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A realistic approach for the assessment of plastic contamination and its ecotoxicological consequences: A case study in the metropolitan city of Milan (N. Italy)

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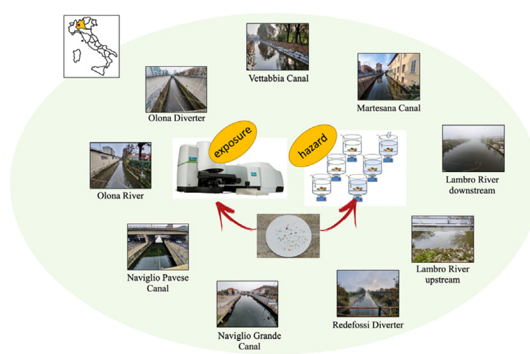
HIGHLIGHTS

- The plastic mixtures were characterized and ecotoxicologically evaluated.
- The plastics sampling was performed with a net with 100 μm mesh.
- The secondary origin of plastics was prevalent.
- Three watercourses showed higher plastics levels than the main European rivers.
- Ecotoxicological results showed a weak oxidative stress.

GRAPHICAL ABSTRACT



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ABSTRACT

The study of the contamination of plastic mixtures sampled in natural environments is currently focused on their qualitative and quantitative assessment, while the evaluation of their effects on organisms is normally performed by experiments carried out at exposure conditions (size, shape, polymers) often far from the environmental ones. To improve the ecological realism, the aim of this study was to collect different plastic mixtures in 9 sampling stations located in 7 watercourses within the metropolitan city of Milan, one of the most anthropized and industrialized European areas, to evaluate both their qualitative and quantitative characteristics and, at the same time, to assess their ecotoxicological effects by exposing for 7 days some specimens of the freshwater bivalve *Dreissena polymorpha* to the mixtures collected in the sampling sites. The plastic characterization was performed by a Fourier-Transform Infrared spectrometer coupled with an optical microscope (μFT-IR), after several stages aimed to sample cleaning, separation of plastics and visual sorting. The possible effects caused by the plastic mixtures were carried out by the measurements of a biomarker suite to evaluate many cellular and molecular endpoints in mussel tissues. The main results showed a widespread and heterogeneous contamination of plastics in the entire metropolitan area, with contamination peaks found above all in the only two rivers of natural origin (Olona River and Lambro River) where comparable or higher values were reached than plastic concentrations measured in several European rivers. Despite this worrying contamination, the ecotoxicological data obtained after the exposures to the plastic mixtures collected in the selected water bodies showed only a mild effect on oxidative stress and on the variation of some antioxidant enzymes.

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1. Introduction

Plastic pollution represents one of the major environmental problems of the 21st century due not only to the constant increase in its world production, but above all to the incorrect disposal of plastic products that end up in any aquatic and terrestrial ecosystems worldwide. The most recent data reports a global plastic production of about 390 million tonnes in 2019, with an increasing annual trend of 2.5% (PlasticsEurope, 2020). The positive news is due to the 7% decrease in annual European plastic production, after the further minor decline of 3% between 2018 and 2019 (PlasticsEurope, 2019) which brings the two-year decrease in plastics production to 10% in Europe. This could be due to bans on the use of microplastics in cosmetic products in many European countries and to recent restrictions on the single-use plastic products as stated by the EU Directive 2019/904.

Despite this encouraging decrease, the presence of plastics in natural ecosystems is now ubiquitous, as synthetic polymeric materials can be dispersed by wind and carried by rivers and ocean currents across the globe, even thousands of kilometers away from the place of release into the environment (Oliveira et al., 2019). This is clear by their presence also in some remote areas, such as Arctic Sea ice (Obbard et al., 2014), submarine canyons (Pham et al., 2014), deep sea sediments (Woodall et al., 2014) and coral reefs (Lamb et al., 2018). A further problem is related to the degradation of larger debris in the environment into the so-called microplastics (MPs) and nanoplastics (NPs) which have recently been reclassified as any synthetic polymeric particle whose largest size is between 1 μm and < 1 mm and between 1 nm and < 1 μm, respectively (Hartmann et al., 2019). These smaller debris can also be directly present in many personal care products, such as scrubs, toothpastes and cosmetics, increasing their presence especially in aquatic environments (Germanov et al., 2018).

While the negative effects of macroplastics (>10 mm; Hartmann et al., 2019), such as entanglement, suffocation, blockages and starvation, are now revealed in many organisms, such as marine mammals (Franco-Trecu et al., 2017; Reinert et al., 2017), turtles (Casini et al., 2018), fish (Alomar et al., 2017) and birds (Good et al., 2020), the ecotoxicological role of the smaller debris is still controversial. This is confirmed by the meta-analysis recently formulated by Bucci et al. (2020) which highlighted how the studies in which effects related to plastics were identified, 42% were due to macroplastics and 58% to MPs, but that the studies in which no effects were observed only 6% were referred to macroplastics, while as many as 94% were due to MPs. This is mainly due to the greater complexity in the study of the (eco)toxicological effects of these physical contaminants compared to chemical pollutants, whose intake is especially mediated by the concentration gradient, as well as by specific active transmembrane transports dependent on the steric hindrance of the chemical. On the contrary, other characteristics such as shape, size, density, crystallinity and even color can play a more important role than the simple concentration of plastics in relation to the capability of intake, accumulation and therefore toxicity (Sherer et al., 2018; Prata et al., 2019a; Muller, 2021). Furthermore, various environmental contaminants can be adsorbed on the surface of plastics, modulating their (eco)toxicological effects since many of these chemicals are listed as priority pollutants by the U.S. EPA. (2014).

A further problem related to the laboratory experiments is represented by the fact that the characteristics of the tested plastics are quite different with respect to those sampled in the environment. For instance, only 17% of the laboratory studies were conducted with environmental relevant concentrations of plastic debris (Bucci et al., 2020). Moreover, plastics with an average size of 0.02 μm are normally tested in laboratory experiments (Bhattacharya et al., 2010), while the smallest mesh size normally used for environmental sampling was 0.45 μm (Barrows et al., 2017). Lastly, although only the 9% of environmental studies were able to collect plastics with a size smaller than 10 μm, as many as 52% of laboratory studies use plastics in this size range (Bucci et al., 2020). This means that, although laboratory experiments are indispensable for evaluating the different conditions of uptake, infiltration and accumulation of plastics in organisms, as well as for studying their mechanisms of action, there is still no direct correlation between the results obtained under laboratory conditions and the effects of plastics in the environment.

In this context, the main goal of this study concerned to achieve the two objectives that an ecotoxicological research should aim also for plastics' contamination, namely the assessment of environmental exposure and the hazard evaluation, in an ecologically more relevant way than laboratory studies. In detail, we perform a qualitative and quantitative assessment of the plastics sampled in 9 different sampling stations of the main watercourses surrounding the metropolitan city of Milan (N. Italy) and at the same time to evaluate the ecotoxicological effects of the same mixtures of sampled plastics. This area extends for about 1575 km^2 and includes 133 different municipalities; with a population of about 3,250,000 inhabitants, it is the second most populous in Italy after the metropolitan city of Rome, constituting 32.6% of the entire regional population and 5.5% of the Italian one. In addition, 42.5% of Lombard companies and 6.6% of Italian ones are concentrated in it (www.cittametropolitana.mi.it). These few data alone can explain how urgent it is to evaluate for the first time the anthropic and industrial impact in the release of plastics in the water bodies of this area at high environmental risk.

The objective of this research was achieved through an experimental design that considered some evidence recently highlighted by other studies, such as the use of sampling nets with 100 μm mesh, as suggested by Covernton et al. (2019) who highlighted as the environmental samples collected with mesh smaller than 100 μm in size contain a quantity of MPs one to four orders of magnitude greater than that sampled with a standard 300-330 μm mesh. Furthermore, the polymeric identification was performed using a Fourier-Transform Infrared spectrometer coupled with an optical microscope (μFT-IR), able to characterize the smaller plastic debris up to 10-20 μm of size.

The second goal of the ecotoxicological approach was reached by *in vivo* laboratory experiments, exposing some specimens of the freshwater bivalve *Dreissena polymorpha* for 7 days to the 9 collected plastic mixtures, using a biomarker suite for the evaluation of many cellular and molecular effects. *D. polymorpha* is an excellent model organism both for the *in situ* monitoring of several environmental contaminants, such as heavy metals, pesticides, hydrocarbons, personal care products, illicit drugs, MPs and for ecotoxicological experiments, as it is very resistant, easy to maintain in artificial conditions, has a high filtration rate and is now a ubiquitous representative of European water bodies (Binelli et al., 2015; Hoellein et al., 2021; Pastorino et al., 2021).

The more realistic approach, which represents an improvement of those already tested by our research group in two recent surveys (Binelli et al., 2020; Magni et al., 2021), could represent the starting point for establishing an experimental design capable of carrying out an environmental risk assessment due to plastic pollution with greater ecological relevance.

2. Materials and methods

2.1. Study area

The hydrography of Milan and the neighboring municipalities is particularly complex, both for natural causes, given the conspicuous presence of rivers, streams and springs, and for the works of canalizations and diversion of watercourses carried out, which led to the construction of many canals, millraces and artificial lakes. Therefore, we have chosen 7 of the main watercourses that cross the metropolitan area, in order to have a picture as complete as possible of the plastic pollution both for the incoming and outgoing watercourses of Milan, as indicated in Fig. 1 in which are also indicated the selected sampling stations. Given the complexity of the watershed of Milan, a brief description is required for each sampled watercourse.

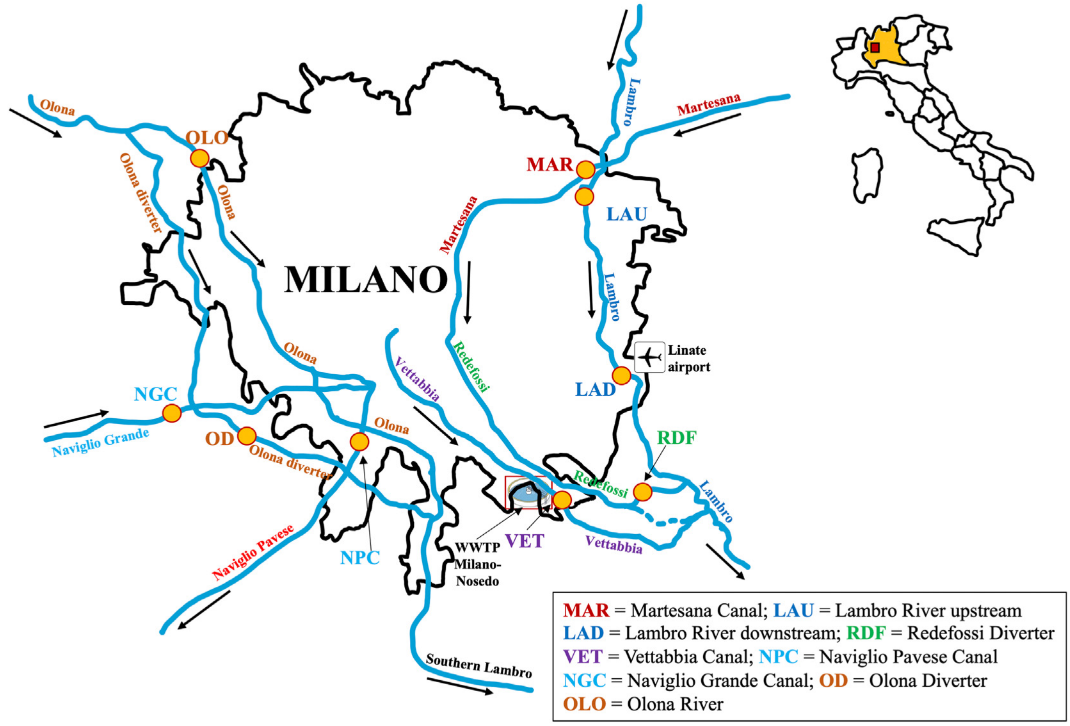


Fig. 1. Sampled hydrographic network of Milan and stations chosen for the collection of plastic mixtures.

2.1.1. Lambro River

It originates in the Lombard Pre-Alps and after about 130 km it flows into the Po River, the main Italian river. The mean flow rate of Lambro River is 34.3 m^3/s and the total area of its basin is approximately 553 km^2, of which 199 km^2 of urban areas and 354 km^2 of extra-urban areas (www.regione.lombardia.it). Lambro River crosses one of the most densely populated and industrialized areas of Italy and has 27 different tributaries along its course. We collected plastics in two sampling sites, named "Lambro River Upstream" (LAU; 45.512222, 9.259377) and "Lambro River Downstream" (LAD; 45.454564, 9.258776) located respectively in its northernmost and southernmost course across Milan (Fig. 1).

2.1.2. Olona River

It rises on the mountains north of the city of Varese, and after over 60 km it passes through the metropolitan area of Milan and then it flows into the Lambro River (www.regione.lombardia.it), representing its main tributary. Just before entering Milan, the river diverts part of its flow into the Olona diverter which rejoins the Olona River, which crosses Milan completely sealed, in the southernmost part of the metropolitan city, and then becomes the so-called Southern Lambro (Fig. 1) that then flows into the Po River. The Olona River has a total length of 71 km with a mean flow rate of 6.2 m^3/s and it flows through very industrialized and urbanized areas located between Varese and Milan. We selected the first sampling station in the Olona River (OLO; 45.510418, 9.085068) just before to bury underground in Milan, while the second was in the Olona Diverter (OD; 45.447052, 9.114980) to also check the other branch of this watercourse (Fig. 1).

2.1.3. Naviglio Grande Canal

The Naviglio Grande is an artificial canal that initially originated from Ticino River in the Province of Varese and ended in the Darsena of Milan, with a total length of 49.9 km. However, due to various territorial changes, today its effective flow derives from the waters of the Industrial Canal (Canale Vizzola) in the Municipality of Turbigo (www.navigliereloading.eu). The average flow rate is very variable along its course and during the year, as 120 secondary branches branch off from the main canal, allowing irrigation of over 50,000 ha of fields (www.etvilloresi.it). The plastics were collected in one sampling station (Naviglio Grande Canal; NGC; 45.430009, 9.104733) just before entering Milan (Fig. 1).

2.1.4. Naviglio Pavese Canal

The artificial Naviglio Pavese originates from the Darsena of Milan, which it leaves in a south-west direction until it flows into the Ticino River, one of the main tributaries of the Po River. The canal is 33 km long and distributes a mean of 10 m^3/s of water. The derived branches exceed 250 km (www.etvilloresi.it). To monitor the plastics exiting the metropolitan area, we performed the sampling in a station located just before the exit of the Canal from Milan (Naviglio Pavese Canal; NPC; 45.437823, 9.174713) as shown in Fig. 1.

2.1.5. Martesana Canal

The artificial canal connects the Adda River, a tributary of Po River, and Milan with a total length of 38 km. Nowadays it is only an irrigation canal, and its derived branches exceeds 500 km and extends an area of over 24,000 ha (www.etvilloresi.it). We sampled the plastics in a station located in the north-eastern area of Milan (Martesana Canal; MAR; 45.509555, 9.247448), near the intersection of the Martesana Canal with the Lambro River (Fig. 1).

2.1.6. Redefossi Canal

It is an artificial canal that originates in Milan from the waters of the Martesana Canal and Seveso River, the bed of which before entering Milan was however dry throughout the sampling period, and flows into the Lambro River south of the metropolitan area. It is about 18 km long, with an annual average flow rate of 1.5 m^3/s due to the contribution of numerous urban discharges (ATO, 2005). The last part of its course no longer follows the old riverbed, which is used exclusively in the event of flood, but since 1970 the Redefossi diverter has been reactivated and now it is the real tributary of the Lambro River. Thus, to monitor the plastics along the Martesana-Redefossi system, we selected the sampling station in the Redefossi diverter (RDF; 45.407846, 9.289111) located close to the outlet to Lambro River (Fig. 1).

2.1.7. Vettabbia Canal

It is a canal (38 km of length) that originates in the subsoil of Milan uptown and flows to the old riverbed of Redefossi Canal just before the outlet to Lambro River (Fig. 1). The Vettabbia Canal, being the receiver of about 1/3 of the flow rates discharged from Milan, is considered to convey a flow rate of the order of 4 m^3/s to which is added the flow of the fields it crosses, which has a value of about 2 m^3/s (ATO, 2005). We sampled the plastics in a site located about 2 km after the sewer of the main wastewater treatment plant of Milan (WWTP of Milano-Nosedo; Fig. 1) in the south-eastern area of Milan (VET; 45.417337, 9.236230).

2.2. Sampling of plastics

We carried out the sampling of plastics within three days (11–13 November 2019) to avoid any meteorological and hydrological changes among the watercourses that could have changed the plastic composition and concentrations. In addition, the most closely related watercourses, such as the system "Martesana Canal-Redefossi diverter", "Naviglio Grande Canal-Naviglio Pavese Canal" and "Olona River-Olona diverter", as well as the two sampling stations located in the Lambro River (Fig. 1), were sampled on the same day to ensure a more direct comparison.

To collect the floating plastics, we lowered for 15 min two twin plankton nets (Scubla S.r.l., Italy) with 100 μm mesh from the bridges present in the selected sampling sites directly in the center of watercourses. One of these nets was equipped with a flowmeter (Model 2030R, General Oceanics Inc., USA) to calculate the volume of filtered water during each sampling, whose values are shown in Table 1.

After sampling, each net was washed with 500 mL of sodium chloride (NaCl) hypersaline solution (1.2 g/cm^3), previously filtered on glass-fiber filters (1.2 μm of mesh; Whatman GF/C 47 mm), and all the sampled material was then placed in 500 mL glass bottles fitted with a metal cap. We used a total of 18 different glass bottles, 9 for the monitoring of plastics and the other 9 for the following ecotoxicity experiments. Samples were stored at 4 °C and then processed for the qualitative and quantification identification, as well as for the evaluation of their effects, as reported in detail by Binelli et al. (2020) and Magni et al. (2021).

2.3. Quantification and characterization of plastics

The supernatant in each sample, obtained after the density separation through the hypersaline solution, was filtered on cellulose nitrate membrane filters with a mesh of 8 μm (Sartorius™ 50 mm) using a vacuum pump. Subsequently, filters were washed with 500 mL of ultrapure water to eliminate the eventual traces of NaCl crystals. The residues of organic matter were digested, directly on the filters, with hydrogen peroxide (H_2O_2, 15% v/v), for 3 days, renewing the H_2O_2 to avoid the sample drying. The visual sorting on the filters was performed using a stereomicroscope to identify, and transfer on a clean filter, the particles with a possible plastic composition. The collected material was quantified and characterized (polymer composition, color and shape) using a μFT-IR (Spotlight 200i equipped with Spectrum Two, PerkinElmer). The infrared spectra of analyzed particles were acquired in Attenuated Total Reflectance (ATR) with 32 scans at 600 and 4000 cm^{-1} and analyzed using the Spectrum 10 Software. The spectra with a matching score ≥ 0.70 compared to standards were considered in the present study (Magni et al., 2019, 2021). The size of plastics was measured using the ImageJ Software (Ferreira and Rasband, 2012), on the images acquired at the μFT-IR, in accordance to the dimensional classification proposed by Hartmann et al. (2019). To avoid plastic contamination of the samples, filters were kept covered when possible and lab cotton coats were worn both during samplings and sample treatments. In addition, 5 filters were processed as blanks.

2.4. Evaluation of plastic effects

After the cleaning procedure of samples to eliminate the coarse materials, described in detail in our previous studies (Binelli et al., 2020; Magni et al., 2021), the supernatants of samples in hypersaline solution were filtered on a 63 μm steel sieve to eliminate the fine organic matter. Subsequently, the collected material on the sieve was rinsed with ultrapure water and plastics were added in the exposure tanks with the specimens of *D. polymorpha*.

Bivalves were collected in the Lake Maggiore by a scuba diver and maintained for two weeks in 10 L acclimation tanks with tap/deionized water in a ratio 1:1 *v*/v ($T = 20 \pm 1$ °C, $[O_2] > 90\%$) and fed with *Spirulina* spp. The water was changed every 3 days. Acclimatization has also allowed the elimination of any type of chemical that may have accumulated in the tissues of the bivalves, in addition to the possible plastic debris present in their digestive tract. Furthermore, it should be emphasized that the plastics present in other specimens of *D. polymorpha* sampled in Lake Maggiore were preliminarily analyzed and counted, showing the presence of plastic debris only in 3% of the bivalves. Together with the acclimatization period, this ensured that the measured ecotoxicological effects were due only to the plastics ingested during the different exposure tests.

Regarding the exposure, we used 10 tanks (1 control and 9 treated with plastics from the sampling stations) of 4 L with tap/deionized water (1:1 v/v; plastic concentrations used in the exposure are reported in Table 1) maintained at 20 ± 1 °C in a thermostatic room. The other main physical-chemical parameters of water were pH = 7.7, hardness = 15 °F, residual chlorine < 0.01 mg/L, nitrates = 29 mg/L, nitrites<0.2 mg/L.

Table 1
Characteristics of the sampling executed in the 9 stations of the metropolitan city of Milan. The concentrations of the plastic mixtures for each sampling site, the flow rate, the quantity of plastics that on average passes each day from each station and the mean concentration of the plastics put in the exposure tanks are shown.

Sampling Station	Water volume filtered m^3	Number of plastics identified	Plastics/m^3	Flow rate m^3/s	Plastics/day	Mean concentration of plastics in the tanks plastics/L
OLO	0.4	222	555	8.7[b]	415,743,840	55.5
LAD	0.8	310	388	29.6[b]	990,338,400	77.5
LAU	3	212	71	29.6[b]	180,603,648	53.0
MAR	1	24	24	1.2[a]	2,488,320	6.0
NGC	5.2	65	13	7.4[a]	7,992,000	16.3
OD	16	187	12	8.7[b]	8,754,966	46.8
NPC	4.5	50	11	4.8[a]	4,603,392	12.5
VET	1.6	11	7	6.0[c]	2,376,000	2.8
RDF	4.1	12	3	1.5[c]	375,840	3.0

[a] Kindly provided by Consorzio Bonifica Est Ticino Villoresi (referred to the days of sampling).
[b] Mean values of November (2001-2015) from ARPA Lombardia (www.idro.arpalombardia.it).
[c] From ATO (2005).

In each tank we put 50 specimens on a metallic net, with a magnetic stirrer and oxygenator to maintain the plastics in the water column and assuring the bivalve exposure. In addition, to avoid atmospheric contamination by plastic fibers, each tank was covered by aluminum sheet. The average length of the bivalves used for the exposure tests was equal to 1.7 ± 0.3 cm, so that there could be no variations in the filtration rate. The exposure was conducted for 7 days in static conditions and the animals were fed daily with *Spirulina* spp. The mortality was checked every day.

The effects of plastics from the different water courses were evaluated through a battery of biomarkers of cellular stress, oxidative damage and cyto-genotoxicity. We used 27 bivalves for the analyses of the different biomarkers, while the remaining bivalves were frozen for the subsequent analyses to evaluate the uptake of plastic debris after the exposure tests. In detail, we collected at the end of exposure ($t = 7$ days) 9 bivalves per treatment to evaluate the activity of antioxidant/detoxifying enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), as well as the quantification of reactive oxygen species (ROS). Specimens were homogenized in 3 pools of 3 bivalves per treatment (3 biological replicates) in 100 mM phosphate buffer, with pH = 7.4, in 1:10 w/v ratio, containing 100 mM potassium chloride (KCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 1:100 v/v protease inhibitors. Samples were centrifuged at 15,000*g* for 30 min at 4 °C and proteins were quantified in triplicate in the S15 fraction through the 6715 UV/Vis spectrophotometer (Jenway; Bradford, 1976). All biomarkers were assessed in triplicate, in particular CAT activity was measured quantifying the consumption of 50 mM H2O2 at 240 nm, SOD activity was evaluated assessing the reduction inhibition of 10 μM cytochrome *c* at 550 nm due to the superoxide anion produced by both xanthine oxidase and 50 μM hypoxanthine, while GPx activity was evaluated through the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of 0.2 mM H_2O_2, 2 mM glutathione, 1 mM sodium azide (NaN_3), 2 U/mL glutathione reductase and 120 μM NADPH at 340 nm. Regarding the detoxifying enzyme GST, its activity was evaluated using 1 mM reduced glutathione and 1-chloro-2,4 dinitrobenzene and reading the absorbance at 340 nm (Orbea et al., 2002; Magni et al., 2016). For ROS quantification we used 10 mg/mL dichlorofluorescein-diacetate (DCFH-DA) in dimethyl sulfoxide (DMSO) and the fluorescence was read at 485 nm (excitation) and 530 nm (emission) using the EnSight™ multimode plate reader (PerkinElmer), as reported by Parenti et al. (2019). As other endpoint of cellular stress, the efflux activity of the P-glycoprotein (P-gp) was assessed on the gills of other 9 organisms per treatment. The P-gp efflux activity was evaluated in 9 gill biopsies incubated for 90 min at room temperature in tap/deionized water (1:1 v/v) with 1 μM rhodamine B and in dark condition with gentle shaking. Subsequently, the biopsies were washed twice, 300 μL of lysis buffer was added to each sample, homogenized and centrifuged for 10 min at 14,000*g* and the fluorescence of rhodamine B was measured at 545 nm (excitation) and 575 nm (emission) through the EnSight™ multimode plate reader (PerkinElmer; Magni et al., 2017). Regarding the oxidative damage, we evaluated the lipid peroxidation (LPO) and protein carbonylation content (PCC) on other 9 bivalves per treatment.

For LPO and PCC bivalves were homogenized in 3 pools of 3 specimens per treatment in 100 mM phosphate buffer, pH = 7.4, 1:10 w/v, with 100 mM KCl, 1 mM EDTA, 1 mM DTT and protease inhibitors (1:100 v/v). Proteins were quantified in the crude homogenate (CH; Bradford, 1976). LPO was evaluated in the CH using the thiobarbituric acid-reactive substances (TBARS) and reading the absorbance at 535 nm (Ohkawa et al., 1979), while PCC was assessed in the CH exploiting the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups and reading the absorbance at 370 nm (Mecocci et al., 1999). From the same specimens used for the oxidative damage, we also collected the hemolymph, using a hypodermic syringe with 100 μL of EDTA/phosphate buffer saline (PBS) 10 mM, to evaluate both cell viability (cytotoxicity) using the Tripan Blue exclusion method (Strober, 2015) and the micronucleus frequency.

The micronucleus frequency was evaluated on hemocytes as reported by Pavlica et al. (2000), counting 400 cells for each of the 9 prepared slides per treatment.

2.5. Evaluation of plastic uptake

At the end of exposure, ten mussels per treatment were pooled and homogenized in NaCl hypersaline solution (1.2 g/cm^3) through a potter. Subsequently, the samples were filtered on cellulose nitrate membrane filters with a mesh of 8 μm and digested with 15% H_2O_2 under a laminar flow hood. The extracted particles by mussels were analyzed using the μFT-IR as described in the Section 2.3.

2.6. Statistical analyses

For all biomarkers the data normality and homoscedasticity were assessed through the Shapiro-Wilk and Levene tests respectively. The significant differences ($^*p < 0.05$; $^{**}p < 0.01$) at the end of exposure were evaluated between mussels exposed to plastic mixtures and relative controls by the one-way analysis of variance (one-way ANOVA), followed by the Duncan's *post-hoc* test. In addition, we measured the covariation between the number of detected plastics in each water course and the relative filtered water volume through the Pearson correlation test ($p < 0.05$). The STATISTICA 7.0 Software was used for these analyses.

3. Results

First of all, we avoided any environmental contamination or due to the clothing of the operators because the 5 filters used as blanks showed the presence of only 12 natural fibers.

The volume of filtered water in each sampling was very variable, going from 0.4 m^3 at OLO up to 16 m^3 at OD (Table 1), as we decided to keep the residence time of the nets in water constant (15 min), depending on both flow rate and mainly by the presence of suspended material in the water that obstructs the mesh of the nets. On the other hand, there is no correlation ($r = 0.04$; $p = 0.889$) between the volumes of water filtered in each sampling station and the number of detected plastics, as well shown in Table 1 in which the two sites (OLO and LAD) where we collected the smallest volume of water were those where we sampled the highest number of floating plastics.

3.1. Evaluation of plastic uptake

At the end of the exposure, we measured the plastic debris inside the exposed organisms to verify their presence into the bivalves. Bearing in mind that this measurement represents only a snapshot of the total intake that took place during the 7 days of exposure, the results showed the presence of 6 debris in the treated bivalves, namely 3 fibers of polyester, one fiber of polyacrylate, one fiber of polyisoprene, as well as one fragment, also of polyisoprene.

Since 90 specimens were used for the assessment of the intake (see Section 2.5), the percentage of uptake was 6.6%, a value more than double compared to that measured in specimens of *D. polymorpha* sampled in the L. Maggiore (see Section 2.4).

3.2. Total plastics abundance

Although the sampled watercourses are all located within the metropolitan city of Milan, we identified an extremely variable number of plastic debris, with the highest values in the two main and longer rivers (Lambro and Olona) in which we identified 310 (LAD) and 212 (LAU) plastics, as well as 222 (OLO) and 187 (OD) debris, respectively

(Table 1). The lowest number of plastics was instead detected at VET (11 debris) and RDF (12 debris).

More interesting than the simple numerical data is to consider their normalized concentration on the volume of filtered water. In this way, for example, the comparison between OLO and LAD overturns (Table 1), as the first sampling site was the one where the highest plastics concentration was measured (555 plastics/m^3), followed by LAD (388 plastics/m^3). The OD site was also no longer the fourth most contaminated site, but its concentration (12 plastics/m^3) makes it one of the least impacted stations.

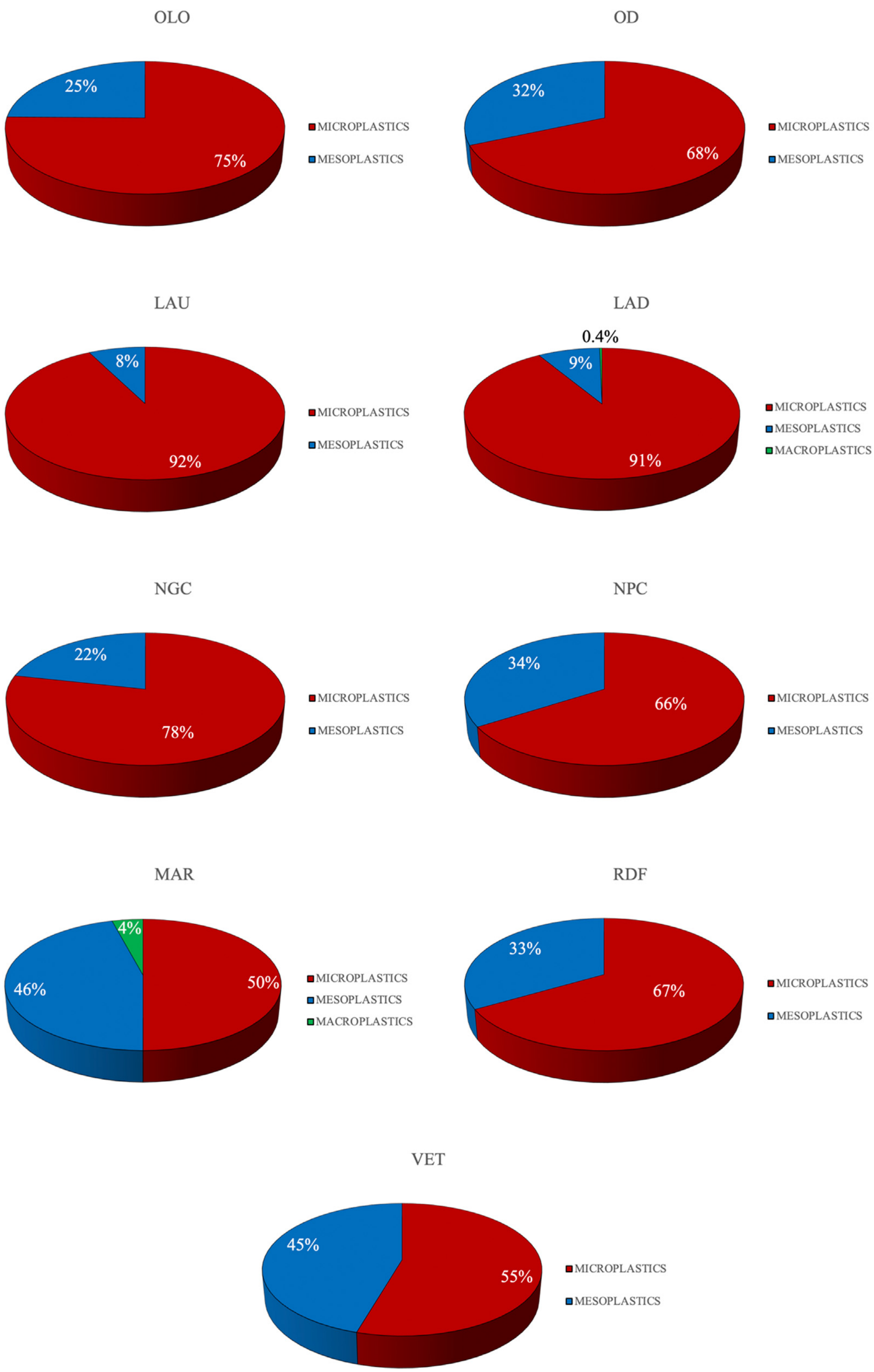


Fig. 2. Characterization of the size of the plastic debris for each single sampling station. OLO: Olona R.; OD: Olona Diverter; LAU: Lambro R. Upstream; LAD: Lambro R. Downstream; NGC: Naviglio Grande Canal; NPC: Naviglio Pavese Canal; MAR: Martesana Canal; RDF: Redefossi Canal; VET: Vettabbia Canal.

Lastly, the debris concentration allows to roughly estimate the quantity of plastics that pass daily in the sampling sites (Table 1). Using the water flow rates actually referred to the sampling day or through the data available in official documents (ATO, 2005; www.idro.arpalombardia.it), it was possible to estimate a daily flow of over 990 million plastic debris in the LAD station, followed by OLO (about 415 million/day) and LAU (about 180 million/day). By contrast, MAR (more than 2.4 million/day), VET (about 2.3 million/day) and especially RDF (about 375,000/day) resulted the watercourses less impacted by these emerging pollutants.

3.3. Qualitative and quantitative assessment of plastics

The size analysis of the collected plastic items showed the presence exclusively of MPs and mesoplastics (MePs; 1 mm < 10 mm) in all sampling points, apart from LAD and MAR sites, where some macroplastics were also detected, albeit in little percentages (Fig. 2). It is very interesting to underline that the watercourses belonging to the same water-system have the same type of size, except for a certain difference for the Martesana-Redefossi system, in which we found that only a third of the plastic debris detected for RDF are represented by MePs, compared to about 50% for MAR (Fig. 2).

We did not obtain the same homogeneity as regards the analysis of the shape of the plastic debris sampled in the different watercourses, as shown in Fig. 3. Indeed, a clear prevalence of pellets is observed in LAD (78.4%), LAU (73.6%) and OLO (64.4%) stations, while fragments prevail at NPC (68%), VET (63.6%) and NGC (53.8%). Lastly, we observed that fibers represent the main shape fraction in the two sampling stations of the Martesana-Redefossi system, with 52.2% for MAR and 50% for RDF, respectively (Fig. 3).

The total polymer composition is just as not homogeneous as the shape category, as shown in Fig. 4. Apart from the two stations LAU and LAD in which polystyrene (PS) prevails as the main polymer with a percentage of 72–75%, the sampling stations placed on the same water-system have very different polymeric percentages. For example, in the OLO site, poly(methyl methacrylate) (PMMA), also known with the commercial name of Plexiglass®, represents about 2/3 of the polymers detected, followed by polyester (PEST, 15%) and polypropylene (PP; 10%). On the contrary, the OD site, placed on the diverter of the same river few kilometers south, showed an extremely diversified polymeric composition, with higher percentages of PEST (28%) and polyamide (PA; 26%), followed by PP (14%), while PMMA represents only 11% of the total polymer composition (Fig. 4).

The Naviglio Grande-Naviglio Pavese system also has the same characteristics, since PP (37%), PEST (29%) and polyethylene (PE; 26%) prevail in NGC, while these percentages change considerably in NPC, as the percentage of PE reverses, reaching a 44% of the polymer composition, followed by PP (20%) which falls by 17% compared to NGC and PEST (18%) which also falls by 11% (Fig. 4).

Compared to a greater polymeric diversity detected at MAR, in which we found a similar percentage (17–25%) of PS, PEST, polyacrylate (PAC) and PA, RDF showed a more homogeneous composition with percentages varying between 17% of PEST and PP and 33% of PA, while only PE showed a lower percentage (8%). Furthermore, PS, which accounted for 17% at MAR, was not detected in the plastic debris sampled at RDF, in which we did not even detect the presence of polyurethane (PU) and polyacrylic rubber (Fig. 4).

Finally, VET showed a clear separation of the frequency among polymers, with four of them (PE, epoxy resin, ethylene-vinyl acetate - EVA and polyvinyl acetate PVAc) which showed identical percentages (9%), while we observed a greater presence of PP (28%), PEST (18%) and PA (18%).

Plastic colors were also very different among the watercourses investigated. For example, OD showed a greater variety of colors for both the MPs and the MePs analyzed. For OLO, most of the MPs and all the sampled MePs were white, while for OD this color did not exceed 50% for the MPs and was less than 40% for MePs (Fig. 5). Even for the MPs collected at LAU and LAD white prevailed, with percentages higher than 80%, while the colors were more heterogeneous for MePs.

NGC and NPC showed the presence of a great variety of colors, with a prevalence for NGC of black (25.5%) and green (23.5%) MPs and of white (35.7%), black (21.4%) and brown (21.4%) MePs, while blue (27.3%) and black (21.2%) MPs prevailed at NPC, as well as black (29.4%) and pink (23.5%) for MePs (Fig. 5)

In relation to the Martesana-Redefossi system, the northernmost sampling point (MAR) showed a greater heterogeneity of colors especially for the MPs, with a prevalence of white, red, transparent and purple, all represented with 16.7%, while for the MePs the two most present colors were white (45.5%) and blue (36.4%). The MePs detected at RDF are equally divided (25%) into the 4 colors indicated in Fig. 5, while for the MPs black and transparent prevailed, both with 37.5%.

Lastly, VET showed the most constant trend in terms of colors, with white (60%) and black (40%) as the only colors for MePs, while red represents half of the sampled MPs, followed by green, white and black, all with 16.7%.

3.4. Results of biochemical analyses

During the exposure we observed a very low mortality of bivalves, with 1 dead organism in the control, OLO and NPC, 2 dead specimens in the NGC and 3 in the RDF. For the other experimental groups, no mortality was obtained. Regarding the cytotoxicity evaluated on *D. polymorpha* hemocytes, we observed a viability always higher than 70%, with a mean value ranging from 75.8 ± 4.7% for VET and 86.0 ± 3.7% for LAD.

The concentration of the plastic mixtures introduced into each of the 4 L tanks is shown in Table 1. As can be seen, it ranges from a minimum of 2.8 plastics/L for VET up to 77.5 plastics/L for the exposure to the plastic mixture collected at LAD.

Concerning the effects, SOD, GST, PCC and P-gp showed levels in the bivalves exposed to the different mixtures similar to the controls and no differences were also detected between the sampling sites of the same water system (Fig. 6).

The biomarkers that responded in the exposure experiments are related to the production of ROS, to the antioxidant activity (CAT and GPx) and to the oxidative damage (LPO; Fig. 6). Going into more detail, the water system that was most impacted by exposure to the mixture of plastics sampled in its two sampling stations was the Olona system. Indeed, we noted a significant decrease of CAT ang GPx activities ($p < 0.05$) in OLO compared to OD, while ROS content was significantly higher in OD with respect to OLO. Besides, CAT and GPx activities were significantly lower in OD compared to control values, while ROS level was significantly higher ($p < 0.05$ for CAT and ROS and $p < 0.01$ for GPx; Fig. 6). As regards the Martesana-Redefossi system, there was a significant decrease ($p < 0.05$) of LPO compared to the controls for both stations and for GPx only for RDF ($p < 0.01$), which was significantly different ($p < 0.05$) also to MAR (Fig. 6). Moving to the Lambro River, LAD reached significantly lower values than the controls for GPx ($p < 0.01$) and an increase of ROS ($p < 0.05$), resulting also different ($p < 0.05$) than LAU for the latter biomarker (Fig. 6). Lastly, in the Naviglio Grande-Naviglio Pavese system, a significant decrease in LPO ($p < 0.01$) was observed for both NGC and NPC compared to controls, as well as for the VET group ($p < 0.05$; Fig. 6).

4. Discussion

This research represents the first Italian study on the monitoring of plastics carried out in a basin heavily anthropized and impacted by numerous industrial activities, which is part of the Po River drainage basin (71,000 km^2), which about 20 million people live.

From a general point of view, it is possible to highlight how the choice of sampling by a plankton net with finer mesh (100 μm) allowed

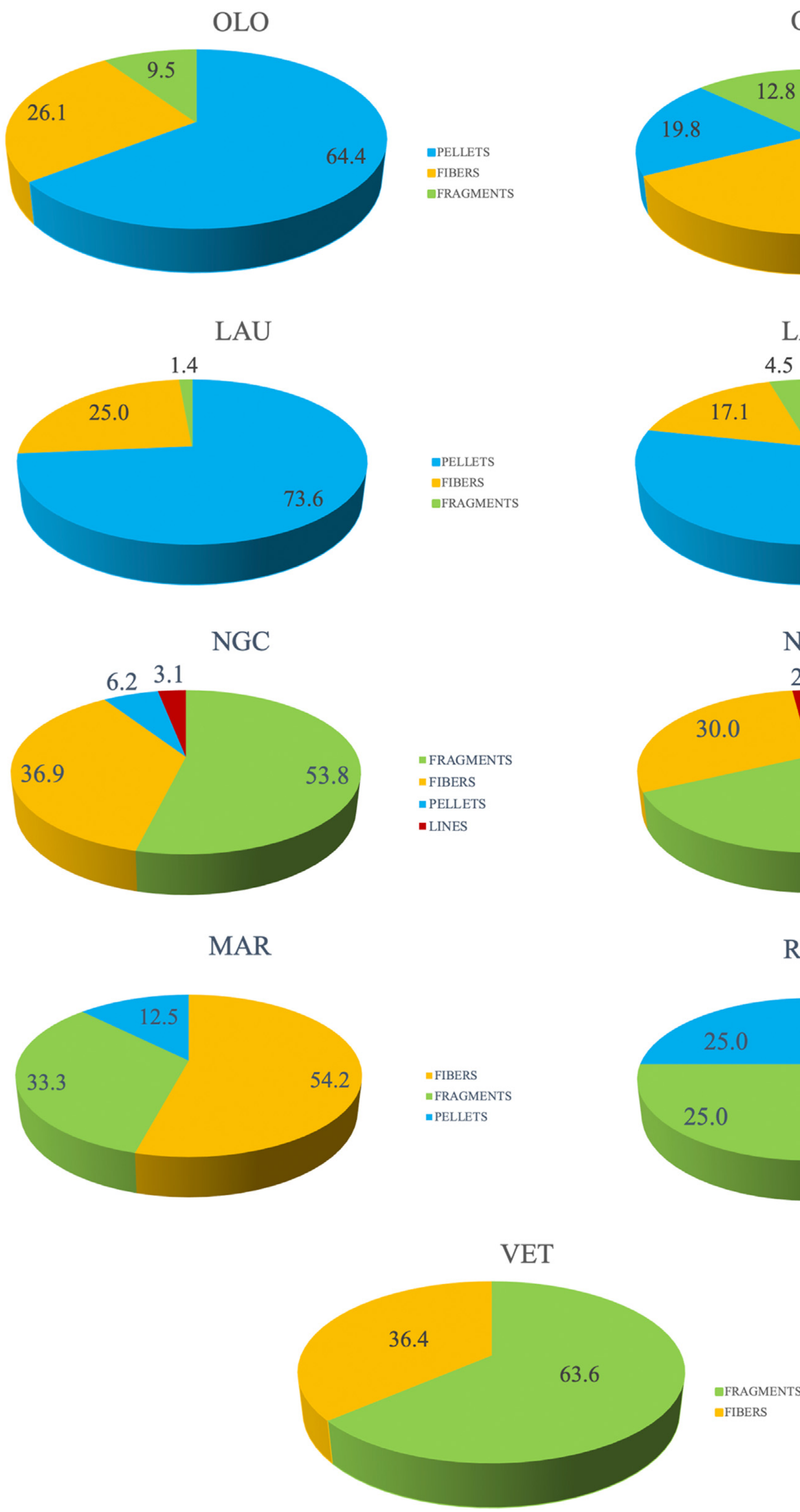


Fig. 3. Shape classification of the plastic debris collected at the 9 sampling stations.
OLO: Olona R.; OD: Olona Diverter; LAU: Lambro R. Upstream; LAD: Lambro R. Downstream; NGC: Naviglio Grande Canal; NPC: Naviglio Pavese Canal; MAR: Martesana Canal; RDF: Redefossi Canal; VET: Vettabbia Canal.

the collection of a greater number of plastics. Indeed, if we compare the concentration of plastic debris sampled in the LAU station (71 plastics/m^3) with that from a site located only 1.5 km south during a previous monitoring campaign conducted with nets of 300 μm (1.7 ± 0.6 plastics/m^3; Magni et al., 2021), we were able to collect a plastic amount of over 40 times higher, in line with what indicated by Coverton et al. (2019).

The extreme complexity of the hydrographic system of the metropolitan city of Milan (Fig. 1) is also reflected in the variability of the results obtained for the qualitative and quantitative characterization

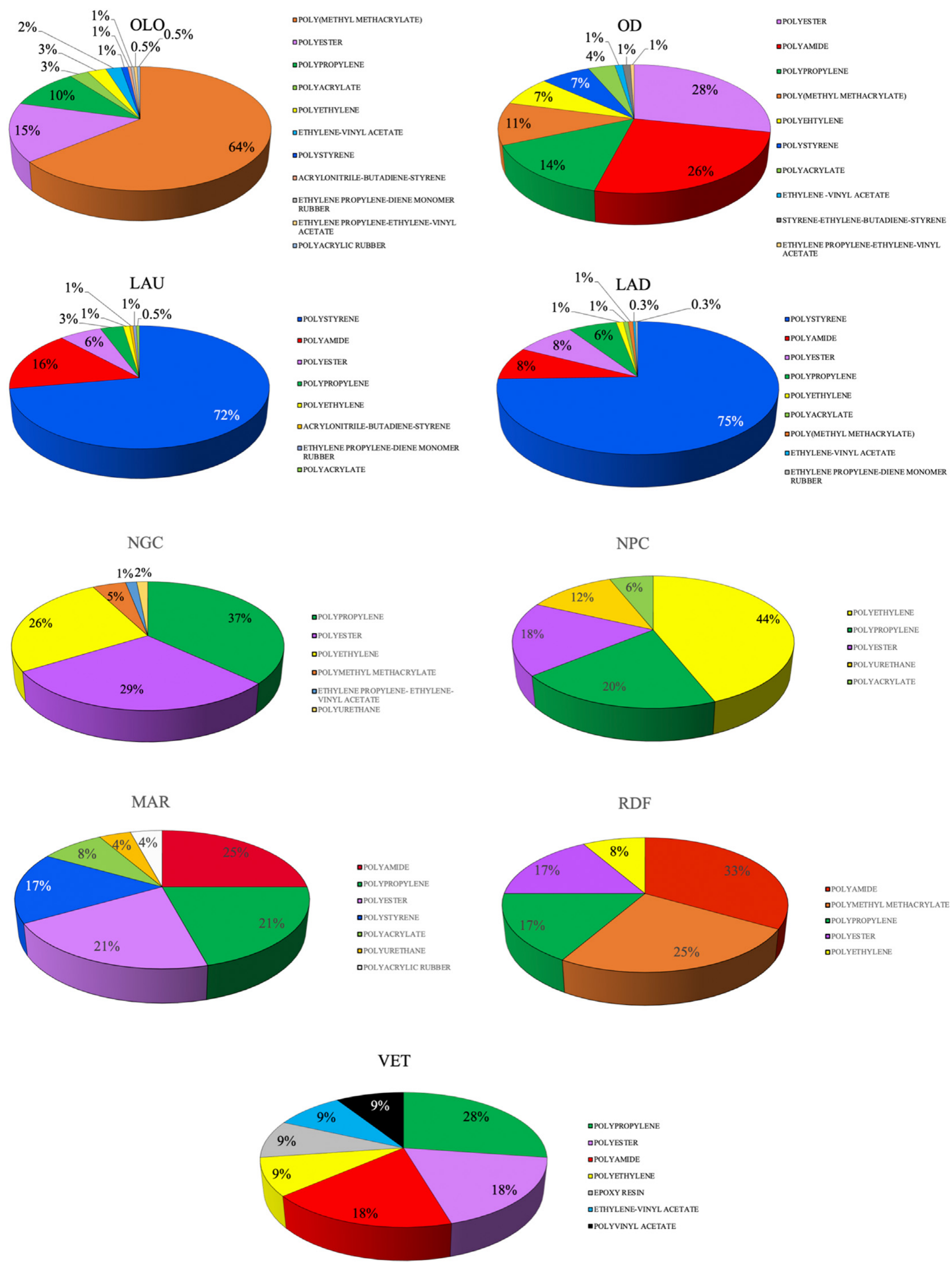
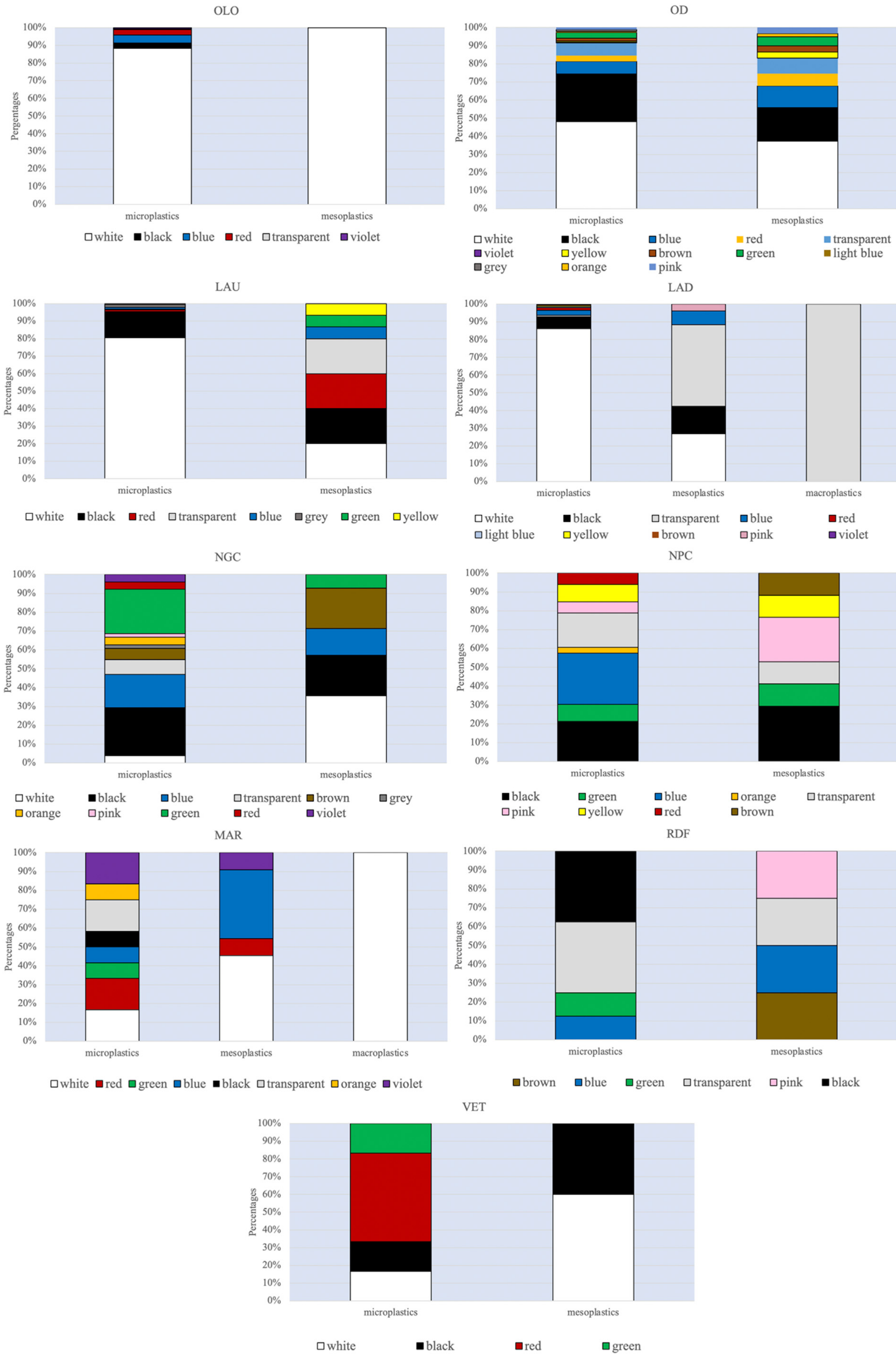


Fig. 4. Polymeric characterization of the plastic debris collected in each sampling site.
OLO: Olona R.; OD: Olona Diverter; LAU: Lambro R. Upstream; LAD: Lambro R. Downstream; NGC: Naviglio Grande Canal; NPC: Naviglio Pavese Canal; MAR: Martesana Canal; RDF: Redefossi Canal; VET: Vettabbia Canal.

of the plastic mixtures collected in the 9 different sampling stations. To give a greater order and allow a simpler explanation of the entire dataset, as well as an immediate comparison, each individual water system will be described separately.

4.1. Olona River system

This water system is certainly the one most affected by plastic contamination, as the station located directly on its main course (OLO; Fig. 1) was the one where we found the highest synthetic polymeric concentration, with 555 plastics/m^3 (Table 1). In the second sampling station (OD), located on its diverter, the concentration of plastics dropped to 12 plastics/m^3, as well as were very different the shape of the plastic debris collected. Indeed, pellets prevail in the OLO site, followed by fibers and fragments, while fibers are the main shape at OD, followed by pellets and fragments (Fig. 3). Fig. 7, that shows the polymeric composition for each shape class, can help us to better explain this specific contamination, since almost all the pellets detected at OLO, which alone constitute more than 64% of all shapes revealed in this site (Fig. 3), are made up of PMMA. Since also the pellets found in OD are formed for about 54% by this specific polymer (Fig. 7), we can hypothesize that these plastics of primary origin are due to an industrial


OLO
Percentages
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
microplastics
mesoplastics
white
black
blue
red
transparent
violet
OD
Percentages
microplastics
mesoplastics
white
black
blue
red
transparent
violet
yellow
brown
green
light blue
grey
orange
pink
LAU
Percentages
microplastics
mesoplastics
white
black
red
transparent
blue
grey
green
yellow
LAD
Percentages
microplastics
mesoplastics
macroplastics
white
black
transparent
blue
red
light blue
yellow
brown
pink
violet
NGC
Percentages
microplastics
mesoplastics
white
black
blue
transparent
brown
grey
orange
pink
green
red
violet
NPC
Percentages
microplastics
mesoplastics
black
green
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orange
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red
brown
MAR
Percentages
microplastics
mesoplastics
macroplastics
white
red
green
blue
black
transparent
orange
violet
RDF
Percentages
microplastics
mesoplastics
brown
blue
green
transparent
pink
black
VET
Percentages
microplastics
mesoplastics
white
black
red
green

discharge located before the separation between the main course of the Olona River and its diverter. The fact that their origin is the same was confirmed because of all the PMMA pellets detected in the two stations are the same white color. Their industrial origin is clear, since this polymer is available as small granules, sheets and just pellets used to produce plastic objects in several fields, such as architecture and construction, lighting, automotive and transportation, electronics, medical and healthcare.

The exact identification of the source of these pellets in the Olona River is currently not easy, as several industrial and civil sewers converge on this watercourse, as many as 9 WWTPs and 19 different tributaries (www.consorziofiumeolona.org). Anyway, this specific pollution due to PMMA pellets represents a very important information for the future management of the problem related to plastic contamination in this river, as it will eventually be possible to carry out targeted samplings on the various sewers to be able to trace the industrial source.

The greater heterogeneity of the plastic characteristics detected at OD, both as polymeric composition and size, is because the Olona diverter, on which this station is placed, also collects the waters of the north-west spillway (NWS), which is the fulcrum of the system for protecting Milan and the northernmost municipalities from floods due to many watercourses (www.regione.lombardia.it). The contribution of NWS to plastic contamination on the Olona diverter seems to be linked more to fibers, which represent about 67% of the polymeric debris found at OD (Fig. 4), in contrast to OLO in which they represented only 26% (Fig. 3). The polymeric composition of fibers, consisting mainly of PEST in both stations, followed by PP at OLO and PA at OD clearly highlights a secondary origin, probably due to synthetic woven and non-woven fabrics' washing (Prata, 2018; Magni et al., 2019).

In conclusion, we can say that the main problem due to plastic pollution in the Olona River is mainly linked to the industrial discharge of PMMA pellets, the origin of which it would be necessary to know in the immediate future to carry out the appropriate risk mitigation, also considering that this water system is part of the Po River basin.

These results confirmed the crucial role of some physical characteristics in determining the effects of plastic on organisms that can dampen or increase the role of concentration alone.

Indeed, Fig. 6 shows how the bivalves exposed to the mixture of plastics from OD, the lower contaminated station, showed significant differences ($p < 0.05$) for ROS, CAT and GPx compared to the controls and also to OLO. This may suggest that it is probably the shape that could cause a higher effect, since the greater difference found between the two sampling stations is that the pellets prevail in OLO, while the fibers are most represented in OD (Fig. 3). On the other hand, there are numerous recent studies that pointed out many effects of microfibers in fish (De Sá et al., 2018; Barboza et al., 2019), zooplankton (Chatterjee and Sharma, 2019; Klein and Fischer, 2019), mussels (Zhao et al., 2019; Mishra et al., 2019) and also humans (Prata et al., 2019b).

Particularly interesting is the ROS increase of about 45% greater than the baseline levels observed for OD, which could lead to a rise of oxidative stress. Neither PCC nor LPO, typical endpoints of oxidative damage, showed a significant increase, while the two antioxidant enzymes CAT and GPx were significantly lower than controls (Fig. 6). The decrease in these enzymatic activities is in line with data obtained in a previous study carried out in the great Italian subalpine lakes, again using *D. polymorpha* as a biological model (Binelli et al., 2020). On the other hand, several recent studies showed a decrease in CAT activity and CAT gene expression after the exposure to nanoparticles and other pollutants (Zhang et al., 2017; Choi et al., 2018; Almeida et al., 2019; Sendra et al., 2021). All these convergent results could indicate a specific effect determined by the exposure to microfibers in decreasing the defense antioxidant systems, even if the possible mechanism of action remains to be elucidated.

4.2. Lambro River system

The plastic contamination present in the stretch of the Lambro River we sampled during this survey was rather high, as the two sampling stations showed the highest concentrations of synthetic polymeric debris after that of OLO (Table 1). The two stations are those that showed a greater degree of similarity both in terms of size (Fig. 2), shape (Fig. 3) and polymeric composition (Fig. 4). The only difference is the concentration of plastic debris detected at LAD (388 plastics/m^3) which is about 5 times higher than that measured at LAU (71 plastics/m^3; Table 1), even if this difference is not so evident if we consider the number of plastics measured in the two sampling sites, as we identified 310 debris at LAD, and 212 debris at LAU. It is therefore probable that the greatest difference in terms of concentration is due to the different volume of filtered water, having been equal to 3 m^3 for LAU and 0.8 m^3 for LAD (Table 1), due to a greater presence of suspended particulate matter at the station located at the exit from Milan, which clogged the mesh of plankton net and therefore determined the collection of a greater quantity of plastics, which could also be the cause of the large difference observed between the two sampling stations of the Olona water system (Table 1). On the other hand, the extreme similarity of the plastic contamination observed at LAU and LAD, which are located a few kilometers away from each other, is due to the lack of civil and industrial discharges in the section that separates them, since the industrial activities, as well as the buildings, located on its banks in the Municipality of Milan are completely collected to WWTPs, while the possible release of plastic debris from the Milano-Linate international airport, located next to the left bank of the Lambro River (Fig. 1), as well as from the Idroscalo, a small artificial basin (1.6 km^2) near the airport, discharge into the Lambro River just south of LAD (SEA, Società per Azioni Esercizi Aeroportuali, 2017).

The origin of the plastic contamination detected in these two stations is double, as the fact of finding more than 70% of pellets (Fig. 3), almost completely in PS in both LAU and LAD (Fig. 7), clearly indicates a primary origin of such plastic debris. Furthermore, the fact that these PS pellets are all white and with an average size of 250 μm also supports their industrial origin. In fact, the PS pellets of an average diameter of around 200 μm are used as raw material (pre-expanded PS beads) of expanded PS (EPS; Howard, 1993), which is one of the most used thermoplastic materials in the world due to its extreme lightweight, thermal insulation and versatile strength. EPS is used as packaging material and to cushion objects during shipping, as insulation material and a filler material in some consumer goods, such as coffee capsules, caps and labels of plastic bottles, fast-food containers for takeaway restaurants and plastic cups, which in 2019 were not yet banned in Italy.

However, the fact that a variable percentage between 21% and 26% was fibers and fragments (Fig. 3) indicates also a smaller, but significant secondary origin of plastic contamination in this stretch of the Lambro River. The PA and PEST fibers, which together represent a percentage variable between about 74% (LAD) and 89% (LAU), underline a civil source, probably due to the washing of synthetic clothes and/or to atmospheric transport. On the other hand, Napper and Thompson (2016) have shown that 6 kg wash of acrylic fabric can release over 700,000 synthetic fibers from domestic washing machines. More in detail, De Falco et al. (2018) demonstrated as a typical 5 kg wash load just of PEST fabrics released over 6,000,000 microfibers, depending on the types of detergent used. Recently, also atmospheric transport has been

Fig. 5. Color definition of the plastic debris from the 9 sampling stations.
OLO: Olona R.; OD: Olona Diverter; LAU: Lambro R. Upstream; LAD: Lambro R. Downstream; NGC: Naviglio Grande Canal; NPC: Naviglio Pavese Canal; MAR: Martesana Canal; RDF: Redefossi Canal; VET: Vettabbia Canal.

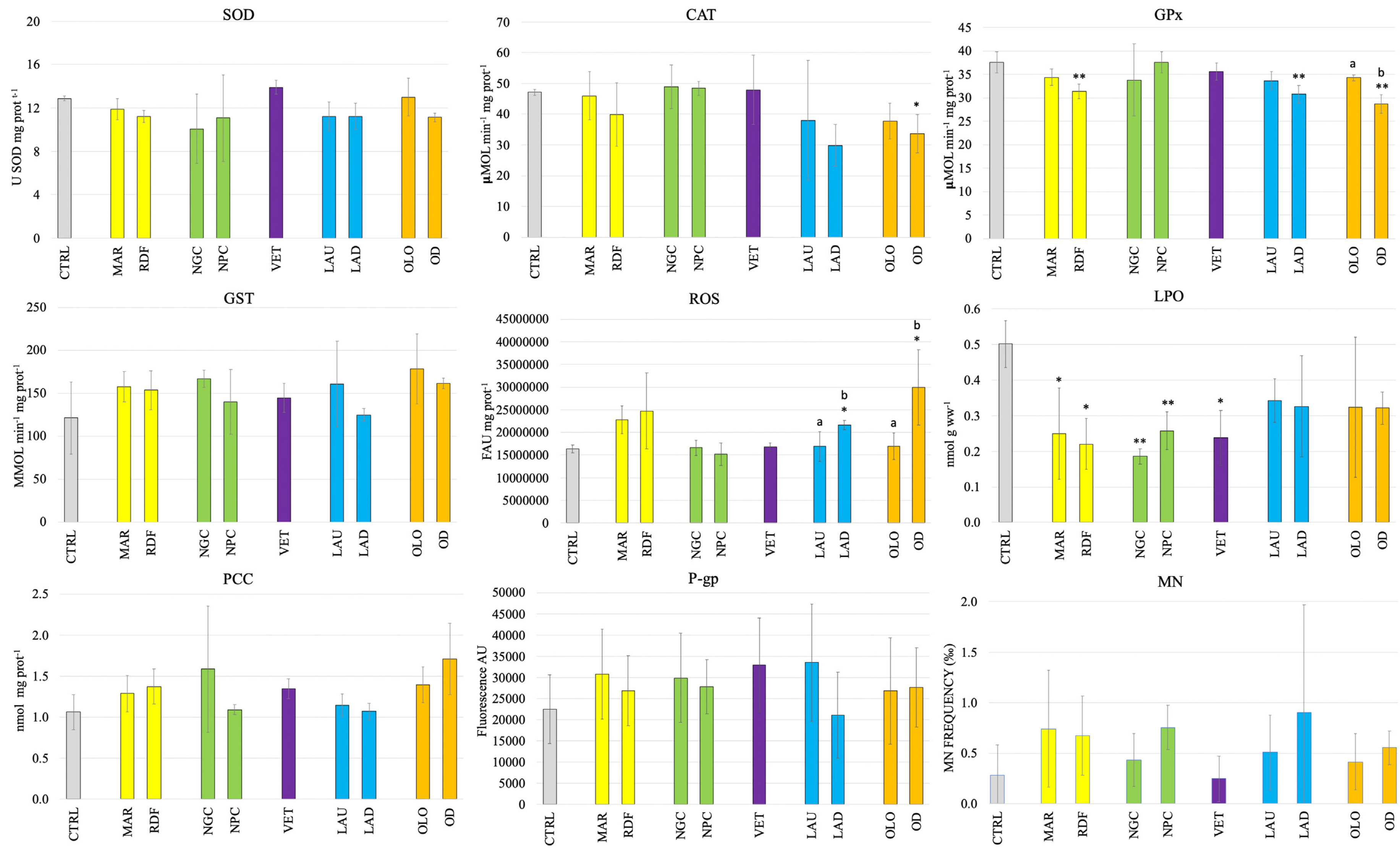

Fig. 6. Mean values (±standard deviations) of the measured endpoints after the exposures to the plastic mixtures collected in each sampling site.
SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GST: glutathione-S-transferase; ROS: reactive oxygen species; LPO: lipid peroxidation; PCC: protein carbonylation content; P-gp: P-glycoprotein; MN: micronuclei.

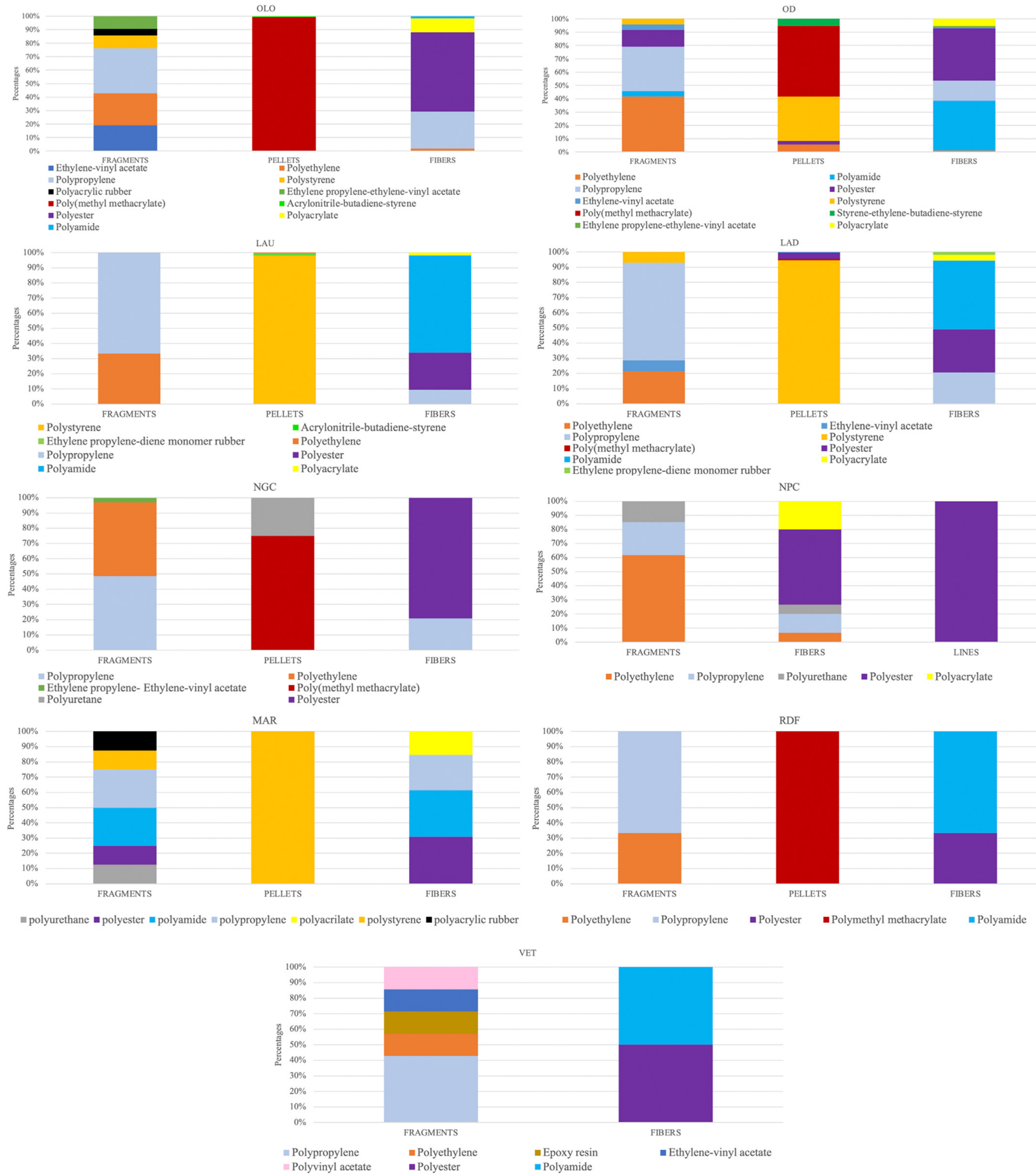

Fig. 7. Polymeric composition of each shape class identified for the 9 sampling stations.
OLO: Olona R.; OD: Olona Diverter; LAU: Lambro R. Upstream; LAD: Lambro R. Downstream; NGC: Naviglio Grande Canal; NPC: Naviglio Pavese Canal; MAR: Martesana Canal; RDF: Redefossi Canal; VET: Vettabbia Canal.

considered an important vector of microfibers that could lead to their deposition to terrestrial and aquatic environments (Zhang et al., 2020). For instance, recent studies carried out in some European cities revealed a mean abundance of MPs from dry and wet deposition ranging from 118 debris/m^2/day measured in Paris, of which more than 60% were fibers (Dris et al., 2015, 2017), and 275 debris/m^2/day in Hamburg (Klein and Fischer, 2019).

The exposure experiments to the two plastic mixtures sampled in the Lambro River highlighted a similarity with the results obtained for the two stations of the Olona River, as LAD showed a decrease in GPx activity ($p < 0.01$) and an overproduction of ROS ($p < 0.05$) (Fig. 6). The activity of CAT was also lower than the controls, as for the two sites of Olona River, but not significant due to the wide standard deviation of LAU.

Since we have observed a contamination by primary plastics of industrial origin in both rivers and a very similar trend with regard to the biomarkers measured, we can hypothesize that these endpoints are possible markers of contamination by these emerging contaminants, mainly due to the fact which result in a non-classical variation from that normally would be expected, especially with respect to the decline in activity of antioxidant enzymes, which confirmed how plastics can cause problems in the response of organisms to the imbalance of redox activity.

4.3. Naviglio Grande-Naviglio Pavese system

Unlike the other two watercourses described above, the concentration of plastics at the two stations located in this watercourse is practically the same, as we measured 13 plastics/m^3 for NGC and 11 plastics/m^3 for NPC (Table 1). On the other hand, the two watercourses are one the continuation of the other (Fig. 1) but, unlike Olona and Lambro rivers, we do not find specific urban or industrial discharges along their course, as they are artificial canals for irrigation purposes.

This water system showed a plastic pollution basically due to a secondary origin, linked to a fragmentation of plastic wastes and not to sources related to industrial wastes. Indeed, in both sampling stations, the fragments and fibers prevail, clear indicators of a fragmentation of plastic wastes and cloth washes, and the pellets represent only about 6% of the plastic shapes at NGC, while they were not even detected at NPC (Fig. 3).

As shown in Fig. 7, almost all the fragments are made up of PP and PE, which further confirm their secondary origin. The presence of a significant aliquot of PP fragments (about 50%, Fig. 7) could indicate how these plastic debris may partially derive also from the international airport of Milano-Malpensa, whose wastewaters of all the buildings are conveyed to the WWTP of S. Antonino Ticino (SEA, Società per Azioni Esercizi Aeroportuali, 2011), the largest sewage treatment unit in the Province of Varese, which has its outlet in the so-called Industrial Canal (www.nordmil.com) that, after a few kilometers, determines the main flow of the Naviglio Grande Canal (www.naviglireloading.eu). On the other hand, PP has numerous uses in the aeronautical field, as technical products with specific characteristics can be obtained, such as high stiffness and chemical resistance, as well as high resistance to high temperatures (Kumar et al., 2013). This polymer is used also as an insulator in electrical cables and in water and gas pipes. Specific and technical plastic materials are now widely used both in the aircraft interior parts and in the aircraft doors and fuselage, as well as in ventilation ducting and seals (Antony et al., 2019; www.acplasticsinc.com). The considerable amount of PE fragments could also come partially from this specific source (Shaikh et al., 2014), as currently it is used to produce the pluriball sheets for the protection of fragile objects during shipping, food films, toys and bottles for detergents or food (Zhong et al., 2017). PE is also the polymer used in the old shopping bags which, although its use has been banned in Italy since 1st January 2011, it is likely that polymer fragments will still end up in the aquatic environments.

Particularly intriguing was the identification of a fragment of the ethylene propylene-ethylene vinyl acetate (EPR-EVA) copolymer (Fig. 7) which has very specific uses, as in the fire retarding cables used for locomotives and coaches, and in nuclear technology (Placek and Bartonícek, 2001). In addition, the generic acronym EPR also includes the elastomer propylene-ethylene diene monomer (EPDM; Southwire Company, 2006) that blended with EVA are being extensively used just in the aeronautics industry, as well as in packaging and other applications (Rigoli et al., 2019). Obviously, the possible origin at least of a part of the plastic debris due to the international airport is only a hypothesis because another pollution source can be the Ticino River that is the main outlet of the Lake Maggiore. Anyway, it would be very interesting to conduct a specific study on the impact of an airport area in the contamination by plastics in surrounding aquatic environments to possibly improve its eco-sustainability.

We can underline that at NPC, after the passage through Milan, PU also appears in the plastic fragments, with a percentage of about 15% of the total polymeric composition (Fig. 7). This is certainly a polymer of urban origin, as it is used in padding for furniture, mattresses and even cars, seals, and bumpers for smartphones. In addition, it is possible to find various PU products in building industry, in the foam of thermal insulation material, in the refrigeration industry or as acoustic insulation. The urban origin of this polymer is also confirmed by its detection in the wastewaters entering the Milano-Nosedo WWTP (Magni et al., 2019).

More than 30% of the plastic debris found in the two sampling stations belong to the class of synthetic fibers (Fig. 3), another proof of their secondary origin. As for their polymeric composition, we found only fibers of PEST (79.2%) and PP (20.8%) at NGC, while after the passage through the downtown of Milan the composition becomes more heterogeneous (Fig. 7), as fibers in polyacrylate (PAK; 20%), PU (6.7%) and PE (6.7%) also appear, although the PEST fibers remain the prevailing ones with a percentage of about 50%.

PEST is one of the most used synthetic fibers in the textile sector, accounting more than 50% of the fiber market worldwide (Lamichhane, 2018), especially for sportswear and as a padding for jackets and winter clothing, thanks to its well-known breathability. Furthermore, we can find it mixed with other fibers, even of natural origin, in any type of clothing, such as shirts, t-shirts and underwear. Also PP fibers are used in the textile field, especially to produce carpets, wallpaper and curtains for outdoor use, blankets, underwear, and technical-sport clothing.

One fifth of the fibers detected at NPC is represented by PAK fibers that have recently been recognized as a new fiber by EU Regulation 122/2018 and identified with the term "polyacrylate" in the mandatory labeling of textile products provided by EU Regulation 2007/2011. PAK confers to fibers a high degree of hygroscopicity which makes it particularly suitable for the production of technical clothing, especially for use in cold climatic conditions. The greater heterogeneity of the polymeric composition detected in this station, located at the exit of Milan, suggested a probable airborne transport of fibers or small discharges in its downtown course due, for example, to road washout. On the other hand, city dust is one of major land-based sources of microfiber pollution (Mishra et al., 2020), as also recently shown by Dris et al. (2017) who found a concentration of 60 synthetic microfibers/m^3 in indoor and outdoor samples in Paris.

From the ecotoxicological point of view, it is interesting to highlight how the different contamination pattern observed for this watercourse system compared to the first two described above is reflected in the effects produced in the specimens of *D. polymorpha* exposed to the two plastic mixtures sampled at NGC and NPC. The only endpoint that is statistically lower than the controls is that of the LPO measured at both NGC and NPC ($p < 0.01$; Fig. 7).

The decrease of LPO noticed for zebra mussels exposed to plastics' mixtures from 5 different sampling sites out 9 (Fig. 7) is quite interesting as normally LPO is a process in which different types of oxidants, such as free radicals and non-radical species, produce lipid peroxyl radicals and hydroperoxides especially on polyunsaturated fatty acids (PUFAs; Ayala et al., 2014). If LPO increases beyond a certain level, the oxidative damage exceeds the homeostatic recovery capacity, inducing cellular apoptotic processes (Volinsky and Kinnunen, 2013). Nevertheless, there are several studies that have highlighted how inhibition of LPO can also occur through several kinds of antioxidants (Niki et al., 2005). For instance, the behavior of nitric oxide (NO) is very particular because, despite being considered a pro-oxidant agent able to act of LPO, it can also have antioxidant activity, decreasing consequently LPO (Violi et al., 1999). The discriminating factor is represented by the presence or absence of the superoxide radical (O_2°), as there is a balance between NO and O_2° which accounted for the antioxidant and pro-oxidant effects of NO, depending upon the production and cellular concentration of NO and O_2° (Rubbo et al., 1994). The lack of an increase in SOD activity compared to controls (Fig. 7) would seem to indicate the absence of an O_2° overproduction, which would stimulate the antioxidant action of NO, with the consequent decrease in LPO observed even beyond the basal levels. Certainly, this is a crucial aspect that will need to be investigated in the future, also measuring the NO concentration after the plastic exposure.

4.4. Martesana-Redefossi system and Vettabbia Canal

These 3 watercourses are discussed together because, although the Redefossi Canal theoretically represents the continuation of the Martesana Canal, from which it originates in the center of Milan (Fig. 1), the plastic contamination observed at RDF seemed to be more similar to that obtained for VET in term of concentration. Indeed, from Table 1 it is possible to point out how the concentration of plastics measured at MAR (24 plastis/m^3) is the fourth most important, while RDF showed the lowest concentration ever (3 plastis/m^3), very similar to VET, which ranks second to last in terms of plastic contamination (7 plastis/m^3). The polymeric composition also confirms this observation since mainly the fibers are made up exclusively of PA and PEST at VET and RDF, while it is more heterogeneous at MAR, where we also found fibers in PP and PAK (Fig. 7). This greater similarity between RDF and VET is probably explained by the fact that the WWTP of Milano-Nosedo discharges the treated wastewaters into the Vettabbia Canal and with a collector of a few kilometers also into the Redefossi Canal, whose outlet is located 4 km further north of RDF (Fig. 1). This water discharge from Vettabbia Canal probably dilutes the contamination of plastics from Martesana Canal, bearing in mind that the other water source for Redefossi Canal is due to the Seveso River that remained dry throughout the entire sampling period, perhaps due to the blockage of the main watercourse due to maintenance and cleaning works that are carried out annually in the autumn.

Data obtained for VET are particularly interesting because the sampling point is located about 1 km after the Milano-Nosedo WWTP, whose outlet had already been sampled in our previous study (Magni et al., 2019). The comparison between the plastic concentrations observed during this survey and data obtained in the previous study, even if obtained in different years and seasons, seem to further confirm the importance in the choice of mesh for plastic sampling systems. In fact, the filtration of the treated wastewaters with a steel sieve with a mesh of 63 μm showed a concentration of plastics in the WWTP outlet equal to 400 ± 100 plastics/m^3, a much higher value than that detected using the net with 100 μm mesh at VET (7 plastics/m^3; Table 1). Surely there is also a dilution effect given by Vettabbia Canal, but not such as to justify a difference of almost 60 times between the plastic concentrations measured in the two surveys.

Since the Vettabbia Canal originates in the center of Milan (Fig. 1) and collects the treated wastewaters of the WWTP of Milano-Nosedo, the characteristics of the plastic mixture sampled at VET confirmed the secondary origin of the contamination, as can be clearly seen by the lack of pellets and the only presence of fibers (36%) and especially fragments (64%; Fig. 3). Even the appearance of a never detected polymer, such as PVAc, or very little found in the other stations, such as EVA, are indexes of the urban origin of the mixture of plastics present in VET. Indeed, PVAc is used to glue several porous materials, such as wood, paper and clothes, but it is also used as a gum base in chewing gum (Amann and Minge, 2012) and as adhesive for cigarette paper (Coggins et al., 2013). Even more urban are the uses for EVA, a polymer that produces materials similar to rubber in terms of softness and flexibility and that has recently become an alternative to polyvinyl chloride (PVC) due to the lack of chlorine as substituent (Meng, 2014). It is mainly used as padding in some sport equipment, as shock adsorber in sport shoes, in eyewear, but also in slippers and sandals and, as for PVAc, it is also used as an adhesive in packaging, textile, metal surfaces and coated paper (Reyes-Labarta and Marcilla, 2012).

The origin of the plastic contamination detected at RDF is more complex because, if it is true that the polymeric composition of the fibers and partially also that of the fragments are similar to VET, the fact that all the pellets, which make up 25% of the plastic shapes (Fig. 3), are in PMMA indicates further contamination of primary and industrial origin.

The greater contamination by plastics detected at MAR, as well as a greater heterogeneity of the shape and polymeric composition, should not be surprising, as the Martesana is an artificial canal much longer than the Redefossi and Vettabbia canals and coming from the Adda River, the only effluent of Lake Como, the third Italian lake by volume and depth. Furthermore, it is one of the watercourses entering the metropolitan city and therefore its contamination pattern does not reflect an urban release of plastics (Fig. 7). The contamination of plastics detected at MAR is dominated by fibers (about 54%) and fragments (about 33%) whose polymeric composition is mainly represented by PU, PA and PEST (Fig. 7). A peculiarity of the mixture of plastics detected at MAR is the non-negligible presence of fragments of polyacrylic rubber (12.5%), detected only at OLO (4.8%; Fig. 7). This polymer is a marker that indicates a specific secondary origin of plastics, namely the automotive industry which exploits the characteristics of polyacrylic rubber linked above all to its resistance to high temperatures. Polyacrylic rubber is recommended for products such as automatic transmission seals, extreme pressure lubricant seals, searchlight gaskets, belting, rolls, tank linings, hose, O-rings, rubber parts, coatings, pigment binders on paper, textiles, and fibrous glass. Since the Martesana Canal has a predominantly irrigation function without apparent significant inlets, the presence of these floating plastic fragments could be due to industrial discharges located upstream of its derivation from the Adda River or even to discharges located in Como Lake, whose main outlet is precisely this river. Another possible origin, linked to automotive use, could be that of a release from agricultural machinery that ends in the Martesana Canal from the numerous canals along its course.

Regarding the ecotoxicological effects, VET showed only a significant reduction ($p < 0.05$) of LPO compared to control. The same result was obtained in the Martesana-Redefossi system ($p < 0.05$), exactly as for NGC and NPC (Fig. 6). This is a further confirmation of the anomaly linked to this biomarker, as all the plastic mixtures administered clearly showed a significant and non-significant decrease in LPO, which is positively correlated ($r = 0.85$) to the number of plastics sampled in the different sites. This particular result could suggest not only investigating the possible formation of NO radicals, as discussed in Section 4.3, which could be the cause of this decrease in LPO, but may also indicate how this could represent a prognostic biomarker of plastic intake in the organisms.

4.5. Final consideration and comparison with other European rivers

From a general point of view, the entire dataset on qualitative and quantitative characterization clearly showed that it could identify not only the different primary or secondary origin of plastic contamination, but also provide a rather precise indication of their industrial or urban origin. Therefore, a detailed analysis of the plastic mixtures can help in the risk assessment and in the subsequent risk management, as it allows to highlight on which types of point or diffuse discharge to focus to possibly carry out a mitigation of the ecological risk associated with these emerging contaminants. This will be important above all considering the future new EU indications based on the research of MPs in drinking water and in surface waters for irrigation use, such as those coming out from several civil WWTPs. It is obvious that to have a valid support from this type of monitoring it is essential to achieve a standardization of collection, sample preparation and analytical phase methods, which are still lacking, as well as a shared definition of MPs and NPs. An example of this are the data shown in Table 2 that compare plastic concentrations recently found in many European aquatic rivers. As we can see, the

Table 2
Concentrations of plastics (plastics/m^3) measured in some European watercourses. Water sampled, mesh of the nets and the equipment for plastic identification are also shown.

Watercourse	Country	Plastics/m^3	Water sampled m^3	Filtration mesh µm	Identification system	References
River Rhine	The Netherlands	102,708–334,667	0.45–0.75	10	LDIR	Mughini-Gras et al., 2021
River Thames	Great Britain	14.2–24.8 (microfibers excluded)	n.a.	250	FT-iR ATR FT-iR µATR FT-iR	Rowley et al., 2020
Lambro River	Italy	0.4 ± 0.2–14.3 ± 11.0	9 ± 6–86 ± 2	300	µATR FT-iR	Magni et al., 2021
Ofanto River	Italy	0.9 ± 0.4–13 ± 5	75-522	333	Py–GC/MS	Campanale et al., 2020
River Dalälven	Sweden	4.5	n.a.	330	NIR ATR FT-iR	Van der Wal et al., 2015
Po River	Italy	14.6	n.a.	330	NIR ATR FT-iR	Van der Wal et al., 2015
Danube River	Romania	10.6	n.a.	330	NIR ATR FT-iR	Van der Wal et al., 2015
River Rhine	The Netherlands	1.85–4.92	n.a.	330	NIR ATR FT-iR	Van der Wal et al., 2015
Ebro River	Spain	3.5 ± 1.4	n.a.	5	µATR FT-iR	Simon-Sánchez et al., 2019
Seine River	France	3–108	0.43–2	80	Microscope[a]	Dris et al., 2015

n.a. = not available; LDIR = Laser Direct Infrared; FT-iR = Fourier-transform infrared spectroscopy; µATR FT-iR = FT-iR attenuated total reflection micro-spectroscopy; Py–GC/MS = Pyrolysis–gas chromatography/mass spectrometry; NIR = Near infrared spectroscopy; ATR FT-iR = FT-iR attenuated total reflection spectroscopy.
[a] Stereomicroscope coupled with image analysis software.

meshes of the nets used for plastic sampling varied from 5 µm (Simon-Sánchez et al., 2019) used for the plastic collection in the Ebro River (Spain) to the 330 µm used in the survey by Campanale et al. (2020) carried out in the Ofanto River (South Italy). The fact that net meshes are the main cause of the different quantity of sampled plastics is confirmed by the negative correlation ($r = -0.55$; $p = 0.083$) between the meshes of the nets used in these monitoring campaigns and the relative concentration of plastics measured. It is true that in the context of the Marine Strategy Framework Directive (2008/56/UE) it is now customary to use Manta net with 300-330 µm of mesh, but with a view to improving environmental monitoring we suggest using nets with finer mesh for the sampling of plastics, in order to be able to take a greater amount of them, even of types that often totally or partially escape from too large mesh, such as fibers.

The equipment used for these evaluations of the polymer composition are also quite heterogeneous, even if most of these monitoring campaigns were performed using ATR FT-IR or µATR FT-IR (Table 2). In this context, it is necessary to use instruments capable of recognizing the smaller plastic debris which represent the most dangerous fraction for ingestion, accumulation capacity and consequently toxicity in organisms. Unfortunately, there are still too many studies or scientific reports carried out with inadequate instrumentation, unable to recognize plastic debris smaller than 1 mm or even based exclusively on visual sorting. The risk of exclusively using visual recognition is to overestimate the amount of plastic detected as, for example, a percentage between 9% (LAD) and even 68% (VET) of the debris from our 9 plastic mixtures by means of visual sorting were not actually synthetic polymers, but only natural fibers, vegetable fragments and fibers, or even minerals.

Considering all these differences, Fig. 8 shows the comparison between the mean of the plastic concentrations obtained in the different surveys in some European rivers and the data obtained for the 9 sampling stations of the metropolitan city of Milan. It is immediately clear how the plastic contamination observed at OLO and in the two sampling sites located in the Lambro River indicate an extremely worrying situation, as they are much higher than the levels detected in all the other European rivers, apart from the River Rhine which was, however, the one in which it has been used the second finest filtration mesh (Mughini-Gras et al., 2021). The plastic contamination found at LAU is comparable to that detected in the Seine River, where the net with the mesh (80 µm) more similar to ours was used, while LAD and especially OLO showed a plastic debris contamination from 4 to 5.5 times higher than the maximum value found in this French river (108 plastics/m^3).

The levels of plastic achieved in the other 6 watercourses of the metropolitan city of Milan are instead comparable to those found in the European rivers, also considering the greater quantity of plastic sampled with our finer mesh net. This is not entirely reassuring in terms of risk assessment, as all these Lombard watercourses are small, short and with a flow rate lower than the great European rivers, such as the Danube, Rhine, Thames, Seine, Ebro and the Po River itself.

The ecotoxicological profile has shown that there is no relationship between the endpoints that have changed and the concentration of plastics detected. Furthermore, all the effects caused, even by the most numerous plastic mixtures detected at OLO, LAD and LAU, are mainly linked to homeostatic responses involved in the antioxidant action and in the control of the redox balance, while was rather interesting the decrease of LPO observed in all the treatments, that could open new windows in the evaluation of some biomarkers for prognostic purposes in the field of plastic contamination. It is certainly necessary, however, to emphasize that the plastic mixtures do not seem to have created heavy ecotoxicological effects on the bivalves exposed, at least as regards the measured endpoints. The imbalance of redox cycle and the activation of some enzymes involved in the antioxidant machinery are

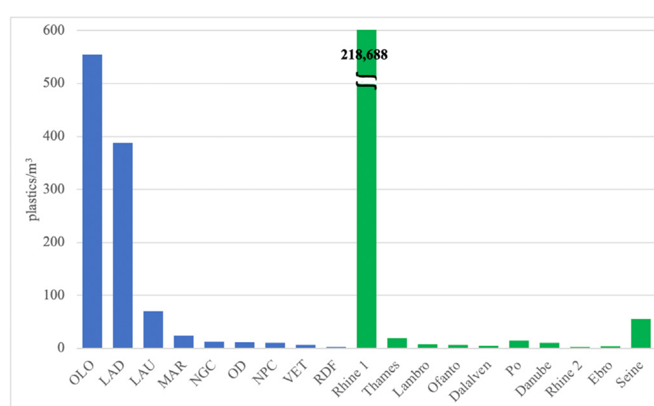

Fig. 8. Comparison between the mean plastic concentrations measured in the 9 sampling sites of the metropolitan city of Milan (in blue) and those obtained in some European watercourses (in green).
OLO: Olona R.; OD: Olona Diverter; LAU: Lambro R. Upstream; LAD: Lambro R. Downstream; NGC: Naviglio Grande Canal; NPC: Naviglio Pavese Canal; MAR: Martesana Canal; RDF: Redefossi Canal; VET: Vettabbia Canal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effects detected in numerous other previous studies performed with different biological models exposed to MPs (Qiao et al., 2019; Xia et al., 2020; Solomando et al., 2020; Tlili et al., 2020), but above all they are effects also observed in the two other monitoring campaigns carried out following the same experimental plan of this survey (Binelli et al., 2020; Magni et al., 2021). In the monitoring of the Lambro River (Magni et al., 2021), we also detected acute effects, linked to a net increase in mortality of *D. polymorpha* specimens exposed to the two most abundant mixtures of sampled plastics. Since the exposure of the bivalves lasted 21 days, it is likely that the exposure time plays a fundamental role in the effects related to the exposure to plastics, as in the present study the exposure to the plastic debris mixtures could have been only one week long and no acute effects have been observed also by exposing the bivalves, whose lifespan ranges between 3 and 9 years (Benson et al., 2020), to higher plastic concentrations.

5. Conclusions

The approach used in this study made it possible to evaluate the contamination by plastics in one of the most industrialized and anthropized European areas, highlighting how their qualitative and quantitative evaluation clearly points out that rivers belonging to the same catchment sub-basin can have different sources of contamination due to plastics, with the sole exception of the two sampling stations located on the Lambro River. Indeed, this survey demonstrated how some rivers are most affected from a release of plastic debris of industrial origin, as in the case of the Olona River and Lambro River, while other watercourses, such as Vettabbia Canal and Redefossi Canal, present a pollution more of urban origin, as it is characterized by polymers present in common use objects. A good characterization of the plastic debris and the correlation between the different characteristics of the plastics detected effectively allows us to suggest how several polymers and shapes could be considered as typical markers of a specific source, as for example fibers and fragments in PU, EVA and PVAc can be clear indicators of an urban origin, while pellets in PMMA or PS are signals of the presence of industrial wastes.

The experimental design performed in this way allowed to highlight the impact that plastic mixtures sampled in these natural environments can have on the aquatic organisms, reaching a higher ecotoxicological realism. This allowed to point out a low toxicity due to the sampled plastics, at least as regards the measured endpoints, the exposure times of the *D. polymorpha* specimens and the selected experimental conditions.

In conclusion, we hope that the results obtained in this study could represent further confirmation of the need for recourse to a new way of conceiving the study of the impact of plastic mixtures in a more realistic manner, which may be useful for a future standardization of correct procedures and experimental plans for determining the effects due to exposure to plastics.

CRediT authorship contribution statement

Andrea Binelli is the corresponding author, and he was responsible for conceptualization, funding acquisition, project administration, supervision and writing.

Stefano Magni was responsible for conceptualization, sampling, analyses, methodology, review and editing.

Camilla Della Torre was involved in sampling, analyses, review and editing.

Lara Nigro was responsible for analyses, review and editing.

Nicoletta Riccardi was involved in sampling and review.

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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