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Multi-level toxicity assessment of different emerging contaminants towards aquatic and terrestrial model organisms

PhD Thesis

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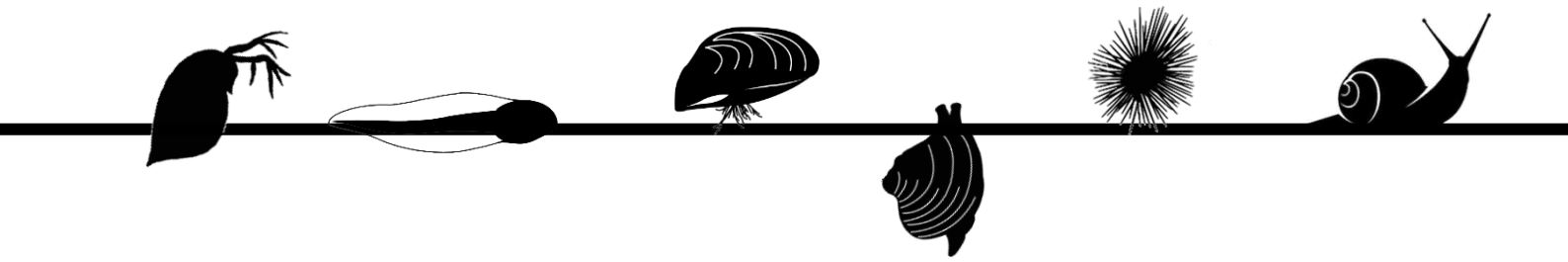

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ABSTRACT

In recent years, emerging contaminants have attracted the attention of scientific community because of their occurrence and potential hazard towards natural ecosystems. Among emerging contaminants, three major classes of molecules can be identified: pharmaceuticals and personal care products (PPCPs), illicit drugs and microplastics (MPs). After anthropic use, these contaminants enter the environment through several routes, such as the sewage systems or direct input, resulting in a widespread contamination of atmosphere, water, soil and biota. Although the presence of these molecules is well-known, there is still a dearth of information regarding their potential negative effects induced towards aquatic and terrestrial non-target organisms. The majority of the studies on this topic have focused on the investigation of effects at low levels of the biological hierarchy, while limited attention has been addressed to the higher ones, such as individual, population or community level. Whilst PPCPs toxicity has been investigated for more than two decades, only in recent years ecotoxicologist focused their attention on the presence and the potential effects of illicit drugs and microplastics. Thus, the aim of this project was to investigate the effects induced by the exposure to different illicit drugs and microplastics. As in environment these contaminants could interact with a wide range of organisms, resulting in species-specific differential effects at different levels of biological organization, their effects have been investigated on six different model organisms, representative for freshwaters (*Daphnia magna* and *Xenopus laevis*), marine (*Mytilus gallopovincialis*, *Ruditapes philippinarum* and *Paracentrotus lividus*) and soil (*Achatina reticulata*) ecosystems. To reach the goal of the project, a multi-level approach based on the application of assays at sub-individual (i.e., biochemical biomarkers), tissue (i.e., histological analysis), individual (i.e., mortality, growth rate and/or swimming behavior) and, when possible, population level (i.e., reproduction) was used.

Regarding illicit drugs, different experiments were planned to evaluate the effects induced by the exposure to the two most used and environmentally detected illicit stimulants, cocaine (COC) and methamphetamine (METH), to the freshwater cladoceran *Daphnia magna*. Moreover, considering that illicit drugs occur in aquatic ecosystems as complex mixtures, an additional experiment was planned to evaluate independent and combined effects induced by the exposure to cocaine and its main metabolite benzoylecgonine (BE), towards the Mediterranean mussel *Mytilus galloprovincialis.* The exposure to environmentally relevant concentrations of COC and METH induced a modulation of the oxidative status, as well as a molecule-specific effect on swimming behavior and reproduction, in *D. magna*. Similarly, the exposure to COC and BE, both independently and in mixture, induced an alteration of the oxidative status of Mediterranean

mussels. These data suggested that illicit drugs might represent a threat for both freshwaters and marine non-target organisms.

Regarding microplastics, the attention was focused on two polymers, polystyrene (PS) and polyethylene terephthalate (PET), having different chemico-physical features and environmental fate. In fact, because of its low density, PS tends to float in the water column, while in contrast high-density PET sinks and accumulates to sediments. For this reason, the administration of PS or PET MPs allowed to investigate the toxicity towards organisms with different feeding strategies and ecological role in ecosystems. The effects of regular PS-MPs were evaluated towards two freshwater organisms, namely the cladoceran *Daphnia magna* and the amphibian *Xenopus laevis*, Moreover, considering that in environment irregular shaped MPs are most common than regular ones, three experiment were planned to investigate the effects of irregular shaped PET-MPs towards two marineorganisms, the Manila clam *Ruditapes philippinatum* and the sea urchin *Paracentrotus lividus*, and a soil organism, the giant snail *Achatina reticulata*. The results obtained in these experiments showed that MPs were efficiently ingested by all the tested organisms, but no or limited adverse effects occurred, depending on the considered model organism.

In conclusion, illicit drugs and microplastics can induce different species-specific adverse effects towards aquatic and terrestrial organisms. Moreover, the project pointed out the usefulness of using a multi-level approach to deeply study the toxicity of emerging pollutants. The integration of information coming from sub-individual and higher levels of the biological hierarchy can allow to shed a light on the propagation of the effects and to explore the complexity of contaminant-induced toxicity. Lastly, the use of different model organisms with different life-history traits and ecological role can allow to explore species-specific differences generated by the exposure to contaminants and to assess the risk of a specific class of contaminations towards the whole ecosystem.

CHAPTER 1 – *State of art*

1.1 Emerging contaminants as environmental pollutants

For a long time, a huge number of studies highlighted the occurrence in environment of several different molecules of anthropogenic origin (Bolong et al., 2009; Rivera-Utrilla et al., 2013; Sauvé et al., 2014). These molecules have been detected in freshwaters, seawaters, soils, atmosphere and even biota and drinking waters worldwide (Tran et al., 2018; Gogoi et al., 2018). To date, among all these molecules, the attention of ecotoxicologists is focused on a group called "emerging contaminants" or "contaminants of emerging concern" (ECE). These so-called emerging contaminants are molecules of new formulation without a regulatory status, whose effects against human health and environment are often still unknown (Gogoi et al., 2018; Lima et al., 2018; Patel et al., 2020).

Among ECEs, three major classes of molecules can be identified: pharmaceuticals and personal care products (PPCPs), illicit drugs and microplastics (MPs). These contaminants enter the environment through many routes, all mediated by anthropogenic activities. Regarding PPCPs and illicit drugs, the main entrance routes are related to industrial, hospital and domestic sewage. After human use these molecules end up in the sewage and, since traditional Waste Water Treatment Plants (WWTPs) cannot efficiently remove them from wastewaters, these molecules enter the surface waters, contributing to the environmental contamination (Richardson et al., 2018; Tran et al., 2018; Rout et al., 2020). In contrast, MPs enter the environments from outlet of WWTPs, the application of sewage sludge as fertilization in agriculture, incidental release during the fabrication process, illegal dumping and the breakdown of larger plastic items present in environment (Duis et al., 2016; De Falco et al., 2019; Chen et al., 2020).

Several monitoring studies have measured the concentration of different ECEs in surface waters worldwide, which for PPCPs and illicit drugs ranged between few ng/L to hundreds of µg/L (Pal et al., 2013; Gogoi et al., 2018; Tran et al., 2019). In contrast, to date no clear indication on the abundance of MPs in environment is available. Once in environment, these molecules could represent a risk to human and ecosystem health (Stuart et al., 2012; Naidu et al., 2016). Although the current environmental concentrations of ECE in ecosystems can be considered low, the risks for the biota cannot be excluded. In fact, regarding PPCPs and illicit drugs their continuative input may result in stable and/or increasing concentrations, conferring to them a sort of pseudo-persistence that could lead to long-term exposure and an increase in the environmental concentrations (Pal et al., 2013; Taheran et al., 2018; Reid et al., 2019). Moreover, the high durability of plastic confers to MPs a persistent nature allowing their long-term accumulation in ecosystems (Andrady et al., 2011; Wagner and Lambert 2018).

To date, the information of the possible negative effects induced by the exposure to ECEs on non-target organisms remains limited (Lei et al., 2015; Pereira et al., 2015). Although some studies pointed out the potential negative effect of ECEs, due to the lack of standardized methods to analyze their impact and fate, most of them still lack of a regulatory status and the working of setting threshold levels is challenging (Teodosiu et al., 2018). Therefore, there are no laws or directives showing the upper limits of concentration of ECEs in wastewater effluents and environment (Gogoi et al., 2018; Teodosiu et al., 2018). At European level, the European Environmental Agency claimed that these contaminants should be monitored in terms of environmental levels and potential toxicity, as they are constantly used and found in aquatic and terrestrial ecosystems worldwide (EEA, 2012). For instance, the Water Framework Directive (WFD) 2000/60/EC (EC, 2000) identifies a list of "priority pollutants" that includes 33 molecules with a high risk based on their significant potential risks for the aquatic environment (Richardson et al., 2007). This directive was followed by the Directive 2008/105/EC (EC, 2008), which claims the need for strict monitoring rules regarding sampling and the standardization of the analytical methods used. Moreover, in 2013, the list was completed by the Directive 2013/39/EU (EC, 2013), which defined a list of 45 priority-emerging pollutants grouped as single or classes of substances. Under the WFD it was established the surface water Watch List (WL), a list of emerging pollutants and substance that may pose a significant risk, at Union level, to or *via* the aquatic environment but for which available monitoring data are not sufficient to show environmental risk (Joint Research Centre, JRC 2020). The first WL was set up in 2015 by Commission Implementing Decision (EU) 2015/495 and contained ten substances or group of substances. The early ECEs identified as a potential threat to aquatic ecosystems, and listed in the WL, were the non-steroidal anti-inflammatory drug, diclofenac, and the hormones, 17-beta-estradiol (E2) and 17-alfa-etiniestradiol (EE2), followed by other seven substances/groups of substances such as antibiotics and pesticides (European Commission, 2015). The list was updated in 2018, by the Commission Implementing Decision (EU) 2018/840, and, during this revision process, it was highlighted that substances as diclofenac, oxadiazon, 2,6-di-tert-butyl-4-methylphenol, tri-allate and 2-ethylhexyl-4-methoxycinnamate should be removed from the WL, while new substances should be added, such as the insecticide metaflumizone and the antibiotics amoxicillin and ciprofloxacin (EU 2018/8402). The newest WL was established by the Commission Implementing Decision (EU) 2020/1161 in august 2020, new substances were included in the WL, such as the sulfonamide antibiotic sulfamethoxazole and the diaminopyrimidine antibiotic trimethoprim, the antidepressant venlafaxine and its metabolite O-desmethylvenlafaxine, a group of three azole pharmaceuticals (clotrimazole, fluconazole and miconazole) and seven azole pesticides (imazalil, ipconazole,

metconazole, penconazole, prochloraz, tebuconazole, tetraconazole), while the fungicides famoxadone and dimoxystrobin were identified as suitable candidates. Moreover, the Directive 2008/105/EC implied that the duration of a continuous WL monitoring period for any individual substance shall not exceed four years. Following this Article (Article 8b(2) of Directive 2008/105/EC) the WL monitoring obligation for the five substances or groups of substances that had been on the list since 2015, namely 17-alpha-ethinylestradiol (EE2), 17-beta-estradiol (E2) and estrone (E1), the group of macrolide antibiotics, methiocarb, and the group of neonicotinoids, ceased in 2019. However, it is important bearing in mind that to date microplastics monitoring was not included in any European action but should require implementation.

1.1.1 Pharmaceuticals and personal care products

Pharmaceuticals and personal care products (PPCPs) measured in environments belong to different classes of human and veterinary drugs, as well as to different products commonly used in human everyday life. These molecules, after human use, are metabolized by the liver and then discharged to the sewage in their parental form or as metabolites (Fent et al., 2006; Liu et al., 2013). Once in the sewage, PPCPs reach the Wastewater Treatment Plants (WWTPs), which are not able to completely remove them from the water and, consequently, these molecules enter surface waters through the effluents, contributing to the contamination of aquatic ecosystems. Several monitoring surveys perform in a number of countries worldwide, including USA (Boyd et al., 2004), Spain (Fernandez-Rubio et al., 2019), Finland (Lindqvist et al., 2005), United Kingdom (Ashton et al., 2004), Italy (Zuccato et al., 2006) and Japan (Nakada et al., 2006), measured the presence of different PPCPs in in wastewaters, surface and drinking waters, whose concentrations ranged between low ng/L to μg/L (e.g., Caracciolo et al., 2015; Gogoi et al., 2018). The PPCPs most commonly found in aquatic environment are non-steroidal anti-inflammatory drugs (NSAIDs), β-blockers, antidepressants and antiepileptics, which are all drugs that do not need to be prescribed and are sold over the counter (Petrie et al., 2015). As a consequence of their presence in aquatic ecosystems, since 1999 (Daughten et al., 1999) a growing number of studies have explored if the exposure to realistic or unrealistic concentrations of different types of PPCPs could induce adverse effects to non-target aquatic organism at different levels of ecological hierarchy. Such studies confirmed that PPCPs exposure, also at low, environmentally relevant concentrations, can induce a plethora of adverse effects at biochemical or molecular (e.g., Parolini et al., 2010; Parolini et al., 2013a), physiological (e.g., Caracon et al., 2012; Pablos et al., 2015) and/or behavioral (e.g.,

Brodin et al., 2014; Saaristo et al., 2019) level, confirming the risk related to these emerging contaminants for natural ecosystems.

In spite of a notable attention that has been focused on PPCPs ecotoxicology, a restricted number of studies have been addressed to explore the environmental presence and potential effects of two other classes of emerging pollutants, such as illicit drugs and microplastics. In detail, since about 15 years ago, illicit drugs began to be recognized as emerging aquatic pollutants, so that they became new target molecules for monitoring (Zuccato et al., 2008; Castiglioni et al., 2011; Pal et al., 2013; Deng et al., 2020), while little is currently known about the potential negative effects they could exert towards aquatic communities. Although the first record of the presence of plastics in environment goes back to 1970s (Colton et al., 1974), these contaminants gained a great attention only from the last 10 years, specifically when Thompson, in 2006 (Thompson 2006) coined the term microplastics. Thus, the present project has identified as target contaminants illicit drugs and MPs.

1.1.2 Illicit drugs

Drugs of abuse represent a global problem with significant adverse impacts, not only towards human health or social welfare but also to the environment (UNODC, 2020). The United Nations Office on Drugs and Crime (UNODC) defined the term 'illicit drug' as a substance whose possession, production, sale or consumption is prohibited by the law, considering the manner in which they are manufactured, distributed and acquired, as well as being used for non-medical purpose (UNODC, 2014). In spite of the difficulties in estimating the production and the consumption of illicit drugs, the newest World Drug Report estimated that more than a quarter of a billion people (i.e., 269 million people), corresponding to the 5.4% of the adult population aged 15 – 64, used drugs at least once in 2018 (UNODC, 2020). Over the past two decades, the use of illicit drugs increased rapidly worldwide, in terms of overall numbers and proportion of world's population drug users. In less than ten years, from 2009 to 2018, the estimated users grew from 210 million (4.8 % of the global population) to 269 million (5.3 % of the global population) people aged 15 – 64 (World Drug Report, 2020). Moreover, in recent years the illicit drugs market suffered a lot of changes with a diversification of the molecules that are present on the market. In detail, in addition to 'classical' illicit drugs related to plant-based substances, such as cocaine, cannabis and heroin, new substances appear on the market, referring to the so-called synthetic drugs, such as the amphetamine like family (ATS) and molecules of new formulation, called new psychoactive substances (NPS). In spite of the wide range of different illicit drugs on the market,

in 2018 cannabis remains the most used illicit drug globally, with an estimated 192 million past-year users, followed by opioids (57.8 million users), opiates (30.4 million users), amphetamines and prescription stimulants (27 million users), "ecstasy" (21 million users) and cocaine (19 million users) (UNODC, 2019). Overall, the second most used class of illicit drugs after cannabis is represented by stimulants, accounting for 68 million past-year users (UNODC, 2019).

Stimulants, or psychostimulant, are a class of molecules characterized by the capacity of acting on the central nervous systems leading to an increase in alertness, heighten arousal and cause behavioral excitement (Jerrold et al., 2019). The general mechanism of action (MoA) of these molecules is related to the increase in the activation of natural stimulating pathways in the brain. In detail, these molecules enhance the function of the three main monoamine neurotransmitters: dopamine, norepinephrine and serotonin (UNODC, 2020). Stimulants can be plant-based substances, such as cocaine, ephedrine and cathinone, or can have a synthetic nature, such as amphetamine, methamphetamine, MDMA (3,4-methylene-dioxymethamphetamine) and MDA (3,4-methylenedioxyamphetamine). Most of stimulants, such as cocaine, amphetamine, methamphetamine and MDMA are controlled at international level during the drug control conventions. The recent data regarding the use of stimulants showed that cocaine and methamphetamine dominate the scene of illicit stimulants worldwide (Figure 1).

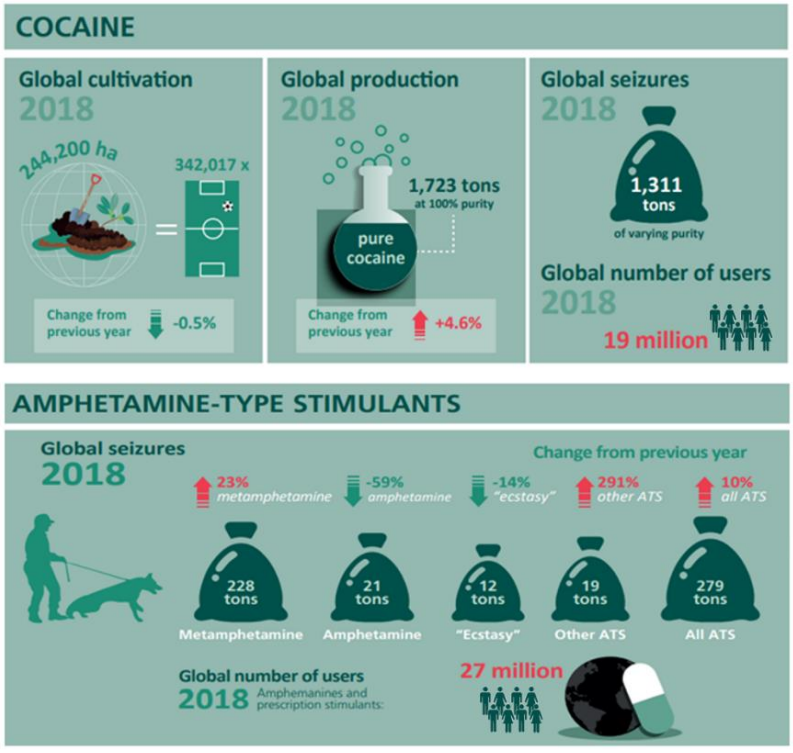

Figure 1: Summary table of the production, use and seizures of the most used stimulants worldwide: the cocaine and the amphetamine-type stimulants (modified from UNODC, 2020).

Indeed, the stimulant effects exerted by these two molecules appear to be similar: cocaine, by blocking the normal recycling process, leads to the inhibition of the re-uptake of the serotonin, dopamine and norepinephrine (Rasmussen et al., 2001), while methamphetamine inhibits the re-uptake of dopamine and norepinephrine, and, to a lesser extent, of serotonin (Freye et al., 2009). The stimulant market is therefore dominated by these two molecules who showed an increasing trend of use when compared with the previous years. Moreover, these molecules could co-exist in some markets, even if usually they are considered as substitutes for each other. The relationship occurring between cocaine and methamphetamine implicates that when the use of one drug rises the other goes down, feeding the same market with parallel increases and declines (UNODC, 2020). For instance, in 2018 the amphetamine-like family (ATS) accounted for 27 million users, while cocaine users were 19 million people (UNODC, 2020).

Furthermore, considering the Coronavirus disease (COVID-19) pandemic, the consumption trends for stimulants are likely to increase, considering that the COVID-19-related restrictions lead to an increase in people buying drugs. Therefore, scientists expect an increase in the use of illicit drugs in the next few years (UNODC, 2020). For instance, focusing on cocaine, even if the manufacture appears to be impeded, especially in South America, the economic crisis may lead more farmers to increase coca cultivation and to increase the maritime drug trafficking, with a consequent expected increase in production and consumption trend. On the other hand, regarding ATS production, the COVID-19 restrictions could have reduced the availability of the precursor molecules affecting the production of these synthetic drugs. Anyway, the available data showed that the production of synthetic drugs is only marginally affected by the restrictions stemming from the measures to control the spread of COVID-19 (World Drug Report, 2020).

Nowadays, the increasing use of illicit drugs shifted the attention from being considered only a socio-economic issue to a global problem, with impacts not only towards human health or social welfare but also to the environment. The continuative use of illicit drugs, coupled with the incapacity to totally remove them from sewage before the input in superficial waters, lead to a situation of widespread contamination. As previously explained, the main source of illicit drugs is the sewage, after human consumption. In fact, traditional WWTPs cannot efficiently remove them from wastewaters and these molecules enter the surface waters, similarly to PPCPs, where they have been measured in concentrations ranging from 100 ng/L to 100 µg/L (Pal et al., 2013; Fontes et al., 2020). In addition, as far as concern the marine ecosystem, intentional and accidental disposal from clandestine laboratories represents another important route for illicit drugs to enter environments, as well as the loss during maritime illegal trade (Zuccato et al., 2008; Pal et al.,

2013; Fontes et al., 2020). Although the current environmental concentrations of these drugs can be considered low, the risks for the aquatic communities cannot be neglected. Indeed, considering the potential toxicity of these molecules, illicit drugs are now included in the list of priority pollutants of Water Framework Directive (Gonzàlez-Mariño et al., 2010). In addition, their continuative input may result in stable and/or increasing concentrations, conferring to these molecules a sort of pseudo-persistence that could lead to long-term exposure and increase the risk for the ecosystems. Lastly, these molecules are generally more polar than traditional pollutants and very stable in water. For these reasons, aquatic ecosystems can be considered the final compartment of contamination by illicit drugs (Pal et al., 2013). Once in environment, these substances, that are specifically designed to act on determinate human enzymatic-pathways and present a high pharmacological activity, could exert their biological activity also through non-target aquatic organisms, since most of the enzymatic pathways are well conserved among organisms, leading to a variety of toxic effects (Abreu et al., 2015). However, although the presence of illicit drugs in aquatic environments and the effects on humans and model organism of toxicology are well documented, the information on the adverse effects towards non-target aquatic organism is still scant (Binelli et al., 2012; Parolini and Binelli, 2013; Parolini et al., 2013; 2014; Liao et al., 2015; Gary et al., 2016; Parolini et al., 2017; Parolini et al., 2018; Capaldo et al.,2019; Hossain et al., 2019). According to the reasons mentioned above, the attention of this project was focused on the analysis of toxic effects induced by the two stimulants most used and commonly found in aquatic environments, namely cocaine, a plant-based drug, and methamphetamine, a synthetic drug, towards opportune model organisms.

1.1.2.1 Cocaine

Cocaine ($C_{17}H_{21}NO_4$, hereafter COC; Figure 2) derives from the leaf of the brush *Erythroxylum coca*.

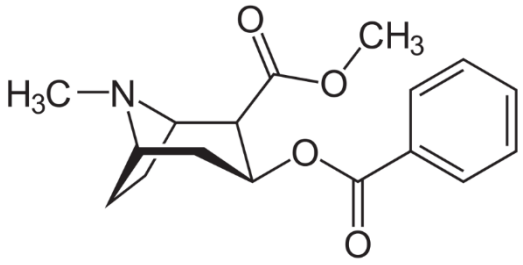

Figure 2: Chemical structure of cocaine.

Primarily, the cultivation of this plant is based in South America, with Colombia being the leader in the cultivation. The cultivation of cocaine suffered a decrease between 2000 and 2013, but this period was followed by a two-fold increase over the period 2013 – 2017 (UNODC, 2019). The newest World Drug Report showed that during 2018 the cultivation of coca seems to have stabilized, reaching the historically highest level of 244,200 ha of coca bush cultivation worldwide (UNODC, 2020). On the basis of preliminary estimates, in 2018 the global manufacture of cocaine reached an all-time high of 1,723 tons, accounting for 4.6% increase compared to the level in 2017 (UNODC 2020). Considering these data, cocaine remains the stimulant most commonly used at a global scale, with an estimated 19 million people of past-year users, corresponding to 0.4% of the global population aged 15 – 64. The main cocaine markets continue to be North America (2.1%), Western and Central Europe (1.4%), Central America (0.7%) and South America (1.0%), although the highest prevalence of past-year cocaine use was in Australia and New Zealand (2.2%).

After human consumption, cocaine is metabolized and bio-transformed by the liver and excreted as parental compound (9 – 20%), benzoylecgonine (hereafter BE; 50 – 60%), ecgonine methyl ester (hereafter EME, 45%) and secondary metabolites (3%) through urines and feces (Maurer et al., 2006). Following the sewage systems, these molecules reach the WWTPs where, after partial removal of the treatment, they are released into the aquatic environment (Zuccato et al., 2008; van Nuijs et al., 2009; Castiglioni et al., 2011). Considering the huge use of this illicit drug globally, cocaine and its metabolites are continuously discharged in the sewage and, according to COC metabolism in human body and its stability in water, the concentration of COC and BE in aquatic ecosystems are always higher compared to other drugs and their metabolites (van Nuijs et al., 2009; Postigo et al., 2010; Castiglioni et al., 2011). A recent review published in 2020 on illicit drugs occurrence has reported that COC was found in both influents and effluents of WWTPs in concentrations up to 4,700 ng/L and 530 ng/L, respectively (Fontes et al., 2020). Likewise, BE was detected at concentrations up to 7,500 ng/L and 1,500 ng/L in WWTPs influents and effluents, respectively (Fontes et al., 2020). Considering the limited efficiency in removing these molecules from sewage by the traditional WWTPs, COC and BE were measured in surface waters in concentrations ranging between 0.4 and 44 ng/L and between 3 ng/L and 316 ng/L, respectively (Pal et al., 2013; Yadav et al., 2017). Considering that surface waters and urban streams flow into oceans and seawater, measurable concentrations of these drugs might occur also in marine ecosystems. However, a limited number of studies have monitored the occurrence of illicit drugs in marine and coastal ecosystems. To date, only two studies have shown the occurrence of COC and BE in marine ecosystems (Santos bay; Santos, Brazil), highlighting a concentration up to 537 ng/L and 20.8 ng/L, respectively (Pereira et al., 2016). In addition, a second study conducted in

the same coastal area, measured concentrations of COC and BE up to 203 ng/L and 38 ng/L, respectively, because of seasonal variability or different seawater parameters (Fontes et al., 2019).

Although the presence of COC and its metabolites in aquatic environments was confirmed, as well as the toxicity of COC on humans (Leri et al., 2003; Spronk et al., 2013) and model organism of toxicology such as the murine model (Brami-Cherrier et al., 2005; Dixon et al., 2010), the information on the adverse effects caused by these molecules towards non-target aquatic organism is still scant (Binelli et al., 2012; Parolini et al., 2017; Capaldo et al., 2019). To date, few studies have been focused on the investigation of the effects induced by COC and its main metabolites, towards freshwater and marine organisms (Parolini et al., 2017; Parolini et al., 2018; dos Santos Barbosa Ortega et al., 2018). For instance, cyto-genotoxic and oxidative stress-related effects were induced by the exposure to different concentrations of COC on the freshwater mussel *Dreissena polymorpha* (range 40 ng/L – 10 µg/L; Binelli et al., 2012), the zebrafish *Danio rerio* embryos (range 0.01 -10 µg/L; Parolini et al., 2017) and the marine mussel *Perna perna* (0.5, 5.0, and 50.0 µg/L; dos Santos Barbosa Ortega et al., 2018). A number of studies performed on the European eel (*Anguilla anguilla*) highlighted that an environmentally relevant concentration of COC (20 ng/L) modulated the levels of both brain dopamine and catecholamines and induced alterations in the hormones levels and histological alteration in different tissues and organs (Gay et al., 2013, 2016; Capaldo et al., 2018, 2019). Furthermore, a redox proteomics study showed that BE (1 µg/L) caused oxidative modifications in different gills proteins involved in energetic metabolism, cytoskeleton, and stress response in *D. polymorpha* (Pedriali et al., 2013), while similar results were obtained in zebrafish larvae after exposure to COC and BE (0.3 – 1 µg/L; Parolini et al., 2018). However, all of these studies have just focused on the effects found at biochemical/molecular (Parolini et al., 2017; Parolini et al., 2018; dos Santos Barbosa Ortega et al., 2018) or tissues and organ levels (Gay et al., 2016; Capaldo et al., 2019, 2018) while, to date, there is still a lack of knowledge on the propagation of the effects to higher levels of biological hierarchy.

1.1.2.2 Methamphetamine

Methamphetamine ($C_{10}H_{15}N$; hereafter METH; Figure 3) is a synthetic illicit drug, belonging to the class of the amphetamine-like substances (ATS) and its peculiarity is a methyl substituent in the amino group of (S)-amphetamine.

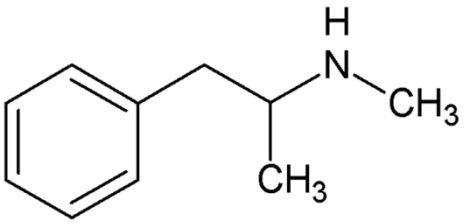

Figure 3: Chemical structure of methamphetamine

From the second part of 1990s the tons of ATS seized increased rapidly at a global scale and, in detail, over the period 2009-2018 a huge peak in the amount of methamphetamine production occurred. Around 27 million people worldwide, corresponding to 0.5 % of the population, are estimated to have used amphetamines-like substances, including amphetamine, methamphetamine and pharmaceutical stimulants, in the past year (UNODC, 2020). The most used ATS was methamphetamine, which accounted for 71% of the total amount of the global ATS seized. Considering the synthetic nature of methamphetamine, the identification of a defined area of manufacture is challenging. The most recent data showed that over the years 2014 – 2018 there were dismantling around 30,000 clandestine laboratories used for ATS preparation and that 95% of those were dedicated to the production of the methamphetamine (UNODC, 2020). Dissimilarly from other ATS, methamphetamine showed a 23% increase in the use, which was doubled in the last 15 years in more than 100 different countries.

After the consumption of a single dose, METH is metabolized in the liver and excreted through feces and urine, mainly as unaltered parental compound (30 – 50%) and limitedly as two metabolites, namely 4-hydroxymethamphetamine (15%) and amphetamine (accounting for 10%) (Cruickshank and Dyer, 2009). As described for cocaine, the modern WWTPs are not designed to efficiently remove METH from sewage; for this reason, a non-negligible amount of METH enters the aquatic ecosystems (Boles and Wells, 2010). A number of monitoring studies have detected measurable concentrations of METH in both wastewaters and surface waters worldwide (Pal et al., 2013). In influents of European and American WWTPs METH concentrations ranged between 2 ng/L and 350 ng/L (Rosi-Marshall et al., 2015; Asimakopoulos and Kannan, 2016), while in the majority of Asiatic WWTPs the concentrations of METH ranged between 100 ng/L and 700 ng/L for effluents, and from 80 ng/L to 1153 ng/L for influents (Zeqiong et al., 2017). In addition, Li et al. (2016) have detected METH in the surface waters from different lakes in concentration up to 95.9 ng/L. To date, no information on the presence of METH in seawater is available. Similarly, there is a scarce information concerning the potential toxicity of METH towards non-target aquatic

organisms. Although the induction of oxidative stress after METH exposure is well known in humans (Zhang et al., 2009; Toborek et al., 2013), only one study shows how METH exposure could induce oxidative stress also in non-target organisms (Liao et al., 2015), highlighting that the exposure to increasing concentrations of METH (range 0.004 - 40 µM) lead to an oxidative stress situation, followed by alteration in development and behavior in the medaka fish *Oryzias latipes*. Moreover, neurophysiological studies focused on the alteration in behavior induced by METH exposure resulted in contrasting results evidencing a decrease in locomotion for some organisms (Rawls et al., 2008) and no effects for others. In detail, a recent study by Hossain and co-authors (2019) showed that METH exposure did not induce an alteration in the swimming activity of the crayfish *Prokambarus clarkii***,** while studies on flatworms showed a decrease in locomotion for *Dugesia dorotocephala* (0.1 - 100 µM of METH; Rawls et al., 2008) and an increase in movement for *Dugesia japonica* 0.03 µM of METH; Tashiro et al., 2014). Lastly, males of the freshwater fish *Poecilia latipinna* showed an increase in the mating activity after exposure to METH (0.1, 0.5 and 1.0 mg/L; Ghazilou and Gazilou, 2011).

1.1.3 Microplastics

In recent years, plastic contamination has raised a worrisome concern for aquatic ecosystems. Even if the first record of the presence of small plastic fragments in the oceans was highlighted in the 1970s (Carpenter and Smith, 1972), only from the last 15 years a growing number of studies began to assess the magnitude of plastic pollution and to analyze the potential consequences connected to the presence of these contaminants in the environment.

In 2018, the global plastic production accounted for 359 million tons (Plastic Europe 2019), several manifold higher than the amount produced in the early 1950s. The production was estimated to have an increase rate of 0.2×10^8 tons/acre, i.e. 0.45×10^8 kg/m^2 and given the remarkable societal benefits plastics provide this trend will probably continue to increase in the future (Li et al., 2020). Plastics present a relatively short use-lifetime: after being used the fate of a plastic item could be the recycling process or the storage in landfills (Li et al., 2020). However, the amount of recycled plastic overall it is relatively small, only the 8.5 – 9 % of the total, corresponding to about three million tons (United States Environmental Protection Agency 2020), while on the contrary the majority of the plastics used end up as litter in municipal solid waste, where they account for the 10 – 15 % by weight (Andrady et al., 2017) or are discharge in illegal dumping site. The presence and the accumulation of plastics in environments, both aquatic than terrestrial, produces several negative consequences from the aesthetic impact of litter to the possible adverse biological and

ecological effects (Avio et al., 2017; Li et al., 2020; Thushari et al., 2020). From the past 15 years the number of papers focusing their attention on the environmental issue of plastic has grown exponentially (e.g., Andrady et al., 2011, Cole et al., 2011; Horton et al., 2017a; Rezania et al., 2018; Xu et al., 2020). The attention that was given to issue of plastics pollution, especially in the marine environments, lead several organizations such as the Marine Debris Program of the US National Oceanographic and Atmospheric Administration (NOAA) to include plastics litter as an emerging form of contamination (Avio et al., 2017; Li et al., 2020).

Plastic waste found in environment can be categorized according to its size in: macroplastics (items > 25 mm), mesoplastics (items 5 - 25 mm), large microplastics (items 1 – 5 mm), small microplastics (items 1 µm – 1 mm) and nanoplastics (items 1 – 1000 nm) (Good Environmental Status 2018; Gigault et al. 2018). Among these different size-classes, recently, ecotoxicology focused their attention on microplastic, defined as any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 µm to 5 mm, which are insoluble in water (Cole et al., 2011; Gigault et al. 2018; Frias and Nash 2019). The little size of this class of plastics facilitates their ingestion by a wide range of organisms whose impact are mostly still unknown representing one of the major modern challenges for ecotoxicologist (Anbumani and Kakkar, 2018).

Microplastics (MPs) can be generally categorized in two classes depending on their manufacturing origins: primary and secondary microplastics. Primary microplastics are manufactured as such and are used in several personal care products, such as facial-cleanser, toothpaste or cosmetics (Cole et al., 2011; Li et al., 2020), in medicine for drug delivery (Wagner and Lambert 2018) or as resin pellets (Cole et al., 2011). After the use of products in which are contained, MPs are discharged to aquatic ecosystems through the sewage systems and reach the environment after the insufficient removal treatment by WWTPs (Druis et al., 2016). In contrast, secondary MPs derive from activities, such as littering or incorrect disposal of plastic waste, as well as from the breakdown of larger plastic items caused by physical, chemical and biological processes of weathering (Thompson et al. 2004; Cole et al., 2011; Horton et al., 2017). In detail, larger plastic items experience weathering processes that induce their fragmentation to smaller items (i.e., microplastics) following different pathways, such as photo-degradation, ultraviolet (UV) radiation or photo-oxidation (Halle et al., 2016; Weinstein et al., 2016; Andrady et al., 2017) and, therefore, enter directly into the environment. In addition, recent studies suggest that the interaction between bigger plastic items and biota could result in the production of microplastics (Hodgson et al., 2018; Mateos-Cárdenas et al., 2020). For instance, recent studies evidenced that amphipods, such as

Orchestia gammarellus and *Gammarus duebeni*, can ingest and shred plastic items, leading to the formation of microplastics fragments (Hodgson et al., 2018; Mateos-Cárdenas et al., 2020).
Microplastics enter environment from various sources through various routes so that performing a homogeneous evaluation of the contamination is challenging. Overall, the most common enter routes for MPs in environment are displayed in Figure 4 and are: (1) release from outlet water of WWTPs, both MP used in personal care products (Duis et al., 2016) or released as fibers from clothes after the use of washing machines (De Falco et al., 2019); (2) application of biosolids or sewage sludge from WWTPs to agricultural lands (Carr et al., 2016; Mintenig et al., 2017); (3) storm water overflow events (Chen et al., 2020); (4) incidental release (e.g. during tyre wear) or break down of larger plastic items present in environment (Hurley et al., 2018; Corradini et al., 2019); (5) release from industrial products or processes; and (6) atmospheric deposition of fibers (Dris et al., 2015).

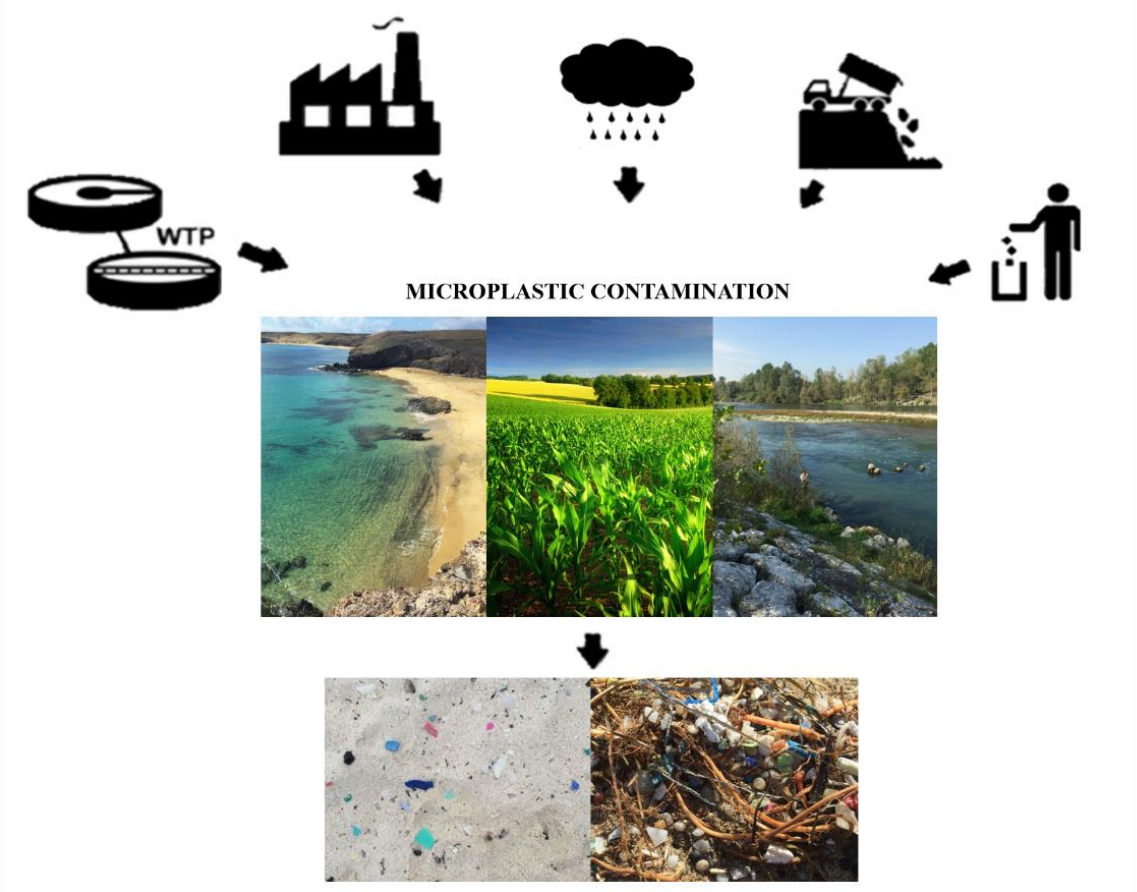

Figure 4: The most common enter route for MPs in aquatic and terrestrial environment.

Furthermore, MPs are not stationary but can move among different environmental compartments according to their physic-chemical (i.e., density) characteristics. For instance, MPs in soil could enter the riverine systems and, depending on weather condition, being transferred to marine

environment where they can float, following the sea current, or sink to the sediments. In detail, the movement of MPs from soil ecosystems to aquatic ones may occur through overland run-off or dispersion (*via* cutting action) to roadside ditches. Recent data showed that MP contamination is particularly severe in estuaries, highlighting that land littering and river input are important sources of microplastics to coastal and marine environments (Sadri and Thompson, 2014; Gallagher et al., 2016; Vendel et al., 2017; Li et al., 2020;).

Nowadays, MPs have been found all over the world, not only along beaches (Imhnof et al., 2013; Lots et al., 2017), shores (Kazour et al., 2019), surface waters (Eriksen et al., 2015; Zhang et al., 2019) and sediments from marine and freshwater environments (Wagner et al., 2014), but also in remote areas, such as deep sea (Wright, Thompson, and Galloway 2013; Law and Thompson 2014) and glaciers (Ambrosini et al., 2019). In spite of these findings, a wide degree of uncertainty concerns the environmental concentrations of MPs and the consequences of their presence towards organisms. Indeed, environmental MPs are defined as a wicked problem, due to the considerable complexity and difficulty to have a standardized protocol to investigate the impact of these synthetic materials on the natural world (Wagner and Lambert 2018). Recent studies showed that MPs in oceans sediments could be more abundant than the ones in the water column (Xiong et al., 2018) and that there are regional differences in the contamination connected by the geographical localization of the analyzed site. For example, sites close to industrialized areas or near an outlet water of a WWTPs might be several folds more contaminated than in remote areas (Guo et al., 2019). However, the isolation and the measure of MPs in the environment could be very challenging, considering the wide range of polymers present and the diversity of the analyzed sample (such as water, sediments and soils). Moreover, considering the variability of microplastics in the aquatic environment, several studies showed how microplastics are distributed along the water column in relationship with their physical and chemical features, size, density and shape (Kooi et al., 2016; Kowalski et al., 2016).

Overall, the concentration of MPs in the water column is measured to range from 1 MPs/m^3 to hundred MP/m^3, however, high variability between-site, as well as the different units used for microplastic quantification, complicate the comparison of MPs levels in ecosystems (Wagner and Lambert 2018). For instance, a recent review by Guo and Wang (2019) highlighted how in Northwestern Pacific the abundance of microplastics is 1.0×10^4 items/km^2 (Pan et al., 2018), while in Atlantic Ocean this value is 1.15 ± 1.45 items/m^3 (La Daana et al., 2017).

As for marine environment, several studies reported a widespread pollution and high heterogeneous concentrations of MPs also for freshwater ecosystems. Indeed, freshwaters act as a

major transport way for plastic pollution and it has been estimated that about 70 - 80% of the plastic found at sea derived by freshwaters (Akdogan et al., 2019). Microplastics presence was confirmed for several rivers across Europe (Mani et al., 2015; Guerranti et al., 2017; Horton et al., 2017b), America (Vermaire et al., 2017; Kapp and Yeatman, 2008) and Asia (Peng et al., 2018; Lahens et al., 2018). Furthermore, over the last decade several studies highlighted the contamination of lakes in each continent. In detail, microplastics presence have been reported in Great Lakes of North America (Eriksen et al., 2013; Hendrickson et al., 2018), in African Great Lakes (Biginagwa et al., 2016), in Italian sub alpine lakes (Fischer et al., 2016; Sighicelli et al., 2018) and in twenty urban lakes of Wuhan (Wang et al., 2017). Overall, the estimated MPs contamination in freshwater was reported to be 0.001 - 0.1 items/m^2 in lake water, 0.1 -1 items/m^2 in rivers, while the estimated contamination in sediments was much higher, with 10 - 10,000 items/m^2 for lakes and 1 -1,000 items/m^2 for rivers (Dris et al., 2015).

Although the major MPs sink appear to be the oceans, followed by freshwaters, the contamination of the terrestrial ecosystems should not be neglected. Recent studies highlighted that soils might be the main sink of MPs and that the total amount of MPs in terrestrial ecosystems could be more than 20-fold higher compared to marine ecosystems (Zhang and Liu, 2018). Microplastics enter terrestrial ecosystems mainly by agricultural practices (e.g., water pipes, plastic mulching and greenhouse covers), as well as water-runoff from urban areas, illegal dumping sites or break-down of larger plastic items (Hurley et al., 2018; Corradini et al., 2019). In addition, recent studies showed that sewage sludge retains the 95% of the MPs entering wastewater treatment plants (Carr et al., 2016; Mintenig et al., 2017). Therefore, the use of sewage sludge and wastewater-irrigation could be considered as the major sources of MPs input in agricultural soils (Li et al., 2020). Despite these findings, the information on MPs abundance in soils is still limited. Nizzetto and co-authors (2016) have estimated that up to 430,000 and 300,000 tons of MPs enter annually agricultural soils in Europe and North America, respectively. *In-situ* studies found that MPs concentration in agricultural soils from Switzerland and Chile were up to 55.5 mg/kg and 12.9 mg/kg, respectively (Scheurer and Bigalke 2018; Corradini et al., 2019). Other studies have shown that the concentration in industrial soils from Australia was up to 67,500 mg/kg (Fuller and Guatam, 2016), while negligible levels have been measured in forests close to agricultural soils in China (Zhang and Liu, 2018).

Monitoring studies showed that microplastic found in both marine, freshwater and soil ecosystems display a wide range of different morphologies going from regular shaped sphere to irregular shaped fragments, having different polymeric composition, form and size (Helm et al., 2017).

Several studies have evidenced how microplastics that can be commonly found in environment could be divided into six basic types, depending on the plastic polymer they are made, such as polyethylene (PE), polypropylene (PP), polyamide (PA), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PUR) and polyethylene terephthalate (PET) (Hidalgo-Ruz et al., 2012; Van Cauwenberghe et al., 2015a; Pitt et al., 2018). Among them, MPs made of polymers having a density lower than water (< 0.99 g/cm^3), such as PP and PS are commonly found floating in the water column, while polymers such as PET and PVC having a density higher than water (> 1.35 g/cm^3), sink, settle and accumulate on sediments (Andrady, 2011). Moreover, in order to categorize the huge diversity of MPs in environment, monitoring studies often subdivide MPs into shape-categories, such as spheres, fibers, foams, and irregular fragments. While regular industrial microplastics sphere only represent a small fraction of the MPs found in environments, recent studies highlighted that fibers are the most dominant plastic form that could be found in environment followed by irregular shaped fragments and that only a little amount is represented by regular spheres (Lusher et al., 2015; Martin et al., 2017; Wagner and Lambert 2018).

Once in environment, considering their small size, MPs are bioavailable for ingestion by wide range of organisms, both aquatic and terrestrial ones, as they overlap with the size range of their food (Galloway et al., 2017). The ingestion of MPs could happen through direct active ingestion or through respiratory surfaces, while dermal uptake is limited (Wagner and Lambert 2018).
Several studies evidenced the presence of MPs in the digestive tract of invertebrates (Lusher et al., 2017; Weber et al., 2018; Messinetti et al., 2018; Windsor et al., 2019; Botterell et al., 2019), as well as vertebrates (do Sul et al., 2014; Lusher et al., 2018), both in aquatic and terrestrial ecosystems (Messinetti et al., 2018; Botterell et al., 2019; Song et al., 2019; Xiang et al., 2019). The ingestion of MPs could result in different effects whose magnitude depends on a number of factors, first of all the size of the ingested MPs. Indeed, smaller MPs are likely to transit into in the digestive tract of organism and to accumulate, while on the contrary bigger MPs could be harder to egest and their permanence into the digestive tract could lead to the starvation of the organisms (Besseling et al., 2013). Besides dimension, also shape can be a determinant of potential negative effects induced by MPs exposure. Irregular shaped MPs, whit needle-like contours, by rubbing against organism tissues (such as gills or digestive tracts) could lead to injuries and inflammation situations and therefore are more likely to cause damage than round, smooth particles. Moreover, considering their irregular shaped form these MPs are likely to remain for long into the digestive tract of organisms, determining stronger negative effects. Indeed, a study by Frydkjær and co-authors (2017) showed that *Daphnia magna* organisms were able to ingest both regular and irregular PE-MPs (concentration range 0.0001 – 10 g/L) but the egestion of

regular MPs was faster compared to irregular MPs, which affected the mobility of the organisms more than regular ones.

Additionally, several studies showed that the feeding strategy is one of the main factors driving the ingestion of MPs. For instance, suspension feeders organisms (e.g., protozoans, rotifers, cladocerans and mussels) by feeding on suspended particulate matter, without discriminating the ingested material, have an higher probability to ingest MPs floating in the water column rather than neustonic or pelagic organisms, which discriminate between edible and non-edible-material. In addition, benthic deposit feeders that forage for food in sediment might mainly ingest MPs that sink and accumulate in sediments (Wright et al., 2013; Setälä et al., 2014). Despite the difference in the feeding strategies, the capability to ingest MPs was confirmed for a wide range of different invertebrate and vertebrate species, both aquatic and terrestrial ones. For instance, the capability to ingest microplastics was pointed out for more than 160 marine (Lusher, 2015 and reference therein) and 39 freshwater species (Scherer et al., 2017), including crustaceans (Cole et al., 2015; Canniff and Hoang, 2018; Eltemsah et al., 2019; Wang et al., 2019), rotifers (Jeong et al., 2016), mollusks (Sussarellu et al., 2015; Weber et al., 2020), echinoderms (Della Torre et al., 2014; Messinetti et al., 2018), fish (Lu et al., 2016; Alomar et al., 2017; Barboza et al., 2018; Ding et al., 2018; Wan et al., 2019), amphibians (Hu et al., 2018; Boyero et al., 2020), marine reptiles (Caron et al., 2018; Duncan et al., 2018), seabirds (Masiá et al., 2019; Carlin et al., 2020) and marine mammals (Besseling et al., 2015; Fossi et al., 2016; Moore et al., 2020) Moreover, the capability to ingest microplastics was confirmed also for several terrestrial organisms such as earthworms (Rodriguez-Seijo et al., 2019; Wang et al., 2019), mites (Zhu et al., 2018), collembola (Maaß et al., 2017) and snails (Panebianco et al., 2019; Song et al., 2019).

The ingestion of microplastics and their presence in the digestive tract of organisms could lead to negative effects at multiple levels of the biological hierarchy, with the final consequence of the death of the organism itself (Akdogan et al., 2019). Several studies showed a wide range of negative effects in marine, freshwater and terrestrial species exposed to different types of MPs at sub-individual level (e.g., imbalance of the oxidative status, DNA damage and differential gene expression; Della Torre et al., 2014; Avio et al., 2015; Karami et al., 2016; Jeong et al., 2016), histological damage and inflammatory response at tissue and organ level (von Moos et al., 2012; Lu et al., 2016; Pedà et al., 2016), as well as changes in the growth rate and some behavioral tasks (de Sà et al., 2015; Watts et al., 2016; Rist et al., 2016; Barboza et al., 2018; Choi et al., 2018).

Although the presence and potential effects of MPs was confirmed by a growing number of studies, these investigations are still in their infancy and deserve to be further improved. Different questions remain unsolved regarding the determinants of MPs potential toxicity towards non-target

organisms. Therefore, considering the wide range of different MPs found in environment, in this project the attention was focused on two of the most commonly found plastic polymers, namely polyethylene terephthalate (PET – Figure 5a) and polystyrene (PS – Figure 5b).

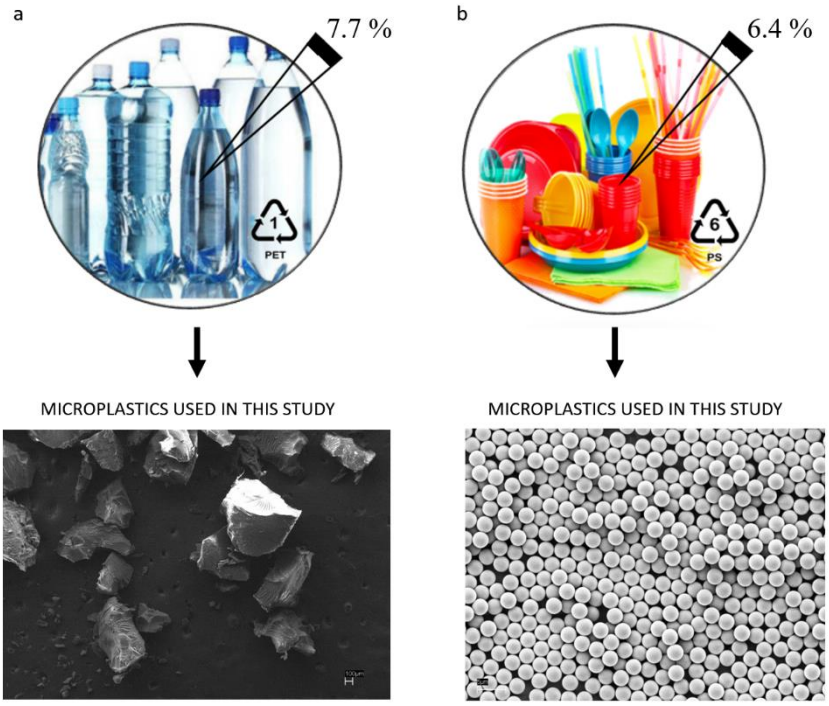

Figure 5: Schematic representation of application and percentage of use of polyethylene terephthalate (b) and polystyrene (a) followed by a Scanning Electronic Microscope image of the microplastics standard used in this project.

This choice was addressed by the different chemical features, and consequently, different environmental fate of these plastic polymers. In fact, because of its low density, PS tends to float in the water column, while on the contrary, because of its high-density PET sinks. For this reason, the administration of PS or PET MPs allowed to investigate the potential adverse effects towards organisms with different feeding strategies and ecological role in ecosystems.

1.1.3.1 Polystyrene

Polystyrene $(C_8H_8)_n$, (PS; Figure 6), is a polymer of styrene (Andrady and Neal, 2009) which has a wide variety of applications ranging from packaging (Marsh and Bugusu, 2007), household appliances (e.g., blenders, air conditioners, refrigerators and microwaves) and electrical and

electronic equipment (Chaukura et al., 2016). Furthermore, PS is also used in toys, televisions, CD or DVD fabrication (Inagaki and Kiuchi, 2001).

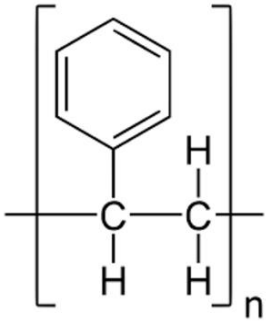

Figure 6: Chemical structure of polystyrene.

The newest Plastic Europe (2019) report showed that the last-year consumption of PS reached the 6.4% of total European plastic consumption. Because of its wide range of use, PS is one of the most commonly found polymer in environment, both as macro- and microplastics (Wagner and Lambert 2018). In fact, PS items own low density and can easily be scattered by wind and move from an environmental matrix to another one, contributing to a widespread contamination (Chaukura et al., 2016). Several monitoring studies have shown the presence of PS in seawaters, freshwaters and also in soils (Wagner and Lambert 2018; Li et al., 2020). The capability to ingest PS microplastics (PS-MPs) was pointed out in invertebrates, such as crustaceans (Cole et al., 2015; Canniff and Hoang, 2018; Eltemsah et al., 2019; Wang et al., 2019), mollusks (Sussarellu et al., 2015; Weber et al., 2020), echinoderms (Della Torre et al., 2014; Messinetti et al., 2018), as well as in vertebrates, such as amphibians (Hu et al., 2016) and fish (Lu et al., 2016; Ding et al., 2018; Wan et al., 2019). Moreover, the ingestion of PS microplastics was observed also in some soil organisms, such as earthworms (Wang al., 2020) and collembolans (Xiang et al., 2019).

The ingestion of this plastic type is known to induce a widespread range of negative effects ranging from the sub-lethal effects, such as the increase in the production of reactive species (Paul-Pont et al., 2016; Yin al., 2018) and the alteration of enzyme activity (Jeong et al., 2016; Gambardella et al., 2017), to the reduction in the growth rate or in the fecundity (Cole et al., 2015; Sussarellu et al., 2015), the alteration in several behavioral tasks (Gambardella et al., 2017; Yin et al., 2018; Wang et al., 2020) and eventually the death of the organisms (Lee et al., 2013). For instance, Cole and co-authors (2015) have shown that the ingestion of 20 µm PS-MPs (75 MPs/mL) by the copepod *Calanus helgolandicus* affected its survival and fecundity. The decrease in fecundity was confirmed also in the copepod *Tigriopus japonicus* exposed to 6 µm PS-MPs (313 µg/mL; Lee et al., 2013). The exposure to PS-MPs (0.125; 1.25, 12.5 and 25 µg/mL) affected the metamorphosis

of the juveniles of the ascidian *Ciona robusta* and the development of sea urchin (*Paracentrotus lividus*) plutei (Messinetti et al., 2018). In addition, Yin and co-authors (2018) recorded a decrease in swimming and exploration ability in the marine jacopever (*Sebastes schlegelii*) after exposure to 15 µm PS-MPs (1 x 10^6 MPs/L), while Sussarellu and co-autors (2015) highlighted a decrease in oocyte number and in sperm velocity in oysters (*Crassostrea gigas*) after the exposure to PS-MPs (6 µm; 0.023 mg/L).

1.1.3.2 Polyethylene terephthalate

Polyethylene terephthalate $(C_{10}H_8O_4)_n$, (PET; Figure 7), is a semi-crystalline, thermoplastic polyester and it is the most representative polymer of the polyester group (Awaja and Pavel, 2005). Its applications include a wide range of fields, including food and beverage packaging, (i.e., bottles for water, soft drinks, juices, cleaners).

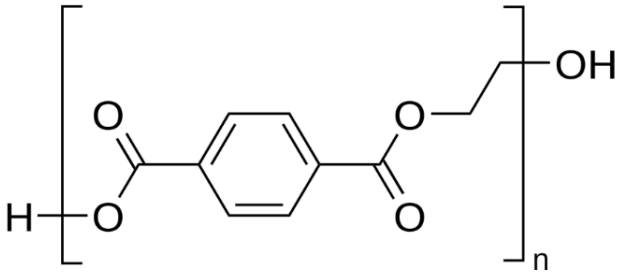

Figure 7: Chemical structure of polyethylene terephthalate.

It was estimated that PET consumption accounts for up to 7.7 % of total European plastic consumption, with a demand of ~4,000,000 tonnes per year (Plastics Europe 2019). Considering its high density (~ 1.37 – 1.45 g/cm^3), in aquatic ecosystems PET is prone to sink and accumulate in sediments where they can represent a potential threat for benthic organisms. PET items represent one of the most common plastic waste found in environment (Sinha et al., 2010), so that PET microplastics were found to significantly contribute to overall MPs load in large European river systems (Klein et al., 2015; Gasperi et al., 2014) and polyester microfibers were found to be the most abundant plastic polymer in deep-sea sediments from the Atlantic and Indian Oceans, and Mediterranean Sea (Woodall et al., 2014). Moreover, focusing on soil ecosystems, several studies highlighted that PET is one of the most widespread polymers found (Duis and Coors, 2016; Ng et al., 2018; Li et al., 2020), deriving from breakage and erosion of large plastic items used in agriculture or contained in sewage sludge (Li et al., 2020).

Differently than PS and other polymers, few studies have focused their attention on PET potential toxicity towards aquatic and terrestrial organisms. The reason can be identified in the lack of an analytical standard that can be purchased on the market. The few studies currently available demonstrated that PET ingestion occurs in crustaceans (Jemec et al., 2016; Weber et al., 2018), sea cucumbers (Mohsen et a., 2019), fish (Alomar et al., 2017) and terrestrial snails (Song et al., 2019). Moreover, the potential negative effect related to PET exposure were investigated only by few works in crustaceans (Jemec et al., 2016; Weber et al., 2018), mussels (Provenza et al., 2020) and towards a soil invertebrate, the giant land snail (Song et al., 2019). In detail, PET exposure (10 – 150 µm; 0.8 - 4,000 MPs/mL) did not affect the survival, the feeding activity and the energy reserves in *Gammarus pulex* (Weber et al., 2018), while the exposure to PET-MPs fibers (12.5 – 100 mg/L; average dimension 300 µm) caused the death of *Daphnia magna* daphnids (Jemec et al., 2016). Moreover, PET-MPs exposure induced oxidative stress in the mussel *Mytilus galloprovincialis* (0.5 – 3 mm; 0.1 g /L; Provenza et al., 2020) and in the land snail, *Achatina fulica* (0.01–0.71 g/Kg dry soil weight; Song et al., 2019).

1.2 Multi-level and multi-organism approach

The need of deeply investigate the propagation of negative effects through different levels of the biological hierarchy rises from the awareness that, to date, the majority of ecotoxicological studies on the toxicity of environmental contaminants focused their attention only on potential negative effects at the lower levels of the biological hierarchy, such as biochemical and molecular levels, while little is known on the potential toxicity at higher levels, such as individual or population levels.

In detail, from the last twenty-five years, short-term effects on low levels of biological organization have been used to explore the early signs of health status impairment caused by the exposure to environmental stressors, including contaminants (Newman and Jagoe, 2006; Dalzochio et al., 2016). This evaluation have relied on the so-called biomarkers, defined as a biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body fluid samples, or at the level of whole organism that provide evidence of the exposure to and/or effects of one or more chemicals and/or radiations (Depledge, 1993). From their first appearance to date, biomarkers have received a great attention because of their peculiar features, such as easy application and quick response, becoming the most commonly tool to investigate the effects of a pollutant and environmental contamination (Newman and Jagoe, 2006). Several studies performed under field

and laboratory conditions, have highlighted the effectiveness and reliability of biomarker use, showing that biological responses seen at the sub-individual level of the bio-ecological hierarchy might be useful indicators of environmental pollution (Dalzochio et al., 2016). For instance, the evaluation of the antioxidant or detoxification enzymatic activity as well as the presence of oxidative damages are some of the most commonly analyzed sub-individual biomarkers, showing early response to pollutant exposure (Bonnail et al., 2016; Moncaleano-Niño et al., 2018). However, one of the major concerns related to the use of biomarkers, is that, the early warning signals pointed out at sub-individual level have a low ecological relevance and return no or limited information on the possible effect at higher level of the ecological hierarchy. Indeed, when an organism is exposed to a pollutant, the adverse effects induced can be classified according to the levels of organization in bio-ecological hierarchy, which are linked mechanistically in a bottom-up order (Figure 4). The adverse effects of toxicants begin at lower level of organization and then this signal tends to propagate to higher hierarchical levels of the bio-ecological organization (from cellular up to community or ecosystem). The propagation of that signals leads to a plethora of effects that can affect the eco/ethological performances of exposed individuals and populations, and in ultimate analysis on the relationships of communities.

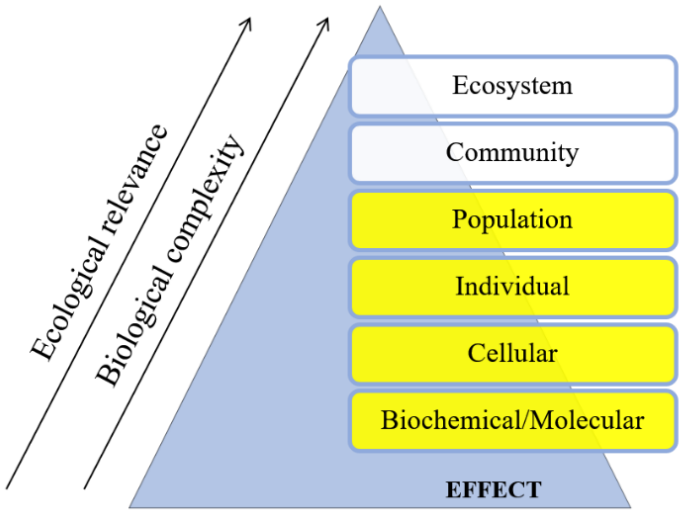

Figure 8: Schematic representation of the propagation of the effect induced by a contaminant at different levels of the biological organization.

However, the extrapolation of robust conclusions concerning the effects at higher levels of the biological hierarchy using information coming only from the biochemical or molecular biomarkers, is difficult, because the links between hierarchical levels are far from straightforward

and sometimes even misleading (Forbes and Calow, 2002; Hagger et al., 2006). In addition, until now, ecotoxicological studies investigated the adverse effects of contaminants by focusing separately on sub-lethal and/or behavioral effects, largely neglecting the possible links underlying the propagation of this effects over the ecological hierarchy.

To overcome this problem, in the last years, some authors suggested to integrate the information coming by the use of biomarkers assessing effects at sub-individual levels with that coming from more ecologically relevant biomarkers, such as those investigating effects at individual and population levels (e.g., change in a specific behavior or in reproduction) in order to obtain a better prediction of the effects and the risks related to the exposure to a specific contaminant at the ecosystem level (Forbes and Calow, 2002). Indeed, a sub-individual effect can be considered as ecologically relevant only if it results in an impairment of the organism function, such as an impairment in fitness or in some other life activities (Lam and Gray, 2003). In order to reach this goal, some authors suggest the necessity to use an integrate and multi-level approach, mixing the information obtained from sub-individual and higher level of the biological organization, in order to shed a light on the propagation of the effects from one level to other ones. Indeed, a multi-level approach including scientifically based combinations of endpoints coming from different levels and of different model organisms it is essential for evaluating the complexity of potential biological and environmental effects induced by a pollutant (Pereira et al., 2014; Reyna et al., 2019).

Another issue to be considered is related to the biological model on which the effects of contaminants are assessed. Classical ecotoxicology studies rely on few model organisms in order to understand how the exposure to different contaminants can affect organisms and environment health status (Jha, 2004, 2008; Cheung et al., 2006;). However, extrapolating the result obtained from one or few species to the whole ecosystem might be speculative and fail to protect the wide range of species present in natural environments. Indeed, in order to assess the effects and related risk of a contaminant towards the ecosystem, species-specific differences in response to the exposure to a specific contaminant need to be taken in account. To date, most of the studies present in literature have investigated the effects induced by contaminants using one model organism only. However, there is a growing concern and awareness that the use of a single model organism to define the effects associated to a specific contaminant is not enough to be used in risk assessment evaluations (Amiard-Triquet 2009). To date, the few studies that have focused on this issue showed that the exposure to the same contaminant, chemical or physical, might induce differential, species-

specific responses, which depend on the sensitivity of the model organism used (Wong et al., 2010; Schiavo et al., 2018).

Thus, the use of a number of different model organisms should be necessary to shed light on differential effects of a specific contaminants towards organisms with different life-history traits and ecological role in the ecosystems. This approach should allow to enlarge the information on the toxicity of a contaminant in the environment and to predict potential adverse consequences on ecosystems.

Chapter 2 – *Aim of this project*

The attention of the present project was focused on two different classes of emerging pollutants, namely illicit drugs (PAPER I, II, III) and microplastics (PAPER IV, V, VI, VII, VIII).
The attention was focused on both illicit drugs and microplastics because, in contrast with PPCPs, they represent understudied classes of emerging contaminants. In fact, although their presence in different ecosystems have been confirmed by a number of studies, there is still a dearth of information regarding their possible negative effects towards non-target organisms. Moreover, the rationale of the present project took in consideration the need for an increase in knowledge regarding the propagation of the effect induced by a pollutant following the bio-ecological hierarchy. Indeed, this is particularly true for emerging contaminants, such as illicit drugs and microplastics, because until now, there is a deficiency of information on how and if the effects measured at a given biological level can spread to the other hierarchical levels.

Thus, the main aim of this Project was to investigate the effects induced by illicit drugs and microplastics towards different aquatic and terrestrial non-target organisms, in order to enlarge the scientific knowledge on the potential hazard of these emerging contaminants. This goal was achieved by performing different experiments aimed at evaluating the negative effects induced by the exposure to illicit drugs (i.e., cocaine and methamphetamine) or microplastics (i.e., PS or PET MPs), at different levels of the biological hierarchy on opportune aquatic and terrestrial biological models. In detail, a multi-level approach based on the investigation of effects at sub-individual, individual and, if possible, population level, was performed in order to investigate the possible propagation of the effect from a low to high levels of the biological hierarchy.

In detail, effects at sub-individual level were evaluated by a battery of different oxidative stress biomarkers, including assays investigating the amount of reactive oxygen species, the modulation of antioxidant and detoxifying enzymes and oxidative damage, while histological analyses were performed to explore potential effects at tissue or organ level. Moreover, effects at individual level were assessed through the evaluation of changes in growth rate and/or locomotor behavior. Lastly, only for experiments having as model organisms *Daphnia magna*, reproductive fitness was investigated to check for potential effects of contaminants at population level. This approach was applied on six different non-target model organisms representative of both freshwater (*Daphnia magna* and *Xenopus laevis)*, marine (*Mytilus gallopovincialis, Ruditapes philippinarum* and *Paracentrotus lividus*) and terrestrial ecosystems (*Achatina reticulata*).

Specifically, the present thesis summarizes the results obtained during the three-year Project. The research activities were grouped in two different sections, with different goals.

The first section includes the results of the studies investigating the adverse effects induced by the exposure to environmentally relevant concentrations of cocaine (COC) or methamphetamine (METH) towards the freshwater cladoceran *Daphnia magna*. In detail, the main goal of the experiments explained in this first section was to expand the knowledge about the propagation of effects of COC and METH from the sub-organismal level up to individual and population level, by linking changes in sub-individual (oxidative stress-related biomarkers), individual (swimming behavior) and population (reproduction) effects in *D. magna*.
Moreover, considering that in environment illicit drugs occur as complex mixtures, whose toxicity cannot be accurately evaluated by analyzing the effects obtained by the exposure to a single molecule, it was performed a second study aimed at assessing the adverse effects induced, at sub-individual level only, by the exposure to an environmentally relevant concentration of COC and its main metabolite (i.e., benzoylecgonine, BE), singularly and in mixture, towards a marine species, the mollusks *Mytilus galloprovincialis*.

The second section includes the results of the studies investigating the adverse effects induced by the exposure to increasing concentrations of regular polystyrene microplastics towards two aquatic species, a freshwater invertebrate (the cladoceran *Daphnia magna*) and a freshwater vertebrate (the tadpoles of the amphibian *Xenopus laeivis*). The main goal of this section was to expand the knowledge about the ingestion and potential toxicity induced by microplastics towards freshwater organisms by using different endpoints at individual (body length and swimming behavior) and, when possible, population level (reproduction). Moreover, considering that in environment the majority of the microplastics own an irregular shape we performed three different experiments to assess the adverse effects induced by the exposure to different concentrations of irregular shaped polyethylene terephthalate microplastics towards two marine species (*Ruditapes philippinarum* and *Paracentrotus lividus*) and a terrestrial one (*Achatina reticulata*). In detail, we investigated the potential negative effects by evaluating changes in sub-individual (oxidative stress related biomarkers or tissue damage) level for *R. philippinarum* and *P. lividus* experiments, while both sub-individual (oxidative stress related biomarkers) and individual (growth rate) level endpoints were investigated for *A. reticulata*.

Chapter 3 – *Publications*

In this chapter, the main results obtained during this three-year project will be grouped in two sections. Section 1 will summarize the results of the studies investigating the adverse effects induced by the exposure to illicit drugs towards opportune model organisms (PAPER I, PAPER II, PAPER III), while section 2 will summarize the results obtained from the experiments aimed at investigating the potential toxicity of microplastics towards aquatic and terrestrial organisms (PAPER IV, PAPER V, PAPER VI, PAPER VII AND PAPER VIII)

Section 1: Illicit drugs toxicity studies

Considering the presence of illicit drugs in environments, in order to enlarge the knowledge regarding the possible negative effects induced by the exposure to these molecules, two studies were performed in order to investigate the adverse effects induced by two of the most commonly found stimulants in aquatic environment, namely cocaine (COC), a plant-based drug, and methamphetamine (METH), a synthetic drug.

Two experiments, whose results are reported in detail in PAPER I and PAPER II, were performed by exposing the freshwater crustacean *Daphnia magna* for 21 days to two ecologically relevant concentrations (50 ng/L and 500 ng/L) of cocaine or methamphetamine. We assessed toxicity of these illicit drugs through a multi-level approach. In detail, adverse effects induced by COC and METH were tested at different levels of the biological hierarchy. Effects sub-individual level were investigated by a battery of oxidative stress-related biomarkers focusing on the amount of reactive oxygen species, the activity of antioxidant and detoxifying enzymes and lipid peroxidation. Behavioral endpoints were used to assess effects at individual and population levels; effects at individual level effects were investigated by a video-tracking analysis, in terms of alteration in the swimming activity of cladocerans, while the alteration in reproductive effort was used as a proxy of effects at population level. Our findings suggested that the exposure to the same ecologically relevant concentrations of both COC and METH altered the oxidative status of treated cladocerans compared to untreated conspecifics. In contrast, molecule-depending effects were observed for behavioral endpoints. Indeed, while COC affected the swimming activity and, consequently decreased the reproductive effort of *D. magna* organisms, METH did not modulate swimming activity but induced an unexpected increase in reproduction. These data suggest that natural and synthetic illicit drugs can elicit a similar mechanism of action, which involved the onset of oxidative stress, returning different behavioral outcomes. Further studies should be necessary to

investigate in depth the molecular pathways determining different behavioral effects in cladocerans exposed to illicit drugs with a similar mode of action at biochemical level.

The studied mentioned above investigated the toxic effects due to the independent exposure to a specific illicit drug. However, in environment, illicit drugs occur as complex mixtures, whose toxicity cannot be accurately evaluated by analyzing the effects cause by the exposure to a single molecule. For this reason, in PAPER III are reported the results of an experiment aimed at investigating the adverse effects induced by independent and combined exposure to an environmental concentration of cocaine and its main metabolite benzoylecgonine (BE) towards the Mediterranean mussel *Mytilus galloprovincialis*. Despite previous papers used as model organism the freshwater cladoceran *D. magna*, the experiment described in PAPER III was performed on *M. galloprovincialis* because previous studies on different mussel species investigated the toxicity of independent exposure to COC and BE (Binelli et al., 2012; Parolini et al., 2013; Pedriali et al., 2013; dos Santos Barbosa Ortega et al., 2019), allowing to formulate *a priori* expectation on the outcomes of the experiments. In detail, we assessed the possible negative effect induced at sub-individual level by a 96-hours exposure to COC (500 ng/L) and BE (20 ng/L), both independently and in mixture. The effects of the exposure to COC, BE and their mixture was assessed in the gills and the digestive glands isolated by mussels through the application of an oxidative stress biomarker battery. In detail, we investigated the changes in the amount of reactive oxygen species, modulation of the activity of the antioxidant and detoxifying enzymes, as well as oxidative damage. Our findings demonstrated that the exposure to an environmentally relevant concentration of COC and BE, both singularly and in mixture, can induce a modulation of the oxidative status of mussels, whereby mixtures induced a more marked effect compared to the exposure to the single molecules.

Overall, the results found in these studies highlighted how environmentally relevant concentration of the most commonly found illicit drugs in aquatic ecosystems can induce detrimental effects in both freshwater and marine invertebrate species, confirming the role of oxidative stress in their mechanism of action. Moreover, considering the continuous and increasing use of illicit drugs worldwide, the current environmental levels might increase, resulting in potentially worse effects at all the levels of the ecological hierarchy. Lastly, considering that in natural ecosystems organisms are exposed for their whole lifespan to different illicit drug mixtures, further studies are necessary in order to investigate long-term consequences of different illicit drug ‘cocktails’ and to enlarge the knowledge on the risk of these contaminants towards non-target organisms.

PAPER I

De Felice B., Salgueiro-Gonzàlez N., Castiglioni S., Saino N., Parolini M.

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Biochemical and behavioral effects induced by cocaine exposure to *Daphnia magna*

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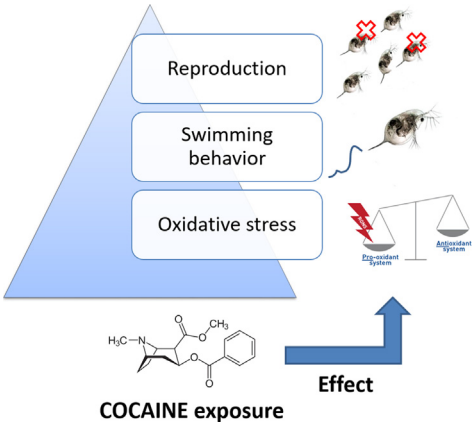
HIGHLIGHTS

- Cocaine exposure altered the *D. magna* oxidative status.
- Cocaine altered the swimming behavior depending on the tested concentration.
- High cocaine concentration reduced the reproductive output of *D. magna*.
- Cocaine might represent a threat for freshwater zooplanktonic species.

GRAPHICAL ABSTRACT



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ABSTRACT

Illicit drugs and their metabolites have been identified as emerging aquatic pollutants. Cocaine (COC) is one of the most used illicit drug worldwide. After human consumption, COC enters the aquatic ecosystems, where it is commonly detected in ng L^{-1} concentration range. Although a number of studies have shown that the exposure to environmental concentrations of COC can induce diverse biochemical, molecular and histological effects on aquatic organisms, the information of COC-induced behavioral alterations is scant. Thus, the present study aimed at exploring both biochemical and behavioral effects induced by the exposure to two environmental concentrations (50 ng L^{-1} and 500 ng L^{-1}) of COC on the freshwater cladoceran *Daphnia magna*. Specimens were exposed to selected COC concentrations for 21 days and the effects on the oxidative status, including the amount of reactive oxygen species and the activity of antioxidant (SOD, CAT and GPx) and detoxifying (GST) enzymes, and swimming activity were investigated after 7, 14 and 21 days of treatment, while effects on reproductive success was assessed after 21-days only.. Exposure to COC induced an overproduction of reactive oxygen species and a modulation of the activity of defense enzymes. Moreover, COC affected the swimming behavior and altered the reproductive success of treated specimens. Our results highlighted that environmental concentrations of COC can cause adverse effects at different levels of the biological hierarchy in a zooplanktonic species, confirming the potential threat due to this illicit drug for the aquatic community.

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1. Introduction

Illicit drugs have been considered for a long time as a dramatic socio-economic and public health problem. However, only recently they have been identified as a serious environmental issue, attracting the interest of analytical and environmental chemistry (Zuccato and Castiglioni, 2009), as well as ecotoxicology (Capaldo et al., 2018; Parolini et al., 2013, 2017). Among illicit drugs, cocaine (COC), a psychostimulant affecting human behavior and brain physiology by the alteration of dopamine release from dopaminergic neurons (Jeon et al., 2008), represents one of the most used illicit drugs worldwide (UNODC, 2018). Indeed, the recent World Drug report (2018) has estimated that the global amount of COC users ranges between 13.9 and 229 million people (age range 15–64), showing an increase of ~7% compared with the previous years (UNODC, 2018). After the ingestion of a COC dose, this drug undergoes hepatic metabolism and is excreted through urine and feces as two main metabolites, namely benzoylecgonine (BE, 45% of the dose) and ecgonine methyl ester (EME, 40%), and limitedly (1–9%) as unchanged parental compound (Baselt, 2004). Thus, COC continuatively enters the sewage, whereby it has been monitored in concentrations up to 420 ng L^{-1} in the inlet water of wastewater treatment plants (WWTPs; Pal et al., 2013 and references therein). Moreover, considering that WWTPs cannot efficiently remove COC from the sewage, it reaches surface waters, whereby it was detected in concentrations ranging between 0.4 and 44 ng L^{-1} (Pal et al., 2013 and the reference therein), although two recent monitoring surveys carried out in Brazilian surface waters reported concentrations up to 5,896 ng L^{-1} (Thomas et al., 2014; Pereira et al., 2016). Despite the low COC concentrations currently found in aquatic ecosystems, the risk for the aquatic communities cannot be neglected. Although the toxicity of COC was well-known on humans (Leri et al., 2003; Spronk et al., 2013) and murine organisms (Brami-Cherrier et al., 2005; Dixon et al., 2010), the information on aquatic organisms is still limited. A preliminary study showed that exposure to three increasing COC concentrations (range 40 ng L^{-1} - 10 μg L^{-1}) induced cytotoxic and genotoxic effects on the freshwater bivalve *Dreissena polymorpha* (Binelli et al., 2012). Similar cyto-genetic effects have been found in 96 hours post fertilization (hpf) larvae of zebrafish (*Danio rerio*) exposed to COC (0.01–10 μg L^{-1} range) and have been caused by an overproduction of reactive oxygen species (ROS) that imbalanced the oxidative status of larvae (Parolini et al., 2017). A companion proteomic study has revealed that the exposure to 0.3 and 1 μg L^{-1} of COC modulated the protein profile of 96 hpf zebrafish larvae, changing the expression of several proteins belonging to different functional classes, including cytoskeleton, eye constituents, lipid transport, lipid and energy metabolism, and stress response (Parolini et al., 2018a). Cyto-genotoxicity has been observed on the brown mussel (*Perna perna*) after the exposure to crack COC (0.5, 5.0, and 50.0 μg L^{-1}; dos Santos Barbosa Ortega et al., 2018). Gay et al. (2013) have demonstrated that an environmental concentration of COC (20 ng L^{-1}) modulated the levels of brain dopamines, catecholamines and pituitary activity, and induced histological alteration in diverse tissues and organs (Capaldo et al., 2019, 2018; Gay et al., 2016) in the European eel (*Anguilla anguilla*). Moreover, the neurotoxicity of COC has been highlighted on planarians (Pagán et al., 2013), while injections of COC (ranging from 2.5 to 10 mg/g body weight) affected the locomotor activity of the crayfish (*Orconectes rusticus*; Nathaniel et al., 2012).

Thus, the present study was aimed to enlarge the knowledge of COC toxicity exploring biochemical and behavioral effects induced by a 21-days exposure to two environmental concentration of COC (50 ng L^{-1} and 500 ng L^{-1}) on the freshwater cladoceran *Daphnia magna*. Previous studies on both murine models (Muriach et al., 2010; Pomierny-Chamioło et al., 2013) and aquatic species have pointed out that COC exposure can induce an oxidative stress situation (Parolini et al., 2018a, 2017). Accordingly, we expect that COC can alter the oxidative status of cladocerans. Thus, a suite of oxidative stress-related biomarkers was evaluated: the amount of reactive oxygen species (ROS) and the activity of antioxidant (superoxide dismutase - SOD; catalase - CAT and glutathione peroxidase - GPx) and detoxifying (glutathione S-transferase - GST) enzymes. Moreover, as previous study on crustaceans have demonstrated that COC altered the locomotor activity of a crayfish species (Nathaniel et al., 2012), we expect changes in swimming activity of *D. magna*, which was investigated by a video-tracking analysis. Effects of COC exposure on biochemical and swimming activity endpoints were investigated after 7, 14 and 21 days of exposures. Lastly, a 21-days reproduction test was performed to assess changes in the reproductive output of the model species and potential consequences at population level after 21 days of exposure. As no study has investigated the reproductive toxicity of COC on invertebrate species so far, we have no a priori expectation on the effects of this illicit drug on *D. magna* reproduction.

2. Materials and methods

2.1. Chemicals and reagents

The analytical standard of cocaine (COC) was purchased from Sigma-Aldrich (Steinheim, Germany), after obtaining the permission for possession and use for scientific purposes by the Italian Ministry of Health (Decree n. SP/177, 11/12/2017). The deuterated analogue cocaine-*d3* (COC-*d3*), used as internal standard (IS), was acquired from Cerilliant Corporation (Round Rock, Texas, USA) as a solution of 0.1 mg/mL in acetonitrile (ACN). All the reagents used for biomarker analyses were purchased by Sigma-Aldrich (Steinheim, Germany). For chemical analysis, analytical grade methanol (MeOH) and hydrochloric acid (HCl, 37%) were purchased from Carlo Erba (Italy), ammonium hydroxide solution (25%) and acetic acid (AA) for LC-MS (>99%) from Fluka (Buchs, Switzerland) and ACN from Riedel de Haen (Seelze, Germany). HPLC grade Milli-Q water was obtained with a MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France). Solid phase extraction cartridges Oasis® MCX (60 mg, 3 cc) were purchased by Waters Corp. (Milford, MA, USA).

2.2. Daphnia magna husbandry

Daphnia magna individuals were cultured in 400 mL beakers (40 individuals L^{-1}) filled with a culture medium made of commercial mineral water (San Benedetto®) and fed ad libitum every other day with a suspension of the unicellular green algae *Pseudokirchneriella subcapitata* and the yeast *Saccharomyces cerevisiae*. The culture was maintained at 20 ± 0.5 °C under a 16 h light:8 h dark photoperiod to allow reproduction, which is parthenogenetic in this species. Details of husbandry conditions are reported elsewhere (Parolini et al., 2018b).

2.3. Experimental design

We planned different exposures, aimed at exposing organisms to investigate biochemical and swimming behavior effects, as well as reproductive alterations. First, a stock solution of COC (1 mg L^{-1}; stock solution 1) was prepared by diluting a commercial standard solution (1 g L^{-1} in MeOH) in the same commercial water used for the culture medium and used to perform exposures for biochemical and swimming behavior analyses. Such exposures were performed in beakers filled with 100 mL of the culture medium to which 5 μL and 50 μL of stock solution 1 were added to reach the selected exposure concentrations, 50 ng L^{-1} and 500 ng L^{-1} of COC, respectively. Moreover, a second stock solution (100 μg L^{-1}; stock solution 2) was prepared to be used for chronic toxicity reproduction test exposures, which were performed in 50 mL of culture medium to which 25 μL and 250 μL of the stock solution 2 were added to reach 50 ng L^{-1} and 500 ng L^{-1} of COC, respectively. The concentration of the stock solution was confirmed by liquid chromatography tandem mass spectrometry (LC-MS/MS; see

Section 2.4 Chemical analysis of COC in stock solution and exposure beakers).

The 50 ng L^{-1} tested concentration was comparable with the maximum level of COC found in surface waters worldwide, while the 500 ng L^{-1} reflected the value found in influents of WWTPs worldwide (Pal et al., 2013). As we planned to investigate COC-induced effects on biochemical and swimming activity endpoints every seven days and we could not perform repeated biomarker measures on the same individuals, we planned three different exposures. In detail, we planned three experimental groups (control, 50 ng L^{-1} and 500 ng L^{-1}), including three independent replicates (beakers) per treatment, which lasted for 7, 14 or 21 days. All the exposures started at the same day and relied on organisms born by the same mothers. Twenty daphnids <24 h old were randomly selected from husbandry beakers and seeded into beakers filled with 100 mL of culture medium and volumes of the stock solution were added up to the selected concentrations (see above). As three beakers containing 20 daphnids each were prepared per treatment, including control, a total of 60 individuals per treatment for each time point (7, 14 or 21 days) were exposed. Overall, 540 daphnids were used to perform exposure for analysis of COC-induced effects on biochemical and swimming behavior endpoints. Moreover, in order to assess the effect of COC on *D. magna* reproduction, a 21-days chronic toxicity reproduction test was performed according to the OECD guidelines (OECD, 2004). Fifteen individuals (<24 h old) per experimental treatment, including control, were exposed individually into 50 mL glass beakers filled with culture medium to which 25 μL and 250 μL of the stock solution 2 were added to reach 50 ng L^{-1} and 500 ng L^{-1} of COC, respectively. The culture medium and the amount of COC were renewed every single day for 21 days, checking for the viability of individuals. The number of offspring born by each single individuals and the number of clutches over the 21 days of the exposure were recorded.

Although the COC standard solution was in MeOH, no solvent control treatment was planned. Considering the dilution performed to obtain the stock solution and to reach the selected concentrations in the test beakers, the estimated concentrations of MeOH in exposure beakers were expected to be negligible (maximum calculated amount of MeOH accounted for 0.03% of the final volume). Moreover, our preliminary analyses did not show significant differences between negative and solvent (MeOH) control for both biochemical and behavioral endpoints tested in the present study (unpublished data). Exposures were performed under semi-static conditions, renewing the culture medium and adding COC solution every day. Daphnids were fed ad libitum over the 21-days exposures as the exposure medium included a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8×10^6 cells ind^{-1} day^{-1} until they were 8-days old; 16×10^6 cells ind^{-1} day^{-1} until they were 21-days old) and the yeast *Saccharomyces cerevisiae* (15×10^6 cells ind^{-1} day^{-1}). After 7, 14 or 21 days of exposure, individuals were video-tracked and then transferred to a 1.5 mL Eppendorf tube and stored at −80 °C until the biochemical analyses. Moreover, to check for the reliability of the exposure, the concentration of COC in exposure medium from control and treatment beakers was measured. Water samples were stored at −20 °C until the chemical analyses were performed.

2.4. Chemical analysis of COC in stock solution and exposure beakers

The chemical analysis of water samples to check COC expected concentrations was carried out by solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). A method published previously was adapted for this analysis (Castiglioni et al., 2011). Different aliquots were prepared for extraction: 25 mL for samples spiked at 50 ng L^{-1} and 2.5 mL for samples spiked at 500 ng L^{-1}. SPE was performed using mixed reverse-phase cation exchange cartridges (Oasis® MCX). Before extraction, the pH of each aliquot was adjusted to 2.0 with 37% HCl and was spiked with the IS (2 ng of COC-d_3). Cartridges were conditioned with 6 mL methanol, 3 mL Milli-Q water, and 3 mL Milli-Q water acidified to pH 2. Samples were passed manually through the cartridges at a flow rate of 5 mL min^{-1}. Cartridges were then vacuum-dried for 10 min and eluted with 2 mL of MeOH and 2 mL of a 2% ammonia solution in MeOH. SPE eluates were pooled and dried under a gentle nitrogen stream. Dried samples were redissolved in 100 μL of Milli-Q water, centrifuged for 2 min at 2500 rpm, and transferred into glass vials for LC injection. LC-MS/MS analysis was performed using an Agilent HP-1200 Series LC system with a binary pump and an autosampler (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source (Applied Biosystems–Sciex, Thornhill, Ontario, Canada). LC separation was performed at room temperature using an Atlantis T3 column (2.1 × 150 mm, 3 μm) from Waters and a mobile phase consisting of A (0.1% AA in Milli-Q water) and B (ACN). The flow rate was 200 μL/min and the injection volume was 4 μL. The MS analysis was done in the positive ion mode with a spray voltage of +5.5 kV and a source temperature of 400 °C. The MS analysis was done in the positive ion mode using the Selected Reaction Monitoring (SRM) acquisition mode. MS/MS parameters and retention time are shown in SM (Table S1). Quantitation of COC was performed using the isotopic dilution method and a 6-point calibration curve was made freshly before each analytical run. Method detection limit (MDL) and method quantitation limit (MQL) are reported in Table S1.

2.5. Biomarker methods

The biomarkers suite applied in the present study was performed on homogenates from pools of all alive specimens found in each exposure beaker at the end of the specific exposures. Three independent experimental replicates (pool of n = 17–20 individuals per replicate) for each treatment were performed. All the biochemical measurements were carried out in duplicate for each pool. According to Parolini et al. (2018b), individuals were homogenized using a motor pestle in a 100 mM potassium phosphate buffer (added with KCl 100 mM, EDTA 1 mM, protease inhibitors 1:100 v/v and dithiothreitol 1 mM, pH 7.4) and centrifuged at 15.000 ×*g* for 10 min. The supernatant was collected and immediately processed to assess protein content and enzyme activity through spectrophotometric methods, while the amount of ROS was assessed through a fluorimetric method. Details of all the biomarker methods applied in the present study are reported by Parolini et al. (2018b). Briefly, SOD activity was measured at λ = 550 nm as the inhibition of cytochrome *c* (10 μM) reduction caused by the superoxide anion generated by the xanthine oxidase (1.87 mU mL^{-1})/hypoxanthine (50 μM) reaction, and expressed as SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). The CAT activity was determined by measuring the decrease of H_2O_2 (50 mM) in potassium phosphate buffer (66.7 mM at pH 7) at λ = 240 nm. The GPx activity was measured by monitoring the consumption of NADPH (0.12 mM) at λ = 340 nm using 0.2 mM H_2O_2 as a substrate in 50 mM potassium phosphate buffer, added with reduced glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U mL^{-1}). The GST activity was measured at λ = 340 by adding reduced glutathione (1 mM) in 80 mM phosphate buffer (pH 7.4) and using CDNB (1 mM) as a substrate. Spectrophotometric reading was performed by a Genova Bio spectrophotometer (Jenway). The amount of ROS was assessed according to a fluorimetric method that relies on the change in fluorescence of the dichlorofluorescein-diacetate (DCFH-DA; 10 mg/ mL in DMSO) in presence of pro-oxidant molecules. The fluorescence intensity was measured by an Infinite® 200 PRO microplate reader (TECAN Life Sciences) with λ = 485 nm as excitation and λ = 536 nm as emission wavelength, respectively.

2.6. Video tracking analysis

To assess changes in swimming activity induced by the exposure to COC, video tracking analyses were performed. At the end of the

exposures lasted 7, 14 or 21 days, ten individuals per treatment for each experimental replicate ($n = 30$ individuals for each treatment) were transferred individually into a 12-well plate (11.5 cm × 8 cm × 1.5 cm), called 'arena', filled with 3 mL of culture medium (without food) and were filmed with an iPhone 6 for 30 s (900 frames, 30 frames per second), obtaining 1080p Full HD videos. Videos were analyzed using the ImageJ plugin AnimalTracker, a tracking application specifically designed to support animal behavioral analyses. We relied on a module that processed the video recordings and provided the observed object's XY coordinates in each frame (see Gulyás et al., 2016 for details). AnimalTracker returned the swimming activity of *D. magna* individuals, in terms of distance moved (mm) and swimming speed (cm s^{-1}) of each individual.

2.7. Statistical analysis

The effects of COC treatment, the exposure time and their interaction on the amount of ROS, enzyme activities of *D. magna* individuals were investigated using general linear models (GLM), while the effects on swimming behavior were assessed by Linear mixed models (LMM), including the identity of the test beaker in the models as a random factor to account for the so-called 'tank effect'. In the models the effect of beaker identity was tested by likelihood ratio test, by comparing the log-likelihood value of the model including or excluding the random effect of beaker identity. The effect of COC on chronic toxicity reproduction test endpoints was analyzed by generalized linear models, assuming a Poisson distribution of data. Fisher's LSD post-hoc test was applied to point out significant differences among treatments, exposure time and treatment × time interactions. Significance was set at $p < 0.05$ (*) and $p < 0.01$ (**). Statistical analyses were performed using IBM SPSS Statistics 25.0 software package.

3. Results

3.1. Concentration of COC in stock solution and exposure beakers

The concentration of COC in the stock solution (nominal concentration: 1 mg L^{-1}) was 1.19 mg L^{-1} (accuracy: 119%). No COC residues were found in control beakers, while concentrations of COC in culture medium from beakers spiked with the lowest (50 ng L^{-1}) and highest (500 ng L^{-1}) tested concentrations were respectively 67 ng L^{-1} (accuracy: 134%) and 634 ng L^{-1} (accuracy: 127%).

3.2. COC-induced effects on oxidative stress-related endpoints

Over the 21 days of the exposure, a mortality of 2.7 (±0.57) %, 6.7 (± 0.95) % and 4 (±0.95) % occurred in the control, 50 ng L^{-1} and 500 ng L^{-1} experimental groups, respectively. These results agreed the OECD guidelines (OECD, 2004), which indicate that the mortality in the control group should not exceed the 10% in order to consider the tests with *Daphnia magna* as valid. Moreover, no significant ($p > 0.05$) differences between treated and control groups occurred.

Results of statistical analyses are reported in Table 1. A significant effect of the time of exposure was noted for all the considered biochemical endpoints, with the exception of GST. It is interesting to note that that both biochemical endpoints and swimming activity changes at different ages of the individuals (Figs. 1–3). For this reason, the effect of COC on these endpoints was highlighted by comparing the responses obtained after the exposure to both the COC concentrations with the corresponding temporal control. A significant effect of COC treatment and time × treatment interaction on the amount of ROS was found. Independently of the time of the exposure, the amount of ROS in 50 ng L^{-1} and 500 ng L^{-1} specimens was 46% and 79% higher compared to controls. Moreover, a significant ROS overproduction was noted after 7 days of exposure to 500 ng L^{-1} (3.6-fold higher) and after 21 days of treatment to 50 ng L^{-1} and 500 ng L^{-1} (1.8-fold higher in both the cases) compared to the corresponding control (Fig. 1). A significant effect on SOD activity was induced by COC treatment, showing a significant 20% activity decrease in specimens exposed to 500 ng L^{-1} COC compared to the control group ($p < 0.001$). Moreover, SOD changed according to a significant time × treatment interaction, with a 40% and 21% decrease in enzyme activity measured after 7 and 21 days of exposure at 500 ng L^{-1}, respectively, with respect to the corresponding controls (Fig. 2a). Although no significant effect of COC on CAT activity was noted, the significant time × treatment interaction showed a decrease of activity at the end of the exposure to 50 ng L^{-1} (−33%) and after 7 days (−31%) to 500 ng L^{-1} compared to the corresponding controls, as well as an increase after 14 days at 500 ng L^{-1} (+19%) (Fig. 2b). Despite a significant effect of COC treatment on the GPx activity, whereby a significant activity increase measured in specimens from 50 ng L^{-1} (+28%) and to 500 ng L^{-1} (+23%) tested concentration compared to the controls ($p < 0.047$ in both the cases) was found, the time × treatment interaction was not significant (Fig. 2c). A significant increase of GST activity (Fig. 2d) was noted, showing an activation (+13%) in specimens exposed to 500 ng L^{-1} COC compared to control ($p = 0.015$; Table 1).

3.3. COC-induced effects on swimming behavior

Log-likelihood ratio test did not show any significant effect of exposure beaker identity for both the considered variables ($\chi^2_1 = 0.00$; $p = 1$ for both the cases). The COC treatment induced a significant effect on the distance moved by *D. magna* specimens. Moreover, a significant effect of the time of the exposure and time × treatment interaction (Fig. 3a) was found, suggesting that swimming activity changed at different ages of the individuals. In detail, the exposure to 500 ng L^{-1} COC caused a significant decrease of the distance moved compared to the corresponding control, accounting for the 19% and 11% after 14 and 21 days of exposure, respectively. Conversely, the exposure to 50 ng L^{-1} COC induced an increase (13.5%) in the distance moved after 21-day exposure compared to control. Although no significant effect of the COC treatment on the swimming speed was found, the significant effect of time × treatment interaction revealed that specimens exposed for 21 days to 50 ng L^{-1} COC were 20% quicker than the corresponding controls, while a slowing down was noted in 14-days old specimens treated with 500 ng $L^{-1}$with respect to the corresponding control (Fig. 3b).

3.4. Chronic toxicity test results

COC treatment induced a significant decrease on the total number of offspring (Wald $\chi^2_{2,31} = 49.417$; $p < 0.001$), with a 38% ($p = 0.005$) and 28% ($p = 0.033$) fecundity reduction compared to the control group in specimens exposed to 50 ng L^{-1} and 500 ng L^{-1}, respectively (Fig. 4). In contrast, no significant effect (Wald $\chi^2_{2,31} = 0.833$; $p = 0.660$) on the number of clutches between treated and control specimens was noted (data not shown).

4. Discussion

The present study showed that the exposure to environmental concentrations of cocaine (50 ng L^{-1} and 500 ng L^{-1}) imbalanced the oxidative status and negatively affected the swimming activity and reproductive effort of *D. magna*.

Many studies have shown that COC exposure can damage the structure and the function of diverse organs through different mechanisms of actions, whereby the majority of the direct toxic effects is mediated by the onset of oxidative stress and mitochondrial dysfunction occurring during the metabolism of this illicit drug (Riezzo et al., 2012 and references therein). Our findings showed that the exposure to COC induced an overproduction of ROS at both the tested concentrations. These results agreed with a previous study on zebrafish larvae (96 h post

Table 1
Effects due to treatment, time of exposure and their interactions on biochemical (SOD, CAT, GPx, and GST) swimming activity and reproduction variables in *D. magna*. Details of statistical approach used to analyse each single variable are reported in Section 2.7 Statistical analysis. Significant effects are reported in bold.

Biochemical effects	F	df	*p*
ROS			
Time	184.110	2,18	<**0.001**
Treatment	25.021	2,18	<**0.001**
Time × treatment	16.573	4,18	<**0.001**
SOD			
Time	217.367	2,18	<**0.001**
Treatment	12.828	2,18	<**0.001**
Time × treatment	4.687	4,18	**0.009**
CAT			
Time	4.783	2,18	**0.022**
Treatment	0.806	2,18	0.462
Time × treatment	6.644	4,18	**0.002**
GPx			
Time	4.790	2,18	**0.021**
Treatment	4.024	2,18	**0.045**
Time × treatment	0.733	4,18	0.581
GST			
Time	2.941	2,18	0.078
Treatment	3.809	2,18	**0.042**
Time × treatment	2.494	4,18	0.080
Swimming activity	**F**	**df**	***p***
Distance moved			
Time	8.381	2,258	<**0.001**
Treatment	3.074	2,258	**0.048**
Time × treatment	4.799	4,258	**0.001**
Swimming speed			
Time	8.807	2,259	<**0.001**
Treatment	2.295	2,259	0.103
Time × treatment	5.149	4,259	**0.001**
Reproduction	**Wald χ^2**	**df**	***p***
Number of offspring			
Treatment	49.417	2,31	<**0.001**
Number of clutches			
Treatment	0.833	2,31	0.660

fertilization), which showed a significant increase in ROS levels after a short-term exposure to increasing COC concentrations, ranging between 0.1 and 1 μg L^{-1} (Parolini et al., 2017). Such ROS overproduction modulated the activity of the *D. magna* antioxidant enzymatic shield, which relies on a cascade mechanism of three main enzymes, namely SOD, CAT and GPx (Lushchak, 2011). The significant decrease of SOD activity found at 500 ng L^{-1} COC might be related to a ROS overproduction (Gonzalez-Rey and Bebianno, 2014) and suggests the accumulation of superoxide anion ($O_2^{\bullet -}$) within the organism (Verlecar et al., 2008). Alternatively, the decrease of SOD activity might be due to the inhibition and/or negative feed-back mechanism related to the byproducts of SOD reaction, suggesting the production of cytosolic hydrogen peroxide (Vlahogianni and Valavanidis, 2007). Moreover, the spontaneous dismutation of superoxide anion by non-enzymatic pathways (Gwoździński et al., 2010) and other cellular enzymes, such as those contained in the peroxisomes (Khessiba et al., 2005), might boost the production of hydrogen peroxide. Despite no activation of CAT, the increase of GPx activity found at 500 ng L^{-1} COC, independently of the time of exposure, supported the hypothesis of the H_2O_2 production. Although GPx and CAT play a complementary role in metabolizing hydrogen peroxide, the divergence in their activity response could be explained by a competition for the same substrate (Kappus, 1985) or, alternatively, by the levels of H_2O_2 that the organism has to face. In fact, while GPx acts at low H_2O_2 levels, CAT is activated only at high concentrations of this pro-oxidant molecule (Pereira et al., 2013). Similar trends of the antioxidant enzymes were found in zebrafish larvae exposed to COC and its main metabolites (Parolini et al., 2017), as well as in *D. polymorpha* specimens exposed to ibuprofen (Parolini et al., 2011) and Δ-9-tetrahydrocannabinol (Δ-9-THC; Parolini and Binelli, 2014). Lastly, the increase of GST activity observed in specimens exposed to the highest treatment suggested the involvement of this phase II enzyme in detoxification processes of COC. These results were in accordance with previous studies showing an increase of GST in murine models exposed to COC (e.g., Devi and Chan, 1997; Uys et al., 2011), as well as in the brown mussel *Perna perna* treated with crack COC (dos Santos Barbosa Ortega et al., 2018).

Overall, our findings suggested that an imbalance in the oxidative status of *D. magna* treated specimens occurred, which could lead to the onset of oxidative stress. Such situation often results in detrimental behavioral effects at individual level (e.g., Hedgespeth et al., 2014; Rivetti et al., 2016). COC exposure caused significant alterations in swimming activity of cladocerans, in terms of both distance moved and swimming speed. Opposite responses were found at either treatment concentrations, whereby on one hand, the exposure to 50 ng L^{-1} COC induced an increase of distance moved and swimming speed, while on the other hand the highest tested concentration negatively affected both the endpoints. This discrepancy suggests a different mechanism of action of COC in *D. magna*, which depends on the administered concentration. In fact, at low concentrations COC might act as a stimulant molecule, boosting the swimming activity, while at high concentrations COC becomes toxic and impairs the swimming behavior. Alternatively, as COC is a psychomotor stimulant drug, at low doses it increases locomotor activity whereas, when the dose increases, the locomotor activity decreases (Grilly and Salamone, 2011). Our interpretation is supported by results from a previous study by Nielsen and Roslev (2018), showing that the exposure to high concentrations (1–10 μg mL^{-1}) of two psychotropic drugs, namely fluoxetine and propranolol, stimulated the swimming activity of *D. magna*, whereas very high concentrations (>100 μg mL^{-1}) inhibited it. However, as fluoxetine is an anti-depressant and propranolol is a β-blocker, their mechanisms of action in modulating swimming activity could be different from that of COC. Changes in the swimming activity can be related to an increased energy demand of the organism to complete the physiological processes needed to counteract COC toxicity. As the swimming behavior integrates physiological, sensorial, nervous and muscular responses (Charoy et al., 1995), our results suggest an overall impairment of the health status of treated *D. magna* specimens, with potentially detrimental consequences to fitness and survival of the organism. In fact, the alteration of the swimming performance could affect the filtering activity

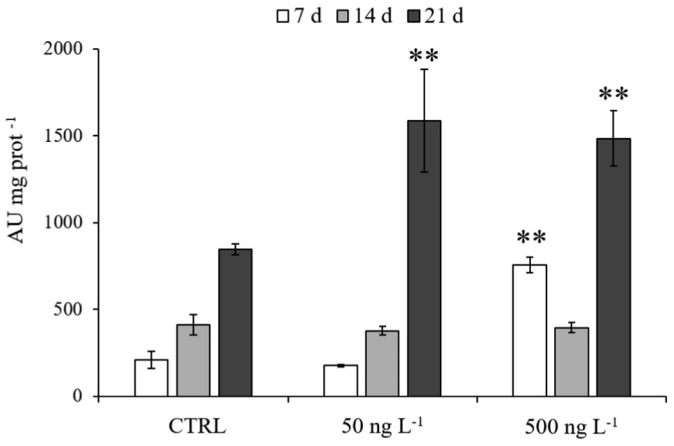

Fig. 1. Mean (±standard deviation) of the amount of reactive oxygen species (ROS) measured in *D. magna* specimens after 7, 14 and 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (**$p < 0.01$).

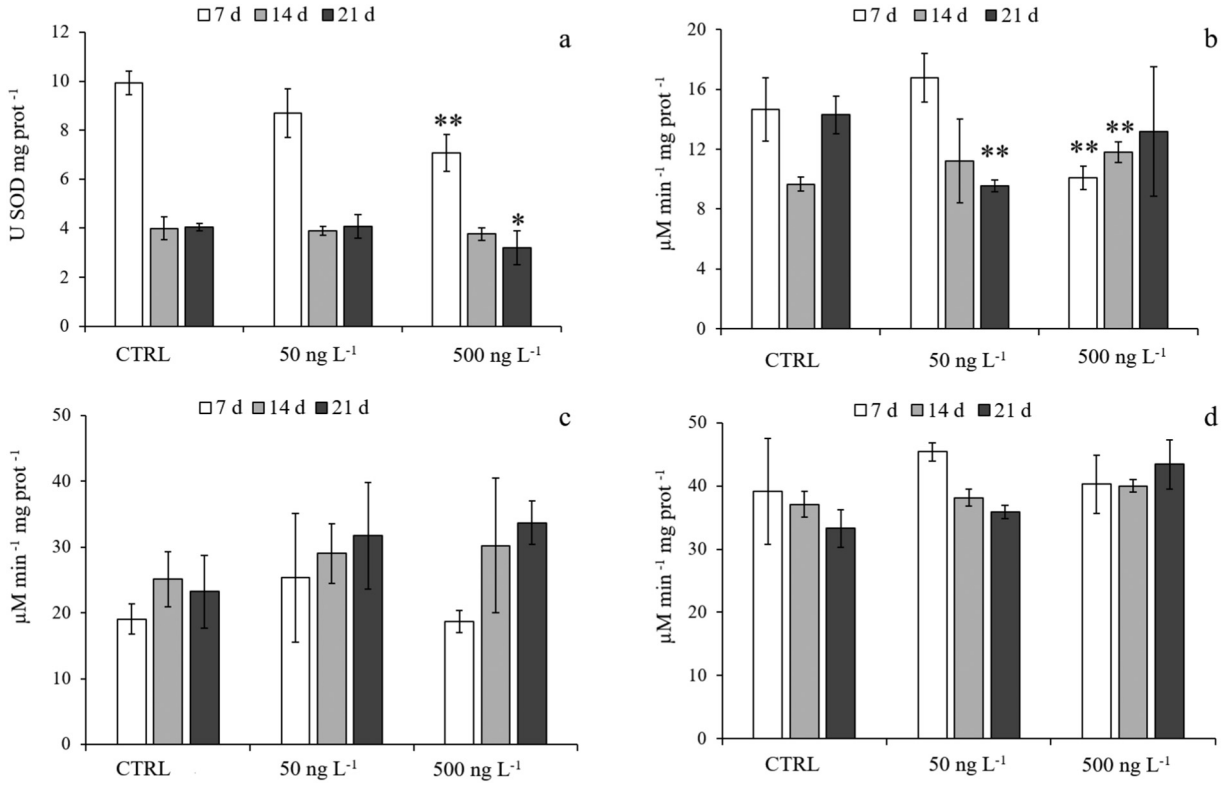

Fig. 2. Mean (±standard deviation) of SOD (a), CAT (b), GPx (c) and GST (d) activity measured in *D. magna* specimens after 7, 14 and 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (*$p < 0.05$; **$p < 0.01$).

and therefore the food uptake, leading to an impairment of reproduction (Baillieul, 1997). Both COC treatments caused a significant decrease in the total number of offspring with respect to the control, while no change in the number of clutches was recorded (data not shown). The decrease in reproductive success of individuals exposed to 500 ng L^{-1} COC was a plausible consequence considering the impairment of swimming performance. These findings agree with those from previous studies that demonstrated changes of *D. magna* reproductive success in response to the exposure to different emerging contaminants, including pesticides (e.g., Villarroel et al., 2009, 2003), pharmaceuticals (de Oliveira et al., 2016) and illicit drugs (Parolini et al., 2018b). In contrast, the adverse effects caused by the exposure to 50 ng L^{-1} COC was unforeseen. In fact, considering that COC boosted the swimming activity, null or positive effects on reproduction was expected. Thus, we may speculate that the decreased reproductive success of individuals treated with the lowest COC concentration depended on a different use of energy obtained by the food uptake, which was diverted to support swimming rather than reproduction, or alternatively on direct, yet unknown, reproductive effects. Overall, our findings suggest that environmental COC concentrations could negatively affect the population dynamic of *D. magna*, with potential detrimental consequences on the whole trophic chain because of the pivotal role of this species in freshwater ecosystems.

5. Conclusion

Our findings showed that the exposure to low concentrations of COC can alter the oxidative status and affect both the swimming and the reproductive behavior of the cladoceran *D. magna*. As the concentrations tested in the present study were similar to those measured in aquatic ecosystems worldwide, our results cannot be underestimated. Moreover, the uninterrupted use of COC and the consequent input in the sewage confer to COC a sort of pseudo-persistence. Thus, aquatic organisms might be exposed to similar or higher COC concentrations for their

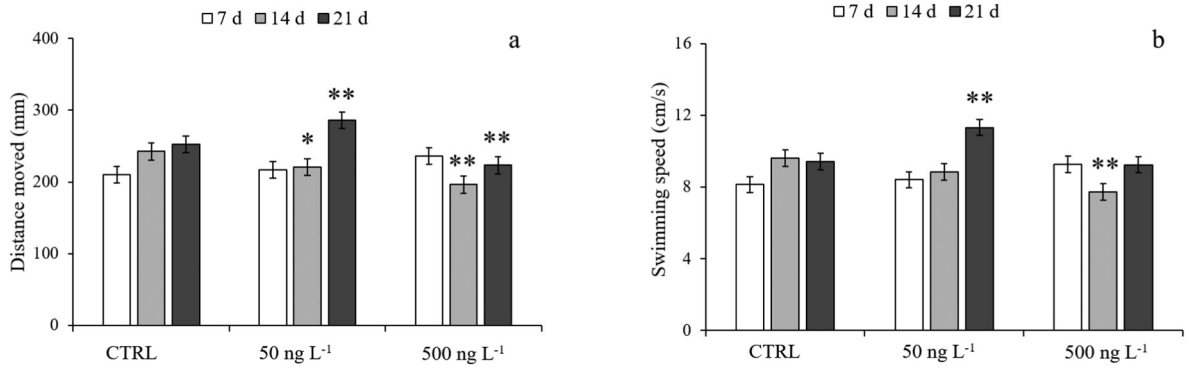

Fig. 3. Mean (±standard deviation) of distance moved (a) and swimming speed (b) measured in *D. magna* specimens after 7, 14 and 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (*$p < 0.05$; **$p < 0.01$).

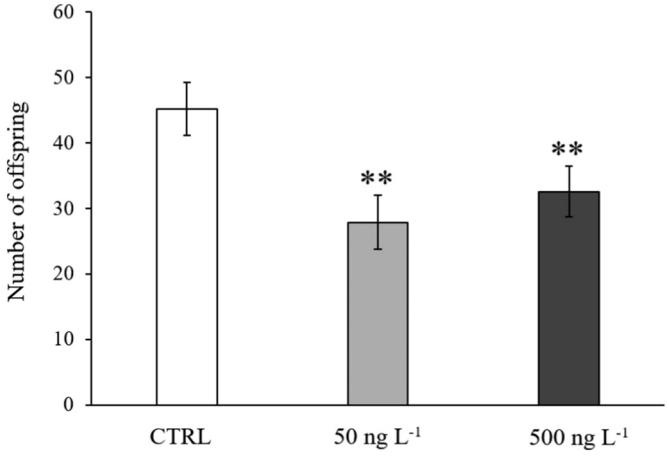

Fig. 4. Mean (±standard deviation) number of offspring of *D. magna* specimens after 21 days of exposure to 50 ng L^{-1} and 500 ng L^{-1} of COC. Asterisks above the histograms show significant differences between treated individuals and the corresponding control ($^{**}p < 0.01$).

whole life-span, resulting in potentially worst adverse effects with respect to those we found in our laboratory exposures. Further studies are therefore recommended to shed light on the toxicity at different level of the ecological hierarchy and on the mechanisms of action of COC in aquatic species and to formulate an accurate risk assessment of this illicit drugs for freshwater ecosystems.

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PAPER II

De Felice B., Mondellini S., Salgueiro-Gonzàlez N., Castiglioni S., Parolini M.

Methamphetamine exposure modulated oxidative status and altered the reproductive output in *Daphnia magna*.

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Methamphetamine exposure modulated oxidative status and altered the reproductive output in *Daphnia magna*

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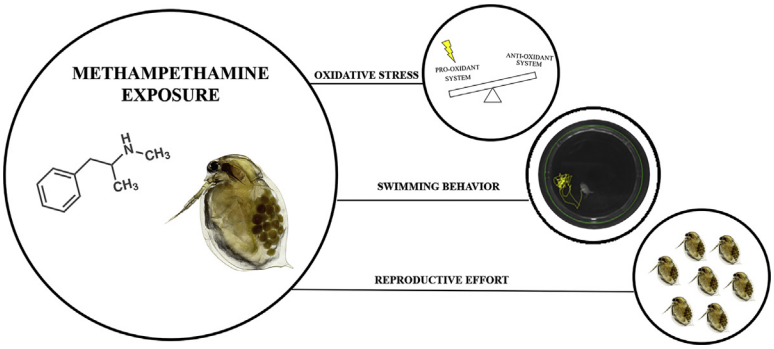
HIGHLIGHTS

- Methamphetamine toxicity was assessed by biochemical and behavioral endpoints.
- Methamphetamine exposure imbalanced the oxidative status of *D. magna*.
- Methamphetamine exposure did not induce any alteration in swimming behavior.
- Increase in reproductive effort was observed from 50 ng/L upwards.

GRAPHICAL ABSTRACT



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Behavior

ABSTRACT

Methamphetamine (METH) is a central nervous system stimulant drug whose use has increased in the last few years worldwide. After the ingestion of even a single dose, METH is excreted by the organism and enters the aquatic ecosystems, whereby concentrations up to hundreds of ng/L were measured in both sewage and surface waters. Although the environmental concentrations are currently quite low, the high biological activity of METH might cause adverse effects towards non-target organisms. However, to date the information on METH toxicity towards aquatic organisms is limited. Thus, the present study aimed at investigating biochemical and behavioral effects induced by METH exposure towards the Cladoceran *Daphnia magna*. A 21-days exposure to two environmental concentrations of METH (50 ng/L and 500 ng/L) was performed. At selected time points (7, 14 and 21 days) the amount of pro-oxidant molecules, the activity of antioxidant enzymes (SOD, CAT, GPx) and levels of lipid peroxidation (LPO) were measured as oxidative stress-related endpoints. Changes in swimming activity and reproductive output were assessed as behavioral endpoints. METH exposure affected the oxidative status of *D. magna* specimens at both tested concentrations, although no oxidative damage occurred. Although METH did not modulate the swimming activity of *D. magna*, a significant, positive effect on reproductive output, in terms of number of offspring was found. Our results showed that low concentrations of METH might represent a threat for *D. magna*, affecting the health status of this aquatic species at different level of biological organization.

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1. Introduction

Amphetamine-type simulants (ATS) are a group of synthetic illicit drugs whose use increased in the last decade, reaching 28.9 million users worldwide in 2017 (UNODC, 2019). ATS are the second most used illicit drugs after cannabis worldwide, while methamphetamine (METH) dominates the ATS market, being the most frequently used (UNODC, 2019). Methamphetamine is a central nervous system stimulant, which induces euphoria and sense of well-being in humans by increasing the neuronal release of monoamines, mainly dopamine (Angilin et al., 2000; Vearrier et al., 2012). After human consumption, METH is metabolized in the liver and excreted through feces and urine, mainly as unaltered parental compound (accounting for 30–50%) and limitedly as two metabolites, namely 4-hydroxymethamphetamine (accounting for 15%) or amphetamine (accounting for 10%) (Cruickshank and Dyer, 2009), entering the sewage. As the wastewater treatment plant (WWTP) efficiency of removal is limited, METH enters surface waters by WWTP effluents or wet-weather run-off (Boles and Wells, 2010). Diverse monitoring surveys have detected measurable concentrations of METH in both wastewater and surface waters worldwide (Pal et al., 2013). In detail, influents of European and Americans WWTPs presented a METH concentration ranging from 2 ng/L to 350 ng/L (Rosi-Marshall et al., 2015; Asimakopoulos and Kannan, 2016), while the majority of Asiatic WWTPs present concentration ranging from 100 ng/L to 700 ng/L for effluents and from 80 ng/L to 1153 ng/L for influents (Zeqiong et al., 2017). In addition, Li et al. (2016) have detected METH in the surface water of several Asiatic lakes in concentration up to 95.9 ng/L.

Although the current environmental levels are quite low, its high biological activity suggests that the risk of METH for the aquatic ecosystem cannot be neglected. Previous studies performed on humans (Davidson et al., 2001; Logan, 2002) and murine models (Barrett et al., 2001; Peerzada et al., 2013) have shed light on the toxicity and the mechanisms of action (MoA) of METH. Methamphetamine exerts its toxicity by modulating the levels of the neurotransmitter dopamine (Iversen, 2006; Krasnova and Cadet, 2009). METH excess induces the overproduction of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide radical ($O_2•^-$), via dopamine auto-oxidation or enzymatic oxidation operated by monoamine oxidase processes (McDonnell-Dowling and Kelly, 2017; Krasnova and Cadet, 2009). The ROS overproduction can induce the onset of oxidative stress, as confirmed by diverse studies that have demonstrated the imbalance of the equilibrium between pro-oxidant and antioxidant molecules in favor of the former, as a consequence of METH exposure (Chen, 2007, Gluck et al., 2001, Harold et al., 2000, Iwazaki et al., 2006; McDonnell-Dowling and Kelly, 2017; Ramkissoon and Wells, 2015).

Considering the presence of METH in aquatic ecosystems and its peculiar MoA, METH might exert its toxicity towards non-target aquatic organism at both biochemical and behavioral level. However, to date the information concerning the potential toxicity of METH on aquatic species is still limited and often contrasting. A study performed by Liao et al. (2015) confirmed that exposure to METH (concentration range 0.004–40 μM) caused the onset of oxidative stress, as well as developmental and behavioral alteration, in early-life stages of the medaka fish *Oryzias latipes*. Conversely, studies focused on METH-induced behavioral changes returned contrasting outcomes. In fact, Hossain et al. (2019) did not find significant alterations in the swimming distance of the crayfish *Procambarus virginalis* exposed to 1 μg/L of METH. In contrast, a significant decrease of locomotor activity was noted in the flatworm *Dugesia dorotocephala* exposed to 0.1–100 μM of METH (Rawls et al., 2008), while the exposure to 0.03 μM induced an increase of spontaneous movements in the flatworm *Dugesia japonica* (Tashiro et al., 2014). Moreover, an increase of sex arousal in males of the sailfin molly *Poecilia latipinna* was noted, resulting in an enhancement of the mating activity after the exposure to 0.1, 0.5 and 1.0 mg/L of METH (Ghazilou and Gazilou, 2011). Despite these findings, the studies assessing the effects induced by METH and their propagation over different levels of the biological organization in aquatic organisms remain scant. The present study aimed at investigating biochemical and behavioral effects induced by 21-days exposure to two concentrations of METH (50 ng/L and 500 ng/L) towards the Cladoceran *Daphnia magna*. As previous studies have demonstrated that METH exposure induced the onset of oxidative stress (e.g., McDonnell-Dowling and Kelly, 2017; Ramkissoon and Wells, 2015; Liao et al., 2015), a suite of oxidative stress-related biomarkers, investigating the amount of reactive oxygen species (ROS), the activity of antioxidant enzymes (superoxide dismutase - SOD; catalase - CAT and glutathione peroxidase - GPx) and the levels of lipid peroxidation was applied to assess METH toxicity at biochemical level. Moreover, behavioral effects were assessed in terms of changes in swimming behavior (i.e., alteration of the distance moved) and in reproductive output (i.e., change in the number of clutches and offspring) in *D. magna*. We expect that the exposure to METH concentrations modulates the oxidative status of *D. magna* specimens, leading to an oxidative stress situation. However, considering the contrasting outcomes of previous behavioral studies, we have no a priori expectations on the behavioral effects induced by METH exposure in Cladocerans.

2. Materials and methods

2.1. Chemicals and reagents

The analytical standard of methamphetamine (METH) used in this study was purchased from Sigma-Aldrich (Steinheim, Germany), after obtaining the permission for the possession and the use for scientific purposes only by the Italian Ministry of Health (Decree n SP/177, 11/12/2017). For the chemical analysis of METH, the deuterated analog methamphetamine-d9 (METH-d9; 0.1 mg/mL in acetonitrile - ACN) was used as an internal standard (IS) and was purchased from Cerilliant Corporation (Round Rock, Texas, USA). Analytical grade methanol (MeOH) and hydrochloric acid (HCl, 37%) were purchased from Carlo Erba (Italy), ammonium hydroxide solution (25%) and acetic acid (AA) for LC-MS (>99%) from Fluka (Buchs, Switzerland) and ACN from Riedel de Haen (Seelze, Germany). HPLC grade Milli-Q water was obtained with a MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France). Solid phase extraction (SPE) cartridges Oasis® MCX (60 mg, 3 cc) were purchased by Waters Corp. (Milford, MA, USA). All the reagents used for biomarker analyses were purchased by Sigma-Aldrich (Steinheim, Germany).

2.2. Experimental design

All the organisms used in this work came from the *Daphnia magna* husbandry located in the laboratory of the University of Milan, generated by a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy). Adult *D. magna* specimens were cultured in 400 mL beakers (30 individuals/L) in a commercial mineral water (San Benedetto®) and fed ad libitum with the yeast *Saccharomyces cerevisiae* (0.5 g/L) and a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8×10^6 cells/individual/day up to the eight day, then 16×10^6 cells/individual/day) following a protocol described by Parolini et al. (2018). Specimens were maintained at 20.0 ± 0.5 °C under a 16 h light:8 h dark photoperiod in order to ensure the amictic parthenogenetic reproduction (Frey, 1982). All the organisms used for the experiment were born from the same mothers and randomly selected to be used for control or METH-treated group. Two concentrations of METH were tested, namely 50 (0.34 nM) and 500 ng/L (3.4 nM). The lower concentration was similar to the level of METH found in surface waters, while the higher one was close to the METH levels measured in influents of WWTPs worldwide (Pal et al., 2013). Two stock solutions were prepared by diluting the standard

solution of METH (1 g/L in MeOH) in the San Benedetto® mineral water, namely stock solution 1 (1 mg/L) and stock solution 2 (100 μg/L). Stock solution 1 was used for exposures devoted to biochemical and behavioral analyses, which were performed in beakers filled with 100 mL of the culture medium in which 5 and 50 μL of the stock solution were added to reach the selected exposure concentrations. Stock solution 2 was used for exposures devoted to chronic toxicity reproduction test, which were performed in beakers filled with 50 mL of culture medium in which 25 μL and 250 μL of stock solution were added up to reaching the selected nominal concentrations.

The concentration of METH in the stock solutions was checked by a liquid chromatography tandem mass spectrometry analysis (LC-MS/MS). Moreover, to confirm the reliability of the exposure conditions, METH concentrations in culture medium sampled from exposure beakers were checked (see Section 2.3 Chemical analysis of METH). Although METH standard solution was in MeOH, we did not plan a solvent control group because the estimated MeOH concentration in exposure beakers was negligible (always lower than 0.03%). Furthermore, our preliminary analyses did not show negative effects induced by the exposure to a concentration of MeOH similar to that estimated in beakers of METH exposure on both biochemical and behavioral endpoints (unpub. data).

In order to assess the METH-induced effects on biochemical endpoints and *D. magna* swimming behavior three different exposures were performed following the rationale described by De Felice et al. (2019). In detail, semi-static exposures lasting 7, 14 and 21-days were planned. For each experimental group (i.e., control, 50 ng/L and 500 ng/L) we performed three independent replicates, seeding twenty daphnids (<24 h old) in each exposure beaker, for a total of 60 daphnids per treatment and time of exposure. Although the previous studies have demonstrated that the degradation rate of METH in water is slow (i.e., 15/30 days; Bagnall et al., 2013; Evans et al., 2016; Wang et al., 2018), to guarantee constant exposure conditions the culture medium and the METH concentrations were renewed every day. Organisms were maintained at the same light and temperature condition and fed ad libitum as reported for the husbandry. At the end of each exposure, the swimming activity of thirty organisms, randomly chosen from the three exposure beakers per each treatment, was assessed through a video-tracking analysis (see Section 2.5 Video tracking analysis). At the end of the behavioral analysis, all the organisms were stored in an Eppendorf tube at −80 °C up to the biochemical analyses.

The METH-induced effects on reproduction were evaluated by a standard 21-days *Daphnia magna* chronic toxicity reproduction test following the OECD guidelines (OECD, 2004). Forty-five daphnids <24 h old were selected and randomly seeded in 50 mL beakers in order to have 15 replicates per each treatment. Exposures were performed under semi-static conditions, with the daily renewal of culture medium and contaminant, and the number of offspring born by each single individual, as well as the number of clutches of each individual, were recorded.

2.3. Chemical analysis of methamphetamine

Culture medium analysis was carried out by SPE using mixed reverse-phase cation exchange cartridges (Oasis® MCX) followed by LC-MS/MS analysis, according to a previously published method (Castiglioni et al., 2006). Briefly, different aliquots (25 mL for samples spiked at 50 ng/L and 2.5 mL for samples spiked at 500 ng/L) were spiked with 2 ng of IS (METH-d9) and the pH was adjusted to 2.0 with 37% HCl. The MCX cartridges were conditioned with 6 mL MeOH, 3 mL Milli-Q water, and 3 mL Milli-Q water acidified to pH 2. Then, samples were manually passed through the cartridges at a flow rate of 5 mL/min. After vacuum-dried for 10 min, MCX cartridges were eluted with 2 mL of MeOH and 2 mL of a 2% ammonia solution in MeOH. SPE eluates were pooled and dried under a gentle nitrogen stream. Dried samples were redissolved in 100 μL of Milli-Q water, centrifuged for 2 min at 2500 rpm, and finally transferred into glass vials for LC injection.

LC-MS/MS analysis was performed using an Agilent HP-1200 Series LC system with a binary pump and an autosampler (Agilent Technologies, Santa Clara, CA, USA) coupled to 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 at room temperature using an Atlantis T3 column (2.1 × 100 mm, 3 μm) from Waters and a mobile phase consisting of A (0.1% AA in Milli-Q water) and B (ACN). The gradient started with 1% B for 3 min, increased to 60% B in a 20-min linear gradient and then to 100% B in a 1-min linear gradient. After 3-min with 100% B (washing step), the gradient returned to the initial conditions (1% B) and was maintained for 8 min (column equilibration). The flow rate was 200 μL/min and the injection volume was 2 μL. The MS/MS analysis was done in the positive ion mode (spray voltage of +5.5 kV and a source temperature of 400 °C) and using a multiple reaction monitoring (MRM) acquisition mode, measuring the most abundant fragmentation products of the protonated pseudo molecular ions of METH and its deuterated analog (METH-d9). METH was quantified by isotopic dilution method and a six-point calibration curve was made freshly before each analytical run. The method quantitation limit (MQL), calculated as the concentration at which the signal-to-noise ratio is 10, was 2.7 ng/L.

Stock solutions used for reaching the nominal METH concentrations (1 mg/mL and 100 μL) in the exposures were diluted and analyzed by direct injection following the previous LC-MS/MS method.

2.4. Biomarker analysis

At the end of the exposure all the alive individuals from each beaker per treatment were pooled. Three independent replicates (i.e., pools of 17–20 organisms per replicate) per treatment were performed and for each pool every biochemical analysis was performed in duplicate. A homogenate of *D. magna* specimens was carried out with a motor pestle in 100 mM potassium phosphate buffer (added with 100 mM KCl, 1 mM EDTA, protease inhibitors 1:100 v/v and 1 mM, dithiothreitol; pH 7.4). The homogenates were centrifuged at 15,000 ×*g* for 15 min at 4 °C and immediately collected and processed to assess the protein content and enzyme activities. The amount of ROS was assessed by a fluorimetric method by Deng et al. (2009). In detail, the change in fluorescence of dichlorofluorescein-diacetate (DCFH-DA; 10 mg/mL in DMSO) caused by the presence of pro-oxidant molecules was recorded by an EnSight multimode plate reader (Perkin Elmer) with $\lambda = 485$ nm as excitation and $\lambda = 536$ nm as emission wavelength, respectively. The ROS concentration was expressed in arbitrary units as AU DCF/mg protein. The activity of antioxidant enzymes was assessed according to methods described by (Parolini et al., 2018; De Felice et al., 2019). Briefly, SOD activity was assessed measuring the inhibition of cytochrome *c* (10 μM) reduction operated by the superoxide anion generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 mM) reaction for 1 min at $\lambda = 550$ nm. The CAT activity was assessed measuring the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (66.7 mM; pH 7) for 1 min at $\lambda = 240$ nm. The GPx activity was assesses measuring the consumption of NADPH (0.12 mM) using H_2O_2 (0.2 mM) as a substrate in a potassium phosphate buffer (50 mM), added with glutathione (2 mM), sodium azide (1 mM) and glutathione reductase (2 U/mL) for 1 min at $\lambda = 340$ nm. The activity of antioxidant enzymes was normalized on protein concentration measured according to the Bradford method. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979) and results were expresses as nmol TBARS/mg protein. All the spectrophotometric analyses were performed by means of a Genova Bio spectrophotometer (Jenway).

2.5. Video tracking analysis

Ten individuals for each experimental replicate (total = 30 individuals for each experimental condition and time of exposure) were randomly transferred into a 12-well plate (well size 11.5 cm × 8 cm × 1.5 cm). Organisms were seeded singularly in a single well of the 12-multiwell plate, called 'arena'. The arena was filled with 3 mL of San Benedetto® mineral water before transferring the organisms and after an acclimation time of 5 min each organism was filmed for 30 s with an iPhone 6. The acclimation time and the duration of the recording were chosen in accordance with our previous studies on *D. magna* swimming behavior (i.e., Parolini et al., 2018; De Felice et al., 2019). The 1080p Full HD videos (900 frames, 30 frames/s) were analyzed using the ImageJ plugin AnimalTraker (Gulyàs et al., 2016), a tracking application specifically designed to support animal behavior analyses. We relied on the 'Tracker module', which assigned for each organism its XY coordinates in each frame, while the 'Tracking Analyzer module' extracted the measured parameters from the trajectory of the tracked object (i.e., individual). Swimming activity, in terms of distance moved (mm) by each single organism, was measured.

2.6. Statistical analysis

The effects of METH treatment, time of exposure and their interaction on the amount of ROS, enzyme activity and lipid peroxidation were investigated by using General Linear Models (GLM), while the effect on swimming activity was assessed through the application of Linear Mixed Models (LMM), including the treatment, the time of exposure as predictors, while the identity of the exposure beaker was included in the model as a random factor. The effect of METH treatment on the reproductive success of *D. magna* specimens was assessed by Generalized Linear Models, assuming a Poisson distribution of the data. Fisher's LSD post-hoc test was applied to point out significant differences among treatments, time of exposure and treatment × time of exposure interaction. Significance was set at $p < 0.05$ (*) and $p < 0.01$ (**). Statistical analyses were performed using IBM SPSS Statistics 25.0 software package.

3. Results

3.1. Analysis of METH levels

The concentration of METH was checked in both the stock solutions and in culture medium collected from the exposure beakers, and measured concentrations were slightly higher than the nominal ones The high accuracy is probably ascribable to standard preparation and dilution together with analytical variability, nevertheless the quality of analyses was checked during analyses and was good. In details, the concentration of METH in the stock solution 1 (nominal concentration: 1 mg/L) was 1.43 mg/L (accuracy: 143%), while the concentration in the stock solution 2 (nominal concentration: 100 μg/L) it was 151 μg/L (accuracy: 151%). No residues of METH were found in control beakers, while the concentration of METH in culture medium from beakers spiked with 50 ng/L and 500 ng/L were 65 ng/L (accuracy: 130%) and 571 ng/L (accuracy: 114%), respectively.

3.2. Biochemical and behavioral effects

A low mortality of *D. magna* specimens was noted in control, 50 ng/L and 500 ng/L experimental group, accounting for 4 ± 1.4%, 12 ± 2.2% and 6.6 ± 2.5% over the 21-days of exposure. In Table 1 are reported the results of the statistical analyses performed on the selected endpoints. A significant effect of time of exposure was noted for all the investigated endpoints (Table 1). In detail, ROS levels measured at 14-days of exposures significantly differed from those measured after 7 and 21 days of exposure. A similar trend was observed also for SOD activity. An overall, significant increase in CAT activity was noted in 14- and 21-days old specimens compared to 7-days old ones, while GPx activity showed a decreasing trend with age. Although an overall, significant decrease of ROS levels was noted in specimens from both treatment groups compared to the control independently of the time of exposure, the time × treatment interaction revealed a significant increase in the amount of pro-oxidant molecules after 21-days of exposure to 500 ng/L of METH with respect to the corresponding temporal control (Fig. 1a). A significant effect of treatment was noted on SOD, whose activity showed a 38% increase in specimens treated with 500 ng/L of METH compared to control (Fig. 1b). Moreover, the time × treatment interaction showed a significant increase of SOD activity after 7 and 21-days of exposure to the highest METH concentration. While a significant decrease of CAT activity was noted after 7- and 14-days of exposure to both the tested METH concentrations, a significant activation of this enzyme occurred after 21-days of exposure to 500 ng/L only, with values 67% higher than those measured in the corresponding temporal control (Fig. 1c). A similar trend was also observed for GPx, with a ~20% decrease of its activity after 7-days of exposure to both the tested concentrations, and 57% and 45% reduction with respect to controls after the 14-days exposure to 50 ng/L and 500 ng/L, respectively. However, GPx activity measured in 21-days old specimens treated with the highest METH concentration resulted as 2-fold higher compared to controls (Fig. 1d). Lastly, a marginally significant effect of METH exposure on lipid peroxidation was noted, independently of the time of exposure (Fig. 1e). In fact, ~50% increase of lipid peroxidation was noted after the exposure to both the tested concentrations compared to controls. METH exposure did not induce any significant effect on swimming activity in terms of changes in the distance moved by treated specimens compared to control ones (Fig. 2).

3.3. Effects on reproductive output

METH treatment induced a significant increase in the total number of offspring. In detail, the mean number of offspring born by specimens exposed to 50 ng/L and 500 ng/L of METH was 28% and 29% higher compared to controls, respectively (Fig. 3). In contrast, no significant effect on the mean number of clutches between treated and control groups was noted (data not shown).

4. Discussion

Our results showed that the exposure to two environmentally relevant concentrations of methamphetamine, namely 50 ng/L and 500 ng/L, imbalanced the oxidative status and altered the reproductive output of *Daphnia magna*. METH exposure significantly modulated the levels of pro-oxidant molecules after the exposure to both the tested concentrations, according to previous study demonstrating time dependency of oxidative stress biomarkers in *D. magna* (Barata et al., 2005). A significant overproduction of ROS was noted after 21-days of exposure to the highest tested concentration compared to controls. These results agreed with previous studies performed on murine models that highlighted an overproduction of ROS after the exposure to METH concentrations (Yamamoto and Raudensky, 2008; Halpin et al., 2014; Johnson et al., 2015; McDonnell-Dowling and Kelly, 2017). For instance, an increase in ROS levels was noted in murine striatal brain tissue after 2 h exposure to 2 mM of METH (Pubill et al., 2005), as well as in different brain regions after 4 h and 24 h after the last of four consecutive injections of METH (10 mg/kg/injection q2h; Gluck et al., 2001). Moreover, a ROS overproduction was also noted in medaka fish embryos after the exposure to 40 μM of METH (Liao et al., 2015). in the different temporal response to METH exposure in terms of ROS overproduction between *D. magna* specimens and murine or fish species might be attributable to the different sensitivity of the biological models to METH or to the dissimilar administration procedures or to the difference in tested concentrations. In fact, the concentration administered in previous investigations were one or two order of magnitude higher

Table 1
Results of the statistical analyses reporting the effects due to METH treatment, time of exposure and their interactions on oxidative stress-related (ROS, SOD, CAT, GPx and LPO) and behavioral (swimming activity and reproductive output) endpoints in *D. magna*. Significant effects are reported in bold. F = Fisher-Snedecor F; df = degrees of freedom; P = P-value associated to F-value.

	F	df	P
Biochemical effects			
ROS			
Time	26.898	2,17	**<0.001**
Treatment	14.212	2,17	**<0.001**
Time × treatment	8.380	4,17	**0.001**
SOD			
Time	3.821	2,16	**0.044**
Treatment	6.805	2,16	**0.007**
Time × treatment	7.935	4,16	**0.001**
CAT			
Time	12.285	2,17	**0.001**
Treatment	3.435	2,17	0.056
Time × treatment	7.296	4,17	**0.001**
GPx			
Time	128.509	2,18	**<0.001**
Treatment	32.886	2,18	**<0.001**
Time × treatment	36.937	4,18	**<0.001**
LPO			
Time	58.473	2,18	**<0.001**
Treatment	2.968	2,18	0.077
Time × treatment	1.316	4,18	0.302
Swimming activity			
Distance moved			
Time	2.195	2237.6	0.114
Treatment	1.388	2237.6	0.321
Time × treatment	2.204	4237.6	0.069
Reproductive output	Wald χ^2	df	P
Number of offspring			
Treatment	28.674	2,31	**<0.001**
Number of clutches			
Treatment	0.213	2,31	0.899

compared to those tested in the present study, explaining the increase of ROS only at the end of the exposure to 500 ng/L of METH. The increase in ROS levels might be the result of METH-induced increase of the neurotransmitter dopamine, which might undergo auto-oxidation or enzymatic oxidation by monoamine oxidase leading to the production of hydrogen peroxide (H_2O_2) and limitedly of hydroxyl radicals (OH•) and superoxide radicals ($O_2^{\bullet -}$) (McDonnell-Dowling and Kelly, 2017).

In accordance with the increase of pro-oxidant molecules, a METH-induced modulation of the activity of antioxidant enzymes was noted. A significant increase of SOD activity was noted in 21-days old specimens treated with the highest METH concentration, suggesting an overproduction of superoxide radicals, whose toxicity was counteracted by the activity of CAT and GPx. A significant increase of the activity of CAT and GPx, which play a complementary role in the detoxification of hydrogen peroxide, was observed in specimens treated with 500 ng/L of METH. A similar trend of CAT activity was observed in the hypothalamus, liver, and kidney of male Sprague Dawley rats injected twice a day over five days period with 10 mg/kg of METH (Koriem et al., 2013), as well as in brain dissected from male Wistar rats treated for 24 h with 5 mg/kg of METH (Shokrzadeh et al., 2015). Furthermore, an increase in CAT activity was also noted in medaka fish embryos after exposure to 4–40 μM of METH (Liao et al., 2015). Thus, the increase in CAT and GPx activity we found at the end of the exposure to 500 ng/L of METH suggests an effective response of the organisms to the toxicity of H_2O_2. However, a significant decrease of CAT and GPx activity was noted after 7 and 14-days of exposure to both the tested concentrations. These results were partially in agreement with previous studies showing that the exposure to METH inhibited the activity of GPx, but not of CAT. For instance, 24 h exposure to 100 μM of METH decreased GPx1 and GPx4 protein levels as well as their enzymatic activity in SH-SY5Y neuronal cells (Barayuga et al., 2013), while the intraperitoneal injection of 10 mg/kg of METH decreased the GPx activity in the hippocampus of rats (Mozaffari et al., 2019). As suggested by some studies, the inhibition of antioxidant enzyme activity might be due to mitochondrial dysfunction (e.g., Cadet et al., 2007; Motaghinejad et al., 2017; Mozaffari et al., 2019) or directly affect cell-signaling pathways (Barayuga et al., 2013), even though further investigations are needed to verify these hypotheses. Overall, these results point to an imbalance of the oxidative status of METH-treated *D. magna* specimens that can lead to an oxidative stress situation and, consequently, to oxidative damage towards the main cellular macromolecules, including lipids, proteins and DNA (Lushchak, 2011). METH exposure induced a notable, but only marginally significant increase in the levels of lipid peroxidation in 21-days old specimens treated with the both the tested concentrations. Significant increase in lipid peroxidation was found in the retina and blood plasma of rats treated with 5 mg/kg every 2 h for a 6 h period (Melo et al., 2010), as well as the brain of male Wistar rats after a 1 day exposure to 5 mg/kg (Shokrzadeh et al., 2015).

Although we have previously demonstrated that contaminant-induced onset of oxidative stress is often related with changes in the swimming behavior of *D. magna* (Parolini et al., 2018; De Felice et al., 2019), exposure to METH did not affect the distance swam by Cladocerans at both the tested concentrations. Previous studies on different model organisms returned contrasting results on the effects of METH exposure on animal movements. For instance, intrapericardial injection of 2 μg/g of METH increased the mobility of crayfish *Orconectes rusticus*, while the injection of a high dose (10 μg/g) promoted immobility (Imeh-Nathaniel et al., 2017, 2016). The exposure to 10–40 μM of METH decreased the activity in medaka fish embryos (Liao et al., 2015), while no changes in distanced moved, velocity or activity were noted in the crayfish *Procambarus virginalis* exposed for 7- and 21 days to 1 μg/L of METH (Hossain et al., 2019). These differences might me due to different sensitivity of the biological models as well as to the dissimilar administration procedures or to the difference in tested concentrations.

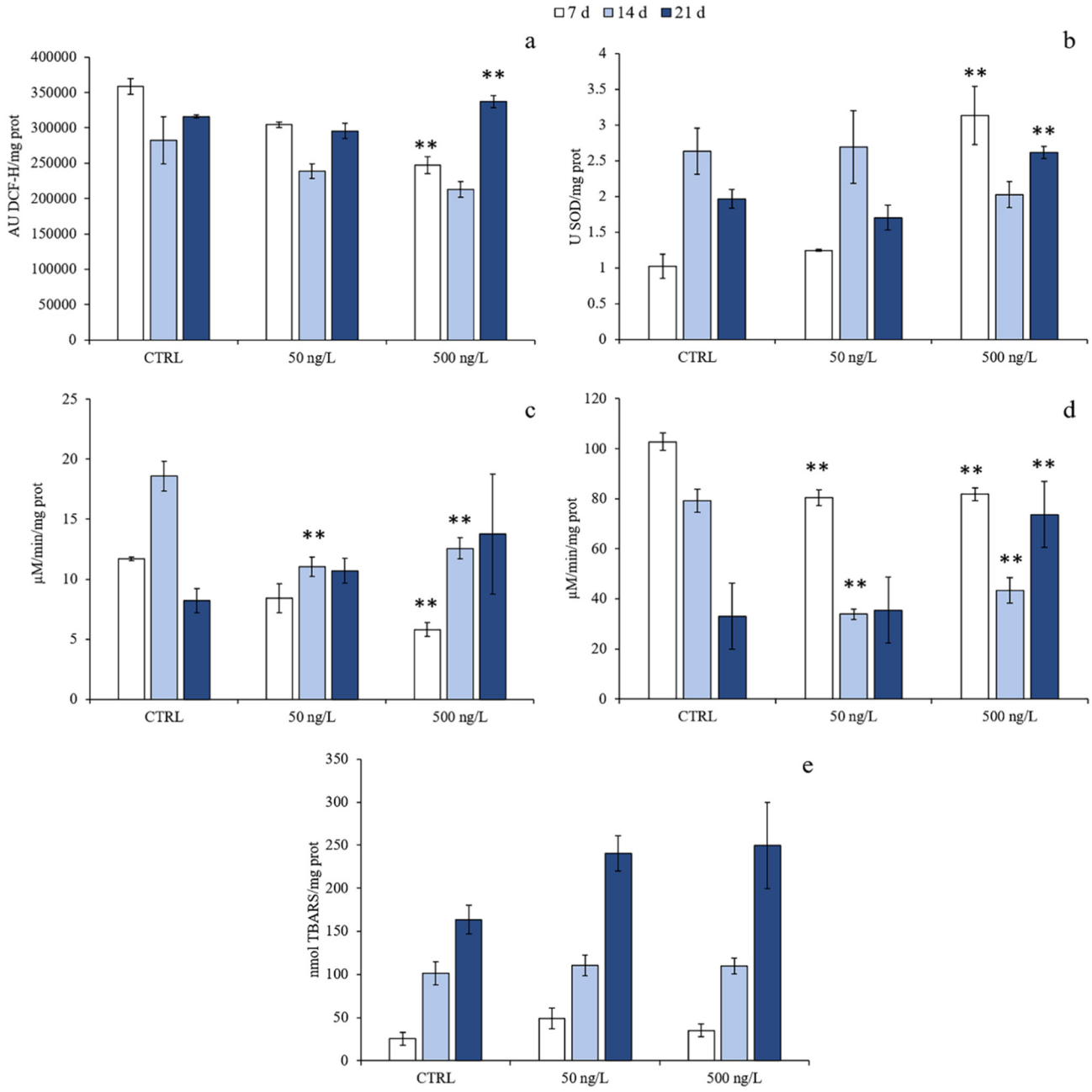

Fig. 1. Mean (±standard error) of the amount of ROS (a), SOD activity (b), CAT activity (c), GPx activity (d), GST activity (e) and lipid peroxidation measured (f) in *Daphnia magna* after 7, 14 ad 21 days of exposure to 50 ng/L and 500 ng/L of METH. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (time per time; $^{**}p < 0.01$).

Both the tested concentrations of METH stimulated the reproduction of *D. magna*, as shown by the significant increase of the mean number of offspring born from treated specimens with respect to controls. To date, only one study analyzed the effect of METH on sexual behavior and reproduction of aquatic models, showing that the exposure to 0.1, 0.5 and 1 mg/L of METH altered the sexual behavior in male sailfin molly *Poecilia latipinna*, stimulating sex arousal, courtship and mating attempt (Ghazilou and Gazilou, 2011). This unexpected result might be due to the specific MoA of METH, which determines the increase of dopamine levels in organisms (Angilin et al., 2000; Vearrier et al., 2012). Several studies have demonstrated the pivotal role of dopaminergic system in controlling the reproductive functions in both invertebrate and vertebrate model organisms (e.g., Baskerville and Douglas, 2008; Giuliano and Allard, 2001; Prasad et al., 2014), suggesting that the increase in dopamine levels stimulates the reproduction (Boulay et al., 2001; Bloch et al., 2000; Kleitz-Nelson et al., 2010). This is particularly true for crustaceans, whose reproduction relies on the release of neurohormones regulated by biogenic amines, such as the dopamine (Sainath and Reddy, 2010; Cahansky et al., 2011; Swetha et al., 2011). Indeed, modulation of dopamine transmission can be considered as a key pharmacological feature mediating the effect of METH on the fitness of organisms (Ghazilou and Gazilou, 2011). Thus, the increase in the reproductive output of METH-treated specimens might be related to the effect of METH on dopamine levels or transmission in *D. magna*.

5. Conclusion

Our results showed that the exposure to two environmentally relevant concentrations of METH induced both biochemical and reproductive output effects in *D. magna* specimens. The exposure to

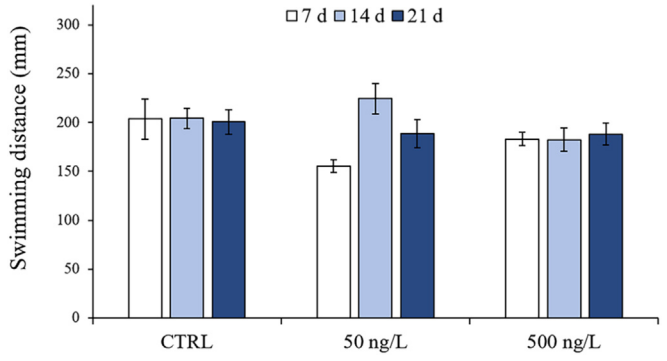

Fig. 2. Mean (±standard error) of distance moved (mm) measured in *Daphnia magna* after 7, 14 ad 21 days of exposure to 50 ng/L and 500 ng/L of METH. No significant differences between treated individuals and the corresponding control were noted.

both the tested concentrations imbalanced the oxidative status of Cladocerans and caused an unexpected increase in the reproductive output that could be due to the effect of METH on dopamine levels or transmission. Moreover, considering the continuous and increasing use of METH worldwide, the current environmental levels might increase, resulting in potentially worse effects at all the levels of the ecological hierarchy. Thus, further studies are necessary to shed light on the toxicity and MoA of METH in *D. magna*, focusing especially on its role in neurotransmitters modulation due to their crucial role in organism behavior.

CRediT authorship contribution statement

Beatrice De Felice: Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Simona Mondellini:** Investigation, Formal analysis, Writing - review & editing.**Noelia Salgueiro-González:** Formal analysis, Writing - review & editing. **Sara Castiglioni:** Formal analysis, Writing - review & editing. **Marco Parolini:** Conceptualization, Methodology, Formal analysis, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

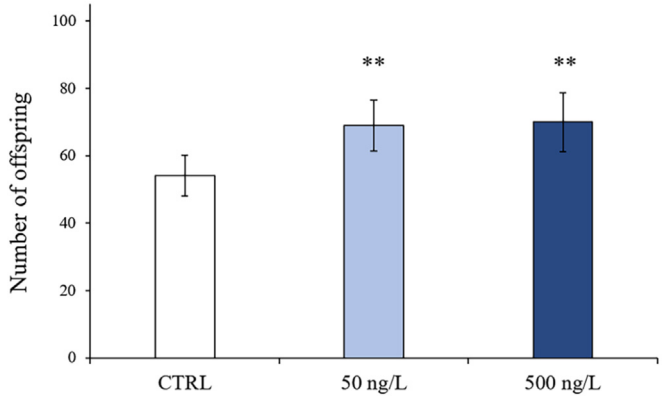

Fig. 3. Mean (±standard error) number of offspring of *Daphnia magna* specimens after 21 days of exposure to 50 ng/L and 500 ng/L of METH. Asterisks above the histograms show significant differences between treated individuals and the corresponding control (**$p < 0.01$).

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PAPER III

De Felice B., Parolini M.

Effects of single and combined exposure to cocaine and benzoylecgonine on the oxidative status of *Mytilus galloprovincialis*.

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Effects of single and combined exposure to cocaine and benzoylecgonine on the oxidative status of *Mytilus galloprovincialis*

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ABSTRACT

The information concerning the effects of single and combined exposure to cocaine (COC) and its main metabolite, the benzoylecgonine (BE), towards marine organisms is still scant. Thus, the aim of this work was to compare the effects induced by 96 -hs exposure to a concentration of COC (500 ng/L) or BE (20 ng/L) and their mixture (500 ng/L of COC and 20 ng/L of BE) on *Mytilus galloprovincialis*. Oxidative stress biomarkers were applied on mussel gills and digestive gland, investigating changes in the amount of reactive oxygen species, activity of antioxidant (SOD, CAT and GPx) and detoxifying (GST) enzymes and lipid peroxidation. Independent exposure to COC and BE slightly altered mussel oxidative status in both the organs, while the mixture induced more marked responses compared to single molecules. Our results suggest the necessity to explore the toxicity of illicit drug mixtures to shed light on the risk of these molecules to marine organisms.

1. Introduction

Illicit drugs are emerging contaminants that can cause both direct and indirect adverse effects towards aquatic ecosystems worldwide (dos Santos Ortega et al., 2019; Parolini et al., 2017; Castiglioni and Zuccato, 2010). Among illicit drugs, cocaine (COC) is one of the most used psychoactive substances, with an estimated amount of 19 million users in 2018 (World Drug Report, 2020). After the assumption of a single dose, COC undergoes human metabolism and is excreted mostly as two main metabolites, the benzoylecgonine (BE, 45 % of the dose) and the ecgonine methyl ester (EME, 40 % of the dose), while is limitedly excreted as parental compound and other metabolites (van Nuijs et al., 2011v). Therefore, cocaine and its metabolites are continuously discharged in the sewage. Two recent reviews on illicit drugs occurrence have reported that COC was found in both influents and effluents of Wastewater Treatment Plants (WWTPs) in concentrations up to 4700 ng/L and 530 ng/L, respectively (Fontes et al., 2020; Yadav et al., 2017). Similarly, BE was detected at concentrations up to 7500 ng/L and 1500 ng/L in WWTPs influents and effluents, respectively (Fontes et al., 2020; Yadav et al., 2017). Because of the limited removal efficiency of the traditional WWTPs, COC and BE were measured in surface waters, in concentrations ranging between 0.4 and 44 ng/L and between 3 ng/L and 316 ng/L, respectively (Fontes et al., 2020; Pal et al., 2013; Yadav et al., 2017). Interestingly, a study by Thomas et al. (2014) detected COC in Brazilian urban streams in concentration up to 5900 ng/L. Considering that surface waters and urban streams flow into seawaters, measurable concentrations of these drugs might occur also in marine ecosystems. However, a limited number of studies have currently monitored the occurrence of illicit drugs in marine, coastal ecosystems. A study by Pereira et al. (2016) showed the occurrence of COC and BE, in the Santos bay (Santos, Brazil) in concentrations up to 537 ng/L and 20.8 ng/L, respectively. In addition, a recent study conducted in the same coastal area measured lower concentration of COC and BE up to 203 ng/L and 38 ng/L, respectively, because of seasonal variability or different seawater parameters (Fontes et al., 2019). Although current environmental levels of COC and BE in aquatic ecosystems are relatively low, because of their high biological activity, adverse effects towards non-target aquatic organisms cannot be underestimated. In fact, a growing number of laboratory studies pointed out the potential toxicity of COC and BE in both invertebrates (Binelli et al., 2012; Parolini et al., 2013; Pedriali et al., 2013; da Silva Souza et al., 2019; De Felice et al., 2019; dos Santos Ortega et al., 2019; Parolini et al., 2018a) and vertebrates (Capaldo et al., 2019, 2018; Parolini et al., 2017, 2018b). All these studies suggested that the mechanism of action of COC and BE relies on the onset of oxidative stress. However, such investigation were focused only on the toxicity of a single molecule, while in environment illicit drugs occur as complex mixtures (Santo et al., 2010), whose toxicity cannot be accurately evaluated by analyzing the effects

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obtained by the exposure to a single molecule (Flaherty and Dodson, 2005). Indeed, the combined exposure to COC and BE might induce additive or synergic effects with unpredictable outcomes (Flaherty and Dodson, 2005; Santo et al., 2010), including the onset of an oxidative stress situation. For these reasons, exploring the toxicity of mixtures represents a challenge in the risk assessment of illicit drugs in order to better understand the risk for non-target aquatic organisms. To date, only two studies investigated the effects of an illicit drug mixture on aquatic organisms by exposing *D. polymorpha* specimens to a mixture of cocaine (50 ng/L), benzoylecgonine (300 ng/L), amphetamine (300 ng/L), morphine (100 ng/L) and 3,4-methylenedioxymethamphetamine (50 ng/L). These studies showed that the illicit drug mixture altered the oxidative status of the zebra mussels, leading to oxidative and genetic damage (Parolini et al., 2015, 2016), supporting the idea that oxidative stress is one of the main mechanisms of action involved in the toxicity of these molecules. In spite of these findings, to date no study explored the toxicity of illicit drug mixtures towards marine organisms, although several studies have pointed out the presence of these molecules also in marine surface water (Fontes et al., 2020, 2019; Pereira et al., 2016). Moreover, considering that the alkaline pH of seawaters could promote the absorption and the bioavailability of illicit drugs, the potential negative effects in marine organism could be worse compared to freshwater ones (da Silva Sousa et al., 2019; Pereira et al., 2016). Thus, considering the limited or no information concerning the toxicity of illicit drugs towards marine organisms singularly or in mixture the aim of this study was to investigate the adverse effects induced by independent and combined 96 -hs exposure to an environmental concentration (Pereira et al., 2016) of COC (500 ng/L) and BE (20 ng/L) towards the Mediterranean mussel (*Mytilus galloprovincialis)*. After 48 and 96 h of exposure, the gills and the digestive glands of mussels were isolated and used to apply a battery of oxidative stress biomarkers. In detail, we investigate the changes in the amount of reactive oxygen species (ROS), the activity of the main antioxidant (SOD, CAT and GPx) and detoxifying (GST) enzymes as well as the onset of oxidative damage (i.e., lipid peroxidation). As previous studies on freshwater mussels showed that COC and BE induced an oxidative stress situation both independently and in mixture, we expect a similar response also in marine mussels.

2. Materials and methods

2.1. Chemicals and reagents

The analytical standard of cocaine (COC - CAS number 50−36-2; molecular weight 303.35 g/mol) and of benzoylecgonine (BE - CAS number 519−09-5; molecular weight 289.33 g/mol) used in this work were purchased from Sigma-Aldrich (Steinheim, Germany), after obtaining the permission for detention and use for scientific purpose by the Italian Ministry of Health (Decree n. SP/177, 11/12/2017). COC standard solution (1 g/L in acetonitrile) and BE standard solution (1 g/L in methanol) were diluted in ultrapure water to obtain a 1 mg/L stock solution for each chemical, whose concentration was validated according to the method reported elsewhere (Parolini et al., 2018a; De Felice et al., 2019).

In detail, a stock solution of COC (1 mg/L) and a stock solution of BE (1 mg/L) ware prepared by diluting 100 μL of the commercial standard solutions in 100 mL of ultrapure water. The obtained stock solutions were maintained at 4 °C in the dark and used as working solutions to perform the experiment during the 96 h of exposure. The reagents and the solvents used for the biomarker analyses were purchased by Sigma Aldrich (Italy).

2.2. Experimental design

Mediterranean mussels (*Mytilus galloprovincialis*) were collected in the gulf of Le Grazie (La Spezia, Italy) on December 2018. Animals were quickly transferred to aquaria located in the facility of the University of Milan. Such facility consists in several aquaria filled with artificial seawater (Instant Ocean, with a salinity of about 37‰) continuously circulating and under constant aeration. The temperature was settled to be 14 °C (±1 °C) with a photoperiod of 16 h of light and 8 h of dark. The temperature used during the experiment was settled at 14 °C (±1 °C) in order to simulate the environmental temperature at the moment of sampling. Animal viability, as well as all water physical and chemical parameters were monitored daily, and death animals were removed from the aquaria. Mussels were maintained in the aquaria for 2 weeks to allow their acclimation to laboratory conditions and depuration from contaminants accumulated in their native place before starting the experiments.

Mussels were exposed independently or in mixture to COC and BE because they are the illicit drugs found at higher concentrations in marine ecosystems (Pereira et al., 2016). In detail, mussels were exposed independently to a concentration of COC (500 ng/L) or BE (20 ng/L) similar to the mean levels detected in seawater samples collected in the Santos Bay (Santos, Brazil) by Pereira et al. (2016). In addition, mussels were exposed to a mixture of COC and BE, which were administered at the same concentrations tested independently. Twenty-five organisms (~ 4 cm in length) were seeded in 5 L glass beakers containing seawater from the facility where mussels were acclimatized and maintained at the same conditions described above. Four experimental groups were planned: a control group in which mussels were maintained in artificial seawater only, a COC-treated group (500 ng/L), a BE-treated group (20 ng/L) and a mixture-treated group (hereafter MIX; 500 ng/L of COC and 20 ng/L of BE). Although the standard solutions were in acetonitrile (COC) and in methanol (BE), no solvent-control group was planned because of the very low amount of acetonitrile and methanol included in the exposure beakers (maximum calculated amount of acetonitrile accounted for 0.03 % of the final volume and maximum calculated amount of MeOH accounted for 0.002 %). We performed two independent experimental replicates per each single experimental group, whereby twenty-five organisms were used for each replicate, for a total of fifty individuals per each treatment. Mussels were placed on a stainless grid close to the bottom of the beakers. The exposure lasted 96 h and was performed under semi-static conditions, whereby the contaminant where renewed every other day. As a previous studies demonstrated that the rate of cocaine reduction in saltwater after 24 h corresponded to 33.17 % (dos Santos Barbosa Ortega et al., 2019), while BE is considered as highly stable than COC (Campestrini and Jardim, 2017: Bijlsma et al., 2013; Gheorghe et al., 2008) the renewal of the exposure medium should allow to maintain constant levels of exposure. One hour before each seawater renewal, mussels were fed with 0.3 g of Algamac2000® (Aquafauna Bio-Marina, USA), an algae replacement-substitute-enrichment medium consisting of spray-dried cells of *Schizochytrium* spp. algae. Before the beginning of the experiment sixteen organisms were randomly chosen from each beaker in order to monitor the baseline levels of oxidative stress biomarkers. After 48 and 96 h of exposure, ten mussels were randomly chosen from each beaker (i.e., replicate). Each organism was dissected, the gills and the digestive glands were isolated, quickly frozen in liquid nitrogen and stored at – 80° until biochemical analyses.

2.3. Oxidative stress biomarker methods

The biochemical analyses were conducted on a pool of gills and digestive glands of mussels according to methods described elsewhere (Parolini et al., 2020). Three independent replicates (i.e., three pools of organs from three organisms) were performed per each beaker, for a total of nine organisms per treatment and time point. All the biochemical analyses were carried out in duplicate for each pool. For each time point and experimental group, gills and digestive gland from three organisms exposed in the same beaker (~ 0.3 *g*) were pooled and homogenized using an automatic motor pestle in 100 mM phosphate buffer (pH 7.4), with the addition of KCl (100 mM), EDTA (1 mM), specific

protease inhibitors (1:100 v/v) and dithiothreitol (DTT, 100 mM). Homogenates were centrifuged at 15.000 × g for 30 min at 4 °C. The supernatant was collected and immediately processed to measure the protein content and oxidative stress biomarkers. The amount of ROS was performed following a fluorometric evaluating the change in fluoresce of dichlorofluorescein-diacetate (DCFH-DA; 10 mg/mL in DMSO) due to the presence of pro-oxidant molecules (Deng et al., 2009). The fluorescence was recorded by an EnSight multimode plate reader (Perkin Elmer) with λ =485 nm as excitation and λ =536 nm as emission wavelength, respectively. Protein content, enzyme activity and lipid peroxidation were assessed through spectrophotometric methods. The SOD activity was evaluated by measuring the inhibition of cytochrome c (10 μM) reduction caused by the superoxide anion formed after the xanthine oxidase (1.87 mU/mL) hypoxanthine (50 mM) reaction at λ =550 nm for 1 min. The CAT activity was evaluated by assessing the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (66.7 mM; pH 7) at λ =240 nm for 1 min. The GPx activity was evaluated by measuring the consumption of NADPH (0.12 mM) towards using the H_2O_2 (0.2 mM) as a substrate in a potassium phosphate buffer (50 mM), containing glutathione (2 mM), sodiumazide (1 mM) and glutathione reductase (2 U/mL) at λ =340 nm for 1 min. The GST activity was evaluated by adding the reduced glutathione (1 mM) in a potassium phosphate buffer (80 mM; pH 7.4) using 1-chloro-2,4 dinitrobenzene (CDNB; 1 mM) as a substrate at λ =340 nm for 1 min. Total protein content was determined following the Bradford method (1976) and used to normalize the activity of the antioxidant enzymes. Lipid peroxidation was evaluated by the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979) and results were expresses as nmol TBARS / mg protein. All the spectrophotometric analyses were performed by means of a Genova Bio spectrophotometer (Jenway).

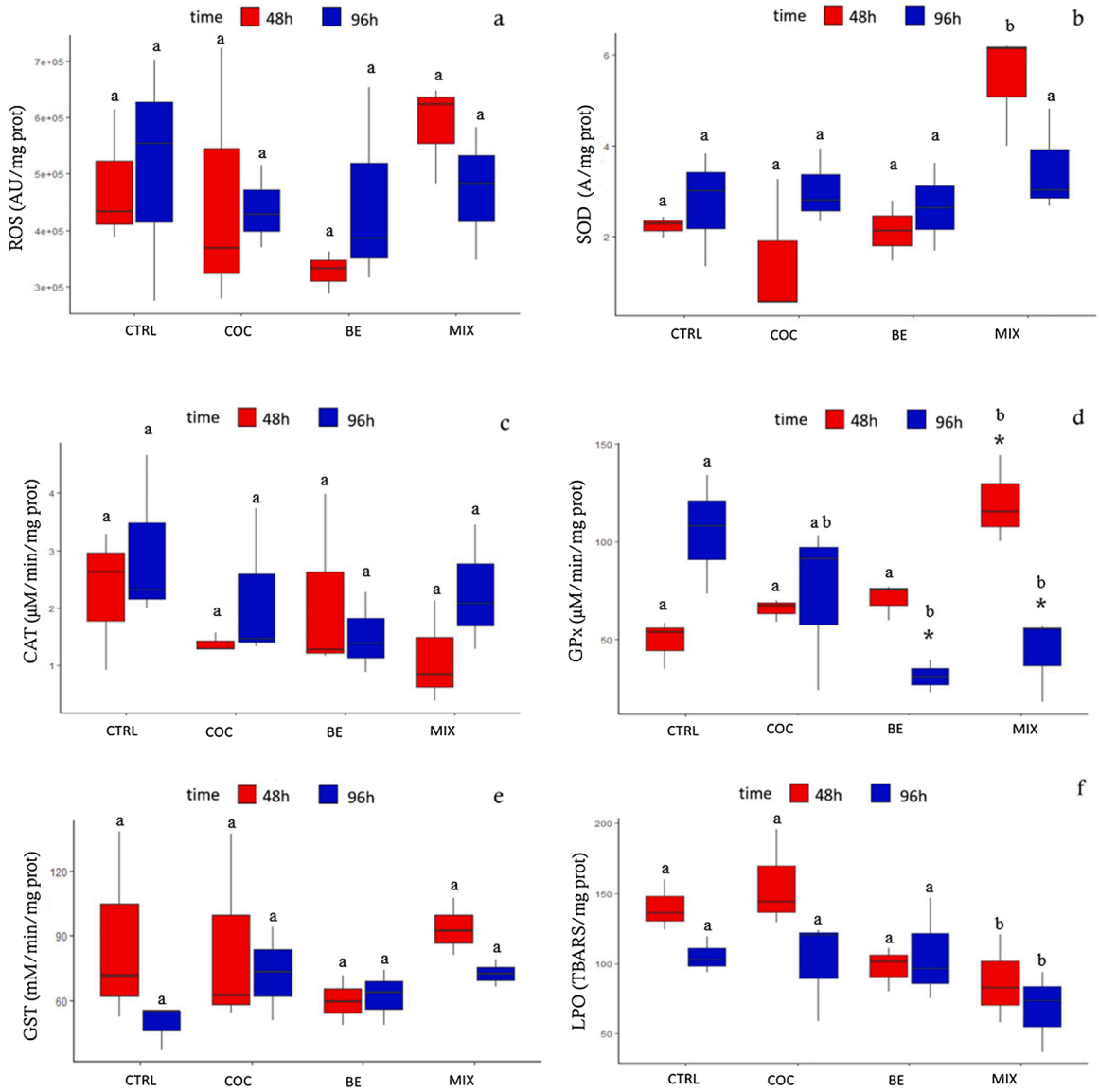

Fig. 1. Box and whiskers plot of the amount of ROS (a), SOD activity (b), CAT activity (c), GPx activity (d), GST activity (e) and lipid peroxidation (f) measured in homogenates of gills dissected from *M. galloprovincialis* after 48 (red) and 96 (blue) hours of exposure to COC, BE and MIX. Asterisks above the box and whiskers plot show significant differences between treated individuals and the corresponding control (* $p < 0.05$). Letters above the box and whiskers plot indicate significant differences among groups and time of exposure. Same letters indicate the lack of significant differences among groups.

2.4. Statistical analysis

The effects of the treatment, time of exposure and their interaction on the amount of ROS level, activity of enzymes and lipid peroxidation were investigated by a two-way ANOVA statistical test, including the treatment and the time of exposure as predictors. When significant differences among treatments, time of exposure or their interaction were found a Tukey post-hoc test was applied. Significance was set at $P < 0.05$ (*) and $P < 0.01$ (**). Statistical analyses were ran in R 3.6.1 (R Core Team 2019).

3. Results

None of the mussels used in the experiment died during the 96-hrs exposure in any experimental group. No significant effects of the treatment ($F_{3,16} = 0.9833$, $P = 0.4254$), the time of exposure ($F_{1,16} = 0.0096$, $P = 0.9233$) and the time × treatment interaction ($F_{3,16} = 0.6710$, $P = 0.5822$) on the amount of ROS in the gills was noted (Fig. 1a). In contrast, a significant effect of the treatment ($F_{3,16} = 5.986$, $P = 0.00618$), but not of the time of exposure ($F_{1,16} = 0.134$, $P = 0.7193$) and time × treatment interaction ($F_{3,16} = 2.949$, $P = 0.0647$) on SOD activity was found. In detail, SOD activity measured in the gills of mussels exposed to the MIX was significant higher compared to that measured in control ($P = 0.022$), COC-treated ($P = 0.010$) and BE-treated ($P = 0.016$) groups (Fig. 1b), independently of the time of exposure. CAT activity did not show any significant modulation (Fig. 1c) related to the treatment ($F_{3,16} = 0.873$, $P = 0,476$), the time of exposure ($F_{1,16} = 1.190$; $P = 0.292$) and their interaction ($F_{3,16} = 0.703$, $P = 0.564$). Although, no significant effect of the treatment ($F_{3,16} = 2.165$, $P = 0.132$) and time of exposure ($F_{1,16} = 2.020$, $P = 0.171$) was noted for the GPx activity, a significant time × treatment interaction ($F_{3,16} = 9.756$; $P < 0.001$) was found (Fig. 1d). Specifically, a significant activation of this enzyme occurred after 48 h of exposure to the MIX ($P = 0.024$), while a significant inhibition was noted after 96 h exposure to both BE ($P = 0.017$) and MIX ($P = 0.048$) compared to the corresponding control. GST did not show any significant effect (Fig. 1e) of treatment ($F_{3,16} = 0,918$, $P = 0.454$), time of exposure ($F_{1,16} = 2.750$, $P = 0.117$) and their interaction ($F_{3,16} = 0.666$, $P = 0.585$). Lastly, a significant effect of treatment ($F_{3,16} = 3.955$, $P = 0.027$) and exposure time ($F_{1,16} = 4.681$, $P = 0.046$), but not of time × treatment interaction ($F_{3,16} = 1.359$, $P = 0.290$), on lipid peroxidation occurred. In detail, levels of lipid peroxidation significantly decrease over the time, with significant differences occurring between mussels exposed to the MIX and the control group ($P = 0.006$; Fig. 1f).

According to results on the gills, no significant effect of the treatment ($F_{3,15} = 1.112$, $P = 0.375$), the time of exposure ($F_{1,15} = 0.189$, $P = 0.670$), as well as the time × treatment interaction ($F_{3,15} = 0.925$, $P = 0.453$) on the hepatic amount of ROS was found (Fig. 2a). A significant effect of treatment ($F_{3,16} = 3.739$, $P = 0.0328$), but not of the time of exposure ($F_{1,16} = 1.508$, $P = 0.2372$) and time × treatment interaction ($F_{3,16} = 0.421$, $P = 0.7404$), on SOD activity was noted (Fig. 2b). In detail, the activity of this enzyme was significant inhibited in mussels exposed to BE compared to the control group ($P = 0.042$). CAT activity (Fig. 2c) did not show any significant modulation due to the treatment

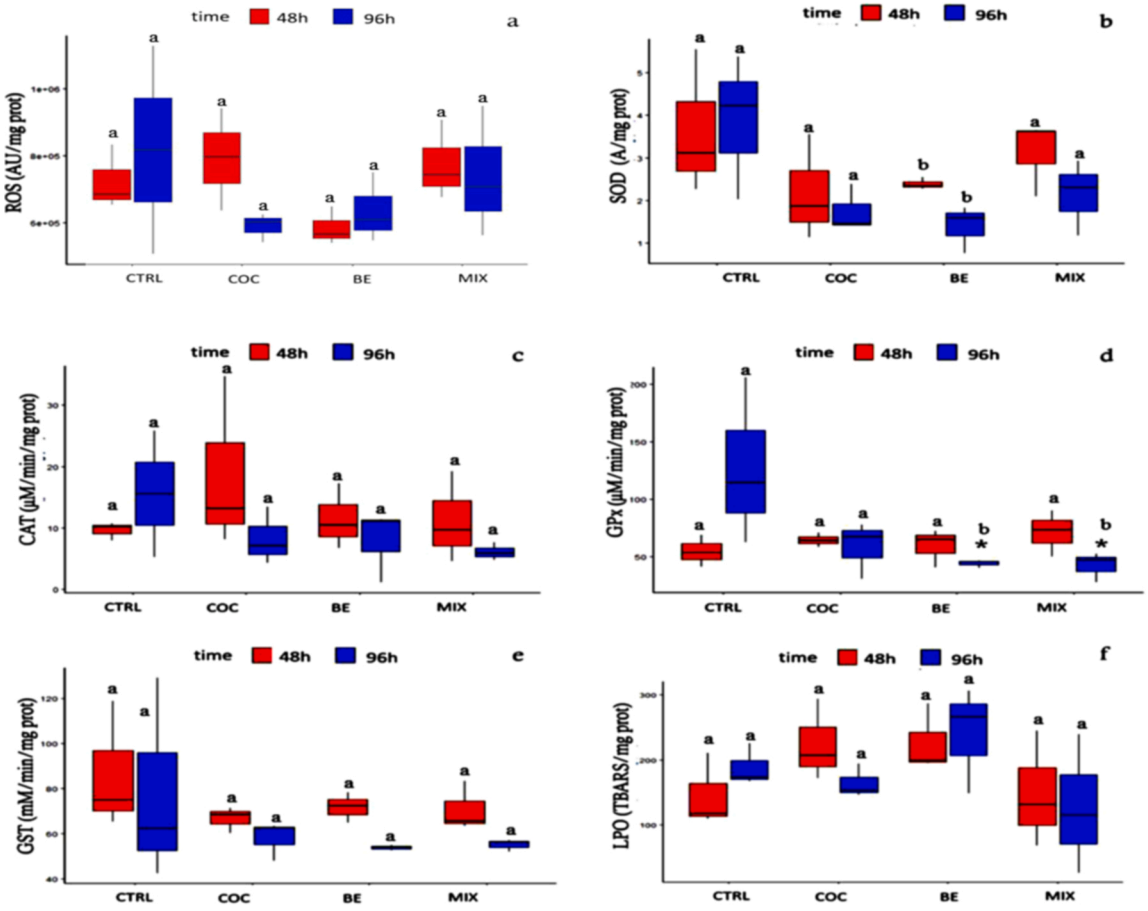

Fig. 2. Box and whiskers plot of the amount of ROS (a), SOD activity (b), CAT activity (c), GPx activity (d), GST activity (e) and lipid peroxidation (f) measured in homogenates of digestive gland dissected from *M. galloprovincialis* after 48 (red) and 96 (blue) hours of exposure to COC, BE and MIX. Asterisks above the box and whiskers plot show significant differences between treated individuals and the corresponding control (* $p < 0.05$). Letters above the box and whiskers plot indicate significant differences among groups and time of exposure. Same letters indicate the lack of significant differences among groups.

($F_{3,15} = 0.477$, $P = 0.703$), the time of exposure ($F_{1,15} = 1.338$, $P = 0.265$) and their interaction ($F_{3,15} = 1.0073$, $P = 0.417$). In spite of no significant effect of treatment ($F_{3,16} = 2.156$, $P = 0.134$) and time of exposure ($F_{1,16} = 0.223$, $P = 0.643$), a significant time × treatment interaction ($F_{3,16} = 3.594$, $P = 0.037$) was found on GPx activity (Fig. 2d). In fact, a significant inhibition of this enzyme was noted after 96 h of exposure to BE ($P = 0.05$) and MIX ($P = 0.043$) compared to the control group. GST activity (Fig. 2e) was not significantly modulated by the treatment ($F_{3,16} = 1.444$, $P = 0.267$), the time of exposure ($F_{1,16} = 2.446$, $P = 0.137$) and their interaction ($F_{3,16} = 0.090$, $P = 0.965$). No significant effect of treatment ($F_{3,16} = 2.119$, $P = 0.138$), time of exposure ($F_{1,16} = 0.048$, $P = 0.829$) and their interaction ($F_{3,16} = 0.619$, $P = 0.613$) on lipid peroxidation occurred (Fig. 2f).

4. Discussion

Independent and combined, short-term exposure to COC and its main metabolite BE induced a slight modulation of the oxidative status in gills and digestive gland of *Mytilus galloprovincialis* individuals. These results were expected because the gills and the digestive gland are considered the main organs that could suffer potential negative effects and an oxidative stress situation induced by the exposure to illicit drugs. In fact, the gills are the first organs to be exposed to an environmental pollutant (Walker et al., 2007; de Oliveira David et al., 2008), while the digestive gland is directly involved in phase I and II pathways of xenobiotic metabolism (Livingston et al., 1994). The exposure to COC, BE and MIX lead to a slight modulation of the activity of antioxidant enzymes in *M. galloprovincialis*, as observed by the changes in SOD and GPx activity for both the analyzed organs. The overall increase of SOD activity observed in gills from MIX-treated mussels compared to the other experimental groups suggest that the mixture induced an overproduction of superoxide anion, whose toxicity was counterbalanced by the SOD. The increase in SOD activity was significantly higher not only compared to the control group but also to COC- and BE-treated groups and could be explained by a higher production of superoxide anion caused by the mixture with respect to the single molecules. The activation of SOD in gills might suggest a production of hydrogen peroxide, which is then degraded by CAT and GPx (Lushchak, 2011). Although no significant modulation of CAT was noted, GPx activity showed significant changes. In detail, a significant increase of the GPx activity compared to all the experimental groups was noted after 48 h of exposure to the MIX. This result agreed with the increase found in SOD activity confirming the overproduction of H_2O_2 and the sub sequential activation of GPx to counteract it. Furthermore, the significant difference in contrast to control, COC and BE supported the previous results and suggested a potential higher modulation of the oxidative status of the mixture when compared with the single exposure. Moreover, a significant inhibition of GPx compared to control was also noted after 96 h in gills of both BE and MIX-treated mussels. The inhibition of the GPx could be due to the duration or to the concentration used in the exposures. Indeed, previous studies demonstrated that the trend of antioxidant enzyme activity can depend on the duration of the exposure and/or on the concentration tested. In fact, short-term exposures or low concentration treatments can lead to an increase in the enzyme activity, while long-term exposure or high concentrations can inhibit it (Valavanidis et al., 2006; Wang et al., 2011). Thus, we might speculate that at the end of exposure to the MIX (and BE), the levels of H_2O_2 were too high to be offset by GPx, but simultaneously not high enough to induce the activation of CAT. The discrepancy in response of CAT and GPx, which play a complementary role in degrading hydrogen peroxide, could be due to the competition for the same substrate (Kappus, 1985) and/or to the difference in the affinity for the H_2O_2 (Pereira et al., 2013). Previous studies demonstrated that GPx is activated by low H_2O_2 levels, while CAT activity is boosted by higher levels of the same molecule (Baud et al., 2004; Pereira et al., 2013). Similar trends of the GPx and CAT activities were found in *D. magna* specimens after the independent exposure to COC (De Felice et al., 2019) and BE (Parolini et al., 2018a), as well as in *D. polymorpha* specimens after the exposure to BE (Parolini et al., 2013). Furthermore, similar antioxidant responses were observed in *D. polymorpha* specimens exposed to an illicit drug mixture, composed by COC, BE, amphetamine, morphine and 3,4-methylenedioxymethamphetamine at their levels found in surface waters (Parolini et al., 2015). Lastly, disaccording to previous studies on mussels showing an induction of GST activity after COC or BE exposure (dos Santos Ortega et al., 2019; Parolini et al., 2013), no significant modulation of GST occurred in both the organs isolated by *M. galloprovincialis* specimens after independent or combined exposure to COC and BE. These results might be explained considering the changes of GPx activity induced by mixture exposure. Indeed, the activity of these two enzymes (i.e., GPx and GST) is related to the presence of the glutathione (GSH). We might speculate that the activation of GPx consumed most of the available GSH stock, compromising the activation of the GST, as suggested by a previous study by Moszczynska et al. (1998). Overall, the discrepancies among studies concerning the response of antioxidant and detoxifying enzymes might be due to differences in tested concentrations of COC and BE or in sensitivity of model organisms. In addition, concerning differences in COC-induced response might be ascribable to the different formulation of this drug used during the experiments, as suggested by dos Santos Barbosa Ortega et al. (2019) in a previous study exposing *P. perna* to crack-COC, a by-product of COC.

In contrast with results obtained for the gills, the exposure to COC, BE and MIX lead only to a slight modulation of the activity of antioxidant enzymes in the digestive gland on mussels. In detail, we noted a significant inhibition in SOD activity after 96 h of exposure to BE, probably due to the inhibition and/or negative feedback mechanism related to the byproducts of SOD reaction (Valavanidis et al., 2006). In addition, GPx activity resulted to be inhibited after 96-hrs of BE and MIX exposures. These results suggest that the gills were more sensitive and quicker to respond to the presence of illicit drugs compared to the digestive gland. Indeed, COC and BE exposure led to a significant modulation of antioxidant enzymes also after a short-term exposure (i.e., 48-hrs), while similar effects might arise in the digestive gland only after a prolonged exposure period (Faggio et al., 2018). Indeed, a previous study of the *D. polymorpha* confirmed that the alteration of the oxidative status occurred only after 96-hrs exposure to BE (Parolini et al., 2013). Furthermore, our results agreed those from a previous study of *P. perna* exposed to crack-COC that showed significant modulations of antioxidant responses in gills after 48-hrs exposure, while no alteration was found in the digestive gland even after a prolonged exposure time (i.e., 168-hrs; dos Santos Barbosa Ortega et al., 2019).

Although our results did not show any significant change in ROS levels, we recorded in both the organs a slight modulation of antioxidant enzyme activity. These modulations, which were more marked after the exposure to the mixture rather than to the single molecules, might suggest an imbalance of the oxidative status of mussel. However, the lack of alteration in ROS levels might suggest that the antioxidant defenses were able to counteract the toxicity of contaminant-induced prooxidant species (Parolini et al., 2020). According to this hypothesis, but in contrast with previous studies on diverse model organisms that highlighted the onset of oxidative damage as a consequence of COC or BE exposure (Pomierny-Chamioło et al., 2013; Parolini et al., 2013, 2018a), no significant increase in lipid peroxidation levels was induced by independent and combined exposure to COC and BE compared to controls. Surprisingly, an overall decrease in the levels of lipid peroxidation of the gills from MIX-treated mussels compared to the control was noted, while no modulation was induced by all the treatments in the digestive gland. We might speculate that both enzymatic and non-enzymatic, exogenous antioxidant defenses play a complementary role to prevent the overproduction of ROS (Prasad et al., 2004; Dietrich et al., 2002; Hoyos et al., 2000) and, consequently, the onset of an oxidative stress situation in treated mussels. Furthermore, our results agree with a previous study of *P. perna* showing that the exposure to

crack-COC did not induce any change in lipid peroxidation levels in mussel digestive gland and gills (dos Santos Barbosa Ortega et al., 2019).

5. Conclusions

Our findings demonstrated that the single exposure to an environmental concentration of COC and BE was not able to alter the oxidative status in the gills and digestive gland of the Mediterranean mussels. In contrast, the combined exposure to these illicit drugs modulated the antioxidant activity in the gills, but not in the digestive gland of treated mussels. These results, although focused only on a battery of some oxidative stress biomarkers, support findings of previous studies focused on other chemicals demonstrating that the exposure to illicit drug mixtures, which reflect the real environmental situation, can induce higher effects than the single molecules. As the concentrations tested in the present study were similar to those measured in marine coastal ecosystems, our results provide the first information regarding the potential toxicity of a mixture of the main illicit drugs found in aquatic ecosystems towards a marine organism. Furthermore, considering the continuative use of COC we could expect an increase of environmental levels of this drug and its metabolite BE that could result in more hazardous effects compared to those observed in the present study. In addition, as in natural ecosystems organisms are exposed for their whole lifespan to different illicit drug mixtures, further studies should be planned to explore long-term consequences of different illicit drug 'cocktails' and their mechanism(s) of action in order to enlarge the knowledge on the risk of these contaminants towards marine organisms and ecosystems.

Credit author statment

Beatrice De Felice: designed the experiments, perform the experiment, data analysis, writing- original draft preparation;

Marco Parolini: conceived the project, designed the experiments, data analysis, writing- reviewing and editing.

All authors edited and contributed to the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Section 2: Microplastic toxicity studies

The presence of microplastics (MPs) in environment is considered a dramatic environmental issue, whose consequences are still unknown. In order to enlarge the limited knowledge on the toxicity of microplastics towards aquatic and terrestrial organisms, five experiments were performed to assess the potential toxicity of different MP types on freshwater, marine and terrestrial model species.

First, two experiments were performed to investigate the potential effects induced by the exposure to increasing concentrations (0.125 µg/mL, 1.25 µg/mL, 12.5 µg/mL) of regular shaped polystyrene microplastics (hereafter PS-MPs) towards two freshwater organisms, namely the crustacean *Daphnia magna* and the amphibian *Xenopus laevis*.
Effects induced by PS-MPs exposure were investigating at individual level, in terms of body growth and swimming activity, and for *D. magna* at population level, in terms of reproductive effort.
The results obtained from these experiments showed that despite an efficient ingestion by both the tested organisms, the consequences due to the presence of MPs into the digestive tracts were different with respect to the model organism. In detail, *Daphnia magna* organisms (PAPER IV) exposed to PS-MPs with different size showed, at the higher tested concentrations, an unexpected increase in body size, swimming and phototactic activity, as well as an in the reproductive effort. These results suggest that the exposure to PS-MPs could affect behavioral traits of this crustacean that might have consequences on population dynamics of this zooplanktonic species. Otherwise, the results obtained for *Xenopus laevis* (PAPER V) showed that PS-MPs can be ingested by tadpoles (from stage 36 to stage 46 of development), but they did not alter the development and the swimming behavior. For this reason, PS-MPs seem do not represent a threat for amphibian larvae, at the concentrations and on the endpoint considered in this study.

The previous experiments tested the potential effects of regular shaped MPs, whereas in natural environments irregular items are more common. For this reason, further experiments, whose results are summarized in PAPER VI, PAPER VII and PAPER VIII, explored the potential effects induced by irregular shaped MPs to aquatic and terrestrial biological models. In these experiments, a polyethylene terephthalate microplastics (PET-MPs) standard was created in laboratory and administered through different approaches to two marine organisms having different feeding strategies, the filter feeder Manila clam *Ruditapes philippinatum* (PAPER VI) and the benthic grazer sea urchin *Paracentrotus lividus* (PAPER VII), as well as to the giant land snail *Achatina*

reticulata (PAPER VIII). PET-MPs were chosen because are the most common MPs that sink in marine ecosystems, as well as one of the most commonly MPs typology found in soils.

In PAPER VI are described the results obtained after 7-days exposure of *R. philipinarum* specimens to two concentrations (0.125 and 12.5 µg/mL) of PET-MPs. A battery of oxidative stress biomarkers was applied to investigate the alteration of the oxidative status and the presence of oxidative damage, while histological analyses were used to investigate the ingestion of PET-MPs and the potential injuries at tissue level. The results showed that the highest tested concentration lead to an imbalance of the oxidative status and the onset of oxidative stress in clam gills, but not in digestive gland. However, no tissue damages were noted.

In PAPER VII are reported the results of a 7-days dietary exposure to three amounts (8, 80 and 800 particles/g of food) of PET-MPs, using as model species the sea urchin *Paracentrotus lividus*. In detail, the possible negative effects were investigated on digestive tract at biochemical (oxidative stress biomarkers) and tissue level (histopathological analyses), as previously described for PAPER VI. In agreement with the result from PAPER VI we highlighted that exposure to PET-MPs induced an alteration in the oxidative status of sea urchin, but no histological alteration. Furthermore, the novelty of PAPER VI it strictly connected to the investigation of the other side of the "plastic coin". Indeed, to date, ecotoxicological studies have focused their attention only on the effect caused by MPs on organisms, entirely omitting the potential biota-induced (physico-chemical) alterations on MPs following the ingestion by organisms. Thus, in PAPER VI besides investigating the possible effect induced by PET-MPs on sea urchin organisms, the attention was focused also on investigating the effect induced by sea urchin on PET-MPs. In detail, it was investigated if the grazing activity and the related transit of PET-MPs within digestive tract might affect PET structure and composition. The analyses of the egested PET-MPs by the SEM (Scanning Electron Microscope) and by FT-IR (Fourier-Transform Infrared Spectroscopy) showed a structural and chemical alteration of PET, indicating a biological weathering of this benthic grazer that might partially contribute to the degradation of PET in marine ecosystems.

Considering the results reported in PAPER VI and PAPER VII, a third experiment was planned with the aim to assess the adverse effects caused by PET-MPs on a terrestrial organism, the giant land snail *Achatina reticulata*.

In PAPER VIII are described the results obtained by 40-days dietary administration of two doses of PET-MPs (1% and 10 % of MPs on the weight of the administered food) on the effects at sub-individual and individual level. According to the results found for *R. philippinarum* the exposure to PET-MPs did not induce an alteration of the oxidative status and oxidative damage in the digestive gland of exposed snails. Surprisingly, the administration of PET-MPs boosted the growth

of treated organisms, suggesting that PET-MPs may act as a mechanical shredder or may promote physiological response that enhanced food assimilation, and consequently the growth of snails.

Overall, the results obtained in these experiments highlighted that both regular and irregular MPs can be efficiently ingested by aquatic and terrestrial organisms. However, the ingestion of MPs lead to differential, species-specific responses. For instance, the discrepancy in responses to the exposure to the same plastic polymer could be explained by different feeding strategies or physiology of different model organisms, as well as to the different experimental set-up and administration pathway of MPs. For instance, the same PET-MPs that induced an imbalance of the oxidative status in *R. philippinarum* and *P. lividus* did not induced the onset of an oxidative stress situation in *A. reticulata*. The negative effect observed in clams and the sea urchins could be due to the rubbing of sharped and needled irregular PET-MPs on tissues of digestive system, while in the giant land snail, the presence of the mucus produced during feeding activity might wrap PET-MPs precluding the rubbing on tissues.

PAPER IV

De Felice B., Sabatini V., Antenucci S., Gattoni G., Santo N., Bacchetta R., Ortenzi M.A., Parolini M.

Polystyrene microplastics ingestion induced behavioral effects to the cladoceran *Daphnia magna*.

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Polystyrene microplastics ingestion induced behavioral effects to the cladoceran *Daphnia magna*

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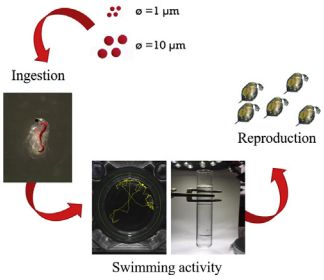
HIGHLIGHTS

- Behavioural effects caused by polystyrene microplastics on *D. magna* were studied.
- Microparticles were observed into the digestive tract of daphnids and adults.
- Unexpected increase in body size of adults and swimming activity was noted.
- An increase in reproductive effort at high microparticle concentration was noted.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic (μPs) contamination represents a dramatic environmental problem threatening both aquatic and terrestrial organisms. Although several studies have highlighted the presence of μPs in aquatic environments, the information regarding their toxicity towards organisms is still scant. Moreover, most of the ecotoxicological studies of μPs have focused on marine organisms, largely neglecting the effects on freshwater species. The present study aimed at exploring the effects caused by 21-days exposure to three concentrations (0.125, 1.25 and 12.5 μg/mL) of two differently sized polystyrene microplastics (PμPs; 1 and 10 μm) to the Cladoceran *Daphnia magna*. The ingestion/egestion capability of daphnids (<24 h) and adults, the changes in individual growth and behavior, in terms of changes in swimming activity, phototactic behavior and reproduction, were investigated. Both particles filled the digestive tract of daphnids and adults within 24 h of exposure at all the tested concentrations. Ingested PμPs remained in the digestive tract even after 96 h in a clean medium. For both particles, an overall increase in body size of adults was noted at the end of the exposure to the highest tested concentrations, accompanied by a significant increase in swimming activity, in terms of distance moved and swimming velocity, and by an alteration of the phototactic behavior. A significant increase in the mean number of offspring after the exposure to the highest PμPs concentrations of different size was recorded. Polystyrene μPs can affect behavioral traits of *D. magna* leading to potentially harmful consequences on population dynamics of this zooplanktonic species.

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1. Introduction

Plastics play a pivotal and irreplaceable role in our society. As plastics are lightweight, durable, inert and corrosion-resistant (Plastic Europe, 2010) they are extensively used in near inexhaustible applications (Andrady, 2011). However, although the undeniable societal benefits of polymers (Andrady and Neal, 2009), the persistence and the inappropriate disposal of plastic materials have raised the worrisome environmental problem of plastic contamination in both anthropic and natural ecosystems. The presence and the impact of the so-called macroplastics (i.e., large plastic debris) have been largely investigated in the marine environment. Such investigations have highlighted that macroplastic contamination represents not only an aesthetic issue with serious economic consequences for tourist and marine-industries, but also a serious threat for the health of marine organisms (Barnes et al., 2009; Sivan, 2011; Cole et al., 2011). In recent years, the attention has been focused on microplastics (μPs), polymer particles smaller than 5 mm that are considered as emerging contaminants of aquatic ecosystems (Avio et al., 2017). Microplastics can be specifically produced to be used in diverse personal care products and as a virgin pellet in different industrial applications (i.e., primary μPS), or can be generated by the break-down of macroplastics (i.e., secondary μPs; Eerkes-Medrano et al., 2015). The main sources of μPs are wastewater treatment plants, inland littering after storm and water overflow events, and industrial processes (BrodhagenPeyron et al., 2015; Dris et al., 2018). A growing number of studies has highlighted the width of microplastic contamination in marine environment, investigating the presence of different plastic polymers in both abiotic (water and sediments) and biotic (zooplankton, mussels and fish) matrices (Cole et al., 2013). However, to date just a limited number of studies has been focused on microplastic contamination in freshwater ecosystems, although they are recognized as the main source of plastic contamination for marine environment (Wagner et al., 2014; Eerkes-Medrano et al., 2015). In fact, microplastics can enter the riverine ecosystems directly, through wastewater effluents, or in refuse site leachates and are transported out to sea and oceans (Browne et al., 2010; Jambeck et al., 2015). In freshwater environmental matrices, recent studies have estimated a μP contamination as high as 0.001–0.1 items/m^2 in lake water, while 0.1–1 items/m^2 in rivers. On the contrary, the estimated contamination in sediments was much higher with 10–10,000 items/m^2 for lakes and 1–1,000 items/m^2 for rivers (Dris et al., 2015). Monitoring surveys of μPs contamination in freshwater ecosystems revealed the presence of diverse plastic polymers, having different environmental fate depending on their physical and chemical features. For instance, polyvinylchloride (PVC) and polyethylene terephthalate (PET) μPs can sink and settle on sediments (Andrady, 2011), while polystyrene (PS), polyethylene (PE) and polypropylene (PP) μPs may float within the water column (Vianello et al., 2013; Hämer et al., 2014; Li et al., 2016). Microplastics have been reported to interact with aquatic organisms through feeding activity (Cole et al., 2013), dermal uptake (Wagner and Lambert, 2017) or respiration (Watts et al., 2014). A wealth of studies has demonstrated that μPs can efficiently be ingested by 160 marine (Lusher, 2015 and reference therein) and 39 freshwater species (Scherer et al., 2017), including invertebrates, such as cladocerans (Canniff and Hoang, 2018), rotifers (Jeong et al., 2016) and molluscs (Imhof and Laforsch, 2016), and vertebrates, such as amphibians (Hu et al., 2016; De Felice et al., 2018), fish (Lei et al., 2018) and marine mammals (Fossi et al., 2012). A growing number of experimental studies performed on diverse aquatic species has demonstrated that the ingestion of μPs induced diverse sub-lethal adverse effects, including decrease of food uptake (Blarer and Burkhardt-Holm, 2016), onset of oxidative stress (Alomar et al., 2017) and inflammation (Lu et al., 2016), as well as decrease in growth and reproduction rate (Sussarellu et al., 2016; Lo and Chan, 2018). On the contrary, other studies have reported slight or null effects due to μPs ingestion (e.g., Hämer et al., 2014; Kaposi et al., 2014; Imhof et al., 2017; Weber et al., 2018). Of particular concern is the exposure and the subsequent effects due to μPs in zooplanktonic filter-feeder species, which indiscriminately ingest μPs during their normal swimming and feeding activity (Gorokhova, 2015). Several studies have demonstrated that the presence of μPs in the digestive tract of diverse zooplanktonic species can result in detrimental effects. For instance, studies of *Daphnia magna* have demonstrated that the ingestion and the egestion of μPs depend on the particle type, size, and shape (Rosenkranz et al., 2009; Jemec et al., 2016; Frydkjaer et al., 2017). Moreover, it has been reported that the ingestion of high concentrations of 1 and 100 μm PE microbeads by daphnids caused a dose and time dependent increase in their immobilization rates (Rehse et al., 2016). However, it must be considered that these results have been obtained by short-term exposures (<48 h), and that in the natural environment organisms are usually exposed to lower concentrations, but for longer periods. A previous long-term exposure to different concentrations of fluorescent green PE microbeads (63–75 μm) has shown that the ingestion did not affect reproduction in *D. magna*, although the digestive tract resulted filled with μPs (Canniff and Hoang, 2018). In spite of these findings, there still is a dearth of information regarding the potential toxicity of plastic polymers to *D. magna*, mainly on swimming activity and reproduction after long-term exposure. The present study was aimed at exploring the ingestion/egestion and possible effects induced by 1 and 10 μm polystyrene microplastic beads (PSμPs) to this species. Daphnids and adults were exposed to 0.125, 1.25 and 12.5 μg/mL PSμPs for 21 days and ingestion/egestion microscopically investigated at selected times. Moreover, individual growth and behavioural endpoints, including changes in swimming activity, phototactic behaviour and reproduction were also considered. Lastly, we used of Size Exclusion Chromatography (SEC) to detect the possible effects due to permanence in *D. magna* digestive tract on PSμPs molecular weight and distribution index.

2. Materials and methods

2.1. *Daphnia magna* husbandry

Daphnia magna used in this work came from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy). Specimens were reared in a commercial mineral water (San Benedetto® - conductivity 415 μmS cm^{-1} at 20 °C, pH 7.42, 301 mg/L HCO_3^-, 48.6 mg/L Ca^{2+}, 28.2 mg/L Mg^{2+}) and maintained in a facility at the University of Milan. Forty individuals/L were maintained in glass beakers at 20.0 ± 0.5 °C under 16 h light: 8 h dark photoperiod, to allow the continuum of the amictic, parthenogenic reproduction (Frey, 1982). Specimens were fed *ad libitum* with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata* (8×10^6 cells for individual per day until they were 8-day old, then 16×10^6 cells for individual per day) and the yeast *Saccharomyces cerevisiae* (15×10^6 cells for mL) three times a week. The culture medium was renewed every second day. Algae were cultured in ISO 892:1989 medium in 2 L flask at 20 ± 2.0 °C under continuous light and shaken through a stirrer. During their exponential growth, algae were left to settle in the dark at 4 °C for a week, then the supernatant was removed and cell density measured under a light microscope by a Burker chamber.

2.2. Polystyrene characterization and stock solution preparation

Red polystyrene microplastics (PSμPs) having two different sizes (Ø = 1 μm and Ø = 10 μm) were purchased from Sigma-Aldrich (Milan, Italy). Polystyrene (PS) was used because it is one of the most abundant plastic type used for food packaging and it is therefore found in freshwaters and marine environments (Li et al., 2016). Moreover, PS has a negligible styrene release in water (Cohen et al., 2002). Chemical-physical properties of the PSμPs beads were provided by the supplier (nominal Ø = 1 μm − calibrated particle diameter = 1.07 ± 0.03 μm, density = 1.51 g/cm^3 and nominal Ø = 10 μm diameter - calibrated particle diameter = 9.86 ± 0.13 μm, density = 1.51 g/cm^3) and confirmed by laboratory analyses. Both commercial standards were chemically characterized by using a Fourier Transformed Infrared Spectroscope (FT-IR) PerkinElmer Spectrum 100. To confirm the reliability of our exposures, the composition of PSμPs was characterized in the stock solution, obtained by diluting the commercial standard 1:1,000 (v/v) with San Benedetto® mineral water, and in the culture medium used for the experiments, comparing them with a standard food-grade PS used for disposables. Moreover, in order to assess the possible degradation over the experiment the composition of microbeads into the exposure medium was characterized at the onset and at the end of the 21-days experiment.

2.3. Exposure to polystyrene microplastics

The experiment was divided into three steps: *1*) an ingestion (24 h) and egestion test (96 h), to investigate if both daphnids (<24-h old) and adults were able to ingest and egest 1 and 10 μm PSμPs; *2*) a 21-day chronic test, to assess the possible effects on reproduction, and *3*) two behavioral assays, to evaluate changes in swimming activity (horizontal swimming) and phototactic behavior (vertical swimming). Each test was performed using PSμPs of both sizes and three different concentrations: 0.125, 1.25 and 12.5 μg/mL. These concentrations were similar to those used in previous studies on other aquatic species, including copepods (Lee et al., 2013), early-life stages of sea urchins and ascidians (Messinetti et al., 2018). All exposures were performed in semi-static conditions, renewing the exposure medium (including control) every single day up to the end of the test, and the beakers were maintained at the same condition as described above. Specimens from all groups were fed every single day until the end of the experiment.

2.4. Size exclusion chromatography (SEC) analyses

Both 1 μm and 10 μm PSμPs were analysed using a SEC system consisting of a Waters 1515 Isocratic HPLC pump, four Waters Styragel columns set (HR3-HR4-HR5-HR2) and a UV detector Waters 2487 Detector (λ = 255 nm). Analyses were performed at room temperature, using a flow rate of 1 cm^3/min and 40 μL as injection volume. Samples were prepared by dissolving 60 mg of polymer in 1 cm^3 of anhydrous dichloromethane (DCM, anhydrous ≥99.8%). Before the analysis, the solution was filtered with 0.45 μm filters. The analyses were performed in order to check the possible detection of additives such as colorants or others in view of future investigation on the effects of PSμPs egestion on the molecular weight of the polymer. No internal reference was used to avoid possible superimposition of peaks due to species with low hydrodynamic volume.

2.5. Light microscopy analyses: ingestion and egestion assessment

Light microscopy analyses were performed to assess the capability of both daphnids (<24-h old) and adults to ingest and egest PSμPs. Fifty specimens were exposed to the three selected concentrations into 100 mL beakers filled with freshly prepared medium together with the opportune amount of PSμPs stock solution to reach the selected concentrations to be tested. A beaker containing the sole culture medium was included as negative control. The ingestion/egestion test was replicated in triplicate. After 1, 3, 5 and 24 h from the beginning of the exposure, five individuals were collected for the microscopy analyses. The remaining specimens were transferred into a clean medium to assess the PSμP egestion (after 1, 3, 5, 24, 48 and 96 h from the beginning of the test). The collected individuals (5 individuals per treatment per each time of analysis) were fixed in paraformaldehyde 4% in 0.1 M phosphate buffered saline (PBS) at pH 7.4. Prior to microscopy analyses, all samples were rinsed in PBS and then examined under a Leica EZ4 D stereomicroscope coupled with an integrated 3 MP digital camera.

2.6. Chronic toxicity test and body growth evaluation

The chronic test was performed according to the standard 21 days chronic reproduction test (OECD, 2004). For each group, control included, 15 replicates (a replicate corresponds to a daphnids less than 24 h) were performed. The exposures run in 50 mL beakers filled with freshly prepared medium and the opportune amount of PSμP stock solution to reach the concentrations to be tested. Exposure medium was renewed daily and every single day the number of offspring was recorded. At the end of the exposure, the 21-days old individuals were fixed in paraformaldehyde 4% in 0.1 M PBS at pH 7.4. All samples were then photographed at the stereomicroscope and their digital images used for the morphometric analyses by the free Fiji software (Schindelin et al., 2012).

2.7. Swimming activity and phototactic behavior

To assess the swimming activity and the phototactic behavior due to PSμPs, five daphnids for each group were exposed to the selected concentrations in 50 mL beaker, and each treatment was replicate six times. To assess changes in swimming activity, 30 individuals per treatment were observed at 7, 14 and 21 days of exposure by a video tracking analysis. Videos were recorded with an iPhone 6 by placing organisms in a 12-well plate (11.5 cm × 8 cm x 1.5 cm), called 'arena', filled with 3 mL of water medium (San Benedetto®). After a 30 min acclimation, the swimming of each organism was tracked for 30 s. The thirty 1,080p Full HD videos acquired for each experimental condition were analyzed using the ImageJ plugin Animal Track (Gulyás et al., 2016). Swimming activity was assessed as the distance moved (expressed in mm) and as the swimming speed (cm/sec). The phototactic behavior test was performed according to Rivetti et al., (2016), with slight modifications. After 7, 14 and 21 days of exposure, all samples were individually transferred to the bottom of a glass cylinder (13 × 100 mm with a concave bottom), where they were left 3 min for acclimatization in the dark. To avoid interference due to other light sources, the test was performed in a dark room and the bottom of the cylinder was covered by a black cardboard to minimize light reflection. An artificial light was placed above the glass cylinder to mimic a light stimulus. The cylinder was filled with mineral water (San Benedetto®) up to 2 cm from the top (~8 mL). After the acclimation period, the light was turned on and the time (in seconds) spent by each sample to reach an arbitrary 'goal line' placed at 6 cm from the bottom of the cylinder was measured. An arbitrary period of 60 s was fixed as a temporal endpoint to allow the organism to swim up to the goal line.

2.8. Statistical analysis

The effects of PSμPs exposure on body length, swimming activity, phototactic behavior, as well as reproductive endpoints, of *D. magna* were investigated by using linear mixed models (LMMs), including the treatment and the time of analysis (for body length, swimming activity and phototactic behavior only) as fixed factor, while the identity of the exposure beaker as a random factor to account for the so-called 'tank effect'. Results of all the final model that were run are reported in Supporting information. All the analyses were run using SPSS 21.0 statistical package.

3. Results

3.1. Characterization of polystyrene microplastics

FT-IR analyses show that both 1 μm and 10 μm PSμPs were very similar to the industrial food grade PS used as a reference standard. Nevertheless, small differences were visible (see Fig. 1a and b), especially in the 1,350-1,000 cm^{-1} range. The peaks observed in PSμP were probably due to additives, such as colorants. No significant difference was detected between 1 μm and 10 μm PSμP. The analysis of hydrodynamic volumes of polystyrene used for PSμP is shown in Fig. 2a and b: species having higher hydrodynamic volumes have lower retention times than species having lower hydrodynamic volume, therefore they appear earlier in the chromatogram. SEC curves detected the presence of the polymer, at about 40 min in 1 μm PSμPs and at about 38 min in 10 μm PSμPs, as well as the presence of an additive in 1 μm PSμPs and of two additives in 10 μm PSμPs. Since UV detector was used, such additives are visible because they were UV-sensitive moieties. Therefore, they might be colorants and/or antioxidants or additives used to increase UV resistance of the material. In view of future analyses of egested PSμPs, also these additives must be taken into account, since they are present in the material used even if they are not the polymer itself.

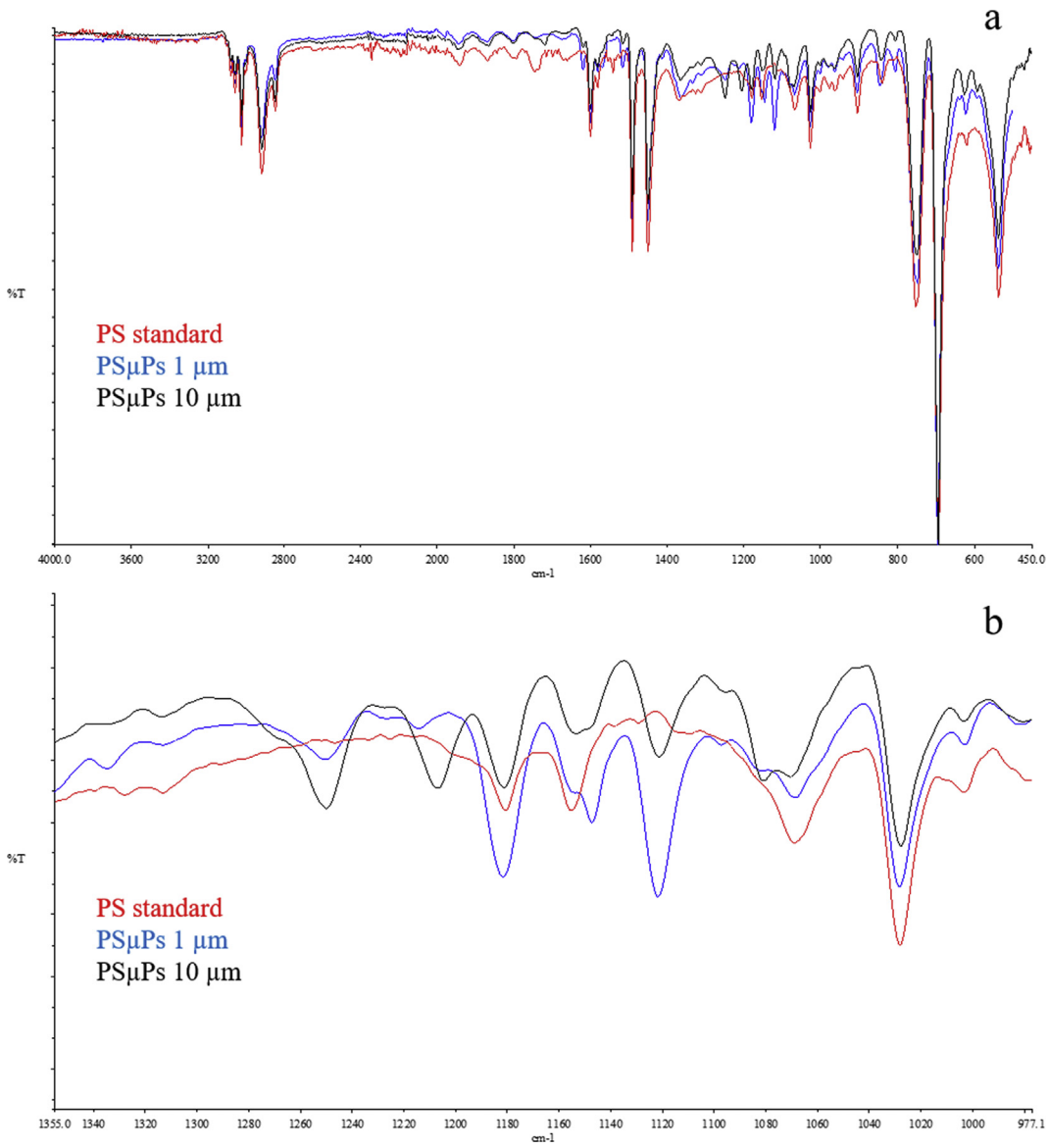

Fig. 1. Panel a) complete FT-IR spectrum of standard PS (red curve), 1 μm and 10 μm PSμP (blue and black curves respectively); panel b) magnification of 1,350-1,000 cm^{-1} range. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

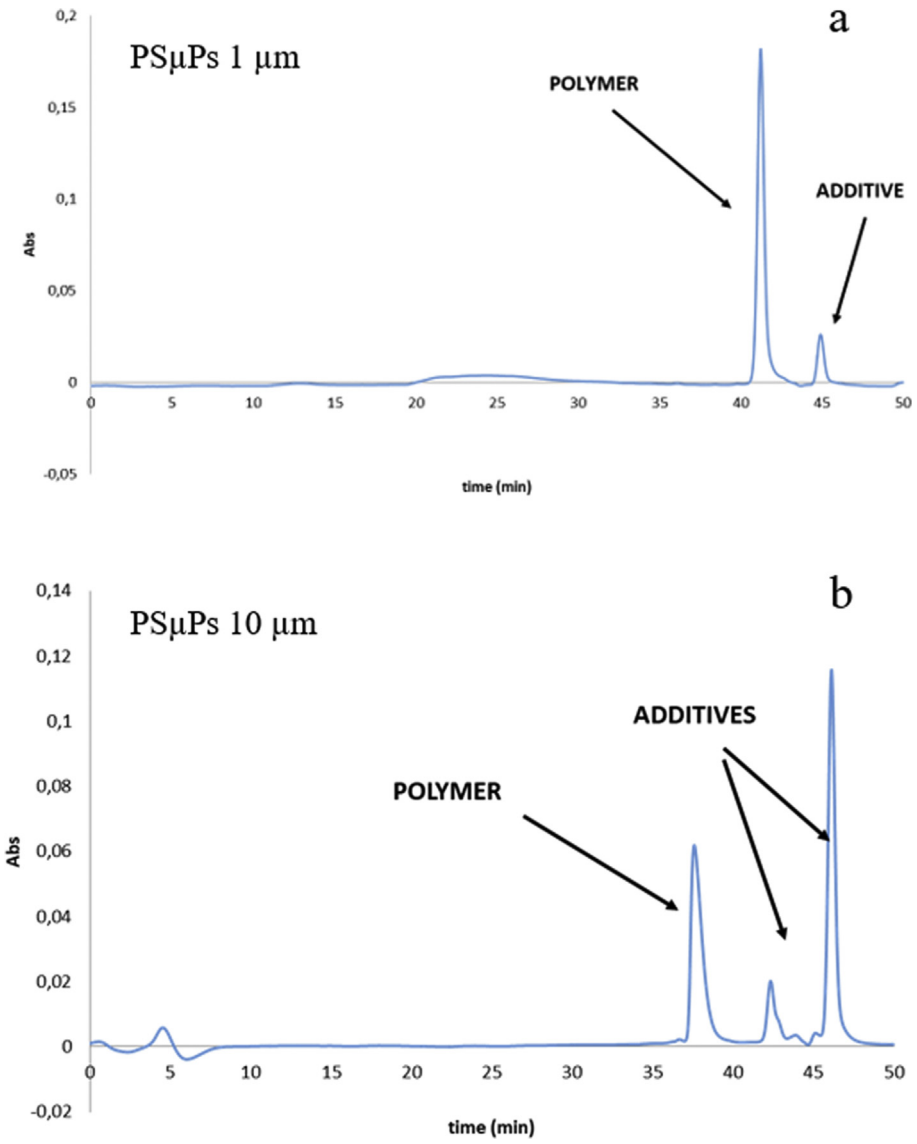

Fig. 2. Size exclusion chromatography (SEC) of 1 μm PSμPs (panel a) and 10 μm PSμPs (panel b).

3.2. *Effects of 1 μm polystyrene microplastics*

No mortality was found over the ingestion (24 h)/egestion (96 h) test in both daphnids and adults. Similarly, no mortality was recorded over the 21-days exposure performed to assess the behavioral effects caused by PSμPs. Microscopy analyses evidenced that 1 μm PSμPs were efficiently ingested by daphnids and adults, both showing their digestive tracts filled with PSμPs already after 1 h of exposure to the highest tested concentration (12.5 μg/L). At 0.125 and 1.25 μg/L, specimens showed their intestines full of PSμPs

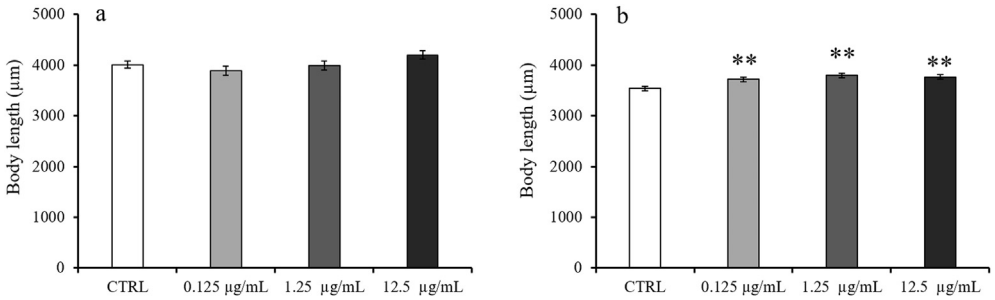

Fig. 3. Estimated marginal means (±SE) of body length of 21-days old *D. magna* individuals exposed to 1 μm (a) and 10 μm (b) PSμPs. Asterisks above the histograms indicate significant differences compared to the control group (**P < 0.01).

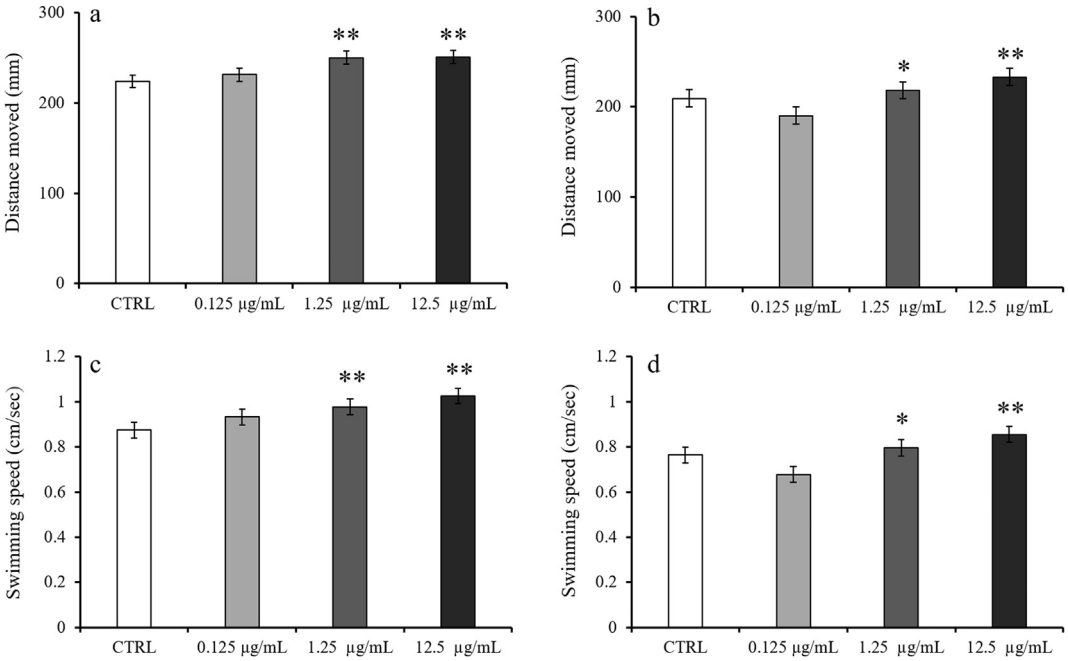

Fig. 4. Estimated marginal means (±SE) of swimming activity, in terms of distance moved (a and c) and swimming speed (b and d), measured in *D. magna* individuals after the exposure to 1 µm (a–c) and 10 µm (b–d) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (*P < 0.05; **P < 0.01).

only after 24 h of exposure (Fig. S1). It is interesting to note that even in a clean medium both daphnids and adults still showed their digestive tracts full of red PSµPs, suggesting that they were unable to purge their gut contents, at least within 96 h. A marginally non-significant increase in body length in 21-days old individuals ($F_{3,25} = 2.322$; $P = 0.099$) was induced by PSµPs exposure (Fig. 3a), while significant effects of PSµPs treatment on the swimming activity was found ($F_{3,19} = 3.779$; $P = 0.028$). In fact, a significant increase in the distance moved by 21-days old individuals exposed to 1.25 and 12.5 µg/L compared to controls was recorded (Fig. 4a). Accordingly, individuals treated with the highest PSµPs concentrations showed a higher speed compared to controls ($F_{3,19} = 4.244$; $P = 0.018$; Fig. 4c). No significant effect of time × treatment interaction was found in the distance moved and in swimming speed. According to the results related to horizontal swimming, a significant effect of PSµPs exposure on phototactic response was found ($F_{3,19} = 9.222$; $P = 0.001$), with individuals exposed to the highest concentration spending more time to reach the top of the cylinder, if compared to control (Fig. 5a). A significant time × treatment interaction effect was found ($F_{9,439} = 5.141$ $P < 0.001$), with a significant alteration of phototactic response after 7 days of exposure to 1.25 and 12.5 µg/L of PSµPs and after 14 and 21 days only at the highest tested concentration (Fig. S2). Lastly, PSµPs treatment induced a significant effect on *D. magna* reproduction, in terms of mean number of offspring ($F_{3,42} = 6.258$; $P = 0.001$), but not of number of reproductive cycles ($F_{3,42} = 1.402$; $P = 0.255$). In detail, a significant increase in the mean number of offspring was found in individuals exposed to the highest tested concentration if compared to the control group (Fig. 6a).

3.3. Effects of 10 µm polystyrene microplastics

According to the experiment performed on 1 µm PSµPs, no mortality of *D. magna* daphnids and adults was found over the ingestion (24 h)/egestion (96 h) test, as well as over the 21-days exposures performed to assess PSµPs-induced behavioral effects. Microscopy analyses showed that 10 µm PSµPs were ingested by daphnids and adults. Already after 1 h of exposure to the highest concentration, the digestive tract of juvenile and adult individuals were full of PSµPs, while 24 h of exposure were needed to fill up the digestive tract of samples at the lower tested concentrations (0.125 and 1.25 µg/L). Also for 10 µm PSµPs, a 96 h period in a clean medium was not enough to completely purge the gut content of both daphnids and adults (data not shown). A significant increase in body length of 21-days old individuals ($F_{3,50} = 6.867$; $P = 0.001$) was induced by PSµPs exposure (Fig. 3b), being the exposed samples about 5% longer than controls. Overall, a significant increase in swimming activity was noted ($F_{3,19} = 3.656$; $P = 0.030$), with individuals exposed to 1.25 and 12.5 µg/L PSµPs that travelled more distance than controls (4 and 11% independently from the time of exposure, respectively (Fig. 4b). Accordingly, a significant increase in swimming speed was recorded in individuals exposed to 1.25 and 12.5 µg/L PSµPs with respect to controls ($F_{3,19} = 4.282$; $P = 0.018$; Fig. 4d), independently from the time of exposure. According to the results on horizontal swimming, a significant effect of PSµPs exposure on phototactic response was found ($F_{3,19} = 4.080$; $P = 0.019$). However, in contrast to the results obtained at the end of the exposure to 1 µm PSµPs, treated individuals spent less time to reach the top of the cylinder if compared to controls (Fig. 5b), independently from the time of exposure. Lastly, a significant increase in the mean number of offspring was induced by the exposure to the highest PSµPs treatment ($F_{3,48} = 3.561$; $P = 0.021$; Fig. 6b), while no significant effect on the number of the reproductive cycles ($F_{3,48} = 2.132$; $P = 0.108$) was found.

4. Discussion

The results of the present study showed that the exposure to 1 and 10 µm PSµP beads were efficiently ingested by *D. magna* and that these particles were able to significantly induced behavioral changes in terms of swimming activity, phototactic behavior and reproduction. Microscopy analyses highlighted the presence of red particles of both sizes in the digestive tract of *D. magna* specimens, including daphnids (<24 h-old), which showed PSµPs already 1 h

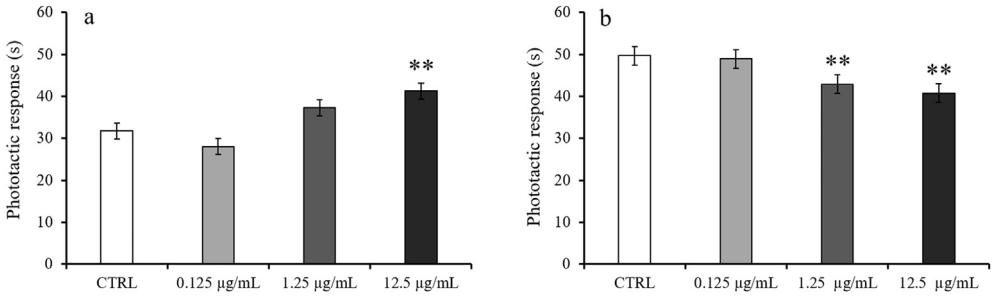

Fig. 5. Estimated marginal means (±SE) of phototactic behavior measured in *D. magna* individuals exposed to 1 µm (a) and 10 µm (b) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (**P < 0.01).

after the onset of the exposure at the highest concentration (Fig. S1). In all treated groups, daphnids and adults efficiently and quickly ingested both 1 and 10 µm PSµPs at all the tested concentrations. These findings are consistent with previous studies which demonstrated that PSµPs of different size can be ingested and accumulated in the digestive tract of *D. magna* (Ma et al., 2016; Rist et al., 2017) and of other zooplanktonic species (Cole et al., 2013). Similarly, also PE particles can be ingested by *D. magna* (Rehse et al., 2016): in a 96 h exposure, these authors not only reported that 1 µm PE particles can be ingested by daphnids, but also that this ingestion results in immobilisation of samples exposed to high PE concentrations. Our results comply with our expectations because the size of PSµPs we tested felt in the same size range of those algae *D. magna* usually feed on (1–50 µm; Ebert, 2005), confirming that 1 and 10 µm PSµPs that can be found in the water column are available for a filter-feeder organisms, such as *D. magna* and other zooplanktonic species. In contrast, a limited, slow egestion of PSµPs was noted when daphnids or adults were transferred to a clean medium. This finding is in accordance with a previous study on 1-week old *D. magna* specimens in which no significant egestion of 100 nm and 2 µm fluorescent PS beads occurred after 1 h of ingestion and 24 h of egestion into a clean medium (Rist et al., 2017). The limited egestion might be due to the absence of food in the clean medium, as the presence of food in the digestive tract is reported to be necessary for the egestion of faeces (Ebert, 2005). Although we did not quantify the ingestion/egestion rate, this hypothesis was supported by the investigation by Rist and coauthors (Rist et al., 2017), who demonstrated that food administration in a plastic-free medium notably affected plastic body burdens, with a decrease of particle mass per individual by 93% and 100% for the 100 nm and 2 µm particles, respectively.

Although PSµPs of both sizes filled up the digestive tract of *D. magna* over the whole experiment, no individuals died over the 21-days exposure period, in all the treatment groups. These results agree with previous studies on different freshwater invertebrate species, in which no mortality was recorded after short-term exposures to µPs at concentrations similar to those tested in the present study (e.g., Imhof et al., 2017; Rist et al., 2017; Weber et al., 2018). Anyway, in spite of no acute toxicity, the ingestion of PSµPs may cause a number of sub-lethal effects (Cole et al., 2015; Xu et al., 2017). For instance, it has been suggested that µP ingestion can decrease the feeding activity in several aquatic species, resulting in long-term alterations of physiology, behaviour and fitness of the organisms due to impairments of the energy budget and the whole metabolism (Cole et al., 2015; Wright et al., 2013). However, a previous study on *D. magna* demonstrated that only the exposure to 100 nm and not to 2 µm PS microbeads significantly affected the feeding rate of 1-week old individuals (Rist et al., 2017) likely because of the interaction of nanometric particles with the filter setae and/or the gut wall, thus disturbing the feeding process. Although we did not specifically investigate the feeding rate, considering that the size of particle we tested was in the same dimensional range of algae and similar to that used by Rist et al. (2017), and that the amount of algae administered to our specimens was above the incipient limiting concentration throughout the test, we could suggest that PSµPs exposure did not negatively affect *D. magna* feeding rate (see also Ebert, 2005; Furuhagen et al., 2014). This hypothesis was indirectly supported by the results on the morphology of *D. magna* adults. In fact, 21-days old individuals treated with the highest concentration of both PSµPs were even longer than controls (Fig. 3). Indeed, also adults treated with the lowest concentrations of 10 µm PSµPs were significantly longer than controls, suggesting that the increase in the size at the end of the exposures might be due to an enhanced food uptake related to an increased filtering activity and/or to a better efficiency of food absorption in presence of microplastics within the digestive tract. The first hypothesis was supported by our data on swimming activity, which showed that the exposure to PSµPs of both size surprisingly increased the swimming activity of *D. magna*, independently from the individual age. In fact, the exposure to the highest tested concentrations of 1 and 10 µm PSµPS significantly enhanced *D. magna* swimming activity in terms of distance moved and swimming speed (Fig. 4). The increase in swimming activity might be explained as an avoidance behavior fulfilled by the organism to swim away from a contaminated environment (Lopes et al., 2004), or as an attempt by the organism to get rid of the particles, as also suggested by a previous study on *D. magna* exposed to fullerene (nano-C_{60}) and a fullerene derivative ($C_{60}HxC_{70}Hx$) (Lovern et al., 2007).

We may speculate that if the organism is able to perceive the presence of particles in the solution, these occluding the digestive tract or hindering the appendage movements, it might increase the swimming activity to look for clean, PSµPs-free water into its carapax, to clean up gut and body cavities or enhance movements of its appendages to rid them of particles. This hypothesis could be particularly true for *D. magna* exposed to 1 µm PSµPs because some particles were found adhering on some external structures of both daphnids and adults, including the carapax and/or the appendages. Interestingly, an increase in phototactic behavior (i.e. vertical swimming activity expressed as the time spent by the individual to travel a vertical path in response to a light stimulus; Fig. 5), was also found at the highest concentration of 10 µm PSµPs, while an opposite response was induced by the exposure to 1 µm PSµPs. The discrepancy might be due to the additional weight represented by 1 µm particles on the body and appendages of *D. magna* specimens that lengthened the time due to respond to a light stimulus. In detail, the strongest effect due to 1 µm PSµPs on the phototactic behaviour was noted after 7 days of exposure to 1.25 and 12.5 µg/mL treatments (Fig. S2). As *D. magna* started reproduction after 7/8 days from birth, this detrimental effect might be explained as an

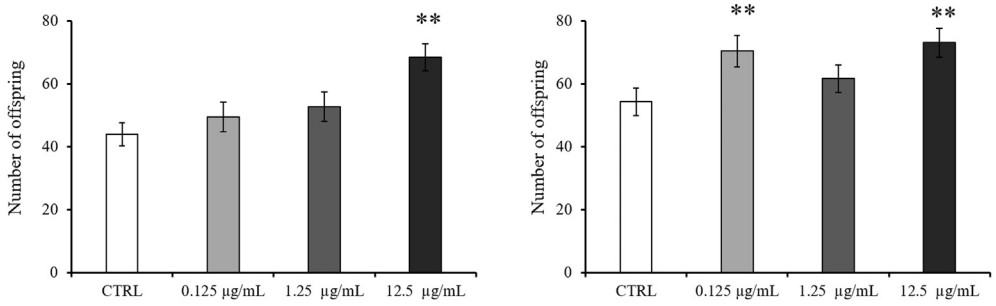

Fig. 6. Estimated marginal means (±SE) of mean number of offspring generated by *D. magna* individuals exposed to 1 µm (a) and 10 µm (b) PSµPs. Asterisks above the histograms indicate significant differences compared to the control group (**P < 0.01).

energy redeployment to the first reproductive event rather than to a light stimulus response. However, this hypothesis needs to be tested in further in-depth experiments. Thus, as the swimming behaviour is strictly related to filter-feeding activity and food uptake is one of the main driving forces of growth and reproduction (Enserink et al., 1993), the enhancement of swimming activity (i.e., horizontal swimming activity) might promote food uptake, body growth and, consequently, reproduction. According to results from body length and swimming activity, an enhanced reproduction, in terms of the mean number of offspring, was induced by the exposure to the highest tested concentrations of 1 and 10 µm PSµPs (Fig. 6), while no effects were induced by the exposure to the lowest concentration. Data from the literature reported contradictory results; Ogonowski et al. (2016) reported no negative effects on the reproductive success of *D. magna* specimens exposed to 4.1 µm beads or 2.6 µm PE fragments at a concentration approaching environmentally realistic values (102-105 particles/mL), while Rist et al. (2017) observed a slight increase in the number of neonates from *D. magna* adults exposed to 100 nm PSMPs. In contrast, Besseling et al. (2014) demonstrated in the same species that the exposure to nanopolystyrene (~70 nm) at concentrations up to 103 µg/mL lowered the number of offspring, increased the occurrence of malformation in daphnids and decreased the growth of adults. These findings suggest that the size and the chemical features of the plastic polymers can affect the reproductive success of *D. magna*, although at the same time they indicate that the reproduction of this cladoceran species is rather robust to micro- and nano-plastic stress at and above environmentally relevant particle concentrations (Jemec et al., 2016). The increase in reproductive success might be explained as a terminal effort accomplished by *D. magna* specimens in an adverse, contaminated environment, preferring to invest energy for its fitness rather than to its survival. Although we did not experimentally investigate this hypothesis in the present study, a preliminary long-term experiment showed that *D. magna* specimens exposed to 3 µm PSµPs died before controls (personal communication, unpublished data). Alternatively, we might speculate that individuals exposed to high concentrations of PSµPs increased the efficiency of food absorption, as demonstrated in Pacific oysters exposed to yellow-green fluorescent PS beads (2 and 6 µm diameter; Sussarellu et al., 2016). The enhanced absorption efficiency suggests a compensation to adjust energy intake in response to a possible interference caused by the presence of PSµPs filling the digestive tract of *D. magna* specimens.

5. Conclusion

Results from the present study showed that 1 and 10 µm PSµPs are quickly ingested by *D. magna* daphnids and adults at all the tested concentrations. In contrast to our expectations, the ingestion of PSµPs induced significant enhancements of body length and swimming activity at higher, unrealistic concentrations, resulting in a surprising increase of reproductive effort, in terms of mean number of offspring. In spite of these findings, the exposure to low, environmentally similar PSµP concentration of both 1 and 10 µm particles did not induce any significant change in the investigated endpoints, suggesting that PSµP contamination does not seem to pose a worrisome risk for zooplanktonic organisms. Further studies should be planned in order to check for the hypotheses concerning the enhancement of efficient absorption of food and of terminal effort of *D. magna* specimens in very contaminated environments by plastic polymers. Moreover, investigations on the potential effects due to smaller PS spherical particles or to fragments, foams and pellets, which are predominant in freshwater ecosystems, should be necessary to shed light on the impact of PSµPs towards aquatic organisms.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.05.115.

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PAPER V

De Felice B., Bacchetta R., Santo N., Tremolada P., Parolini M.

Polystyrene microplastics did not affect body growth and swimming activity in *Xenopus laevis* tadpoles.

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RESEARCH ARTICLE

Polystyrene microplastics did not affect body growth and swimming activity in *Xenopus laevis* tadpoles

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Abstract

A growing number of studies have highlighted the contamination and the effects towards organisms of diverse microplastics (μPs) in the marine environment. Surprisingly, although the main sources of μPs for marine environments are inland surface waters, the information on the occurrence and the effects of μPs in freshwater ecosystems is still scant. Thus, the aim of the present work is to investigate the ingestion and possible adverse effects due to the exposure to polystyrene μPs (PSμPs; $\varnothing$ = 3 μm) on tadpoles of the Amphibian *Xenopus laevis*. Larvae at the developmental stage 36, prior to mouth opening, were exposed under semi-static conditions to 0.125, 1.25, and 12.5 μg mL^{-1} of PSμPs, and allowed to develop until stage 46. At the end of the exposure, the digestive tract and the gills from exposed and control tadpoles were microscopically examined, as well as changes in body growth and swimming activity. PSμPs were observed in tadpoles' digestive tract, but not in the gills, from each tested concentration. However, neither body growth nor swimming activity were affected by PSμPs exposure. Our results demonstrated that PSμPs can be ingested by tadpoles, but they did not alter *X. laevis* development and swimming behavior at least during early-life stages, also at high, unrealistic concentrations.

Keywords Polystyrene microplastics · *Xenopus laevis* · Ingestion · Microscopy · Swimming activity

Introduction

Plastic contamination is a worrisome environmental problem gripping aquatic ecosystems worldwide. Over the past 50 years, an unfathomable amount of plastic debris has reached the marine environment, representing a serious hazard for seas and oceans at all latitudes (Thompson et al. 2004). Although the negative impact of big plastic debris (i.e., macroplastics; > 25 mm in size) on marine ecosystems has been highlighted since the 1980s (Stefatos et al. 1999), a growing scientific interest has recently raised on microplastics. Microplastics (μPs) are small plastic particles (< 5 mm in size) that are produced *ex novo* to be used in cosmetics, industrial or medical applications, or derive from macroscopic debris after chemical, physical, and biological breakdown (Barnes et al. 2009). A number of studies have identified marine ecosystems as hotspots of μPs pollution (Wright et al. 2013 and references therein), where they have been recorded up to a maximum estimated density of 100,000 particles m^{-3} in surface waters and in the range of 100,000 items m^{-2} on shorelines (e.g., Desforges et al. 2014).

In spite of these findings, the contamination of freshwaters cannot be underestimated. In fact, freshwaters are the primary source of μPs entering seas and oceans through household sewage discharge (e.g., Fendall and Sewell 2009), direct input in water run-off or via storm-7water and wastewater treatment plant outlets (Dris et al. 2015), spillage of plastic resin powders or pellets used for airblasting (Gregory 1996), and feedstocks used to manufacture plastic products (Zbyszewski et al. 2014) or, alternatively, from the breakdown of larger plastic items. Microplastic contamination of surface waters that has been reported was in the 0.001–0.1 items m^{-2} range for lakes and 0.1–1 items m^{-2} range for rivers, while in the 10–10,000

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items m^{-2} and 1–1000 items m^{-2} for lake and river sediments, respectively (Dris et al. 2015). The presence of μPs in different environmental matrices and their small size can result in the ingestion by organisms. A wealth of studies has demonstrated the ingestion of different μP items in 160 marine species (see Lusher 2015 and reference therein), including fish (Collard et al. 2017), seabirds (Lavers et al. 2014), mammals (Fossi et al. 2012), and invertebrates (Graham and Thompson 2009; Cole et al. 2013; Messinetti et al. 2018), as well as in 39 freshwater species (Scherer et al. 2017). Experimental studies have also demonstrated that μPs ingestion might negatively affect the health status of aquatic species, including fish (e.g., Lei et al. 2018), molluscs (e.g., Sussarellu et al. 2015), and crustacean (e.g., Frydkjaer et al. 2017). However, such investigations have returned contrasting results mainly depending on μP size and shape, as well as the tested concentration (Lee et al. 2013; Wright et al. 2013; Scherer et al. 2017).

Whilst evidence of strong negative effects, including intestinal damage, inhibition of feeding activity, and reduction of survival rates and body growth have been found (Lei et al. 2018; Murphy and Quinn 2018), some studies have pointed out slight or null adverse effects due to μPs ingestion (Hämer et al. 2014; Imhof et al. 2017; Weber et al. 2018). In spite of these findings, information on the impact of μPs on swimming activity of aquatic organisms are still limited. However, this effect cannot be neglected because ingestion of plastic microparticles could constrain organisms' movements in water.

To the best of our knowledge, only two studies have been focused on μPs ingestion on amphibian species even though these organisms can be a target of μPs contamination, being exposed both in aquatic and terrestrial ecosystems. Moreover, as amphibians are filter feeders until they complete their metamorphosis, tadpoles are excellent models to investigate the ingestion of μPs and the subsequent effects during early-life periods. A first laboratory study demonstrated the uptake, accumulation, and elimination of polystyrene μPs in *Xenopus tropicalis*, showing their presence in both the digestive tract and on the gills (Hu et al. 2016). Similarly, a recent field work performed by Hu et al. (2018) confirmed that tadpoles can ingest μPs from their surrounding environment, showing the presence of different μPs typologies in the digestive tract of tadpoles belonging to four different species sampled in small waterbodies of the Yangtze River Delta (China). Despite of these findings, no study was focused on the potential adverse effects induced by μPs ingestion in tadpoles. Thus, the present study was aimed at investigating the ingestion and the possible negative caused by polystyrene spherical microplastics (PμPs; $\varnothing = 3$ μm) on *Xenopus laevis* tadpoles. We exposed *X. laevis* tadpoles to three increasing concentration of PSμPs (0.125, 1.25, and 12.5 μg mL^{-1}) from stage 36, prior to mouth opening, to stage 46 (Nieuwkoop and Faber 1994). At the end of the exposure, we assessed the ingestion of PSμPs in tadpoles' digestive tract and gills, as well the effects on survival, body growth, and swimming activity.

Materials and methods

Chemicals and polystyrene microplastic preparation

All analytical grade reagents, L-cysteine, 3-amino-benzoic acid ethyl ester (MS222), salts for FETAX solution, and blue polystyrene microplastics (PSμPs; $\varnothing = 3$ μm) were purchased from Sigma-Aldrich, Milano, Italy. Chemical-physical properties of the μP beads were tested. The size of polystyrene μPs was assessed by measuring size of 500 particles on different pictures captured with a scanning electron microscope (SEM) (Fig. S1) using Fiji freeware software (Schindelin et al. 2012), resulting in 2.75 ± 0.09 μm of diameter. Polystyrene μPs were chemically characterized by using a Fourier transformed infrared spectroscope (FT-IR) PerkinElmer Spectrum 100: PSμPs were analyzed as received. Subsequently, 10 mL of FETAX solution was dried at room temperature overnight (16 h) together with the same volume of a FETAX solution containing the PSμP (50 μg mL^{-1}). The two residues were compared with the PSμPs. In Fig. 1, the spectra obtained are overlapped and signals showing the presence of PSμP are indicated. We focused on PSμPs because this polymer is one of the most abundant in both marine and freshwater ecosystems (Li et al. 2016). Moreover, polystyrene has a negligible styrene release in water solution; therefore, we can be reasonably sure that possible effects are due to the physical presence of μPs and not to monomer release (Cohen et al. 2002). The commercial standard was an aqueous suspension (50 mg mL^{-1}) that was diluted in culture medium to obtain a stock solution of 50 μg mL^{-1} concentration. Three PSμPs concentrations, namely 0.125 (1×10^5 particles mL^{-1}), 1.25 (2.833×10^5 particles mL^{-1}), and 12.5 μg mL^{-1} (8.666×10^5 particles mL^{-1}), were tested according to previous works on other aquatic organisms (Lee et al. 2013; Messinetti et al. 2018).

Animals and experimental design

Adults of *Xenopus laevis* were maintained at the University of Milan in aquaria filled with dechlorinated tap water at 22 ± 2 °C, with a 12 h light/dark cycle and fed a semi-synthetic diet (Mucedola S.r.L., Settimo Milanese, Italy). Embryos were obtained from natural breeding of adult pairs and the experiment run according to the Frog Embryo Teratogenesis Assay-Xenopus, FETAX, protocol (ASTM 1998), lightly modified. In particular, we planned a late exposure, being interested in the possible effects of ingested PSμPs and not to their developmental toxicity. Embryos were thus exposed prior to mouth opening, which happens at stage 40 (Nieuwkoop and Faber 1994), and not at the classic midblastula stage (stage 8). At the end of the test (stage 46), FETAX endpoints, i.e., mortality

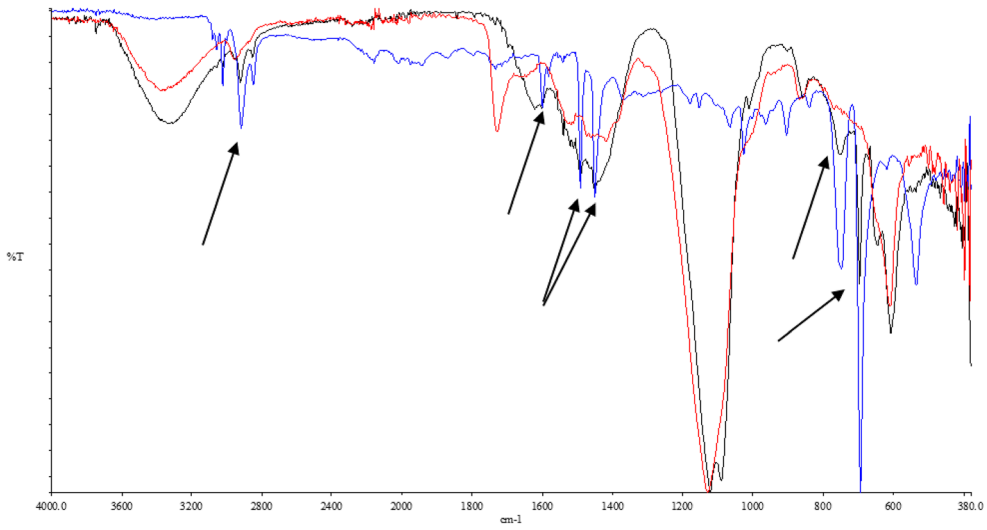

Fig. 1 Chemical characterization of blue PSμPs by a Fourier transformed infrared spectroscope (FT-IR). Spectra of PSμPs (blue), FETAX solution (red), and FETAX solution containing PSμPs (black) are reported. Black arrows indicate the specific peaks of polystyrene

and growth inhibition, were considered. Exposure tests were performed in FETAX solution (0.01 M NaCl, 1 mM $NaHCO_3$, 0.4 mM KCl, 0.1 mM $CaCl_2$, 0.35 mM $CaSO_4$ and $2H_2O$, and 0.6 mM $MgSO_4$, at pH 7.6–8.0).

After breeding, adults were removed and embryos collected in plastic Petri dishes. Fertilized eggs were dejelled with 2% L-cysteine solution (pH 8.0) and rinsed several times with FETAX solution. Normally, cleaved embryos were selected, transferred to plastic Petri dishes filled with 10 mL of FETAX solution, and allowed to develop until stages 36–37, according to Nieuwkoop and Faber (1994). Thirty tadpoles at stages 36–37 were seeded in Petri dishes and exposed to a nominal concentration of 0.125, 1.25, and 12.5 μg mL^{-1} PSμPs in FETAX. The test was performed in semi-static conditions every single day. All groups were incubated in a thermostatic chamber at 22 ± 0.5 °C, and both control and PSμPs exposure groups duplicated. Tadpoles were not fed during the experiment and allowed to develop until stage 46, end of the exposure test. At this point, 20 tadpoles from each group were transferred to a small Petri dish filled with 5 mL of culture medium to be video-tracked. Then, all tadpoles were anesthetized with MS222 at a final concentration of 100 mg L^{-1} and evaluated for single malformations under a dissecting microscope. At the end of the analysis, all samples were fixed in 2%

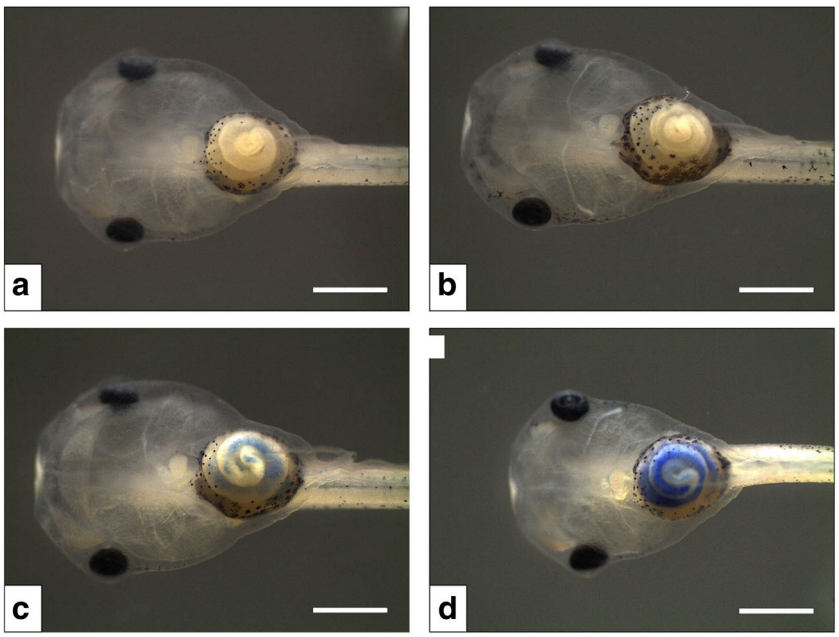

Fig. 2 Ventral view of *X. laevis* tadpoles (stage 46) at the steromicroscope. **A** Control sample. **B** Sample exposed to 0.125 μg mL^{-1} PSμPs showing no sign of blue beads in the digestive system. **C**, **D** Samples exposed to 1.25 (**C**) and 12.5 μg mL^{-1} (**D**) showing large amounts of PSμPs in their gut. Scale bar = 1 mm

glutaraldehyde in 0.1 M sodium cacodylate buffer solution (pH 7.4) for growth retardation measurements and for the subsequent microscopical analyses.

Microscopy analyses

For light microscopy analyses, 26 tadpoles per replicate were dehydrated in ethanol (EtOH) up to 70% and examined under a Leica DMRA2 microscope. Images were collected with a Leica DC300F digital camera and tadpole body lengths measured using Fiji freeware software (Schindelin et al. 2012). For electron microscopy analyses, 10 tadpoles from each treatment group were randomly selected, post-fixed in 1% OsO_4 for 2 h at 4 °C, and critical-point dried in a Balzers Unions CPD 020 apparatus (Balzers Unions, Lichtenstein). Under a stereomicroscope, the digestive tract and gills of each tadpole were dissected, mounted onto standard SEM stubs, gold sputtered, and observed under a Zeiss LEO 1430 SEM at 20 kV.

Swimming activity analysis

The effects on swimming activity of tadpoles were evaluated by a video tracking analysis. Twenty tadpoles per treatment, including control, were randomly selected from exposure Petri dishes and individually transferred to another Petri dish (Ø = 60 mm) filled with 5 mL of culture medium. Because of their high motility, tadpoles were enclosed in a small arena (Ø = 10 mm) placed in the center of the Petri dish where they

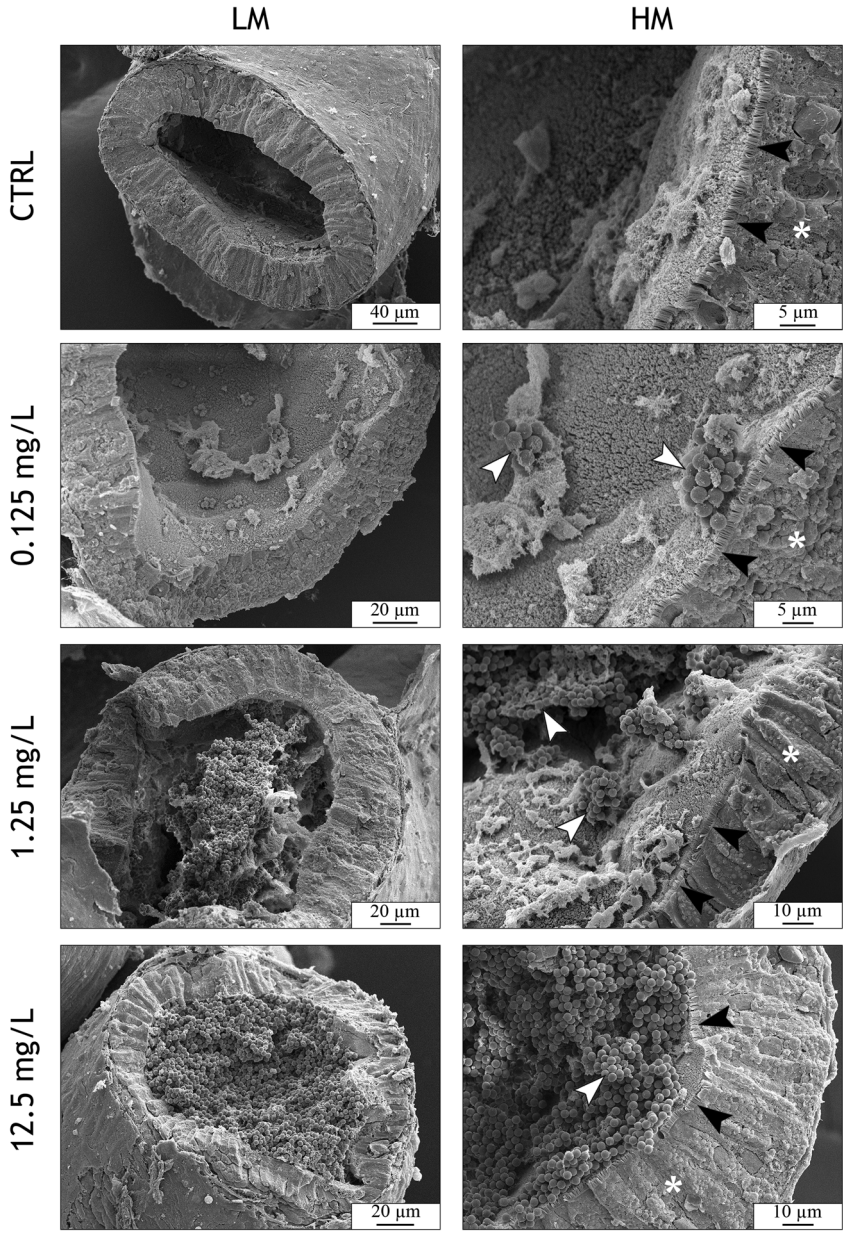

Fig. 3 SEM images from the digestive epithelium of *X. laevis* tadpoles showing the increasing presence of PSμPs into the lumen. *LM* low magnification, *HM* high magnification. White arrowhead indicates PSμPs, black arrowhead indicates brush border, and asterisk indicates the intestinal wall

stopped movements and acclimatized for 3 min to new conditions. After acclimatization, the small arena was removed, the tadpoles restarted to swim, and its movements were filmed by an iPhone 6 for 10 s. The obtained 1080p Full HD videos were analyzed by using the ImageJ plugin Animal Track (Gulyás et al. 2016). The distance moved (mm) and mean swimming speed (cm s^{-1}) were considered as swimming activity endpoints.

Statistical analysis

The effect of PSμPs exposure on the body length and the swimming activity of tadpoles was investigated by using linear mixed models (LMMs) including the treatment as fixed factor, while the identity of the Petri dish as a random factor. As no mortality occurred in all the experimental groups, no statistical analysis on this endpoint was performed. The analyses were run using SPSS 21.0 statistical package.

Results and discussion

The present work showed that *X. laevis* tadpoles can ingest polystyrene microplastic beads during early-life stages, even though these particles did not significantly affect survival, body growth, and swimming activity. Stereomicroscopy analyses showed the presence of PSμPs in the whole digestive tract of tadpoles, mostly after the exposure to 1.25 μg mL^{-1} and 12.5 μg mL^{-1}, while the lower concentration seems not to show the presence of PSμPs (Fig. 2). However, the SEM analyses showed the presence of PSμPs in the digestive tract of tadpoles from all the treatments, including 0.125 μg mL^{-1} (Fig. 3). As expected, the digestive tract from tadpoles exposed to 12.5 μg mL^{-1} of PSμPs was completely full of particles, while the amount of microbeads was notably lower in the individuals from the other treatments. SEM analyses suggest the absence of mechanical damage to the walls of the epithelium. Our findings are in agreement with previous studies demonstrating that polystyrene μPs of different size can be easily ingested and accumulated in the digestive tract of different aquatic organisms. For instance, PSμPs (1.7–30.6 μm in size) were observed in the digestive tract of 13 marine zooplanktonic organisms (Cole et al. 2013), while 100 nm–10 μm PSμPs filled up the digestive tract of the cladoceran *Daphnia magna* (Ma et al. 2016; Rist et al. 2017). Moreover, similar results were also obtained in *Xenopus tropicalis*, whereby fluorescent polystyrene μPs (1 and 10 μm in size) were clearly observed in alimentary canal, stomach, and intestine of tadpoles already after 1 h of exposure (Hu et al. 2016). However, in our study, no PSμPs were found on tadpole gills at each tested concentration (Fig. S2), contrasting previous results on *X. tropicalis* that showed the presence of 1 and 10 μm on the gills of tadpoles (Hu et al. 2016). Similarly, 8–10 μm PSμPs were found on the gills of the crab *Carcinus maenas* (Watts et al. 2014). Such findings suggested that the ingestion, the transfer, and the accumulation of different μPs in specific body districts greatly depend on the concentration and the size of the particles, as well as on the size of the focal model species (Wright et al. 2013). Thus, we suppose that the discrepancy in the presence of μPs on the gills of two *Xenopus* species might be due to their different body size. In fact, *X. tropicalis* is smaller than *X. laevis*, and consequently, it owns smaller gills and thick filaments, which allowed a more efficient trapping of PSμPs. This anatomic feature could also explain the higher accumulation of PSμPs in *X. tropicalis* compared to *X. laevis* although the exposure concentration selected by Hu et al. (2016) was notably lower (concentration range of 1 μm PSμPs 10–10^5 particles mL^{-1} and concentration range of 10 μm PSμPs 0.1–10^3 particles mL^{-1}) than those tested in our study (concentration range of 3 μm PSμPs 1×10^5–8.6×10^5 particles mL^{-1}.

Although PSμPs filled up the digestive tract of tadpoles, no tadpole died over the exposure period neither in the control nor in all the treatment groups. Our results are consistent with previous studies showing no mortality on diverse aquatic organisms after the exposure to diverse concentrations of

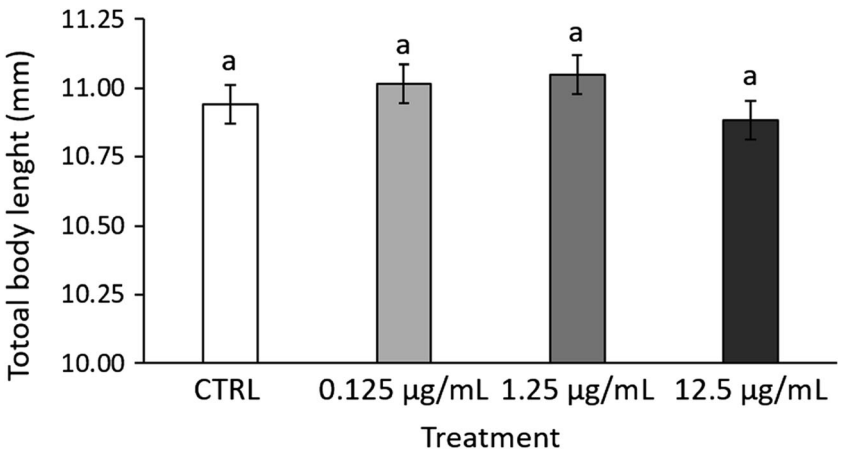

Fig. 4 Estimated marginal means (± standard error) of total body length of *X. laevis* tadpoles (stage 46). Letters above histograms indicate differences between groups, whereby similar letters indicate no significant differences. No significant differences were found ($p > 0.05$)

dissimilar μP polymers, including invertebrates (e.g., Imhof et al. 2017; Rist et al. 2017; Weber et al. 2018) and vertebrates (e.g., Hu et al. 2016; Chen et al. 2017). However, the ingestion of PSμPs might cause sub-lethal effects, including the reduction of food assimilation and body growth (Cole et al. 2015; Xu et al. 2017). No significant differences in body length of tadpoles at stage 46 were noted between the treatment groups and the control ($F_{3,203} = 1.137$; $P = 0.335$; Fig. 4), suggesting that PSμPs ingestion did not affect body growth of tadpoles during early-life stages. Our results are in contrast with previous studies demonstrating that the ingestion of PSμPs negatively affected body growth of diverse organisms (Besseling et al. 2014; Lo and Chan 2018). These discrepancies might be due to the duration of the exposure and/or the size of the tested μPs. In fact, 14-day exposure to PSμPs (Ø = 2–2.4 μm) reduced the growth of the onyx slipper snail *Crepidula onyx* (Lo and Chan 2018). Moreover, the 21-day exposure to polystyrene nanoplastics (Ø = 70 nm) reduced the growth of *Daphnia magna* (Besseling et al. 2014), while the exposure up to 7 days post-fertilization to polystyrene nanoplastics (Ø = 50 nm) altered the early development of *X. laevis* (Tussellino et al. 2015). We may suppose that ingested PSμPs did not affect body growth of *X. laevis* tadpoles because they do not interfere with the assimilation of yolk reserves used during early-life stages. Alternatively, polystyrene microbeads were ingested and egested quickly by tadpoles (Hu et al. 2016) and did not affect the development.

Despite no developmental effects, the ingestion of μPs could affect tadpole swimming activity because particles can represent an additional weight for tadpoles and consequently a high energy demanding effort to be supported. According to results on body growth, PSμPs ingestion did not affect the swimming activity of tadpoles (Fig. 5a, b); no significant differences in terms of distance moved ($F_{3,73} = 0.677$; $P = 0.569$) and mean swimming speed ($F_{3,73} = 0.196$; $P = 0.899$) occurred between the treatment groups and the control. On the contrary, a previous study of the amphipod *Platorchestia smithi* showed that the ingestion of polyethylene μPs (Ø = 35–45 μm) caused a decrease of the jump height (Tosetto et al. 2016). This discrepancy can be due to species-specific differences, different ontogenetic stage, and/or to the type of analyzed swimming activity of the model organisms. In fact, in the present study, we monitored the horizontal swimming of tadpoles, while Tosetto et al. (2016) monitored the vertical hopping of amphipods. In addition, the rate of μP ingestion/egestion, the size and the composition of plastic used for exposures (3 μm polystyrene used in our study versus 35–45 μm polyethylene particles used by Tosetto et al. 2016), and their exposure concentration can affect the swimming activity and explain the differences of the responses after μP exposure. Lastly, Tosetto et al. (2016) "doped" the polyethylene μPs administered to amphipods with contaminated marine water and doped μPs adsorbed on their surface 0.007 μg g^{-1} of

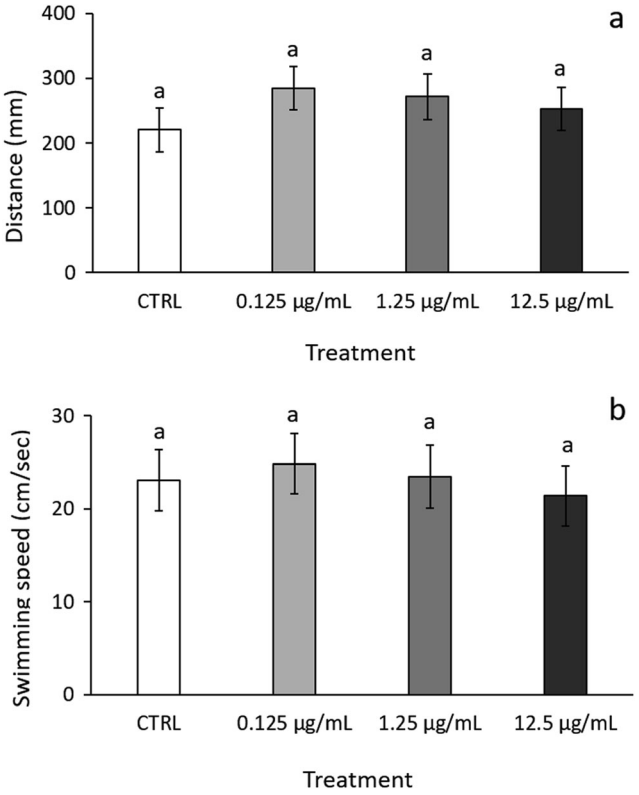

Fig. 5 Estimated marginal means (± standard error) of distance moved (**a**) and swimming speed (**b**) measured in *X. laevis* tadpoles (stage 46). Letters above histograms indicate differences between groups, whereby similar letters indicate no significant differences. No significant differences were found ($p > 0.05$)

PAHs, which could cause the observed behavioral changes. This hypothesis is supported by a previous study of zebrafish larvae showing that negative effects on swimming activity occurred only when organisms where co-exposed to μPs and α-ethynylestradiol, while no swimming alteration was noted when larvae were exposed to μPs alone (Chen et al. 2017).

Conclusion

Our findings showed that 3 μm PSμPs are quickly ingested by *X. laevis* tadpoles at all the tested concentrations, but the exposure period does not induce negative effects on the body growth and swimming activity, also at high unrealistic concentrations. Further studies should be planned in order to evaluate if long-term exposure can impact the development and post-metamorphic stages of *X. laevis*. Lastly, investigations on the potential effects due to smaller polystyrene spherical particles or to fragments, foams, and pellets, which are predominant in freshwater ecosystems, should be necessary to understand the real impact of PSμPs on aquatic organisms.

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PAPER VI

Parolini M., **De Felice B.,** Gazzotti S., Annunziata L., Sugni M., Bacchetta R., Ortenzi M.A

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Oxidative stress-related effects induced by micronized polyethylene terephthalate microparticles in the Manila clam

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ABSTRACT

Microplastic (MP) contamination represents a serious threat for marine organisms. Several lab studies demonstrated adverse effects induced by exposure to different MP polymers toward diverse marine species. However, the information regarding toxicity of polyethylene terephthalate (PET) MPs is largely unknown. The present study was aimed at investigating the adverse effects induced by 7-day exposure to two concentrations (0.125 or 12.5 µg/ml) micronized, irregular shaped and variable size PET microparticles (PET-MPs) toward Manila clam (*Ruditapes philippinarum)*. Histological analyses were performed to assess tissue damage on digestive glands, gonads, gut and gills, whereas oxidative stress-related effects, including the concentration of pro-oxidant molecules, activity of antioxidant (superoxide dismutase – SOD, catalase – CAT and glutathione peroxidase – GPx) and detoxifying (glutathione S-transferase – GST) enzymes, as well as levels of lipid peroxidation, were determined in gills and digestive gland. Our results showed that clams ingest and egest micronized PET-MPs, but no marked histological alterations to bivalve tissues occurred. Although PET-MPs did not produce oxidative stress in the digestive gland, these materials significantly altered oxidative status of gills, leading to lipid peroxidation. No apparent clear indication of a weakness of bivalve health status was obtained in this study.

KEYWORDS

Manila clam; microplastics; oxidative stress; polyethylene terephthalate (PET)

Introduction

In recent years, plastics contamination has raised a worrisome concern for aquatic ecosystems. Almost 10% of the annual plastic production contributes to marine contamination, and consequently, accumulation of plastic debris was identified as a global environmental problem (Barnes et al. 2009). Growing interest has been raised regarding microplastics (MPs), which are classified as any plastic item < 1 mm (Browne et al. 2008). Microplastics are ubiquitous in marine ecosystems and represent one of the main threat that aquatic organisms have to face (Eriksen et al. 2014). Over 5 trillion microscopic plastic fragments are estimated to float into the oceans worldwide (Eriksen et al. 2014), conferring to these materials the role of the most numerically abundant oceanic debris (Law and Thompson 2014). A growing number of investigators reported the presence of different MP polymers in both abiotic (water and sediments) and biotic (zooplankton, mussels and fish) matrices (Cole et al. 2013), from beaches and coastlines, to subtropical oceanic gyres and remote areas, including polar ice caps and the deep ocean (Cole et al. 2014; Law and Thompson 2014; Wright, Thompson, and Galloway 2013). Depending upon their size, shape and chemical composition, MPs assume different positions and behaviors along the water column, from involuntary ingestion or predation by a vast range of marine species that mistake them for natural food/preys (Galloway, Cole, and Lewis 2017). Several investigators demonstrated that ingestion of MPs induce a series of sub-lethal effects toward aquatic organisms though direct and indirect processes, including decrease of food uptake (Blarer and Burkhardt-Holm 2016), onset of oxidative stress (Alomar et al. 2017; Magara et al. 2018, 2019) and inflammation (Lu et al. 2016), as well as developmental alterations (Messinetti et al. 2018) and reduction in growth and reproduction rate (Lo and Chan 2018; Sussarellu

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et al. 2016). In contrast, other studies reported slight or null effects due to MPs ingestion (De Felice et al. 2018; Hämer et al. 2014; Imhof et al. 2017; Kaposi et al. 2014; Weber et al. 2018). A recent meta-analysis of the effects attributed to MPs, showed negative effects for consumption, growth, reproduction, and survival of fish and aquatic invertebrates but, simultaneously, many of the effects were neutral, confirming an inconsistency and a high variability of responses across taxa (Foley et al. 2018). Based upon these findings, it is important to examine the influence of diverse MP polymers represent a priority to understand realistic risk toward aquatic organisms.

Of particular concern is exposure and subsequent effects attributed to MPs in filter-feeder species such as bivalves, whose high filtration activity during normal breathing and feeding activity confers them a unique capability to ingest large quantities of MPs. For this reason, bivalves were proposed and used as suitable indicator organisms of MP pollution (Van Cauwenberghe et al. 2015; Wesch et al. 2016). The presence of MPs has been predominantly documented in mussels, whereby up to approximately 2 particles/g of diverse μP items were detected in both farmed and wild mussels from European countries and fishery market of China (Li et al. 2015; Mathalon and Hill 2014; Van Cauwenberghe et al. 2015; Van Cauwenberghe and Janssen 2014). However, MPs were also found in other bivalve species, including Manila clam (*Ruditapes philippinarum*), ark clam (*Scapharca subcrenata*) and oysters (*Alectryonella plicatula*), in concentration ranging between approximately 3 – 11 particles/g (Li et al. 2015). Considering the amount of MPs present in bivalves tissues, several lab studies investigated the adverse effects produced by ingestion of MPs, differing in polymeric composition and size. Most of such studies were performed on mussels, exploring the uptake and the sub-lethal toxicity of uniform, spherical polyethylene (PE) or polystyrene (PS) MPs (Lusher 2015), two of the most abundant polymers in the environment (Wagner et al. 2014). However, ingestion and toxicity of MPs exhibiting different polymeric composition rather than PE and PS have been scarcely examined because of lack of commercial standards (Paul-Pont et al. 2018). Thus, to mimic real particles in the environment, MPs of various size and shape may be created to reproduce exposure conditions that organisms experience in the wild. The aim of the present study was to assess ingestion and potential adverse effects induced by exposure to micronized polyethylene terephthalate (PET) particles toward *R. philippinarum*. Considering the high density of PET (1.38 g/cm^3), it was decided to test the potential toxicity of PET-MPs toward Manila clam, a bivalve species living sunken in marine sediments that might be exposed to this type of polymer. Manila clams were exposed for 7 days to two concentrations (0.125 or 12.5 μg/ml) of PET microparticles (PET-MPs). The ingestion of PET-MPs and tissue damage were investigated by histological analyses, while oxidative stress-related effects were investigated in gills and digestive gland by measurement of a suite of six different biomarkers including (1) amount of pro-oxidant molecules, (2) activity of antioxidant (superoxide dismutase – SOD, catalase – CAT and glutathione peroxidase – GPx) and detoxifying (glutathione S-transferase – GST) enzymes, and (3) levels of lipid peroxidation.

Materials and methods

Experimental plan

Specimens of *R. philippinarum* were purchased on May 2019 in a fish shop and quickly transferred to aquaria located in the facility of the University of Milan. Aquaria were filled by circulating artificial seawater (Instant Ocean; salinity about 37‰) under constant aeration, temperature (14°C) and photoperiod (16/8 hr light dark cycle). Clams were left in aquaria without sediment for 1 week to enable acclimation to lab conditions. Clams displayed good health status and a low mortality occurred during the acclimation period (<3%). Clam were exposed to PET microplastics because PET is predominantly used as packaging material and accounts for up to 7.1% of total European plastic consumption (Plastics Europe 2014). Klein, Worch, and Knepper (2015) and Gasperi et al. (2014) found that PET microplastics, though not as dominant as PE and PP polymers, contribute significantly to overall MP load in large European river systems. However, PET microparticles are one of the least used plastic polymers with respect to known MP effects toward marine organisms (de Sá et al. 2018). As no consistent information concerning the concentration of PET-

MPs in marine environments is available, it was arbitrarily decided to test two concentrations similar to those administered to other aquatic organisms in previous laboratory studies assessing the toxicity of MPs (De Felice et al. 2018, 2019; Messinetti et al. 2018). Fifteen specimens (approximately 4 cm in length) were seeded in 5 L glass beakers filled with 4 L of the same artificial seawater circulating in the acclimation aquaria and exposed for 7 days to two concentrations (0.125 or 12.5 µg/ml) of micronized PET-MPs. Specimens from the control group were maintained in 5 L glass beakers with artificial seawater only. The exposures were performed under semi-static conditions. Specimens of *R. philippinarum* were placed on a stainless grid close to the bottom of the tank. Artificial seawater was renewed every single day and the selected amount of PET-MPs was added to the exposure aquaria. Clams were fed for 1 hr with Algamac2000® (Aquafauna Bio-Marina, USA), an algae replacement-substitute-enrichment medium consisting of spray-dried cells of *Schizochytrium* spp., before renewal of exposure conditions. Three independent replicates (= beakers) per experimental group were performed. Further, considering the low amount of PET-MPs used in the present study, in order to confirm the MPs uptake by *R. philippinarum*, an additional 1-day exposure to a highest, unrealistic PET-MPs concentration (50 µg/ml) was performed. Because of the lack of a PET-MP analytical standards, particles used in the present study were obtained by mechanically grinding commercial bottle-grade PET chips (Invista 1101 PET) with a blade grinder. This procedure allowed us to obtain MPs mimicking a realistic exposure scenario, whereby secondary PET-MPs derive from breakage and erosion of plastic bottles. A commercial bottle-grade PET chips was frozen in liquid nitrogen and after grinding, particles were passed through a 1 mm sieve to select only items included in the MPs range (Browne et al. 2008). Resulting PET-MPs had an irregular shape and a size ranging between 8 and 1,054 µm in length (mean length 220 µm; Figure 1a). The relative % for each dimensional class composing our PET-MPs standard was measured in a subsample of the resulting grinded MPs (n = 500 particles) and was the following: <10 µm = 1%, <50 µm = 28%; 50µm < 100 = 24%; 100 µm < 1,000 = 47%. The polymeric composition of micronized PET-MPs was assessed by using a Fourier Transformed Infrared Spectroscope (FT-IR) Perkin Elmer Spectrum 100 (Figure 1b).

Histological analyses

For the histological analyses, five clams from each experimental group were randomly sampled and whole body was fixed in Bouin's fluid for approximately 1 week. Then, the remaining proteinic shell portions were removed under the stereomicroscope, the whole specimens dehydrated in an ascending alcohol series and embedded in ParaPlast Plus tissue embedding medium (Sigma-Aldrich, Italy). Using a Reichert rotary microtome, all fixed clams were cut in 7 µm transverse sections at different levels throughout the whole specimen. Ten serial sections

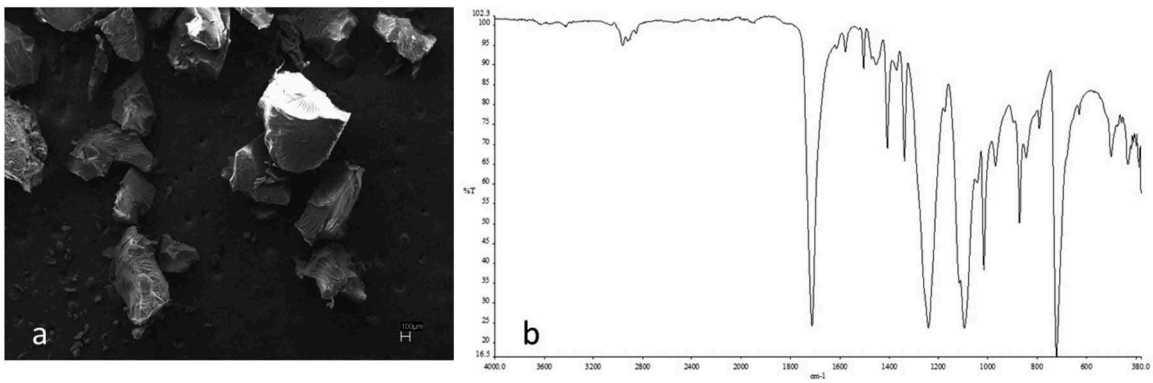

Figure 1. Scanning electron microscopy (SEM) image (a) and Fourier Transformed Infrared Spectroscope (FT-IR) spectrum (b) of micronized polyethylene terephthalate microplastics (PET-MPs).

after approximately every 150 sections, i.e., 1 mm, were placed on microscope slides and left to dry overnight at 37°C. Slides were stained with Mayer's Haemalum and Eosin, mounted in Eukitt (Kindler GmbH, Freiburg) and observed under a Leica DMRA2 light microscope equipped with a Leica DC300 F digital camera. All clams were observed and analyzed microscopically for the presence of diseases and inflammatory response.

Methods of oxidative stress biomarkers

Nine clams per each treatment were dissected under the stereomicroscope, gills and digestive glands isolated, quickly frozen in liquid nitrogen and maintained at −80°C until analysis of oxidative stress biomarkers. The suite of biomarkers was applied to homogenates of gills and digestive glands extracted from Manila clams according to the methods described by Parolini et al. (2013); Parolini et al. (2017). Gills or digestive glands from three specimens exposed in the same beaker (approximately 0.5 g) were pooled and homogenized by an automatic homogenizer in 100 mM phosphate buffer (pH 7.4), with addition of KCl (100 mM), EDTA (1 mM), protease inhibitors (1:10 v/v) and dithiothreitol (100 mM). Three pools were performed per each treatment, for a total of nine organisms per treatment. The amount of reactive oxygen species (ROS) was measured according to the dichlorofluorescein-diacetate (DCFH-DA, 10 mg/ml in DMSO) method (Deng et al. 2010). The activity of SOD, CAT, GPx and GST was assessed using the supernatant (S9 fraction) obtained by the centrifugation at 16,000 × g for 30 min of raw homogenate for both organs according to spectrophotometric methods (Parolini et al. 2010). Total protein content was determined according to the Bradford method (1976). In brief, the inhibition of cytochrome c (10 μM) reduction due to the superoxide anion generated by the reaction between xanthine oxidase (1.87 mU/mL) and hypoxanthine (50 μM) was assessed at λ = 550 to measure SOD activity. The CAT activity was assessed by monitoring the consumption of H_2O_2 (50 mM) at λ = 240 nm. The consumption of NADPH (120 μM) in a solution containing 0.2 mM H_2O_2 as a substrate in 50 mM potassium phosphate buffer (pH 7) containing glutathione (2 mM), sodium azide (NaN_3; 1 mM), glutathione reductase (2 U/mL) was monitored at λ = 340 nm to assess GPx activity. The GST activity was determined by monitoring the reaction among the sample, reduced glutathione (1 mM) and 1-chloro-2,4 dinitrobenzene for 1 min at λ = 340 nm. Lipid peroxidation was measured according to the thiobarbituric acid reactive substances (TBARS) method (Ohkawa, Ohisi, and Yagi 1979).

Statistical analysis

Linear mixed models (LMMs) were used to assess the effects induced by the exposure to PET-MPs. The treatment was included in the models as a fixed effect factor, while the identity of the exposure beaker as a random factor. Biomarker responses were employed by processing of three pools of gills or digestive glands isolated from three organisms sampled in each of the three experimental replicates (i.e., exposure tanks) prepared for each experimental condition (n = 3 replicates). All the analyses were run by means of SPSS 21.0 statistical package.

Results

Histological analysis did not show the presence of PET-MPs in the digestive tract of Manila clams exposed to both the tested concentrations. No marked alterations of histological structure of the digestive tract walls were noted. In fact, samples from both controls and treated groups displayed a regular monolayer of high columnar epithelial cells with eosinophilic cytoplasm and elliptical nuclei (Figure 3a–c). The basal portion of these cells laid on a thin basement membrane which identified an underneath layer of connective tissue (lamina propria). In contrast, the cell membrane along the apical portion of the cells appeared as a thick line, with overlying bristle-like cilia that extended into the gut lumen.

The analysis of the suite of oxidative stress biomarkers performed on the gills and digestive gland presented contrasting results. Exposure to PET-MPs did not induce significant modulation of the oxidative status of clam digestive gland (Figure 4a–f). PET-MPs treatments did not significantly affect the production of ROS (Figure 4a). Similarly, except for a significant inhibition of GPx activity (Figure 4d) at the end of the

exposure to the highest tested concentration, the activities of SOD (Figure 4a), CAT (Figure 4b) and GST (Figure 4e) was not markedly altered by PET-MPs exposure. In addition, no significant alterations in lipid peroxidation levels were noted (Figure 4f). Histological analyses of the digestive glands also did not reveal significant differences between controls and exposed groups, both showing intact digestive tubules with regular and well distinguishable digestive and basophilic pyramidal cells. In samples from all experimental groups epithelium of digestive gland did not exhibit any alteration but appeared well comparable to that of controls with well-nourished digestive cells and basophilic pyramidal cells assembled to form crypts.

Exposure to PET-MPs exposure significantly altered the oxidative status of clam gills (Figure 5a–f). Although the exposure to PET-MPs did not significantly modulate ROS levels, the activity of antioxidant enzymes was significantly modulated by treatment with the highest PET-MPs concentrations (Figure 4b–e). Whilst the activity of SOD (Figure 4c) and GST (Figure 5e) did not significantly differ from control, a significant increase of CAT activity (Figure 5c) was found. In contrast, a significant inhibition of GPx (Figure 5d) was detected in gills from clams exposed to the highest tested concentration compared to control. A significant elevation in lipid peroxidation levels was found (Figure 5f) in gills from clams treated with the highest PET-MPs concentration compared to controls. Despite these findings, no histological effects on gill structure and integrity were noted. Gills from both controls and treated groups showed typical structure of the bivalve *ctenidium*, with strictly folded filaments lined by a single layer of ciliated and secretory cells. Inside the filaments, the water channels and the hemal sinuses were evident, these latter only occasionally displaying few hemocytes (Figure 3d–f). No marked differences in the number of hemocytes among the experimental groups were recorded. Histological analyses were also performed on gonads of all samples, in order to look for possible effects of PET-MPs on reproduction. Samples from both treated groups demonstrated testes and ovaries comparable to those observed in controls with no evident signs of hemocyte infiltration or gonad degeneration. Examination of gonadal stages of the samples revealed no marked differences among the groups suggesting that 7-day exposure to PET-MPs did not significantly alter reproductive biology of *R. philippinarum*.

Discussion

Data demonstrated that micronized PET-MPs exhibiting irregular shape and variable length was ingested and induced imbalance in oxidative status and onset of oxidative damage in gills but not digestive gland of Manila clam. Histopathological analyses of gills, digestive gland, gonads and gut did not reveal differences with respect to controls. The uptake and tissue distribution of MPs were reported in diverse mussel species, mainly in blue mussel, in both field and lab studies (Li et al. 2015; Mathalon and Hill 2014; Van Cauwenberghe et al. 2015; Van Cauwenberghe and Janssen 2014), demonstrating that gills, stomach, intestine and digestive tubules are the main sites of MPs accumulation for high-density PE (HDPE) and PS (Avio, Gorbi, and Regoli 2015; Browne et al. 2008; Von Moos, Burkhardt-Holm, and Köhler 2012). These investigators observed aggregates of MPs within the intestinal lumen and digestive tissues, while a limited presence was found in branchial epithelial cells. Further, plastic items smaller than 3.0 or 9.6 μm were also found in the hemolymph and within hemocytes after translocation from the gut cavity (Browne et al. 2008). Similarly, PS microplastics accumulated in gills and digestive gland of peppery furrow shell *Scrobicularia plana* (Ribeiro et al. 2017), while PS microbeads (2 and 6 μm in diameter) were detected only in digestive gland of Pacific oyster *Crassostrea gigas* (Sussarellu et al. 2016). In the present study, PET-MPs were not found in gills Manila clams treated with the low-tested concentrations, while the presence of PET-MPs was observed in the digestive tract of specimens exposed to the highest concentration (50 μg/ml; Figure 2). However, it needs to be considered that the highest concentration was unrealistic, but also that the exposure to such a concentration was short. The low amount of particles found in clams might be due to the low concentrations of MPs used in our experiments compared to studies performed on other mussel species and/or to the different density of PET (1.38 g/cm^3) with respect

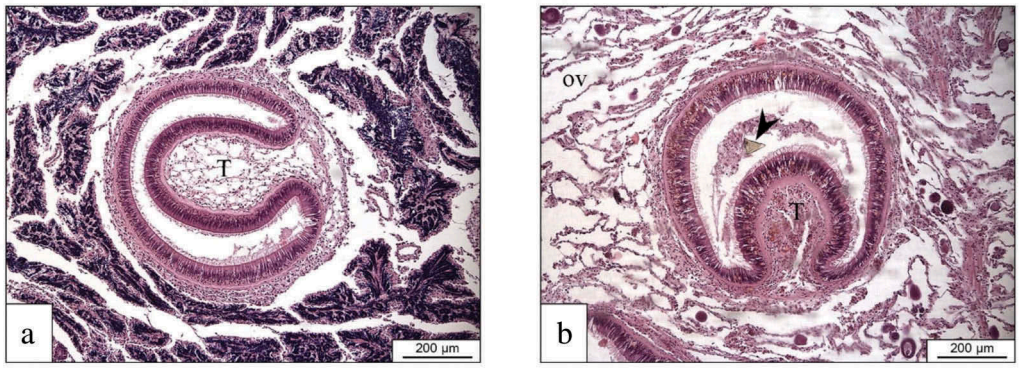

Figure 2. Histological cross sections of the digestive system from control (a) and micronized 50 µg/mL PET-MPs exposed *Ruditapes philippinarum* specimen (b). A PET-µP fragment is visible in panel (b) inside the gut lumen (arrowhead). T = typhosole; t = testis; ov = ovary.

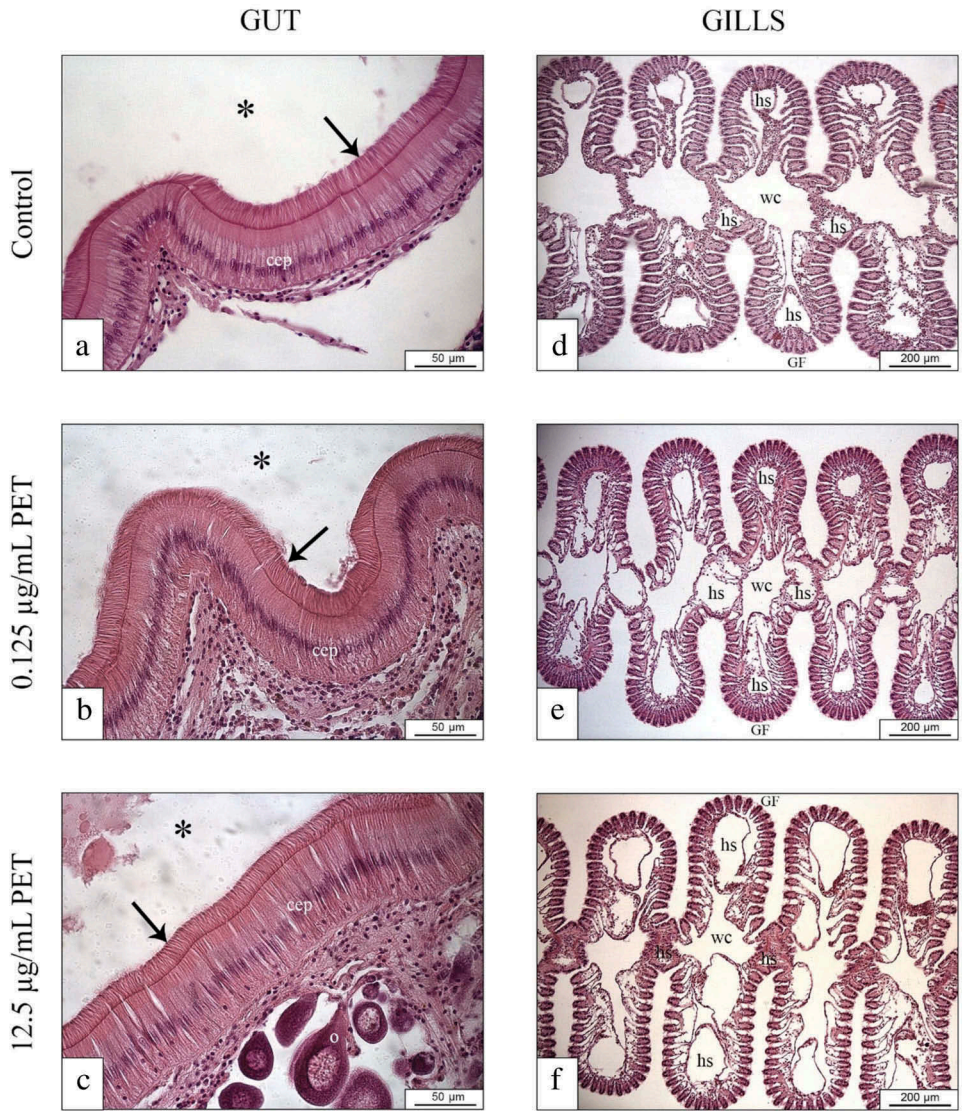

Figure 3. Details of the type 1 epithelium at the crystalline-style sac level (A-C), and the gill filaments (D-F) of *Ruditapes philippinarum*. The digestive epithelium shows the characteristic columnar cells with their bristle-like cilia, and gills their internal structure. * = lumen; → = bristle-like cilia; cep = columnar epithelium; o = oocyte; GF = gill filament; wc = water channel; hs = hemal sinus.

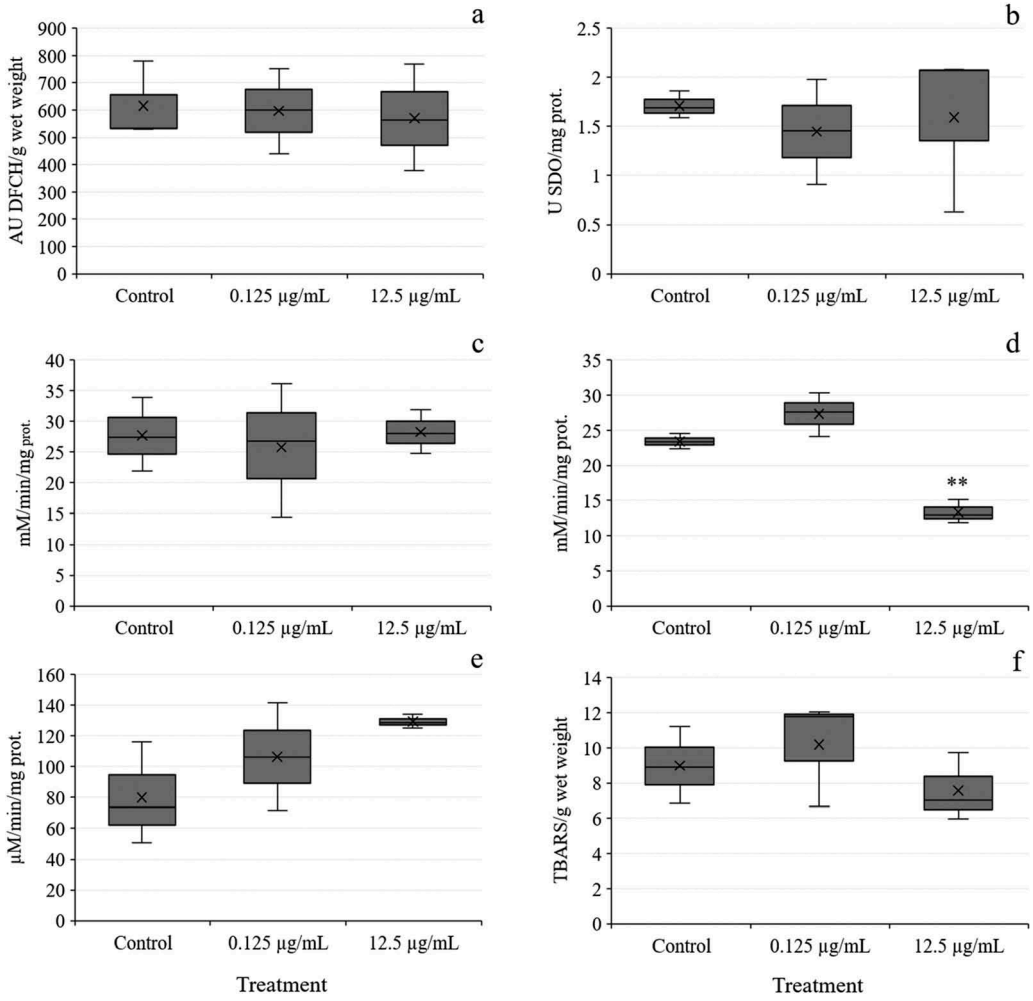

Figure 4. Box and whiskers plot of the total amount of reactive oxygen species (a), activity of superoxide dismutase (b), catalase (c), glutathione peroxidase (d), glutathione S-transferase (e) and lipid peroxidation levels (f) measured in homogenates of digestive gland dissected from clams exposed to two concentrations of micronized PET-MPs. The '×' symbol within the box-plots represents the mean of values, while asterisks above the box-plots show significant differences in the biomarker response between treated and control group (* $p < .05$).

to PS (1.04 g/cm^3) and PE (0.88–0.96 g/cm^3). Because of the high density of PET, MPs (predominantly the larger ones) rapidly sink at the bottom of the exposure tanks, resulting in less available for filter-feeder species than PE and PS, which are both prone to float in the water column and do not (PE) or need up 24 hr to sediment (PS; Ribeiro et al. 2017). Alternatively, as bivalves exert a limited control on the types of filtered particles, the limited amount of PET-MPs found in the digestive tract, coupled with lack of particles on gill surface, suggest a sorting capacity of clams, enabling them to discriminate prior to ingestion unfavorable particles that are subsequently rejected as pseudofeces (Gosling 2003; Ward and Shumway 2004). According to this hypothesis, it is conceivable that PET-MPs were ingested and egested by clams. In fact, a number of PET-MPs visible to the naked eye and wrapped in mucus were ejected as pseudofeces through the inhalant siphon by treated clams. As most of PET-MPs were >100 µm n length, it is not possible to exclude that a limited amount of small MPs were ingested by clams. According to this hypothesis, the additional exposure to a highest, unrealistic concentration (50 µg/ml) indicated the capability of clams to ingest PET-MPs, as evidenced by the presence of microparticles within their digestive tract (approximately 60 µm PET-MPs as depicted in Figure 2).

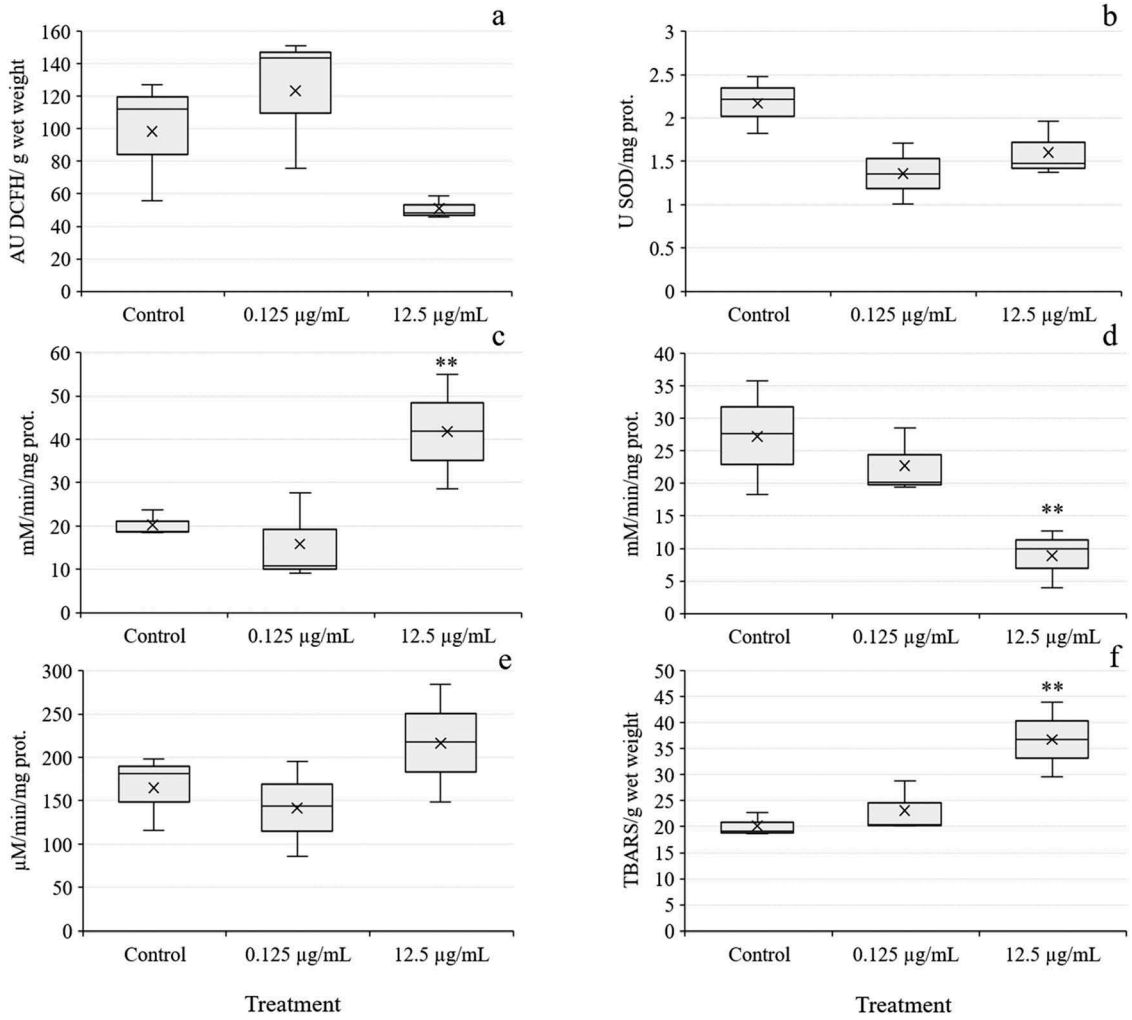

Figure 5. Box and whiskers plot of the total amount of reactive oxygen species (a), activity of superoxide dismutase (b), catalase (c), glutathione peroxidase (d), glutathione S-transferase (e) and lipid peroxidation levels (f) measured in homogenates of gills dissected from clams exposed to two concentrations of micronized PET-MPs. The '×' symbol within the box-plots represents the mean of values, while asterisks above the box-plots show significant differences in the biomarker response between treated and control group (* $p < .05$).

Considering the negligible amount of PET-MPs ingested by clams over the 7-day exposure, it is not possible to exclude that their presence in the digestive tract might impair the digestive system with a consequent decrease in feeding behavior and growth as noted in previous studies of invertebrates (Foley et al. 2018). However, ingestion of PET-MPs may result in detrimental sub-lethal effects due to chemical or physical damage, as demonstrated previously in other mussel species (Avio, Gorbi, and Regoli 2015; Nobre et al. 2015; Von Moos, Burkhardt-Holm, and Köhler 2012; Wright, Thompson, and Galloway 2013). In fact, in marine environments, MPs occur in a wide range of shapes, from fibers to irregular fragments, spheres and rods, which affect potential for physical adverse effects induced by different plastic polymers toward aquatic organisms (Wright, Thompson, and Galloway 2013). Most studies that investigated the potential toxicity of MPs toward marine organisms used particles possessing regular shape and reported null or negligible effects (Foley et al. 2018). For example, a 7-day exposure of *M. galloprovincialis* specimens to 20 g/L of virgin PE and PS microparticles (<100 µm) showed that both polymers were ingested and accumulated in hemolymph, gills and digestive gland, leading to a significant reduction in lysosomal membrane stability and enhanced DNA fragmentation in

hemocytes (Avio, Gorbi, and Regoli 2015). Although destabilization of lysosomal membrane might be related to over-production of pro-oxidant molecules (Canesi et al. 2002; Jovanović and Palić 2012), the antioxidant defenses were not significantly modulated in the mussel digestive gland, except for a significant inhibition of Se-dependent GPx observed at the end of the exposure to virgin PS microparticles (Avio, Gorbi, and Regoli 2015). In the present study, the onset of oxidative stress was investigated on the gills because, being involved in nutrient uptake, digestion and gas exchange, gills are continuously brushed by seawater and represent the first organ to be exposed to different waterborne contaminants (de Oliveira David, Salaroli, and Fontanetti 2008), while the digestive gland is the main organ involved in digestion/assimilation and in phase I and II pathways of xenobiotic metabolism (Livingston, Förlin, and George 1994). For these reasons, both these organs might be prone to suffer contaminant-induced oxidative stress. However, exposure to irregular shaped PET-MPs might exhibit different results as PET-MPs administered to clams displayed a variety of shapes with serrated or sharp extremities (Figure 1a), which might result in physical damage in clam tissues and simultaneously to onset of inflammatory responses and oxidative stress. In contrast to our expectation, treatment with PET-MPs failed to induce oxidative stress in the digestive gland of Manila clams, as overproduction of pro-oxidants or modulation of antioxidant responses and GST, with the exception of a significant inhibition of GPx, or a change in the levels of lipid peroxidation were observed (Figure 4). However, opposite responses were noted in gills, where exposure to the highest PET-MPs concentration produced a modulation in oxidative status, as evidenced by significant elevation in CAT and inhibition of GPx activity (Figure 5). Changes in the activity of these enzymes, which play a concurrent role in the removal of hydrogen peroxide (H_2O_2) (Regoli and Giuliani 2014), suggest a production of this pro-oxidant molecule. The lack of activation of SOD might suggest an inhibitory effect and/or negative feed-back initiated by H_2O_2, indicating that SOD reaction already produced cytosolic H_2O_2 (Vlahogianni and Valavanidis 2007). Alternatively, H_2O_2 might originate from spontaneous dismutation of superoxide radical through non-enzymatic pathways (Gwoździński et al. 2010) or by other cellular enzymes like those included in peroxisomes (Khessiba, Roméo, and Aïssa 2005). While GPx is mainly responsible for eliminating H_2O_2 produced by metabolic processes, CAT acts also toward the exogenous source of this molecule (Avio, Gorbi, and Regoli 2015). The contemporary, opposite modulation of these enzymes in gills suggests that PET-MPs induced an overproduction of H_2O_2. It is conceivable that activation of CAT was able to efficiently counteract onset of pro-oxidants induced by PET-MPs, explaining the lack of changes in ROS levels observed in treated groups. The inhibition of GPx activity may be due to the competition for the same substrate with CAT (Kappus 1985). Alternatively, as CAT is activated only at high H_2O_2 concentrations (Pereira, Fernandes, and Martinez 2013), while GPx acts also at lower substrate levels, the significant decrease in GPx activity suggest a possible excess of H_2O_2 is present that this enzyme cannot offset. This hypothesis was supported by similar trends of CAT and GPx reported in gills of *M. galloprovincialis* exposed to 250 ng/L of ibuprofen (Gonzalez-Rey and Bebianno 2011), as well as in the freshwater zebra mussel (*Dreissena polymorpha)* after the exposure to 8,000 ng/L of the same pharmaceutical (Parolini, Binelli, and Provini 2011). The imbalance in oxidative status induced by a short-term exposure to PET-MPs in Manila clam gills might lead to oxidative stress situation with detrimental consequences to cellular macromolecules, including lipids, proteins and DNA. The significant increase in lipid peroxidation levels observed in clam gills at the end of the exposure to the highest tested concentration (Figure 5) suggest that treatment with PET-MPs might induce oxidative damage to lipids and lead to cellular alterations, including disruption of the cell membrane (Yajima et al. 2009). Thus, evidence indicates that the contact or rubbing of PET-MPs with the gill surface induces an inflammatory, and consequently, oxidative stress response in this organ although no histological effects were noted.

Conclusions

Our results suggest that short-term exposure to low concentrations of micronized, irregular-shaped and variable size PET-MPs might represent a threat for the health status of Manila clams.

Although these MPs did not modulate oxidative status of clams' digestive gland, an oxidative stress situation occurred in gills, which did not show histological alterations. Considering that organisms are exposed to MPs for their whole lifespan, long-term exposure to PET-MPs may be necessary to confirm the hazard of these particles toward filter-feeders organisms. Further, because of the high density of PET, PET-MPs tend to deposit on marine sediments which represents a potential threat for benthic organism with different feeding strategy such as grazers. For these reasons, further investigations on potential adverse effects initiated by exposure to irregularly shaped PET MPs toward marine organisms represent a priority in marine ecotoxicology. Indeed, irregular shaped with different size MPs are predominant in marine ecosystems and might represent a major threat for free-living organism compared to regular shaped MPs, whose toxicity was more frequently tested under controlled lab conditions.

Author contribution

Marco Parolini: Conceptualization, Writing – Original Draft, Supervision; Beatrice De Felice: Investigation; Stefano Gazzotti, Luisa Annunziata: Investigation; Michela Sugni: Investigation, Renato Bacchetta: Investigation; Marco Aldo Ortenzi: Investigation.

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PAPER VII

Parolini M., Ferrario C., **De Felice B.**, Gazzotti S., Bonasoro F., Candia Carnevali M.D., Ortenzi M.A., Sugni M.

Interactive effects between sinking polyethylene terephthalate (PET) microplastics deriving from water bottles and a benthic grazer.

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Interactive effects between sinking polyethylene terephthalate (PET) microplastics deriving from water bottles and a benthic grazer

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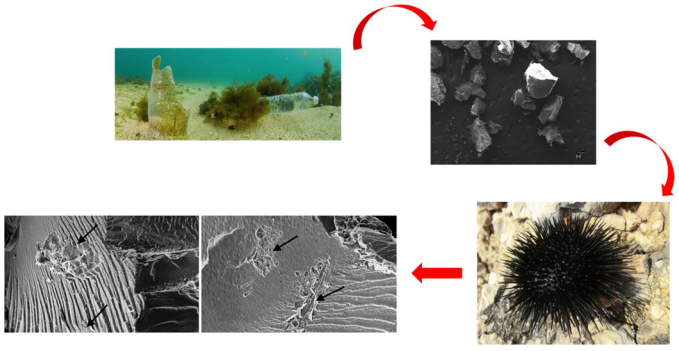
GRAPHICAL ABSTRACT

For the image of sinking water bottles: Plastic Bottles on sea floor in Malta. Photo David Jones



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ABSTRACT

The information concerning the toxicity of sinking microplastics (MPs) on benthic marine animals, particularly benthic grazers, is still scant. No study focused on biological weathering of sinked MPs operated by benthic organisms. This study aims at investigating the ingestion and the effects induced by 7-days dietary exposure to environmentally relevant amount (8, 80 and 800 particles/g of food) of irregular shaped and sized (diameter 12.6 − 1,065 μm; mean diameter 316 ± 12 μm) polyethylene terephthalate microplastics (PET-MPs) on a common marine benthic grazer, the sea urchin *Paracentrotus lividus*. Adverse effects were investigated on digestive tract at biochemical (oxidative stress biomarkers) and tissue level (histopathological analyses). Potential alteration of MP structure/surface and PET macromolecules due to the ingestion of PET-MPs within the sea urchin digestive tract were investigated. Results showed that PET-MPs were efficiently egested by sea urchins without producing histological alterations on digestive tract tissues, only inducing a slight modulation of oxidative status. Sea urchin grazing activity and the related transit of PET-MPs within animal digestive tract slightly affected MP structure and PET composition. These findings suggest that PET-MPs might represent an hazard for benthic grazer organisms, which can partially contribute to the degradation of PET in marine ecosystems.

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Introduction

The imprudent use of plastics in our everyday life, exacerbated by the current throw-away culture of our society and the inappropriate end-use disposal, has led to a widespread accumulation of plastic debris in our ecosystems. This situation is particularly worrisome for marine ecosystems, whereby both large plastic items (*i.e.*, macroplastics) and mainly microplastics (MPs), i.e. any plastic item < 1 or < 5 mm (Browne et al., 2008; Eerkes-Medrano et al., 2015), are ubiquitous contaminants of Seas and Oceans worldwide (e.g.,Barnes et al., 2009; Eriksen et al., 2014; Zeng, 2018). Over 5 trillion of MPs have been estimated to float on the surface of the oceans worldwide (Eriksen et al., 2014), and this huge amount is destined to increase because of the weathering of macroplastics (Cózar et al., 2014). Given their low density compared to seawater, polyethylene (PE) and polypropylene (PP) are the dominant polymers floating in marine ecosystems and are commonly found in marine surface water, as well as in pelagic and filter-feeder animals (Qiu et al., 2015; Cole et al., 2013; Gasperi et al., 2014; Klein et al., 2015). However, a recent study has suggested that the amount of plastics floating on surface waters represents a minimal part of plastics that entered the marine ecosystems (Cózar et al., 2014). Indeed, MPs can mutually aggregate (homo-aggregation) or aggregate with suspended solids or organisms (hetero-aggregation; Besseling et al., 2017) so that, positively buoyant plastics can become negatively buoyant and sink (Lobelle and Cunliffe, 2011). For instance, different studies have reported the accumulation of large plastic items and microscopic fragments of low-density polymers in the deep sea (e.g., Van Cauwenberghe et al., 2015). Moreover, some plastic polymers having higher density than seawater are intrinsically negatively buoyant and can sink and accumulate in benthic ecosystems (Woodall et al., 2014). Among these, polyethylene terephthalate (PET), a semi-crystalline, thermoplastic polyester being the most representative polymer of the polyester group (Awaja and Pavel, 2005), is of great concern. PET is primarily used in the form of fibres, sheets and films, and in diverse applications, including food and beverage packaging, such as water bottles (Sinha et al., 2010), which represent a very common environmental waste. PET comprises up to 7.7 % of the totalEuropean plastic consumption, with a demand of ~4000, 000 tonnes per year (Plastics Europe, 2019). Because of its high density (about 1.37–1.45 g/cm^3) PET is prone to sink, thus representing a potential threat for benthic organisms. Indeed, polyester microfibers were found as the most abundant plastic polymer in deep-sea sediments from the Atlantic and Indian Oceans and Mediterranean Sea (Woodall et al., 2014). Several experimental studies have shown that the exposure to MPs induced toxic responses towards marine animals, from suspension- and filter-feeders to deposit feeders (e.g., Cole et al., 2011; Paul-Pont et al., 2018). However, the majority of these studies was focused only on the investigation of the potential toxicity of PE and PS on some nektonic or filter-feeders benthic animals (i.e. fish, crustaceans and molluscs; de Sá et al., 2018d). However, to date there is a dearth of information on the ingestion and toxicity of sinking MPs, including PET (de Sá et al., 2018d) on marine organisms and, specifically, on benthic grazers, which represent key elements of marine ecosystems. In fact, to date only two studies have investigated the uptake and potential effects induced by polyester or PET textile fibres towards soil invertebrates, namely enchytraeids (*Enchytraeus crypticus*), springtails (*Folsomia candida*), isopods (*Porcellio scaber*) and oribatid mites (*Oppia nitens*; Selonen et al., 2020) and the freshwater zooplanktonic species *Daphnia magna* (Jemec et al., 2016).

Moreover, ecotoxicological studies on MPs have been focused only on the effects caused by exposure to plastic items on the organisms, utterly neglecting the potential biota-induced (physico-chemical) alterations of MPs following ingestion by animals, which can affect their environmental fate. Thus, the present study was aimed at exploring both sides of the same “plastic coin”, investigating the potential effects of a MP polymer towards a benthic organism and *vice versa*. First, we investigated the ingestion and the potential adverse effects induced by 7-days dietary exposure to three amounts (8; 80 and 800 particles/g of food) of micronized, irregularly shaped and sized polyethylene terephthalate microparticles (hereafter PET-MPs) on adults of the sea urchin *Paracentrotus lividus* Lamarck, 1816. The *P. lividus* was used as a model organism because represents one of the main benthic grazer of the Mediterranean and north-east Atlantic sea, where it plays a crucial ecological role (Palacin et al., 1998; Privitera et al., 2008). Its continuous foraging activity on rocky bottoms or *Posidonia oceanica* meadowsone and its presence also in highly anthropic areas makes it a potential target of MPs sinking and accumulation in marine benthic ecosystems. Moreover, although some studies demonstrated the ingestion and the adverse effects of floating MPs, such as polystyrene (PS) and PE, on sea urchin planctotrophic larvae (e.g., Nobre et al., 2015; Messinetti et al., 2018), no information is available on the effects induced by sinking MPs on benthic adults although they can represent an excellent model organism in marine ecotoxicology (Sugni et al., 2007, 2010; Sugni et al., 2014). At the end of the exposure, the ingestion of PET-MPs and tissue alterations at the level of the oesophagus were investigated by histological analyses, whereas oxidative stress-related effects were investigated in parallel on homogenates of the same tissue by the application of a suite of six different biomarkers assessing both the imbalance of the oxidative status and oxidative damage. Lastly, the potential structural and chemical alterations of the macromolecular chain in PET-MPs caused by sea urchin feeding activity, including the grazing process and transfer/permanence within the digestive tract, were assessed through Scanning Electron Microscopy (SEM) and Fourier Transformed Infrared Spectroscope (FT-IR) analyses of PET-MPs collected in fecal pellets.

Materials and methods

Animal collection and maintenance

Adult sea urchins (4 − 5 cm in diameter, 30–40 g total weight) were manually collected by snorkeling in La Spezia Gulf (Ligurian Sea, Italy, 44° 04′ 12″ N; 9° 50′ 20″ E) at 1 − 2 m depth. They were immediately transported in cooled boxes to the University of Milan, where they were transferred to 50 L glass aquaria filled with artificial sea water (Instant Ocean, salinity 37 − 38 ‰, temperature 16 − 17 °C) and provided with chemical-physical filters (activated charcoal, filter wool, ceramic rings) and internal circulation systems (Micra Plus pump, 400 L/h). The animals were left to acclimatize for a time period at least corresponding to the duration of the experiment (7–10 days). Previous studies on MPs that used sea urchin species as model organism, acclimatized specimens for similar time intervals (Porter et al., 2019; Suckling and Richard, 2020). During this period animals were starved to allow the emptying of the digestive tube. On the basis of our long-lasting experience this time is sufficient to empty most of the digestive tract. Throughout the experiments standard water chemical-physical parameters were daily checked, including: salinity (Milwakee refractometer), temperature (analog thermometer), pH, KH, GH, NO_2, NO_3 (Tetra test strips). Water parameters were promptly adjusted if necessary, by adding distilled water (for salinity), drops of CaOH (for pH), partial water renewal (for KH, GH, NO_2, NO_3).

Experimental design

Two different experiments were performed. The first one was addressed to evaluate the effects of PET-MPs dietary administration on sea urchin digestive tissues (oesophagus) in terms of histological alterations and oxidative stress, the second one to explore the effects of sea urchin feeding activity (ingestion and digestive processes) on PET-MPs, in terms of surface structural alterations (e.g., scrapes, scratches, dental marks) and changes of PET at chemical level. In both experiments PET-MPs were obtained by mechanical grinding of PET bottles, in order to mimic a realistic environmental shaping and sizing of particles. Our

Table 1
Dimensional and shape features of polyethylene terephthalate microplastics (PET-MPs) used in the experiments with the sea urchins. Mean (± standard error of the mean; SEM) of the area, perimeter, length, circularity, aspect-ratio (AR) and roundness of polyethylene terephthalate microplastics (PET-MPs) measured in control and egested PET-MPs are reported. Student *t*-test for independent samples was applied to check for significant differences between Control and Egested PET-MPs. Different letters as apex of different parameters show significant differences between groups ($P < 0.05$).

	Area (μm^2)	Perimeter (μm)	Diameter (μm)	Circularity	AR	Roundness
Control (n = 298)	110,529 ± 8010[a]	1385 ± 50[a]	316 ± 12[a]	0.53 ± 0.01[a]	1.69 ± 0.05[a]	0.66 ± 0.01[a]
Egested (n = 363)	97,776 ± 7370[a]	1293 ± 48[a]	287 ± 11[a]	0.51 ± 0.01[b]	1.78 ± 0.04[a]	0.62 ± 0.01[b]

experimental design allowed to bypass two of the main weakness in the assessment of MPs toxicity on marine organisms, namely testing of non-realistic concentrations and the use of standard particles (e.g., commercially produced microbeads), which are not representative of the real environmental conditions, where particles, fragments and fibres of different shapes and sizes are commonly found (Paul-Pont et al., 2018). In our experiment, PET-MPs were obtained by consecutive freezing (in liquid nitrogen) and grinding cycles. At the end of the process, PET-MPs were passed through a 1 mm sieve in order to obtain particles in the range of MPs. Produced particles showed an extremely irregular shape (see also Parolini et al., 2020) and variable size. The features of PET-MPs used in the present experiment (i.e., control group) were reported in Table 1. We also grouped PET-MPs in four different size classes (% frequency) on the basis of the calculated diameter (see *2.3 Scanning Electron Microscopy (SEM) analyses for PET-MPs characterization* paragraph for details), which can be considered as a proxy of the length of the particles: < 50 μm (6.4 %); 50 < x < 100 μm (8.1 %); 100 < x < 500 μm (68.5 %); 500 < x < 1000 μm (17.1 %). The polymeric composition of micronized PET-MPs was chemically characterized by using a Fourier Transformed Infrared Spectroscope (FT-IR) Perkin Elmer Spectrum 100 (Parolini et al., 2020).

Experiment 1: thirty-two sea urchins were subdivided in four experimental groups (N = 8 sea urchins/group), each one placed in a 50 L glass aquarium: three groups were fed with pellets containing increasing amount of PET-MPs, whereas pellets of the control animals lacked PET-MPs. The selected amounts of PET-MPs were 8, 80 and 800 particles/g of food. The lowest concentration was selected to match mean environmental values observed in some costal sediments (e.g., Mathalon et al., 2014), while 80 and 800 particles/g of food represented a 'worst case' scenario. The medium and the highest doses were obtained increasing this value by a 10 and 100 factor. Pellets were prepared by mixing PET-MPs with minced artificial sea urchin feed (Wenger Manufacturing, Inc. Patent WO1997018719A1; nutritional values: 12–24 % total crude protein, 30–60 % carbohydrates, 12–40 % moisture) and commercial agar agar (Biovegan, nutritional values for 100 g: carbohydrates 10 g, proteins 2 g, lipids 0,3 g, fibres 70 g, salts 3,1 g). Considering that about 100 plastic particles weighted 3.5 mg, the following recipe was used to prepare the pellets: 0.03 - 0.3–3 g PET-MPs, corresponding to about 760 – 7600 – 76,000 particles, respectively; 7.5 g of minced artificial sea urchin feed; 7.5 g of commercial agar agar and 80 mL of distilled water. In detail, the agar powder was added to cold water, boiled for 1 min and then partially cooled. Minced sea urchin feed and PET-MPs were subsequently added and the obtained mixture was used to prepare pellets of about 1 g each. These were left in the fridge for about 2 h and then stored at −20 °C. Each animal was daily fed with one pellet (corresponding to 2,5 %–3,3 % of the animal weight) and the dietary administration lasted 7 days. Uneaten food, if present, was carefully removed from the aquaria. A 7-days exposure was performed because a previous experiment on the Manila clam *Ruditapes philippinarum* showed that a one week exposure to low concentrations of PET-MPs was enough to induce an alteration of the oxidative status of exposed organisms (Parolini et al., 2020). Moreover, the exposure time we planned was similar to those used in other experiments performed on different sea urchin species aimed at assessing the potential effects of MPs on physiological and behavioural endpoints (Suckling and Richard, 2020), as well as the effects of grazing on the integrity of plastics (Porter et al., 2019).

The pellet consumption was daily checked by visual inspection. At the end of the experimental period animals were sacrificed, dissected and the oesophagus was collected. The proximal (to the mouth) part was frozen in liquid nitrogen and stored at −80 °C for biomarker analyses (see *2.5 Biomarker methods*), the distal one was immediately immersed in cold (4 °C) Bouin's fixative and processed for histological analyses (see *2.3 Histological analyses*). We selected the oesophagus as the target tissue since it is the first and narrowest part of the digestive tract and it could be therefore most probably affected by the transit of irregular MPs.

Experiment 2: four sea urchins were transferred to a 15 L glass aquarium provided with filters and circulation system (see above). Pellets were prepared according to the following recipe: 8 g of minced artificial sea urchin feed, 9 g of PET-MPs, corresponding to about 257,000 particles; 4.3 g of commercial agar agar (Biovegan) and 80 mL of distilled water. The preparation procedure was the same described above. The high concentration of PET-MPs used in this experiment was necessary to maximize the recovery of egested MPs to be used for the different subsequent analyses (see below). For this reason PET-MPs concentration was much higher than environmental levels. Each sea urchin was daily fed with one PET-MPs containing pellets (about 1 g, equal to 2,5−3,3 % of the sea urchin weight) and the dietary administration lasted for seven days. The pellet consumption was daily checked by visual inspection and uneaten food was promptly removed from the aquarium Fecal pellets were daily collected from the aquarium, manually minced with scissors and left in hydrogen peroxide 30 % (H_2O_2; Sigma-Aldrich) for 6 days at room temperature (23 °C) to dissolve organic matter and recover PET-MPs (Nuelle et al., 2014). The digestion was performed in glass vials filled with H_2O_2 at the following indicative ratio: 0,5 g pellet/50 mL H_2O_2. The recovered PET-MPs were dried at 20 °C in a drying oven (Roshan Enterprises, India) and stored in a glass container until the subsequent scanning electron microscope (SEM) or Fourier Transformed Infrared spectroscopy (FT-IR) analyses. As a control, some of the PET-MPs containing pellets were left in a different compartment of the aquarium, isolated from the sea urchins, for 24 h. Then they were collected and processed exactly as the fecal pellet (minced, treated with H_2O_2 for 6 days and dried). At the end of the exposures (7 days) sea urchins were sacrificed and dissected, the digestive tube was partially removed and further dissected to observe in detail and photograph its content under a stereomicroscope (Leica MZ75, provided by a Leica EC3 Camera and Leica Application Suite LAS EZ Software (Version 1.8.0).

Scanning Electron Microscopy (SEM) analyses for PET-MPs characterization

Subsamples of virgin (immediately after their production), egested and control PET-MPs were randomly distributed on 2,5 cm x 2,5 cm stubs provided with carbon double-sided tape, covered with a thin film of pure gold (Agar SEM Auto Sputter), observed and photographed under a scanning electron microscope (LEO1430, Zeiss, Oberkochen, Germany). Measurements of PET-MPS were performed according to Winkler et al. (2019), with slight modification. Two random vertical

transects was performed along the stub, for a total of 12 SEM images acquired (magnification 25 X, consistent all over the sample) per experimental group. Perimeter, area, circularity, roundness and aspect-ratio of each particle was automatically measured by means of the freeware software ImageJ. Circularity was calculated as 4π*area/perimeterˆ2: a value of 1.0 indicates a perfect circle, while a value approaching to 0.0 indicates an increasingly elongated shape. Aspect-ratio (AR) was calculated as major_axis/minor_axis ratio of the best fitting ellipse. In addition, to account for the extremely high irregularity of the particles, their size was calculated as the diameter of a spherical particle of the same area as the detected irregular particle. Both control and egested PET-MPs, were grouped in four size classes according to the calculated diameter : < 50 μm; 50 < x < 100 μm; 100 < x < 500 μm and 500 < x < 1000 μm. Lastly, higher magnification SEM observations and photos were performed to detect any sign of sea urchin teeth scrapes.

Histological analyses

Oesophagus samples were left in Bouin's fixative for 24 h at 4 °C. They were washed several times in tap water, dehydrated in an increasing ethanol series (70 %, 90 %, 95 %, 100 %) and cleared with xylene. They were left in a xylene:paraffin mixture (1:1) overnight at room temperature and then embedded in pure paraffin (56 − 58 °C). Transverse sections (about 8 μm in thickness) were cut with a Leitz 1512 rotary microtome and stained according to Milligan's Trichrome technique (Milligan, 1946). All the reagents used for histological analyses were purchased by Sigma-Aldrich. The obtained sections were observed and photographed under a Jenaval light microscope provided with a Leica EC3 Camera and Leica Application Suite LAS EZ Software (Version 1.8.0). Each oesophagus sample was sectioned at two different levels to investigate the potential effects of PET-MPs on different part of the oesophagus. For each level at least 2 glass slide were prepared and observed, each one containing 5–6 histological sections, for a total of about 20–24 sections per each single specimen.

Methods of oxidative stress biomarkers

The suite of oxidative stress biomarkers was applied on homogenates of the proximal part of the oesophagus collected by the eight sea urchin specimens per experimental treatment. Considering the limited amount of tissue, all the biochemical measurements were carried out in duplicate. Oesophagus samples (~ 0.05 – 0.2 *g*) were homogenized (1:5 wt/volume) using a motor pestle in a 100 mM potassium phosphate buffer (added with KCl 100 mM, EDTA 1 mM, protease inhibitors 1:100 v/v and dithiothreitol 1 mM, pH 7.4). One hundred μL of raw homogenates were used for lipid peroxidation analysis, whereas the remaining part was centrifuged at 15.000 × g for 10 min in an Eppendorf centrifuge. The supernatant was then collected and immediately processed to assess protein content and enzyme activities, which were applied according to spectrophotometric methods described elsewhere (Parolini and Binelli, 2014; Parolini et al., 2016, 2017). The superoxide dismutase (SOD) activity was measured at λ = 550 nm as the inhibition of cytochrome c (10 μM) reduction caused by the superoxide anion generated by the xanthine oxidase (1.87 mU mL^{-1})/hypoxanthine (50 μM) reaction, and expressed as SOD units (1 SOD unit = 50 % inhibition of the xanthine oxidase reaction). The catalase (CAT) activity was determined by measuring the decrease of H_2O_2 (50 mM) in potassium phosphate buffer (66.7 mM at pH 7) at λ = 240 nm. The glutathione peroxidase (GPx) activity was measured by monitoring the consumption of NADPH (0.12 mM) at λ = 340 nm using 0.2 mM H_2O_2 as a substrate in 50 mM potassium phosphate buffer, added with reduced glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U mL^{-1}). The glutathione S-transferase (GST) activity was measured at λ = 340 by adding reduced glutathione (1 mM) in 80 mM phosphate buffer (pH 7.4) and using chlorodinitrobenzene (CDNB; 1 mM) as a substrate. Lipid peroxidation was assessed through the thiobarbituric acid-reactive substances (TBARS) method (Ohkawa et al., 1979). All the spectrophotometric readings were performed by a Genova Bio spectrophotometer (Jenway). The amount of reactive oxygen species (ROS) was assessed according to a fluorimetric method that relies on the change in fluorescence of the dichlorofluorescein-diacetate (DCFH-DA; 10 mg mL^{-1} in dimethyl sulfoxide) in presence of pro-oxidant molecules. The fluorescence intensity was measured by an Infinite® 200 PRO microplate reader (TECAN Life Sciences) with λ = 485 nm as excitation and λ = 536 nm as emission wavelength, respectively. All the reagents used for biomarker analyses were purchased by Sigma-Aldrich.

Fourier transform infrared spectroscopy (FT-IR) analyses

Fourier transform infrared spectroscopy (FT-IR) spectra were obtained on a Spectrum 100 spectrophotometer (Perkin Elmer) in attenuated total reflection (ATR) mode using a resolution of 4.0 and 256 scans, in a range of wavenumber between 4000 and 400 cm^{-1}, using air at standard temperature and environmental moisture (23 °C and 50 % RH) as background. The pressure applied was checked and maintained at about 50 N for all samples. A single-bounce diamond crystal was used with an incidence angle of 45°. FT-IR spectra were collected on samples dried overnight before the analysis: no further treatment was performed.

Statistical analysis

The effects of PET-MPs dietary exposure on oxidative stress biomarkers were investigated by a one-way analysis of variance (ANOVA), after checking for normality and homoscedasticity of data with Shapiro–Wilk and Levene's tests, respectively. As the Shapiro-Wilk test showed that data for SOD, CAT and LPO were not normally distributed, we log-transformed them before running the ANOVA. Significant differences among groups were checked through the application of the Tukey *post- hoc* test. All the analyses were run using RStudio statistical package.

Results

No mortality was observed in any of the experiment performed and the sea urchins displayed optimal health condition (e.g., climbing ability, straightened spines) throughout the experimental period. These information supported the reliability of the experimental set up (from the point of view of animal health status). Overall, the time needed for the complete transition through the digestive tract (from the beginning of pellet ingestion to the observation of the first fecal pellets containing MPs) was less than 24 h.

Experiment 1: Effects of PET-MPs on sea urchins

Overall, no differences were observed at the tissue-level between control and treated groups (Fig. 1). The different oesophagus layers (inner and outer epithelium, connective tissue, muscular layer) as well as the epithelial architecture, the number, distribution and thickness of oesophagus criptae were comparable among the different experimental animals. No evident signs of inflammation (*i.e.*, edema, hypertrophy or accumulation of immune cells) was observed in any analysed histological sample. Results of biomarker analyses on the oesophagus of sea urchin specimens exposed *via* diet to increasing amount of PET-MPs are reported in Fig. 2. A significant effect of PET-MPs on the amount of reactive oxygen species (ROS) was found ($F_{3,26}$ = 12.46; $p < 0.001$), with a significant increase observed in specimens exposed to the highest tested concentration compared to the control group ($p = 0.025$). No significant effect of the treatments on SOD ($F_{3,27}$ = 1.64; $p = 0.2034$), CAT ($F_{3,19}$ = 0.5732; $p = 0.6396$) and GST ($F_{3,28}$ = 2.629;

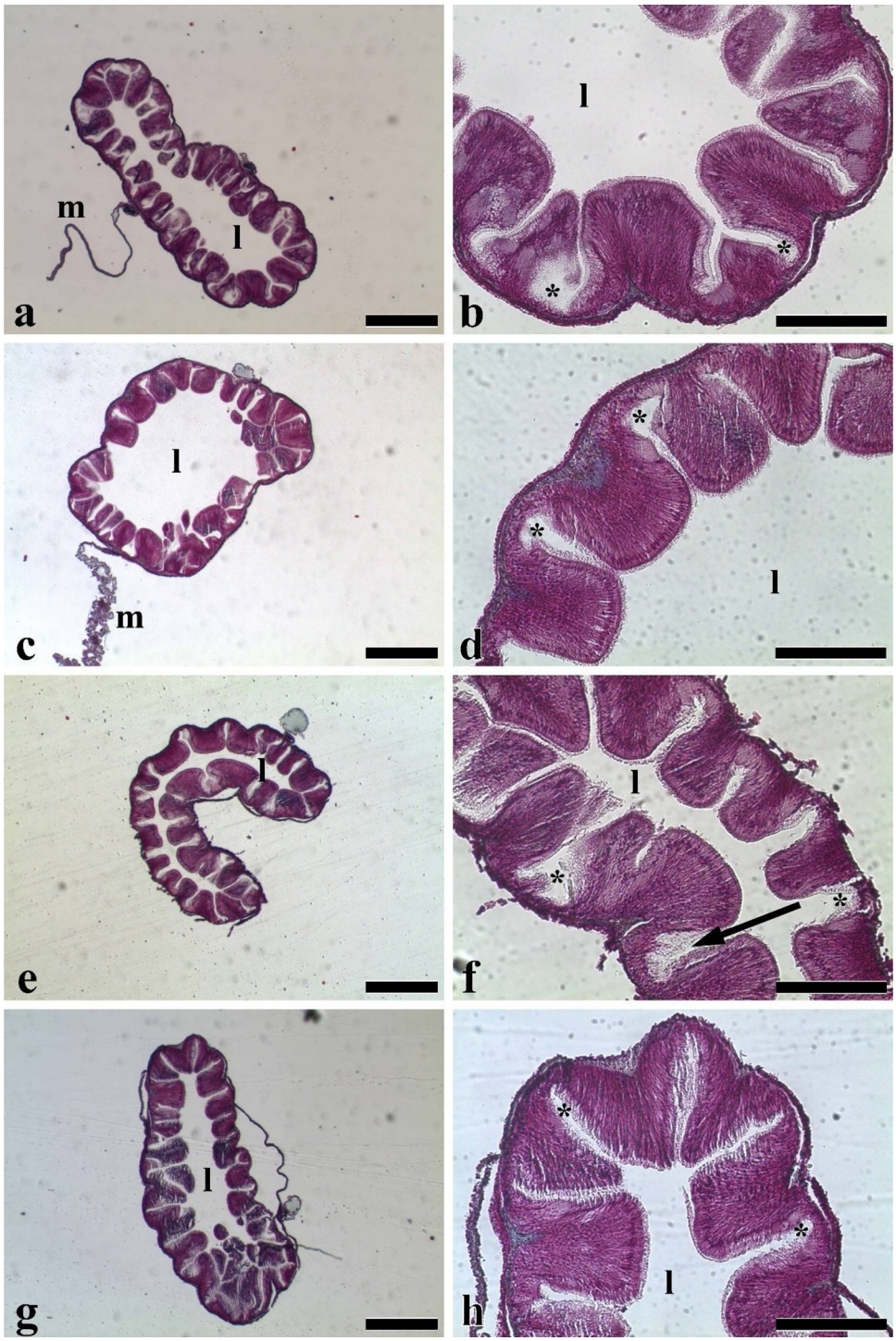

Fig. 1. Histological cross sections of the oesophagus (paraffin, Milligan's trichrome staining). a-b) Control samples. c-d) sea urchin fed with 8 particles/g. e-f) sea urchin fed with 80 particles/g. h-g) sea urchin fed with 800 particles/g. The microscopic anatomy of the different samples is comparable. The lumen (l) is always clearly visible, the intestinal wall (inner epithelium, connective tissue, muscular layer and outer mesothelium) does not show any pathological alteration. No differences are visible in number or architecture of oesophagus criptae (*), at the bottom of which mucous material can be observed (d, black arrow). m = intestinal mesentery. a,c,e,g: Scale bar = 100 μm. b,d,f,h: Scale bar = 25 μm.

$p < 0.0697$) activity was noted. However, a significant effect of treatment on GPx activity ($F = 25.145$; $p < 0.001$) was found, showing significant 1.76- and 2-fold increase at the end of exposure to low ($p < 0.001$) and mid ($p < 0.001$) tested concentration, respectively. No significant increase of lipid peroxidation levels was induced by any treatment with PET-MPs ($F_{3,26} = 1.3979$; $p = 0.2657$).

Experiment 2: Effects on PET-MPs induced by sea urchins

Stereomicroscope analyses of dissected digestive tracts showed that most plastic particles were embedded in the faecal pellets, mixed with mucus and undigested organic material, and only few PET-MPs were freely present in the lumen (Fig. 3). Faecal pellets containing PET-MPs were accumulated in the intestine, whereas no one was observed in the 'stomach' (Fig. 3a and personal observations). No statistically significant differences (Studend *t*-test for independent samples) was found between control and egested PET-MPs in the most morphometric parameters (i.e., area, perimeter, diameter and AR) (Table 1). However, circularity ($P = 0.037$) and roundness ($P = 0.004$) of egested PET-MPs was significantly lower than control MPs. Similarly, the distribution of PET-MPs among size classes was comparable between the two experimental groups (Fig. 4). No further ultrastructural difference was present between control and egested PET-MPs when observed at high magnification (Fig. 5 a,b): regardless the experimental condition, the surfaces

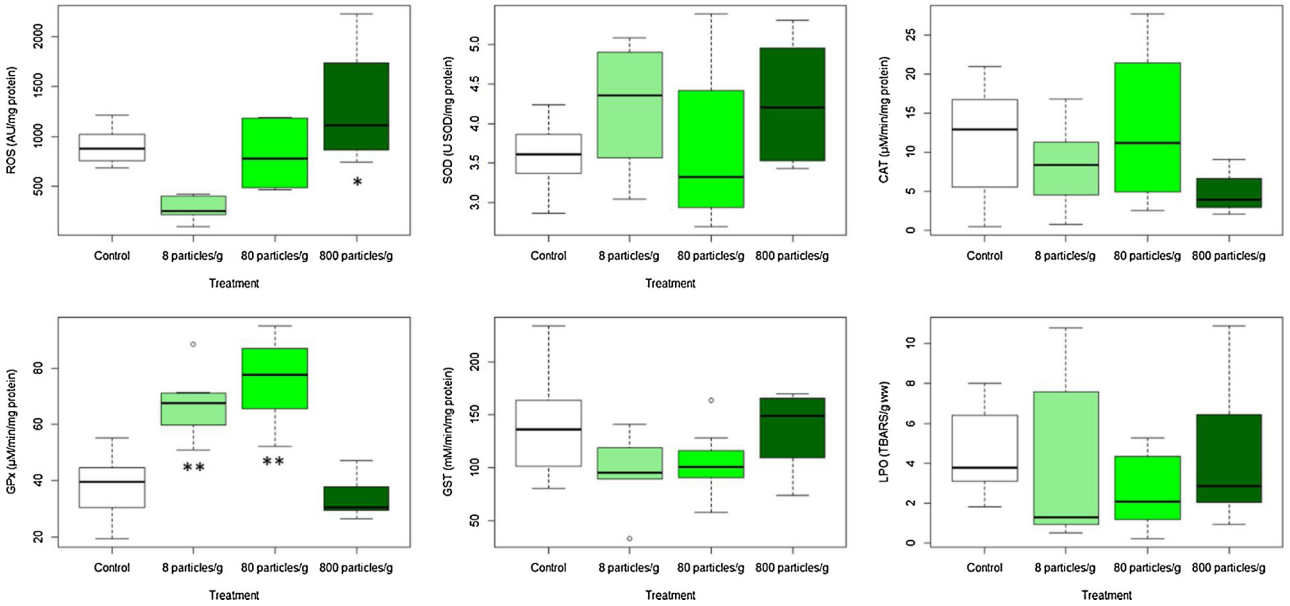

Fig. 2. Box-plot representing data of the total amount of reactive oxygen species (ROS), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST) and lipid peroxidation (LPO) measured in the oesophagus of sea urchin specimens exposed via diet to increasing concentrations of micronized PET particles. Asterisks below the box-plot show significant differences in the biomarker response between treated and control group (* $p < 0.05$; ** $p < 0.01$; one-way ANOVA; Tukey *post-hoc* test).

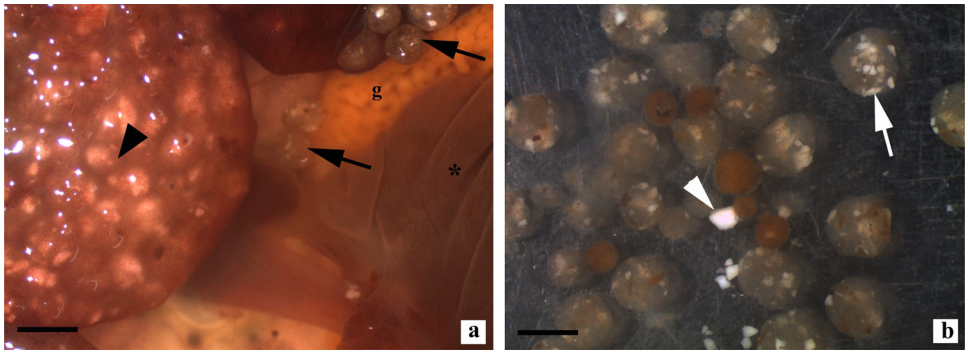

Fig. 3. Stereomicroscopic views of the digestive tube of a sea urchin fed with PET-MPs (2nd experiment). a. Roundish fecal pellets containing PET-MPs completely fill the intestine (arrowhead) and are clearly visible upon rupture of the intestinal wall, from which they pour out (black arrow). On the contrary the "stomach" (*) appear swollen and empty b) Several mucous fecal pellets (white arrow) of different size and containing several plastic particles can be collected from the intestine. Few PET-MPs, not embedded in the pellets, can be occasionally found (white arrowhead). g: gonad. Scale bar = mm.

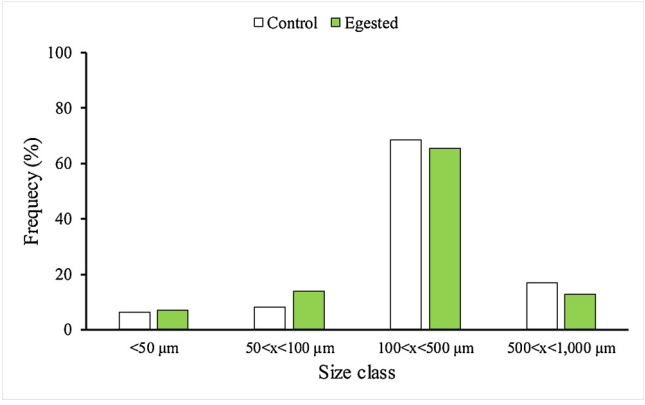

Fig. 4. Frequency (%) of polyethylene terephtalate microplastics (PET-MPs) grouped in five size classes based on their diameter: < 50 μm; 50 < x < 100 μm; 100 < x < 500 μm; 500 < x < 1000 μm.

were always highly irregular. However in some of the ingested particles we observed superficial signs compatible with sea urchin teeth scrapes (Fig. 5 c,d). An analysis *via* FT-IR was performed to check if any sign of possible chemical degradation was visible even after a single passage in the digestive tract. Fig. 6 shows the comparison between the spectrum of control PET-MPs and those obtained from the fecal pellets of sea urchins: the spectra were scaled according to the main peak (*i.e.*, the one at about 700 cm^{-1}).

As expected, the two spectra were very similar: some differences could be observed in terms of small peaks in control PET-MPs due to some organic residue of food (e.g., the peak at about 1400 cm^{-1}) and due to the intensities of some peaks. In general, control PET-MPs showed higher intensities in the 1700 cm^{-1} peak (*i.e.*, carbonyl stretching) and in the 1020 cm^{-1} peak (*i.e.*, vibration of ethylene glycol moiety in PET ester bond; Ward and Girme, 1958). Also the peaks around 3000 cm^{-1}, related to aromatic and aliphatic C–H stretching, were more intense in control PET-MPs.

Discussion

The present study was addressed to study two opposite but linked aspects of the MP issue in marine ecosystems: 1) the potential adverse effects of realistic PET-MPs, in terms of exposure concentrations, shape and size, on a benthic grazer macroinvertebrate; and 2) the potential biological weathering of PET-MPs induced by ingestion and transit in the digestive tract of the same animal. A growing number of studies on marine organisms exposed to different MP polymers demonstrated that the ingestion of MPs can result in detrimental sub-lethal effects, at both histological and biochemical level, due to chemical or physical damage

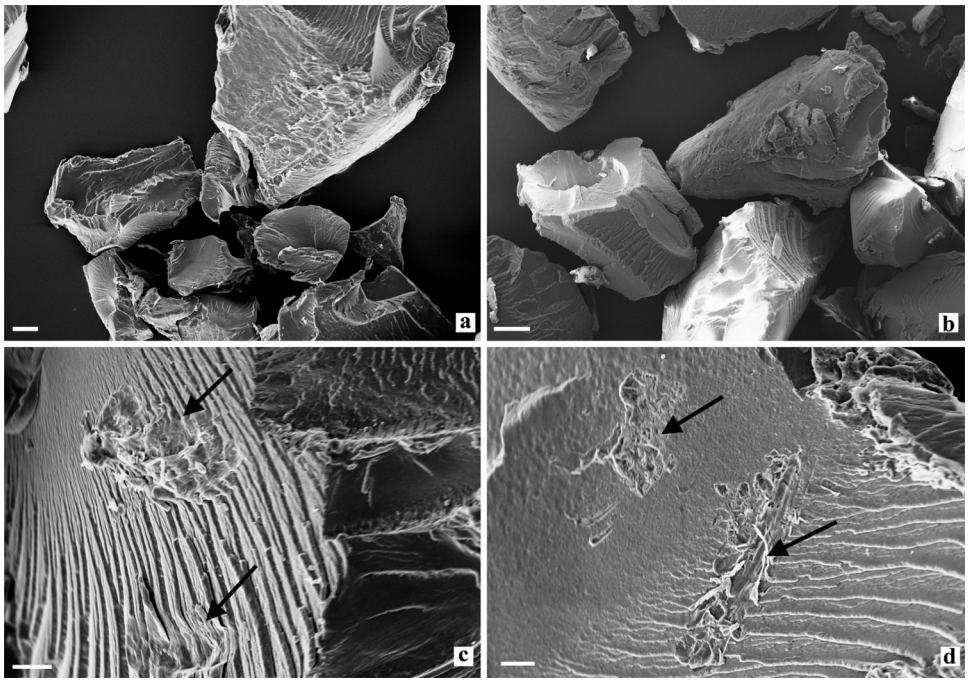

Fig. 5. SEM micrographs of control (a) and ingested/egested PET-MPs (b, c, d). No differences were visible in the overall superficial structure between control and ingested/egested particles. c, d) detail of ingested/egested PET-MPs with putative teeth marks (black arrows). As expected from the structural organization of the sea urchin masticatory apparatus (penta-radial arrangement of the teeth) a major and a minor dental marks are visible. Scale bar = 100 μm (a,b); 40 μm (c,d).

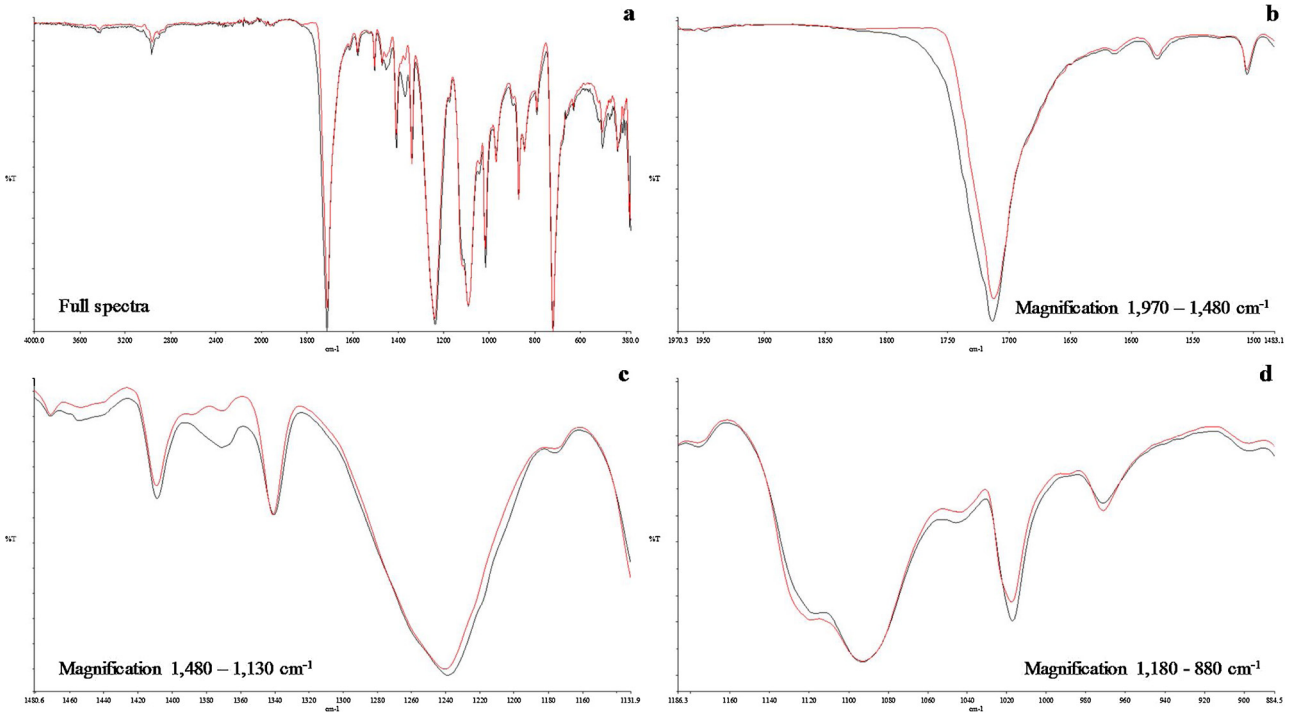

Fig. 6. FT-IR analysis of control PET-MPs (black line) and PET-MPs isolated from fecal pellets (red line). Full spectra (panel a), magnification 1970 – 1480 cm^{-1} (b), magnification 1480 – 1130 cm^{-1} (c) and magnification 1180 – 880 m^{-1} (d) are reported.

(e.g., von Moos et al., 2012; Avio et al., 2015; Nobre et al., 2015). However, these effects were caused by the use of environmentally unrealistic concentrations (de Sà et al., 2018) or exposure to artificially regular (in shape and size) MPs (e.g., commercially produced microbeads), which are in contrast to natural conditions of marine environment where MPs can be found in a wide range of size and shapes, ranging from fibres to irregular fragments, spheres and rods. Therefore, in order to mimic actual environmental situations, in the present study, environmentally relevant concentrations were used as well as more realistic MPs types. Indeed we produced and tested highly irregular MPs obtained from mechanical grinding of a very common plastic waste found in marine ecosystems, such as PET water bottle (e.g., Ioakeimidis et al., 2016). The exposure to irregular MPs might exacerbate the adverse effects induced by regular items because of different interactions within the organism. The administration of irregular PET-MPs, with serrated or sharp extremities, might result in physical damage/alteration of sea urchin tissues and, simultaneously, in the onset of inflammatory responses and oxidative stress conditions. Nevertheless, our histological analyses showed that the ingestion of irregular PET-MPs did not cause any significant tissue alteration and histologically visible

inflammation processes at the level of sea urchin digestive tract (Fig. 1). This might be related to both a real ineffectiveness of such MPs, in terms of shape, size and tested concentrations, or to the limited exposure period (seven days), which did not allow the onset of damage at tissue level. Alternatively a possible explanation for the lack of effects can be related to the peculiar mechanism of food ingestion by sea urchins. Indeed, *in vivo* observations suggest that sea urchins use their teeth for scraping, grasping and tearing the food from the substratum. Then the animals form boluses thanks to the action of five fleshy structures, the so-called paradental tongues, which are covered by a soft mucosa (Bonasoro and Candia Carnevali, 1994). In practice sea urchin processes the fragmented and irregular material, plastic debris included, as if it was kneading soft meatballs, thus possibly mitigating the mechanical effect on the tissues of the digestive tract.

In contrast, PET-MPs administration induced on the sea urchin a slight modulation of the oxidative status of the oesophageal proximal part, as revealed by the increase of the amount of pro-oxidant molecules at the end of the exposure to the highest tested PET-MPs dose (800 particles/g of food). The observed overproduction of reactive oxygen species might suggest a modulation of the antioxidant enzymatic shield led by SOD, CAT and GPx, which act according to a cascade mechanism to prevent the onset of an oxidative stress situation. Although no modulation in SOD and CAT activity was noted at the end of the exposure to all the tested doses, a significant increase in GPx activity was noted as a consequence of the exposure to 8 and 80 PET-MP particles/ g food (Fig. 2). Since GPx acts for eliminating the H_2O_2 produced by metabolic processes and/or at low substrate levels (Pereira et al., 2013), we might suppose that low doses of PET-MPs induced a slight production of pro-oxidant molecules that this enzyme can efficiently counteract. Our findings agree with other studies showing that the activity of antioxidant enzymes increases when the animal is exposed to low xenobiotic doses, whereas may decrease, or even be inhibited, at higher dosages (Valavanidis et al., 2006; Chen et al., 2011), when the overproduction of pro-oxidant molecules exceeds the capability of the organism to synthetize antioxidant enzymes. Thus, the lack of activation of all the antioxidant enzymes observed at the end of the exposure to the highest dose suggests a disequilibrium in the sea urchin oxidative status, which could, in the long term, cause oxidative damage to cellular macromolecules. However, no significant increase of lipid peroxidation was induced by PET-MPs dietary exposure. We might speculate that non-enzymatic antioxidants were able to efficiently protect macromolecule from the attack of the excess of pro-oxidant molecules, preventing the onset of oxidative damage. As an alternative hypothesis, the levels of pro-oxidant molecules generated by the exposure to the highest tested PET-MPs could be not high enough to cause oxidative damage in a short-term exposure (7-days), a possible damage induced by prolonged exposures being not excluded. Overall, our results are in accordance with a previous study of the sea urchin (*Arbacia punctulata*) showing that both short-term exposure to polystyrene (PS) spheres of 9 μm in diameter (25,000 spheres/L) did not affect physiology of adult specimens in terms of righting time and oxygen consumption rate (Suckling and Richard, 2020). In addition, PS-MPs did not block the madreporite pores of sea urchins, indicating the active removal of particles by cilia and pedicellariae (Suckling and Richard, 2020).

The most original finding of our research concerns the effects induced by the ingestion of PET-MPs by sea urchins on their structure and macromolecular chain. The grazing and tearing activity, as well as the transit within the digestive tract could in fact alter the structure or chemical composition of MPs, contributing to their weathering in the marine ecosystem and environmental degradation. A recent experiment demonstrated that the sea urchin *Paracentrotus lividus* can act as a bioeroder of macroplastics; sea urchins readily grazed on a polyethylene (PE) plastic trays and their grazing activity generated a great amount of PE-MPs (Porter et al., 2019).

Signs of potential teeth marks were observed on the surface of some PET-MPs (Fig. 5), suggesting that the long-term grazing activity of the sea urchin might contribute to the breakdown of particles through mechanical fragmentation and wear, to produce smaller particles, including nanoplastics. Moreover, slight, but statistically significant, modification in circularity and roundness of egested PET-MPs compared to control ones (Table 1) showed that the grazing activity of the sea urchin might alter the shape of ingested MPs. Indeed, the sea urchin masticatory apparatus (the so-called Aristotele's lantern) is an extremely potent device, designed to crumble hard materials (e.g., encrusting algae), including rocks (Candia Carnevali et al., 1991). On the other hand, sea urchin feeding activity apparently induced chemical modifications to the macromolecular chain of PET-MPs. We might speculate that the transit through the alkaline microenvironment likely present in sea urchin stomach (Stumpp et al., 2013, 2015), coupled with digestive enzymes and/or the microbiome activity (Danso et al., 2019) favoured the breakage of some ester bonds, that are prone to undergo hydrolysis under basic conditions (Kamei et al., 1992) returning some slight differences in FT-IR spectrum of 'digested' PET compared to the control polymer.Signals related to ester bonds in PET isolated from fecal pellets, namely the ones at about 1020 cm^{-1}, associated to vibration ester bonds, and at about 1240 cm^{-1}, associated to terephthalate bonds (dos Santos Pereira et al., 2017; Edge et al., 1996; Silverstein and Webster, 1998), were slightly less intense than in control PET, as well as the intense stretching of the ester carbonyl at about 1730 cm^{-1}. Our results were in agreement with pprevious literature showing that progressive degradation of PET was predicted by changes in FT-IR peaks intensity over years of exposure to marine environment (Ioakeimidis et al., 2016). The slight PET degradation can be expected considering that the time of permanence of PET-MPs within the *P. lividus* digestive system was rather limited (< 24 h), although in accordance with the digestion time reported in the literature for sea urchins (8–50 hrs; Lawrence, 1975). In fact, PET-MPs were not found in the first part of the digestive tube, suggesting a quick transfer to the distal (to the mouth) part of the digestive system. Thus, this short permanence might explain the lack of marked structural alterations of PET but could be sufficient to initiate chemical modification of the polymer. Lastly, it is noteworthy that PET chemical modifications occurred after only a single transit in sea urchin's digestive system, whereas in marine ecosystems PET-MPs can be ingested and egested many times by sea urchins, as well as by other benthic animals, leading to a potentially higher and diversified degradation of PET polymer. These hypotheses need to be verified by further studies investigating the conditions experienced by PET-MPs within the digestive system of sea urchins (and other animals) and enhancing the number of ingestion/egestion processes of the same particles in order to mimic a more realistic situation.

Overall, our results suggest that short-term exposure to low concentrations of irregular shaped PET-MPs might represent a threat for marine benthic grazer animals. In fact, although MPs ingestion was able to modulate the sea urchin oxidative status, no oxidative damage and histological alterations were observed. However, considering that organisms are exposed to MPs for their whole lifespan, we cannot exclude that long-term exposure to PET-MPs enhance the risk for sea urchins and other benthic organisms, resulting in the onset of adverse consequences. Despite the limited adverse effects induced by PET-MPs on sea urchins, a structural and chemical alteration of PET was caused by sea urchins, indicating that biological weathering of benthic grazers might contribute to the degradation of MPs that sink and accumulate at bottom sediments. This underlines the importance of animals in influencing plastic and MPs environmental fate. Furthermore, degradation of MPs due to the biological activity might represent an additional risk for marine organisms because of the formation of smaller and more bioavailable particles (including nanoplastics); the latter could easily pass through biological epithelia and induce even more hazardous adverse effects not only on benthic grazers but also on other relevant components of the marine ecosystem.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Marco Parolini: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing - original draft, Writing - review & editing, Resources. **Cinzia Ferrario:** Formal analysis, Investigation, Visualization, Resources. **Beatrice De Felice:** Data curation, Investigation, Visualization, Resources. **Stefano Gazzotti:** Investigation, Writing - review & editing, Resources. **Francesco Bonasoro:** Investigation, Software, Resources. **Maria Daniela Candia Carnevali:** Funding acquisition, Resources. **Marco Aldo Ortenzi:** Conceptualization, Funding acquisition, Project administration, Validation, Writing - original draft, Writing - review & editing, Resources. **Michela Sugni:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing - original draft, Writing - review & editing, Resources.

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PAPER VIII

De Felice B., Ambrosini R., Bacchetta R., Ortenzi M.A., Parolini M.

Dietary exposure to polyethylene terephthalate microplastics (PET-MPs) induces faster growth but not oxidative stress in the giant snail *Achatina reticulata*.

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Dietary exposure to polyethylene terephthalate microplastics (PET-MPs) induces faster growth but not oxidative stress in the giant snail *Achatina reticulata*

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HIGHLIGHTS

- PET_MPs ingestion and toxicity were investigated in *Achatina reticulata.*
- MPs were observed in the faeces and in the gastrointestinal tract of snails.
- Microplastics did not induce mortality or an oxidative stress situation.
- Unexpected increase in the growth of treated organisms.

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ABSTRACT

Polyethylene terephthalate (PET) is one of the main plastic polymers contaminating natural ecosystems. Although PET microplastics (PET-MPs) have been found in both aquatic and terrestrial ecosystems, the information concerning their potential toxicity towards terrestrial organisms is limited. The present study aimed at investigating the ingestion and the possible adverse effects induced by a 40-days exposure to irregular shaped PET-MPs toward the giant snail *Achatina reticulata*. Giant snails were exposed via the diet to two concentrations (1% and 10% w/w; i.e., g of PET-MPs/g of the administered food) of PET-MPs and their capability to ingest and egest PET-MPs was assessed together with an evaluation of their potential effects at biochemical and individual levels. Oxidative stress-related biomarkers (i.e., the amount of reactive oxygen species, the activity of antioxidant enzymes and lipid peroxidation) and DNA fragmentation were measured in the digestive gland isolated from snails as biochemical endpoints. Changes in growth trajectories, in terms of body weight and shell size, were considered as morphometric endpoints. Our results demonstrated that *A. reticulata* can efficiently ingest and egest PET-MPs. Whilst giant snails did not experience an oxidative stress condition, significant changes in their growth trajectories were observed, with PET-MPs-treated snails grew more and more quickly than the control group. Our results suggest that PET-MPs might represent a risk during early-life stages for terrestrial organisms.

1. Introduction

A blooming number of studies has documented the presence of microplastics (MPs) in atmosphere, water, soil and biota, confirming that these contaminants are of serious concern for natural ecosystems (He et al., 2018; Panebianco et al., 2019; Lindeque et al., 2020; Li et al., 2020). To date, the vast majority of the studies investigated the MPs contamination in marine and freshwater ecosystems (Lindeque et al., 2020; Li et al., 2020), while only the 5% of them has focused on terrestrial ecosystems (Li et al., 2020). However, soils are considered as the main sink of MPs and the total amount of MPs in terrestrial ecosystems could be more than 20-fold larger than in marine ecosystems (Zhang and Liu, 2018). Microplastics enter terrestrial ecosystems mainly by agricultural

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practices (e.g., water pipes, plastic mulching and greenhouse covers), as well as from water-runoff from urban areas, from illegal dumping sites, or break-down of larger plastic items (Hurley and Nizzetto 2018; Corradini et al., 2019). In addition, recent studies showed that sewage sludge retains the 95% of the MPs entering wastewater treatment plants (Carr et al., 2016; Mintenig et al., 2017). Therefore, the use of sewage sludge and wastewater-irrigation could be considered as the major sources of MPs input in agricultural soils (Li et al., 2020). Microplastics that have been found in agricultural and natural soils were mostly made of polyethylene (PE), polystyrene (PS), polypropylene (PP), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) (Rillig, 2012; Duis and Coors, 2016; Ng et al., 2018; Li et al., 2020). Despite these findings, the information on MPs abundance in soils is still limited. Nizzetto and co-authors have estimated that up to 430,000 and 300,000 tons of MPs enter annually agricultural soils in Europe and North America, respectively (Nizzetto et al., 2016). *In-situ* studies have found that MPs concentration in agricultural soils from Switzerland and Chile were up to 55.5 mg/kg and 12.9 mg/kg, respectively (Scheurer and Bigalke 2018; Corradini et al., 2019). Other studies have shown that the concentration in industrial soils from Australia were up to 67,500 mg/kg (Fuller and Guatam, 2016), while a negligible concentration have been measured in forests close to agricultural soils in China (Zhang and Liu, 2018).

The presence of MPs in soils could cause adverse effects to terrestrial organisms. Different soil organisms, such as earthworms (Huerta Lwanga et al., 2016; Rodriguez-Seijo et al., 2019; Wang et al., 2019), mites (Zhu et al., 2018), collembola (Maaβ et al., 2017) and snails (Panebianco et al., 2019; Song et al., 2019) can ingest MPs, which can exert diverse adverse effects, from imbalance of the oxidative status, tissue damage and changes in the growth rate, up to the death (Li et al., 2020). For instance, the exposure to PET-fibers (0.71 g/kg of soil w/w) imbalanced the oxidative status, altered the immune system and damaged the gastroenteric tract of giant snail *Achatina fulica* specimens (Song et al., 2019), while the exposure to 20% (w/w of soil) of PE- and PS-MP induced similar effects in the earthworm *Eisenia fetida* (Wang et al., 2019). In addition, a reduction of growth rate and an increase in mortality was observed in earthworm *Lumbricus terrestris* exposed to PE-MPs (up to 28% μPs w/w dry soil; Huerta-Lwanga et al., 2016) and in springtail *Folsomia candida* exposed to PVC-MPs (1 g/kg w/w dry soil; Zhu et al., 2018). Despite these findings, the information on the toxicity of MPs towards soil organisms is still scant.

The aim of the present study was to assess the ingestion/egestion and potential toxicity of irregularly shaped PET-MPs towards the giant snail *Achatina reticulata*. Among terrestrial organisms, land snails recently gained attention as suitable model organisms for laboratory studies (De Vaufleury et al., 2006; Gattoni et al., 2018; Song et al., 2019). In particular, we relied on snails belonging to the genus *Achatina*, which are endemic of Africa (Wang et al., 2014; Li et al., 2015) but became highly invasive in other continents (Odaibo and Olayinka 2019), where they can be commonly found in gardens, woodlands, agricultural areas and fields (Song et al., 2019). Thus, *A. reticulata* snails can be exposed to the MPs contaminating different terrestrial ecosystems. Moreover, this species can be considered as a good model organism in ecotoxicology because it is easy to be maintained and reared under controlled laboratory conditions, has an adequate size that allows the isolation of tissues and organs and follows a rapid growth, mainly in early-life stages, allowing to investigate the effect on growth rate in an acceptable time period.

Giant snails were dietary exposed for 40 days to two doses (1% and 10% w/w of the administered food) of PET-MPs. The ingestion of PET-MPs was checked by visual inspection of the faeces, while their toxicity was investigated at biochemical and individual level. The amount of reactive oxygen species (ROS), the activity of antioxidant (superoxide dismutase - SOD and catalase - CAT) and detoxifying (glutathione S-transferase - GST) enzymes, as well as the lipid peroxidation and DNA fragmentation were investigated in the digestive gland isolated from each giant snails as biochemical endpoints, while changes in growth trajectories in terms of body weight and shell size were used as individual endpoints. Oxidative stress-related biomarkers were chosen because previous studies have demonstrated that the exposure to different MPs can induce the onset of oxidative stress in diverse aquatic and terrestrial organisms (e.g., Wang et al., 2019; Song et al., 2019; Jiang et al., 2020; Parolini et al., 2020a,b). Focusing on PET microplastics, previous studies have demonstrated that the exposure to irregular shaped PET-MPs similar to those used in the present experiment caused the imbalance of the oxidative status in the Manila clam *Ruditapes philippinarum* (Parolini et al., 2020a) and in the sea urchin *Paracentrotus lividus* (Parolini et al., 2020b). Moreover, an oxidative status modulation, in terms of decrease in GPx activity and total antioxidant capacity, coupled with an increase in lipid peroxidation, occurred in the digestive gland isolated from giant snail *Achatina fulica* specimens exposed to PET-fibers (Song et al., 2019). Thus, considering these findings we expect that *A. reticulata* specimens suffer an oxidative stress condition due to PET-MPs dietary exposure. In contrast, we have no *a priori* expectation concerning the potential effects on growth trajectories.

2. Materials and methods

The PET-MPs used in the present work were obtained by mechanically grinding commercial bottle-grade PET chips (Invista 1101 PET) with a blade grinder, as previously described by Parolini and co-authors (2020a,b). PET-MPs were selected because PET is one of the most widely used plastic type worldwide, mostly for food and beverage packaging, and accounts for up to 7.1% of total European plastic consumption (Plastics Europe, 2014). In addition, several studies highlighted that PET is one of the most widespread plastic polymers found in terrestrial ecosystems (Rillig, 2012; Duis and Coors, 2016; Ng et al., 2018; Li et al., 2020). Indeed, considering that in a terrestrial ecosystem the majority of the microplastics are secondary microplastics, with different shapes and size (Paul-Pont et al., 2018), deriving from breakage and erosion of plastic items used in agriculture or contained in sewage sludge (Zubris and Richards 2005; Rillig 2012) our irregularly shaped PET-MPs can be representative of a real environmental situation. We performed the experiments using the same PET-MPs standard obtained and characterized in terms of shape, size and chemical composition by Parolini et al. (2020b). Overall, the PET-MPs standard included particles with heterogeneous shape and size, whose diameter ranged between 12.6 and 1065 μm (mean diameter 316 ± 12 μm). The size of PET-MPs was calculated as the diameter of a spherical particle of the same area as the detected irregular particle, as described by Parolini et al. (2020b). PET-MPs were attributable to four different size classes, with different (%) frequency within the standard used in the experiments: <50 μm (6.4%); 50–100 μm (8.1%); 100–500 μm (68.5%); 500–1000 μm (17.1%). The composition of PET-MPs was the same for both the experimental treatments.

2.1. Experimental design

A. reticulata snails were obtain from a private farmer, all the organisms were coeval snails from the same parents. At the beginning of the experiments the mean (±standard deviation) weight of snails was 1.48 ± 0.24 g, while the mean (±standard deviation) shell length and shell width were 14.71 ± 1.25 mm and

21.55 ± 1.58 mm, respectively. Snails were kept in 5.7 L plastic terraria (355 × 125 × 215 mm) with the bottom covered with 3 cm of coconut fiber compost. In detail, the temperature was settled at 24 ± 1 °C with a photoperiod of 16 h light and 8 h dark, while water was nebulized every second day to maintain the coconut fiber wet (Song et al., 2019). The snails were left to acclimatize for two weeks to laboratory conditions before starting the exposure; during this time period, snails were fed with shredded cucumbers and displayed good health status so that no mortality occurred. The exposure was performed under semi-static conditions. Fifteen coeval snails (1-month old), experimental replicates, were seeded in terraria and dietary exposed, every second day, for 40 days to two doses of irregularly shaped PET-MPs, consisting of 1% and 10% of PET-MPs w/w of the administered food, respectively. The duration of the experiment was arbitrarily set to 40 days in order to allow the snails to grow sufficiently to accurately estimate their growth rate. Considering the inconsistency of data regarding the concentrations of MPs in soils, the amount of PET-MPs administered in the present study was decided arbitrarily, but was in the same range of those used in a previous study with *Achatina fulica* snails that were fed on PET-fibers mixed in fodder/lettuce (6.4% of mass rate; Song et al., 2019). Food administered to snails was prepared as follows: commercial agar powder (0.33 g) was added to cold water, boiled for 1 min and then mixed with shredded cucumbers and micronized PET-MPs 1% or 10% of PET-MPs w/w; i.e., g of PET-MPs/g of the administered food of the food, respectively) to obtain 7.5 g of food. In detail, contaminated food (7.5 g) included 0.075 g or 0.75 g of PET-MPs depending on the treatment (1% or 10% of PET-MPs w/w of administered food, respectively). Control group was fed with the same amount of food without the addition of PET-MPs. In order to take into account the growth of snails and the consequential increase in food request, every second day up to the end of the exposure we administered an amount of food that was double than the mean weight of snails in each terrarium. Before the administration of food, all the snails were weighed, and the amount of food was calculated accordingly. Food was seeded into petri dishes (⌀ = 60 mm) and left in the fridge for about 3 h for solidification. Then, it was placed in the middle of the terraria to allow the snails to feed on it. Every day, the Petri dish with uneaten food, if present, was removed and renewed with fresh food. Moreover, three times a week we integrated the diet of snails with calcium carbonate ($CaCO_3$) obtained by the shredding of hen eggshell, to allow the growth of the shell.

During the 40-days exposure, snails were checked every other day to monitor the ingestion/egestion of PET-MPs, as well as their growth or mortality. The ingestion of PET-MPs was visually checked by collecting the faeces of the snails that were removed every day using metal tweezers. In order to analyze the changes in the growth trajectories of treated snails compared to controls, every other day the weight (g) of each individual was measured with a standard laboratory scale (±0.01 g), while the shell size (i.e., shell length and width) was measured with a caliper (±0.01 mm). To identify each individual within the terraria, the snails were marked on the shell with non-toxic nail polish. At the end of the exposure, ten snails per treatment were sedated with diethyl ether in a desiccator and their digestive gland was isolated, frozen in liquid nitrogen and stored at −80 °C until biochemical analyses.

2.2. Biochemical analyses

A battery of oxidative stress and genetic biomarkers was measured on the cytosolic fractions of the digestive gland isolated from ten *Achatina reticulata* specimens according to methods previously used for mollusks and described in detail elsewhere (Parolini et al., 2020a). Briefly, the digestive gland from each single snail was homogenized by an automatic homogenizer in 100 mM phosphate buffer (pH 7.4), added with 100 mM KCl, 1 mM EDTA, 100 mM dithiothreitol and protease inhibitors (1:10 v/v). The homogenates were centrifuged at 15,000×*g* for 20 min at 4 °C and the supernatant was immediately collected and processed to assess protein content and oxidative stress biomarkers. All biomarkers were measured in duplicate for each specimen. The amount of ROS was assessed adapting the fluorimetric method by Deng et al. (2009) to snail homogenates, using the dichlorofluorescein-diacetate (DCFH-DA) method. The change in fluorescence was recorded by an EnSight multimode plate reader (PerkinElmer) with λ = 485 nm as excitation and λ = 536 nm as emission wavelength, respectively. The ROS amount was expressed in arbitrary units as AU DCF/mg proteins. Protein content, enzyme activity and lipid peroxidation were assessed through spectrophotometric method. Briefly, SOD activity was assessed by measuring for 1 min at λ = 550 nm the inhibition of cytochrome *c* (10 μM) reduction operated by the superoxide anion that was generated by the xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 mM) reaction. SOD activity was expresses as SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction) as SOD U/mg proteins. The CAT activity was assessed by measuring for 1 min at λ = 240 nm the consumption of H_2O_2 (50 mM) in potassium phosphate buffer (100 mM; pH 7). The GST activity was assessed for 1 min at λ = 340 nm by adding reduced glutathione (1 mM) in phosphate buffer (80 mM; pH 7.4) and using 1-chloro-2,4 dinitrobenzene (CDNB; 1 mM) as substrate. Activity of CAT and GST was expressed as μM/min/mg proteins. Total protein content was determined according to the Bradford method (1976) using the bovine serum albumin as standard, and it was used to normalize the activity of the antioxidant and detoxifying enzymes. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979) and results were expresses as nmol TBARS/mg protein. All the spectrophotometric analyses were performed by means of a Genova Bio spectrophotometer (Jenway). DNA damage was assessed by the DNA precipitation assay (Olive, 1988). The change in fluorescence was recorded by an EnSight multimode plate reader (PerkinElmer) with λ = 360 nm as excitation and λ = 450 nm as emission wavelength and results were expressed as μg DNA fragmented/mg proteins.

2.3. Statistical analyses

The effects of PET-MPs on biomarkers of oxidative stress and genotoxicity were investigated by a one-way analysis of variance (ANOVA) using the treatment as predictor. When significant differences among treatments were found, Tukey post-hoc tests were applied. Significance was set at $P < 0.05$ (*) and $P < 0.01$ (**). The effects of PET-MPs on growth trajectories were investigated with non-linear mixed models (NLMM). Based on visual inspection of the data, we assumed growth trajectories to follow a four-parameter logistic curve. In detail, the equation used for the curve was:

$$y = A + \frac{B - A}{1 + e^{\frac{X_{mid} - t}{Scal}}}$$

whereby t indicated the time (to ease model fit, the first day of measurement was set as $t = 0$). Parameters A and B indicated the lower and the upper asymptote of the curve, respectively, while *Xmid* indicated the day of the inflection point of the curve that fitted our data. Moreover, the parameter *Scal* was inversely related to the growth rate of the curve. Temporal autocorrelation and non-homogeneity of the variances were included in the model, while for the fixed part of the model we considered that every single

parameter of the logistic curve could vary among experimental group, besides Parameter *A*. Statistical analyses were performed using the nlme package (Pinheiro et al., 2020) of R 3.6.2 software package (R Core Team 2019).

3. Results

A. reticulata specimens efficiently ingested PET-MPs as confirmed by the presence of several particles both in the faeces and in the gastrointestinal tract of snails after dissection (Fig. 1a and

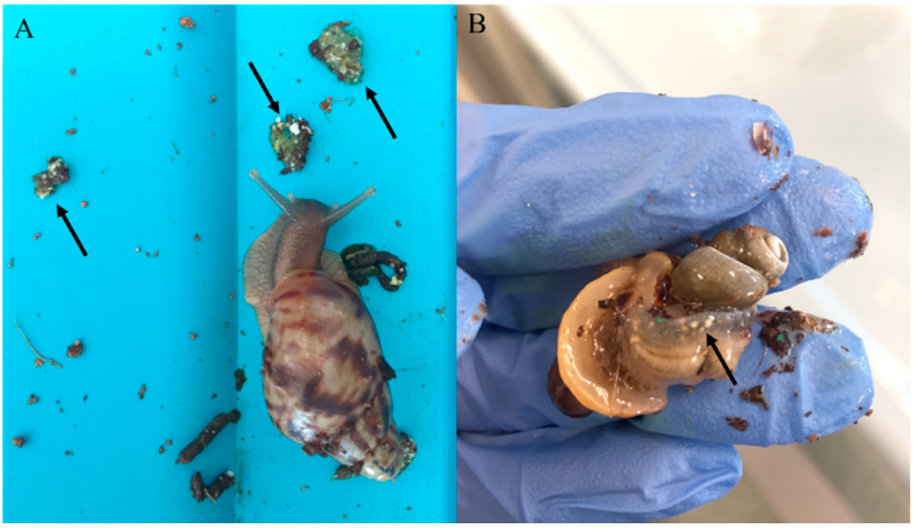

Fig. 1. Black arrow indicated the PET-MPs irregular fragments in faeces (A) and gastrointestinal tract (B) of *A. reticulata* snails.

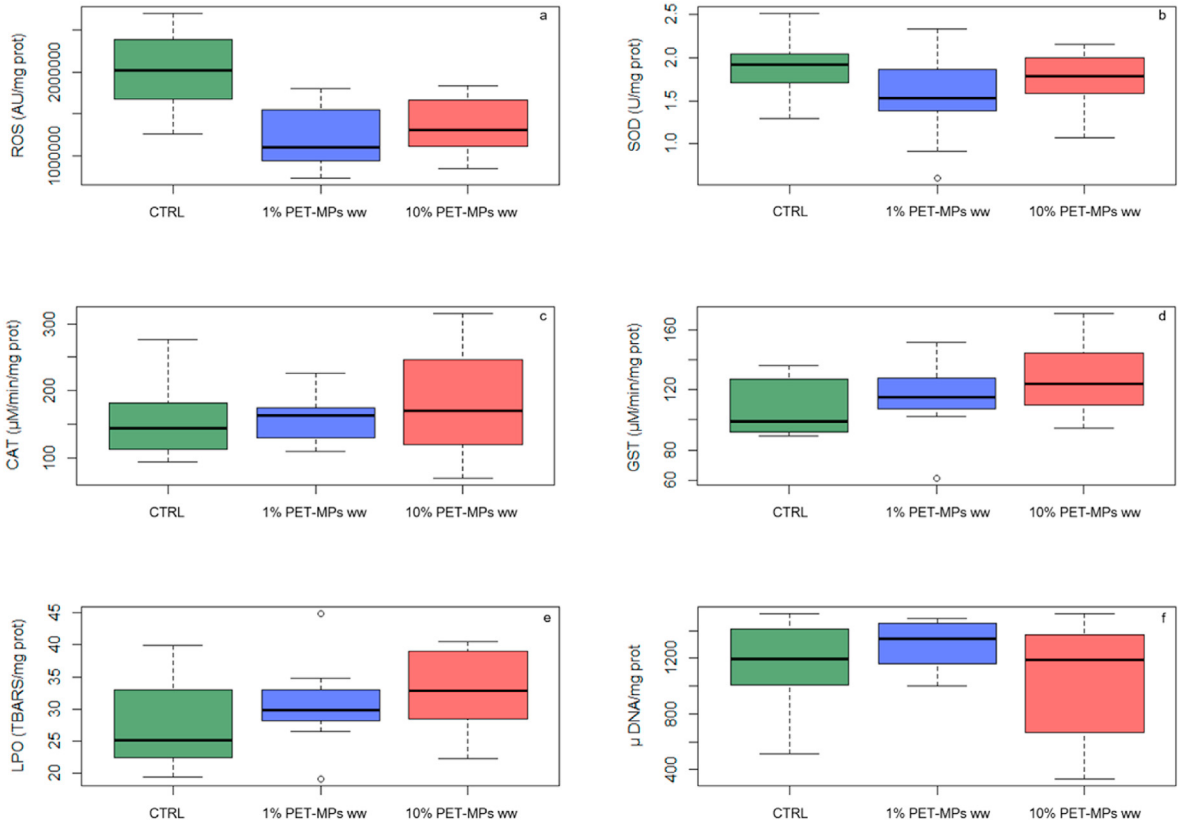

Fig. 2. Box and whiskers plot of the total amount of reactive oxygen species (a), activity of superoxide dismutase (b), catalase (c), glutathione S-transferase (d), lipid peroxidation levels (e) and DNA damage (f) measured in the cytosolic fraction of digestive glands from *A. reticulata* individuals (n = 10 per treatment) exposed to two doses of micronized PET-MPs. Green = control group, Blue = 1% PET-MPs and Red = 10% PET-MPs. The bold line in the box indicate the median value while the lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper and the lower whiskers extend from the hinge to the largest/smallest values and the white dots indicate an extreme value but not a significant outlier. No significant effect of the treatment was found ($P > 0.05$) for all the biomarkers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

b). Negligible mortality was observed in all the experimental groups during the 40 days of the experiment; only 1 snail died in control and 10% PET-MPs groups, while none in the 1% PET-MPs group.

The 40-days dietary exposure to PET-MPs did not induce the onset of oxidative stress in the digestive gland of the snails. PET-MPs treatment did not significantly affect ROS levels ($F_{1,25} = 2.050$, $P = 0.165$, Fig. 2a), or the activities of SOD ($F_{1,24} = 0.012$, $P = 0.914$; Fig. 2b), CAT ($F_{1,24} = 1.156$, $P = 0.293$; Fig. 2c), GST ($F_{1,25} = 3.063$; $P = 0.092$; Fig. 2d) and the levels of LPO ($F_{1,25} = 2.034$, $P = 0.166$; Fig. 2e). Similarly, no significant effect on DNA damage ($F_{1,25} = 1.423$, $P = 0.244$; Fig. 2f) was observed at the end of the PET-MPs exposure.

Significant differences in growth trajectories for body weight and shell length (Table 1; Fig. 3a and b, respectively), but not in shell width (Fig. 3c) occurred between untreated and treated snails. Specifically, a significant effect ($F_{2,663} = 3.921$, $P = 0.020$) of the treatment was found in the model concerning the body weight for the upper asymptote of the growth curve (parameter *B* of the formula, see *2.6 Statistical analyses* section), whereby individuals treated with 10% PET-MPs tending to a higher final weight than those of the control group (Fig. 3a). In addition, a significant effect on shell length was found for the X_{mid} parameter describing the day when the maximum growth rate was attained. In detail, this variation was observed between the curves of both treat groups and the control group curves, with individuals from the control group that showed a delay in shell length growth ($F_{2,645} \geq 5.406$, $P < 0.01$) compared to both the treated groups (Fig. 3b).

4. Discussion

The present study demonstrated that *A. reticulata* was able to efficiently ingest and egest irregular shaped and sized PET-MPs and that these, did not induce mortality or an oxidative stress condition in treated organisms. However, PET-MPs ingestion caused significant variation in growth trajectories of snails, whereby treated specimens grew more than control ones.

The presence of several PET-MPs observed in snail faeces and in their gastrointestinal tract provided direct evidence of the ingestion, permanence and egestion of these particles by the snails. These results supported those obtained in previous studies showing that diverse soil invertebrates are able to ingest MPs

Table 1
Results of the statistical analyses reporting the effects due to PET-MPs treatment on growth trajectories in *A. reticulata*. Significant effects are reported in bold. Right show the values of the parameters estimated by the model for each group and the relative standard errors. F = Fisher-Snedecor F; df = degrees of freedom; P = P-value associated to F-value; Treatment = CTRL = control, 1% = 1% PET-MPs and 10% = 10% PET-MPS; SE = Standard Error. Different letters indicated significant differences among groups. φ is the temporal autocorrelation coefficient.

Snail weight							
Parameter	F	df	P	Treatment	Coef	SE	
A					1.469	0.104	
B	**3.921**	**2**	**0.020**	**CTRL**	**3.256**	**0.324**	**a**
				1%	**4.182**	**0.398**	**ab**
				10%	**4.646**	**0.475**	**b**
X_{mid}	0.074	2	0.928	CTRL	27.546	3.620	
				1%	27.114	3.540	
				10%	25.544	4.019	
Scal	0.535	2	0.586	CTRL	8.986	2.609	
				1%	11.025	2.311	
				10%	11.937	2.352	
residual df = 663	φ = 0.951						

Shell length							
Parameter	F	df	P	Treatment	Coef	SE	
A					21.289	0.276	
B	1.094	2	0.336	CTRL	28.056	0.748	
				1%	28.486	0.498	
				10%	29.248	0.474	
X_{mid}	**5.406**	**2**	**0.005**	**CTRL**	**22.261**	**2.009**	**c**
				1%	**15.149**	**1.055**	**d**
				10%	**15.438**	**0.711**	**d**
Scal	2.818	2	0.061	CTRL	9.346	1.629	
				1%	7.050	0.806	
				10%	5.747	0.526	
residual df = 645	$\varphi_1 = 0.612$	$\varphi_2 = 0.367$					

Shell width						
Parameter	F	df	P	Treatment	Coef	SE
A				CTRL	11.685	1.374
B	0.971	2	0.419	CTRL	22.369	1.519
				1%	22.281	0.636
				10%	23.375	0.790
Xmid	1.393	2	0.249	CTRL	18.619	4.289
				1%	10.926	3.332
				10%	10.735	3.408
Scal	0.345	2	0.708	CTRL	16.444	5.066
				1%	13.731	2.849
				10%	15.844	2.992
residual df = 676	φ = 0.956					

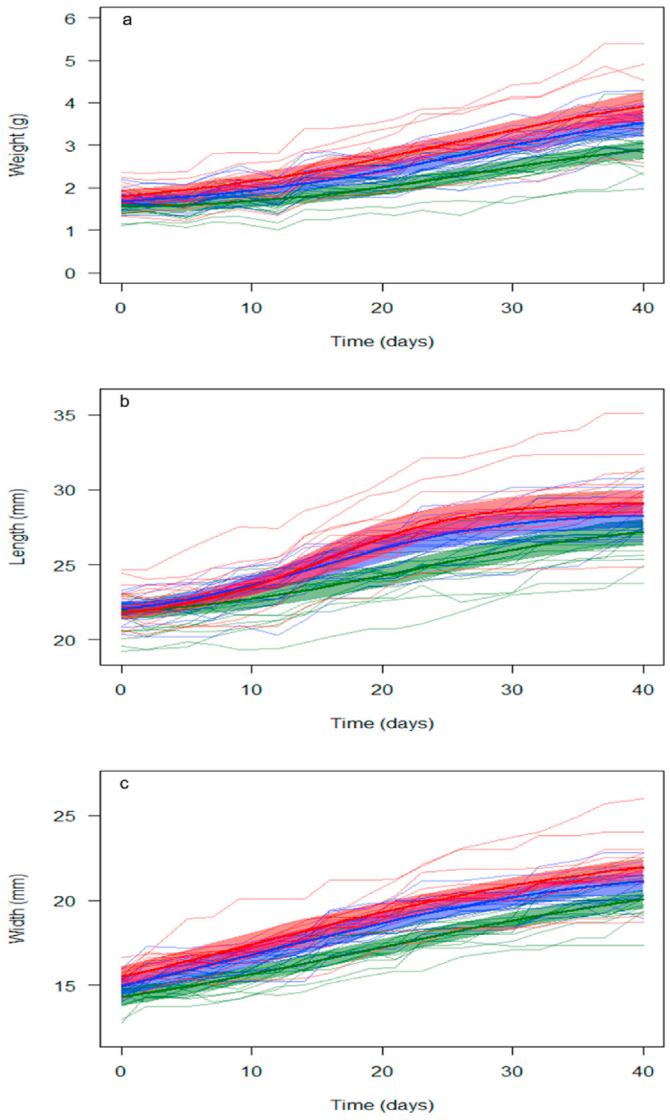

Fig. 3. Variation in growth trajectories of the curve describing weight (a), shell length (b) and shell width (c) of *A. reticulata* individuals (n = 15 per treatment) dietary exposed for 40 days to two doses of PET-MPs. Green curves = control group, Blue curves = 1% PET-MPs and Red curves = 10% PET-MPs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

having different shape, size and polymeric composition (Huerta Lwanga et al., 2017; Hurley and Nizzetto 2018; Rodriguez-Seijo et al., 2019; Song et al., 2019). The transit of PET-MPs in the digestive tract of snails could result in the onset of different sub-lethal effects, including the alteration of the oxidative status or the alteration of the immune systems, as previously demonstrated in other soil organisms (Hodson et al., 2017; Zhu et al., 2018; Wang et al., 2019; Song et al., 2019; Jiang et al., 2020). However, the 40-days dietary exposure to irregular shaped and sized PET-MPs did not induce an oxidative stress condition in the digestive gland of treated snails compared with those from the control group. Our results therefore contrast with a those of previous study by Wang and co-authors (2019) performed on the earthworm *Eisenia fetida*, whereby a 14-days exposure to microplastics made by PE and PS (1%; 5%; 10% and 20% MPs w/w in soil) lead to the modulation of antioxidant (SOD, CAT and GPx) and detoxifying (GST) enzymes, as well as to the increase of lipid peroxidation. Another study on the same earthworm species highlighted the alteration of SOD activity and glutathione level, as well as the onset of DNA damage after the exposure to 100–1000 μg/kg in soil of PS-MPs (Jiang et al., 2020). A modulation of the oxidative status coupled with the onset of oxidative damage was also observed in *A. fulica* snails exposed for 28 days to PET-fibers (0.71 g/kg dry soil weight; Song et al., 2019). In addition, our results differed from those obtained in two previous studies testing the toxicity of the same irregular shaped and sized PET-MPs towards marine invertebrates. In fact, a 7-days exposure to 12.5 μg/mL PET-MPs significantly altered the oxidative status of gills leading to an increase in lipid peroxidation in the clam *R. philippinarum* (Parolini et al., 2020a), while the exposure to 8, 80 and 800 particles/food caused a slight modulation of the oxidative status in the sea urchin *P. lividus* (Parolini et al., 2020b). These discrepancies of results could be due to differences in MPs concentrations, size, shape, polymer, duration and pathway of exposure, as well as to the sensitivity of different model organisms. Furthermore, the lack of biochemical alteration we observed could be related to the mucus produced by our model organism. Indeed, previous studies highlighted that, besides facilitating the locomotion and the defense against predator, mucus is used by snails also to prevent infections and to facilitate wound healing (Zhong et al., 2013; Pitt et al., 2015; Cilia and Fratini 2018). The mucus might thus act as a protector for the gastrointestinal tract bypassing the direct contact with PET-MPs and helping the healing if needed. According to this hypothesis, we may speculate that the mucus protected the snails form the contact or the rubbing operated by ingested irregular shaped PET-MPs, preventing the onset of inflammation and oxidative stress.

Despite no effects at biochemical level, the ingestion of PET-MPs by snails could cause other detrimental consequences due to chemical or physical damage, including the reduction of the available energy for the correct growing, as showed in other non-target soil organisms exposed to MPs made by different polymers (Hodson et al., 2017; Zhu et al., 2018; Wang et al., 2019; Song et al., 2019). The permanence of MPs in the gastrointestinal tract of snails could cause a sense of false fullness and, consequently, the reduction of food assimilation and growth. However, a previous study by Song and co-authors (2019) did not show any change in food intake and growth in *A. fulica* specimens exposed to PET fibers. Surprisingly, our results were in contrast with our expectations, as PET-MPs-treated snails grew more and more quickly than the conspecific from the control group. The exposure to 10% w/w PET-MPs boosted the weight gain, suggesting that treated snails tended to a higher final weight than the control. At the same time, both the administered doses induced a higher growth of the shell in length, suggesting that treated specimens tended to reach the maximum length quicker than conspecifics from the control group (Fig. 2). Our result differed from previous studies that showed a reduction in growth rate after the exposure to MPs in marine organisms (Lei et al., 2018; Lo and Chan 2018) and in the terrestrial collembolan *Folsomia candida* (Zhu et al., 2018). However, our results agree with a recent study of the earthworm *Eisenia fetida*, whose growth rate was promoted by the exposure to 100 and 1000 PS-MPs/kg soil (Jiang et al., 2020), as well as with findings obtained on *Daphnia magna* specimens exposed to 0.125–12.5 μg/mL of polystyrene MPs with 10 μm in diameter (De Felice et al., 2019). Treated snails could grow more than untreated ones because they were able to better assimilate the ingested food, as suggested by a previous study of Pacific oysters (*Crassostrea gigas*) exposed to yellow-green fluorescent PS beads (2 and 6 μm in diameter; Sussarellu et al., 2016). The improved assimilation of the food could be determined by the presence of PET-MPs in the gastrointestinal tract of treated snails that could have enhanced the shredding of food, promoting its efficient assimilation. Alternatively, as terrestrial organisms

increase the production of mucus after the ingestion of exogenous material (Huerta-Lwanga et al., 2016), the ingestion of PET-MPs could promote the production of mucus rich in enzymes and microorganisms that can both enhance the digestion and the assimilation of food (Cardoso et al., 2012; Pawar et al., 2015; Dar et al., 2015). In addition, a recent study by Song and co-authors (2020) demonstrated that the exposure to PS-MPs induced a shift in gut microbiota of *A. fulica* snails, which could contribute to improve digestion of food, as well as to the biodegradation of microplastics.

5. Conclusions

Our results showed that a 40-days dietary exposure to irregular shaped and sized PET-MPs were efficiently ingested and egested by the giant snail *Achatina reticulata,* but they did not induce biochemical changes in their digestive gland. Surprisingly, the administration of PET-MPs boosted the growth of treated organisms, suggesting that microplastics may act as a mechanical shredder or may promote physiological response that enhanced food assimilation. Both these hypotheses need to be verified by further studies. In addition, considering the effects on growth that was observed in the early life period of the snails, long-term exposures to PET-MPs should be crucial to explore long-lasting effects at both biochemical and individual levels, as well as to investigate potential fitness-related consequences. Lastly, considering the presence of microplastics made by different polymers in terrestrial ecosystems, further studies should be a priority to shed light on the toxicity of these contaminants towards soil organisms.

Credit author statement

Beatrice De Felice: designed the experiments, perform the experiment, data analysis, Writing – original draft; Roberto Ambrosini: data analysis, Writing – review & editing; Renato Bacchetta: Resources, Writing – review & editing. Marco Aldo Ortenzi: perform the experiment, Writing – review & editing. Marco Parolini: conceived the project, designed the experiments, data analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 4 – *Conclusions*

The results obtained by this project confirmed that the presence of illicit drugs and MPs in environment might represent a threat for non-target organisms.

In detail, the exposure to both cocaine and methamphetamine altered the oxidative status of the exposed organisms leading to differential molecule-depending effects, at the higher level of the biological hierarchy (i.e., swimming activity and reproduction). The effects observed for the higher levels of the biological hierarchy, such as swimming behavior and reproduction, are of a great concern, suggesting that the exposure to these molecules could result in negative consequences also for the entire ecosystem. Indeed, an effect at population level might affect the population dynamic, while an effect on swimming behavior, by causing a disadvantage in the escape from a predator, might result in an alteration of the prey-predation relationships, resulting in an alteration of the community and ecosystem. These hypotheses will be analyzed in further researches by performing experiments aimed at evaluating potential effects at community level. Moreover, the experiment with *M. galloprovincialis* showed that illicit drugs mixtures could be more toxic than the single molecules. This result is of great importance considering that in natural ecosystems organisms are simultaneously exposed to a wide range of different molecules. For this reason, investigating the effects of the exposure to a realistic environmental mixture of illicit drugs still remains one of the big challenges of ecotoxicology.

The experiments on MPs confirmed that aquatic and terrestrial organisms can ingest both regular and irregular MPs, but the effects induced by their ingestion are contrasting and strictly related to the polymer and the species used. In detail, exposure to PS-MPs lead to an unexpected increase in body size, swimming and phototactic activity, as well as in the reproductive effort, in *Daphnia magna* organisms, while no effects on body growth and swimming activity were showed on *X. laevis*. Moreover, the exposure to PET-MPs lead to the alteration of the oxidative status without histological damages in *R. philippinarum* and *P. lividus*. In contrast, no alteration of the oxidative status and oxidative damage but a, surprisingly, boost of the growth of treated organisms was found in *A. reticulata*.

The results obtained in these experiments showed that the negative effects are connected to the model organisms used, as well as to the endpoint analyzed and the MPs used. Indeed, the criticalities in the investigation of the environmental issues of microplastics are due to the heterogeneity of MPs, that complicate the attempt to obtain univocal information regarding their toxicity. Thus, as highlighted by our results, MPs different in polymer composition, shape and concentration could result in differential result. Therefore, considering the heterogeneity of the

MPs present in environment, made of different polymers and having different dimension and shape, investigating the effects of the exposure to microplastics remains one of the big challenges of ecotoxicologists.

Overall, the results obtained in these works are of great importance and evidence that, in order to deeply investigate this environmental issue, ecotoxicologists need to integrate information coming not only from different levels of the biological hierarchy, but also from the exposure of different non-target model organisms. Indeed, only by using an integrate approach, and mixing the information coming from more than one biological level, as well as, from more than one model organism, it would be possible to obtain information with a more ecological relevance in order to be able to better understand the consequences connected to the presence of these pollutants in the environment.

Lastly, our data pointed out the potential threat of few illicit drugs molecules (COC, METH and BE) and only on two plastic polymers (PS and PET), but in environment monitoring studies evidenced a wide range of different illicit drugs and plastic polymers often present in mixture. To date investigation of the possible effects induced by these molecules is still very challenging considering the wide range of molecules present in environment whose ecological fate could change depending not only from considered the environment but also from the co-presence of other contaminant as well as from the interaction with the biota. Moreover, according to the data regarding production, selling and use of these emerging contaminants the increase of their concentration in environments is expected, with the consequent enhancement of risk toward the biocenosis. At the same time also the continue formulation of new molecules and new plastic types, whose presence is expected in environment, exacerbate these issues. Thus, regarding illicit drugs, further studies should be aimed at the investigation of the possible effects induced by different illicit drug 'cocktails' made by environmentally relevant illicit drugs mixture, as well as to investigate their mechanism(s) of action in order to enlarge the knowledge on the risk of these contaminants towards non-target organism and ecosystems. At the same time, further studies aimed at investigating the presence and the toxicity of polymers having different physico-chemical characteristic (such as density, shape or color), including the so-called "bioplastics" (such as the polylactic acid or the polyhydroxyalkanoates) and the non-homogeneous fractions of mixed polymers, should be a priority.

Chapter 5 – *References*

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Appendix – *List of other publications*

PAPER IX - Ferrario C., Parolini M., **De Felice B.**, Villa S., Finizio A. 2018 Behavioural disturbances: linking sub- and supra-organismal biomarkers in *Daphnia magna* exposed to sublethal concentrations of chlorpyrifos. Environmental Pollution 235, 411-418.

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PAPER XII - Parolini M., Ghilardi A, **De Felice B.**, Parolini M., 2019. Environmental concentration of fluoxetine disturbs larvae behavior and increases the defense response at molecular level in zebrafish (*Danio rerio*). Environmental Science and Pollution Research 26, 34943–34952.

PAPER XIII - Parolini M., Panseri S., Håland Gaeta F., Ceriani F., **De Felice B.**, Nobile M., Rafoss T., Schnell J., Herrada I., Arioli F., Chiesa L.M., 2020. Incidence of persistent contaminants through blue mussels biomonitoring from Flekkefjord fjord and their relevance to food safety, Food Additives & Contaminants: Part A 37, 831-844.

PAPER XIV - Valsecchi S., Babut M., Mazzoni M., Pascariello S., Ferrario C., **De Felice B.**, Bettinetti R., Veyrand B., Marchand P., Polesello S., 2020. Perfluoroalkyl Substances (PFAS) in Fish from European Lakes: Current Contamination Status, Sources, and Perspectives for Monitoring. Environmental Toxicology and Chemistry 1-19.

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