight), which could only be performed once. To quantify the protein content, we removed the shell and byssus and subsequently homogenized the soft tissues in a solution of phosphate buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.4) and 0.1% Triton. The samples were placed at 4 °C in a refrigerated centrifuge at 12,000 rpm for 15 min. The proteins were quantified by the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA) using bovine serum albumin (BSA) as the standard. For the quantification of lipids, the mussel soft tissues were lyophilized and extracted using 100 mL of an acetone/*n*-hexane (1:1 v/v) mixture in a Soxhlet apparatus (FALC Instruments, Lurano, Italy). The samples were subsequently reduced in volume with a rotary evaporator, transferred to 5 mL tubes and gently evaporated under a nitrogen stream. The lipid content was then measured by gravimetric determination.

2.4. Evaluation of the zebra mussel removal efficiency

Because some environmental pollutants are bound to the particulate matter, we chose the measurement of COD as preliminary markers of the possible chemical removal. Thus, to evaluate the efficiency of *D. polymorpha* in removing the selected compounds, we compared the COD changes obtained after the tests with and without mussels in the pilot-plant. For these tests, performed under

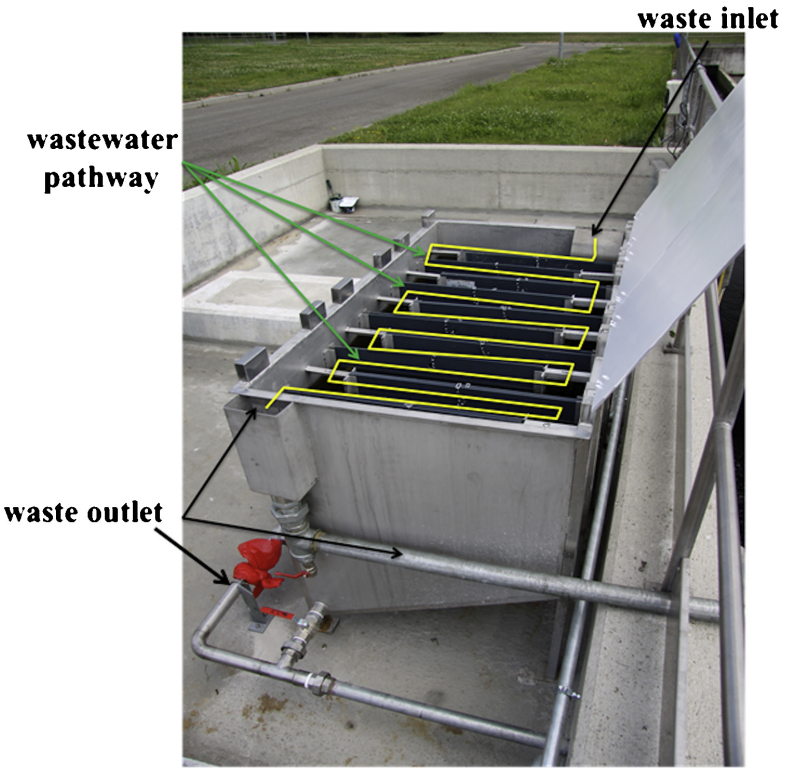

Fig. 2. The pilot-plant with the Plexiglas® panels placed inside. The yellow broken line indicates the zigzag flow direction that maximizes the contact between the bivalves and wastewater.

the re-circulation condition, we added approximately 40,000 animals to the pilot-plant, which was self-powered solely by the outflow waste pumped from the post-sedimentation canal (waste flux = 3500 L/h), allowing a residence time of 15 min in the pilot-plant. The time of complete re-circulation was 18 min as we added other 200 L of waste to the re-circulation tank (Fig. 1). This experimental design enabled a longer contact of the waste with the mussels because the waste re-circulates approximately 14 times in 4 h. Because the COD value in the outflow is normally very low (approximately 10–15 mg/L), we first added 32 g of *Spirulina* spp. to the pilot-plant to increase the starting COD to approximately 50 mg/L, after mixing the algal suspension with waste in the re-circulation tank for 20 min to reach a good homogenization. After these preliminary tests, we conducted other assays with different wastewaters obtained by mixing the inflow and the outflow of the WWTP in different proportions. In detail, we performed four tests in triplicate using the following waste ratios: (1) the outflow effluent from the WWTP, (2) the inflow waste of the WWTP, (3) 50% inflow and 50% outflow and (4) 25% inflow and 75% outflow. Before the addition of the inflow waste to the treated effluent, we preliminarily filtered the former with a bag filter (mesh = 1 mm) to eliminate the gross particulate matter. In each test, we measured the COD every 30 min for 4 h to obtain the removal slope for both assays, with and without mussels. In detail, we collected 50 mL of the mixture from the pilot-plant every 30 min for a total of 4 h to follow the two COD decrease slopes. The samples were placed in plastic bottles, immediately acidified with 1 mL H_2SO_4 and transported in refrigerated bags to the laboratory, where the samples

Table 1
Mean starting values of the chemical oxygen demand (COD), total suspended solids (SST) and colony-forming units (cfu) of *Escherichia coli* measured in the wastewater mixtures used in the preliminary tests.

	Wastewater mixture	COD mg/L	TSS mg/L	*E. coli* cfu/100 mL
Without mussel	100% OUT	15 ± 2	7 ± 3	$6 \times 10^3 \pm 2 \times 10^3$
	25% IN	38 ± 15	20 ± 6	$2 \times 10^5 \pm 8 \times 10^4$
	50% IN	59 ± 8	35 ± 11	$4 \times 10^5 \pm 9 \times 10^4$
	100% IN	132 ± 43	101 ± 24	$2 \times 10^6 \pm 6 \times 10^5$
With mussel	100% OUT	12 ± 1	5 ± 1	$6 \times 10^3 \pm 4 \times 10^3$
	25% IN	37 ± 10	32 ± 18	$2 \times 10^5 \pm 6 \times 10^4$
	50% IN	52 ± 10	26 ± 10	$5 \times 10^5 \pm 1 \times 10^5$
	100% IN	94 ± 23	97 ± 30	$2 \times 10^6 \pm 5 \times 10^5$

were analyzed within 24 h. For the COD measurement, we used the Hach Lange kit (LCK414 COD; 5–60 mg/L) coupled with the Hach Lange LT 200 digester and the Hach Lange DR 3900 spectrophotometer.

2.5. Evaluation of the abatement of pharmaceuticals and illicit drugs

These tests were carried out in re-circulation conditions with a constant flow rate of 3500 L/h. We covered the re-circulation tank with a dark plastic bag to protect the waste from light and the pilot-plant was already equipped with three plastic hard tops. First, we performed four assays without mussels in the pilot-plant for 6 h to verify the possible role played by photo- and bio-degradation of these pollutants: (1) the treated effluent that flows from the sedimentation tank of the WWTP; (2) the inflow waste of the WWTP; (3) 50% of inflow and 50% of treated effluent and (4) 25% of inflow and 75% of treated effluent. Before the addition of the inflow waste to the treated effluent, we preliminarily filtered the former with a bag filter (mesh = 1 mm) to eliminate the gross particulate matter. Then, to evaluate the abatement of pharmaceuticals and drugs of abuse contained in the wastewater, we performed four assays with the same types of wastes described above to investigate the possible role played by the different quantity of suspended particulate matter in these processes. Since the residence time of the wastewater in the WWTP of Milano-Nosedo is 24 h, these assays were conducted initially for 4 h in order to maintain a temporal condition compatible with the possible future engineering of the process. Additionally, we decided the extension of the exposure time until 24 h for the last two mixtures (50% inflow/50% outflow; 25% inflow/75% outflow), which showed the better performances, in order to investigate the possible relationship between filtration rate and time of contact with the chemicals present in the wastewaters. Then, we tested the eventual decrease in the chemical concentration in the pilot-plant without mussels (controls) for all the wastewater mixtures. In these tests, performed in November 2012, we equipped the re-circulation tank with four submerged heaters to maintain the temperature of the wastewater at 15 °C also during the night. From the analytical point of view, the water samples (2 L) were first stored at −20 °C until analysis and successively filtered on a mixed cellulose membrane filter 0.45 μm (Whatman, Kent, U.K.). Samples (50 mL for raw wastewater and 100 mL for treated wastewater) were solid-phase extracted by a mixed reversed-phase cation exchange cartridge (Oasis-MCX). Before extraction, the pH was adjusted to 2 with 37% HCl, and the samples were spiked with the labeled deuterated compounds used as internal standards: atenolol d7, carbamazepine d10, ciprofloxacin d8 and ibuprofen d3 (Sigma Aldrich, Steinheim, Germany) as well as cocaine d3, benzoylecgonine d3, methamphetamine d9 and methadone d3 (Cerilliant Corporation, Round Rock, TX, USA). The cartridges were conditioned before use by washing with 6 mL methanol, 3 mL MilliQ water and 3 mL water acidified to pH 2. Samples were then passed through the cartridges at a flow rate of 5–10 mL/min. Cartridges were vacuum-dried for 10 min and eluted with 2 mL of methanol and 2 mL of a 2% ammonia solution in methanol. The eluates were dried under a nitrogen stream. The dried samples were redissolved in 200 μL of MilliQ water, centrifuged, transferred into glass vials and analyzed by HPLC–MS/MS (high-pressure liquid chromatography-tandem mass spectrometry) following previously validated analytical methods (Castiglioni et al., 2005, 2011; Zuccato et al., 2008). In the last assays, we measured also two other pharmaceuticals: paracetamol and ibuprofen, not preliminary considered in the photo- and bio-degradation tests.

2.6. Statistical analyses

Data normality and homoscedasticity were verified using the Kolmogorov–Smirnov and Levene's tests, respectively. We performed a statistical comparison (SPSS 21 IBM software package) between tests carried out with and without mussels in the pilot-plant. For all these cases, we conducted the comparison using the one-way analysis of variance (ANOVA; $p < 0.05$; $p < 0.01$).

3. Results and discussion

As no data are available about the possible use of *D. polymorpha* for wastewater treatment purposes, conducting some preliminary tests was necessary to investigate some operative problems and to organize in the most appropriate way the project plan to reach the correct data interpretation in the final assays. Thus, we showed first data from these preliminary tests, and then results obtained with pharmaceuticals and illicit drugs.

3.1. Adhesion of the mussels to panels and adaptation of individuals to the effluent

One of the first crucial problems faced in our project was how to attach thousands of mussels to each panel without damaging them or decreasing their filtration capability. To solve this problem, we decided to exploit the natural behaviour of *D. polymorpha* to produce the byssus. Thus, the collected specimens were simply placed on plastic panels stored horizontally in the attachment tanks (Fig. 3) filled with tap water, waiting the time required to establish a sufficient physical bond by the byssus. We evaluated the best conditions that guaranteed the minor stress to bivalves by taking advantage of this natural adhesion. We conducted several tests that highlighted the need to observe few simple precautions: (1) the transport of mussels from the sampling site to the pilot-plant should be performed as fast as possible and (2) the time of adhesion must be sufficient to increase the resistance of the byssus, which will be able to support the weight of the animal then positioned vertically in the pilot-plant. Indeed, although we observed that 6–7 days were generally sufficient to guarantee an adequate adhesion to the substrate, we preferred to wait at least two weeks before the final placement in the pilot-plant. Furthermore, in the final tests in which these two simple conditions have been maintained, we noticed a very low mussel mortality rate during the entire test time.

To evaluate the health status of the *D. polymorpha* specimens exposed to the outflow effluent of WWTP, we measured the total content of lipids and proteins in their soft tissues over the next three months after their sampling. Fig. 4A shows only a slightly decreasing trend in the percentage of lipids, reaching a final percentage of approximately 10% of the dry weight, which is absolutely comparable with that found in several surveys performed using this biological model in the Italian sub-alpine great lakes (Binelli and Provini, 2003; Binelli et al., 2006, 2008). Furthermore, no significant ($p > 0.05$) increase was noticed in the protein content (Fig. 4B). The obtained data confirmed the acclimatization and adaptation of these mollusks to the physico-chemical characteristics of the final waste, showing a remarkable resistance of the bivalve.

3.2. Evaluation of the COD removal efficiency

Because the preferential food of this filter-feeder is algae and suspended particulate matter, to which some pollutants are bound, the measurement of COD can indirectly highlight the potential removal efficiency of *D. polymorpha* with respect to these

Fig. 3. Plexiglas® panels with bivalves placed horizontally inside the attachment tanks.

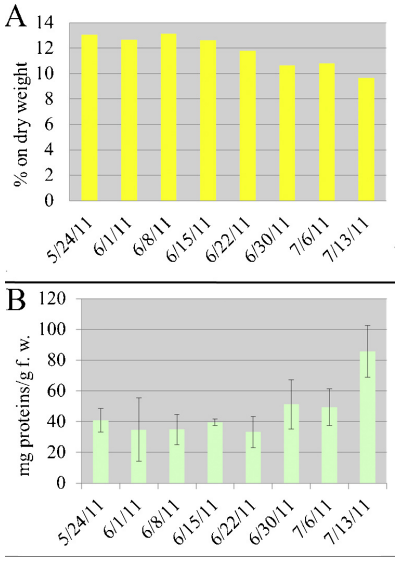

Fig. 4. Lipid (%; (A)) and protein (mg/mL ± standard deviation; (B)) content measured in the soft tissues of *D. polymorpha* exposed to the WWTP effluent for two months after the sampling.

recalcitrant chemicals. Thus, preliminary tests were conducted considering the decrease of COD due to the mussel filtration compared with the control tests performed without bivalves in the pilot-plant.

Fig. 5 clearly shows the higher and significant COD removal rate (one-way ANOVA, $F = 809.9$; $p < 0.001$) observed in the experiments conducted with the zebra mussel specimens in the pilot-plant compared with the controls without mussels. After a linear starting phase of 60 min, in which the two slopes were completely overlapped, the removal rate due to the zebra mussel filtration continued to be linear ($R^2 = 0.95$), with an increase of 42% after 4 h. In contrast, the COD slope obtained without mussels tended to an asymptote ($R^2 = 0.90$). The percentage of COD abatement without the mussels was only 19%, which was produced by the natural algal and particulate matter sedimentation. Therefore, it can be concluded that *D. polymorpha* is able to break down by bio-filtration approximately 20% of the COD in the filtered wastewater supplemented with *Spirulina* spp. after 4 h and potentially the same percentage of the pollutants bound to the suspended particulate matter.

Once the feasibility of the *D. polymorpha* to filter its food has been established, the final preliminary step was the evaluation of the COD removal in wastewaters with different physical–chemical properties, in which the organic/inorganic matter ratio is very different (Fig. 6). The choice to investigate the potential bio-filtration potency with four different wastewaters' mixtures (the outflow effluent from the WWTP; the inflow waste of the WWTP; 50% inflow and 50% outflow and 25% inflow and 75% outflow) allowed the investigation of both the presence of toxic substances in the wastes that decrease the pumping rate of mussels and/or increase their mortality and the possible change in the removal efficiency due to the different quantities of suspended particulate matter. Because the starting COD is different for each test due to the high intrinsic variability of the wastewater, we should normalize the data to compare the results obtained with the simple sedimentation and with the filtration effect by the mussels. Fig. 6 shows the comparison between the specific removal rates obtained with

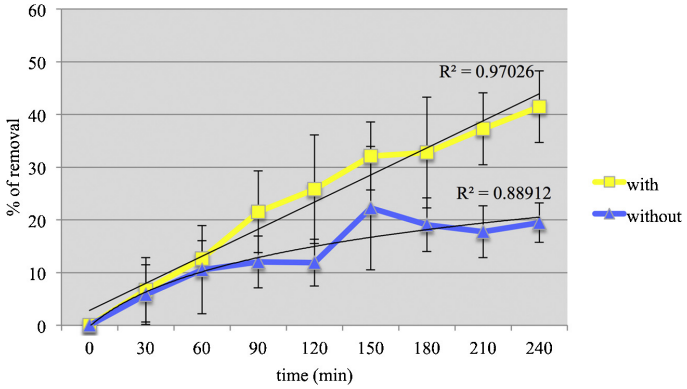

Fig. 5. Percentage of COD (±standard deviation) removed from the outflow with the addition of *Spirulina spp*. The yellow curve showed the COD removal with *D. polymorpha* specimens in the pilot-plant, whereas the blue curve showed the COD decrease in the control test (without zebra mussels).

and without mussels in the pilot-plant for four hydraulic retention times (HRTs; 60, 120, 180 and 240 min) related to the COD (mg/L) measured at these HRTs. No differences were noticed in the outflow effluent tests, as expected, because the starting COD value (mean = 12 mg/L) was too low to highlight any variation. Moreover, as expected, the specific removal rates were increased with the increasing COD in the wastewater and are higher for the early times both in the controls and in the presence of *D. polymorpha*. We can highlight that the higher COD removal trend was always obtained by the additional filtration effect of the bivalves compared with the removal rates produced only by the natural sedimentation, although there were no significant differences ($p > 0.05$) between the two treatments. As the COD concentration measured at the single HRT for the test pairs (with and without zebra mussels) is different, we can use the equations describing the trend lines to compare the effect of only the sedimentation with that obtained by the filtration activity of *D. polymorpha*. For example, the mussels were able to remove from the 50% inflow/50% outflow a mean of 40.8 g of COD/h at 40.7 mg of COD/L (Fig. 6, 60 min), whereas the simple sedimentation can remove only 23.0 g of COD/h at the same COD concentration, based on data from the equation describing the COD removal rate. This finding corresponds to a removal due to the mussel filtration of 44% higher than that obtained solely with the natural sedimentation. Thus, the zebra mussel specimens could be able to increase, in the same manner, the removal of the chemicals associated with the particulate matter from the wastewater.

Finally, because these preliminary results showed that the better performance was obtained mainly for the wastewater mixture of 50% inflow/50% outflow (Fig. 6), the zebra mussels' filtration basically appears to be more effective with higher values of

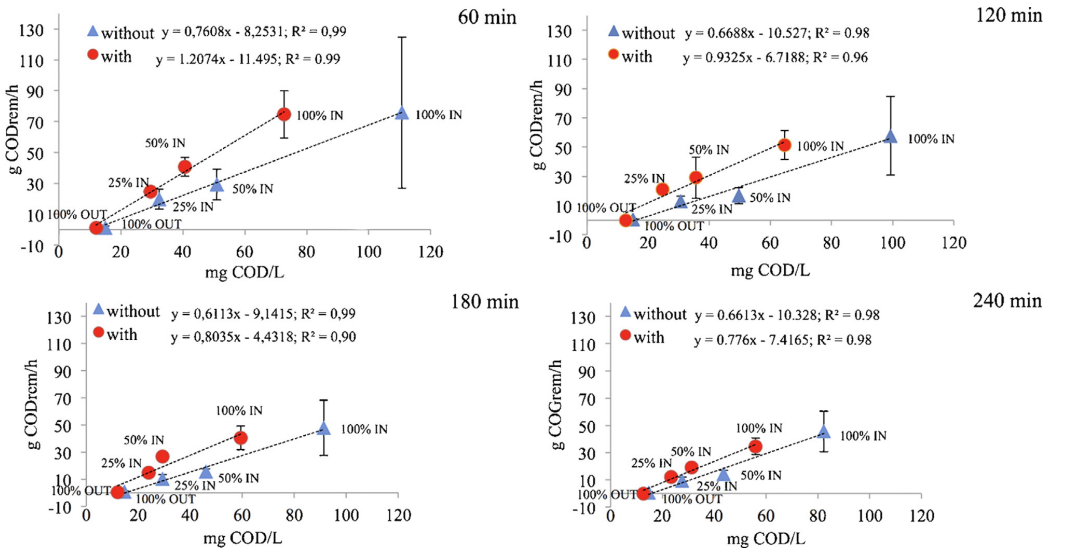

Fig. 6. Removal rate (g of COD removed/h ± standard deviation) related to the measured COD concentration (mg/L) evaluated for each of the fourth tests conducted with different waste mixtures (100% WWTP outflow, 25% inflow/75% outflow, 50% inflow/50% outflow and 100% inflow). The equations of the trend lines and the related R^2 are also shown.

initial COD (30–40 mg/L) than those usually found in the very clean outflow from the WWTP of Milan-Nosedo (10–20 mg/L). However, the European Union Municipal Wastewater Directive fixed 125 mg COD/L as the limit for WWTP outflow into the freshwater, and several outflows generally present a COD value of 60–70 mg/L, comparable with the apparent best level for filtration by the *D. polymorpha* specimens.

3.3. Measurements of the abatement of pharmaceuticals and illicit drugs

These environmental pollutants represent the new challenge in the field of wastewater treatments because some of the most widely used pharmaceutical classes, such as antibiotics and non steroidal anti-inflammatory drugs (NSAIDs), reach levels comparable with those of many POPs both in freshwaters, wastewaters and groundwater. The fate and transformation of pharmaceuticals depend on the basis of their special physical–chemical and biological characteristics, which are very complex and different from other industrial chemical compounds (Fatta-Kassinos et al., 2011). These characteristics include polymorphism, their continuous introduction into the environment after human metabolism, their chemically complex structure and the fact that they can be ionized and have multiple ionization sites spread throughout the molecule (Cunningham, 2008). Particular attention should be made to polymorphisms that arise when a molecule has the ability to crystallize in more than one form, giving to polymorphic forms with different physical, chemical, electrical and thermal properties. Thus, the polymorphs usually differ in bioavailability, solubility, dissolution rate, chemical and physical stability. Moreover, pharmaceuticals may be acid, basic or neutral and of variety of chemical forms, such small organic molecules, large polymers, carbohydrates and other compounds with complex chemistry (Fatta-Kassinos et al., 2011). These very different physical–chemical properties make very difficult the comprehension of their fate and behaviour especially in wastewaters, where the bacterial community can also transform the parent compound into a variety of degradation products. In fact, because of the great availability of glucose in biological systems, glucoronide formation is one of the most common mechanisms of pharmaceutical metabolism (Fatta-Kassinos et al., 2011). Therefore, the administered parent compound may be excreted unchanged, as a glucoronide or sulfate conjugate and as a complex mixture of many metabolites. There is evidence that glucoronides, which are the simplest and most common form of conjugated pharmaceuticals excreted by humans, are capable of being deconjugated to the parent compound during municipal sewage treatment (Kozak et al., 2001). For serotonin reuptake inhibitors, benzodiazepines, carbamazepine and macrolide antibiotics negative removal is often observed during conventional treatments. This is usually attributed to biotransformation of conjugates (Gros et al., 2010). Finally, the degree of ionization of pharmaceuticals at a particular pH will affect its bioavailability to organisms, its physical and chemical activity and its ultimate environmental fate. For example, an ionized molecule will generally have greater water solubility and will be less likely than its non-ionized form to partition to lipid-like substances (Cunningham, 2008). All these particular characteristics make difficult both a correct qualitative and quantitative evaluation of these pollutants in wastewaters, and the achievement of a single abatement system able to decrease their levels before the following outflow in the environment.

Results obtained by our tests were in some cases extremely variable and sometimes contrasting, pointing out the difficulties in data treatment of this pollutants' class. This lack of homogeneity seems not due to photo- and biodegradation that can interfere on the quali–quantitative evaluation of these classes of chemicals in the aquatic environments (Konstantinou et al., 2010). In fact, Fig. 7 shows the concentrations of pharmaceuticals and drugs of abuse at the beginning (T0) of the assays and after 6 h (T6) of recirculation measured in the pilot-plant without zebra mussels and filled with 100% WWTP outflow (Fig. 7A), 25–75% (v/v inflow:outflow; Fig. 7B), 50–50% (v/v inflow:outflow; Fig. 7C) and 100% inflow (Fig. 7D), respectively. These data pointed out that the precaution taken in our tests (see Section 2.5) make irrelevant these environmental processes because of no significant variations ($p > 0.05$) of the chemicals' concentration were noticed at the end of these assays. Obviously a slight decrease due to natural sedimentation was present, since we obtained after 6 h of recirculation an average decrease of 12%, 11% and 9% for 50% inflow–50% outflow, 25% inflow/75% outflow and 100% outflow mixtures, respectively. By contrast, results obtained with the last test (100% inflow) were different because a mean increase of 9% in respect with the starting chemicals' concentrations was noticed. In fact, only carbamazepine (−8.5%) and furosemide (−10.8%) showed the expected decrease, while all the other compounds slightly raised its level in the wastewater. This can be due to the higher amount of particulate matter, especially formed by the finest materials that cannot be able to sediment rapidly.

Overall, results highlighted a great variability in the pharmaceutical abatement in the final tests conducted until 4 h not only with the mussels in the pilot plant, but also in the controls, and mainly for the two extreme wastewater mixtures (100% inflow and 100% outflow). This is probably due to two opposite reasons: the final outflow of the WWTP contains very poor quantity of suspended matter, which can be filtered by mussels with the bounded chemicals, while the sewage from Milan (100% inflow) probably had the contrary physical and chemical characteristics, with an excessive quantity of suspended particulate matter that block the gills with the consequent death of the mussels. Another hypothesis should be due to the possible presence of specific toxicants for *D. polymorpha* in the sewage. On the other hand, after this last test, carried out at the end of the entire campaign, we observed a great mortality of zebra mussel specimens and an increase of the byssus detachment from the panels, a clear stress symptom for this species.

The variability observed after only 4 h of exposure was probably due to the peculiar chemical and physical properties and the unexpected behaviours above-mentioned. Thus, we showed only data from the intermediate wastewaters' mixtures (25% inflow/75% outflow and 50% inflow/50% outflow) carried out for 24 h, which showed a more homogeneous behaviour. Fig. 8A (25% inflow/75% outflow) points out that the biofiltration due to the mussels is able to decrease the level of several pharmaceuticals and drugs of abuse better than the simple natural sedimentation (controls). In detail, paracetamol seems to be the chemical more prone to the abatement because the daily decrease due to the effect of the mussels' filtration is double than that found after the control tests (Fig. 8A). However, the greatest difference between the biofiltration effect and the natural sedimentation is not obtained for paracetamol, but for other pharmaceuticals: biofiltration determined an abatement of the β-blocker atenolol four times higher than controls, over than three times for the dehydro-erythromycin, the main metabolite of the antibiotic erythromycin, and eight times higher for the ibuprofen, one of the most used NSAIDs worldwide. The abatement noticed for carbamazepine and ofloxacin is more difficult to explain because controls showed pharmaceuticals levels slightly higher than the starting concentrations, even if the biofiltration effect determined a clear decrease of their final concentrations (52 ± 11 μg/24 h and 141 ± 48 μg/24 h, respectively). The slight rise of levels observed for controls probably points out that these two molecules do not possess a great capability to

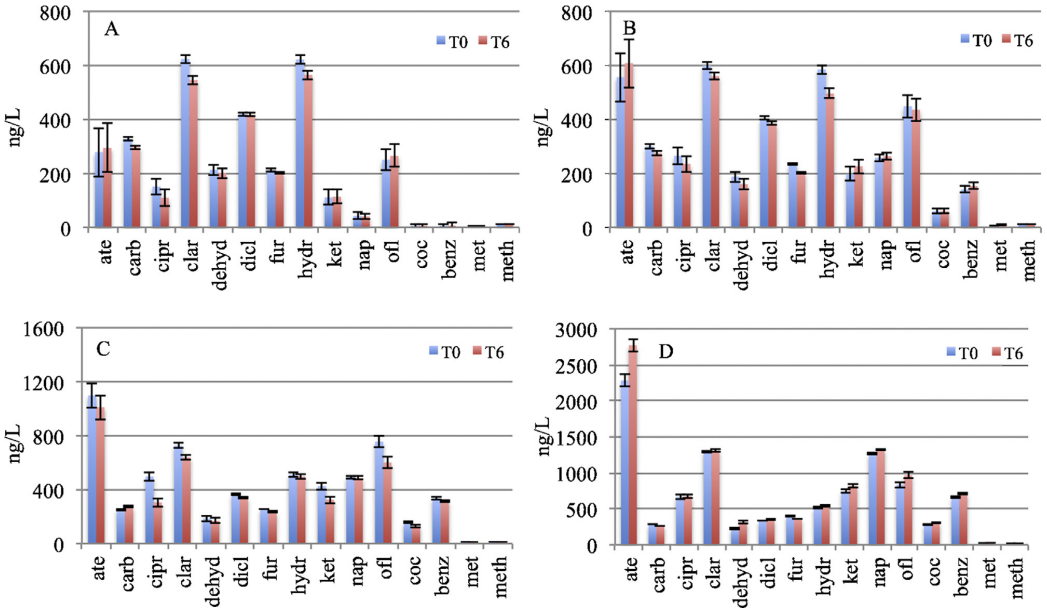

Fig. 7. Concentration (ng/L) of pharmaceuticals and drugs of abuse at the beginning (T0, blue histogram) of the tests and after 6 h (T6, red histogram) measured in the water from the pilot-plant without zebra mussels and filled with 100% WWTP outflow (A), 25–75% (v/v inflow:outflow; (B)), 50–50% (v/v inflow:outflow; (C)) and 100% inflow (D), respectively. Standard deviations from the marginal means were also shown.
ate = atenolol; carb = carbamazepine; cipr = ciprofloxacin; clar = clarithromycin; dehydr = dehydroerythromycin; dicl = diclofenac; fur = furosemide; hydr = hydrochlorothiazide; ket = ketoprofen; nap = naproxen; ofl = ofloxacin; coc = cocaine; benz = benzoylecgonine; met = methamphetamine; meth = methadone.

sedimentation and the only process able to decrease their concentrations is just the biofiltration. The illicit drugs showed a more homogeneous trend with a final decrease more than double to controls for cocaine, over six times higher for its main metabolite (benzoylecgonine) and amphetamine, and about 1.5 times higher for methadone, even if the measured concentrations of the last two drugs were very low. On the contrary, the effect of the biofiltration seems to be irrelevant for other pharmaceuticals, namely diclofenac and naproxen. Finally, we must highlight the more controversial cases observed for four pharmaceuticals (ciprofloxacin, furosemide, hydrochlorothiazide, ketoprofen), in which the final concentrations were sharply higher than those measured at the start of the assays (Fig. 8A). Since these rises of levels are observed also in the test carried out with a second wastewater mixture (Fig. 8B), but not in the controls, this points out a clear role of zebra mussel. Due to the great variability in the behaviour of some pharmaceuticals and the very different chemical–physical characteristics, we can only suggest some hypotheses to explain this apparent contradiction. Ciprofloxacin, furosemide and hydrochlorothiazide are generally excreted in unconjugated form. Moreover, these molecules possess in their structures an acid and a basic group that determine two different dissociation constants at least. This can heavily interfere on the molecules' interaction with the suspended particulate matter in relation to pH changes. Although this parameter was not measured during the 24 h assays, there are predictable little pH variations due to mussels' metabolism, which can modify the ionic state of these pharmaceuticals. Thus, we can suppose that these compounds were mainly bound to suspended particulate matter at the start of the assays and less present in the aqueous phase. The possible change of their ionic states, due to pH variations made by zebra mussels' metabolism, should increase their affinity to the aqueous phase with a consequent partial or total detachment of the molecules from the particulate matter. Thus, this affinity modification contemporarily does not allow to mussels the decrease of pharmaceutical concentration in the wastewater, since they become not bioavailable with the suspended particulate matter, increasing at the same time their instrumental reconnaissance. However, this suggestion cannot be applied to ketoprofen because it does not possess any acid or basic groups and it is excreted as conjugated form (about 85% of ketoprofen-glucuronide; Baselt, 2004). Thus, zebra mussel can be able to de-conjugate this chemical, using the glucoronide for its metabolism, increasing the concentration of ketoprofen in the wastewater by means the excretion. The great transformation of ketoprofen-glucuronide to the parent compound can be also promoted by the re-circulation conditions followed in these tests. In confirmation of the unexpected behaviour of some pharmaceuticals, depending both of their very particular physical–chemical characteristics and some environmental factors, such as pH, temperature and the quantity of suspended particulate matter, we can highlight that the same four pharmaceutics involved in the concentration increase showed the same trend also in the test carried out with the second wastewater mixture (Fig. 8B). Surprisingly, other molecules (ibuprofen, naproxen, ofloxacin, paracetamol) showed the same behaviour only in the control assays, carried out without mussels in the pilot-plant. Since ibuprofen, naproxen and paracetamol were excreted in conjugated form, the bacterial community, greater than that present in the waste mixture above-described, could de-conjugate the metabolites, increasing their concentration in the waste. This means that the biofiltration should be more efficient for these pharmaceuticals because mussels' action was able to decrease them after 24 h.

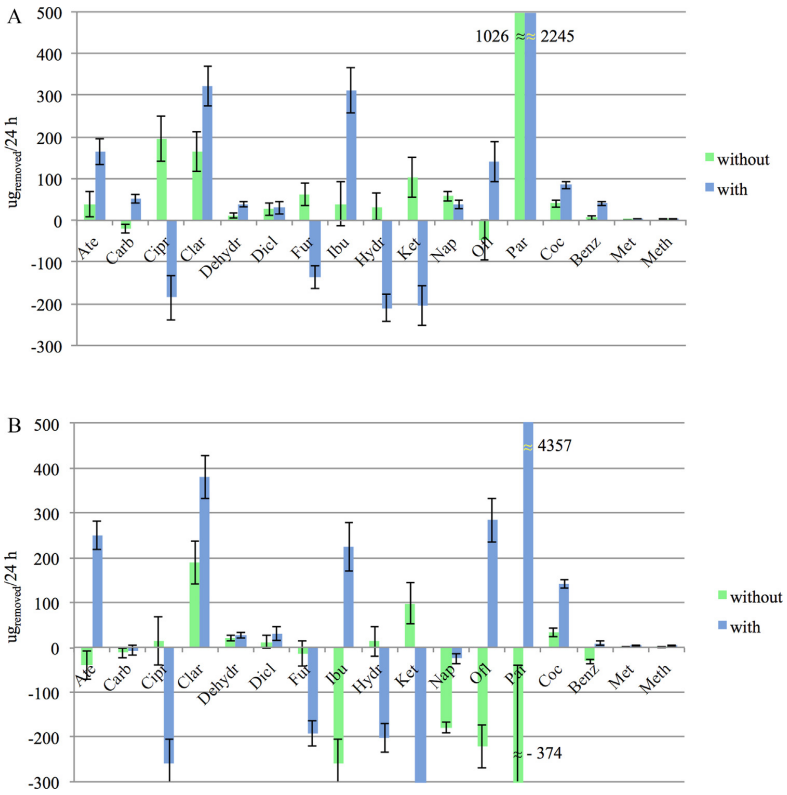

Fig. 8. Daily removal rate (μg/24 h) of pharmaceuticals and drugs of abuse obtained with and without mussels in the pilot-plant filled with 25–75% (v/v inflow:outflow; (A)) and 50–50% (v/v inflow:outflow; (B)). Standard deviations from the marginal means were also shown.
ate = atenolol; carb = carbamazepine; cipr = ciprofloxacin; clar = clarithromycin; dehydr = dehydroerythromycin; dicl = diclofenac; fur = furosemide; ibu = ibuprofen; hydr = hydrochlorothiazide; ket = ketoprofen; nap = naproxen; ofl = ofloxacin; par = paracetamol; coc = cocaine; benz = benzoylecgonine; met = methamphetamine; meth = methadone.

On the contrary, this explanation is not valid for ofloxacin that is excreted in non-conjugated form. The only current possible suggestion of its particular behaviour can be due to the property of similar fluoroquinolones (ciprofloxacin and norfloxacin), which are mainly bound to sludge (Golet et al., 2003) and consequently to suspended particulate matter. Thus, a change of particulate content in the tested wastewater mixture (from 25% inflow/75% outflow to 50% inflow/50% outflow) can modify the bioavailability of ofloxacin to bacteria.

Notwithstanding the evident difficulties in data interpretation, we can point out as the biofiltration effect seems remarkable for many compounds: paracetamol confirmed surely the best abatement performance previously noticed with more than 4000 μg eliminated from the wastewater in 24 h. Other significant abatements were registered for clarithromycin ($380 \pm 48\ \mu g_{removed}/24\ h$), ofloxacin ($284 \pm 48\ \mu g_{removed}/24\ h$), atenolol ($250 \pm 31\ \mu g_{removed}/24\ h$) and ibuprofen ($225 \pm 54\ \mu g_{removed}/24\ h$), as well as the cocaine ($142 \pm 9\ \mu g_{removed}/24\ h$).

The mechanism of the clear abatement of several pharmaceuticals and drugs of abuse observed after 24 h due to *D. polymorpha* biofiltration can be only presumed since, for instance, only very recently a method to measure their levels in mussels is available (Klosterhaus et al., 2013). We hypothesized that zebra mussels can catch the SPM from wastewater and put them through faeces and pseudofaeces into the bottom of the pilot plant together with the adsorbed chemicals. Since we previously filtered (0.45 μm) the samples, we can suggest that these pollutants are bound to the finest SPM, as occur for some POPs. On the other hand, we preliminary tested both the filtered fraction and the part of SPM remained on the filter, obtaining a negligible concentration of the studied chemicals in this last fraction (data not shown). Moreover, also Darwano et al. (2014) assert that the portion of suspended particulates in a WWTP, that would be more recalcitrant to sedimentation, would be smaller and lighter and thus remain in the effluent without other treatments. Furthermore, such smaller SPM would tend to have a greater specific surface area to which these chemicals can be bound at higher concentration. Other possible hypotheses can be due to the bioaccumulation and/or metabolism of certain chemicals. Klosterhaus et al. (2013) found several pharmaceuticals and drugs of abuse in the soft tissues of the benthic ribbed horsemussel (*Geukensia demissa*) from San Francisco Bay (CA, USA). Interestingly, since the calculated bioaccumulation factor (BAF) was much higher than that the model-predicted BCF (bioconcentration factor), the Authors explained this apparent discrepancy through the partitioning or binding of these particular chemicals to a non-lipid compartment (e.g. protein binding or active transport processes rather than passive diffusion). Finally, zebra mussel can be able

to biotransform the parent compounds into different metabolites, which cannot be longer revealed by instrumental determinations. Therefore, when it is establish that *D. polymorpha* could be useful for the abatement of pharmaceuticals and drugs of abuse, it will be necessary other studies in order to investigate the mechanism to which it is able to do it.

4. Conclusions and future prospects

Although the reported results are only preliminary, they highlight for the first time a natural and inexpensive method to polish biologically treated effluents from some recalcitrant pollutants. The idea to try this new type of treatment based on the natural behaviour of a species, considered in several countries to be an alien species, appears to be encouraging even if many other tests must be conducted to verify this possibility before the engineering of the entire process. During the project, we improved our knowledge about many technical and logistic problems, not possible to foresee before the starting of the assays. Since the better performances were obtained with certain characteristics, namely a greater time of exposure and the right quantity of suspended particulate matter, we concluded that the next step to the possible grand-scale use of the biofiltration technique shall be tested in small reservoirs, such as the phytodepuration and lagooning systems, where the residence time are generally a few days long. Moreover, one of the crucial problems to solve in the next future will be the need to collect many zebra mussels, since each of our tests was carried out with about 40,000 specimens in the pilot plant. This number is doomed to increase proportionally to the volume of the reservoir, forcing a fast method to collect hundreds of thousand zebra mussels in the natural ecosystems. In this context, there are just encouraging studies about the possible cultivation of this species by very simple methods in a cost-effective way (McLaughlan and Aldridge, 2013). Another problem to engage for a possible future engineering of this process is the management of mussels, which can bioaccumulate not only these particular chemicals, but also other environmental pollutants from wastewaters (e.g. POPs and heavy metals). Thus, mussels must be regularly substituted with clean animals both to maintain a higher concentration gradient between biota and wastewater and contemporarily eliminate the polluted mollusks. On the other hand, also sludge from the WWTPs and the vegetable biomass in the phytodepuration plants must be treated as dangerous wastes, but the advantage made by the use of *D. polymorpha* is surely be due to the lower weight of waste to dispose.

Finally, we must avoid the escape from the plant of adult specimens and larvae of this alien species. Thus, the placement of the pilot plant before the sand filters and the peracetic treatment resulted essential and must be followed with similar expedients in a possible engineering of the system. In fact, we would not advocate the introduction of this invasive species for water quality management, but rather propose that we consider embracing their positive attributes in controlled systems, positioned in the areas where they are already established. In fact, the recourse for these purposes to zebra mussel, considered an alien species in many countries, seems to be encouraging only if many precautions are made to avoid not only any mussel outflow, but also the escape of the veliger larvae that were produced up to 1 million per individual per year (McIsaac et al., 1992). Obviously, in a conservation context, the ideal situation would be to use native mussels, such as freshwater unionids, but unfortunately they are in decline worldwide since several years (Bogan, 1993) and its reproduction depends on parasitic larval stage in fish that can represent a sure disadvantage in a possible future industrialization of the biofiltration process.

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Chapter 3

PAPER 2

Removal of heavy metals from wastewater using zebra mussel bio-filtration process

(Journal of Environmental Chemical Engineering. Under review)

Removal of heavy metals from wastewater using zebra mussel bio-filtration process

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ABSTRACT

The heavy metal pollution is a serious environmental problem but still unsolved since these contaminants are released mainly by human activity, reaching all the environmental compartments. Traditional wastewater treatment plants are very efficient in removing heavy metals only when their concentration is in the order of mg/L, but are not able to remove them till µg/L, as it would be needed to cope with the water quality standards in low flow receptors. Therefore, the aim of our study was to evaluate the potential removal of some heavy metals recalcitrant to the classical treatments, by the natural process of bio-filtration performed by the invasive zebra mussel (*Dreissena polymorpha*). For this purpose we built a pilot-plant at the Milano-Nosedo wastewater treatment plants, where we placed about 40,000 *D. polymorpha* specimens appointed to the wastewater bio-filtration. The heavy metal removal due to zebra mussel activity was evaluated in the treated wastewater with a plasma optical emission spectrometry (ICP-OES). Data obtained in these experiments showed an encouraging heavy metal removal due to *D. polymorpha* activity; in particular, the total abatement (100%) of Cr after one day of bio-filtration exposure is remarkable. Therefore, this study encourages further research related with the use of bivalves as a new tool for the wastewater depuration process.

Keywords: zebra mussel; bio-filtration; wastewater treatment; heavy metals

ABBREVIATIONS: Heavy metals (HMs); wastewater treatment plants (WWTPs)

1 INTRODUCTION

Heavy metal (HM) pollution is a major global concern since these inorganic contaminants are continuously released into the environment by human activities (Williams *et al*., 1998; Kadirvelu *et al*., 2001). The ability of these compounds to accumulate in the organisms and to trig the onset of diseases and disorders makes HMs very dangerous for many organisms, including humans, at very low concentrations (Inglezakis *et al*., 2003). In particular, the water pollution due to HMs is a serious and partially unsolved issue because the removal needed to reach acceptable concentrations in the receiving waters (in the order of μg/L) is well over the efficiency of wastewater treatment plants (WWTPs), normally reported as between 40 and 90% (Mailler *et al*., 2014). For this reason, alternative methods for the HM abatement have been identified in order to be complementarily applied to traditional wastewater treatment processes. However, most of these techniques, such as precipitation/neutralization, ion exchange, membrane separation, reverse osmosis, electrodialysis and activated carbon adsorption (Matheickal *et al*., 1987; Atkinson *et al*., 1998; Ahluwalia and Goyal 2007) have high costs for the regeneration of resins or activated carbon and/or for the disposal of chemical sludge or concentrates (Guibal *et al*., 1992). Therefore, the attention of the scientific community has to be focused on the development of natural methods which were more eco-sustainable and, possibly, less expensive. In this regard, biosorption is a possible natural method for HM elimination; this term defines the passive pollutants uptake from an aqueous solution by a dead or non-growing microbial biomass (Beveridge and Doyle, 1989; Volesky, 1990). Although this treatment has the advantage to not undergo inhibition due to the pollutants' toxicity, the early biomass saturation by adsorbing contaminants represents an important limitation for the further exploitation of this process (Ahluwalia and Goyal, 2007). In addition to the biosorption, the bioaccumulation process to which many organic and inorganic contaminants are subject to some aquatic viable microorganisms such as fungi, algae, bacteria and yeast (Galun *et al*., 1987; Gadd, 1992) may be considered. In particular, bioaccumulation due to microorganisms living on aquatic macrophyte tissues is correlated with HM removal in constructed wetlands. This methodology is certainly the most used natural system of wastewater treatment, which couples accumulation in microbial biomass and in macrophytes such as *Phragmites australis*, *Eichhornia crassipes* and *Lemna* spp. (Reddy, 1984; Pip and Stepaniuk 1992; Certini and Scalenghe, 1999; Dhote and Dixit, 2009). This alternative method, in addition to the removal of HMs, also reduces organic matter and nutrients from wastewater (Dhote and Dixit, 2009). Despite the existence of these eco-friendly methodologies, in recent years, more studies have been conducted to identify new methods for natural water purification from some recalcitrant pollutants. In this regard, it is of great interest the research carried on by Ledda and co-workers (2012) aimed at assessing how small breeding of

Mediterranean sponges *Ircinia variabilis* and *Agelas oroides* could remove some contaminants from marine waters. In the same way, the use of other filtering organisms can be interesting for the improvement of waters quality. In this context, the freshwater bivalve *Dreissena polymorpha* has some characteristics that would make it suitable for the above mentioned purpose: an enormous filtering capacity, ranging from 5 to 400 mL/bivalve/h (Ackerman, 1999; Baldwin *et al*., 2002), a high population density, with more than 700,000 individuals/m^2 (Pathy, 1994), and the ability to produce faeces and pseudofaeces to which many contaminants are adsorbed. Moreover, taking into account the indirect ability of bivalves to bioaccumulate many environmental contaminants, including HMs (Naimo, 1995), we can point out the potential of *D. polymorpha* to this purpose (Reeders and Bij de Vaate, 1992; Silverman *et al*., 1996; Elliott *et al*., 2008; Sousa *et al*., 2009). In this regard, a study conducted in 1983 by Piesik highlighted how *D. polymorpha* is able to remove nutrients from eutrophic waters and a following research confirmed the potential of *D. polymorpha* in the reduction of algal density (Richter, 1986). In the last two decades, several other studies have demonstrated the filtering capacity of this bivalve, whose breeding could be developed for an alternative treatment of polluted freshwaters (Elliot *et al*., 2008; Stybel *et al*., 2009; McLaughlan and Aldridge, 2013; Sousa *et al*., 2014). In this regard, a recent study conducted by Binelli and co-workers (2014) showed the ability of this mollusk to remove from wastewaters different types of emerging contaminants, such as pharmaceuticals and drugs of abuse. Nevertheless, it is important to take into account that *D. polymorpha* is considered an invasive alien species all over Europe and United States, even if this mollusk was present in Europe before the last glaciation (Starobogatov and Andreeva, 1994) and was then bounded in some basins of Eastern Europe in the post-glacial period until the 18th century (Olenin *et al*., 1999). The human activity then favored the distribution of *D. polymorpha* in its original European areal; in Italy, for example, this bivalve is found since 1973 (Giusti and Oppi, 1973) and its presence in the Italian inland waters has been confirmed by subsequent studies (Spilinga *et al*., 2000; Bodon *et al*., 2005; Cianfanelli *et al*., 2007). Therefore, the idea of using this invasive species for anthropic purposes (bio-filtration, human food, animal feed, fertilizer and biogas; Stybel *et al*., 2009) would be of huge interest, especially in the economic sphere. On the basis of these above mentioned studies on *D. polymorpha*, we assessed the efficiency of this bivalve as a new biological method as the last step of wastewater treatment in a conventional WWTP. For this purpose, we built at the Milano-Nosedo WWTP (Northern Italy) a pilot-plant in which 40,000 *D. polymorpha* specimens were added in order to filtrate some types of wastewaters and evaluate the possible abatement of some HMs, such as Aluminum (Al), Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni) and Lead (Pb).

2 MATERIALS AND METHODS

2.1 *Pilot-plant construction and placement at the Milano-Nosedo WWTP*

A scuba diver collected the bivalves from the Lake Maggiore and Lake Lugano, both located close to the Italy-Switzerland border. Since it is well-known that *D. polymorpha* is a biofouling organism (Arpita *et al*., 2013), we placed approximately 40,000 specimens in a special attachment tank in order to let them naturally re-adhered to twenty Plexiglas® panels (size: 70x40 cm; Figure 1) via their *byssus* over a period of two weeks. During this acclimatization period, the bivalves were kept in tap water and fed with the green-blue alga *Spirulina* spp. The Plexiglas® panels were then placed into the pilot-plant (Figure 1), a stainless steel tank with a volume of about 1000 L (L=154.0 cm, h=102.0 cm, w =80.5 cm) where the panels are disposed following a zig-zag pathway (yellow line, Figure 2), in order to increase both the surface and the contact time between the wastewater and each bivalve. In addition to the steel tank, we installed a recirculation tank (Figure 1) with a volume of 200 L with a submerged pump to allow a constant wastewater flow (3,500 L/h) into the pilot-plant. The recirculation tank further increases the contact time between the wastewater and the filter-feeding bivalves placed into the pilot-plant, as well as limits the efficiency of settling which would remove part of the contaminants adsorbed on suspended solids. The pilot-plant can directly collect the effluent from the canal placed between the sedimentation tanks and the sand filters of the Nosedo WWTP using a submersible pump (0-5000 L/h). The installation site of the pilot-plant allows to test a clarified effluent and to avoid thus the risk that suspended solids can not only compromise the filtration capability of bivalves but also cause the animals death due to gill occlusion. Moreover, the pilot-plant position into the Nosedo WWTP guaranteed the lack of any possible accidental release of *D. polymorpha* specimens into the surrounding environment because the sand filters and the following process of disinfection with peracetic acid stopped and killed any possible leaked organism.

2.2 *Experimental design and sample collection*

The preliminary tests designed to evaluate the filtering and purifying performance of *D. polymorpha* have been described in detail by Binelli and co-workers (2014). In that study, the following issues have been discussed: 1) the adaptation of *D. polymorpha* to wastewater; 2) the estimation of *D. polymorpha* filtering efficiency; and 3) the analysis of *D. polymorpha* capacity in the removal of a new class of environmental pollutants (pharmaceuticals and illicit drugs). Only after these issues have been clarified, we evaluated also the HM abatement. As previously mentioned, an important point was the need to evaluate the removal efficiency of *D. polymorpha*

independently from any other settling process, which would remove the metals adsorbed on suspended solids. The filtering action of *D. polymorpha* was first evaluated on the effluent outflowing secondary settling. However, since this effluent had a very low COD (≈10 mg/L) and, consequently, a low suspended solid concentration (on which a relevant amount of HMs is normally adsorbed; Tessier and Campbell, 1987; Calmano *et al*., 1993), the following tests were performed with other three different wastewater mixtures, previously filtered through a 1 mm mesh bag filter to remove coarse matter. This allowed us to evaluate the filtration efficiency of *D. polymorpha* on wastewater having a polluting load and a different quantity of suspended particulate, also taking into account that this bivalve selects particles for food with a diameter range between 15-40 μm (Winkel and Davids, 1982). The mixtures used in the tests, in addition to 100% outlet, are the following: 25% inlet/75% outlet and 50% inlet/50% outlet and 100% inlet (wastewater incoming at WWTP). The HM removal evidence from wastewater were carried out through the measurement of their concentration in the water samples taken from the pilot-plant with bivalves inside; at the same time, control tests were conducted into the pilot-plant without adhering animals. All tests were performed in triplicate. The HM removal progress was monitored for 4 hours, by sampling the wastewaters every 30 min, which enabled to obtain the removal slope for each HMs. We chose to evaluate the HM removal within 4 hours, taking into account that the treated wastewater remain in the Milano-Nosedo WWTP for about 24 h; thus, the selected time seemed a fair compromise in view of integrating the conventional treatment with limited dimensional requirements. To check the practicability of such assumption, we carried out further final tests in single for a period of 24 h, making only two samples, one at the beginning and one at the end of the tests. The tests were conducted with an initial flow rate corresponding to 3,500 L/h, which would imply 18 minutes contact time, recirculating the effluent in the pilot-plant 84 times to obtain an overall 24 h contact time. After each test, the entire pilot-plant was washed with tap water, to avoid memory-effects related to the previous tests. For this reason, to minimize this problem, as well as to decrease the bivalve stress, the test schedule was organized starting with the most diluted waste (100% outlet) and gradually increasing its concentration till 100% inlet. We monitored the wastewater temperature both at the beginning and end of each tests to take into account its possible interference with the filtration activity of zebra mussels. Wastewater temperature within the pilot-plant ranged from 14 to 24 °C, comparable with the optimal values for *D. polymorpha* filtration activity (10-20 °C; McMahon, 1996). Samples were taken from the pilot-plant at the selected times by 250 mL plastic bottles, acidified with 1% of HNO_3 and stored at 4 °C at dark until analysis.

2.3 *Evaluation of HM abatement*

We evaluated the removal of some HMs relatively abundant in urban wastewaters: Aluminum (Al), Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni) and Lead (Pb). The samples, taken from the pilot-plant, were treated according to the CNR IRSA 3010 method. Briefly, an aliquot of each sample was transferred into a flask and heated up to 100 °C to remove turbidity. After cooling, samples were brought back to the starting volume with distilled water. Samples were analyzed in a plasma optical emission spectrometer (ICP-OES; OPTIMA 2100 DV, Perkin Elmer; detection limits for each HM: Al 0.5 µg/L; Fe 0.2 µg/L; Mn 0.1 µg/L; Ni 0.5 µg/L; Pb 1.0 µg/L; Cu 0.5 µg/L; Cr 0.2 µg/L) equipped with ultrasonic nebulizer (CETAC Ultrasonic Nebulizer, model U5000AT +). The HM concentrations were quantified by a calibration curve at two points, starting from appropriate dilutions of mixed certificate standard (AccuStandard MES 16-1).

2.4 *Statistical analyses*

Data normality and homoscedasticity were verified using the Shapiro-Wilk and Levene's tests, respectively. We performed a statistical comparison (SPSS 21 IBM software package) between tests carried out with and without mussels in the pilot-plant, where the dependent variable is the HM concentration in the wastewater and the fixed factors are the treatment and the exposure time. For all these cases, we conducted the comparison using the two-way analysis of variance (two-way ANOVA; *$p<0.05$; **$p<0.01$).

3 RESULTS AND DISCUSSION

3.1 *Evaluation of D. polymorpha filtering ability in the HM removal*

The results obtained from the tests carried out with a 25% inlet/75% outlet mixture (Figure 3A, B, C, D, E, F, G) showed a good performance due to the bio-filtration effect, probably because of the suitable concentration of suspended matter. The removals obtained for each HM tested through the filtering activity of bivalves are always greater than those related to the natural sedimentation evaluated in controls. In fact, for the majority of the analyzed HMs, the contribution of the zebra mussel filtration is evident, since the difference between the removal percentage with and without bivalves in the pilot-plant was statistically significant: Al ($F=36.809$, $p<0.01$); Fe ($F=62.686$, $p<0.01$); Mn ($F=125,452$, $p<0.01$); Ni ($F= 5,695$, $p<0,05$); Pb ($F=16,645$, $p<0.01$); Cu ($F=6.220$, $p<0.05$). In detail, observing the trends reported in Figure 3, it has to be highlighted that the difference between the removal percentages measured at the end of the tests reached the 30%, for Fe and Pb, while for Al, Ni and Mn the removal was about 20-25% higher than controls. Thus, in

only 4 h, zebra mussels have been able to significantly decrease levels of most of the tested HMs, even if the removal of Cu was only 8% higher than natural sedimentation. On the other hand, the time selection to conduct the tests is crucial for the possible engineering of the process that cannot be longer than few hours, since the entire cycle of the wastewater treatment ends in about 24 h. Tests carried out by adding 50% of inlet to the WWTP outlet (Figure 4A, B, C, D, E, F, G) showed a lower difference compared to control than the previous tests, probably due to an excessive presence of suspended particulate matter that determines a stress condition to the animals, which may require a longer time than 4 h to acclimate and begin the filtering process. Moreover, we cannot exclude the possible presence of toxic compounds into the inlet of WWTP that could have led to a further decrease in the filtration activity. Despite these possible interfering processes, we found significant difference between tests carried out with bivalves in the pilot-plant and their respective controls for Al ($F=68.587$ $p<0.01$), Mn ($F=38.710$, $p<0.01$), Pb ($F=26.183$, $p<0.01$), Cu ($F=22.861$, $p<0.01$) and Cr ($F=4.729$, $p<0.01$). In this regard, at the end of the test the removal was around 20-25%, comparable to the results obtained for the mixture 25% inlet/75% outlet for Al, Mn, Pb and Cu, whilst for the other tested metals it decreased dramatically. The fluctuating values obtained for Ni could be due to the low concentration of this metal in the analyzed wastewater (<10 µg/L), taking into account the huge variability of pollutant load in the inlet wastewaters. The role of the initial concentration must always be considered when drawing conclusions in terms of percent removal: if these are very low, small variations (which could also be partly due to analytical reasons) assume relevant percent weight. In both the considered tests (25% inlet/75% outlet and 50% inlet/50% outlet mixtures) negative values of sedimentation, comprised in a range of -5 and -10%, are observable; these values are likely to be related to the coefficient of variation of the method used to perform the wastewater HM quantification. These data do not appear to be random, because, except for the fluctuating values of Ni (Fig. 4E), Mn and Pb showed null sedimentation values in both tests performed (Fig. 3D,F and 4D,F). This result can be reasonably related to the chemical speciation phenomenon because these metals can probably be dissolved in water and not bounded to the particulate. Therefore, the observed Mn and Pb removal process carried out by D. *polymorpha* could mainly be related to bioaccumulation. Further studies are needed in order to deepen the knowledge about some of the above-mentioned aspects, as also suggested by Camusso and co-workers (2001). In this regard, the wastewater pH value, which influences the metal speciation, is kept constant in WWTPs and, therefore, should not compromise the *D. polymorpha* purification activity. Finally, with regard to the test with 100% inlet, there has been a serious decline in the bivalves' performance related to a high mortality of the animals (data not shown). This result further confirms how an excessive suspended particulate matter amount and the possible presence of toxic

substances into the WWTP can decrease the bivalves filtering capacity and even compromise their health status. However, this aspect does not limit the possible engineering of this method, since it would be sufficient to control the particulate matter of the wastewater, as suggested by Binelli and co-workers (2014). Moreover, despite the suspended matter concentration represents a limiting factor of *D. polymorpha* filtering capacity, it should be noted that the specimens used in this study are the same used in the pharmaceuticals and illicit drugs removal process, described by Binelli and co-workers (2014). Despite an exposure to multiple pollutants, the bivalves purifying ability is stable during the whole experimental trial, representing a sure advantage in the use of this very resistant organism. Furthermore, the data shown refer to the HM removal within the first 4 h of wastewaters exposure to *D. polymorpha*, and that the bivalves' performance can be improved with increasing contact time between mollusk and wastewater, as described below.

3.2 *Time influence on the HM removal by D. polymorpha*

Data obtained by the above-mentioned tests suggested that the contact time between wastewater and the filter-feeding bivalves was probably one of most crucial parameters, affecting the extent of HM removal from wastewater. As previously mentioned, although the increase of contact time could be almost impossible at full scale, we decided to carry out tests 24 h long. On the basis of the results obtained at 4 h, the 24 h tests were performed only on 25% inlet/75% outlet and 50% inlet/50% outlet mixtures. For most HMs, the removal due to mussel filtration was about 70% with the 25% inlet/75% outlet mixture (Figure 5A). The natural sedimentation, at the same time, removed 50% of Cr and Fe and, surprisingly, only 10-25% of Cu, Mn and Pb (Figure 5A). Thus, zebra mussels' filtration is able to increase the removal of Pb and Mn by about 60% with respect to the settling effect in blanks. Notably, Cr removal appeared very interesting because of its high toxicity for aquatic organisms (Nriagu and Nieboer, 1988; Chandra and Kulshreshtha, 2004); in fact, *D. polymorpha* removed it completely in 24 h, while the blank removal was only 50%. Therefore, contact time seems to affect significantly the extent of HM removal by the filter-feeding bivalves, considering that at the end of the first 4 h the mean removal was 20% higher with *D. polymorpha* than in the blank tests. This was also confirmed in the test performed with the 50% inlet/50% outlet mixture (Figure 5B), where the HM removal due to *D. polymorpha* was always over 70%. In particular, for Cu, Mn and Pb the net removal due to *D. polymorpha* (calculated as the difference from the blank removal) was 50%, 70% and 60%, respectively. At the same time, the high removal observed for Ni contradicts the results obtained in the experimental data set. The 24 h tests, although only performed in single and therefore needing further confirmation, provide first evidence that better HM removal performances may be obtained by increasing the contact time

between the bivalves and the feed. Further, the obtained data may indicate that the bivalve could need a period of acclimation to the wastewater, especially if characterized by a considerable amount of suspended particulate material, before starting the filtration process.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

This work, to our knowledge, represents one of the few studies concerning the possibility to use bivalves in wastewater treatment process to remove some HMs. The results appear to be very encouraging, considering that the use of non-native species, such as *D. polymorpha*, for anthropogenic purposes, could have interesting economic implications and represent an important starting point for the alien species exploitation, as suggested by Stybel and co-workers (2009). In this regard, the prevention strategies regarding the non-native and invasive species introduction determine complex social and ethical implications; furthermore, while the procedures on how to respond to invasions have been delineated, their application is still severely limited (Simberloff *et al*., 2013). Therefore, in the exclusive case of *D. polymorpha*, it may be advantageous to exploit the potential of this bivalve, now present in the almost all Europe inland waters. This will certainly not be an easy process; in fact, being *D. polymorpha* considered a serious threat for the aquatic environment and a dangerous fouling agent of many industrial structures (Claudi and Mackie, 1994; Elliott *et al*., 2005), is poorly perceived by the scientific community as a valid filtering factor, despite the presence of encouraging results in the depuration context (Richter, 1986; Antsulevich, 1994; Elliot *et al*., 2008; Schernewsk *et al*., 2012; McLaughlan and Aldridge, 2013; Binelli *et al*., 2014). In this regard, the construction of appropriate facilities for bio-filtration, followed by further downstream treatment aimed to contain bivalves accidentally leaked from the plant (such as the peracetic acid treatment and sand filters) would avoid the problem related to fouling, as suggested by previous studies (Elliott *et al*., 2005; 2008). The ideal condition would be to use native bivalves, such as unionids; however these mollusks, besides being affected by a serious population decline (Bogan *et al*., 1993), have a parasite larval stage that would be disadvantageous for the engineering of the bio-filtration process. At the same time, due to the scarcity of scientific data, we faced many technical and logistical problems during our research, not foreseeable during the experimental design drafting; the best performances of bio-filtration were in fact obtained with prolonged exposure times (24 h) and with moderate amounts of particulates. The ability of *D. polymorpha* to remove certain types of pollutants from pretreated wastewater could suggest, in a possible engineered scenario, the placement of this filter-feeding bivalve as the last step of conventional wastewater treatment sequence or to include them in other natural systems, such as constructed wetlands or lagooning, where the hydraulic retention time is of one or more days, and thus a longer

contact time between wastewater and the bivalves is allowed. Apparently, the high number of animals required could represent a limitation to the engineering of this process, but is important to keep in mind that *D. polymorpha* is a fast growing invasive species; this is a pivotal feature in order to preserve the animal turnover among the depuration tank. Once contaminated by the filtration process, in fact, the specimens undergo dehydration treatment followed by incineration or storage in dedicated landfills, as it is currently the case for sewage sludge. Therefore, the results presented in this study encourage future research regarding the use of *D. polymorpha* in the civil depuration field and stimulate insights aimed at understanding the mechanisms underlying observed removal process, investigating the fate of removed HMs by analysis of soft tissue, shell, feces and pseudofaeces.

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7 FIGURES

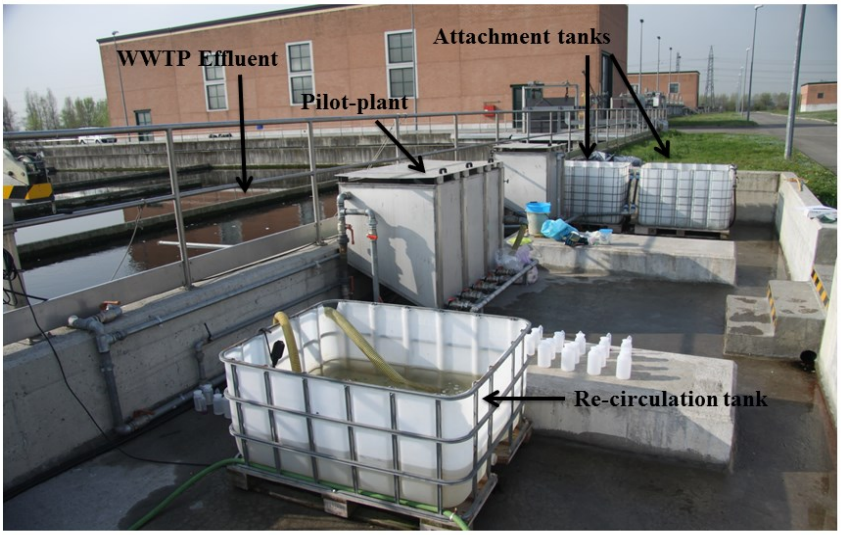

Figure 1: Structure of the pilot-plant located at the Milano-Nosedo WWTP.

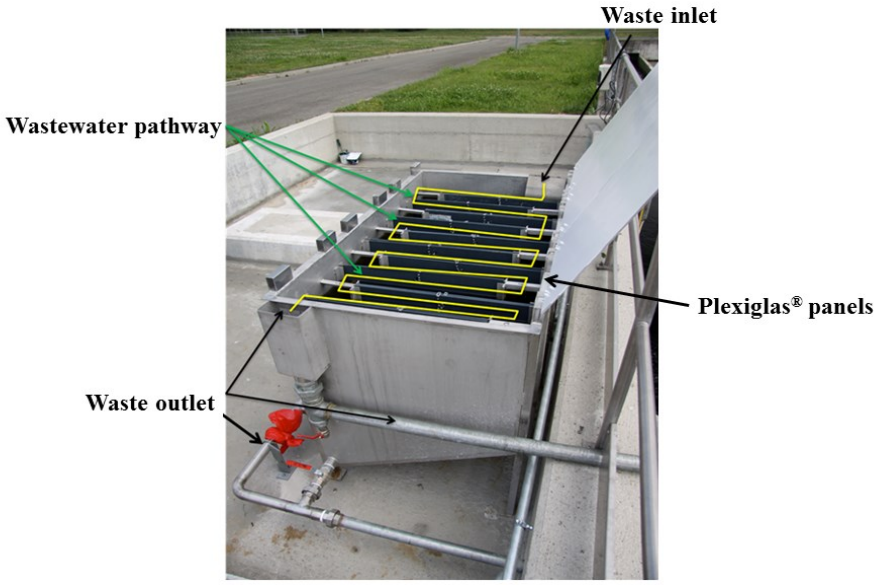

Figure 2: Plexiglas® panels placed into the pilot-plant. The yellow line indicates the zig-zag flow pathway of wastewater within the pilot-plant.

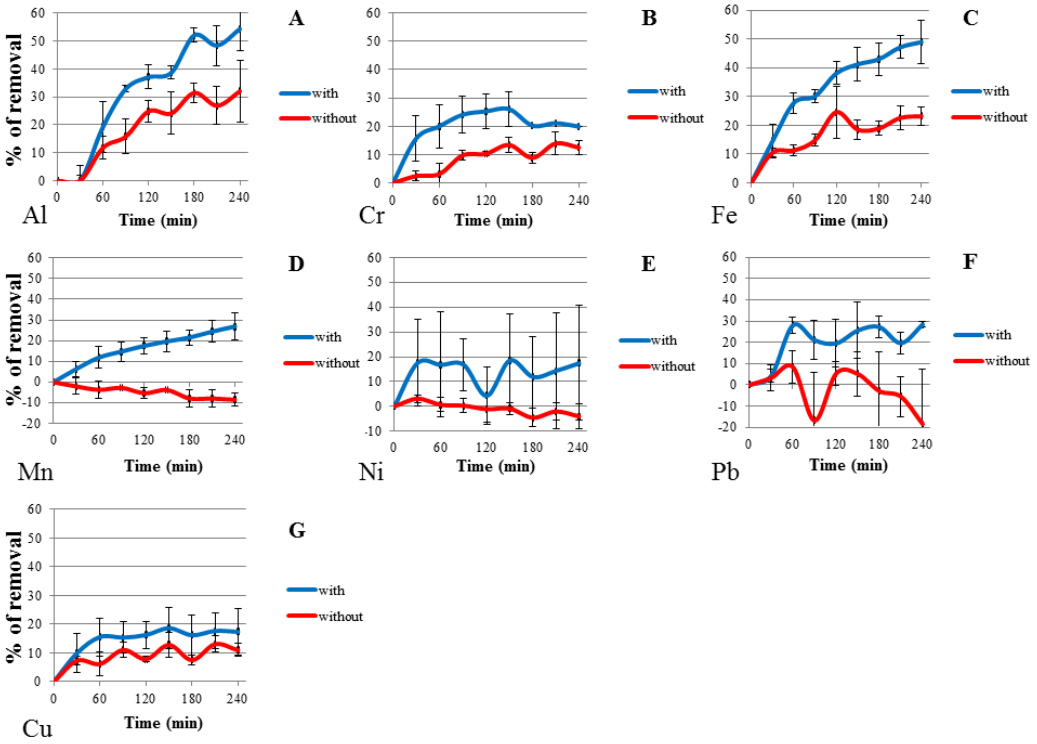

Figure 3: Mean trends (± SEM) of heavy metal removal during the first 4 h (240 min; Aluminum, A; Chromium, B; Iron, C; Manganese, D; Nickel, E; Lead, F; Copper, G) with *D. polymorpha* (blue curve) and without bivalves (red curve) inside the pilot-plant for the 25% inlet/75% outlet mixture. The differences between controls and treated, with the exception of Chromium, were statistically significant (Two-way ANOVA).

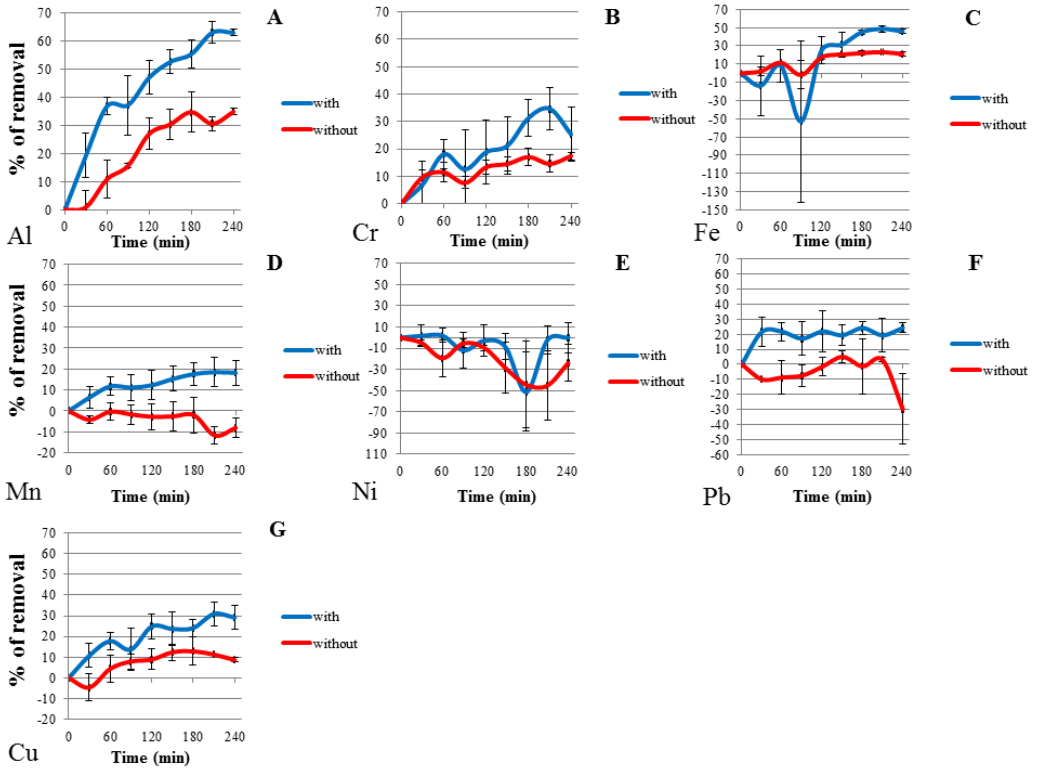

Figure 4: Mean trends (± SEM) of heavy metal removal during the first 4 h (240 min; Aluminum, A; Chromium, B; Iron, C; Manganese, D; Nickel, E; Lead, F; Copper, G) with *D. polymorpha* (blue curve) and without bivalves (red curve) inside the pilot-plant for the 50% inlet/50% outlet mixture. The differences between controls and treated, with the exception of Nickel and Iron, were statistically significant (Two-way ANOVA).

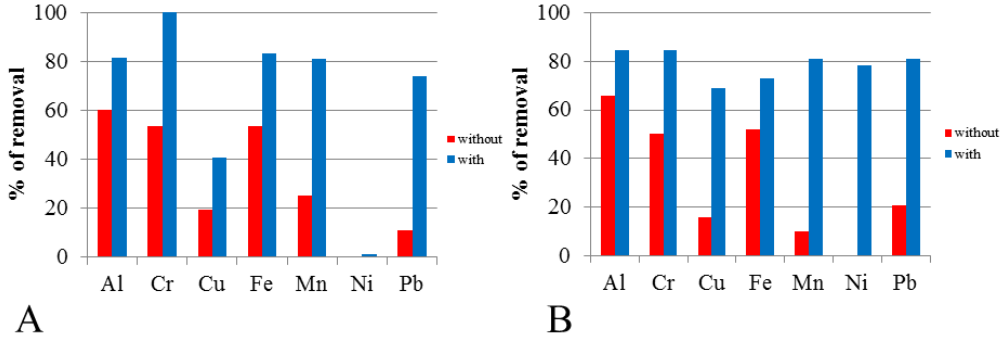

Figure 5: Removal percentage of heavy metals from wastewater after 24 h in the 25% inlet/75% outlet (A) and 50% inlet/50% outlet (B) mixtures.

Chapter 4

PAPER 3

Does zebra mussel (*Dreissena polymorpha*) represent the freshwater counterpart of *Mytilus* in ecotoxicological studies? A critical review

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Review

Does zebra mussel (*Dreissena polymorpha*) represent the freshwater counterpart of *Mytilus* in ecotoxicological studies? A critical review

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ABSTRACT

One of the fundamentals in the ecotoxicological studies is the need of data comparison, which can be easily reached with the help of a standardized biological model. In this context, any biological model has been still proposed for the biomonitoring and risk evaluation of freshwaters until now. The aim of this review is to illustrate the ecotoxicological studies carried out with the zebra mussel *Dreissena polymorpha* in order to suggest this bivalve species as possible reference organism for inland waters. In detail, we showed its application in biomonitoring, as well as for the evaluation of adverse effects induced by several pollutants, using both *in vitro* and *in vivo* experiments. We discussed the advantages by the use of *D. polymorpha* for ecotoxicological studies, but also the possible limitations due to its invasive nature.

1. Introduction

The application of the European Water Framework Directive (WFD; Directive 2000/60/EC) for the surveillance of chemical contamination of surface waters involves two main objectives based on the assessment of the chemical status of water bodies, by determining whether contamination levels are compliant with the regulatory Environmental Quality Standards (EQSs), and the evaluation of temporal trends of the contamination in the different environmental compartments of aquatic ecosystems (Besse et al., 2012). Although to reach the first objective, checking compliance with EQSs, the chemicals' analyses in water are a requirement for all the priority substances, biota is starting to be a pivotal matrix as demonstrated by the addition of three biota EQSs (mercury, hexachlorobenzene and hexachlorobutadiene) in the Directive 2008/105/EC. The same Directive promotes the use of the so-called "integrating matrices", such as biota and sediments, to reach the second objective of the WFD, contamination trend biomonitoring. In particular, biota is recognized as a preferential matrix for 14 and an optional matrix for 12 of the 41 substances listed under Directive 2008/105/EC. Furthermore, the physic-chemical characteristics of the 15 newly proposed substances also point to biota as a relevant matrix for 12 of these (Besse et al., 2012). In this context, it is crucial the selection of the proper biological model, which should possess several fundamental characteristics. Looking at the choice of organisms, aquatic macroinvertebrates emerge as one of the most valuable option, as they enable robust control of biotic factors, by using size homogenous indicator species that lend themselves well to practical and easy-to-handle biological models. Moreover,

Abbreviations: MTT, 3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazolium bromide); MDMA, 3,4-methylenedioxymethamphetamine; AChE, acetylcholinesterase; ABM, active biomonitoring; ALP, alkaline phosphatase; AP, alkylphenols; Al, aluminum; Am, americium; As, arsenic; AhR, aryl hydrocarbon receptor; ATL, atenolol; Ba, barium; Bq, becquerel; B[α]P, benzo[α]pyrene; BE, benzoylecgonine; 2DE, bidimensional electrophoresis; BLM, bleomycin; BTs, butyltins; Cd, cadmium; Ca, calcium; CBZ, carbamazepine; CAT, catalase; Ce, cerium; Cs, cesium; ChEs, cholinesterases; Cr, chromium; Co, cobalt; Cu, copper; COES, crude organic extract of sediments; Cox I, cytochrome c oxidase subunit – I; DBT, dibutyltin; DDTs, dichlorodiphenyltrichloroethane and relative homologues; DCF, diclofenac; DMAA, dimethylarsinic acid; *dl*-PCBs, dioxin-like polychlorinated biphenyls; EDs, endocrine disruptors; ENDO, endosulphan; EROD, ethoxyresorufin-O-deethylase; EST, expressed sequence tag; HHCB, galaxolide; GEM, gemfibrozil; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-*S*-transferase; Hsp70, heath shock protein 70; HCB, hexachlorobenzene; HCHs, hexachlorocyclohexanes; I, iodine; LDH, lactate dehydrogenase; Pb, lead; LPO, lipid peroxidation; Mg, magnesium; Hg, mercury; MT, metallothionein; MeHg, methylmercury; MN, micronuclei; MMC, mitomycin C; MOA, mode (mechanism) of action; MBT, monobutyltin; MXR, multixenobiotic resistance; NRRA, neutral red retention assay; Ni, nickel; Nb, niobium; NSAIDs, non steroidal anti-inflammatory drugs; OCs, organochlorine compounds; PBM, passive biological monitoring; PMF, peptide mass fingerprint; POPs, persistent organic pollutants; PCPs, personal care products; P-gp, pglycoprotein; PPCPs, pharmaceutical and personal care products; PBDEs, polybrominated diphenyl ethers; PCBs, polychlorinated biphenyls; PCDDs, polychlorinated dibenzodioxins; PCDFs, polychlorinated dibenzofurans; PAHs, polycyclic aromatic hydrocarbons; K, potassium; PCR, potassium chromate; PCC, protein carbonyl content; PP2A, protein phosphatase 2A; Rb, rubidium; Se, selenium; Ag, silver; SCGE assay, single cell gel electrophoresis assay; SB, strand breaks; Sr, strontium; SOD, superoxide dismutase; Th, thorium; Sn, tin; AHTN, tonalide; TBT, tributyltin; TCS, triclosan; TPT, triphenyltin; U, uranium; VTG, vitellogenin-like proteins; Zn, zink; Zr, zirconium.

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invertebrates represent about 95% of animal species, have an important ecological role and could be potential transfer of pollutants through the food web (Baun et al., 2008). For all these reasons, mussels are widely used as sentinel organisms to monitor chemical pollution in the aquatic environment. In fact, mussels are filter feeding, sessile bottom dwellers that bioaccumulate many contaminants with little metabolic transformation and provide time-integrated observations of chemical contamination in the environment (Roesijadi et al., 1984; Sericano, 1993). For instance, the US NOAA (United States National Oceanic and Atmospheric Administration) Mussel Watch Program is the nation's longest running continuous coastal contaminant monitoring program. In particular, different *Mytilus* species (*Mytilus edulis*, *Mytilus californianus*, *Mytilus galloprovincialis*, *Mytilus trossulus*) and oysters (*Cressostrea virginica*) have been used to evaluate the contamination status of the US coasts. On the other hand, specimens from the genus *Mytilus* are historically used as sentinel organisms worldwide to monitor the contamination of some persistent organic pollutants (Monirith et al., 2003; Carro et al., 2005; Kožul et al., 2011), heavy metals and organotin compounds (Chase et al., 2001; Furdek et al., 2012; Edwards et al., 2014), radionuclides (Rožmarić et al., 2013; Kiliç et al., 2014) and more recently also emerging pollutants, such as pharmaceuticals (McEneff et al., 2014). *Mytilus* is used also to evaluate the effect of pollutants on biota, which represents the second part of the environmental risk assessment (Forbes and Forbes, 1994). Several studies have been conducted with different approach, such as classical bioassays (Beiras and Bellas, 2008; Marcheselli et al., 2010; Paredes et al., 2014), biomarkers (Canesi et al., 2010; Gonzalez-Rey and Bebianno, 2014; Franzellitti et al., 2014) genomics (Ioannou et al., 2009; Canesi et al., 2014; Liu et al., 2014) and proteomics (Schmidt et al., 2014; Hu et al., 2014). Although *Mytilus* is the most used biological model for the monitoring of coastal waters and for the effect evaluation of marine pollutants, no specific organism has been proposed for freshwaters. Thus, one of the future crucial challenges in the biomonitoring and ecotoxicological studies on the inland water bodies should be the identification of suitable organisms with convenient characteristics like *Mytilus*. Actually, a very similar biological model is present in the European and United States freshwaters, namely the invasive mollusc zebra mussel (*Dreissena polymorpha*; Pallas, 1771). This bivalve is a relatively widespread macrobenthic species in both lentic and lotic waters and is able to live in estuarine environments because it tolerates salinity up to 5‰. It is unique among the freshwater bivalves because, like marine ones, it releases gametes freely into the surrounding waters (Neumann and Jenner, 1992). Its ecological success is due mostly to its ability to develop through several larval stages (trochophora, veliconcha, pediveliger) and to attach itself by the bissus to hard substrates in both lakes and river beds. These ecological characteristics point out a phylogenetic affinity with *Mytilus* narrower than that with the native freshwater bivalves, such as *Unionids*. This is indirectly supported by studies of Stepien et al. (1999) and Orlova et al. (2005), in which *M. edulis* was used as the out group to evaluate phylogenetic relationships among dreissenids.

The following characteristics make the zebra mussel particularly useful as bioindicator: wide distribution, continuous availability throughout the year, adequate body size, sessile organism, ease of sampling, high longevity (3–5 years; Stanczykowska, 1977), great resistance at laboratory conditions. Moreover, zebra mussel has an enormous filtering capacity, ranging from 5 to 400 mL/bivalve/h (Baldwin et al., 2002) that allows a fast intake of environmental pollutants. This is a great advantage for the biomonitoring purposes because this sentinel-organism responds very quickly to contamination changes. Furthermore, the rapid intake of toxicants allows

Table 1
List of many biomonitoring studies using *D. polymorpha*. See the Glossary for the abbreviations' meaning.

Study area	Chemical	Range	Reference
Mosel River, France	^{110m}Ag	<d.l.–6 Bq/kg d.w.	Mersch et al., 1992
	^{58}Co	<d.l.–160 Bq/kg d.w.	
	^{60}Co	<d.l.–6 Bq/kg d.w.	
	^{134}Cs	<d.l.–2 Bq/kg d.w.	
	^{137}Cs	6–11 Bq/kg d.w.	
	^{40}K	25–54 Bq/kg d.w.	
	^{210}Pb	<d.l.–30 Bq/kg d.w.	
	^{232}Th	25–40 Bq/kg d.w.	
	^{238}U	6–24 Bq/kg d.w.	
Kiev Reservoir, Ukraine	^{140}Ba	<d.l.–2294 Bq/kg	Frantsevich et al., 1996
	^{141}Ce	<d.l.–2183 Bq/kg	
	^{144}Ce	28–1036 Bq/kg	
	^{134}Cs	11–666 Bq/kg	
	^{131}I	17–370 Bq/kg	
	^{103}Ru	<d.l.–2220 Bq/kg	
	^{106}Ru	<d.l.–740 Bq/kg	
	^{90}Sr	662–1273 Bq/kg	
	^{95}Zr, Nb	<d.l.–3219 Bq/kg	
Mirgenbach Reservoir, France	Cd	0.5–1.7 μg/g	Mersch et al., 1996a
	Cu	32.0–238.0 μg/g	
Buffalo, New York	Ag	<0.04 mg/kg w.w.	Roper et al., 1996
	As	0.97 mg/kg w.w.	
	Ba	7.00 mg/kg w.w.	
	Cd	0.47 mg/kg w.w.	
	Hg	0.03 mg/kg w.w.	
	Pb	3.28 mg/kg w.w.	
	Se	0.90 mg/kg w.w.	
	PAHs	6.58 mg/kg w.w.	
	PCB Aroclor 1248	1.64 mg/kg w.w.	
Lake Erie, Canada–United States	HCBs	19.1–41.3 μg/kg lip. w.	Roe and MacIsaac, 1998
	PCBs	5367.3–10465.7 μg/kg lip. w.	
Vienna, Austria	Cd	0.50–1.30 μg/g d.w.	Gundacker, 1999
	Cu	6.9–12.3 μg/g d.w.	
	Pb	0.18–1.11 μg/g d.w.	
	Zn	98.0–128.0 μg/g d.w.	
Lake Maggiore, Italy	DDTs	18.5–134.1 ng/g d.w.	Binelli et al., 2001a,b
Subalpine Lakes, Italy	Cd	0.60–3.44 μg/g d.w.	Camusso et al., 2001
	Co	0.88–1.51 μg/g d.w.	
	Cr	2.03–4.97 μg/g d.w.	
	Cu	14.6–26.3 μg/g d.w.	
	Hg	0.049–0.158 μg/g d.w.	
	Ni	11.9–24.2 μg/g d.w.	
	Pb	1.96–5.87 μg/g d.w.	
	Zn	158.00–346.00 μg/g d.w.	
St. Lawrence River, Canada–United States	DBT	<1–158 Sn/g w.w	Regoli et al., 2001
	MBT	<1–134 Sn/g w.w	
	TBT	37–1078 ng Sn/g w.w.	
	TPT	<1–52 ng Sn/g w.w.	
Buffalo, New York	DBT	0.47–2.25 ng Sn/g d.w.	Roper et al., 2001
	MBT	<1.97 ng Sn/gd.w.	
	TBT	3.50–5.88 ng Sn/g d.w.	

(*continued on next page*)

Table 1 (*continued*)

Study area	Chemical	Range	Reference
Great Lakes, North America	Ag	0.02–0.066 μg/g d.w.	O'Connor, 2002
	As	6.20–9.80 μg/g d.w.	
	Cd	2.60–8.40 μg/g d.w.	
	Cu	14.00–34.00 μg/g d.w.	
	Cr	4.20–10.00 μg/g d.w.	
	Hg	0.041–0.082 μg/g d.w.	
	Ni	18.00–33.00 μg/g d.w.	
	Se	3.50–7.90 μg/g d.w.	
	Pb	0.77–4.60 μg/g d.w.	
	Zn	98.00–160.00 μg/g d.w.	
	DDTs	9.80–96.00 ng/g d.w.	
	BTs	35.00–240.00 ng/g d.w.	
	PAHs	270.00–650.00 ng/g d.w.	
	PCBs	110.00–470.00 ng/g d.w.	
	Chlordane	4.20–18.00 ng/g d.w.	
	Dieldrin	4.20–14.00 ng/g d.w.	
Lake Geneva, France–Switzerland	Cd	0.9 μg/g d.w.	Berny et al., 2003
	Pb	3.1 μg/g d.w.	
	HCH	6.6 ng/g d.w.	
Lake Maggiore, Italy	DDTs	612.6–3119.6 ng/g lip. w.	Binelli and Provini, 2003
St. Lawrence River, Canada–United States	As	2.49–8.23 μg/g d.w.	Kwan et al., 2003
	Ca	54.90–142.00 μg/g d.w.	
	Cd	1.41–7.43 μg/g d.w.	
	Cr	0.46–9.45 μg/g d.w.	
	Cu	14.20–35.90 μg/g d.w.	
	Hg	0.10–0.22 μg/g d.w.	
	Mn	35.00–96.00 μg/g d.w.	
	Ni	8.84–52.50 μg/g d.w.	
	Pb	0.31–1.78 μg/g d.w.	
	Se	4.05–7.36 μg/g d.w.	
	Zn	140.00–340.00 μg/g d.w.	
Flanders, Belgium	Ag	0.01–0.09 μg/g d.w.	Bervoets et al., 2004
	Al	22.30–140.00 μg/g d.w.	
	As	1.73–9.84 μg/g d.w.	
	Cd	0.40–17.50 μg/g d.w.	
	Co	0.15–0.56 μg/g d.w.	
	Cu	7.08–16.90 μg/g d.w.	
	Cr	1.73–6.40 μg/g d.w.	
	Hg	0.11–0.83 μg/g d.w.	
	Mn	26.00–89.70 μg/g d.w.	
	Ni	1.94–7.54 μg/g d.w.	
	Pb	0.42–7.94 μg/g d.w.	
	Zn	73.50–114.00 μg/g d.w.	
	HCB	0.25–0.58 ng/g w.w.	
	pp'-DDE	0.47–2.19 ng/g w.w.	
	PBDEs	0.22–1.82 ng/g w.w.	
	PCBs	6.21–66.60 ng/g w.w.	
Lake Maggiore, Italy	DDTs	4–5 μg/g lip. w.	Binelli et al., 2004
Hudson River, United States	PCBs	1.28–6.80 μg/g mussel	Cho et al., 2004
Flanders, Belgium	Ag	1.0–269.0 μg/g d.w	Bervoets et al., 2005
	Al	12.0–256.0 μg/g d.w	
	As	0.5–22.0 μg/g d.w	
	Cd	0.29–21.0 μg/g d.w	
	Co	0.29–32.0 μg/g d.w	
	Cu	5.5–173.0 μg/g d.w	
	Cr	1.4–8.9 μg/g d.w	
	Hg	0.01–0.75 μg/g d.w	
	Mn	7.4–227.0 μg/g d.w	
	Ni	1.2–112 μg/g d.w	
	Pb	0.27–18.0 μg/g d.w	
	Zn	54.0–1230.0 μg/g d.w	
	HCB	0.23–5.2 ng/g w.w.	
	pp'-DDE	0.51–8.3 ng/g w.w.	
	PCBs	8.6–168.0 ng/g w.w.	
Subalpine Lakes, Italy	DDTs	62.3–1417.3 ng/g lip. w.	Binelli et al., 2005
	HCB	<0.05–21.3 ng/g lip. w.	
	PAHs	32–200 ng/g lip. w.	
	PCBs	428.5–2508.5 ng/g lip. w.	
	Chlorpyrifos	<0.05–31.9 ng/g lip. w.	
	Chlorpyrifos oxon	<0.05–53.8 ng/g lip. w.	
Niagara River, Canada–United States	Cd	1.6–11.0 μg/g d.w.	Richman and Somers, 2005
	Cu	18.0–42.0 μg/g d.w.	
	Hg	0.05–0.12 μg/g d.w.	
	Mn	110.0–540.0 μg/g d.w.	
	Ni	31.0–56.0 μg/g d.w.	
	Pb	0.55–14.0 μg/g d.w.	
	Zn	65.0–200.0 μg/g d.w.	
	PCDDs and PCDFs	10.0–19.0 pg/g d.w.	
	Cd	1.8–3.5 μg/g	

Table 1 (*continued*)

Study area	Chemical	Range	Reference
Riou-Mort and Lot Rivers, France	Zn	12.0–152.0 μg/g	Marie et al., 2006a
Seine Estuary, France	PAHs	1000 ng/g d.w.	Minier et al., 2006
	PCBs	800 ng/g d.w.	
Flanders, Belgium	Cd	1.17–23.55 μg/g d.w.	Voets et al., 2006
	Cu	6.00–28.47 μg/g d.w.	
	Ni	2.18–10.64 μg/g d.w.	
	Pb	1.08–15.17 μg/g d.w.	
	Zn	73.7–185.1 μg/g d.w.	
	pp'-DDE	0.54–6.49 ng/g w.w.	
	HCB	<d.l.–0.36 ng/g w.w.	
	PBDEs	0.10–0.96 ng/g w.w.	
	PCBs	4.22–179.7 ng/g w.w.	
Visnes, Norway	Cu	13.53–183.63 g/g d.w.	Zorita et al., 2006
Lakes Como and Iseo, Italy	DDTs	6.11–11.18 mg/kg lip.w.	Bettinetti et al., 2008
	PCBs	0.42–0.70 mg/kg lip.w.	
Lake Maggiore, Italy	PBDEs	39.5–446.5 ng/g lip.w.	Binelli et al., 2008a
Ebro River, Spain	Hg	0.02–0.81 μg/g w.w.	Carrasco et al., 2008
	MeHg	0.22–0.60 μg/g w.w.	
Subalpine Lakes, Italy	DDTs	729.9–1386.2 ng/g lip.w.	Riva et al., 2008
	HCB	0.3–14.6 ng/g lip.w.	
	HCHs	0.1–76.7 ng/g lip.w.	
	PCBs	365.9–2508.6 ng/g lip.w.	
Flanders, Belgium	Cd	0.69–40.6 nmol/g w.w.	Voets et al., 2009
	Cu	16.9–70.0 nmol/g w.w.	
	Zn	136.8–284.5 nmol/g w.w.	
Gulf of Finland, Russia	^{241}Am	7–84 kBq/g d.w.	Zuykov et al., 2009
	^{137}Cs	7–9 kBq/g d.w.	
	^{85}Sr	27–53 kBq/g d.w.	
Orge River basin, France	Cd	0.6–1.0 μg/g d.w.	Bourgeault et al., 2010
	Co	0.8–1.3 μg/g d.w.	
	Cr	0.7–1.2 μg/g d.w.	
	Cu	8.5–12.5 μg/g d.w.	
	Mn	27.9–116.6 μg/g d.w.	
	Ni	2.6–8.4 μg/g d.w.	
	Zn	84.8–125.3 μg/g d.w.	
	PAHs	1122.0–2336.0 μg/g d.w.	
Ebro River, Spain	As	4.84–6.19 μg/g d.w.	Faria et al., 2010
	Cd	0.31–3.96 μg/g d.w.	
	Cr	0.62–1.30 μg/g d.w.	
	Cu	7.91–35.75 μg/g d.w.	
	Hg	0.10–3.01 μg/g d.w.	
	Ni	9.77–37.77 μg/g d.w.	
	Pb	0.50–0.77 μg/g d.w.	
	Zn	89.11–136.38 μg/g d.w.	
	DDTs	5.34–317.16 ng/g w.w.	
	HCB	0.41–78.41 ng/g w.w.	
	HCHs	0.34–6.08 ng/g w.w.	
	PCBs	6.82–109.16 ng/g w.w.	
Ebro River, Spain	As	3.68–5.80 μg/g d.w.	Alcaraz et al., 2011
	Cd	0.13–2.34 μg/g d.w.	
	Cr	1.68–4.83 μg/g d.w.	
	Cu	6.83–47.24 μg/g d.w.	
	Hg	0.03–0.60 μg/g d.w.	
	Mn	20.41–518.31 μg/g d.w.	
	Ni	3.85–14.94 μg/g d.w.	
	Pb	0.48–2.32 μg/g d.w.	
	Ti	16.68–61.54 μg/g d.w.	
	Zn	78.98–100.44 μg/g d.w.	
Vesle River, French	Cd	0.05–0.09 μg/g w.w.	Palais et al., 2011
	Cu	2.36–6.21 μg/g w.w.	
	Pb	0.07–0.49 μg/g w.w.	
	Ni	0.92–2.08 μg/g w.w.	
	Zn	13.14–22.02 μg/g w.w.	
Lake Maggiore, Italy	PAHs	93.58–1200.10 ng/g lip.w.	Parolini and Binelli, 2014

d.w. = dry weight; w.w. = wet weight; lip. w. = lipid weight.

the fast identification of their negative effects, awarding to this biological model the capability to be used as early-warning system. Moreover, *D. polymorpha* has been in depth studied because its physiology, anatomy, ecology and ethology are well known from several decades thanks to its wide distribution in many European countries since two centuries. These are crucial characteristics in the right selection of a good bioindicator since the lack of information like that can represent a confounding effect, producing a misunderstanding in data interpretation, mainly for in field studies.

The main drawback in the extensive use of zebra mussel could be due to its invasive behavior that obviously limits its application in active biomonitoring only in water bodies in which its presence is hailed. On the contrary, this apparent disadvantage could represent one of the pivotal reasons in order to use *D. polymorpha* both in biomonitoring and in studies on the toxicant effect evaluation, since the sampling of this invasive, numerous and wide distributed bivalve can safeguard the native species. For instance, the *Unionids'* species (e.g. *Unio* spp., *Anodonta spp.*) are in decline worldwide since several years (Bogan, 1993), in so much as the taxon *U. elongatulus* is protected under the two main pieces of European biodiversity conservation legislation: the Habitats Directive and the Bern Convention (Araujo et al., 2005).

Furthermore, the very interesting and recent paper of McLaughlan et al. (2014) points out the impacts on the ecosystem services due to the European top ten invasive species, showing that a number of these services were actually positive for ecosystems and human well-being. On the other hand, the NOAA's Mussel Watch Program collects zebra and quagga mussels since 1992 for the monitoring of the Great Lakes, notwithstanding they have been considered two of the main invasive species in the United States. A not insignificant aspect is that the possible recourse to invertebrates in laboratory studies could increase after the application of the Directive 2010/63/EU on the protection of animals used for scientific purposes, which asks to improve the welfare of vertebrates and cephalopods used in scientific procedures. Thus, the aim of this review is to illustrate all the different ecotoxicological applications of zebra mussel made until now to suggest this invertebrate as possible reference biological model for ecotoxicological studies on freshwaters.

2. Application of zebra mussel in biomonitoring

The measurement of pollutant concentrations in bioaccumulator organisms has been recognized as highly relevant in ecotoxicological terms because of the reflection of their bioavailability in the ecosystem (Phillips and Rainbow, 1992). Since the end of '70s, *D. polymorpha* specimens have been extensively used in Europe as model organism for the quality assessment and the biomonitoring of freshwaters (Table 1). In fact, field studies have established that *D. polymorpha* is able to accumulate large amounts of mineral and synthetic organic trace pollutants within its soft tissues (Gillet and Micha, 1987; Yevtushenko et al., 1990; Bush and Schuchardt, 1991), making them suitable organisms for the biomonitoring of heavy metals and organic compounds. The first study using the zebra mussel for biomonitoring have been carried out at the end of the '70s to assess the presence of metal pollution in different freshwater ecosystems. Levels of many heavy metals, typically As, Cd, Cr, Cu, Hg, Mg, Ni, Pb, Se and Zn, were also investigated in soft tissues of bivalves from freshwater environment of Central Europe, in which zebra mussel was widely diffused for a long time. A notable contamination by such metals (Cd, Cu, Pb and Zn) was found in many water streams flowing across some industrialized areas of Central Europe, such as the R. Mosel and R. Meuse in France (Mersch et al., 1992, 1993), the R. Mosel and R. Rhine in Germany (Kraak et al., 1991; Mersch et al., 1992), the Wiltz River in Luxemburg (Mersch and Pihan, 1993), the Danube River in Austria (Gundacker, 1999), as well as lakes, such as the Markermeer Lake in Netherlands (Kraak et al., 1991, 1994). Afterward, the diffusion of *D. polymorpha* in other European countries such as Italy, where was found for the first time at the end of '60 in the Lake Garda (Giusti and Oppi, 1973), encouraged researchers to use this mollusk to monitor the pollution by trace elements in high populated and industrialized areas. For example, Camusso et al. (2001) measured the concentrations of Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn in zebra mussels from the main Italian subalpine lakes, namely Lake Maggiore, Lugano, Como, Iseo and Garda, which are located in one of the most populated and industrialized Italian areas. Data analyses allowed to authors to point out that Lake Maggiore was the most polluted basin for all the monitored metals, while animals from Lake Garda and Lake Lugano showed the lowest metal levels. Moreover, thanks to the sessile status of zebra mussel, the most contaminated sites and possible local source for metals were identified for each lake, which were classified into quality classes for heavy metal pollution. Similarly, the recent diffusion of *D. polymorpha* in Spain has encouraged Carrasco et al. (2008) to monitor the levels of Hg and methylmercury (MeHg) in an area impacted by industrial hazardous dumps located in the Ebro River basin. Overall, the level of total Hg found in this area (0.16–6.81 μg/g, dry weight, dw) was much higher than those that have previously been reported for zebra mussels anywhere in the world. Overseas, in 1992 the US NOAA began to sample this bivalve in order to monitor the metal pollution status of Great Lakes (O'Connor, 2002). A six-years (1992–1998) monitoring survey highlighted notable levels of Cd, Cr, Cu, Ni and Se in zebra mussel from the Great Lakes (O'Connor, 2002), with values of Cd and Cr higher than those measured in blue mussels, according to higher zebra mussel efficiencies of their uptake from both water and food (Roditi and Fisher, 1999). Side by side to heavy metal monitoring, the zebra mussel was extensively used for the assessment of persistent organic pollutants (POPs) contamination in European lakes and rivers (Binelli et al., 2001a,b, 2004, 2005; Bervoets et al., 2005; Voets et al., 2006; Zorita et al., 2006; Guerlet et al., 2007; Bacchetta and Mantecca, 2009; Bourgeault et al., 2010). One of the best examples of the use of the zebra mussel in this field was reported by a long-term biomonitoring program of the Lake Maggiore, the second widest Italian lake. After the discovery of a contamination by DDT homologues in edible fish from the Lake Maggiore at levels exceeding the European legal limits for edible fish (Ceschi et al., 1996), since 1996 a series of international projects have been established by Italian and Swiss authorities (International Commission for the protection of Suisse-Italy waters – CIPAIS) to monitor the trend of DDTs in various environmental matrices (sediments from lake and several inlets, lake and river water, fish, bivalves, dry and wet depositions; CIPAIS, 1999, 2002, 2003, 2008, 2009, 2010, 2011, 2012; Riva et al., 2010). The zebra mussel was chosen as reference organism to evaluate the pollution trends of p,p'-DDT and relative homologues in different sampling stations of the Lake Maggiore, obtaining an interesting historical series on DDT contamination that is likely the most complete one among the few available uninterrupted contamination dataset in Western countries. The long-term biomonitoring program highlighted a notable transient DDT contamination in the Lake Maggiore depending on both anthropogenic and natural factors (i.e. the heavy flood occurred in the autumn of 2000), which interests all the lacustrine areas (CIPAIS, 2003). Moreover, the quali-quantitative analysis of zebra mussel body burden showed also a notable polychlorinated biphenyls (PCBs) pollution, with levels three-to seven-fold higher than those measured in other Italian subalpine lakes (Binelli and Provini, 2003; Riva et al., 2008), polybrominated diphenyl ethers (PBDEs – Binelli et al., 2008a), polycyclic aromatic hydrocarbons (PAHs – Parolini and Binelli, 2014), hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs), as well as some organophosphate insecticides (carbaryl, chlorpyriphos and its oxidized metabolite chlorpyriphos-oxon) at low or negligible concentrations (Binelli et al., 2004; Riva et al., 2008). Lastly, the zebra mussel was also used to assess radioactivity contamination by measuring concentration of different natural and artificial radioisotopes. The distribution of radionuclides within bivalve soft tissues and the factors responsible for their bioaccumulation by whole bivalves are relatively well described. Mersch et al. (1992) measured the radioactivity in zebra mussel specimens collected from two rivers flowing in a high industrialized area of the central Europe: the Mosel River (France) and the Rhine River (Germany). Measurable concentrations of the natural radioisotopes ^{238}U, ^{232}Th, ^{40}K and ^{210}Pb were detected, higher than those of artificial ones (^{134}Cs, ^{137}Cs, ^{110m}Ag, ^{58}Co and ^{60}Co). Similarly, the ability of zebra mussel to accumulate radionuclides, e.g. ^{90}Sr, ^{137}Cs, ^{65}Zn, ^{86}Rb, ^{131}I and others, has been demonstrated in studies by Kinney et al. (1994) and Frantsevich et al. (1996). A laboratory study by Zuykov et al. (2009) described the accumulation of ^{241}Am in shells and soft tissues of *D. polymorpha*, confirming that radioecological studies and measurement of bulk activity of radionuclides in

bivalve shells are simple tools for radioactivity pollution assessment.

As well explained above, many biomonitoring studies have been used indigenous zebra mussel specimens to evaluate the contamination of different aquatic ecosystems by several environmental pollutants, by the so-called passive biological monitoring (PBM). However, the feasibility of PBM depends on the presence of resident organisms of the same species at all the investigated sites and in sufficient biomass or number of individuals. Transplanting organisms within close cages combines the advantages of controlled laboratory experiments with the traditional environmental biomonitoring (Salazar and Salazar, 1997). With the so-called active biomonitoring (ABM), the sampling sites can be chosen and studied independently of the presence of resident indicator species. Bivalves can be exposed for a time-limited period, so recent contamination can be detected. Several studies have used transplanted mussels for biomonitoring purposes, including the zebra mussel. As for PBM, the first application of ABM regarded the accumulation of heavy metals. Camusso et al. (1994) transplanted *D. polymorpha* specimens in cages for one month in two different sites along the lower course of the Po river (Northern Italy) demonstrating that Cd, Pb, Cr and Ni concentrations in mussel tissues were notably increased with respect to the beginning of the experiment and that levels of metals accumulated in transplanted mussels were comparable to those from the native ones. Similarly, Mersch et al. (1996a) transplanted zebra mussels from three different populations of the Moselle River into the Mirgenbach Reservoir in order to evaluate Cu accumulation. Results showed that indigenous and introduced zebra mussels exposed in the same environmental conditions provided similar qualitative Cu patterns, emphasizing the technical feasibility of the transfer method and its suitability for active monitoring programs. In the same way, recent experiment used the ABM approach to monitor the pollution by different POPs. Bervoets et al. (2004) compared the accumulation of polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and hexachlorobenzene (HCB), as well as trace metals, between indigenous and transplanted zebra mussels from 12 canals and lakes situated in Flanders (Belgium). The results showed that transplanted zebra mussels exposed for a period of six weeks accumulated micropollutants up to a level comparable to the levels measured in resident mussels, reflecting the local situation. Moreover, since levels measured in bivalves covered a wide range of pollution, these results confirmed that the ABM approach could be applied at both polluted and unpolluted sites, proving the applicability of transplanted mussels as a biomonitoring tool for the accumulation of xenobiotics, always bearing in mind the invasive nature of this organism that must limit its use in ABM only in aquatic ecosystems when it is formerly present.

3. Application of biomarkers in the zebra mussel

As stated above, bioaccumulators provide the opportunity to determine simultaneously the presence of several pollutants in the environment. However, even if the simple quali-quantitative analysis of bioaccumulated pollutants has the great benefit of quantifying the bioavailable fraction of the xenobiotic having the potential to cause damage, no information on effect is obtained. For this reason, the relationship between exposure to chemicals and the subsequent induced adverse effects is of growing importance in environmental risk assessment and management (Segner and Braunbeck, 1998). To this aim, the use of biological markers or biomarkers measured at different levels of the biological organization is of extreme importance as sensitive 'early warning' tools to understand the possible biological adverse effects of pollutants and to obtain useful information for environmental quality assessment (Cajaraville et al., 2000). In contrast to the simple measurement of contaminants accumulating in body tissues, biomarkers can offer a more complete and biologically relevant information on the potential impact of pollutants on the health status of organisms (Van der Oost et al., 2003). In the last decades, many different biomarkers at different levels of organization, from subcellular to whole-organisms, and having diverse specificity in responding to chemicals have been measured in different groups of aquatic organisms, including the zebra mussel.

3.1. Application of biomarkers under controlled laboratory conditions

At the beginning of the biomarker application, genetic biomarkers have been probably the most used in assessing the toxicity of xenobiotics considering the zebra mussel as biological model. This choice was likely due to the fact that among the various anthropogenic pollutants found in freshwater ecosystems, one of the main representative classes comprehends the genotoxic compounds. The Micronucleus (MN) test was the first genetic biomarker applied on the zebra mussel (Table 2), as reported by Mersch et al. (1996b), who investigated the induction of micronuclei (MN) in *D. polymorpha* hemocytes and gill cells exposed to four different clastogen compounds (mitomycin C, bleomycin, dimethylarsinic acid and potassium chromate). Accordingly, further researches have been used this biomarker for testing genotoxicity of environmental pollutants (Table 2), showing significant increases of MN frequency in zebra mussel hemocytes caused by the exposure to various chemicals, such as B[α]P (Binelli et al., 2008b), p,p'-DDT and its p,p'-homologues (p,p'-DDD and p,p'-DDE, respectively; Binelli et al., 2008c), several PBDEs (BDE-47, -100, -154, -209; Riva et al., 2007; Parolini and Binelli, 2012a), personal care products (Binelli et al., 2008d), pharmaceuticals (Binelli et al., 2009a; Parolini et al., 2010, 2011a,b; Parolini and Binelli, 2012b) and, recently, also some illicit drugs (Binelli et al., 2012; Parolini et al., 2013a; Parolini and Binelli, 2013, 2014). MN test shows a fixed genetic damage, but the exposure to pollutants can also induce primary lesions, inducing DNA fragmentation as single or double strand breaks. The increase of DNA fragmentation is commonly assessed by using the Single Cell Gel Electrophoresis (SCGE assay), also known as Comet test. Pavlica et al. (2001) firstly applied this simple, sensitive and rapid technique for detection of DNA damage in zebra mussel cells under laboratory condition. Authors exposed bivalves to different concentrations of pentachlorophenol to evaluate if it might induce DNA strand breaks (SB) in hemocytes and pointed out significant increase in DNA primary lesions at high doses. Following findings by Pavlica et al. (2001), the genotoxic potential of several persistent organic pollutants, including pesticides (Binelli et al., 2008b,c), PAHs (Binelli et al., 2008b), PBDEs (Riva et al., 2007; Parolini and Binelli, 2012a), as well as emerging aquatic pollutants such as pharmaceutical and personal care products (PPCPs; Binelli et al., 2008d; Parolini et al., 2010, 2011a,b; Parolini and Binelli, 2012) was investigated in *D. polymorpha* cells by using the SCGE assay, pointing out the high sensitivity of this technique to detect DNA fragmentation. In addition, DNA strand breaks can be also quantified using a fluorescence technique adapted from the alkaline precipitation assay (Olive, 1988). Quinn et al. (2011), for instance, used this assay to assess the primary lesions induced by gemfibrozil and diclofenac to the zebra mussel, while Parolini et al. (submitted for publication) showed the capability of environmental concentrations of two synthetic musks, namely galaxolide (HHCB) and tonalide (AHTN), to increase DNA primary lesions. Another biomarker applied to zebra mussel cells is the DNA diffusion assay, whose method was developed from the SCGE assay by Singh (2000) and can be used to

Table 2
In vivo and *in vitro* studies by biomarkers' measurement in zebra mussel at laboratory conditions. See the Glossary for the abbreviations' meaning.

Chemical	Concentration (nM, if available)	Exposure time	Tested biomarkers	Reference
***In vivo* laboratory studies**				
Mitomycin C (MMC), bleomycin (BLM), dimethylarsinic acid (DMAA) and potassium chromate (PCR)	40 μg/L for MMC and BLM and 100 μg/L for DMAA and PCR	12 days	MN test	Mersch et al., 1996
Acridine	0.16 mg/L, 0.31 mg/L, 0.63 mg/L, 1.25 mg/L, 2.50 mg/L	48 h–10 weeks	Filtration rate	Kraak et al., 1997
Pentachlorophenol	10, 80, 100, 150 μg/L	7 days	SCGE assay	Pavlica et al., 2001
Atrazine	0.003 mg/L, 0.05 mg/L, 0.5 mg/L, 5 mg/L	21 days	Histological analyses	Zupan and Kalafatiç, 2003
Menadione and Lawsone	0.56 mg/L and 1 mg/L	48 h and 20 days	DT-diaphorase, NADPH-cytochrome c reductase and NADH-cytochrome c reductase	Osman et al., 2004
Cadmium	10 mg/L and 200 mg/L	3 weeks	b-Glucuronidase activity, lysosomal surface density, lysosomal surface to volume ratio, and lysosomal numerical density	Giamberini and Cajaraville, 2005
B(α)P and crude organic extract of sediments (COES)	5 and 50 μg/L of B(a)P; 100 μL/L of COES	5 days	32P-postlabelling for DNA adducts	Le Goff et al., 2006
Paraquat	0.125, 0.250, 0.500 mg/L	7–14 days	Histological analyses and MN test	Mantecca et al., 2006
4-Nonylphenol	0.1 mg/L, 1 mg/L, 5 mg/L, 10 mg/L and 5 μg/L, 500 μg/L	15, 35 and 50 days	attachment and siphon extension; Vitellin (Vn)-like protein	Quinn et al., 2006
Arochlor 1260, CB-153 and CB-126, p,p'-DDT, chlorpyrifos, carbaryl	100 ng/L	96 h	EROD, AChE	Binelli et al., 2006
BDE-209	0.1, 2 and 10 μg/L	96–168 h	SCGE assay, MN test	Riva et al., 2007
Butylated hydroxyanisole and lead	1 mg/L	24–48 h	DT-diaphorase	Osman and van Noort, 2007
Palladium	0.05 μg/L – 5 μg/L – 50 μg/L – 500 μg/L	10 weeks	Metallothionein	Frank et al., 2008
p,p'-DDT, p,p'-DDD and p,p'-DDE	0.1, 2 and 10 μg/L (0.3, 5.6, and 28.2 nM for the p,p'-DDT; 0.3, 6.2, and 31.2 nM for p,p'-DDD; and 0.3, 6.3, 31.4 nM for p,p'-DDE)	168 h	SCGE assay, MN test	Binelli et al., 2008c
B(α)P and p,p'-DDE	0.1 μg/L, 2 μg/L and 10 μg/L (0.4 nM, 7.9 nM and 40 nM for B(a)P; 0.3 nM, 6.2 nM and 31 nM for p,p'-DDE)	96-168 h	SCGE assay, MN test	Binelli et al., 2008b
Triclosan	290 ng/L (1 nM) – 580 ng/L (2 nM) – 870 ng/L (3 nM)	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test	Binelli et al., 2008d
Trimethoprim	0.29 μg/L (1 nM) – 0.87 μg/L (3 nM) – 2.9 μg/L (10 nM)	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx and GST	Binelli et al., 2009
Mercury chloride, methyl mercury, cadmium and Aroclor 1260	40 μg/L for mercury chloride, 40 μg/L for methyl mercury, 34 μg/L for cadmium and 150 ng/L for Aroclor 1260	5 days	EROD, GST, SOD, CAT, GPx, GSH, GR, metallothionein, LPO and DNA precipitation assay	Faria et al., 2009
Paracetamol	0.154 μg/L (1 nM) – 0.75 μg/L (5 nM) – 1.51 μg/L (10 nM)	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx, GST	Parolini et al., 2010
Cadmium, Copper and Pentachlorophenol	10 μg/L, 50 μg/L, 100 μg/L, 500 μg/L for Cd – 10 μg/L, 30 μg/L, 50 μg/L, 80 μg/L for Cu – 1 μg/L, 10 μg/L, 100 μg/L for PCP	7 days	Metallothionein	Ivankovic et al., 2010
Diclofenac	95 ng/L (0.3 nM) – 318 ng/L (1 nM), 637 ng/L (2 nM)	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx, GST	Parolini et al., 2011b
Ibuprofen	0.2 μg/L (1 nM) – 2 μg/L (9 nM) – 8 μg/L (35 nM)	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx, GST	Parolini et al., 2011a
Triclosan	290 ng/L (1 nM) – 580 ng/L (2 nM) – 870 ng/L (3 nM)	96 h	SOD, CAT, GPx, GST	Binelli et al., 2011
Gemfibrozil and Diclofenac	1 μg/L and 1000 μg/L	24–96 h[a]	Lipid peroxidation, DNA precipitation assay, metallothionein, GST	Quinn et al., 2011
4-Nonylphenol	1 μg/L, 10 μg/L, 50 μg/L	7 days	CAT, GPx, GST, 17-β-estradiol and testosterone levels	Riva et al., 2011
NSAID mixture	0.1 μg/L DCF; 0.1 μg/L IBU; 0.5 μg/L PCM – 0.5 μg/L DCF; 1 μg/L IBU; 1 μg/L PCM – 1.5 μg/L DCF; 9 μg/L IBU; 13 μg/L PCM	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx, GST	Parolini and Binelli, 2012b
BDE-47, BDE-100, BDE-154	0.1 mg/L – 0.5 mg/L – 1 mg/L	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test	Parolini and Binelli, 2012a
BDE-154	0.1 mg/L – 0.5 mg/L – 1 mg/L	96 h	SOD, CAT, GPx and GST	Parolini et al., 2012
Cocaine	40 ng/L (0.13 nM) – 220 ng/L (0.73 nM) – 10 μg/L (32.96 nM)	96 h	NRRA, SCGE assay, DNA diffusion assay, MN test	Binelli et al., 2012
Benzoylecgonine	0.5 μg/L (1.7 nM) – 1 μg/L (3.4 nM)	14 days	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx and GST, LPO and PCC	Parolini et al., 2013a
Ecgonine methyl ester	0.15 mg/L (0.75 nM) – 0.5 mg/L (2.5 nM)	14 days	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx and GST, LPO and PCC	Parolini and Binelli, 2013

Table 2 (*continued*)

Chemical	Concentration (nM, if available)	Exposure time	Tested biomarkers	Reference
Δ-9-tetrahydrocannabinol	0.05 μg/L (0.16 nM) – 0.5 μg/L (1.6 nM)	14 days	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx and GST, LPO and PCC	Parolini and Binelli, 2014
3,4- methylenedioxymethamphetamine (MDMA)	0.05 μg/L (0.25 nM) – 0.5 μg/L (2.5 nM)	14 days	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx and GST, LPO and PCC, filtration rate	Parolini et al., 2014
Morphine	0.05 μg/L (0.17 nM) – 0.5 μg/L (1.7 nM)	14 days	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx and GST, LPO and PCC, filtration rate	Magni et al., 2014
Galaxolide (HHCB) and Tonalide (AHTN)	100 ng/L (0.39 nM) and 500 ng/L (1.93 nM) for HHCB 20 ng/L (0.08 nM) and 80 ng/L (0.31 nM) for AHTN	21 days	DNA precipitation assay, MN test, LPO and PCC	Parolini et al., submitted for publication
***In vitro* laboratory studies**				
Triclosan and Trimethoprim	0.1 μM, 0.15 μM, 0.2 μM, 0.3 μM for TCS – 0.2 μM, 1 μM, 5 μM for TMP	1 h	NRRA, SCGE assay and DNA diffusion assay	Binelli et al., 2009b
Diclofenac, Ibuprofen and Paracetamol	60 μg/L (0.2 μM), 156 μg/L (0.5 μM), 250 μg/L (0.8 μM) for DCF – 30 μg/L (0.2 μM), 150 μg/L (1 μM), 450 μg/L (3 μM) for IBU – 45 μg/L (0.2 μM), 450 μg/L (2 μM), 909 μg/L (4 μM)	1 h	NRRA, SCGE assay and DNA diffusion assay	Parolini et al., 2009
Atenolol, Carbamazepine, Diclofenac and Gemfibrozil	0.001 mg/L, 0.01 mg/L, 0.1 mg/L, 1 mg/L, 10 mg/L	96 h	Trypan blue exclusion method, MTT assay	Parolini et al., 2011c

[a] Exposure by injection.

evaluate the possible induction of apoptotic and/or necrotic cells caused by pollutants. Recent laboratory studies have used this test to investigate the capability of some PPCPs, namely triclosan (Binelli et al., 2008d) and ibuprofen (Parolini et al., 2011a), to trigger the apoptotic process in bivalve hemocytes. Lastly, Le Goff et al. (2006) firstly measured the bulky DNA adducts in the zebra mussel using a 32P-postlabelling protocol with nuclease P1 enrichment after the exposure to B[α]P. A dose-dependent accumulation of B[α]P and a clear induction of DNA adduct formation in the digestive gland of mussels were noticed, demonstrating the applicability of this new method for bulky adduct detection in the digestive gland of *D. polymorpha*.

Another category of biomarkers commonly applied in laboratory studies on the zebra mussel comprehends biochemical parameters, which are considered the most sensitive and the earliest response due to the exposure to pollutants and therefore could be used as early warning systems (Rodrıguez-Ortega et al., 2002). Among them, enzymes represent one of the most suitable biomarkers, since they are involved not only in the biotransformation of pollutants but also could be susceptible to induction or inhibition by contaminants. Indeed, enzyme activity variations are used as indicators of exposure to contaminants. The first studies to assess the suitability of these techniques tested the variations of enzyme activities after the exposure of bivalve to proclaimed toxics (Table 2). For instance, Osman et al. (2004) firstly investigated the activity of the two-electron quinone oxidoreductase (DT-diaphorase), the one-electron catalyzing quinone reductases NADPH-cytochrome c reductase and NADH-cytochrome c reductase in the gills and the rest of soft tissue (excluding gills) after *in vivo* exposure to two quinones, namely menadione (2-methyl-1,4-naphthoquinone) and lawsone (2-hydroxy-1,4-naphthoquinone). Results showed that high concentrations of both the quinones induced a reduction of the activity of NADPH-cytochrome c reductase in the gills and in the rest of the soft mussel tissues, while the DT-diaphorase and NADH-cytochrome c reductase activity was not significantly affected (Osman et al., 2004). Binelli et al. (2006) found significant variations in ethoxyresorufin-O-deethylase (EROD) and acetylcholinesterase (AChE) activity after treatment with different pollutants (Arochlor 1260, PCB 153 and 126, p,p'-DDT, chlorpyrifos, carbaryl). Faria et al. (2009) found notable contaminant-related changes in phase I and II metabolizing enzymes (EROD and glutathione S-transferase), pro-oxidant/antioxidant processes (antioxidant such as SOD, CAT, GPx, glutathione (GSH) levels and glutathione reductase), metallothionein proteins, as well as markers of oxidative tissue damage (lipid peroxidation and DNA strand breaks) in *D. polymorpha* specimens exposed to metals (mercury chloride, methyl mercury, cadmium) and PCBs (Aroclor 1260). Considering the responsiveness, the reliability, the easiness and the quickness of the enzymatic methods, they are currently used to assess the potential toxicity of emerging pollutants towards the zebra mussel. Recent studies demonstrated that both 96-h and 14-day exposure to environmental concentrations of PBDE congener BDE-154 (Parolini et al., 2012) and of some common PPCPs, including paracetamol (Parolini et al., 2010), ibuprofen (Parolini et al., 2011a) and triclosan (Binelli et al., 2011), and illicit drugs as well, namely benzoylecgonine (Parolini et al., 2013a), ecgonine methyl ester (Parolini and Binelli, 2013), Δ-9-tetrahydrocannabinol (Parolini and Binelli, 2014) and 3,4-methylenedioxymethamphetamine (MDMA-Parolini et al., 2014) imbalanced the activity of antioxidants and GST in treated *D. polymorpha*. Nowadays, biomolecular and biochemical assays are the main biomarkers applied on the zebra mussel to assess the toxicity of aquatic pollutants. However, toxic effects can be shown also at higher levels of the biological organization, such as at tissue and organ level. Despite of this assumption, just few studies investigated the morphological alteration of *D. polymorpha* tissues and organs by using histological analyses. For instance, Zupan and Kalafatiç (2003) showed the histological effects caused by low atrazine concentrations, while Mantecca et al. (2006) reported severe lesions, including cellular vacuolation, lysis and thinness of the germinative epithelia, in the digestive

gland and testis of bivalves after paraquat exposure. Moreover, a positive trend between the number of granulocytes and paraquat concentrations was observed in gonads and digestive glands, pointing out the inflammatory capacity of this herbicide on bivalve tissues (Mantecca et al., 2006). These results, in addition to the remarkable cytotoxicity of paraquat observed in *D. polymorpha*, also suggested a larger use of this species in laboratory to study in depth the physiological and morphological responses due to stressors in an aquatic non-target species. Lastly, laboratory experiments also investigated pollutant-induced alteration of physiological parameters at organism level. The analysis of filtration rate was one of the first example of the application of biomarker on the zebra mussel to assess the toxicity of xenobiotics (Kraak et al., 1997), likely because it is a sensitive sub-lethal parameter having a great importance considering the ecological role that the zebra mussel fulfills in ecosystems (Kraak et al., 1994). Authors exposed bivalves to five increasing acridine (benzo[b]quinoline) concentrations and measured the variation of filtration rate of treated mussels, showing a clear dose–response relationship. Recently, alteration of the *D. polymorpha* filtration rate was investigated after exposure to MDMA (Parolini et al., 2014) and morphine (Magni et al., 2014) but no significant alteration of this physiological parameter was induced by the exposure to these illicit drugs. Another biomarker investigated at organism level is the so-called MXR or MXD (multixenobiotic resistance or defense), which points out the protection of the cells against the xenobiotics. Since the defense is modulated by chemicals, the MXR is used as a biomarker of organisms' exposure to environmental contamination (Pain and Parant, 2007). MXR related gene and protein expression, as well as MXR transporter efflux activities, have been studied in adult specimens (Smital et al., 2003; Pain and Parant, 2007; Tutundjian and Minier, 2007) and also in early life stages of zebra mussels (Faria et al., 2011) highlighting their usefulness in ecotoxicological studies.

3.2. Application of biomarkers in field studies

Following the application and the validation under controlled laboratory conditions, the most of the above mentioned biomarkers have been also applied in field studies sometimes paired to chemical analyses, in order to assess the contamination status of freshwater ecosystems and the potential hazard of the pollution towards the aquatic biocenosis (Table 3). Among these, mainly biomarkers based on responses at the molecular and cellular level are commonly used for biomonitoring, since they represent the earliest signals of environmental disturbance (Depledge, 1994; Lowe et al., 1995). Initially, for *in situ* validation, only one or two biomarkers have been assessed. For instance, Pavlica et al. (2001) evaluated the potential application of the comet assay on mussel hemocytes for genotoxicity monitoring of freshwater environment. Zebra mussels were exposed for seven days in the Sava River (Croatia) downstream from Zagreb municipal wastewater outlet. The significant increase in DNA damage that was observed confirmed that the comet assay applied on zebra mussel hemocytes might be a useful tool in determining the potential genotoxicity of water pollutants. Similarly, an in the field-based experiment, bivalves collected in the upper part of the Seine estuary (France) exhibited elevated levels of DNA adducts (up to 4.0/108 nucleotides), demonstrating that DNA adduct measurement in the zebra mussels could be another suitable biomarker to monitor PAH-exposure and evaluate genotoxicity in freshwater ecosystems. Binelli et al. (2005) evaluated the EROD activity and the acetylcholinesterase (AChE) inhibition as specific biomarker to confirm the presence of a contamination by planar compounds and organophosphates in the Lake Maggiore. Despite the promising results of these studies, in the real environment zebra mussel specimens are exposed to a plethora of diverse chemical pollutants, which can produce a great amount of adverse effects. For this reason, the use of single or limited number of biomarker should not useful to obtain a comprehensive picture of the real hazard of the contamination to aquatic organisms. Indeed, a suite of biomarkers investigating adverse effects at different levels of biological organization have to be applied in order to gain in-depth information on the dangerousness of environmental contaminants and the variation in the health status of target organisms for ecosystems, as tools in environmental risk assessment. For this reason, the recent field monitoring studies applied a suite of diverse biomarkers to study thoroughly the hazardous effects of the pollution. A multi-biomarker approach based on a set of at least 4-6 parameters was proposed as a useful tool in ecotoxicological programs for identifying the level of physiological impairment of an organism (Viarengo et al., 2000; Giamberini and Cajaraville, 2005). For this reason, different biochemical, biomolecular, cellular and physiological responses have been linked in field studies. Effects of pollution characterizing the lower Ebro River (Catalonia, Spain) were assessed by integrating chemical analyses and several biological responses, including levels of metallothioneins, activities of EROD, SOD, CAT, GST, GPx and GR, glutathione content, levels of lipid peroxidation and DNA strand breaks, in native zebra mussel (Faria et al., 2010). The results evidenced an increasing toxic stress from upper parts of the river towards the most polluted site located near to a waste dumps. Parolini et al. (2013b) applied a multi-biomarker battery to zebra mussel from the Lake Maggiore to investigate if the well-known POP contamination measured within this lake could pose a serious hazard to native specimens. The significant alterations in the activity of SOD, CAT, GPx and GST, as well as by the increase in oxidative and genetic damage, confirmed that the health status of mussels sampled from the most polluted sites was worst compared to those from less polluted ones. Biomarker data agreed those from a previous study indicating the serious hazard of the Lake Maggiore contamination to *D. polymorpha*, which showed spawning behavior changes and histological alterations in mussels from Pallanza Basin (the most polluted area within the lake) compared to a less contaminated area (Binelli et al., 2001a,b).

Similarly to the biomonitoring of xenobiotic levels, when the zebra mussel population is present in the study area in exiguous number, the ABM approach can be useful to assess the hazard of pollution. Several studies pointed out the usefulness of transplanted zebra mussels as active biomonitors in aquatic environments. Smolders et al. (2002) investigated the responses of bivalves transplanted in an effluent-dominated stream, showing that an exposure period of few weeks should be required to detect significant changes in condition status or scope for growth. Klobucar et al. (2003) collected zebra mussels from the R. Drava (Croatia) and transplanted them into cages to four monitoring sites having a different pollution degree in the R. Sava (Croatia). Results showed significant increase of both MN frequency and DNA fragmentation (SCGE assay) according to a pollution gradient confirming that both these tests are sensitive tools to be used in pair for the freshwater genotoxicity monitoring.

Biotransformation and antioxidant enzymes of *D. polymorpha* were used to evaluate the extent of urban water pollution. Activity changes of soluble and membrane bound glutathione S-transferase (s- and mGST), SOD, CAT and GPx to environmental stress were explored in pre-cultured mussels exposed for one day to one week in sites with different levels of anthropogenic pollution in watercourses of Berlin (Germany; Contardo-Jara et al., 2009). Bourgeault et al. (2010) transplanted zebra mussels along a metal and organic pollution gradient in different locations from the Seine river basin

Table 3
In situ studies carried out in North American and European freshwater ecosystems by biomarkers' evaluation. See the Glossary for the abbreviations' meaning.

In situ studies			
Study area	Chemicals (concentrations, if available)	Tested biomarkers	Reference
Meuse River, France	–	Fitness, MN	Mersch and Beauvais, 1997
Riou-Mort River, France	Cd (0.5–13 μg/L), Zn (118–896 μg/L)	MT	Marie et al., 2006a
Moselle River, France	–	MXR	Minier et al., 2006
Moselle River nuclear power plant, France	–	Lysosomal and peroxysomal system, lipofuscin and neutral lipids contents	Guerlet et al., 2007
River Fench/River Moselle confluence, France	Cd (0.5–0.82 μg/g d.w.), Pb (0.88–2.76 μg/g d.w.), Ni (5.96–12–11 μg/g d.w.), Cu (17–25.9 μg/g d.w.), Zn (87–128 μg/g d.w.), Fe (341–2093 μg/g d.w.), (PAHs 9.17–121 μg/g d.w.)	Lysosomal and peroxysomal system, lipofuscin and neutral lipids contents, condition index	Guerlet et al., 2010
Seine Estuary, France	PCBs (6.2–23.5 ng/g w.w.) PAHs (290 ng/g w.w.)	MXR, AChE, NRRA	Minier et al., 2006
Seine River, France	Zn (84.8–125.3 μg/g d.w.), Cr (0.8–1 μg/g d.w.), Co (0.8–1.3 μg/g d.w.), Mn (27.9–116.6 μg/g d.w.), Cu (8.5–12.5 μg/g d.w.), Cd (0.6–1 μg/g d.w.), Ni (2.6–8.4 μg/g d.w.), PAHs (1122–2336 μg/g d.w.)	SCGE assay, MN test, GST, digestive enzymes	Bourgeault et al., 2010
Vesle River, France	PAHs (26.57–48.34 ng/L), pesticides (0.015–1.01 μg/L), Cu (0.07–0.36 μg/L), Pb (0.02–0.07 μg/L), Ni (0.17–1.37 μg/L), Zn (0.62–8.95 μg/L)	AChE, GST, MT, amylase, endocellulase, energy metabolism	Palais et al., 2012
St Lawrence River, Canada	Metals, POPs	SCGE assay, MT, EROD, DNA strand LPO, vitellogenin-like proteins (VTG)	De Lafontaine et al., 2000
St Lawrence River, Canada	Butyltin (262.0–2088.0 ng/g d.w.)	histopathology	Regoli et al., 2001
Sava River, Croatia	–	SCGE assay	Pavlica et al., 2001
Drava River, Croatia	–	SCGE assay, MN test	Klobucar et al., 2003
Campine, Belgium	–	condition status or scope for growth	Smolders et al., 2002, 2004
Sewage treatment works in Athlone, Ireland	EDs	Condition index, ALP, Vt-like, cholesterol	Quinn et al., 2004
Lake Maggiore, Italy	DDTs (18–134 ng/g d.w.)	Spawning behavior, histology	Binelli et al., 2001a,b
Lake Maggiore, Italy	DDTs (238.9–701.86 ng/g lip.w.), PAHs (93.58–410.62 ng/g lip.w.), PCBs (158.71–306.35 ng/g lip.w.), HCB (2.39–4.38 ng/g lip.w.), HCHs (1.52–3.43 ng/g lip.w.)	SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx, GST, LPO, PCC	Parolini et al., 2013b
Italian sub-alpine Lakes Lake Maggiore, Italy	Chlorpyrifos (0.05–31.9 ng/g lip. w.), Chlorpyrifos oxon (0.05–53.8 ng/g lip. w.), PCBs, (429.2–2508.5 ng/g lip. w.) HCB (0.05–17.4 ng/g lip. w.), DDTs (62.3–945.33 ng/g lip. w.) DDTs (116.8–1416.3 ng/g lip. w.), PCBs (365.9–1846.0 ng/g lip. w.), CP + CPO (5.0–72.7 ng/g lip. w.), HCB (0.3–21.3 ng/g lip.w.)	EROD, AChE EROD, AChE	Binelli et al., 2005, Ricciardi et al., 2006
Lake Iseo, Italy	DDTs	Histopathology, reproductive behavior	Bacchetta and Mantecca, 2009
River Lambro/River Po confluence, Italy	DDTs (171.3–2922.9 ng/g lip. w.), PAHs (235.5–881.6 ng/g lip. w.), PCBs (226.7–599 ng/g lip. w.), HCB (1–2.8 ng/g lip. w.), HCHs (3.8–18.1 ng/g lip. w.)	NRRA, SCGE assay, DNA diffusion assay, MN test, SOD, CAT, GPx, GST, LPO, PCC	Binelli et al., 2010
Berlin watercourse, Germany	PCBs (4.5–30.6 ng/L), HCHs (4.2–34.5 ng/L), DDTs (14.5–67.9 ng/L), Al (74.1–613.2 μg/L), Pb (1–2.5 μg/L), Zn (30.9–46.2 μg/L), Cr (10.8–17.4 μg/L), Ni (6.6–10.6 μg/L), Cu (20.5–61.2 μg/L), Cd (0.9–1.4 μg/L)	GST, SOD, CAT, GPx	Contardo-Jara et al., 2009
Lake Walenhoek, Lake Nekker, Albert canal, Beverlo canal, Belgium	Cd (0.23–1.30 μg/L) Cu (0.57–16.4 μg/L) Zn (0.25–103.2 μg/L) Cd (0.17–1.49 μg/g sed.) Cu (4.83–57.3 μg/g) Zn (7.17–4410 μg/g)	MT	Voets et al., 2009
Ebro River, Spain	Cr (0.6–1.3 μg/g d.w.), Ni (9.77–37.77 μg/g d.w.), Cu (7.91–35.75 μg/g d.w.), Zn (89.1–136.4 μg/g d.w.), As (4.84–6.19 μg/g d.w.), Cd (0.31–3.96 μg/g d.w.), Pb (0.50–0.77 μg/g d.w.), Hg (0.1–3.01 μg/g d.w.), HCB (0.41–78.41 ng/g d.w.), DDT (5.34–317.1 ng/g d.w.), HCH (0.34–6.08 ng/g d.w.), PCBs (6.82–109.2 ng/g d.w.)	SCGE assay, MT, EROD, SOD, CAT, GPx and GST, GR, GSH, LPO, ChEs	Faria et al., 2010
Ebro and Mijares Rivers, Spain	Cr (2.8–10.4 μg/g d.w.), Ni (3.5–20 μg/g d.w.), Cu (20–168.5 μg/g d.w.), Zn (73.1–265.9 μg/g d.w.), As (5.3–21.7 μg/g d.w.), Cd (0.36–4.06 μg/g d.w.), Pb (0.48–15.51 μg/g d.w.), Hg (0.07–1.23 μg/g d.w.), PCBs (6.2–825 ng/g d.w.), PAHs (38.6–815.6 ng/g d.w.), AP (78.1–889 ng/g d.w.), ENDO (5.1–20.4 ng/g d.w.)	SCGE assay, SOD, CAT, GSH, GST, MXR, LDH, LPO, histology, lipid content	Faria et al., 2014

d.w. = dry weight; w.w. = wet weight; lip. w. = lipid weight.

for two months. Significant variations in condition index, GST activity and in genotoxicity parameters (MN and SCGE assay) were noted in mussels transplanted downstream compared to upstream. A 12-month ABM performed on the Vesle river basin (France) transplanting *D. polymorpha* specimens in four sites with different pollution levels pointed out changes in response of AChE, GST, metallothionein, as well as digestive enzyme activities (amylase, endocellulase) and energy reserve concentrations (Palais et al., 2012). According to Smolders et al. (2002), all the studies mentioned above highlighted the use of transplanted mussels as a sensitive and easily applicable tool that can be used to assess water quality, pollution and subsequent recovery through self-purification in field situations. Lastly, an alternative ABM approach was applied by Binelli et al. (2010) investigating the hazard of the chemical pollution of the River Lambro/River Po confluence (Northern Italy). Instead of transplant mussels into an

environment in which probably they could not live, bivalves were exposed under controlled laboratory conditions in water collected in spring and autumn from three sites (upstream and downstream the confluence and at the confluence of the rivers). Significant increases in DNA strand breaks, apoptosis and micronuclei were observed downstream the confluence compared to upstream of the confluence of the two rivers. This approach allows to exclude from consideration most of the environmental variables that could affect the biomarker responses and to focus on the hazard of chemical pollution characterizing the aquatic environment. Moreover, it could solve the drawback due to the invasive behavior of *D. polymorpha*, eliminating any possible release and/or escape from cages to uninhabited ecosystems.

4. *In vitro* approach on the zebra mussel

In the previous paragraphs we listed the studies using the zebra mussel to assess the toxicity of aquatic pollutants in both laboratory and field conditions. All these studies exposed bivalves *in vivo* because this approach provides a more general ecotoxicological screening (Ching et al., 2001), assessing the pollutant effect on the organism. However, considering the great variability in biomarker responses due to both experimental conditions in laboratory experiments (i.e. time and concentration of exposure, as well as considered end-points) and environmental factors in field experiments (i.e. temperature, water chemical parameters, age of bivalves and food availability), the evaluation of the toxicity and the mechanism of action of xenobiotics, as well as the comparison of their toxicity, is often very difficult. In order to enlarge the knowledge regarding the adverse effects of pollutants on organisms, the use of *in vitro* methods in ecotoxicology is recommended but rarely considered (Laville et al., 2004). Several ethical, scientific and economic reasons support the efforts to develop and to apply *in vitro* techniques in aquatic ecotoxicology, since they allow 1) to reduce the use of test organisms; 2) to perform a rapid, low cost and reliable screening to evaluate the toxic effect of many chemicals with great precision and reproducibility (Olabarrieta et al., 2001); 3) to rank the toxic potential of diverse xenobiotics and to compare their effects towards different species at the cellular level under equivalent conditions of exposure; 4) to understand the mechanisms involved in cellular and molecular responses to environmental pollutants (Gagnaire et al., 2004) in order to justify more intensive *in vivo* studies with whole organisms or mesocosms (Blaauboe, 2008; Gura, 2008). Considering all these reasons, cultures of different vertebrate cell lines have been standardized and widely applied in ecotoxicology, but only recently cells from different organs of marine bivalves, including gills (Gómez-Mendikute et al., 2005), mantle (Koyama and Aizawa, 2000; Barik et al., 2004; Cornet, 2006), digestive gland (Le Pennec and Le Penec, 2001, 2003; Chelomin et al., 2005) and hearth (Domart-Coulon et al., 2000) have been cultured. To fill this gap for freshwater environments, Quinn et al. (2009) developed an *in vitro* technique for culturing cells in suspension and tissue explants from the gill, digestive gland and mantle of the zebra mussel, showing their successful maintenance in culture for up to 14 days. The final goal of this technique development was for its potential use in toxicity tests to assess the mechanistic effect of different environmental pollutants on isolated cells and tissues (Quinn et al., 2009). To date, the *in vitro* approach on the zebra mussel was applied as a first screening to assess the toxicity of pharmaceutical and personal care products. The first *in vitro* studies directly exposed hemocytes of the zebra mussel to different concentrations of triclosan and trimethoprim (Binelli et al., 2009b), and of three common NSAIDs (diclofenac, ibuprofen and paracetamol; Parolini et al., 2009) pointing out their potential cyto-genotoxicity. Following the development of the culture method (Quinn et al., 2009), it was applied by Parolini et al. (2011c) to investigate the cytotoxicity of four pharmaceutical compounds, namely atenolol (ATL), carbamazepine (CBZ), diclofenac (DCF) and gemfibrozil (GEM), on culture of hemocytes, gill and digestive gland cells. Increasing concentrations of CBZ, DCF and GEM significantly decreased the viability of each cell type, while the MTT (3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay highlighted only a slight reduction of mitochondrial activity of gill and digestive gland cells. Overall, DCF was the most cytotoxic drug for zebra mussel cells, followed by GEM and CBZ, while ATL has negligible cytotoxicity. All these results showed that different zebra mussel cell typologies are excellent and sensitive models for toxicity assessment of xenobiotics and demonstrated that *in vitro* exposures can be used in screening strategies for the characterization of risk and impacts of aquatic pollutants towards freshwater bivalves.

5. Application of *D. polymorpha* for omics studies

In the last decade there has been an improvement in the development of omics techniques in ecotoxicology, as transcriptomics and proteomics, since changes in environmental conditions and exposure to pollutants influence the expression of gene transcripts, which in turn has effect on protein synthesis. Such high throughput technologies represent powerful diagnostic tools addressing the links between environmental conditions, harmful effects and responses in the organisms (Suárez-Ulloa et al., 2013). The integration of omics approaches enables to move a step forward in environmental toxicology allowing the identification of molecular events that are involved in the responses to environmental stressors and that might be responsible of the adverse effect at higher levels of biological organization (Ankley et al., 2006). Molecular techniques have been widely applied in marine bivalves, particularly in *Mytilus* spp., to evaluate sub-lethal effects of environmental stressors (Milan et al., 2011; Place et al., 2012; Li et al., 2013), including metals (Ciacci et al., 2012; Banni et al., 2014), pharmaceuticals (Franzellitti et al., 2013; Milan et al., 2013) and POPs both in laboratory and natural exposure conditions (Dondero et al., 2006; Venier et al., 2006). On the contrary, the recourse to these omics technologies in freshwater mussels is still scarce. The application of genome-based tools in zebra mussel has been facilitated by the recent availability of several EST (expressed sequence tag) collections and mRNA clones from *Dreissena* spp. (Bultelle et al., 2002; Xu and Faisal, 2009a,b). Transcriptomic analyses using *D. polymorpha* as biological model have been performed to evaluate the toxicological mechanism of a broad range of hazardous chemicals in laboratory and field studies (Table 4). For instance, the expression profile of several genes was characterized to assess the toxicity mechanism of some metals: a significant induction of metallothionein (MT) and cytochrome c oxidase subunit I (Cox I) was observed in gills of zebra mussels after 10 days of exposure to Cd, while any modulation was elicited by Zn (Marie et al., 2006b; Achard-Joris et al., 2006). In a more recent paper, adults of zebra mussel were exposed to Cd, Hg and Cu and collected after 1 and 7 days (Navarro et al., 2011). After 7 days of exposure the gene encoding for MT was significantly up-regulated by Hg and Cd in gills and digestive gland, but any modulation was observed upon Cu exposure. Furthermore, the Hsp70 gene resulted significantly up-regulated by Cu in gills only after 1 day of exposure and in the digestive gland at 7 days. A transient induction of Cox I was also observed in gill tissues. Since a different induction profile to metal responses in gills and digestive gland was observed, the authors suggested a more susceptibility of gills to short-term exposure than digestive gland. In addition, zebra mussel larvae at pediveliger

Table 4
List of transcriptomics studies using *D. polymorpha*. See the Glossary for the abbreviations' meaning.

Chemical	Concentrations	Exposure time	Tissue	Tested genes	Reference
***In vivo* exposure studies**					
Cd Zn	0.133 μM 15.3 μM	10 days	Gills	MT	Marie et al., 2006b
Metropolol	2 nM–2 μM	7 days	Gills digestive gland	Hsp70, AhR, CAT, SOD, MT, piGST, P-gp, PP2A	Contardo-Jara et al., 2010
Ibuprofen Carbamazepine bezafibrate	1 nM–1 μM	7 days	Gills digestive gland	Hsp70, AhR, CAT, SOD, MT, piGST, P-gp, PP2A	Contardo-Jara et al., 2011a
Levonorgestrel	1–10–20 nM	7 days	Gills digestive gland	hsp70, AhR, CAT, SOD, MT, piGST, P-gp, PP2A	Contardo-Jara et al., 2011b
Cd–Cu–Hg	20 μg/L 1 μg/L	7 days 24 h	Gills digestive gland larvae	Hsp70-90, AhR, CAT, SOD, GPx, MT, piGST, P-gp, Cyt C ox	Navarro et al., 2011
B[a]P	10–100 μg/L	96 h	Gills digestive gland	Hsp70, AhR, CAT, SOD, piGST, P-gp	Châtel et al., 2012
Dactal Hg^{2+}	0.5–2 mg/L 1–20 μg/L	24 h	Gills Larvae	Abcb-Abcc-like	Navarro et al., 2012
***In situ* studies**					
Renaturated sewages	OCs (ng/L) metals (μg/L)	7 days	Gills	Hsp70, Pgp, GSTp, Cat	Contardo-Jara and Wiegand, 2008
Seasonal and environmental variations	Gills	Custom microarray	Navarro et al., 2013a		
Pollution gradient	Metals (μg/g d.w.) POPs (ng/g d.w.)	Gills	Custom microarray		Navarro et al., 2013b

stage (10 days post fertilization) were exposed to the same metals for 24 h, but only a slight up-regulation of MT and Hsp70 in response to Hg and Cd was observed.

Molecular markers from zebra mussel were recently applied also for environmental management purposes (Table 4). In particular, some genes involved in antioxidative stress response, detoxification/metabolism and excretion process, receptor interactions and cell signaling have been measured to assess the success of a renaturation process of an urban stream formally used to discharge treated sewage waters (Contardo-Jara and Wiegand, 2008). In detail, zebra mussels were transplanted both into the renaturated urban stream and in a nearby semi-natural stream for comparison. Individuals were collected after 1, 4 and 7 days. The expression of CAT, GSTp, Hsp70 and P-gp genes in zebra mussel collected at the renaturated site was comparable or even better than the nearby semi-natural stream, in line with levels of metals and organochlorines (OCs) measured in such waters and consequently validating the success of the renaturation operation. The same set of genes was further applied to investigate the effects of different pharmaceuticals, as metoprolol (Contardo-Jara et al., 2010), carbamazepine, ibuprofen, bezafibrate (Contardo-Jara et al., 2011a) and levongestrel (Contardo-Jara et al., 2011b). Individuals were waterborne exposed to increasing concentrations (in the nanomolar range) of pharmaceuticals in a flow-through system for 1, 4 and 7 days. Final results showed that the β-blocker metoprolol determined a significant up-regulation of Hsp70, GSTp, P-gp and MT genes in gills and a transient induction of Hsp70, MT, PP2A, AhR, SOD and CAT in the digestive gland of zebra mussel (Contardo-Jara et al., 2010). In another study, a 7-days exposure to carbamazepine produced a significant down-regulation of SOD gene expression in gills, while CAT and P-gp showed this effect in the digestive gland (Contardo-Jara et al., 2011a). Moreover, similar concentrations of ibuprofen inhibited AhR gene expression and CAT in gills already after 1 d of exposure, while in the digestive gland a transient induction of SOD and CAT was observed. In the same study, bezafibrate exposure produced a significant up-regulation of CAT, Hsp70 and SOD in gills and an increased AhR and GSTp m-RNA abundance in the digestive gland; on the contrary MT gene resulted significantly down-regulated in the digestive gland (Contardo-Jara et al., 2011a). The contraceptive steroid levongestrel determined a significant down-regulation of SOD, AhR and Hsp70 after 1 day followed by an up-regulation after 7 days in gills. In the digestive gland a significant induction of P-gp, SOD, MT and CAT was observed. GSTp gene was up-regulated in both tissues only after 1 day of exposure (Contardo-Jara et al., 2011b). Overall results of these studies underlined the suitability of mRNA-based analysis to reveal a stress conditions related to the exposure to pharmaceuticals at environmental concentrations. The tested pharmaceuticals modulated significantly genes expression with different time and concentration-dependent pattern. As demonstrated for other species, the different trends of gene expression observed in response to the tested pharmaceuticals confirmed a specific mode of action of the compounds affecting different molecular targets also in zebra mussel. Moreover, this study highlighted a tissue-specific profile of gene expression according to the different physiological role of gills and digestive gland. In mussels, in fact, gills localize at the interface between the external environment and the organism being first target of environmental pollutants while the digestive gland is the main metabolic tissue.

A deep characterization of genes encoding for the ABC transport proteins has been performed on zebra mussel larvae and adults (Faria et al., 2011; Navarro et al., 2012). Abcb1 and Abcc homologs were identified in larvae and in gills from adults. The basal gene expression, measured during embryo development, showed an induction of Abcs genes from 1 hpf until trocophora and veliger stages. A significant increase of Abcb1 and Abcc transcripts abundance was observed in larvae upon exposure to the herbicide dacthal (dimethyl tetrachloroterephthalate) and Hg treatments. As well Abcb1 transcription was enhanced in gills in response to dachtal, while Abcc mRNA was increased only by Hg. Results of these studies confirm the role of the MXR as a first defense mechanism towards toxic compounds also in zebra mussel, as well established in *Mytilus* sp. (Luckenbach and Epel, 2008; Della Torre et al., 2014).

To better characterize the metabolic response to B[α]P, Châtel et al. (2012) investigated the expression of genes involved in the detoxification pathway and antioxidant response in zebra mussel waterborne exposed for 4 days followed by 28 days of recovery, and paralleled with the measurement of DNA adducts. Results showed a significant increase in the expression of almost all tested genes confirming that B[α]P is actively metabolized by zebra mussel. A different induction profile was observed in gills and digestive gland correlated with the occurrence of genotoxic effects suggesting gills as more sensitive target of B[α]P toxicity.

A custom microarray was developed to assess the transcriptome changes related to seasonal variations in a natural population from the Ebro River (Navarro et al., 2013a). Individuals were collected in February, June and September to cover the annual cycle of *D. polymorpha*. A significant modulation in the abundance of more than 700 genes was observed related to the reproductive maturation status. During the pre-spawning and spawning season, genes involved in proliferation and gametogenesis were significantly up-regulated while stress response genes were overexpressed during

the resorption period. A further application of this microarray technique was to investigate the differences in the transcriptomic profiles of zebra mussel populations living under different environmental conditions and to characterize the mechanisms besides acclimation to environmental stressors (Navarro et al., 2013b). The approach highlighted significant variations in the transcriptomic profile of the different population investigated. Such differences of genes transcription positively correlated with the body burden of several metals, but not with Hg or OCs. Modified genes are related mostly to mitochondrial functions, cell proliferation, ribosomal biogenesis, structural function and stress response. Therefore, authors suggested that modulation at the transcriptional level might allow acclimation of the species to extremely polluted conditions. Both these pioneering studies highlighted the potential of microarray technology to show the variability in the expression of large amount of genes related to physiological condition of the organisms, as well as the response to environmental stress in natural conditions.

The proteomics is another powerful omics tool with the potential to reveal new and unexpected associations between proteins and toxicant exposure, without *a priori* hypotheses. This approach provides insights into the mechanisms underlying the toxic action of pollutants. So far, the proteomic studies in bivalves aimed to generate hypothesis about the mode of action (MOA) of harmful chemicals (Campos et al., 2012; Suarez-Ulloa et al., 2013). Furthermore, proteomic techniques have been applied to study the adaptive response to different sources of environmental stress, as for example ocean acidification. As for other non-target species, the large-scale application of proteomic approach in *D. polymorpha* is hindered by the lack of complete genomic data available in public databases, necessary for automated protein identification (Campos et al., 2012). However, although this drawback, several proteomic studies are performed on zebra mussel to identify biochemical pathways involved in the toxicity mechanism of environmental pollutants (Table 5). The first study in which *D. polymorpha* was used as a suitable biological model for proteomics has been shown in the paper of Riva et al. (2011). The authors evaluated the role of gender and exposure concentrations by exposing zebra mussel to waterborne B[α]P environmental concentrations for 7 days. Proteins associated with cytoskeletal functions, cell redox homeostasis, cell signaling and metabolism resulted significantly modulated by B[α]P in a concentration-depending manner, but the main result is related to different protein changes obtained in males and females, respectively. This baseline study underlined the importance of the selection of toxicant concentrations for *in vivo* proteomics studies, as well as the influence of gender in the modulation of the protein pattern, characteristic often neglected in this kind of research. The proteomics analysis was performed also as the last step of a multi-tiered study on the effects of triclosan (TCS) on zebra mussel specimens (Riva et al., 2012). TCS induced the expression of proteins related to oxidative damage, calcium homeostasis, cell survival and stress response, confirming this anti-bacterial compound as a potent oxidative agent and also provided useful information to characterize the cellular mechanism underlying the observed TCS toxicity (Binelli et al., 2008d, 2009b).

Proteomics has been also involved in the evaluation of the possible effects of drugs of abuse in the protein pattern of non-target organisms. In particular, the redox proteomics was applied in zebra mussel for an in-depth knowledge of the toxicity mechanism of benzoylecgonine (BE), the main metabolite of cocaine (Pedriali et al., 2012). Individuals were exposed to BE for 14 days under semi-static conditions. BE did not produce oxidation of thiols but determined a significant increase in carbonyl groups. This approach showed also oxidative modifications in proteins involved in cytoskeletal components, energy metabolism and stress response. A further 2DE analysis on the same samples highlighted the alteration of proteins involved in cytoskeleton, calcium homeostasis, amino-acidic and energy metabolism and stress response upon BE exposure (Binelli et al., 2013). Since this paper represented the last stage of a stepwise approach (involving biomarkers, redox and functional proteomics analyses) to evaluate the effects of BE on *D. polymorpha*, this is one of the first examples of the usefulness of a multidisciplinary approach applied for ecotoxicological evaluation.

The study of Riva and Binelli (2014) is the last paper currently available in the contest of proteomics by using *D. polymorpha*. In detail, the proteine profile of zebra mussel waterborne exposed to a mix of the 12 dioxin-like PCBs (IUPAC N° 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) was investigated to get information about their MOA. Final analysis showed that exposure to dl-PCBs for 7 days induced significant modulation of the proteome in gill tissue. Results indicated that dl-PCBs toxicity was not dependent on AhR binding whereas involved mostly proteins related to glycolysis pathway, cytoskeletal functions, cellular metabolism and stress response. Gender-related differences in the protein expression profiles were also underlined, confirming data from the previous paper of Riva et al. (2011).

Overall, these studies highlighted the potential of omics approaches using *D. polymorpha* as model for the mechanism-based evaluation of stress response in freshwater bivalves. Nevertheless, it is absolutely needed an in-depth approach with the help of the state-of-the-art technologies available especially in the field of the

Table 5
List of proteomics studies using *D. polymorpha*. See the Glossary for the abbreviations' meaning.

Chemical	Exposure time	Tissue	Gender	Modified proteins	Method/protein identification	Reference
B[a]P (0.1–2.0 μg/L)	7 days	Gills	M/F	DUPD1, N-acetyltransferase 8-like protein, Aspartate aminotransferase, Actin, Alcohol dehydrogenase class-3, Peroxiredoxin-6	2DE/ PMF	Riva et al., 2011
TCS (580 ng/L)	7 days	Gills	–	HSP70, Tubulin, Omega-crystallin, GAPDH, Myosin, Cytosolic malate dehydrogenase, Sarcoplasmic calcium-binding protein, Annexin B9, 14-3-3	2DE/ PMF *de novo* sequencing	Riva et al., 2012
BE (0.5–1 μg/L)	14 days	Gills	–	HSP70, Tubulin, GAPDH, Fructose-bisphosphate aldolase, Cytochrome c subunit I, Phosphoenolpyruvate, Carboxykinase, Actin, Galectin7	Redox proteomics 2DE/ *de novo* sequencing	Pedriali et al., 2013; Binelli et al., 2013
dl-PCBs (200 ng/L)	7 days	Gills	M/F	Actin, Tubulin, GAPDH, Cathepsin B-like cysteine proteinase 6, Enolase	2DE/ *de novo* sequencing	Riva and Binelli, 2014

protein identification when we used a non-sequenced organism. In fact, Riva et al. (2011) identified only 37% of the protein changed by B[α]P because of they used the peptide mass fingerprint (PMF) for the protein identification, which is very useful only when the genome of the biological model is fully sequenced. By contrast, in the further studies, the recourse to *de novo* sequencing approach allowed the identification for homology of a higher percentage of protein varied, ranging from 62% (Riva and Binelli, 2014) to even the 100% of identification (Riva et al., 2012).

6. Final remarks

Overall, all the studies mentioned above highlighted the potential of *D. polymorpha* in diverse ecotoxicological applications aimed to both the monitoring of the contamination due to different aquatic pollutants and the evaluation of their effects. Zebra mussel does not only appear very useful for these purposes, but it also demonstrated a great plasticity, accordingly to its marine counterpart *Mytilus*, since it can be used both in laboratory and field studies, through in *in vitro* and *in vivo* approaches. On the other hand, the rising interest in its application in environmental toxicology is proven by the increasing number of papers concerning this topic. In fact, considering only the whole environmental sciences/ecology area, the total number of scientific papers relating to zebra mussel is now more than 2000 (McLaughlan et al., 2014). We can also noticed that biomonitoring studies carried out with zebra mussel tend to maintain themselves constant from the beginning of the century, while we point out a clear recent increase in the studies in which *D. polymorpha* has been used for biomarker measurement and omics techniques.

In our opinion, the invasive behavior of this species should be not considered a drawback in its employment as reference organism, even if several precautions must be taken before its use. In fact, we would not advocate the introduction of this invasive species for the evaluation of freshwater quality, but rather propose that we consider embracing their positive peculiarities in ecosystems where they are already established. Obviously we cannot consider these precautions in experiments carried out under controlled laboratory conditions, which can employ this organism instead of some other endangered species.

However, in order to suggest this species as model-organism for freshwater studies, several improvements should be made in all fields of environmental toxicology to provide a homogeneous data comparison. For instance, for biomonitoring purposes, it is crucial the definition of the right number of yearly samplings to define the health status of a freshwater ecosystem, since the POP concentrations measured after the spawning period of zebra mussel is lower than those evaluated before the spawning, due to the natural depuration of these chemicals bound to lipids contained in gametes (Binelli et al., 2001a,b). Another standardization improvement should affect the sampling, transport and conservation of zebra mussel specimens, as well as the draft of guidelines that well specify the case in which the use of this invasive species is allowed in order to avoid its dispersion. Particular attention must be taken when biomarkers are investigated in field, since environmental parameters can greatly interfere mainly on the response of biochemical biomarkers, as recently demonstrated by Faria et al. (2014). In laboratory biomarker studies, some standardization could affect the number of specimens and replicates needed to reach good quality data. Another crucial point is the selection of chemicals' concentrations tested in this kind of studies, since many experiments are carried out at concentrations much higher than environmental levels. In our opinion, the selection of these high toxicant concentrations is justified only when the aim of the study is exclusively the evaluation of the MOA of tested pollutants. On the contrary, if the scope of research is the environmental risk assessment of specific pollutants, the selection of environmental concentrations of toxicants must be the priority. Finally, several improvements are needed mainly for the applications of 'omics' techniques on the zebra mussel. Although the use of the *de novo* sequencing allowed the identification of a reasonable number of proteins changed in response to a chemical administration, it is absolutely needed the genome sequencing to suggest *D. polymorpha* as reference organism in proteomics. This can be obtained more quickly than past by the next generation sequencing, which is an ever increasing techniques and also a cost-effective approach. Nevertheless, for the successful application of 'omics' in environmental monitoring, more studies focusing on deep characterization of genes/proteins functions and regulation mechanisms in this species are recommended. Finally, we hope that this review should suggest to consider *D. polymorpha* not only as a pest to fight, but also a possible useful tool in many fields of environmental toxicology, bearing in mind obviously its invasive nature that implies some particular ethic behaviors.

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We dedicate this review to the memory of Prof. Alfredo Provini.

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Chapter 5

PAPER 4

Sublethal effects induced by morphine to the freshwater biological model *Dreissena polymorpha*

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Sublethal Effects Induced by Morphine to the Freshwater Biological Model *Dreissena polymorpha*

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ABSTRACT: Opioids are considered as emerging contaminants in aquatic ecosystems, mainly due to their large illicit consume worldwide. Morphine (MOR) is the main opiate and it was commonly found at measurable concentrations in freshwaters. Even though its occurrence is well documented, just limited information is available regarding its hazard to nontarget organisms. The aim of this study was of the evaluation of sublethal effects induced by MOR to the freshwater bivalve *Dreissena polymorpha*. We exposed mussels to two MOR concentrations (0.05 μg/L and 0.5 μg/L) for 14 days and we investigated the sublethal effects by a suite of biomarkers. The Neutral Red Retention Assay (NRRA) was used as a test of cytotoxicity, while the oxidative stress was evaluated by the activity of antioxidant and detoxifying enzymes, namely catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST), and by measuring the levels of lipid peroxidation (LPO) and protein carbonylation (PCC). The genetic damage was assessed by the Single Cell Gel Electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test). Finally, the filtration rate of *D. polymorpha* was evaluated in order to investigate possible physiological effects. Both tested concentrations reduced the lysosome membrane stability of bivalves, but only the highest MOR concentration induced significant changes in the activity of antioxidant enzymes (SOD, CAT, and GPx) and increase in lipid peroxidation levels. Slight increase in primary DNA fragmentation was noticed, while no fixed genetic damage and alterations of the filtering rate were found. Environ Toxicol 00: 000–000, 2014.

Keywords: morphine; biomarkers; *Dreissena polymorpha*

INTRODUCTION

The scientific community has recently shown a growing interest for problems related to the presence of new contaminants such as pharmaceuticals and personal care products (PPCPs) and illicit drugs (Pal et al., 2013) in the aquatic environment. Once used, these chemicals and their metabolites enter the sewage waters through urines and feces (Ternes, 1998; Zuccato et al., 2000; Heberer, 2002; Castiglioni et al., 2006). The wastewater treatment plants (WWTPs) are built to remove the organic matter and nutrients and are not suitable for the removal of most PPCPs and drugs of abuse (Reungoat et al., 2011; Pal et al., 2013). Many monitoring studies showed measurable concentrations (in the ng/L-μg/L range) of several PPCPs and illicit drugs in both European and US WWTP effluents and surface waters (Fent et al., 2006; Santos et al., 2010). Among pharmaceuticals, analgesics are topical pain relievers and can be divided into two groups: nonopioids (nonnarcotic analgesics) and opioids (narcotic analgesic). The first family reduces pain and inflammation interfering with the synthesis of prostaglandin hormones (Julien, 1997), while the latter group causes a muscular relaxation interacting with specific opioid receptor (MOP), a class of G-protein-coupled receptors (Suzuki and Misawa, 1997). Considering their pharmacological features, opioids are used as pharmaceuticals in human medicine, but also as drugs of abuse. The latest World Drug

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Report (UNODC, 2013) has estimated that about 16.5 million people worldwide, accounting for the 0.4% of the population aged 15 to 64, have used opiates as drugs of abuse at least once in 2012. These chemicals are not the most common illicit drugs used worldwide, since cannabis (3.9% of the global population) and amphetamines (0.7% of the global population) showed a higher use prevalence (UNODC, 2013). However, their use trend remains stable over the last years, with high prevalence in South-Western and Central Asia, Eastern and South-Eastern Europe and North America (UNODC, 2013). Opiates are opium derivatives, a substance extracted from *Papaver somniferum* and *Papaver setigerum*, historically prescribed for the care of cough, anemia and diarrhea (Nicholson, 2003). Opium contains many active alkaloid compounds, mainly morphine (MOR). In humans, MOR acts on the nervous system by binding to opioid receptors, reducing pain and smoothing muscle contraction (Zhu et al., 2005). MOR is metabolized for 87% from hepatic carboxylase, and the main metabolite is represented by morphine-3β-D-glucuronide (75%, Baselt et al., 2004). In addition, MOR is a metabolite of heroin, which has low affinity for opioid receptors and only when it is converted into MOR (4%), 6-acetylmorphine (1.3%) and morphine-3β-D-glucuronide (38%) performs a pharmacological action (Baselt et al., 2004; Maurer et al., 2006). For many decades, studies on vertebrates were focused on the pharmacological effects of exogenous MOR and exogenous morphine-like compounds, but after the discovery of the binding of MOR with opioid receptors, endogenous opioids have been identified (Lord et al., 1977). The presence of endogenous MOR is not a prerogative of vertebrates, as shown by studies performed on different species of invertebrates (Stefano et al., 2000). For instance, it is known that mussels have opioid receptors in their nervous system (Stefano and Scharrer, 1996). The MOR-opioid receptors interaction in these bivalves involves a release of dopamine (Zhu et al., 2005), a crucial neurotrasmitter involved in oogenesis. Despite the abovementioned evidences, very few studies have been carried out on aquatic organisms to evaluate the effects of MOR towards nontarget organisms. Mantione et al. (2002) showed that nitric oxide (NO) is released by the pedal ganglia in *Mytilus edulis*, after the stimulation by the interaction between opioid receptors and MOR metabolites. Other studies performed on microglia and immunocytes of *M. edulis* suggested an immunosuppressive activity of MOR (Stefano 1989; Stefano et al., 1993), similar to that described in humans (Stefano et al., 1994; Makman et al., 1995). A recent investigation by Gagné et al. (2010) showed the neurochemical consequence of MOR exposure to the freshwater bivalve *Elliptio complanata*. After injections of increasing MOR doses in the adductor muscle (0.07; 0.15 and 0.75 mg/g wet weight), reductions in levels of serotonin and acetylcholinesterase (AChE), as well as increases in dopamine and γ-aminobutyric acid (GABA) levels, were noticed. Similar effects were obtained in the same mussel species exposed to a WWTP effluent extract, in which MOR was detected at 0.1 μg/L concentration (Gagné et al., 2004). However, to date no one investigation was performed to study neither the MOR cyto-genotoxicity nor the involvement of oxidative stress in the mechanism of action of this drug towards nontarget organisms. Considering that MOR is frequently detected in European surface waters with an average concentration of 50–55 ng/L (Karolak et al., 2010; Terzic et al., 2010; Martinez Bueno et al., 2011; Rosa Boleda et al., 2011; Jurado et al., 2012), and that the continual input of this drug can lead to the exposure for the entire life-cycle of aquatic organisms, the investigation of its potential sublethal toxicity is new and pivotal in freshwater ecotoxicology. The aim of this study was to investigate the effects of MOR on the zebra mussels *Dreissena polymorpha*, using an *in vivo* multi-biomarkers approach. Thanks to its physioecological features, this bivalve species is commonly used in ecotoxicology, showing a good sensitivity to different emerging aquatic pollutants (Binelli et al., 2009a,b; Parolini et al., 2010; Parolini and Binelli 2011, 2012), including illicit drugs (Parolini et al., 2013; Parolini and Binelli, 2013, 2014). Moreover, this filter-feeding species has a great filtration rate (mean = 200 mL/h/mussels) and it is more prone than other biological models to introduce the aquatic pollutants into the organism, pointing out rapidly their potential toxic effects. We exposed *D. polymorpha* specimens for 14 days to two low MOR concentrations: 0.05 μg/L and 0.5 μg/L. The end-points of twelve different biomarkers were measured to assess MOR sublethal effects. Cytotoxicity was evaluated on hemocytes by the Neutral Red Retention Assay (NRRA), while the activity of antioxidant and detoxifying enzymes, namely catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST), as well as the lipid peroxidation (LPO) and the protein carbonyl content (PCC) were applied as indices of oxidative stress in mussel homogenates. Primary (DNA strand breaks) and fixed (apoptotic and micronucleated cell frequency) genetic damage was investigated on *D. polymorpha* hemocytes by the Single Cell Gel Electrophoresis (SCGE) assay, the DNA diffusion assay and the micronucleus test (MN test), respectively. Finally, the filtration rate was evaluated as physiological biomarker.

MATERIALS AND METHODS

The MOR standard (CAS number 57-27-2) was purchased from Alltech-Applied Science (State College, PA), while all the reagents used for biomarker analyses were purchased from Sigma-Aldrich (Steinheim, Germany). We diluted the methanol stock solution (1 g/L) to 10 mg/L in ultrapure water (working solution), which was then used to obtain the MOR concentration in experimental aquaria.

Experimental Design

D. polymorpha specimens were collected in September 2012 by a scuba diver at a depth of 4 to 6 m in Lake Lugano

(Northern Italy), which is considered a reference site due to its low drug pollution (Zuccato et al., 2008). The 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. Mussel ($n = 60$), having the same shell length (15 ± 4 mm), were placed within 5 L beakers filled with 4 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). In order to avoid the so-called tank effect, we prepare three beakers per treatment, including control. The bivalves were fed daily with lyophilized algae *Spirulina* spp., and the water was regularly renewed every two days for 2 weeks to gradually purify the mollusks by any possible pollutants that had previously accumulated in their soft tissues. Only specimens that were able to re-form their byssus were used in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method and was 93±2%, whereas biomarker baseline levels were checked weekly. Mussels were exposed to MOR concentrations only when target biomarker levels were comparable with baseline ones obtained in our previous laboratory studies (Parolini et al., 2010, 2011a,b,2013; Parolini and Binelli, 2013). Exposure assays were performed under semi-static conditions for 14 days. Control and exposure beakers were processed at the same time and the whole water volume (4 L) was renewed on a daily basis. Mussels were exposed to 0.05 μg/L (0.17 nM, Low) and 0.5 μg/L (1.7 nM, High) of MOR. The first concentration was similar to the levels found in European surface waters (Pal et al., 2013), while the second one was the same tested in previous studies investigating the toxicity of cocaine metabolites, benzoylecgonine (BE; Parolini et al., 2013), ecgonine methyl ester (EME; Parolini and Binelli, 2013), and Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli, 2014) in order to allow a comparison among drug toxicity administered at the same dose. Exact volumes of working solution (10 ± 0.6 mg/L) were added daily to the exposure aquaria until reaching the selected concentrations. Specimens were fed 2 h before the daily change of water and chemicals to avoid the adherence of the drugs to food particles and to prevent the reduction of their bioavailability. Every 3 days, 8 specimens were randomly collected from each tank (24 specimens per treatment) to evaluate MOR-induced sublethal effects. Hemolymph was withdrawn by 10 bivalves and cyto-genotoxicity was evaluated on hemocytes. After the withdrawal, the soft tissue of mussels was immediately frozen in liquid nitrogen and stored at −80°C until LPO and PCC analyses. Lastly, the soft tissue of the other 14 specimens was frozen in liquid nitrogen and stored at −80°C until the enzymatic activity was measured. Simultaneously, 10 zebra mussels were placed in other control and exposure 500 mL beakers (three replicates per treatment), maintained at the same condition described above and exposed at the same concentrations to assess the variation in filtration rate due to MOR treatments.

Evaluation of MOR Concentrations

In order to guarantee the reliability of the experimental design, the MOR concentration in both working solution and exposure beakers were measured. At each time of biomarker analysis, water was sampled 1 h after the contamination from both the three control and exposure beakers and integrated in a unique sample (100 mL) per treatment. Water samples were spiked with 0.2 μg/L of MOR-D_3 as internal recovery standard. The concentration of the MOR was checked in LC-MS/MS by using a HCT Ultra (Bruker, Germany) using a Phenomenex Luna PFP (2×50 mm to 5 μm) column after purification and concentration by SPE (HLB 1 cm^3, Waters). After cartridge activation (2 mL methanol and 3 mL of water), 5 mL of each sample with internal standard were load on SPE and then resuspended with 50 μL of water; 20 μL of each sample were then injected and analyzed in LC-MS/MS. MOR quantification in water was performed by a calibration curve (0.025–1 μg/L; $R^2 = 0.99$) and internal standard recoveries were >90%. The analysis of MOR concentration in working solution, control and exposure beakers was performed in triplicate.

Biomarkers of Cytotoxicity

The NRRA was performed to assess cytotoxicity following the method proposed by Lowe and Pipe (1994) and applied on mussel hemocytes. 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 then calculated from five replicates.

Oxidative Stress Biomarkers

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) with dithiothreitol (DTT, 100 mM 1:10 v/v) using a Potter homogenizer. Specific protease inhibitors (1:10 v/v) were also added to the buffer: phenanthroline (Phe, 10 mM) and trypsin inhibitor (Try, 10 mg/mL). The homogenate was centrifuged at $15{,}000 \times g$ 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 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. LPO and 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 × 105 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. (1999). 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).

Genotoxicity Biomarkers

Since methods and procedures of genotoxicity biomarkers applied in this study were described in detail by Parolini et al. (2010), only a brief description of the followed techniques was reported here. The alkaline (pH > 13) SCGE assay was performed on hemocytes according to 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 SCGE assay 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%).

Filtration Rate

Mussel filtration rate was measured according to a procedure adapted from Faria et al. (2009) and Palais et al. (2012) and based on the loss of neutral red dye particles from the water column as a result of mussel filtration activity (Coughlan, 1969). Ten zebra mussels per beaker (three replicates per treatment) were placed in 500 mL and were exposed to 0.05 μg/L and 0.5 μg/L MOR concentrations. Every 3 days for 14 days a 250 μg/L neutral red solution was added in the beakers. After 15 min of acclimation, bivalve were allowed to filter for 3 h in the dark at 20°C. Dye particle concentration in the test beakers was then measured spectrophotometrically (540 nm) at the beginning and at the end of the experiment, using a standard curve for neutral red solution. The filtration rate (f), expressed in mL of water per individual and per hour (mL/ind/h), was calculated using the following formula:

$$f = [V/(n \times t)] \times \log (C_0/C_t)$$

where V is the volume (mL) of the dye solution in the beaker, n the number of mussels, t the duration of the filtration period (h), C_0 and C_t the initial and final dye particle concentrations in the beaker (Coughlan, 1969).

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 MOR concentrations as variables, while biomarker end-points served as cases. The ANOVA was followed by a Fisher 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. All statistical analyses were performed using the STATISTICA 7.0 software package.

RESULTS

MOR Concentration in Exposure Tanks

The MOR concentration in the control tanks was <0.025 μg/L, which is the limit of detection of the used equipment. In the exposure tanks, MOR level was close to the nominal values (0.05 μg/L and 0.5 μg/L). We found an average value of 0.045 ± 0.005 μg/L for the lower concentration and a value of 0.35 ± 0.01 μg/L for the higher tested concentration, accounting for the 90% and 70% of the nominal values, respectively. Considering that the coefficient of variation of

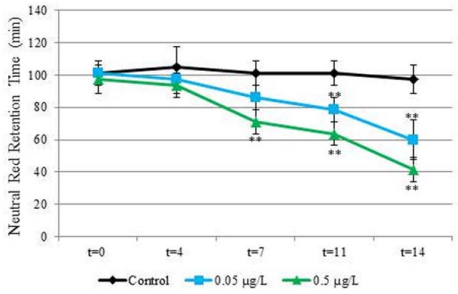

Fig. 1. Assessment of lysosomal membrane stability (neutral red retention time-mean ± SEM) found in the hemocytes of treated bivalves ($n = 5$). Asterisks indicate significant differences between the treated and the corresponding controls (two-way ANOVA, Fisher LSD *post hoc* test, $*p < 0.05$, $**p < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the method was ±20%, our analyses confirmed the reliability of the whole experimental design.

Baseline Levels of Applied Biomarkers

During the 14-day experiment, very low mortality was observed in the control (0.6%) and exposure (<3%) tanks. Baseline levels of cyto-genotoxic and oxidative stress biomarkers were similar to those obtained in our previous laboratory studies (Binelli et al., 2009a,b; Parolini et al., 2010, 2011a,b, 2013; Parolini and Binelli, 2011, 2013). The baseline filtration rate of zebra mussel specimens ranged between 0.74 ± 0.29 and 1.92 ± 0.17 mL/individual/h, according to values measured in the same species with a similar method by Palais et al. (2012).

Biomarkers of cytotoxicity

The NRRA showed a significant destabilization of hemocytes lysosome membranes (Fig. 1) according to time- ($F = 27.87$, $p < 0.01$) and concentration-dependent ($F = 48.56$, $p < 0.01$) relationships. Both the MOR concentrations were able to significantly increase generic cellular stress in mollusks: 0.05 µg/L treatment caused a significant decrease ($p < 0.01$) of NRRT starting to 11 days of exposure, while at 0.5 µg/L a significant destabilization ($p < 0.01$) of the lysosomal membranes was notice as early as 7 days of exposure.

Biomarkers of Oxidative Stress

The activity of antioxidant enzymes (SOD, CAT, and GPx) and detoxification enzymes (GST) showed some significant changes compared with controls during the exposure tests [Fig. 2(A–D)]. Although the activity of GST was not significantly altered ($p > 0.05$) after exposure to the two MOR tested concentrations [Fig. 2(A)], the activity of SOD showed a significant time-dependent ($F = 3.07$, $p < 0.05$) and dose-dependent ($F = 11.54$, $p < 0.01$) inhibition already after 4 days of exposure to 0.5 µg/L [Fig. 2(B)], reaching at the end of the test values lower than 40% compared with controls. Regarding the GPx, a significant time- ($F = 4.64$, $p < 0.01$) and concentration-dependent ($F = 10.46$, $p < 0.01$) increase was noticed after 7 days of exposure at the highest MOR concentration [Fig. 2(C)]. Accordingly, CAT showed significant activity increase ($p < 0.05$) compared with baseline values [Fig. 2(D)] at the end of the exposure to both the treatments. Finally, significant time-dependent ($F = 5.37$; $p < 0.01$) and concentration-dependent ($F = 4.36$; $p < 0,05$) differences were found in the lipid peroxidation levels [Fig. 3(A)] with an increase of 15% compared with controls at the end of exposure. In contrast, no significant differences ($p > 0.05$) of protein carbonylation compared with baseline levels were found, except for a single value obtained after 11 days at the lowest treatment [Fig. 3(B)].

Biomarkers of Genotoxicity and Filtration Rate

Even if no significant increase in LDR parameter was found (data non shown), significant increase in DNA fragmentation was noticed at the end of the 0.5 µg/L exposure, as pointed out by the raise of percentage of DNA in the hemocyte comet tail with respect to control [$F = 2.57$, $p < 0.05$; Fig. 4(A)]. No significant increase ($p > 0.05$) in frequencies of apoptotic [Fig. 4(B)] and micronucleated cells [Fig. 4(C)], whose levels were similar to the baseline ones throughout the test, even if a significant time-dependent relationship ($F = 2.91$, $p < 0.05$) was noticed for the latter end-point. Lastly, the filtration rate (ranged between 1.43 ± 0.25 and 3.11 ± 0.28 mL/individual/h for 0.05 µg/L MOR concentration and ranged between 1.19 ± 0.17 and 3.05 ± 0.66 for 0.5 µg/L) followed a bell-shaped curves for both MOR tested concentrations, with no significant changes ($p > 0.05$) compared with controls (data not shown).

DISCUSSION

Sublethal Effects of MOR

Although the lowest MOR concentration did not induced any significant variation ($p > 0.05$) for all the investigated end-points, the exposure to 0.5 µg/L MOR caused a notable cytotoxicity to *D. polymorpha* specimens, as pointed out by the significant ($p < 0.01$) time- and concentration dependent decrease of NRRT (Fig. 1). This showed a progressive aggravation of the bivalve health status, suggesting that bivalves suffer a situation of general cellular stress, which could be linked to the induction of oxidative stress (Lowe et al., 1995). The destabilization of lysosome membranes in aquatic organisms could be caused by the production of

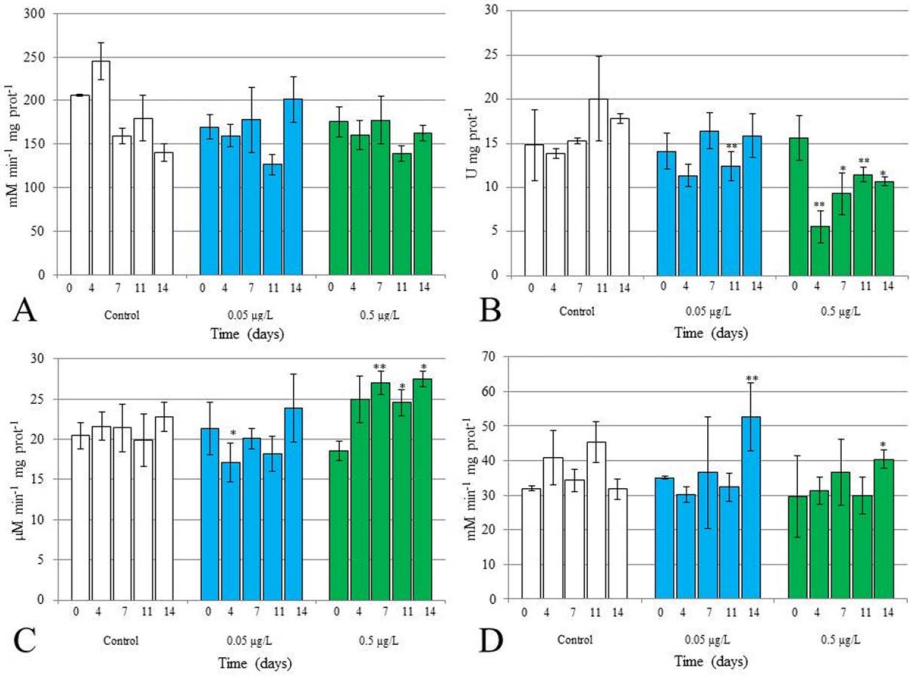

Fig. 2. Mean values (±SEM) of the activity of glutathione-*S*-transferase (GST, A), superoxide dismutase (SOD, B), glutathione peroxidase (GPx, C) and catalase (CAT, D), measured in the bivalves ($n = 3$, pool of three individuals) exposed to both MOR concentrations. The significant differences (two-way ANOVA, Fisher LSD *post hoc* test, $^{*}p < 0.05$; $^{**}p < 0.01$) refer to the comparison of exposed with the corresponding baseline value. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

reactive oxygen species (ROS) following exposure to pollutants (Regoli et al., 1998). The ROS are mainly produced as side-products of oxygen metabolism and among these the biotransformation of xenobiotics is an oxidative process in which the production of ROS and the formation of more polar (reactive) intermediates occur (Gagné et al., 2010). In

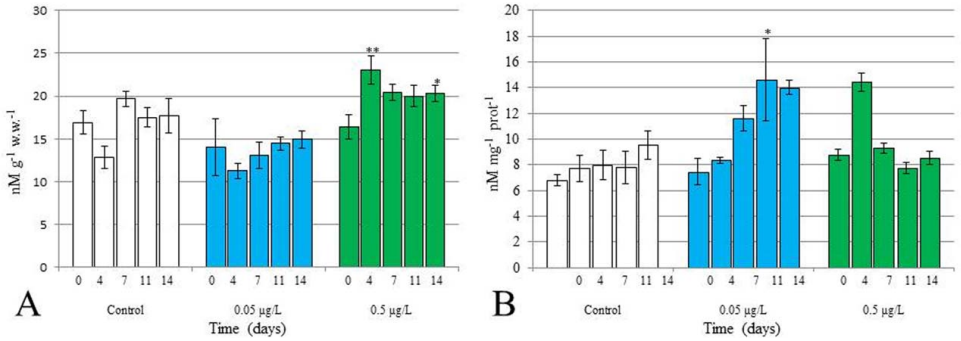

Fig. 3. Mean values (±SEM) of lipid peroxidation levels (LPO, A) and protein carbonylation (PCC, B) found in the bivalves ($n = 3$, pool of three individuals) exposed to both MOR concentrations. The significant differences (two-way ANOVA, Fisher LSD *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$) relate to the comparison between the exposed and corresponding baseline value. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

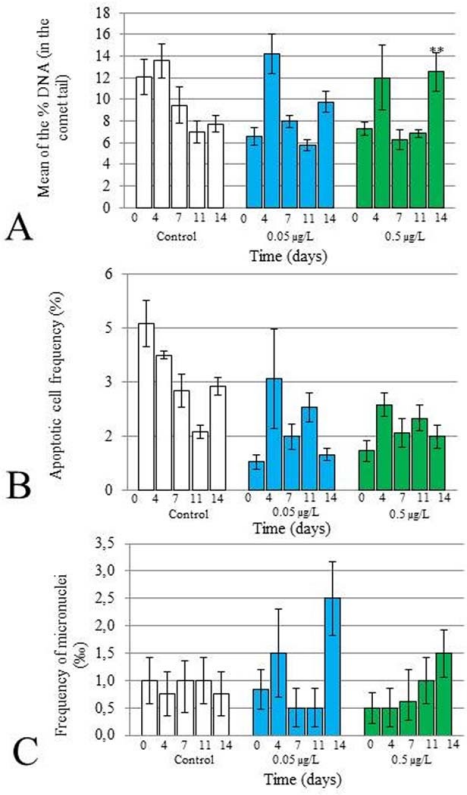

Fig. 4. DNA percentage in the comet tails (A; mean ± SEM) of the bivalve hemocytes ($n = 8$) exposed to both MOR concentrations. The significance (two-way ANOVA, Fisher LSD *post hoc* test, $^{**}p < 0.01$) refers to the comparison between the exposed and controls. Mean (±SEM) apoptotic cells frequency (%; B) and micronucleated frequency (‰MN; C) showed no significant differences compared with the corresponding controls (two-way ANOVA, $p > 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

vertebrates, MOR is biotransformed by cytochrome P450 3A4 and 2C19, which involves oxidative N-dealkylation, hydroxylation and conjugation to glucuronide (Charney et al., 2001). Even if no information regarding the biotransformation of MOR in the zebra mussel is currently available, the observed trends of antioxidant enzymes suggested that this drug could induce the production of ROS. Variation in antioxidant levels, in fact, indirectly supply information on the changes of pollutant-induced reactive oxygen species (ROS) levels in different aquatic organisms (Viarengo et al. 2007), including *D. polymorpha* (Parolini et al., 2010, 2013). The inhibition of SOD activity [Fig. 2(B)] suggested an increase of superoxide anion in bivalves ($\bullet O_2^-$; Verlecar et al., 2008), as found in our previous study exposing zebra mussel to cocaine metabolites (Parolini et al., 2013; Parolini and Binelli, 2013). Since dismutation of $\bullet O_2^-$ leads to the production of hydrogen peroxide, this particular trend could be due to a phenomenon of product inhibition, according to a negative feedback mechanism (Vlahogianni and Valavanidis, 2007). The inhibition of SOD should therefore indicate both an accumulation of $\bullet O_2^-$ and an overproduction of H_2O_2, which could be also produced through the spontaneous conversion of superoxide anion mediated by nonenzymatic pathways (Gwoździński et al., 2010). The significant time-dependent trend in the levels of GPx and CAT [Fig. 2(C,D)] confirmed that MOR was able to increase the levels of H_2O_2, whose toxicity seems to be counterbalanced by the antioxidant shield of bivalves. However, it is important to note that the accumulation of superoxide radical caused by SOD inhibition, combined with the increase of H_2O_2 caused by the activation of CAT and GPx, could lead to the formation of hydroxyl radicals through the Haber-Weiss reaction, with the consequent increase in the levels of lipid peroxidation and protein carbonylation (Verlecar et al., 2008). The analysis of lipid peroxidation levels and protein carbonyl content just partially confirmed this hypothesis, since we observed a significant ($p < 0.01$) increase in the levels of lipid peroxidation [Fig. 3(A)], despite no variations in protein carbonylation were noticed [Fig. 3(B)]. Accordingly, MOR treatments caused negligible genotoxic effects to zebra mussels, since just slight significant ($p < 0.01$) increase of DNA fragmentation was found at the end of exposure to 0.5 µg/L [Fig. 4(A)]. Although several studies showed that the increase in DNA fragmentation is one of the main factor leading to the onset of fixed genetic damage in *D. polymorpha* specimens (Binelli et al., 2009a,b; Parolini and Binelli, 2012), no significant ($p > 0.05$) increases in frequency of apoptotic cells and MN were found [Fig. 4(B,C)]. Lastly, even if our data showed that low MOR concentrations could alter antioxidant status of the zebra mussel leading to low oxidative damage, no physiological effect was noticed, as pointed out by the lack of significant ($p > 0.05$) alteration of bivalve filtration rate (data not shown). However, despite MOR causes an increase in dopamine levels correlated with ciliary beating inhibition, as observed in *M. edulis* (Aiello et al., 1986), in our study were not observed effects of this drug at physiological level. Despite of the moderate MOR-induced adverse effects found in the zebra mussel, the potential toxicity of this illicit drug cannot be neglected since it could cause other deleterious effects that further studies should have to investigate. For instance, being a psychotropic substance, MOR could act as neurotoxic compound, as pointed out by a recent *in vivo* study on the freshwater bivalve *Elliptio complanata* in which the exposure to three MOR concentrations (0.07; 0.15, and 0.75 mg/g wet weight) induced decreases in serotonin and AChE, and increases in

dopamine and GABA levels, suggesting the induction of a relaxation state in mussels (Gagnè et al., 2010).

Comparison of MOR Toxicity with the Effects of Other Illicit Drugs

Considering the moderate sublethal effects of MOR to treated zebra mussel, the comparison between its toxicity and that of previously analyzed illicit drugs, namely benzoylecgonine (BE; Parolini et al., 2013), ecgonine methyl ester (EME; Parolini and Binelli, 2013) and Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli, 2014), should allow the drawing of a toxicity scale to individuate the most dangerous compound towards our biological model, laying the bases for further in-depth investigations. Although it is well-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 considered insufficient to rank the hazard of different pollutants because of the wide variability in biomarker responses. For example, by comparing present data and those from our experiments on the sublethal effects induce by other illicit drugs (Parolini et al., 2013; Parolini and Binelli, 2013, 2014) we can note a remarkable variability in most of investigated end-points, depending on tested compound and probably due to dissimilarities in their mechanism of action, which prevents from ranking their toxicity. In fact, even though all psychotropic substances were able to induce significant destabilization of lysosome membranes, substantial differences in the activity of antioxidant/detoxification enzymes, as well as in oxidative and genetic damage were found. For this reason, the application of procedures able to integrate the biomarker responses within a simple synthetic index could help to minimize the variation of responses, allowing to draw an accurate scale of toxicity. To compare the sublethal toxicity of tested illicit drugs we integrated the whole biomarker dataset obtained at 0.5 μg/L for MOR into a synthetic index called Biomarker Response Index (BRI), previously described by Parolini et al. (2013). We excluded from integration analysis the results from filtration rate since it is not assessed in previous studies. Briefly, since changes in each specific biomarkers follow different trends (increasing, decreasing or bell-shaped curves, Hagger et al., 2010), we calculated the percentage of alteration level (AL) of each biomarker per exposure time compared with the correspondent control. To calculate the BRI, we attributed a specific score to each obtained AL value according to Parolini et al. (2013) and each biomarker was then weighted in relation to its level of biological organization (Hagger et al., 2010). Finally, we compared the BRI value obtained for MOR with those calculated for BE and EME (Parolini and Binelli, 2013), and Δ-9-THC (Parolini and Binelli, 2014), deriving the following toxicity scale:

$$\Delta-9-\mathrm{THC} > \mathrm{BE} \approx \mathrm{EME} \gg \mathrm{MOR}$$

The toxicity of Δ-9-THC (BRI = 8.78; Parolini and Binelli, 2014) seems to be slightly higher than that of BE (BRI = 8.22) and EME (BRI = 8.06; Parolini and Binelli 2013), while MOR (BRI = 6.17) showed the lowest value among the tested illicit drugs, suggesting its possible lowest hazard towards the zebra mussel for the measured end-points at least.

CONCLUSION

Our findings showed that MOR exposure could induce moderate adverse effects to this freshwater bivalve species, highlighting its possible hazard to freshwater communities. Even if current environmental MOR levels seem not cause any deleterious effect to bivalves, 14-day treatment to 0.5 μg/L concentration affected the oxidative status of bivalves and induced slight oxidative damage to cellular macromolecules. Although our findings suggest that oxidative stress seems to be involved in the mechanism of action of MOR in zebra mussel, further studies using powerful techniques, as well as the analysis of different end-points (i.e. neurotoxicity parameters), should be necessary to confirm it. Although the integrated MOR toxicity obtained at 0.5 μg/L treatment resulted lower compared with that of other common illicit drugs previously tested at the same experimental conditions, its environmental hazard cannot be underestimated. In fact, considering that in the real environment organisms are exposed to MOR concentrations for their whole life span, and its levels could increase due to the stable use of opiates worldwide, MOR effects could be more deleterious with respect to those highlighted in the present study. For this reason, further investigations should be necessary to enhance knowledge on MOR sublethal effects and its mechanism of action in nontarget aquatic organisms.

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Chapter 6

PAPER 5

Environmental concentrations of 3,4-methylenedioxymethamphetamine (MDMA)-induced cellular stress and modulated antioxidant enzyme activity in the zebra mussel

(Environmental Science and Pollution Research 21, 11099-11106)

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SHORT RESEARCH AND DISCUSSION ARTICLE

Environmental concentrations of 3,4-methylenedioxymethamphetamine (MDMA)-induced cellular stress and modulated antioxidant enzyme activity in the zebra mussel

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Abstract Recent monitoring studies showed measurable levels of the 3,4-methylenedioxymethamphetamine (MDMA) in aquatic environments. However, no information is currently available on its potential hazard to aquatic non-target organisms. The aim of this study was to investigate the potential sub-lethal effects induced by 14-day exposures to low MDMA concentrations (0.05 and 0.5 μg/L) to zebra mussel (*Dreissena polymorpha*) specimens through the application of a biomarker suite. The trypan blue exclusion method and the neutral red retention assay (NRRA) were used to assess MDMA cytotoxicity. The activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST), as well as the lipid peroxidation (LPO) and protein carbonyl content (PCC), were measured as oxidative stress indexes. The single cell gel electrophoresis (SCGE) assay, the DNA diffusion assay, and the micronucleus test (MN test) were applied to investigate DNA damage, while filtration rate was measured as physiological parameter. Despite significant decrease in lysosome membrane stability, hemocyte viability and imbalances in CAT and GST activities pointed out at the end of the exposure to 0.5 μg/L, no significant variations for the other end points were noticed at both the treatments, suggesting that environmentally relevant MDMA concentrations did not induce deleterious effects to the zebra mussel.

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Keywords 3,4-Methylenedioxymethamphetamine (MDMA) · Sub-lethal effects · *Dreissena polymorpha*

Introduction

The 3,4-methylenedioxymethamphetamine (MDMA), also known as "ecstasy," is a synthetic amphetamine derivative with a range of psychotropic actions, including amphetamine-like stimulant and "entactogen" effects. Its characteristics of low addictive power, low acute toxicity, few unpleasant effects, strong stimulant effects, and "positive" psychological feelings have led to the perception among users that MDMA is a relatively benign substance (Barenys et al. 2009), contributing to its wide popularity mostly among young people during rave parties. The use of MDMA has reached epidemic proportions in the USA and in Europe, since its repeated usages pose significant social and public health problems (Reid et al. 2007). According to the consumption trend, the MDMA issue has recently become also an environmental problem. Drugs of abuse such as MDMA, in fact, are one of the latest groups identified as emerging aquatic pollutants (Kasprzyk-Hordern et al. 2010). Aquatic ecosystems are the ultimate collectors for all these compounds following their metabolism in the human body and/or their accidental or deliberate disposal. Similar to pharmaceuticals, a large proportion of drugs may be excreted as parent compound and/or as metabolites through human urine, feces, saliva, and sweat and discharged into the sewage system (Castiglioni et al. 2011). Several monitoring surveys showed the occurrence of illicit drugs and their metabolites in both surface and wastewaters worldwide in nanograms per liter (ng/L) concentrations (Pal et al. 2013), matching levels of common pharmaceuticals used for therapeutic purposes (Santos et al. 2010). Since illicit

drugs may have high pharmacological and biological activities, their presence in surface waters even at low concentrations may cause toxic effects to aquatic organisms. Recent investigations, in fact, pointed out that low environmentally relevant concentrations of benzoylecgonine (Parolini et al. 2013), ecgonine methyl ester (Parolini and Binelli 2013), and Δ-9-tetrahydrocannabinol (Parolini and Binelli 2014) induced sub-lethal effects to *Dreissena polymorpha*. Even if measurable MDMA concentrations were found both in influents (range 2–15,380 ng/L) and effluents (range 2–10,955 ng/L) of wastewater treatment plants (WWTPs), as well as in surface water (range 0.2–14.1 ng/L) worldwide (Pal et al. 2013), the information on its potential hazard to non-target organisms is completely lacking. Hence, the aim of this study was the evaluation of oxidative and genetic damage induced by MDMA to the zebra mussel through an in vivo multi-biomarker approach. We focused on these end points since some evidences in different vertebrate cells and tissues suggested that MDMA administration can induce cytotoxicity (Nakagawa et al. 2009; Antolino-Lobo et al. 2010), overproduction of reactive oxidative species (ROS), lipid peroxidation, depletion of antioxidant systems, and DNA damage (Barenys et al. 2009; Alvarenga et al. 2010; Cerretani et al. 2011). Mussels were exposed for 14 days to two environmentally relevant MDMA concentrations (0.05 and 0.5 μg/L). The trypan blue exclusion test and the neutral red retention assay (NRRA) were applied to evaluate MDMA cytotoxicity. The activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), and the levels of lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured as oxidative stress indexes. Primary and fixed genetic damage was investigated by the single cell gel electrophoresis (SCGE) assay, the DNA diffusion assay, and the micronucleus test (MN test), respectively, while the evaluation of filtration rate was used to assess possible variations in the physiological status of bivalves.

Materials and methods

Experimental design

Zebra mussels were collected in September 2012 in Lake Lugano (northern Italy) because of its low drug pollution (Zuccato et al. 2008). Mussels (n=60) having the same shell length (15±4 mm) were placed in 5-L beakers filled with 4 L of tap and deionized water (50:50 *v*/*v*) and maintained according to Parolini et al. (2010). We prepared three beakers per treatment, including controls, to avoid the so-called tank effect. Exposure assays were performed under semi-static conditions for 14 days according to Parolini et al. (2013). Control and exposure beakers were processed at the same time; the whole water volume and MDMA concentrations were renewed on a daily basis. The complete water and chemical renewal should guarantee a constant MDMA concentration over a 24-h period, since MDMA degradation in wastewater does not occur up to or even longer than 28 h (van Nuijs et al. 2012). Mussels were exposed to 0.05 μg/L (0.25 nM, low) and 0.5 μg/L (2.5 nM, high) of MDMA. The first concentration was similar to levels found in European surface waters (Pal et al. 2013), while the second one was the same tested in previous studies investigating the toxicity of other illicit drugs (Parolini et al. 2013; Parolini and Binelli 2013, 2014). Every 3 days, eight mussels were randomly collected from each tank (24 specimens per treatment) to evaluate sub-lethal effects. Hemolymph was withdrawn from the sinus near the posterior adductor muscle of 10 bivalves, and cyto-genotoxicity biomarkers were applied on hemocytes. After the withdrawal, the soft tissues of mussels were immediately frozen in liquid nitrogen and stored at −80 °C until LPO and PCC analyses. Lastly, the soft tissues of other 14 specimens were frozen in liquid nitrogen and stored at −80 °C until the enzymatic activity was measured. Simultaneously, 10 zebra mussels were placed in other control and exposure beakers (500 mL; three replicates per treatment) maintained at the same conditions and exposed at the same concentrations described above to assess the variation in filtration rate due to MDMA.

Evaluation of MDMA concentrations

MDMA concentrations in the working solution, control, and exposure beakers were measured in triplicate. Water was sampled 1 h after the contamination from both the three control and exposure beakers and integrated in a unique sample (100 mL) per treatment. Samples were spiked with 0.2 μg/L of 3,4-methylenedioxymethamphetamine-D_5 (MDMA-D_5) as internal recovery standard. The concentration of the MDMA was checked in LC–MS/MS by using a HCT Ultra (Bruker, Germany) equipped with a Phenomenex Luna PFP (2×50 mm–5 μm) column after purification and concentration by SPE (HLB 1 cm^3, Waters). MDMA quantification in water was performed by a calibration curve (0.025–1 μg/L; R^2=0.99), and internal standard recoveries were >90 %.

Biomarker methods

Since biomarker methods were reported in detail elsewhere (Parolini et al. 2010, 2013), we just briefly described them, while detailed protocols were reported in Supplementary information. The trypan blue exclusion method was assessed on hemocytes (n=3), while the NRRA followed the protocol by Lowe and Pipe (1994) and was applied on hemocytes too (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 by spectrophotometric analyses according to Orbea et al. (2002). Lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured in triplicate (n=3) from a pool of three whole mussels. LPO level was assayed by the determination of thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. (1979), while for carbonyl quantification, the reaction with 2,4-dinitrophenylhydrazine (DNPH) was employed according to Mecocci et al. (1998). The alkaline SCGE assay was performed on hemocytes according to the method adapted for the zebra mussel by Buschini et al. (2003), and DNA fragmentation was measured as the percentage of DNA in tail (n=10). The apoptotic cell frequency (n=5) was evaluated through the protocol described by Singh (2000), while the MN test (n=10) was performed according to the method of Pavlica et al. (2000), and micronuclei (MN‰) were identified using the criteria proposed by Kirsch-Volders et al. (2000). Lastly, filtration rate of zebra mussels (n=3) was measured according to the procedure adapted from Palais et al. (2012) and based on the loss of neutral red dye from the water, as a result of mussel filtration activity (Coughlan 1969).

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 MDMA concentrations as variables, while biomarker end points served as cases. The ANOVA was followed by a Fischer 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 performed using all measured variables in the exposure assays to investigate possible correlations between the different biological responses. Statistical analyses were performed using the STATISTICA 7.0 software package.

Results

MDMA concentrations in water

The concentration of the MDMA working solution was checked in triplicate and was 10±0.8 mg/L. No MDMA residues (<0.025 μg/L) were found in the control beakers during the 14-day exposure, while concentrations in the exposure beakers were close to the nominal ones. In detail, MDMA levels measured in water from the 0.05- and 0.5-μg/L beakers were on average 0.033±0.006 μg/L and 0.34±0.05 μg/L, respectively, accounting for about the 70 % of the nominal concentrations. Considering that the coefficient of variation of the method was ±20 %, our analyses confirmed the reliability of our exposures.

Zebra mussel baseline levels

Very low mortality was found both in control (0.6 %) and in exposure beakers (<3 % in both the treatments) all over the exposures. The 14-day average hemocyte viability of bivalves from control was 93.01±1.14 %, and it was similar to that measured at the beginning of the experiment in specimens from beakers used for 0.05 μg/L (95.31±2.37 %) and 0.5 μg/L (94.01±0.81 %) MDMA exposure. Baseline levels of all tested biomarkers were similar to those obtained in previous laboratory studies (Binelli et al. 2009a, b; Parolini et al. 2010, 2011a, b, 2013; Parolini and Binelli 2013, 2014). The baseline filtration rate of zebra mussel specimens ranged between 0.74±0.29 and 1.92±0.17 mL/ind/h, according to values measured in the same species with a similar method by Palais et al. (2012).

MDMA-induced sub-lethal effects

MDMA exposure induced a significant time (F=16.99; p<0.01)- and concentration-dependent (F=7.26; p<0.05) decrease in zebra mussel hemocyte viability. Significant (p<0.01) reduction of cell viability was noticed after 11 days of exposure to 0.5 μg/L, with values 15 % lower than the control ones at t=14 days (Fig. 1a). The NRRA showed a significant time-dependent (F=9.24; p<0.01) decrease of lysosome membrane stability at 0.5 μg/L treatment, with values 36 % lower than the corresponding control at the end of the exposure (Fig. 1b). No significant (p>0.05) variations of SOD and GPx activity were noticed during 14-day exposure to both the MDMA concentrations (Fig. 2a, b), while significant time-dependent (F=4.17; p<0.01) increase of CAT was noticed already after 4 days of exposure to 0.5 μg/L, with values 47 % higher than the controls (Fig. 2c). GST activity showed a significant (p<0.01) increase at the end of the 0.05 μg/L exposure, with values 32 % higher than the corresponding control, while a significant (p<0.01) decrease was noticed after 11 days of exposure to the highest treatment. In Table 1 are reported results for oxidative and genetic damage end points and filtration rate. Exposure to both the MDMA treatments did not induce significant (p>0.05) increases in lipid peroxidation levels and protein carbonyl content. No significant (p>0.05) increase in the percentage of DNA in the comet tail, apoptotic, and micronucleated cell frequency with respect to

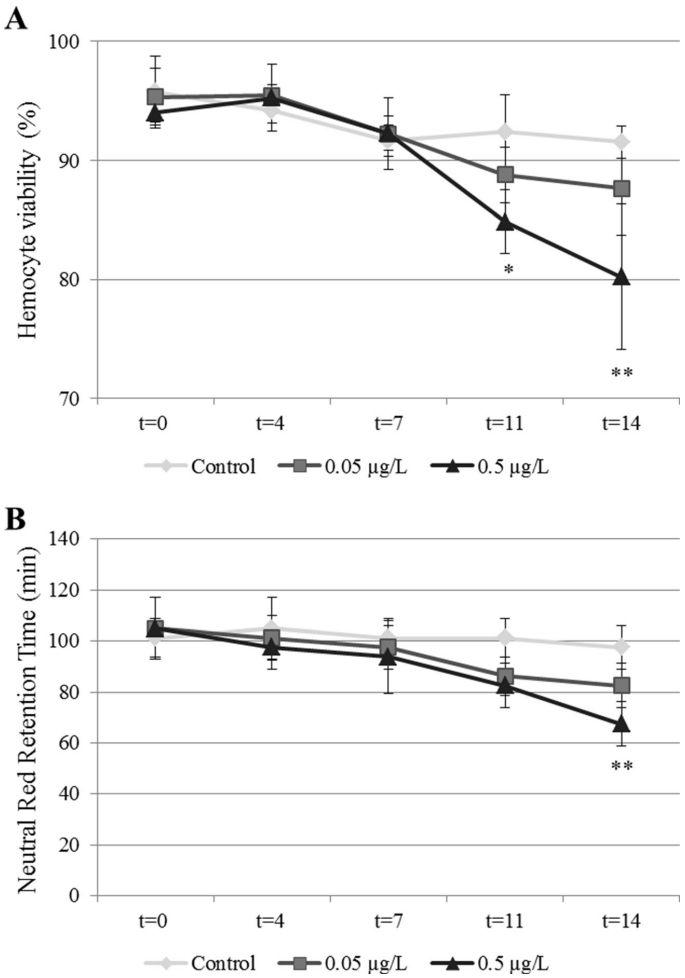

Fig. 1 Effects of MDMA treatments to hemocyte viability (mean%±SEM; **a**) measured by the trypan blue exclusion test (n=3) and lysosome membrane stability (NRRA; **b**) of zebra mussel haemocytes (n=5). Significant differences (two-way ANOVA, Fischer's LSD post hoc test, *p<0.05; **p<0.01) were referred to the comparison between treated mussels and the corresponding control (time to time)

baseline levels was induced by both MDMA concentrations. Lastly, the filtration rate of zebra mussel did not show significant (p>0.05) variations compared to controls during both the exposures.

Discussion

Our 14-day exposures showed that low environmentally relevant MDMA concentrations induce slight sub-lethal effects to the zebra mussel. Despite of the lack of significant variation (p>0.05) in all the investigated end points induced by the 0.05 µg/L MDMA treatment, the significant (p<0.01) increase in hemocyte mortality noticed at the highest tested concentration (0.5 µg/L) pointed out the cytotoxic potential of this molecule to *D. polymorpha* (Fig. 1a). Similarly, the significant (p<0.01) time- and concentration-dependent decrease of neutral red retention time (NRRT) (Fig. 1b) showed a progressive aggravation of the bivalve health status, suggesting that bivalves suffered a situation of general cellular stress, potentially linked to the induction of oxidative stress (Lowe et al. 1995). The destabilization of lysosome membranes in mussels, in fact, is affected by the production of oxyradicals generated by the exposure to contaminants, and it is often correlated with the activity of antioxidant enzymes, whose imbalances indirectly give information on the changes of pollutant-induced ROS levels in aquatic organisms (Viarengo et al. 2007). Significant correlations between the NNRT, antioxidant, and GST activities in the zebra mussel

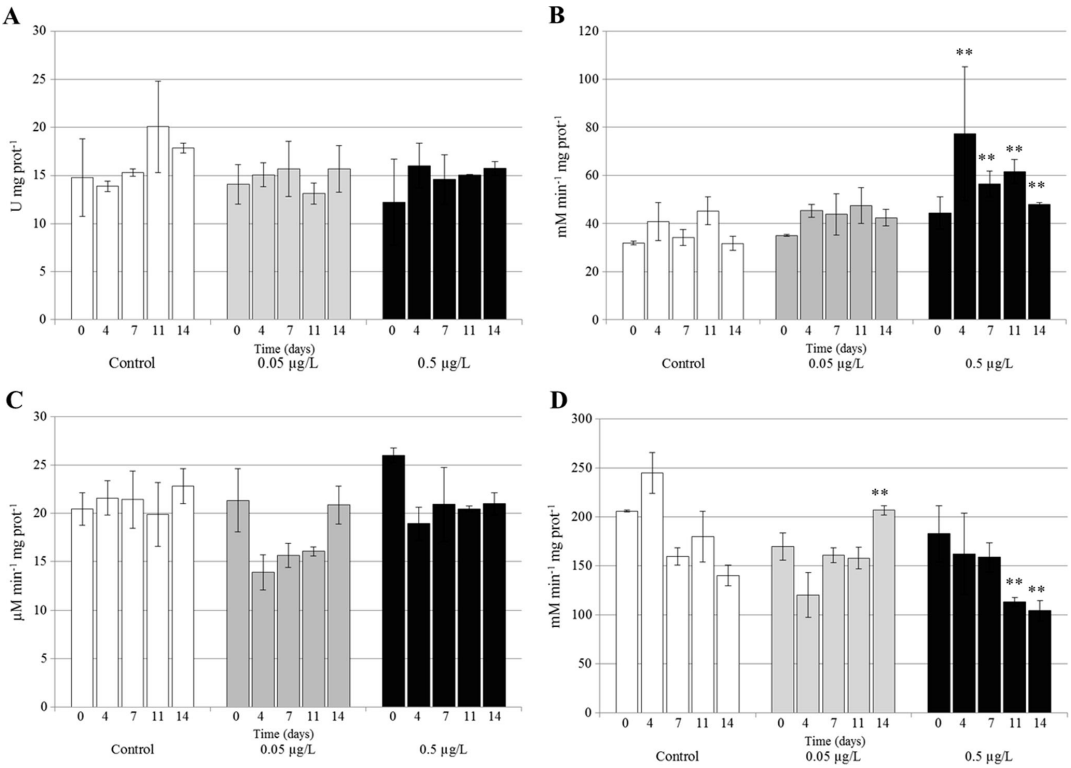

Fig. 2 Effects of MDMA treatments on the activity (mean±SEM) of superoxide dismutase (SOD; **a**), catalase (CAT; **b**), glutathione peroxidase (GPx; **c**), and glutathione S-transferase (GST; **d**), measured in the whole soft tissue of zebra mussels (n=3; pool of three specimens). Significant differences (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)

exposed to different illicit drugs were noticed (Parolini et al. 2013; Parolini and Binelli 2013, 2014). Accordingly, a significant negative correlation between the NRRT and CAT (r=−0.77; p<0.05) and a positive correlation between NRRT and GST (r=0.83; p<0.05) were found, while SOD and GPx did not show any relationship with the lysosome membrane stability. Despite the lack of variation in SOD and GPx activity (Fig. 2a, b), the significant (p<0.01) increase of CAT (Fig. 2c) compared to controls indirectly suggested the production of hydrogen peroxide, agreeing findings from Alvarenga et al. (2010), which showed greater amounts of H_2O_2 and nitrites produced from the mitochondria of MDMA-exposed rats compared to controls. Since SOD was not activated, H_2O_2 production was probably due to the spontaneous dismutation of superoxide radical by non-enzymatic ways (Gwoździński et al. 2010) and/or to other cellular enzymes like those contained in peroxisomes (Khessiba and Roméo 2005). Our data are partially consistent with many studies on vertebrate biological models and/or cell lines showing impairments of enzymatic defense system upon MDMA exposure (Carvalho et al. 2004a; Cerretani et al. 2011). These findings highlighted the crucial role of MDMA metabolism in the onset of toxic effects (Antolino-Lobo et al. 2010), including damage to different tissues (Song et al. 2010), through the increased production of ROS and/or toxic oxidation products (Montiel-Duarte et al. 2004) linked with glutathione (GSH) depletion (Carvalho et al. 2004a, b) and the formation of MDMA protein adducts (Fisher et al. 2007). Besides the slight imbalances of antioxidants, the significant (p<0.01) decrease in GST found at the end of exposure to 0.5 µg/L (Fig. 2d) should suggest a depletion of GSH levels (van Iersel et al. 1996), agreeing the marked GSH depletion accompanied by decrease in GR, GPX, and GST activities in rat hepatocytes exposed to a MDMA metabolite, the α-methyldopamine (α-MeDA; Carvalho et al. 2004a). Although the MDMA metabolism in the zebra mussel is currently unknown, our data should suggest its involvement in MDMA toxicity, but it has to be confirmed by further in-depth studies. Despite the imbalances of enzyme activities, MDMA did not induce oxidative damage to cellular macromolecules since no significant

Table 1 Levels (mean±SEM; $n=3$; pool of three specimens) of lipid peroxidation (LPO, nM/g wet weight), protein carbonylation content (PCC, nM/mg protein), percentage of tail DNA (%DNA; $n=10$), percentage of apoptotic hemocytes (%Apo; $n=5$), frequency of micronucleated hemocytes (‰MN; $n=10$), and filtration rate (mL/ind/h; $n=3$) in zebra mussel specimens treated with MDMA concentrations

	Time (t)	LPO	PCC	%DNA	%Apo	‰MN	Filtration rate
Control	0	16.9±1.3	6.8±0.4	12.1±1.6	4.6±0.6	1.0±0.4	0.7±0.2
	4	12.8±1.3	7.7±1.0	13.6±1.5	3.8±0.1	0.8±0.4	1.1±0.2
	7	19.7±0.9	8.0±1.1	9.5±1.5	2.8±0.5	1.0±0.6	1.3±0.3
	11	17.5±1.1	7.8±1.3	6.9±1.0	1.6±0.2	1.0±0.4	1.3±0.2
	14	17.7±2.0	9.5±1.1	7.7±0.8	2.9±0.2	0.8±0.4	1.9±0.1
MDMA 0.05 μg/L		14.0±2.1	8.6±1.2	11.0±0.9	3.2±0.7	1.3±0.4	0.9±0.2
	4	16.9±0.7	12.3±0.3	12.8±1.5	3.4±0.7	1.5±0.6	1.1±0.2
	7	21.2±2.5	11.6±1.5	8.5±1.3	4.9±0.2	0.8±0.6	1.5±0.2
	11	17.4±1.3	11.8±0.9	7.7±0.8	3.6±2.0	1.8±0.6	1.6±0.1
	14	13.5±0.5	11.0±0.3	5.8±0.6	3.6±0.9	0.3±0.2	2.2±0.2
MDMA 0.5 μg/L	0	10.0±1.7	10.7±1.0	10.4±0.8	3.3±0.5	1.3±0.4	0.7±0.2
	4	9.6±0.8	12.5±1.1	8.9±0.7	3.7±1.4	1.3±0.4	1.3±0.2
	7	15.5±1.2	10.0±0.6	9.6±0.9	4.5±0.9	2.2±0.7	1.4±0.3
	11	13.2±1.0	10.0±0.5	7.2±0.3	3.9±0.6	1.7±0.6	1.1±0.2
	14	15.0±2.0	8.6±0.3	9.8±0.8	2.7±0.3	0.8±0.2	0.9±0.1

No significant differences (two-way ANOVA, $p>0.05$) were found for all the end points by the comparison between treated mussels and the corresponding control (time to time)

increase ($p>0.05$) in both lipid peroxidation and protein carbonyl levels was found in treated bivalves all over the 14-day exposure (Table 1). Similarly, MDMA did not induce genetic damage, as showed by the lack of significant increase ($p>0.05$) in DNA fragmentation, as well as in apoptotic and micronucleated cell (MN) frequency, compared to controls (Table 1). Our results disagreed most of the information available in the scientific literature regarding MDMA toxicity, which pointed out its capability to overproduce ROS (Nakagawa et al. 2009), leading to oxidative damage to lipids (Moon et al. 2008; Alves et al. 2009) and to DNA in different murine cells (Barenys et al. 2009; Nakagawa et al. 2009; Alvarenga et al. 2010). The lack of notable adverse effects induced by MDMA to *D. polymorpha* could be explained by the deep differences between vertebrate and invertebrate metabolism and/or in the exposure concentrations. All the aforementioned works investigating MDMA toxicity to vertebrates treated organisms/cells with the highest MDMA concentrations (in the mM range), while we exposed bivalves to MDMA concentrations (in the nM range) similar to those measured in aquatic environments, to give a high ecological relevance to our research and to provide useful information for environmental risk assessment. However, despite our findings, we cannot exhaustively state that MDMA is not a hazardous substance to the zebra mussel and aquatic non-target organisms, since we investigated just a limited part of its potential toxic effects. For instance, MDMA shows serotonergic neurotoxicity by reversing the serotonin (5-HT) reuptake, which leads to a massive efflux of 5-HT into the synaptic cleft (Parrott 2013). The MDMA neurotoxicity may be induced as consequence of its metabolism (Mueller et al. 2009) and/or by ROS production, since oxidative stress appears to be one of the main factors involved in the serotonergic and dopaminergic terminal injury induced by this drug (Riezzo et al. 2013). 5-HT regulation has been associated to the modulation of important functions in hormonal and neuronal mechanisms in both vertebrates and invertebrates (Stanley et al. 2007; Styrishave et al. 2011). Exposure to MDMA could affect 5-HT regulation, leading to toxic effects and/or physiological, behavioral (mobility, feeding habits, and aggression), and reproductive fitness alterations to bivalves, as found for other neurotoxic drugs such as the fluoxetine, a selective serotonin reuptake inhibitor (Fong 1998; Fong et al. 2003; Gonzalez-Rey and Bebianno 2013). Since MDMA favors 5-HT release and inhibits its reuptake (Garcia Cabeza et al. 2005), it could alter the 5-HT regulation of *D. polymorpha*, inducing one of the different adverse effects mentioned above. Unfortunately, to date, no study was focused on the investigation of neurotoxic and serotonergic effects of MDMA to the zebra mussel and, in general, to aquatic non-target organism. This issue should be pivotal for further studies mainly considering that one of the neurotoxic mechanisms of MDMA involves oxidative stress. Since our data showed slight imbalances of bivalve oxidative status, MDMA-induced alterations in serotonin levels and in physiological/reproductive parameters cannot be excluded. However, since no variations in the zebra mussel filtration rate were found (Table 1), current environmentally relevant MDMA concentrations seem to be not able

to alter serotonin levels and to induce physiological changes to this bivalve species. In conclusion, our findings showed that low MDMA concentrations induced just slight sub-lethal effects to the zebra mussel but do not cause deleterious effects to cellular macromolecules and physiology of mussels. Despite these findings, the potential hazard of MDMA cannot be neglected considering that its growing consumption worldwide could lead to continuous input to freshwaters, with the consequent increase of environmental concentrations. This issue is pivotal in risk assessment since aquatic organisms are exposed to low MDMA concentrations for their whole life span, so longer exposures to higher MDMA concentrations could result in higher effects compared to those pointed out by the present study. For these reasons, further investigation should be necessary to enhance the knowledge on MDMA sub-lethal effects, metabolism, mechanism of action in aquatic organisms, and possible consequences on population dynamics, to clarify the MDMA ecological hazard.

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Chapter 7

PAPER 6

Realistic mixture of illicit drugs impaired the oxidative status of the zebra mussel (*Dreissena polymorpha*)

(Chemosphere. Under review)

Realistic mixture of illicit drugs impaired the oxidative status of the zebra mussel (*Dreissena polymorpha*)

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ABSTRACT

Illicit drugs are considered emerging aquatic pollutants since they are commonly found in freshwater ecosystems in the high ng/L to low µg/L range concentrations. Although the environmental occurrence of the most common psychoactive compounds is well-known, just recently some investigations have showed their potential toxicity towards non-target aquatic organisms. However, to date these studies completely neglected that in real environment organisms are exposed to complex mixture, which could lead to dissimilar adverse effects. The present study was aimed at investigating the oxidative alterations induced by a 14-day exposure to an environmentally relevant mixture of the most common illicit drugs found in aquatic environment, namely cocaine (50 ng/L), benzoylecgonine (300 ng/L), amphetamine (300 ng/L), morphine (100 ng/L) and 3,4-methylenedioxymethamphetamine (50 ng/L), on the freshwater bivalve *Dreissena polymorpha*. The imbalance of the bivalve oxidative status was investigated by evaluating the variation in the activity of antioxidant enzymes (SOD, CAT and GPx) and GST, while oxidative damage was assessed by measuring levels of lipid peroxidation and protein carbonylation. Significant time-dependent increases of all the antioxidant activities were induced by the mixture, while no alteration of GST was noticed. Moreover, the illicit drug mixture significantly increased the levels of carbonylated proteins and caused slight variation in lipid peroxidation. Our results showed that a mixture of illicit drugs at realistic environmental concentrations can impair the oxidative status of the zebra mussel, posing a serious hazard to the health status of this bivalve species and, likely, to the aquatic communities.

Keywords: illicit drugs; mixture; oxidative stress; zebra mussel

1 INTRODUCTION

Since the ’90, the scientific community has focused its attention on the environmental issue regarding the occurrence and the potential hazard of Pharmaceutical and Personal Care Products (PPCPs) in aquatic ecosystems. PPCPs are a diverse group of compounds that are routinely and extensively used in both human and veterinary medicine (Fent *et al*., 2006). After their consumption, these molecules are excreted unaltered or as metabolites via urine and faeces entering the sewage. Since the classical wastewater treatment plants (WWTPs) are not designed to remove these chemicals, many of them escape degradation during wastewater treatment and enter surface water. Thus, hundreds of distinct compounds belonging to dissimilar classes are currently detected in concentrations ranging between the high ng/L to low μg/L worldwide (Santos *et al*., 2010). Since PPCPs are biologically-active molecules exploiting their action at low concentrations, recent studies pointed out their potential hazard towards diverse non-target aquatic organisms at different levels of the biological organization, including biochemical, biomolecular and cellular alterations (Triebskorn *et al*., 2004; Binelli *et al*., 2009 a, b; Parolini *et al*., 2010; 2011; Quinn *et al*., 2011), behavioral changes (Brodin *et al*., 2013; Jonsson *et al*., 2014), as well as modifications in the structure of aquatic communities (Munoz *et al*., 2009; Drury *et al*., 2013) and in the ecosystem functions, such as primary production and microbial respiration (Rosi-Marshall *et al*., 2013) and invertebrate secondary production (Hoppe *et al*., 2012). Despite of these findings, ecotoxicological investigations have been focused on a limited number of drugs belonging to few therapeutic classes, mainly antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs) and blood lipid lowering (Santos *et al*., 2010), excluding from considerations many other potentially hazardous molecules. Among these, to date PPCPs studies inexplicably excluded from consideration the so-called illicit drugs and/or illegally used prescription drugs. This exclusion was likely due to the ambiguity in the definition of illicit drug: although the most of them are illegal, many others are licit medical pharmaceuticals having valuable therapeutic use, such as morphine and oxycodone, so they have to be included into the PPCP group. Moreover, nonetheless they belong to structurally diverse group of chemicals, they share several features with PPCPs, including the extremely high potential for biological activity and the pathway to enter the aquatic environment (Castiglioni *et al*., 2011). For these reasons, recent investigations evaluated the occurrence of diverse illicit drugs, including cocaine, amphetamine type stimulants (ATS), cannabinoids and opiates, as well as of their metabolites, in both WWTP effluents and surface waters worldwide in ng/L concentrations (Pal *et*

al., 2013). Although current environmental levels are relatively low, their continuous manufacture and use/abuse realistically corresponds to their continual input to environment, imparting them with 'pseudo-persistence' that lead to a long-term exposure to aquatic communities. Considering that illicit drugs are biologically-active and neurologically-addictive substances whose aquatic occurrence in environment is well known worldwide, the risks for the aquatic ecosystems cannot be excluded, but it is largely understudied. The first ecotoxicological study concerning this issue showed that the exposure to high cocaine (COC) concentrations (10 µg/L) induced remarkable cyto-genotoxic effects to freshwater bivalve *Dreissena polymorpha* (Binelli *et al*., 2012). Further investigations highlighted that also low environmental concentrations of some psychotropic substances, namely the main COC metabolites, the benzoylecgonine (BE - Parolini *et al*., 2013a) and the ecgonine methyl ester (EME - Parolini and Binelli, 2013), as well as the Δ-9-tetrahydrocannabinol (Δ-9-THC - Parolini and Binelli, 2014), could represent a threat for the health status of this aquatic species, since 14-day exposure showed their capability to significantly imbalanced the oxidative status of the zebra mussel, causing notable oxidative and genetic damage. Moreover, Binelli and co-workers (2013) showed that two realistic BE concentration (0.5 and 1 µg/L) caused significant alteration in the expression of some proteins from zebra mussel gills, whose functions are crucial for the overall metabolism, including calcium homeostasis and oxidative stress responses. In addition, a parallel redox proteomic study (Pedriali *et al*., 2013) supported biomarker data on BE and confirmed that its mechanism of action in the zebra mussel involves the oxidative stress, since oxidative modifications in diverse classes of gill proteins were noted after 14-day exposure to the same concentrations mentioned above. In contrast, low concentrations of 3,4-methylenedioxymethamphetamine (MDMA - Parolini *et al*., 2014) and morphine (Magni *et al*., 2014) slightly altered the enzyme activity of *D. polymorpha* specimens and caused negligible damage to cellular macromolecules with respect to that induced by COC metabolites and Δ-9-THC at the same doses. Overall, all these findings pointed out the potential toxicity of single illicit drug residues towards a freshwater non-target organisms, showing the capability of these molecules to modulate the antioxidant activity and to induce oxidative damage to cellular macromolecules also at environmentally relevant concentrations. However, drugs of abuse occur in the environment as complex mixtures of diverse active substances, metabolites and residues of many other therapeutics (Santos *et al*., 2010), which may lead to unforeseeable pharmacological interactions and to more dangerous toxic effects to aquatic organisms compared to the single compounds. Mixture of active substances, in fact, may induce different effects than single molecules in different aquatic organisms (Cleuvers, 2003; DeLorenzo and Fleming, 2008; Quinn *et al*., 2009), but the knowledge regarding their toxicity to non-target organisms are currently still very

scant. For instance, some studies showed that mixture of pharmaceuticals at environmentally relevant concentrations exhibited additive effects (Pomati *et al*., 2007; DeLorenzo and Fleming, 2008), pointing out that the simultaneous presence of several pharmaceuticals in the aquatic environment could result in a greater toxicity to non-target organisms than the predicted one for single compounds (Santos *et al*., 2010). Since illicit drugs share several features with pharmaceuticals, it is plausible that their toxicity in mixture can result in higher damage compared to that of the single compounds. However, to date no one study investigated the possible adverse effects, including oxidative ones, caused by the exposure to a mixture of illicit drugs towards a non-target aquatic species. This research was aimed at investigating the oxidative alteration induced by a 14 day exposure to a realistic mixture of the five main illicit drug residues found in surface water worldwide, namely cocaine, benzoylecgonine, amphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and morphine, to the freshwater mussel *Dreissena polymorpha*. The activity of antioxidant enzymes, namely catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), as well as the glutathione S-transferase (GST), was measured to assess imbalances in the oxidative status of bivalves, while the lipid peroxidation (LPO) and protein carbonyl content (PCC) investigated the oxidative damage induced by the mixture of illicit drugs.

2 MATERIALS AND METHODS

2.1 *Experimental plan*

Zebra mussel specimens were sampled in October 2013 in the Lake Lugano since it is considered a reference site due to its negligible illicit drug pollution (Zuccato *et al*. 2008). Mussels (n=60) having the same shell length (16±3 mm) were placed within 5 L beakers filled with 4 L of tap and deionized water (50:50 v/v), previously de-chlorinated by aeration, and maintained under the controlled conditions described by Parolini *et al*. (2014). Three beakers *per* treatment were prepared, including controls, to avoid the tank effect. The bivalves were fed daily with algae *Spirulina spp*., and the water was renewed every two days for 2 weeks to purify them of pollutants previously accumulated in soft tissues. Only specimens able to re-form their byssi were used in the experiments. Biomarker baseline levels were checked weekly and mussels were exposed to illicit drug mixture only when they were comparable with those obtained in previous laboratory studies (Parolini *et al*., 2010; 2011a, b; 2013a; 2014). Exposures were performed under semi-static conditions for 14 days. Control and exposure beakers were processed at the same time and the water volume was daily renewed, to guarantee constant concentration of drugs in mixture over a 24-h period. Mussels were exposed to a mixture of the following illicit drugs: cocaine (50 ng/L),

benzoylecgonine (300 ng/L), amphetamine (300 ng/L), MDMA (50 ng/L) and morphine (100 ng/L). The choice of these drugs in composing the mixture was based on the following two reasons: they are the main illicit drug residues commonly found in surface water worldwide and their sub-lethal toxicity to the zebra mussel was singularly tested in some previous studies (Binelli *et al*., 2012; Parolini *et al*., 2013a; 2014; Parolini and Binelli, 2014; Magni *et al*., 2014). It is important bearing in mind that the concentration of each single drug into the mixture was similar to its current maximum levels found in surface water worldwide (see the review by Pal *et al*., 2013). A stock solution (10 mg/L) of each drug was prepared dissolving the analytical standard (1 g/L in methanol; Alltech-Applied Science, State College, PA, USA) in bidistilled water. Exact volumes of each stock solution were added daily to the exposure aquaria until reaching the selected concentrations. Specimens were fed daily 2 h before the change of water and drug administration. Every 3 days, 8 mussels were randomly collected from each tank (24 specimens *per* treatment) and their soft tissues were immediately frozen in liquid nitrogen and stored at -80 °C until the biomarkers' levels were measured.

2.2 *Chemical analysis of illicit drugs*

To guarantee the reliability of the experimental plan, the concentration of all the drugs composing the mixture was measured in both the stock solutions and the exposure beakers. At each time of biomarker analysis, water was sampled 1 h after the spiking of illicit drug mixture from the three control and exposure beakers and integrated in an unique sample (100 mL) *per* treatment. The stability of drugs' concentrations during exposure was checked using solid phase extraction (SPE) and high performance liquid chromatography tandem mass spectrometry analysis (HPLC-MS/MS). The analysis was performed adapting previously validated methods (Castiglioni *et al*., 2006; 2008). Aqueous samples collected during the experiment were stored at -20 °C until analysis, and they were then solid-phase extracted by a mixed reversed-phase cation exchange cartridge (Oasis-MCX). Solid phase extraction (SPE) was performed using an automated system, GX-274 ASPEC (Gilson, Middleton, WI, USA). Before extraction, 25 mL aliquots for the mixture of drugs spiked at 50-300 ng/L and for control samples were prepared for extraction. Each aliquot was spiked with 2 ng of labeled deuterated compounds used as internal standards and the pH was adjusted to 2.0 with 37% HCl. The cartridges were conditioned before use by washing with 5 mL methanol, 3 mL MilliQ water and 3 mL water acidified to pH 2. Samples were then passed through the cartridges at a flow rate of 5 mL/min. Cartridges were vacuum-dried for 10 min and eluted with 2 mL of methanol and 2 mL of a 2% ammonia solution in methanol. The eluates were pooled and dried under a nitrogen stream. Dried samples were re-dissolved in 100 µL of Milli-Q water, centrifuged for 2 min at 2500

rpm (Megafuge 1.0, Heraeus Instruments) and transferred into glass vials for instrumental analysis. The HPLC system consisted of a 1200 Series Binary Pump SL and Autosampler (Agilent Technologies, Santa Clara, CA). The MS system was an API 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source (Applied Biosystems - Sciex, Thornhill, Ontario, Canada). The chromatographic separation was performed by gradient elution using acetic acid 0.1% in Milli-Q water as solvent A and acetonitrile as solvent B at a flow rate of 70 µL/min. The analysis started with 98% of eluent A, followed by a 3-min isocratic gradient, a successive 17 minutes linear gradient to 50% of eluent B, a 1-min linear gradient to 100% of eluent B, a 3-min isocratic washing step with eluent B and a 1-min linear gradient to 98% of eluent A, which was finally maintained for 14 minutes to equilibrate the column. The injection volume was 2 µL and the column was kept at room temperature. The MS analysis was done in the positive ion mode with a spray voltage of 5.5 kV and a source temperature of 350 °C. Mass spectrometer analyses were done in the multiple reaction monitoring (MRM) mode, choosing the two most abundant fragmentation products of the protonated pseudo-molecular ions of each substance and each deuterated analog. Quantification has been performed using the isotopic dilution method and calibration curves were made freshly before each analytical run.

2.3 *Enzymatic and oxidative damage biomarkers*

The activity of SOD, CAT, GPx, and GST was measured in triplicate (n=3) in the cytosolic fraction 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 v/v – 10 mM phenanthroline-Phe and 10 mg/mL trypsin inhibitor-Try) and dithiothreitol (DTT, 100 mM) were added to the buffer. The homogenate was centrifuged at 15.000 g for 1 hour at 4 °C. The sample was immediately processed for the determination of protein content according to the Bradford method (1976) using bovine serum albumin as a standard and enzymatic activities, while enzymatic activities were determined spectrophotometrically as described by Orbea *et al*. (2002). CAT activity was determined measuring the consumption of H_2O_2 at 240 nm using 50 mM of H_2O_2 as substrate in 67 mM potassium phosphate buffer (pH 7). SOD activity, expressed in SOD units (1 SOD unit=50% inhibition of the xanthine oxidase reaction), was assessed measuring the inhibition of cytochrome c (10 µM) reduction at 550 nm due to the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 µM) reaction. The GPx activity was measured by monitoring the consumption of NADPH at 340 nm using 0.2 mM H_2O_2 as substrate in 50 mM potassium phosphate buffer (pH 7) containing glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL) and NADPH (120 µM). 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 DTT (1 mM) and PMSF (1 mM) using a Potter homogenizer. LPO was assessed according to Ohkawa *et al*. (1979) as the measure of the thiobarbituric acid-reactive substances (TBARS) and it was 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) and PCC levels were expressed as nmol/(mg protein).

2.4 *Statistical analysis*

Data normality and homoscedasticity were verified using the Shapiro-Wilk and Levene's tests, respectively. To assess the differences between control and treated group, a two-way analysis of variance (ANOVA) was performed using time and mixture treatment as variable, while biomarker end-points served as cases. The ANOVA was followed by a Fisher LSD *post-hoc* test to investigate significant differences (*p<0.05; **p<0.01) between treated samples and related control (time to time). The Pearson's correlation test was carried out on all the measured end-points to investigate the correlations between the different biological responses. Statistical analyses were carried out using the STATISTICA 7.0 software package.

3 RESULTS

3.1 *Concentration of illicit drugs in water*

To guarantee the reliability of the exposure, the concentration of each illicit drug composing the mixture was measured in triplicate. No residues of all the illicit drugs were found in the control tanks over the exposure period. The mean concentration of cocaine (42.67±6.43 ng/L), benzoylecgonine (312.90±4.77 ng/L), amphetamine (342.40±54.33 ng/L), MDMA (25.20±3.02 ng/L) and morphine (91.07±12.60 ng/L) accounted for more that the 83% of the nominal ones, with the exception of MDMA, whose concentration in water was halved with respect to the nominal one.

3.2 *Zebra mussel baseline levels*

Low mortality (<5%) was found both in control and exposure tanks during the 14-day experiment. The average hemocyte viability of bivalves from control tanks was 91.6±2.3% and it was similar to that measured at the beginning of the experiment in specimens sampled from the exposure tanks

(mean 87.6±5.5%). The mean baseline levels of SOD (9.4±3.8 U/mg protein), CAT (44.6±13.8 µM/min/mg protein), GPx (21.3±0.6 µM/min/mg protein) and GST (161.6±37.1 mM/min/mg protein), as well as of LPO (12.6±4.1 nM/g wet weight) and PCC (5.91±2.09 nM/mg protein), were in the same range of those obtained in previous laboratory studies on the zebra mussel (Parolini *et al*. 2013a; Parolini and Binelli, 2013; 2014).

3.3 *Oxidative stress biomarker results*

Levels of antioxidant (SOD, CAT and GPx) and GST activity are reported in Figure 1. The exposure to the mixture of illicit drugs induced a significant ($p<0.01$) increase in SOD activity already after 7 days of exposure, reaching values 2-fold higher than the corresponding control at the end of the experiment (Figure 1a). A significant time-dependent ($F=14.54$; $p<0.01$) increase in CAT activity was noticed, showing levels significantly ($p<0.01$) 3-fold higher than baseline ones at the end of exposure (Figure 1b). GPx followed an increasing trend, showing a significant time-dependent ($F=23.95$; $p<0.01$) activation already after 7 days of exposure, reaching its maximum expression at the end of the experiment with values 3-fold higher than the baseline ones (Figure 1c). Differently from antioxidant enzymes, GST levels did not follow a clear trend during the 14-day exposure and no significant ($p>0.05$) alterations were noticed with respect to corresponding controls (Figure 1 d). In Figure 2 are reported the results of oxidative damage biomarkers. Lipid peroxidation levels did not shown significant increase with respect to controls during the 14 days of exposure despite of the significant time-dependent relationship ($F = 4.36$; $p<0.01$) and the 30% increase of LPO values obtained at the end of the exposure compared to baseline ones (Figure 2a). Similar time-dependent increase ($F =22.03$; $p<0.01$) of protein carbonylation was found all over the exposure to the illicit drug mixture, with significant increase of PCC levels after 11 ($p<0.01$) and 14 days ($p<0.05$) of treatment, accounting for the 57% and 27% of the correspondent control, respectively (Figure 2b).

4 DISCUSSION

Previous studies highlighted the serious threat that illicit drugs could pose to non-target freshwater organisms, as shown by the significant alterations of several sub-lethal endpoints, including biochemical variations and macromolecular damage, induced by the exposure to diverse psychotropic substances to the zebra mussel (Binelli *et al*., 2012; Parolini *et al*., 2013a; Parolini and Binelli 2013, 2014). However, for an exhaustive risk assessment of these emerging aquatic pollutants towards non-target species, it is important bearing in mind that in natural ecosystems, organisms are exposed to complex mixture of illicit drugs, whose toxicity cannot be accurately

estimated by analyzing the effects obtained for single compounds (Flaherty and Dodson, 2005). It has been demonstrated, in fact, that the joint toxicity of such chemical mixtures, including pharmaceutical mixtures, is typically higher compared to that of each individual compound (Kortenkamp *et al*., 2009). For example, the exposure to a mixtures of quinolone antibiotics and to a set of 14 pharmaceuticals having diverse mode of action showed higher toxicity than the single compound to *Vibrio fischeri* (Backhaus *et al*., 2000 a, b), while a mixture of cimetidine, fenofibrate, furosemide and phenazone was more toxic than individual drugs to *Daphnia magna* (Fent *et al*., 2006). In addition, although the compounds occur within a mixture at concentrations that should not cause toxic effects, a joint effect cannot be excluded. For instance, a mixture of fluoxetine and clofibric acid killed more than 50% of a *D. magna* population after a 6-day exposure, although the pharmaceuticals occurred at concentrations that did not provoke significant adverse effects individually (Flaherty and Dodson, 2005). Similarly, a mixture of non-steroidal anti-inflammatory drugs (NSAIDs), namely diclofenac, ibuprofen and paracetamol, induced high genetic damage to zebra mussel hemocytes, nonetheless each single drug was present at a concentration that did not affect DNA integrity individually (Parolini and Binelli, 2012). All these findings suggest that the interactions among biologically active illicit drugs occurring in mixture at low realistic concentrations could result in unexpected toxicological behaviors, leading to higher adverse effects compared to those induced by the single molecules. Our previous investigations showed that environmental concentrations of some of the most common illicit drugs found in surface waters worldwide, namely ecgonine methyl ester (150 ng/L - Parolini and Binelli, 2014), Δ-9-THC (50 ng/L - Parolini and Binelli, 2014), MDMA (50 ng/L - Parolini *et al*., 2014) and morphine (50 ng/L - Magni *et al*., 2014), did not alter neither the antioxidant defense nor the levels of lipid peroxidation and the protein carbonyl content in treated zebra mussel specimens over a 14-day exposure. Only the lowest tested concentration (500 ng/L) of BE, the main COC metabolite, significantly activated defense enzymes and caused a slight and non-significant increase in oxidative damage to lipids and proteins, as well as significant DNA fragmentation (Parolini *et al*., 2013a). In spite of these results, the 14-day exposure to a realistic 'cocktail' of illicit drugs, whose concentrations within the mixture were similar to those found in surface water worldwide and tested in our previous studies mentioned above, significantly imbalanced the activity of defense enzymes, excluding GST, and caused a remarkable oxidative damage to *D. polymorpha* specimens. The increased activity of all the investigated antioxidants indirectly pointed out that the drug mixture promoted the overproduction of reactive oxygen species (ROS). The significant ($p<0.01$) time-dependent increase of SOD activity (Figure 1a) noticed over the 14-day experiment suggested its activation in counterbalancing the production of the superoxide anion. SOD, in fact, is the first enzyme involved

in the reactions against oxyradicals and it catalyzes the dismutation of two superoxide anions ($\bullet O_2^-$ and •OH) into O_2 and H_2O_2. Hence, the SOD activation suggested the production of H_2O_2, whose toxicity is counterbalanced by CAT and GPx. The significant ($p<0.01$) increase of CAT and GPx (Figure 1b and c) indirectly confirmed the production of hydrogen peroxide by SOD and pointed out their complementary role in its conversion into H_2O and O_2 (Box *et al*., 2007) to complete the defense chain against ROS. The significant correlation ($p<0.05$) among all the antioxidant activities (Table 1) confirmed the activation of the whole *D. polymorpha* enzymatic chain against mixture-induced ROS, whose compensatory capacity should have to efficiently counteract their hazardousness and to prevent the raise of an oxidative stress situation. However, during metabolic processes a small proportion of free radicals may escape from the protective shield of antioxidants, causing oxidative damage to cellular macromolecules, such as lipids of membranes, proteins and DNA. We found a slight time-dependent increase in lipid peroxidation levels (Figure 2a), while the protein carbonyl content was significantly increased already after 11 days of exposure (Figure 2b). The increase in lipid peroxidation and protein carbonylation represent a serious threat to the organism health status since these processes affect the integrity of cell membranes (Yajima *et al*., 2009) and lead to the loss of efficiency in protein structure and function (Dalle-Donne *et al*., 2006), respectively. The significant ($p<0.05$) correlations between PCC and SOD, CAT and GST (Table 1) highlights the strict relationship between antioxidant enzymes and oxidative damage biomarker in the zebra mussel. Our results were similar to those obtained after a 14-day exposure to a similar concentration of BE (500 ng/L) to the zebra mussel, which induced significant activation of antioxidants and GST, as well as slight increases in levels of lipid peroxidation and protein carbonylation (Parolini *et al*., 2013a). Since the concentration of BE within the tested mixture (300 ng/L) was similar to that tested in our previous work, these findings should suggest that BE could be the main responsible of the oxidative alterations induced by the mixture, while the contribution of other drugs could be considered negligible. This hypothesis should be also supported by a redox proteomic investigation confirming that 500 ng/L BE induced protein carbonylation and oxidative modification to 34 proteins in gills dissected from treated zebra mussel specimens (Pedriali *et al*., 2013). Among the varied proteins, some of them have been identified to be involved in cytoskeletal functions, energetic metabolism and stress response, suggesting the involvement of oxidative stress in the BE mechanism of action. On these bases, in order to highlight the role of BE to the mixture toxicity, we compared the mixture-induced adverse effects with those caused by BE exposure (Parolini *et al*., 2013a), expressing them as the percentage alteration level (AL) compared to baseline levels at the beginning of the exposure. Although the treated bivalves used in the experiments have been sampled in diverse periods of time and belong to different populations from

the same lake, they were maintained and exposed under the same laboratory conditions. Moreover, the baseline levels of investigated biomarkers measured during both the independent experiments were extremely similar (no significant differences were found between baseline levels of the two experiments, with the exception of GST activity), facilitating the comparison between induced adverse effects. At the end of the exposure (t=14 days) to the illicit drug mixture, the activity of SOD, CAT, GPx and GST was 59%, 181%, 308% and 126% higher than the corresponding baseline levels, respectively. In contrast, after the BE exposure the increase of the same enzymes was 138%, 81%, 175% and 32% higher than the t=0 day levels, respectively. Hence, with the exception of SOD, whose mixture-induced increase was lower than the BE one, the activity of the other enzymes should suggest a higher capability of the mixture to produce ROS compared to BE, suggesting and additive effect of all the psychotropic substances present in the mixture. Similarly, the increase of lipid peroxidation levels compared to baseline ones induced by the mixture was higher with respect to the BE one (40% for mixture and 16% for BE), while the increase of protein carbonyl content was quite similar between the two exposures (36% for mixture and 48% for BE). Overall, although the comparison of AL value obtained for the tested biomarker should suggest that the illicit drug mixture caused higher effects than BE, the opposite responses of SOD and PCC prevent from attesting it with certainly. For this reason, we integrated the whole biomarker dataset obtained for the mixture and for the similar concentration of BE into a biomarker response index (BRI), according to the procedures described by Parolini *et al*. (2013b). The integration of biomarker data into a synthetic index, in fact, is a useful tool to minimize the variation of responses (Hagger *et al*., 2006) and to easily comprehend complex ecotoxicological data (Sforzini *et al*., 2011). Similarly to the procedure described above, the AL value of each oxidative stress biomarker for each exposure time (from t=4 to t=14 days) was calculated. To calculate the BRI, similarly to Parolini *et al*. (2013b) we gave a specific score to each response depending on its AL, and each biomarker was weighted according to its level of biological organization according to Hagger *et al*. (2010). The BRI approach confirmed that the oxidative toxicity induced by the illicit drug mixture was higher compared to that induced by the exposure to a similar concentration of BE individually (Figure 3). These findings suggest that nonetheless BE at the tested environmental concentration is probably the most toxic among the drugs into the mixture, the contribution of the other molecules cannot be underestimated since it lead to additive effects and higher toxicity with respect to the single drugs.

5 CONCLUSIONS

Our data showed that the exposure to a realistic mixture of illicit drugs significantly altered the oxidative status of the zebra mussel, leading to oxidative damage to cellular macromolecules. Moreover, exposure to the mixture caused higher alterations compared to those provoked by similar concentration of each drug tested individually under the same experimental conditions. These results lay the foundations for further risk assessment studies on this emerging class of aquatic pollutants. In fact, since organisms are exposed to similar 'cocktail' for their whole life span, longer exposures to similar mixture could result in higher effects compared to those pointed out by the present study. In addition, considering the continuous human consumption of illicit drugs worldwide and the consequent input to freshwaters, the environmental concentrations of the single molecules occurring into the mixture could increase, likely leading to more hazardous adverse effects to aquatic species. For all these reasons, further investigation should have to be a priority in ecotoxicology to enlarge the inadequate knowledge regarding the toxicity of illicit drug mixtures towards non-target aquatic species, highlighting the complex interactions among the single molecules and their behavior in causing oxidative damage and, in general, sub-lethal adverse effects, which could have negative consequences from organism to ecosystem health status.

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7 FIGURES

	CAT	GPx	GST	SOD	LPO	PCC
CAT		**0.85**	**0.44**	**0.45**	0.10	**0.47**
GPx			**0.57**	**0.53**	0.09	**0.48**
GST				**0.54**	0.08	0.32
SOD					-0.03	**0.43**
LPO						0.01
PCC						

Table 1: Pearson's correlation matrix of oxidative stress biomarker responses. Significant correlations ($p<0.05$) are indicated in bold.

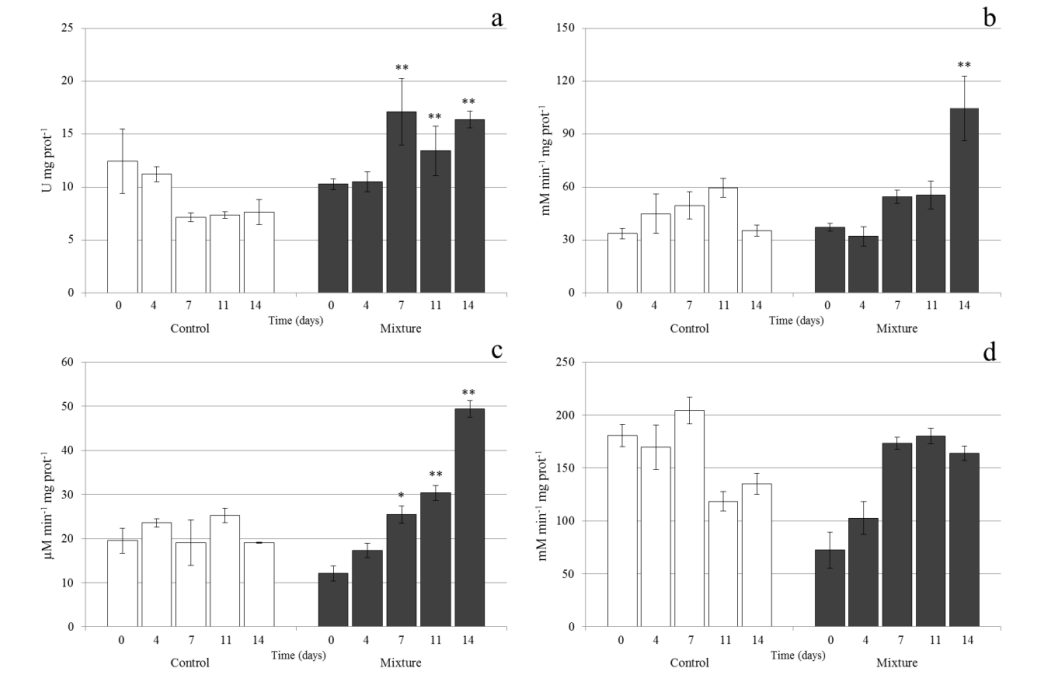

Figure 1: Effects of illicit drug mixture exposure on the activity (mean±SEM) of superoxide dismutase (SOD; a), catalase (CAT; b), glutathione peroxidase (GPx; c), and glutathione S-transferase (GST; d), measured in the whole soft tissue of zebra mussels (n=3; pool of 3 specimens). Significant differences (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).

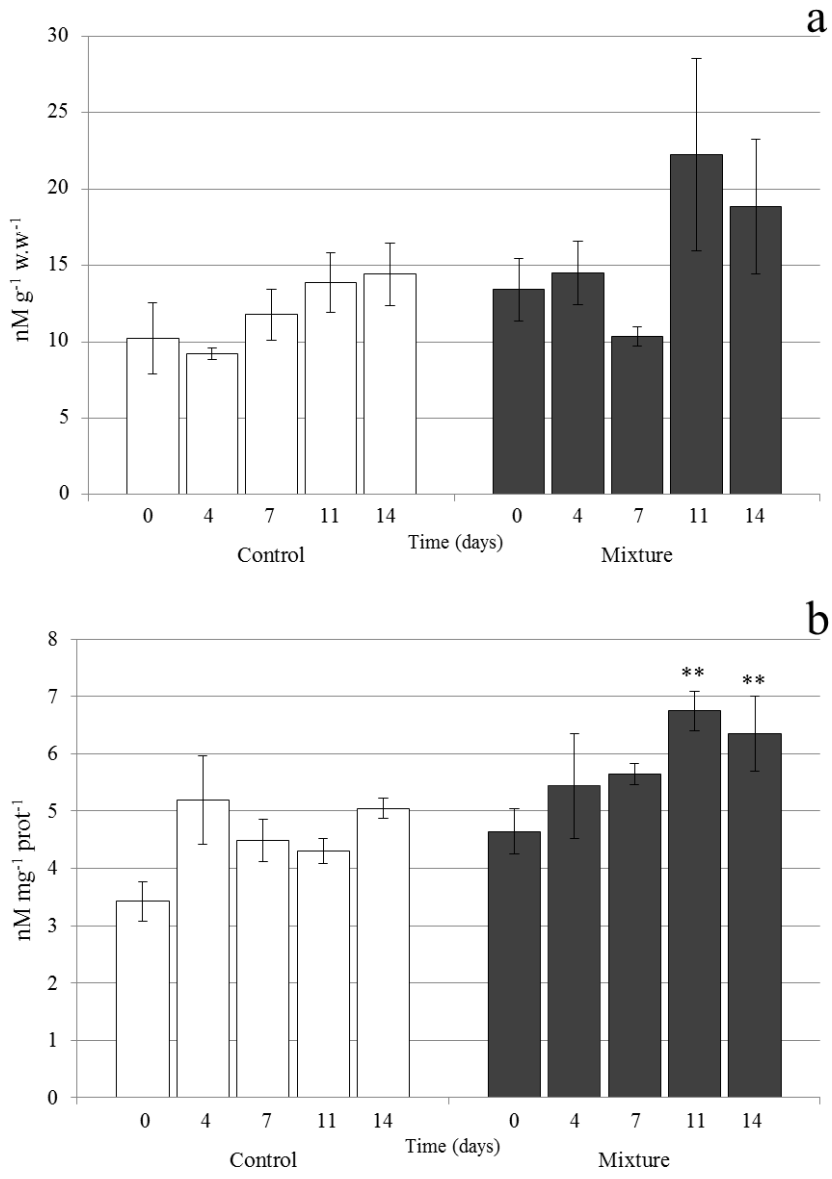

Figure 2: Measure (mean±SEM) of lipid peroxidation (a) and protein carbonyl content (b) in zebra mussels (n=3; pool of 3 specimens) specimens treated with the illicit drug mixture. Significant differences (two-way ANOVA, Fischer's LSD post-hoc test, **$p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

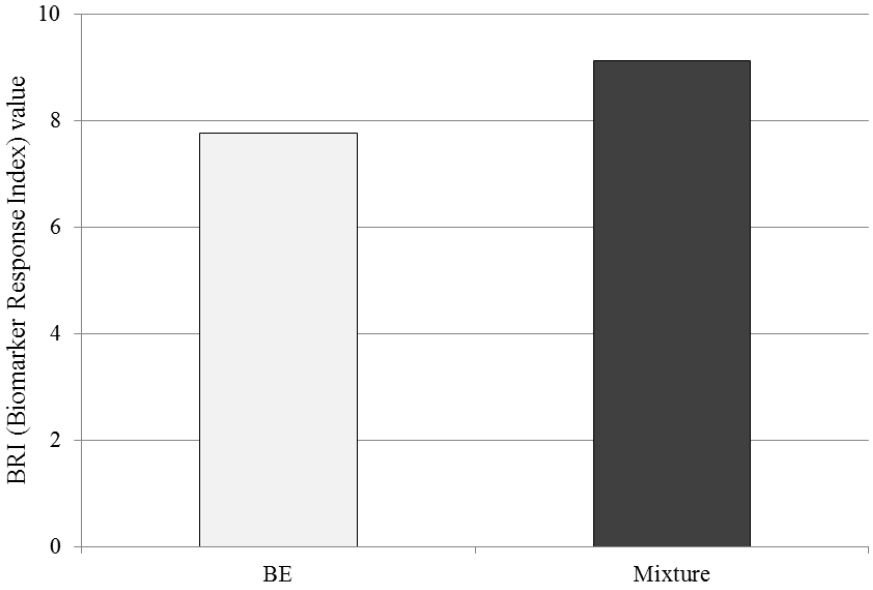

Figure 3: Comparison of oxidative toxicity induced by illicit drug mixture and 0.5 µg/L of benzoylecgonine by integrating biomarker responses into a biomarker response index (BRI) according to Parolini *et al.* (2013b).

Chapter 8

PAPER 7

Environmentally relevant concentrations of galaxolide (HHCB) and tonalide (AHTN) induced oxidative and genetic damage in *Dreissena polymorpha*

(Journal of Hazardous Materials. Accepted)

Environmentally relevant concentrations of galaxolide (HHCB) and tonalide (AHTN) induced oxidative and genetic damage in *Dreissena polymorpha*

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ABSTRACT

Synthetic musk compounds (SMCs) are extensively used as fragrances in several personal care products and have been recognized as emerging aquatic pollutants. Among SMCs, tonalide (AHTN) and galaxolide (HHCB) are extensively used and have been measured in aquatic ecosystems worldwide. However, their potential risk to organisms remains largely unknown. The aim of this study was to investigate whether 21-day exposures to AHTN and HHCB concentrations frequently measured in aquatic ecosystems can induce oxidative and genetic damage in *Dreissena polymorpha*. The lipid peroxidation (LPO) and protein carbonyl content (PCC) were measured as oxidative stress indexes, while the DNA precipitation assay and the micronucleus test (MN test) were applied to investigate genetic injuries. HHCB induced significant increases in LPO and PCC levels, while AHTN enhanced only protein carbonylation. Moreover, significant increases in DNA strand breaks were caused by exposure to the highest concentrations of AHTN and HHCB tested in the present study, but no fixed genetic damage was observed.

Keywords: galaxolide (HHCB), tonalide (AHTN), oxidative and genetic damage; *Dreissena polymorpha*

Capsule: The exposure to environmental concentrations of galaxolide (HHCB) and tonalide (AHTN) induced significant oxidative and genetic damage to the zebra mussel.

1 INTRODUCTION

Synthetic musk compounds (SMCs) are a group of lipophilic chemicals extensively used as fragrances in several types of personal care products (PCPs), including cosmetics, soaps and perfumes [1]. SMCs are generally divided into three categories of substances sharing similar properties but with different chemical structures, namely nitromusks (NMs), polycyclic musks (PCMs) and macrocyclic musks (MCMs). Due to their environmental persistence and potential toxicity to aquatic species, NMs have been phased out from the market [2], and since 1990, they have been replaced by MCMs and PCMs. The latter, predominantly 1,3,4,6,7,8-hexahydro-4,6,6',7,8,8'-hexamethylcyclopenta-(c)-2-benzopyran (HHCB-galaxolide) and 7-acetyl-1,1',3,4,4',6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN-tonalide), became the most important commercial SMCs [3]. AHTN and HHCB use accounts for 95% of the total market volume of PCMs [4], with estimated amounts in Europe of 358 ton/y and 1473 ton/y in 2000, respectively [5]. Even though the use of both compounds was reduced in EU during the 1990s [6], countries in southern Europe (particularly Italy) still have a high consumption, at 7.23 g/y *per capita* of HHCB and 1.81 g/y *per capita* of AHTN [6,7]. After their use, these substances enter the environment basically unchanged. In fact, because they are intended for external application, unlike pharmaceuticals, they do not undergo metabolic transformations [8]. Moreover, because they are only partially eliminated by wastewater treatment plants (WWTPs), they enter aquatic systems, leading to exposure to organisms [8]. The highest concentrations of AHTN and HHCB have been detected in wastewater treatment plants (WWTPs) worldwide (i.e., HHCB range in influent 4772-13399 ng/L and in effluent 2928-10525 ng/L, and AHTN range in influent 509-2337 ng/L and in effluent 328-1754 ng/L) [9], while low levels have been found in surface waters (HHCB range <0.05-12,500 ng/L and AHTN range <0.25-6800 ng/L) [10,11]. In addition, due to their lipophilic nature and moderate persistence, both substances have been found in sediments (HHCB range $<0.74E^{-4}$-17,993 ng/g dry weight (d.w.) and AHTN range $<3.5E^{-4}$-4,321 ng/g d.w.) [11] and have been shown to bioaccumulate to a great extent in wildlife, including in freshwater organisms, such as the zebra mussel (HHCB range 10.3-19.3 ng/g lipid weight (l.w.) and AHTN range 42.2-65.9 ng/g l.w.) [12] and different fish species (HHCB range <1-125 ng/g l.w. and AHTN range <1-71.5 ng/g l.w.) [12], as well as in marine mussels (HHCB range 110-3300 ng/g l.w. and AHTN range 37-860 ng/g l.w.) [13] and mammals (HHCB range 2.2-80 ng/g l.w. and AHTN range 0.6-36 ng/g l.w.)

[14-17]. Lastly, both AHTN and HHCB have been detected in maternal (HHCB range 0.17-1.4 ng/g l.w. and AHTN range <0.17-1.4 ng/g l.w.) and umbilical cord (HHCB range 0.67-2.7 ng/g l.w. and AHTN range <0.67-2.7 ng/g l.w.) serum [18], as well as in breast milk (HHCB range 0.05-456.7 ng/g l.w. and AHTN range 0.01-794.2 ng/g l.w.) [18,19]. Although current environmental concentrations of these contaminants are relatively low, their continual input to aquatic ecosystems imparts them with 'pseudo-persistence' [2], leading to the long-term exposure of aquatic communities. For this reason, an increasing amount of research has been aimed at evaluating their environmental risk and potential toxicity to organisms. Although some adverse effects and ecotoxicological concerns of musks have been indicated [20], the available data are still unsatisfactory and heterogeneous because they were focused on a limited number of species and end-points. These studies showed that acute toxicity to aquatic organisms is not of concern because effect concentrations are generally several orders of magnitude higher than environmental levels [21]. In contrast, diverse sub-lethal effects can occur at concentrations approaching those commonly found in the aquatic environments [22-25]. Despite these findings, limited information is available on oxidative and genetic damage induced by HHCB and AHTN exposure to organisms. To our knowledge, no investigation has been carried out to assess the capability of AHTN and HHCB to induce oxidative and genetic damage in aquatic species. Hence, this study was aimed at investigating oxidative and genetic damage in the freshwater bivalve *Dreissena polymorpha* induced by exposure to two HHCB and AHTN concentrations through a multi-biomarker approach. In order to assess the long-term sub-lethal consequences caused by these synthetic musks, unlike previous experimental studies that investigated the toxicity of emerging aquatic pollutants to the zebra mussel during 96-h and/or 14 day periods [26-29], we exposed bivalves for 21 days. Lipid peroxidation (LPO) and protein carbonyl content (PCC) assays were used as biomarkers of oxidative damage, while primary (DNA strand breaks) and fixed (frequency of micronuclei and nuclear buds) genetic damage was investigated by the DNA precipitation assay and the micronucleus test (MN test), respectively. Moreover, we measured HHCB and AHTN concentrations both in water and in mussel soft tissues in order to check the level of exposure during the experiment and to evaluate the bioaccumulation that occurred in zebra mussels.

2 MATERIALS AND METHODS

The certified standard of HHCB (IUPAC name: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexa-methylcyclopenta-[γ]-2-benzopyran - galaxolide) and AHTN (IUPAC name: 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphtalene - tonalide) were purchased from Dr. Ehrenstorfer GmBH.

All the reagents used for biomarker and chemical analyses were purchased from Sigma-Aldrich (Steinheim, Germany).

2.1 *Experimental design*

Zebra mussel specimens were collected by a scuba diver in Lake Lugano (Northern Italy-Switzerland). The mussels were quickly transferred to the laboratory and maintained in the same conditions described in a previous study [29]. Briefly, mussels (n=200) having similar shell lengths (15±4 mm) were placed within 12 L 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 *Spirulina* spp. algae, and the water was regularly renewed every two days for 2 weeks to purify the mollusks of any possible pollutants accumulated in their soft tissues. Only specimens that re-formed their byssi were used in the experiments. Mussel viability was checked daily by the Trypan blue exclusion method, whereas baseline levels of tested biomarkers were checked weekly. Mussels were exposed to musks only when levels of tested biomarkers were included in the range of baseline values obtained in our previous laboratory studies, namely 12.8-22.38 nM/g w.w for LPO and 3.7-9.5 nM/mg protein for PCC [29-31]. We considered DNA strand breaks baseline level values <6000 µg/g wet weight in accordance with previous studies on the zebra mussel [32,33]. Exposure assays were performed under semi-static conditions for 21 days. We prepared five aquaria, one for solvent control (acetone) and one for each concentration of AHTN and HHCB (two *per* musk). All the aquaria were processed at the same time, and the whole water volume was renewed 2 hours after feeding on a daily basis. Mussels were exposed separately to 100 ng/L (0.39 nM – Low HHCB) and 500 ng/L (1.93 nM – High HHCB) of HHCB and to 20 ng/L (0.08 nM – Low AHTN) and 80 ng/L (0.31 nM – High AHTN) of AHTN. It is important to bear in mind that both HHCB and AHTN exposure concentrations were similar to those commonly found in Italian (HHCB range 0.05-1141 ng/L, median concentration 463 ng/L; AHTN range <0.25-364 ng/L, median concentration 97 ng/L) [11] and European surface waters (HHCB range 5-610 ng/L and AHTN range 2.3-400 ng/L) [10,34,35], giving a notable ecological relevance to our research. Exact volumes of working solutions (HHCB: 10 mg/L; AHTN: 1 mg/L – both were diluted in acetone) were added daily to the exposure aquaria until reaching the selected concentrations. Concentration of acetone in the solvent control aquarium was equal to the highest concentration used in our experiments (0.01%). The complete daily renewal of both water and AHTN and HHCB concentrations in the exposure tanks should allow the maintenance of a constant musk

concentration over a 24-h period and should prevent losses of contaminants. However, the levels of both of the musks in the water tanks were checked according to the method developed by Villa et al. [11] to guarantee exposure reliability. Every 4 days, 15 specimens were randomly collected from each aquarium to evaluate oxidative and genetic damage induced by musks. Hemolymph was withdrawn by 8 bivalves, and micronuclei frequency was evaluated in hemocytes. After the withdrawal, the soft tissues of all 15 mussels were immediately frozen in liquid nitrogen and stored at -80 °C until the lipid peroxidation (LPO), protein carbonylation content (PCC) and DNA precipitation assay analyses were conducted. Moreover, we randomly sampled 10 specimens, whose soft tissues were quickly stored at –20 °C until the chemical analyses to evaluate the bioaccumulation of HHCB and AHTN were performed, from each aquarium every few days (t=0, 1, 2, 3, 4, 11, 15, 18 and 21 days).

2.2 *Evaluation of musk concentrations in water and mussel soft tissues*

In order to guarantee the reliability of the experimental design, the HHCB and AHTN concentrations in both the stock solution and the exposure aquaria were measured. Each time a biomarker analysis was performed, water (100 mL) was sampled 1 h after contamination from the control and from all the exposure aquaria and stored at -20 °C until analyses were conducted. Concentrations of musk in water were measured by the method developed and described in detail by Villa et al. [11]. Briefly, water samples were extracted using 500 mg OASIS HLB cartridges (Waters, Hertfordshire, UK). After the extraction, the cartridges were dried using N_2 flux and subsequently eluted (under gravity) with 10 mL of n-hexane and 5 mL of ethyl acetate. Samples were then further concentrated using N_2 flow to 1 mL, transferred to GC micro-vials and further reduced to 0.5 mL, and internal standards (PCB 30) were added, followed by analysis by GC-MS. Mussel samples were lyophilized, placed in a cellulose thimble lodged into a glass vial filled with n-hexane, sonicated and left overnight. After the extraction, samples were concentrated up to 2 mL under a gentle N_2 flow and subsequently split in two fractions (1 mL). The first aliquot was used to determine the lipid content gravimetrically, while the second one was purified using both SPE florisil cartridges (Supelclean ENVI-florisil, 6 mL tubes, Supelco) and an automated combination of gel permeation chromatography (GPC) and normal-phase HPLC. GPC purification was performed using DCM as an eluent and the Waters EnvirogelTM gpc cleanup (19x300 i.d.) as the column phase. The eluate was concentrated up to 50 µl and then analyzed using GC-MS (internal standard PCB 30). Recoveries of the surrogate spiked fragrances both in water than in suspended solid were satisfactory averaging >80%. Water and soft tissue samples were analyzed by GC-MS (Agilent

Technologies, Santa Clara, CA, USA) in the SIM (Single Ion Monitoring) mode. Identification and quantification ions were 243 and 258 for HHCB and AHTN, respectively. Samples (1 µL) were injected by an automatic injector (Agilent Technologies 7683 Series Injector), and analyte separation was achieved using a 30 m Rxi – 5Sil MS capillary column (0.25 mm i.d. - Restek, Bellefonte, PA, USA). Samples were run in a splitless mode using helium as a carrier gas (flow 1 mL/min). The injection port was kept at 250 °C under a pressure of 60 kPa. The oven program was the following: starting temperature 50 °C, hold for 4 min, increase rate of 30 °C/min to 170 °C and a rate of 1.0 °C/min to 160 °C, then a rate of 40 °C/min to 280 °C hold for 5 min. The transfer line and detector temperature were both maintained at 280° C.

2.3 *Oxidative stress and genotoxicity biomarkers*

Because the methods for tested biomarkers were reported in detail elsewhere [26,29,33], we only briefly describe them. 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). LPO levels were assayed by the determination of thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. [36], while the reaction with 2,4-dinitrophenylhydrazine (DNPH) was used to measure PCC [37]. DNA strand breaks were measured in triplicate (n=3) from a pool of three whole mussels (≈0.3 g fresh weight) using a fluorescence technique adapted from the alkaline precipitation assay [38]. The MN test was performed on mussel hemocytes according to the method of Pavlica et al. [39]; four hundred cells were counted *per* each slide (n=8), and micronuclei were identified according to the criteria proposed by Kirsch-Volders et al. [40].

2.4 *Statistical analysis*

Data normality and homoscedasticity were verified using the Shapiro–Wilk and Levene's tests, respectively. To identify differences between control and treated groups, a two-way analysis of variance (ANOVA) was performed using time and HHCB and AHTN concentrations as variables, while biomarker end-points served as cases. The ANOVA was followed by a Fisher LSD *post-hoc* test to evaluate significant differences (*$p<0.05$; **$p<0.01$) between treated samples and related controls (time to time). Statistical analyses were performed using the STATISTICA 7.0 software package.

3 RESULTS

3.1 *Concentrations of HHCB and AHTN in water and zebra mussel tissues*

Levels of HHCB and AHTN in control and exposure aquaria over the experiment period are reported in Table 1. Low levels of HHCB (mean = 12.64±3.67 ng/L) and AHTN (mean = 0.94±0.75 ng/L) were measured in the control aquarium, while concentrations in the exposure tanks were close to the nominal concentrations. The mean HHCB concentrations in the 100 ng/L and 500 ng/L exposure tanks were 84.48±38.3 ng/L and 361.9±130.3 ng/L, accounting on average for 97% and 67% of the selected nominal concentrations, respectively. AHTN levels in 20 ng/L and 80 ng/L exposure aquaria were 20.51±8.44 ng/L and 63.25±16.59 ng/L, accounting on average for 103% and 79% of the selected nominal concentrations, respectively. HHCB and AHTN levels accumulated in zebra mussel soft tissues over the exposure are reported in figure 1. After the exposure to low concentrations of both of the musks (100 ng/L of HHCB and 20 ng/L of AHTN), we measured the highest levels of HHCB (86 ng/g lipid weight (l.w.)) and AHTN (81 ng/g l.w.) at t=18 day. Despite the increasing trend of accumulated concentrations of both musks up until the 18th day of exposure, we noticed an unexpected decrease in AHTN and HHCB body burdens at the end of the experiment, most likely due to analytical problems that could be considered as outlier data. At the highest tested musk concentrations (500 ng/L for HHCB and 80 ng/L for AHTN), we observed a progressive increasing trend until the end of exposures both for HHCB and AHTN, reaching levels of 150 ng/g l.w. and 117 ng/g l.w., respectively.

3.2 *Biomarker baseline levels*

Negligible mussel mortality was found in the control aquarium (0.5%), while it was <2% for specimens in both AHTN and HHCB exposure aquaria during the whole exposure period. The 21-day average hemocyte viability of bivalves from the control tank was 93.81±1.24%, and the value was similar to that measured at the beginning of the experiment in specimens sampled from all of the exposure aquaria (mean 94.14±2.04%). Mean baseline levels of LPO (15.57±2.60 nM/g wet weight), PCC (5.18±0.57 nM/mg protein), DNA precipitation assay (43.47±8.11 μg/g wet weight) and MN frequency (0.58±0.45 ‰) were similar to those obtained in previous laboratory studies [29-31,33], confirming that this acetone concentration did not cause either oxidative or genetic damage in the zebra mussel, as shown in previous studies on the same model species [41,42].

3.3 *Oxidative damage results*

The low HHCB treatment (0.39 nM) did not induce any significant ($p>0.05$) variation in LPO levels. In contrast, the high HHCB tested concentration (1.93 nM) caused significant time-dependent ($F=4.57$; $p<0.01$) increases in lipid peroxidation even after 4 days of exposure, showing values 86% higher than the corresponding controls at the end of the treatment (Figure 2A). Contrarily, both of the AHTN concentrations did not significantly alter lipid peroxide levels in zebra mussels during the 21 days of exposure (Figure 2B), with the exception of a significant ($p<0.01$) 2-fold increase compared to the controls found after 7 days of exposure to the high AHTN treatment (0.31 nM). The lowest tested concentrations of both HHCB and AHTN did not induce any significant ($p>0.05$) variations in protein carbonylation in bivalve tissues. In contrast, significant increases in protein carbonyl contents were induced by both High HHCB and High AHTN treatments, showing values 50% and 40% higher than the baseline levels at t=21 days, respectively (Figure 3A and B).

3.4 *Genetic damage results*

HHCB induced significant increase in DNA fragmentation at both of the tested concentrations (Figure 4A), according to time ($F=4.47$; $p<0.01$) and concentration ($F=35.46$; $p<0.01$) dependencies. The Low HHCB treatment increased levels of DNA strand breaks even after 4 days of exposure, with values 60% higher than control values, while a clear time-dependent increase in fragmentation was found at the 1.93 nM exposure, showing values 2.5-fold higher with respect to the controls at the end of the experiment. Similarly, AHTN exposures altered the integrity of bivalve DNA (Figure 4B). Slight variations in DNA strand breaks were induced by the Low AHTN treatment, while significant time-dependent ($F=3.83$; $p<0.01$) effects were seen after 4 days of exposure to 0.31 nM concentration, showing values 3.6-fold higher than corresponding controls at t=21 days. The mean value of MN frequency induced by a 21-day exposure to Low AHTN and Low HHCB was 1.7±0.4‰ and 1.5±1.1‰, respectively, while it was 1.1±0.5‰ and 1.0±0.5‰ for High AHTN and High HHCB, respectively. No significant ($p>0.05$) increases in MN frequency compared to baseline levels (21-day mean value 1.0±0.5‰) were observed during the entire exposure period for either of the tested musk concentrations (data not shown).

4 DISCUSSION

A growing number of ecotoxicological studies have shown that exposure to concentrations of AHTN and HHCB, which can be found in aquatic systems, could produce diverse sub-lethal effects

on organisms. For instance, Breitholtz et al. [43] observed impairments in the larval development of the copepod *Nitocra spinipes* exposed to concentrations of 20 μg/L of HHCB, while similar effects were noticed in the copepod *Acartia tonsa* specimens treated with 26 μg/L AHTN and 59 μg/L HHCB. Luckenbach et al. [22] demonstrated that four PCMs, including AHTN and HHCB, affected the multixenobiotic defense systems (MXR transporters) of the marine mussel *Mytilus californianus* in the μM range (200–2000 μg/L). Moreover, recent studies have shown that exposure to PCMs could exert estrogenic, anti-estrogenic, anti-androgenic and anti-progestagenic activities in the zebrafish [23] and in the medaka *Oryzias latipes* [24], while reproductive disorders and population declines in the polychaete *Capitella* species I have been found [25]. Furthermore, recent investigations have shown that these synthetic musks can imbalance antioxidant responses in diverse aquatic organisms. Chen and coauthors [44] showed that both HHCB and AHTN exposures induced a bell-shaped response of SOD and CAT in the earthworm *E. fetida*, with an initial increase in activity due to the activation of enzyme synthesis followed by a decrease due to the enhanced catabolic rate and/or a direct inhibitory action of these chemicals on the enzymes. A similar trend of these antioxidants was also noted in the liver of *Carassius auratus* specimens during a 21-day exposure to 0.15-150 μg/L of HHCB [45], as well in HHCB-treated wheat [46]. These findings showed the capability of HHCB and AHTN to alter the activity of antioxidants in terrestrial and aquatic organisms, providing a warning signal of an oxidative stress situation that could disrupt the integrity and the structures of cellular macromolecules [47,48]. However, considering the peculiar bell-shaped trend and the huge variability of enzymatic responses, these biomarkers have to be considered as an early warning signal of a potentially harmful situation, as they do not provide information on oxidative damage, which could affect an organism's health status.

To investigate this issue, lipid peroxidation and protein carbonyl content are two independent measures commonly used to assess the involvement of oxidative stress in the mechanism of action of dissimilar environmental pollutants towards animal species [49,50]. In fact, the alteration of membrane phospholipids induced by lipid peroxidation is considered one of the primary key events in the increase in oxidative damage [51] and may exert deleterious effects on cells and tissues through the induction of genetic and/or protein damage [52]. In addition, carbonyl groups are irreversible protein alterations arising as a consequence of oxidative modifications of amino acids and proteins [53], which can result in a variety of effects on protein properties, such as aggregation, inactivation or degradation [54]. Although previous studies noted the capability of HHCB and AHTN to alter antioxidant levels, they largely neglected the deleterious effects caused by the oxidative stress situation suffered by treated organisms. Our data showed that exposure to HHCB

and AHTN can induce serious oxidative damage in zebra mussel specimens. Although 0.39 nM of HHCB did not significantly ($p>0.05$) alter LPO levels, the highest treatment (1.93 nM) induced an early and significant ($p<0.01$) increase in lipid peroxidation in treated bivalves with respect to the controls (Figure 2A). These data were consistent with previous studies performed on *E. fetida* specimens that showed significant time- and concentration-dependent increases in MDA, a secondary lipid peroxidation product considered an important indicator of an oxidative stress situation [55], after exposure to increasing concentrations of HHCB [44,56]. In contrast, neither AHTN treatments caused lipid peroxidation to mussels (Figure 2B), disagreeing with results from a previous study on earthworms showing significant increases in MDA levels after just 12 h of exposure to 6 $\mu g/cm^2$ of AHTN in soil [44]. The PCC results indicated a different picture: although HHCB was confirmed to cause oxidative damage in zebra mussel specimens, as shown by the significant ($p<0.01$) increase in protein carbonyls at the end of the exposure to the High HHCB treatment (Figure 3A), 0.31 nM of AHTN also significantly increased ($p<0.01$) protein carbonylation after 15 days of exposure (Figure 3B). The results of LPO and PCC highlighted that zebra mussel specimens treated with high environmental concentrations of HHCB and AHTN suffered a serious oxidative stress situation, likely as consequence of ROS overproduction, which could also lead to genetic injuries.

DNA damage can be caused by the direct reaction between DNA and free radical species and involves diverse structural alterations, including damaged bases, strand breaks and/or inter- and intra-strand crosslinks. Many studies have shown significant correlations between pollutant-induced ROS and changes in the integrity of DNA in different aquatic species [57,58], including the zebra mussel [26,29]. Moreover, the covalent binding of DNA to certain breakdown products of lipid hydroperoxides, such as MDA, can result in strand breaks and crosslinks [59], whereas carbonyl compounds may be toxic because of their carcinogenic properties [60]. Indeed, significant ($p<0.01$) increases in DNA strand breaks were found in *D. polymorpha* specimens exposed to the highest tested concentrations of HHCB and AHTN (Figure 4 A, B), indicating that the oxidative stress situation suffered by bivalves can induce DNA fragmentation. In addition, the significant positive correlations between LPO and DNA strand breaks ($r=0.422$; $p<0.01$) and between PCC and DNA strand breaks ($r=0.431$; $p<0.01$) found at the highest concentrations of HHCB suggested the possible involvement of lipid peroxidation and protein carbonyl byproducts in the onset of primary genetic damage in the zebra mussel. In contrast, the lack of significant correlation ($p>0.05$) between LPO and DNA strand breaks ($r=-0.116$; $p>0.05$), as well as between PCC and DNA strand breaks ($r=0.208$; $p>0.05$) found at 0.39 nM of AHTN seemed to indicate that both lipid peroxidation

byproducts and carbonyl derivatives did not contribute to genetic damage. Indeed, DNA fragmentation could be produced by direct interaction with ROS, but this hypothesis has to be confirmed by further in-depth analyses. However, although both of the musks induced primary DNA lesions, at least at the highest tested concentrations, neither AHTN nor HHCB exposures caused significant ($p>0.05$) increases in irreversible fixed genetic damage, as shown by the frequency of micronuclei that was lower than 2‰ in both control and treated specimens (data not shown). Our results were not consistent with those from previous studies pointing out a strong link between DNA strand breakage and the induction of micronuclei in different mussel species after exposure to aquatic pollutants [61-63], suggesting the lack of cause-effect relationships between DNA strand breaks and MN formation in the onset of genetic damage due to musk exposure. However, our results agreed with those from a previous *in vitro* study revealing that neither HHCB nor AHTN induced significant alterations in the frequency of micronuclei in either human lymphocytes or hepatocytes (concentration range 0.1-387 μM) [64].

Overall, our data seem to show the higher toxicity of AHTN compared to HHCB in the zebra mussel. Comparing the effects caused by similar concentrations (0.39 nM of HHCB and 0.31 nM of AHTN) at the end of the exposures, the levels of protein carbonyl content induced by AHTN were 2.7-fold higher than those induced by HHCB, although neither chemical caused lipid peroxidation. Similarly, AHTN-induced DNA strand breaks were 60% higher compared to those measured in HHCB-treated bivalves. Moreover, this hypothesis is also confirmed by the levels of these chemicals in the mussels' soft tissues because the AHTN levels caused greater damage to zebra mussels even though they were below HHCB levels. These data are in contrast with those from a previous study on terrestrial earthworms showing that HHCB seems to be more toxic than AHTN because it induced significant increases in lipid peroxidation and imbalances in antioxidant activities at concentrations half those of AHTN [44]. This discrepancy could be explained by the differences in tested concentrations and the duration of exposure, but more likely, it is due to the differences in the accumulation pathways between terrestrial and aquatic species. In fact, in other aquatic organisms, AHTN was more toxic than HHCB. For instance, the toxicity of AHTN was considerably higher to the glochidia of the freshwater mussel *Lampsilis cardium* than HHCB because AHTN 24-h median lethal concentrations (LC_{50}) ranged from 454 to 850 μg/L AHTN and from 1000 to >1750 μg/L HHCB [21]. Moreover, the effect concentrations of AHTN (range 244-314 μg/L) towards *Daphnia magna*, *Oncorhynchus mykiss* and *Danio rerio* were lower compared to those of HHCB (range 282-452 μg/L) during 21-day exposures [65]. Similar findings were also obtained using marine copepods as model species because the 96-h LC_{50} of AHTN and HHCB for

Nitocra spinipes were 610 µg/L and 1900 µg/L, respectively [43], while the inhibition of *Acartia tonsa* larval development after 5 days of exposure (5-d EC_{50}) was induced by exposure to 26 µg/L and 59 µg/L of AHTN and HHCB, respectively [66]. Although the acute toxicity levels found in these studies were up to three orders of magnitude higher than the sub-lethal ones and can hardly be seen as good indicators of chronic toxicity, they should suggest that, regardless of the tested concentrations, AHTN is more toxic than HHCB in aquatic organisms.

5 CONCLUSIONS

Our findings indicate that the concentrations of HHCB and AHTN that can be found in aquatic systems induced oxidative and genetic damage in the zebra mussel, suggesting the involvement of oxidative stress in the mechanism of action of these aquatic pollutants to this freshwater bivalve. Overall, although the lowest tested concentrations of these musks did not cause serious oxidative or genotoxic effects in *D. polymorpha* specimens, 21-day exposures to the highest HHCB and AHTN concentrations induced lipid peroxidation, protein carbonylation and primary genetic damage. AHTN was the more toxic musk to the zebra mussel because it induced more damage than HHCB when the effects caused by similar concentrations were compared. Based on our results, the hazard of both of the musks to aquatic invertebrates should not be underestimated, as bivalves have been exposed to concentrations of HHCB and AHTN comparable to current environmental levels. Moreover, in natural ecosystems, aquatic organisms are exposed to HHCB and AHTN for their entire life span, resulting in possible higher toxicity. Indeed, in-depth investigations on musk toxicity should be pivotal in environmental risk assessments in order to enhance knowledge of their possible sub-lethal effects, their mechanism of action in aquatic organisms and their possible negative effects on population dynamics, which will clarify the true ecological hazard of HHCB and AHTN to aquatic communities.

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7 FIGURES

	Time (days)	Control	100 ng/L	500 ng/L
Galaxolide (HHCB)	t=1	13.51	101.48	492.57
	t=2	7.11	86.29	202.74
	t=3	n.d.°	n.d.°	220.60
	t=4	15.45	69.87	322.81
	t=8	8.97	120.34	376.19
	t=11	14.56	120.71	546.49°
	t=15	17.45	85.96	468.59
	t=18	11.40	91.21	265.34
	Mean± Std. Dev.	**12.64±3.67**	**96.55±18.85**	**335.55±115.43**
	Time (days)	Control	20 ng/L	80 ng/L
Tonalide (AHTN)	t=1	1.14	12.43	45.79
	t=2	1.14	9.93	52.28
	t=3	n.d.°	n.d.°	52.86
	t=4	1.82	13.84	62.34
	t=8	0.10	31.84	95.05
	t=11	1.37	28.11	61.06
	t=15	1.80	23.87	207.00°
	t=18	0.18	23.56	73.41
	Mean± Std. Dev.	**1.08±0.70**	**20.51±8.45**	**63.25±16.59**

n.d.= not detected; ° =outlier

Table 1: Concentration (ng/L) of HHCB and AHTN in water from control and exposure tanks measured over the 21-day exposures. The mean was calculated excluding the outliers (°) values that were shown by a box-plot analysis.

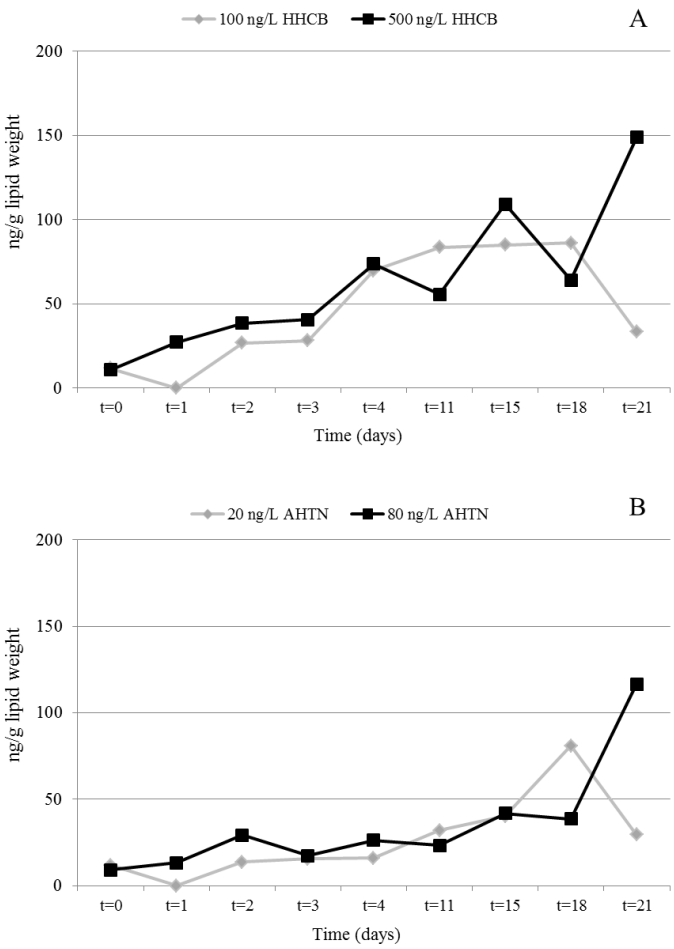

Figure 1: Concentrations (ng/g lipid weight) of HHCB (A) and AHTN (B) measured in zebra mussel soft tissues over the 21-day exposures.

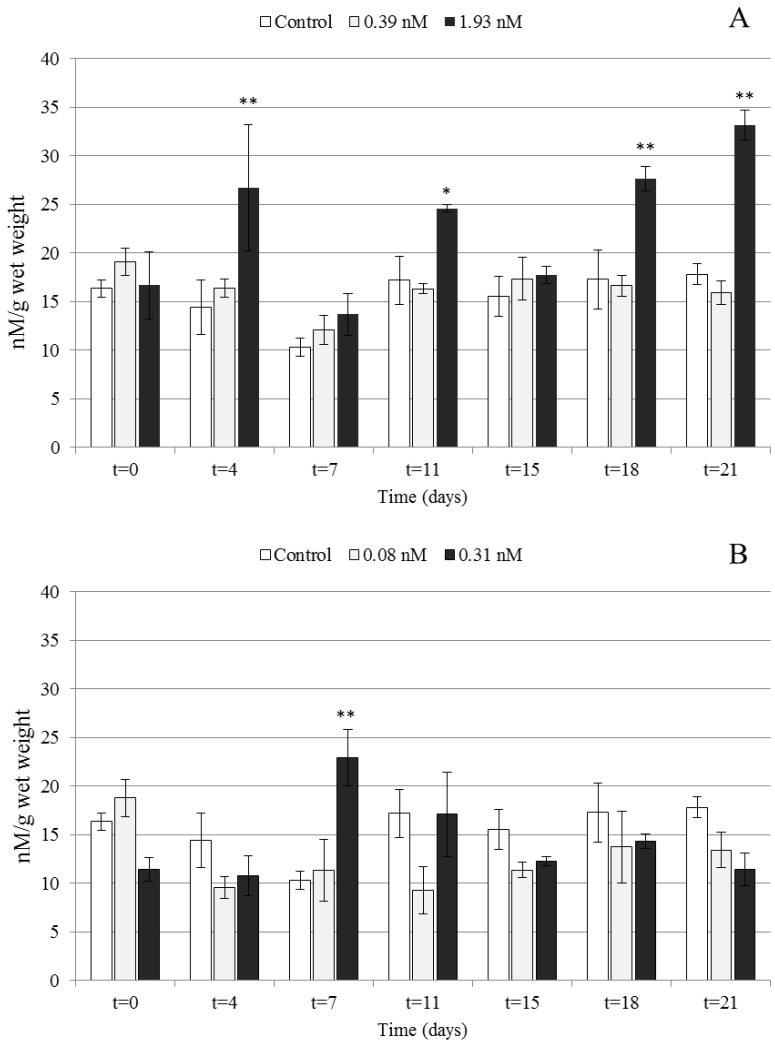

Figure 2: Measure (mean±SEM) of lipid peroxidation in zebra mussels specimens (n=3; pool of 3 specimens) treated with two environmentally relevant concentrations of HHCB (A - 0.39 nM and 1.93 nM) and AHTN (B - 0.08 nM and 0.31 nM) for 21 days. Significant differences (two-way ANOVA, Fischer's LSD post-hoc test, *$p>0.05$; **$p<0.01$) were referred to the comparison between treated mussels and the corresponding control (time to time).

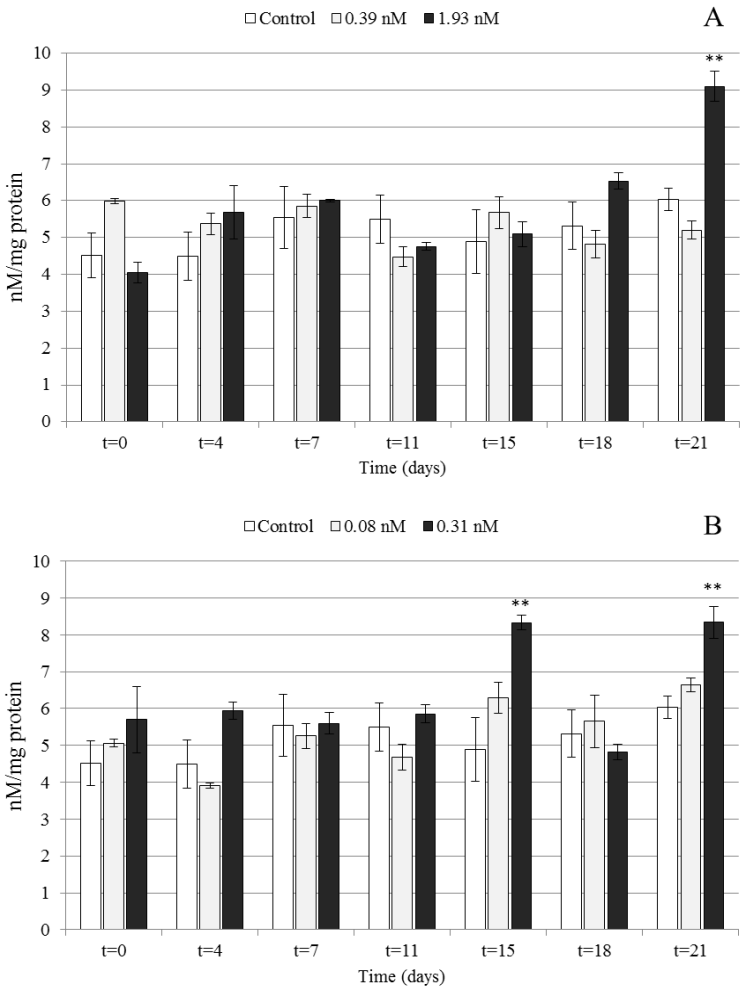

Figure 3: Measure (mean±SEM) of protein carbonyl content in zebra mussels specimens (n=3; pool of 3 specimens) treated with two environmentally relevant concentrations of HHCB (A - 0.39 nM and 1.93 nM) and AHTN (B - 0.08 nM and 0.31 nM) for 21 days. Significant differences (two-way ANOVA, Fischer's LSD post-hoc test, *p>0.05; **p<0.01) were referred to the comparison between treated mussels and the corresponding control (time to time).

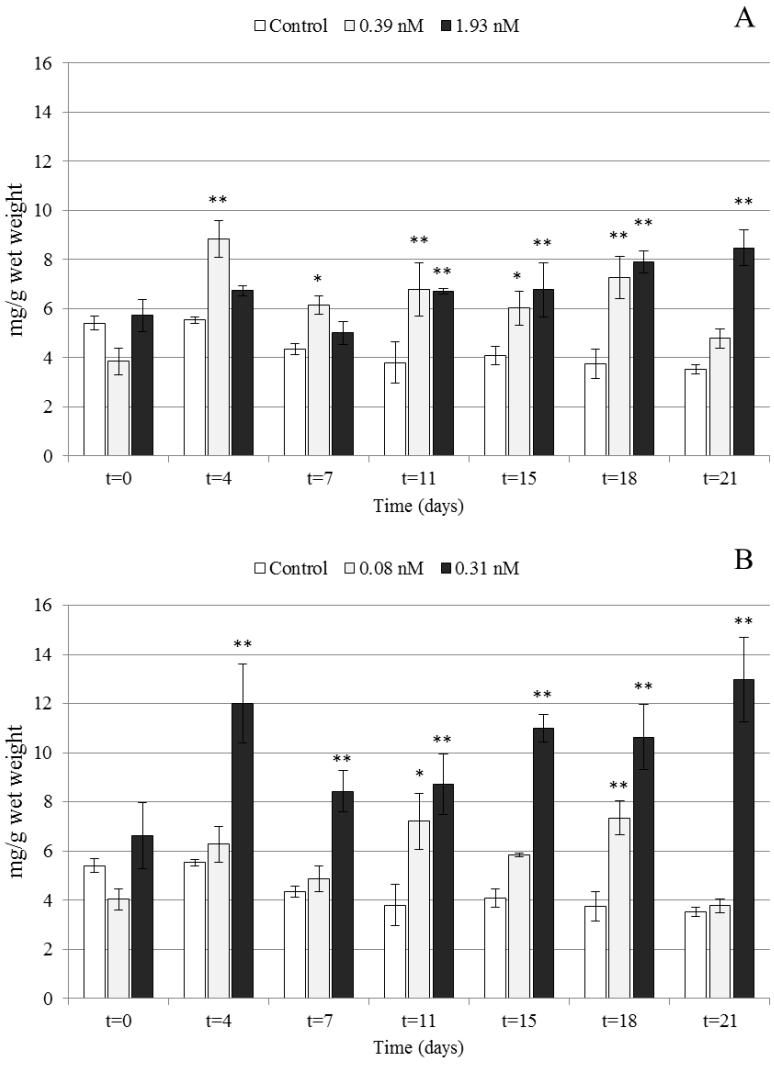

Figure 4: Measure (mean±SEM) of DNA strand breaks in zebra mussels specimens (n=3; pool of 3 specimens) treated with two environmentally relevant concentrations of HHCB (A - 0.39 nM and 1.93 nM) and AHTN (B - 0.08 nM and 0.31 nM) for 21 days. Significant differences (two-way ANOVA, Fischer's LSD post-hoc test, *p>0.05; **p<0.01) were referred to the comparison between treated mussels and the corresponding control (time to time).

Chapter 9

CONCLUSIONS

This PhD project has been divided into two steps: the first one aimed to identifying a new natural removal methods of emerging pollutants (PPCPs and illicit drugs) and heavy metals from wastewater, the second one aimed to assess the ecotoxicological effects of the same emerging contaminants on aquatic organisms.

In the first part of this study, the freshwater bivalve *D. polymorpha* was added to an experimental treatment within the Milano-Nosedo WWTP, in order to remove the wastewater contaminants by means of its natural filtration process. The results obtained regarding the removal of both emerging pollutants and heavy metals appear to be very interesting, highlighting an active role of *D. polymorpha* in the contaminant abatement especially after 24 h of wastewater exposure. In this context, it is important to take into account that data interpretation regarding both PPCP and illicit drug abatement has been very difficult, due to their chemical and physical properties that make the prediction of their behavior in water extremely variable. However, the amount of suspended particulate matter has been found to be essential for the bio-filtration process of bivalve; the high concentrations of the suspended particulate, in fact, limits the mollusk performance, probably due to the mechanical occlusion of the gills. On the other hand, the best results were obtained where low suspended solids concentrations were present, showing, also the great adaptation of *D. polymorpha* to wastewaters and its high filtering efficiency. Although it is plausible the use of the bio-filtration process as the last treatment on the WWTP wastewater output, in our opinion the best performance could be reached in a constructed wetland or lagooning plant. In fact, although there are a number of experimental evidence about the constructed wetland effectiveness in the organic matter and heavy metal removal, and few study on the pharmaceutical abatement (Matamoros and Bayona, 2006; Matamoros *et al*., 2007), the synergy of these two techniques would be an interesting and innovative integrated natural system, able to implement the single effects of these two methodologies.

At the same time, the use of alien species as *D. polymorpha*, that can cause some ecosystem problems and are difficult to eliminate, could have interesting economic implications and developing a new ecological approach aimed at the non-native species exploitation. This procedure has to be supplemented with restraint protocols, such as those we adopted within the Milano-Nosedo WWTP, to prevent further spills of non-native species into the environment. In this context, a native bivalve, such as the Unionidae, could also be used as bio-filtering agent; however, this mollusk family includes species with declining populations that cannot be used for the abovementioned purposes.

In this regard, the identification of an appropriate *D. polymorpha* reproduction methods should be crucial, in order to support the turnover between the bivalves removed from the pilot-plant and stored in dedicated landfills, and animals introduced in the tanks for the bio-filtration process.

Therefore, this work represents an important starting point for future studies aimed at the natural removal of many emerging contaminants from wastewater, taking into account their ecotoxicological effects on aquatic community. In fact, although a statistically significant increase in the antioxidant enzyme activity has been shown after 14 exposure days of *D. polymorpha* at morphine and MDMA, no significant damages at the DNA level are present. These results were also confirmed by the exposition to the illicit drug mixture, that showed oxidative damage at lipidic and protein levels. At the same time, the toxicity evaluation on *D. polymorpha* of synthetic musks galaxolide and tonalide after 21 exposure day showed significant genotoxic effects, in addition to a significant lipidic and protein oxidative damage.

These data are of great interest, because of the lack of research carried on the ecotoxicological effects of these compounds, especially for drugs of abuse. Considering these results, it is important to take into account that the time of exposure of 14 days was chosen to conform these studies to previous research, but it may be a too short time period to induce more significant effects at genetic or physiological levels.

Furthermore, future studies will be conducted analyzing additional end-points, such as neurotoxicity. Illicit drugs have target effects on the nervous system and it would be interesting to investigate their action mechanism on aquatic organisms; *D. polymorpha* could be once again an excellent model organism, taking into account that recent studies on another freshwater bivalve, *Elliptio complanata*, showed neurotoxic effects due to morphine exposure (Gagné *et al*., 2010).

The results obtained during these three years of PhD constitute an important starting point for future research and studies in the wastewater depuration and ecotoxicology.

Chapter 10

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Websites:

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https://www.uni-due.de (micronucleus image on the thesis cover)

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