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Research Paper

Characterization of plastics and their ecotoxicological effects in the Lambro River (N. Italy)

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ABSTRACT

This study had the dual objective of both the qualitative and quantitative assessment of plastic mixtures sampled in 5 different sites located along the Lambro River (northern Italy), and the contemporarily determination of the ecotoxicological effects of the same mixtures sampled, through 21-day laboratory exposures of the freshwater bivalve *Dreissena polymorpha*. The monitoring survey was carried out by a Fourier Transform Infrared Microscope System, while the ecotoxicological assessment was performed by the mussel mortality, a biomarker suite and the proteomics. The main results of the monitoring have highlighted some critical points, related to the concentration of plastics detected at Milan and, especially at the southernmost sampling station, where a daily flow of more than 6 million plastic debris has been estimated, ending directly into the Po River, the main Italian river. The ecotoxicological analysis highlighted how the toxicity is not exclusively due to the plastic concentration, but that the different characteristics of the polymers probably become more important. Furthermore, we observed an extensive mortality of bivalves exposed to the sampled mixtures in the two southernmost sampling stations, while the battery of biomarkers and the results of proteomics have highlighted how the sampled plastic mixtures caused an imbalance in the redox state, already indicated as a classic effect due to plastic exposure, but also an impact on energy stock and on some fundamental cellular pathways always linked to energy metabolism.

1. Introduction

It has recently been suggested to call the current geological unit of time as Anthropocene, a term used to describe the most recent period in Earth's history when human activity began to have a significant impact on the climate and ecosystems (Zalasiewicz et al., 2019). Some phenomena associated with the Anthropocene include erosion due to urbanization and agriculture, anthropogenic perturbations of element cycles, global warming, ocean acidification, habitat loss and, lastly, the global dispersion of plastics.

The increasing production of plastics worldwide, which reached 359 million tonnes in 2018 (PlasticsEurope, 2019), and especially the improper release of plastic items mainly into aquatic ecosystems are currently one of the biggest environmental problems. In addition to the fact that the so-called macroplastics cause known damage to aquatic organisms, the plastic items can be also fragmented into smaller debris, forming microplastics (MPs) and nanoplastics (NPs), for whose definition a modification has recently been suggested (Hartmann et al., 2019) consistently with the International System of Units (SI), as macroplastics ($\geq$ 1 cm), mesoplastics (1 mm $<$ 10 mm), MPs (1 μm $<$ 1 mm) and NPs (1 nm $<$ 1 μm). This is the definition followed in our study.

Because of their small size and ubiquity, MPs and NPs are more prone to enter the aquatic organisms (Besseling et al., 2015; Webb et al., 2019; Moore et al., 2020; Kazour and Rachid, 2020) and to be ingested and accumulated within the digestive tract of marine and freshwater organisms (Magni et al., 2018; Lefebvre et al., 2019; Sun et al., 2019). There are also several studies which demonstrated their capability to translocate in all the internal tissues (Ding et al., 2018; Magni et al., 2018; Parenti et al., 2019a; Elizarde-Velàzquez et al., 2020). In relation to the adverse effects due to these emerging contaminants, there is a plethora of ecotoxicological studies showing several damages ranging from physical injuries, such as intestinal blockage and villi disruption (Lei et al., 2018), changes in gills and digestive gland (Bråte et al., 2018), to molecular effects mainly reflected in an increase of oxidative stress (Magni et al., 2018; 2019a; Qiao et al., 2019; Xia et al., 2020), changes in immune responses (Limonta et al., 2019), neurotoxicity (Barboza et al., 2018), altered gene expression (Granby et al., 2018) and modulation of proteins involved in many cellular pathways (Green et al., 2019; Magni

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et al., 2019a).

In this new ecotoxicological field, one of the first steps to take is certainly the identification of the mechanisms of interaction with organisms to highlight which type of physical and chemical properties (size, shape, color, density, crystallinity, stability, surface change) could increase absorption, translocation and accumulation of MPs and NPs. To do this, it is necessary to carry out experiments conducted at laboratory conditions, in order to eliminate any environmental interference, and using high concentration of MPs and NPs to simplify the observation of their transport and accumulation in the body districts. However, almost all recent studies aimed to describe the adverse effects of these physical contaminants have been carried out considering concentrations far from the experimental and expected levels in the field. Lenz et al. (2016) pointed out that the experimental exposure concentrations tested to evaluate the impact of MPs on marine organisms are between two to seven orders of magnitude higher than environmental levels. Moreover, many experiments have been conducted using only one or few sizes and shapes of MPs and NPs, mainly micro- or nano-beads, which do not reflect the complexity of plastic mixtures found in the environment, also considering the number of polymers collected in natural samples. At present, it appears that the numerous studies relating to the qualitative and quantitative assessment of MPs in aquatic ecosystems do not fit with the evaluation of their effects conducted by laboratory experiments, which simplify too much the complexity of this environmental contamination. This is also due to the discrepancy between the size of plastics normally collected by a Manta-trawl, whose net have a mesh of 300–330 μm, and laboratory studies that often investigated the impact of smaller plastic debris.

In this context, we tried to connect the environmental monitoring of plastics in one of the most urbanized and industrialized European freshwater basins with the direct evaluation of the effects made by the collected plastic mixtures, in order to assess their environmental hazard. In detail, we collected the plastic debris from 5 sampling points along the Lambro River (N. Italy), one of the main tributaries of the longest Italian river (R. Po). The survey was conducted in 3 different days of a week, sampling each day the selected locations, for a total of 30 samples. The plastic mixtures were then quantified and characterized by a Fourier Transform Infrared Microscope System (μFT-iR), while the effect evaluation was obtained by laboratory exposures of the freshwater bivalve *Dreissena polymorpha* (zebra mussel) to the 5 plastic mixtures for 21 days.

A multi-step approach was used to identify the impact due to plastics in zebra mussels, measuring at the end of exposure several endpoints covering many levels of the biological organization, from the molecular and cellular ones to organism. In detail, mussel mortality was measured during the exposures to check the acute toxicity of plastic mixtures, while a biomarker suite was used to identify many cellular and molecular effects. We also applied a high-throughput technology, as the gel-free proteomics, for the evaluation of protein modulation on zebra mussels collected at the end of exposures.

In this way, we have achieved the two components necessary for the environmental risk assessment, represented both by the evaluation of the levels of plastic mixtures in an aquatic ecosystem and by the simultaneous identification of their adverse effects on a species that lives in the studied catchment basin. This approach based on the risk evaluation of plastics directly sampled in aquatic ecosystems, with the opportune improvements, should be the starting point for this kind of studies, also bearing in mind other possible interferences generally not considered, or too simply handled, in laboratory experiments, such as the plastic weathering and the adsorption of many environmental pollutants which can heavily change the toxicological behavior of plastics.

2. Materials and methods

2.1. Sampling of plastics and sample pre-treatment

Lambro River, along its course of about 130 km, crosses a great industrialized and urbanized area of the Po Valley, receiving the effluents of more than 30 wastewater treatment plants (WWTPs), as well as several artificial or natural tributaries, as the Naviglio Martesana, Seveso and Olona Rivers and Addetta Canal (IRSA, 1997). For this heterogeneous situation, we decided to monitor the plastic contamination in 5 different points along its course: 1) we considered as northernmost sampling point the station of Merone (latitude: 45.786809, longitude: 9.245879, Como, Italy,), at about 20 km from the Lambro source, which represents its outlet from the Alserio and Pusiano Lakes, 2) Brugherio (45.550943, 9.268330, Monza-Brianza, Italy), that is located after the outlet of one of the greatest WWTPs of the northern area of Milan; 3) Milano (45.498669, 9.248415), selected to investigate the impact of the second most populated Italian city; 4) Melegnano (45.355903, 9.328401, Milan, Italy), located at few kilometers south of the main WWTP of Milan; 5) Graffignana (45.210606, 9.460534, Lodi, Italy), near the closing station of Lambro River (Lambrinia) and located at about 15 km from its inlet into the Po River.

To perform the sampling of floating plastics for both monitoring and ecotoxicity evaluation, we used simultaneously two plankton nets (mesh of 300 μm), dropped by bridges in the center of the water flow for 30 min. One of these nets was equipped with a flowmeter (General Oceanics, Inc., Model 2030R) to calculate the volume of filtered water during each sampling. To reduce the intrinsic variability of samples, we performed an integrated sampling for 3 days during the same week in December 2018.

For each sampling point, the following water volumes (mean values on the 3 days of sampling ± standard deviation, SD) were filtered in 30 min: 40 ± 6 m^3 for Merone, 86 ± 2 m^3 for Brugherio, 45 ± 12 m^3 for Milano, 19 ± 14 m^3 for Melegnano and 9 ± 6 m^3 for Graffignana.

The collected material was recovered in 0.5 L glass bottles with metal cap, washing the nets with 500 mL of sodium chloride (NaCl) hypersaline solution (1.2 g/cm^3) previously filtered on glass-fiber filters with a mesh of 1.2 μm (Whatman GF/C 47 mm) to eliminate any impurity. The hypersaline solution allowed to separate the floating plastics from the great amount of suspended matter present in the samples.

Samples (recovered in 30 glass bottles, 15 for monitoring and 15 for the ecotoxicity evaluation) were transported to laboratory and then stored at 4 °C. Subsequently, samples were processed as reported by Binelli et al. (2020). In detail, samples in the glass bottles (the hypersaline solution and the other interfering materials collected) were filtered on a steel sieve with a mesh of 63 μm to retain plastics and the coarse matter, as leaves, branches and insects. The hypersaline solution, passed through the mesh, was collected in an aluminum container. The collected coarse materials on the sieve were washed by another aliquot of fresh hypersaline solution into the aluminum container to avoid the loss of eventual plastics adhered on their surface, and then manually eliminated through metal tweezers. The recovered plastics on the steel sieve, as well as the hypersaline solution filtered on the sieve, which contains the recovered plastics from the coarse materials, were re-collected in the glass bottles to allow the density separation between the synthetic debris and the suspended organic/inorganic matter. The eventual sludge formation on the bottom of glass bottles was eliminated by siphoning (Binelli et al., 2020). As reported in the next paragraphs, two quite different methods were followed to obtain the samples dedicated both to monitoring and ecotoxicological assays, respectively.

2.2. Plastic monitoring: quantification and characterization

The steps above described had the main function to simplify the filtration of the hypersaline solution supernatant, which contains the floating plastics, avoiding the filter occlusion. After this pre-treatment,

samples for plastic monitoring (15 bottles) were filtered on cellulose nitrate membrane filters (mesh of 8 μm, Sartorius™ 50 mm) using a vacuum pump. Filters were then washed with 500 mL of ultrapure water to remove all traces of NaCl. Subsequently, to degrade any residues of organic matter, the filters were digested with 15% solution of hydrogen peroxide (H_2O_2) for 3 days, renewing the H_2O_2 solution when needed, avoiding the sample drying. This procedure was conducted maintaining the filters in Petri dishes under a laminar flow hood, in order to avoid any atmospheric contamination by plastics (Magni et al., 2019b). In this regard, 5 cellulose nitrate membrane filters, one for each sampling station, were processed as blanks to monitor any possible contamination during the entire sample treatment.

Filters were then observed through a stereo-microscope to identify the particles with a suspected plastic nature (visual sorting). Recognized particles were placed on clean filters to be quantified and characterized in terms of chemical composition, shape, color and size. Regarding the polymer characterization, we used a μFT-iR (Spotlight 200i equipped with Spectrum Two, PerkinElmer) and the infrared spectra were obtained in Attenuated Total Reflectance (ATR) with 32 scans and wavelengths between 600 and 4000 cm^{-1}, analyzed using the Spectrum 10 Software and matched with standards found by the PerkinElmer libraries. Furthermore, the relative peaks of each spectrum were carefully checked by the operator to avoid errors of identification. Only the spectra with a matching score $\geq$ 0.70 were considered acceptable (Magni et al., 2019b).

Collected particles were subsequently classified according to their shape (fragments, films, fibers, pellets/beads and lines) and color. Lastly, using the ImageJ Software (Ferreira and Rasband, 2012), and in accordance with the dimensional classification proposed by Hartmann et al. (2019), all collected debris were characterized on the basis of their size, measuring only the major length (mm) and considering two decimals in the results (Table S1).

2.3. Evaluation of plastic ecotoxicity

Regarding the preparation of samples for ecotoxicity (15 bottles), after the cleaning procedure reported in the paragraph 2.1, the supernatant of each sample was filtered again on a 63 μm mesh sieve to eliminate the fine suspended particulate matter that could have interfered with the ecotoxicological results, being possible carrier of chemical contaminants. Indeed, since the particulate matter in suspension was commonly defined as the material filtered off with a 0.45 μm filter (Eisma, 1981), our sieving at 63 μm surely eliminated this possible interfering fraction, retaining only few natural coarse materials, whose larger visible pieces have been eliminated. Then, sieve was rinsed with ultrapure water, adding the plastics directly in the exposure tanks with the zebra mussel specimens. Animals were collected in January 2019 in the same site of Lake Maggiore by a scuba diver and transported to the laboratory in bags with lake water. Mussels were maintained for two weeks in 10 L acclimation tanks with tap/deionized water (1:1), at 20 ± 1 °C, in saturating oxygenation conditions (> 90%), and fed with a water suspension of *Spirulina spp*. The water of tanks was changed every 3 days (Magni et al., 2016, 2017, 2018, 2019a, 2020). This maintenance step allowed also the elimination of any eventual chemical and physical contaminants present in the mussels.

For the exposures, we used 6 tanks (1 control and 5 treated with plastics from Merone, Brugherio, Milano, Melegnano and Graffignana) of 4 L filled with plastics and tap/deionized water (1:1). The tested concentrations of plastics for each experimental group were those detected through the monitoring process in each sampling station, since the two plankton nets were put in the water contemporary: 4.9 plastics/L for Merone, 8.4 plastics/L for Brugherio, 19.2 plastics/L for Milano, 4.3 plastics/L for Melegnano and 24.9 plastics/L for Graffignana.

In each tank we put 75 bivalves placed on a metallic net, with a magnetic stirrer and oxygenation to maintain homogenously the plastics in the water column. The tanks were then covered with an aluminum sheet during the entire exposures avoiding any contamination mainly by atmospheric microfibers. We performed an exposure of 21 days (from t = 0 to t = 21), in semi-static condition, renewing the water and plastic suspensions at the end of each week (t = 6 and t = 14) with the plastics collected in each of the 3 days of sampling. During the exposure, the animals were fed daily with a suspension of *Spirulina spp*.

2.3.1. Acute toxicity and biomarker evaluation

Mussel mortality was assessed as endpoint of acute toxicity during the entire exposure. For the biomarker evaluation, the methods on zebra mussels are reported in our previous studies (Magni et al., 2016, 2017, 2018, 2020). Briefly, the organisms were collected from the acclimation tanks to evaluate the basal level (t = 0) for each endpoint of chronic toxicity to compare with those found in our previous experiments. In detail, we used the following number of mussels: a pool of 5 mussels from the acclimation tanks for the antioxidant/detoxifying enzymes (superoxide dismutase; SOD, catalase; CAT, glutathione peroxidase; GPx, and glutathione S-transferase; GST) and reactive oxygen species (ROS) evaluation, a pool of 5 animals for the oxidative damage (lipid peroxidation, LPO; protein carbonyl content, PCC), the hemolymphs of these specimens was used for the cyto- and genotoxicity assessment, gills from 5 animals for P-glycoprotein (P-gp) measurement and a pool of 5 animals for the neuro-enzyme monoamine oxidase (MAO) assessment (total of 20 mussels).

For the evaluation of the effects made by plastics, we collected at the end of exposure (t = 21) 9 mussels/measurement, instead of 5, from each exposure tank to evaluate the same biomarkers described above. This increase in the number of animals in comparison with the check of baseline levels was necessary to obtain 3 biological replicates. In detail, the antioxidant/detoxifying enzyme activities (SOD, CAT, GPx; GST) and ROS were evaluated in triplicate (technical replicates) on 3 pools of 3 mussels per treatment (biological replicates).

Firstly, mussels were homogenized using a potter in 100 mM phosphate buffer (pH = 7.4), 1:10 w/v ratio, with potassium chloride (KCl) 100 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, dithiothreitol (DTT) 1 mM and protease inhibitors (1:100 v/v). Homogenates were then centrifuged at 15,000*g* for 30 min at 4 °C (S15 fraction). Proteins were quantified using the Bradford (1976) method, to normalize the enzyme kinetics, at the 6715 UV/Vis spectrophotometer (Jenway). More in detail, SOD activity was assessed measuring the inhibition of 10 μM cytochrome c reduction at 550 nm due to the superoxide anion originated by the xanthine oxidase and 50 μM hypoxanthine. CAT activity was evaluated measuring the consumption of 50 mM H_2O_2 at 240 nm, while GPx activity was measured evaluating the nicotinamide adenine dinucleotide phosphate (NADPH) consumption at 340 nm with 0.2 mM H_2O_2, 2 mM glutathione, 1 mM sodium azide (NaN3), 2 U/mL glutathione reductase and 120 μM NADPH. Lastly, GST activity was measured adding to the S15 the 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 the ROS quantification, 10 mg/mL of dichlorofluorescein-diacetate (DCFH-DA) in dimethyl sulfoxide (DMSO) was used. In particular, 20 μL of S15 fraction were added to a 96-well plate and incubated for 5 min at 37 °C. Subsequently, 100 μL of phosphate buffer saline (PBS) and 8.3 μL of DCFH-DA were added to each well, then incubated at 37 °C for 30 min. The fluorescence was read at 485 nm (excitation) and 530 nm (emission) at the EnSight™ multimode plate reader (PerkinElmer; Parenti et al., 2019b).

Regarding the P-gp, the efflux activity was evaluated on mussel gills (Navarro et al., 2012). In particular, 9 biopsies from the gills of 9 animals per treatment were incubated in tap/deionized water (50:50 v/v) with the fluorescent substrate rhodamine B (RhB; 1 μM), for 90 min at room temperature (RT) and in dark condition with gentle shaking. After this procedure, the biopsies were washed twice and stored at −80 °C. Subsequently, 300 μL of tap/deionized water (50:50 v/v) were added to each biopsy, homogenized and centrifuged for 10 min at 14,000*g*. The

RhB fluorescence was read in triplicate at 545 nm (excitation) and 575 nm (emission) through the EnSight™ multimode plate reader (PerkinElmer; Magni et al., 2017).

The LPO and PCC were measured in triplicate on 3 pools of 3 mussels per treatment. Mussels were homogenized 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 directly in the crude homogenate using the Bradford (1976) method. We evaluated the LPO and PCC in accordance with Ohkawa et al. (1979) and Mecocci et al. (1999), and the absorbance was read using the 6715 UV/Vis spectrophotometer (Jenway). In particular, LPO was measured through the evaluation of thiobarbituric acid-reactive substances (TBARS) and reading the absorbance at 535 nm, while for PCC the reaction of carbonyl groups with the 2,4-dinitrophenylhydrazine (DNPH) was exploited. The absorbance was read at 370 nm. Regarding the cyto-genotoxicity, the hemolymph was collected from the abductor muscle of 9 mussel per treatment (the same specimens used for LPO and PCC) using a hypodermic syringe with 100 μL of EDTA/PBS 10 mM to avoid cell agglutination. The hemocyte viability was evaluated using the Tripan Blue exclusion method (Strober, 2015). The micronuclei assays (MNs) were assessed on zebra mussel hemocytes as reported by Pavlica et al. (2000) and 400 cells for each slide were counted (9 slides per treatment). The apoptotic and necrotic frequencies were measured in accordance with Singh (2000) and 300 cells for each slide (5 slides per treatment) were counted. Regarding the neurotoxicity, 3 pools of 3 mussels per treatment, without gills, were homogenized in 100 mM phosphate buffer (pH = 7.4), 1:10 w/v ratio, with 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and protease inhibitors (1:100 v/v). Homogenates were then centrifuged at 1000*g* for 20 min at 4 °C (S1 fraction). Proteins were quantified using the Bradford (1976) method to normalize the neuro-enzyme kinetic. The activity of MAO was measured in S1 fraction using tyramine 1 mM as substrate, DCFH-DA 10 μM in NaCl 140 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/-sodium hydroxide (HEPES-NaOH) buffer 10 mM, pH = 7.4, peroxidase 1 mg/mL and 3-amino-1,2,4-triazole10 mM. The fluorescence was read for 3 min at 485 nm (excitation) and 530 nm (emission) at the EnSight™ multimode plate reader (PerkinElmer; Gagné, 2014, Magni et al., 2018).

2.3.2. Gel free proteomics

The analysis was conducted on the gills of exposed specimens, using a gel free method as reported by Magni et al. (2019a). In detail, considering that the activity of MAO was evaluated on the soft tissues of mussels without gills (Magni et al., 2018), we used these organs to perform the proteomic analysis (3 pools of 6 gills per treatment, with 3 technical replicates for each sample).

Gills were homogenized using a potter in a buffer with HEPES 20 mM pH 7.5, sucrose 320 mM, EDTA 1 M pH 8.5, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 5 mM pH 8.1, sodium orthovanadate (Na3VO4) 1 mM, β-glycerophosphate 10 mM, sodium fluoride (NaF) 10 mM, sodium pyrophosphate (NaPPi) 10 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM in ethanol, DTT 5 mM and protease inhibitors (Roche) in ultrapure water. Homogenates were centrifuged at 15,000*g* for 10 min at 4 °C. Proteins were quantified using the Bradford (1976) method.

Subsequently, in each sample, 300 μg of proteins were precipitated using methanol/chloroform/ultrapure water mixture (4:1:3 v/v). The pellets were suspended in urea 8 M in tris hydrochloride (Tris-HCl) 50 mM with NaCl 30 mM pH 8.5 and protease inhibitors (Roche). Samples were then centrifuged at 14,000*g* for 30 min at 4 °C. Proteins were re-quantified through the Bradford (1976) method. Then, DTT 50 mM in ammonium bicarbonate (AMBIC) 50 mM was added to 10 μg of proteins for each sample and incubated for 30 min at 52 °C under stirring at 600 rpm. Iodoacetamide ($IANH_2$) 100 mM in AMBIC 50 mM was subsequently added and incubated for 20 min at RT. Proteins were digested using trypsin (Trypsin Sequencing Grade, Roche, Italy) in AMBIC 50 mM and incubated over-night at 37 °C under stirring at 400 rpm. Peptides were purified using Zip Tips (μ-C18; Millipore, Milan, Italy).

Protein characterization (5 μL of each sample, in triplicate) was performed at UNITECH OMICs (University of Milan, Italy) through a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with nanoelectrospray ion source. Proteins were identified using the Proteome Discoverer Software 2.2 (Thermo Scientific), selecting the Uniprot-Bivalvia database and trypsin as digestive enzyme (Magni et al., 2019a).

2.3.3. Uptake evaluation

At the end of exposure (t = 21 days) we processed 10 mussels from each exposure tank for the evaluation of plastic uptake. As describe in Binelli et al. (2020), the specimens were pooled and homogenized in NaCl hypersaline solution (1.2 g/cm^3) using a potter. The obtained supernatants were filtered on cellulose nitrate membrane filters. Samples were then digested with 15% H_2O_2 under a laminar flow hood. All particles extracted by mussels were quantified and characterized using the μFT-iR (Spotlight 200i equipped with Spectrum Two, PerkinElmer) with the same instrumental setting used for the characterization of plastics (Section 2.2).

2.4. Statistical approach

Data normality and homoscedasticity were assessed using the Shapiro–Wilk and Levene tests respectively. We evaluated the covariation between the volume of filtered water and the relative number of detected plastics by means of a Pearson correlation test. This aspect was important to exclude that a maximum quantity of plastic in the exposure tanks corresponded to a sample derived from a high volume of filtered water.

To evaluate the significant differences (*$p < 0.05$; **$p < 0.01$) between the plastic amount in the different stations along the Lambro River, the one-way analysis of variance (one-way ANOVA), followed by the Fisher LSD *post-hoc* test, was performed. In the same manner, we used the above-mentioned tests to evaluate the significant differences between treated and control, at the end of exposure (t = 21 days), in the context of biomarker evaluation. The STATISTICA 7.0 Software was used in these analyses.

Regarding the gel free proteomics, only the proteins with a coverage score ≥ 1% with at least 2 identified peptides were considered in the study. In addition, the differences in abundance ratio (AR) of proteins, between treated and control, were considered only with at least a 2-fold change and with a standard deviation between replicates less than 20%. Lastly, as further refine, a Student T-test was performed to consider only the proteins with a significant AR variation (*$p < 0.05$).

3. Results

First of all, the analyses of blanks confirmed the absence of accidental contamination by plastics in our samples, considering that no plastics were detected on the 5 filters analyzed as controls (only 15 cellulose fibers in the total of 5 filters were observed). Based on the volumes of water filtered in each sampling site and day (see Section 2.1), no significant correlation (r = 0.23) with the number of detected plastics was obtained, underlining the goodness of our decision to base our samplings on the sampling time rather than on the volume of water collected.

3.1. Qualitative and quantitative assessment of sampled plastic mixtures

Entering in the context of the plastic mixtures found in all the 5 sampling sites, a total of 59 plastic debris were quantified and characterized in the sample from Merone in the 3 days of sampling (Table S1) with a mean value of 19.7 ± 14.2 plastics, which corresponded to the quantity of plastics put in the 4 L tank of Merone group during the 21

days of zebra mussels' exposure (4.9 plastics/L). On the basis of filtered water volume, we calculated a concentration of 0.5 ± 0.3 plastics/m^3 in this sampling point (Fig. 1), corresponding to about 215,000 plastics that pass daily through Merone, if we consider as 5 m^3/s the mean flow rate of Lambro River (Calamari et al., 2003; Castiglioni et al., 2011).

In detail, the MPs were the main size of debris detected at Merone (63%; Fig. 2), as well as fragments were the main shape (52%; Fig. 3). About color, white debris were the principal collected ones (54%), while the principal represented polymer class was polypropylene (PP – 58%; Fig. 4).

A total of 101 plastic debris were quantified at Brugherio in the 3 days of sampling (Table S1) reaching a mean value of 33.7 ± 21.1 plastics, which corresponded to the mean quantity of plastics put in the tank of this experimental group (8.4 plastics/L). We calculated an amount of 0.4 ± 0.2 plastics/m^3 in this sampling point, not significantly different to Merone (Fig. 1), and represented by 57% MPs (Fig. 2). As observed for Merone, fragments were the main plastic shape (57%; Fig. 3). The concentration of fibers increased in Brugherio, reaching the 14% and doubling that found in the northernmost station. Regarding the polymer composition, the main detected polymer was polyethylene (PE-36%; Fig. 4). The white was the color most found in the sampled plastics, as for Merone, reaching the 64% of detected debris. Considering the above-mentioned results, we calculated that about 170,000 plastics cross daily this sampling point, an amount almost equivalent to that found in the previous site.

Samples from Milano started to show an increase in plastic pollution although not significant in comparison with the two northernmost stations, since a total of 231 plastic debris were quantified and characterized in the 3 days of sampling (Table S1), corresponding to a mean value of 77.0 ± 36.3 plastics (19.2 plastics/L added in the exposure tank). In detail, we calculated 1.7 ± 0.6 plastics/m^3 in this sampling point (Fig. 1), represented by 75% MPs (Fig. 2). Differently to the two northernmost sites, the pellets/beads were the main shape of plastics (55%; Fig. 3). The white was confirmed as the main color of synthetic debris, while polystyrene (PS) was the main detected polymer (48%; Fig. 4) The daily amount of plastics that cross this point increased to about 730,000 debris.

Moving further south along the course of the Lambro River, we sampled the site of Melegnano in which a total of 52 plastic debris were quantified and characterized in the 3 days of sampling (Table S1). A mean value of 17.3 ± 4.5 plastics was calculated, which corresponded to the mean quantity of plastics put in the exposure tank for this site (4.3 plastics/L). Regarding the plastic amount found here, we obtained a concentration of 1.3 ± 0.7 plastics/m^3 (no significant differences in comparison with the other 3 sampling stations were reported; Fig. 1), with 52% MPs and 48% mesoplastics (Fig. 2). The main shape of plastics were fragments (69%; Fig. 3), while transparent (56%) was the main observed color.

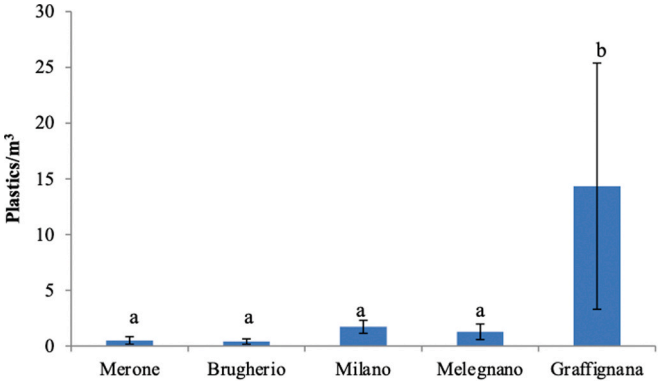

Fig. 1. Amount of plastics (plastics/m^3) detected in the 5 different sampling stations along the Lambro River. The letters indicate the significant differences between the sampling stations (one-way ANOVA).

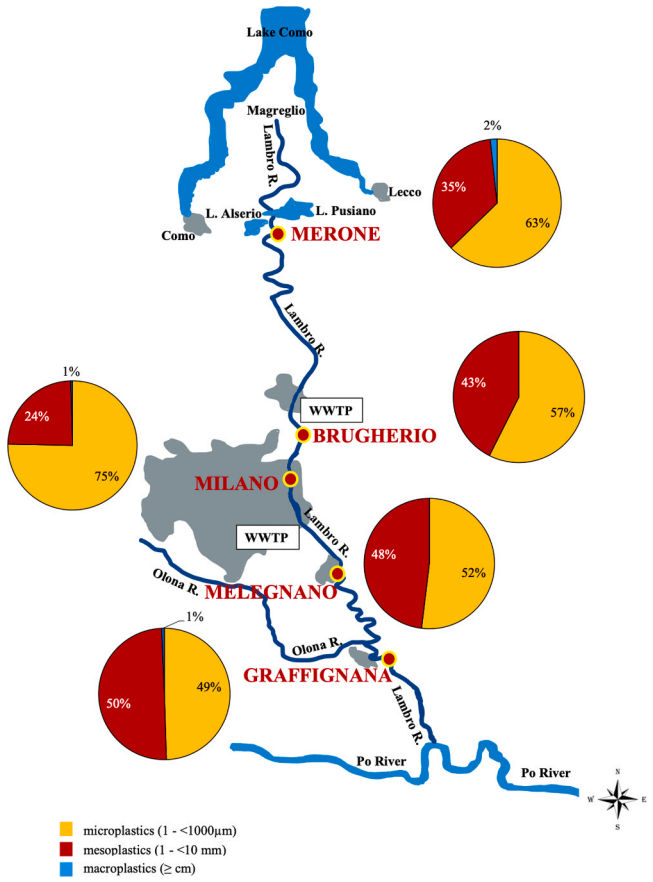

Fig. 2. Plastic size – percentage of micro-, meso- and macroplastics detected along the Lambro River. The two main WWTPs that reverse the treated effluents in the Lambro Rivers are reported.

Lastly, we observed at Melegnano a high concentration of PE (42%; Fig. 4), and we calculated that about 560,000 plastics cross daily this station.

At the southernmost sampling point of Graffignana, a total of 299 plastic debris were quantified in the 3 days of sampling (Table S1) and a mean value of 99.7 ± 67.3 plastics was obtained, which corresponded to the mean quantity of plastics put in the exposure tank for this group (24.9 plastics/L). On the basis of the filtered water volume in this sampling point, we calculated the presence of 14.3 ± 11.0 plastics/m^3 at the end of Lambro course (Fig. 1), with a similar percentage of MPs (49%) and mesoplastics (50%; Fig. 2). The fragments were the main observed shape (73%; Fig. 3). As for the color, transparent synthetic materials were the main collected ones, while we sampled mainly plastic of PE (65%; Fig. 4).

We observed a significant increase ($F_{4,10} = 4.39$; $p < 0.05$) of plastics in this last sampling point (Fig. 1) in comparison with the other 4 northernmost sampling stations. The evident rise of plastic contamination revealed at Graffignana drives to a crucial consequence, because we calculated a daily release of about 6,150,000 plastic debris from Lambro River into the Po River.

3.2. Baseline levels of measured biomarkers

The following baseline levels (mean ± SD) for all the considered biomarkers were measured: 18.3 ± 2.2 µmol min^{-1} mg prot^{-1} for CAT, 19.7 ± 1.4 U mg prot^{-1} for SOD, 10.1 ± 0.0 µmol min^{-1} mg prot^{-1} for GPx, 113.6 ± 15.8 mmol min^{-1} mg prot^{-1} for GST, 4455,236 ± 15,041 AU DCF mg prot^{-1} for ROS, 99,073 ± 28,606 fluorescence AU for P-gp,

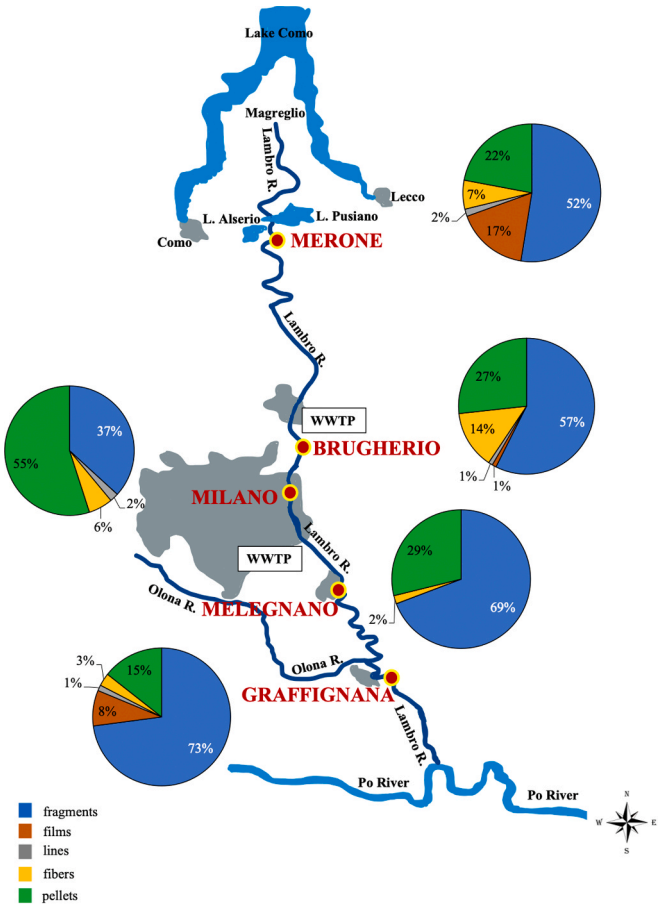

Fig. 3. Plastic shape – percentage of fragments, films, lines, fibers and pellets/beads detected along the Lambro River.

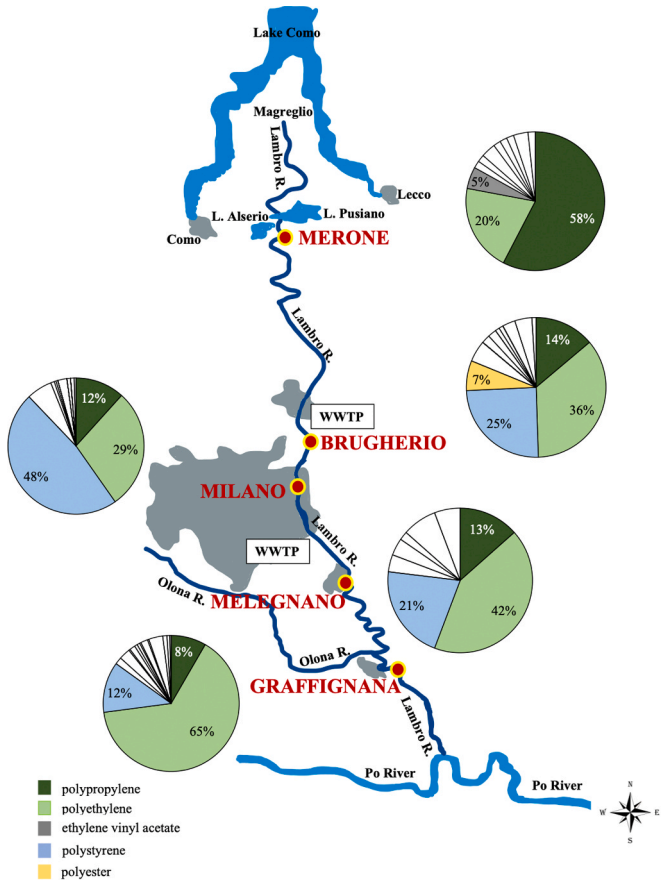

Fig. 4. Plastic (co)polymers – percentage of plastic chemical classes detected along the Lambro River. The white slices of the pie charts indicate the less abundant polymers along the Lambro River (see Table S1 for more information).

27.1 ± 2.8 nmol g ww^{-1} for LPO, 7.2 ± 0.6 nmol mg prot^{-1} for PCC, 82.2 ± 4.8% for cell viability, 1.2 ± 1.7‰ for MN frequency, 1.5 ± 1.5% for apoptotic cells, 0.3 ± 0.4% for necrotic cells, 127,976 ± 16,690 fluorescein produced min^{-1} mg prot^{-1} for MAO. Presented values were comparable to those measured in our previous studies carried out on zebra mussels (Magni et al., 2016, 2017, 2018, 2020).

3.3. Mussel mortality and hemocyte viability

The mussel mortality after 21 days of exposure was only 8% in the control tank, with similar values (11–12%) for the three northernmost sites, while we noticed a large threshold between Milano and Melegnano, in which about a quarter (23%) of zebra mussels died (Fig. 5A). The southernmost sampling point of Graffignana showed the worst case with 31% of mortality measured at the end of the plastic exposure, about 3 times higher than levels of the 3 northernmost sites.

The trend observed for mussel mortality was confirmed by the percentage of hemocyte viability aimed to investigate the cytotoxic effect of plastics (Fig. 5B). In detail, compared to 76% of the baseline levels, Merone, Brugherio and Milano ranged between 75% and 86% of hemocyte viability, while Melegnano (61% of viability) and Graffignana (54% of viability) showed a significant ($p < 0.05$ and $p < 0.01$, respectively) decrease of about 20% and 30% than controls, respectively (significant effect of treatment with $F_{5,47} = 11.85$; $p < 0.01$), following the similar threshold observed for mussel mortality.

3.4. Detoxification and antioxidant enzymes

The GST, the main enzyme of detoxification phase II, showed a significant effect of treatment ($F_{5,12} = 5.99$; $p < 0.01$) and a significant ($p < 0.05$) increase of its activity, compared to control, only at Merone, followed by a slow, but constant decrease until baseline levels in the next sampling stations (Fig. 6).

The enzymatic activities of the antioxidant machinery pointed out contrasting results (Fig. 6): SOD and GPx did not show any significant variation against controls (Fig. S1), while CAT exhibited a significant effect of treatment ($F_{5,12} = 3.58$; $p < 0.05$) and a significant increase of its activity at Merone ($p < 0.05$), Milano ($p < 0.01$) and Graffignana ($p < 0.05$; Fig. 6).

Related to the antioxidant enzymes is the measurement of ROS, which showed a similar behavior because of the lack of significant alterations (Fig. S1).

3.5. Multi-xenobiotic transporter and oxidative damage

We did not observe significant variation of the P-gp activity measured in the mussel gills (Fig. S1), while the PCC highlighted a significant effect of treatment ($F_{5,12} = 8.50$; $p < 0.01$) and a high significant ($p < 0.01$) increase in the carbonylation of proteins at Milano, Melegnano and Graffignana (Fig. 6), clear index of irreversible oxidative damage.

By contrast, LPO, the other main biomarker of oxidative damage, showed a lack of significant variations against controls (Fig. S1).

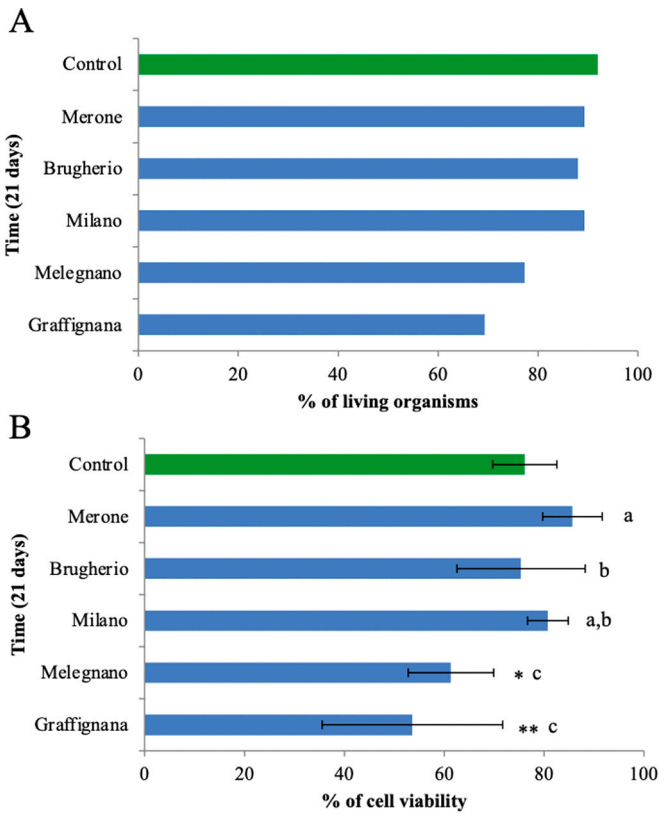

Fig. 5. (A) Percentage of living mussels observed during the entire exposure to plastics from the 5 different sampling stations along the Lambro River. (B) Percentage of cell (hemocytes) viability observed in exposed organisms (n = 9 mussels per treatment) at the end of exposure (t = 21 days). The letters indicate the significant differences between the sampling stations, while the asterisks indicate the significant differences (*p < 0.05; **p < 0.01; one-way ANOVA) between treated and control.

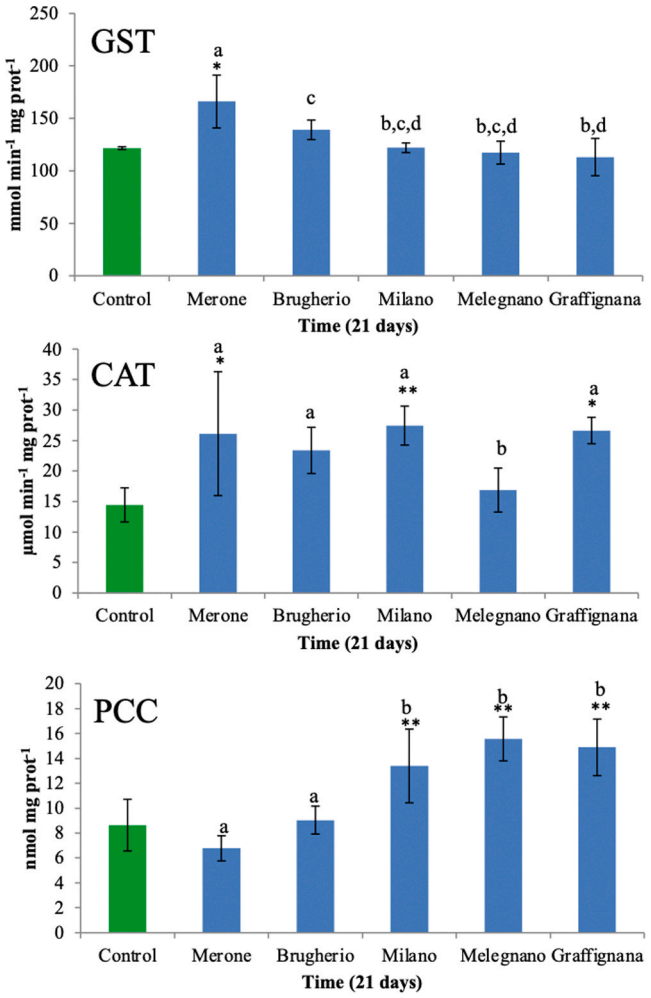

Fig. 6. Activity of GST and CAT and level of PCC (mean ± SD) observed in zebra mussel soft tissues (n = 3 pools of 3 mussels per treatment) at the end of exposure (t = 21 days) to plastics from the 5 different sampling stations along the Lambro River. The letters indicate the significant differences between the sampling stations, while the asterisks indicate the significant differences (*p < 0.05; **p < 0.01; one-way ANOVA) between treated and control.

3.6. Neurotoxicity and genotoxicity

The MAO kinetic revealed a constant and non-significant variation in comparison with controls (Fig. S1), as well as all the measured endpoints of genotoxicity (Fig. S1). We found only a significant effect of treatment for MN ($F_{5,48} = 23.30$; $p < 0.01$), with a significant increase ($p < 0.01$) for the MN frequency at Milano, but with levels not biologically relevant because a mean of 3 micronuclei falls into physiological variability.

3.7. Proteomics

The proteomic analysis identified 308 different proteins in the gills of zebra mussels sampled in the 5 sites along the Lambro River, 288 of which were subsequently quantified.

Using the selected double cut-offs (2-fold changes and significant differences to controls), zebra mussels from Merone revealed 8 modulated proteins than controls, 3 of them up-regulated and 5 down-regulated (Table S2). The plastic mixture collected at Brugherio was able to change 8 proteins, equally divided in up- and down-regulated, while we obtained the highest number of modulated proteins (12) from the site of Milano, by which 4 were up-regulated and 8 down-regulated. This value represented about the 4% of the total quantified gill proteins. After the passage of the Lambro River through the largest metropolitan area in Italy, the number of changed proteins decreased to 7 at Melegnano (4 proteins up-regulated and 3 down-regulated) and 9 at the southernmost station of Graffignana, with 3 proteins up-regulated and 6 down-regulated.

The Venn's chart revealed that only 2 proteins were in common among the 5 sampling sites (Fig. S2), suggesting a different and specific effect due to plastic mixtures for each station, or a possible effect of other concomitant contaminants (e.g., chemicals adsorbed on plastic surface) that vary between locations. Milano showed the highest number of changed proteins (6) not modulated by plastic mixtures collected in the other locations, while Melegnano had only 1 protein not in common with the other sites. The other 3 sampling stations showed an intermediate behavior instead.

Very interestingly, the station with the greatest variability in the modified protein classes was that of Brugherio (Fig. 7), whose sampling was carried out immediately after the outlet of one of the largest WWTPs located in the northern part of the Milan metropolitan area. On the other hand, this sampling site also had the highest variability in the polymeric composition, mainly for fibers, that showed the highest percentage (14%) than the other stations (Fig. 3), suggesting a direct influence of the WWTP outlet.

The most represented class of modulated proteins for all the sites belonged to cytoskeleton with a percentage ranging from 25% (Merone) to 57% (Melegnano) of the total changed proteins (Fig. 7). Even the ATP-binding proteins have been strongly influenced by plastic mixtures, with a minimum of 12% modulated proteins at Brugherio up to a maximum of 37% at Merone. Not negligible effect on DNA-binding proteins was observed both for Merone (25%) and Milano (17%), as well as also for

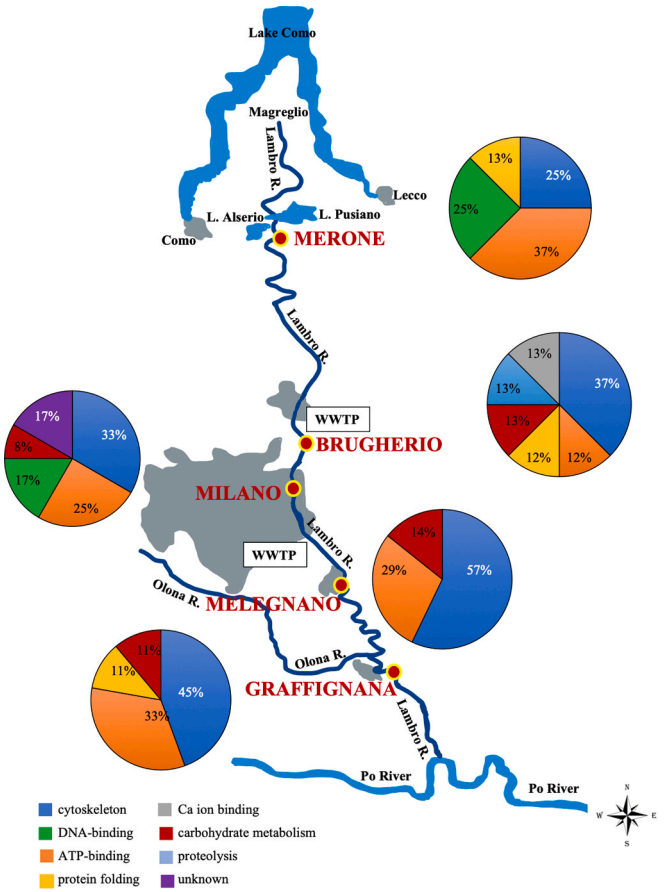

Fig. 7. Percentage of classes of modulated proteins in zebra mussels (3 pools of 6 gills per treatment) exposed to plastics from the 5 different sampling stations along the Lambro River (see Table S2 for more information).

the protein folding class, with 11–13% at Merone, Brugherio and Graffignana (Fig. 7). The last class in common among some sites was that of proteins involved in carbohydrate metabolism, for which we obtained 8% of the total changed proteins at Milano, 11% at Graffignana, 13% at Brugherio and 14% at Melegnano, while Merone, the northernmost sampling station, seemed not to be affected by the variation of this kind of proteins (Fig. 7).

3.8. Plastic uptake by mussels

We reported in Table 1 the plastic amount found in the pools of 10 mussels per treatment. Detected debris confirmed the intake of these contaminants in the exposed organisms at the end of exposures (t = 21 days). In particular, mussel exposed to plastics from the two southernmost sampling stations revealed the main number of internalized particles, with 4 plastics of epoxy resin, PP and polyurethane (PU) for Melegnano and 5 plastics of PP, polyester (PEST) and polycarbonate (PC) for Graffignana.

Table 1
Plastics detected at t = 21 days in zebra mussels (1 pool of 10 mussels per treatment, 6 pools in total) used in control and exposure experiments.

	Shape	Polymer
Control	fragment	polyamide (PA)
Merone	fiber	polyester (PEST)
Brugherio	fragment	epoxy resin
	fiber	polyacrylate (PAK)
Milano	fragment	polyethylene (PE)
	fiber	polyamide (PA)
Melegnano	fragment	epoxy resin
	fragment	epoxy resin
	fiber	polypropylene (PP)
	fragment	polyurethane (PU)
Graffignana	fragment	polypropylene (PP)
	fragment	polypropylene (PP)
	fiber	polyester (PEST)
	fragment	polycarbonate (PC)
	fragment	polycarbonate (PC)

4. Discussion

4.1. Monitoring of plastics along the Lambro River

The plastic amount calculated in the first sampling station of Merone was 0.5 ± 0.3 plastics/m^3, corresponding to 215,000 plastics/day (Fig. 1) and it could directly derive from the upstream area of the Alserio and Pusiano Lakes, from which the Lambro River comes out. Despite this station was located at few kilometers from the river source, different plastic polymers were detected, as PP, PE and the co-polymer ethylene-vinyl acetate (EVA; Fig. 4). This result can be associated to the large use of these chemical classes of plastics in packaging, in the production of bottle caps, labels and shoppers, as well as in adhesives and sealants.

No significant differences in terms of plastics amount were noted between Merone and the second sampling station of Brugherio, where 0.4 ± 0.2 plastics/m^3 (170,000 plastics/day) were found. As observed at Merone, in the second sampling point MPs were the main detected plastics and fragments were the main shape (57%; Fig. 3). At the same time, the concentration of fibers increased at Brugherio, reaching the 14% and doubling that found in the northernmost site. This growth could be associated with the entry of a WWTP (650,000 inhabitant equivalent) effluent (Fig. 2) just located few meters upstream this station, that may release the detected plastic fibers of polyester (PEST), polyamide (PA) and polyacrylate (PAK; Table S1) most likely derived from synthetic cloth washing (Magni et al., 2019b).

Moving along the Lambro River, we observed the impact due to Milan, one of the main metropolitan area of Italy, where we measured an increasing plastic pollution of 1.7 ± 0.6 plastics/m^3 (730,000 plastics/day), even if not significant with Merone and Brugherio. Differently to the two northernmost sites, the pellets/beads were the main shape of plastics (55%; Fig. 3). In this context, it is important to note that the majority of pellets/beads were white MPs of PS with a mean size of 370 μm (Table S1), suggesting the presence of their point-sources between Brugherio and Milano. Thus, other investigations are needed to clarify the origin of these shapes of plastics, probably related to personal care product (PCP) use, considering that the sizes of collected pellets/beads were compatible with those products (Sun et al., 2020).

We detected 1.3 ± 0.7 plastics/m^3 (560,000 plastics/day) at Melegnano, located few kilometers southern than one of the main WWTP of Northern Italy (WWTP of Milano Nosedo; 1,200,000 inhabitant equivalents; Fig. 2) that puts indirectly from the Vettabbia Stream its treated effluent in the Lambro River. However, despite WWTPs seem to be an important source of plastics toward aquatic ecosystems (Lares et al., 2018; Magni et al., 2019b), no significant increase in plastic concentration was observed in comparison with the previous 3 sites (Fig. 1). Perhaps, the further entrance of waters from Naviglio Martesana, Seveso River and Addetta Canal just before Melegnano could dilute the plastic pollution revealed at Melegnano. This hypothesis requires more confirmations, considering that no evidence about the plastic contamination of these Lambro tributaries is available until now.

At the southernmost sampling point of Graffignana we detected a concentration of 14.3 ± 11.0 plastics/m^3, that means as 6,150,000 plastic/day were reversed into the Po River. Our hypothesis for the great and significant increase ($F_{4,10} = 4.39$; $p < 0.05$) of plastics in this last sampling point (Fig. 1) is associated to the inlet of Olona River (also known as Southern Lambro), that ends few kilometers upstream Graffignana. Indeed, the Olona River seems to be highly contaminated by plastics, since we detected from 11.7 to 555 plastics/m^3 (sampling mesh

of 100 μm) in a recent survey (data not published).

Another important point concerning both Melegnano and Graffignana was related to the increasing percentage of fragments and mesoplastics, in comparison with the other 3 northernmost stations (Figs. 2 and 3). Indeed, many fragments, the typical shapes obtained after mechanical abrasion of larger plastics, were mesoplastics (Table S1), suggesting an increase of plastic degradation along the river that could produce debris with a secondary origin.

Making a summary of the more general results obtained through this survey, we can emphasize how the monitoring showed that there is a significant increase of plastic concentration in the last sampling station of Graffignana, where we detected a concentration of MPs 8.4 times higher than that of Milano, the second most contaminated site monitored, and about 29 times higher than the northernmost sampling site. However, this is not the consequence of a slow, but constant increase in contamination by plastics from the rest of the river, but rather the presence of a point source of contamination, probably identified in the inlet of the Olona River, which runs through an enormous industrialized and urbanized area throughout its course. On the other hand, the similar amount of plastics in the first 4 northernmost stations, despite the presence of potential point and diffuse plastic sources, could be also associated to the sedimentation of floating debris along the Lambro River, as a consequence of plastic surface colonization by microorganisms, which could increase their density (Yang et al., 2021).

Regarding the plastic size, considering all identified particles in the 5 different sampling stations, the largest detected plastic measured 19.00 mm, while the smallest one measured 0.15 mm, indicating that also smaller particles can be collected despite the mesh of 300 μm used for sampling, maybe due to net occlusion by suspended particulate matter. Moving to the shape, the fragment percentage increased in the 2 last sampling stations, while for the polymer composition we did not detect any clear trend of the plastics sampled in the 5 different stations. The first part of the Lambro River seems to be more contaminated by PP plastic wastes, while in its southernmost part we found a greater presence of PE debris, passing through Milano, where a high percentage of PS wastes was observed, a feature never found in the other 4 sampling stations. Lastly, the fact that we observed a great variability in the quantity of plastics sampled in the 3 days of the weekly sampling (Table S1) underlines how it is necessary to carry out an integrated sampling, perhaps also taking into account seasonal variations in the release of plastics.

To get an idea of the extent of the contamination found in the area of study, which we remember being studied from this point of view for the first time, the measured plastic amount was compared with other available surveys carried out in several European, Asiatic and American water courses (Table 2). Despite the difficulties in the comparison of results due to different sampling and analytical methods, it is possible to observe that the contamination of the Lambro River is absolutely comparable with plastic amounts monitored in several European and American rivers, where values from 0.28 to 108 plastics/m^3 were detected (Table 2), if we eliminate the lowest value of 0.05 plastics/m^3 found in the Rhine River, but limited only to microbead monitoring (Mani et al., 2019). In particular, the plastic contamination of Lambro River (from 0.4 ± 0.2 plastics/m^3 to 14.3 ± 11.0 plastics/m^3) is completely superimposable to that of Ofanto River (from 0.9 ± 0.4 plastics/m^3 to 13 ± 5 plastics/m^3 sampled with 333 μm mesh; Campanale et al., 2020), the only other Italian river in which the contamination of plastics has so far been evaluated. Regarding the Asian water courses, with the exception of some lower values in the Pearl River delta (Table 2; Mai et al., 2019), there seem to be a higher plastic contamination than the other continental areas (Pan et al., 2020; Wong et al., 2020), with values up to 6517 plastics/m^3 in the Qiantang River (Table 2; Zhao et al., 2020).

Table 2
Comparison of plastic amount detected in the Lambro River with other water courses in Europe, America and Asia. Data are reported with increasing values of plastic contamination.

River	Plastics/m^3	Filtration mesh (μm)	Citation
Seine River (France)	0.28–0.47	330	Dris et al., 2015
Pearl River delta (China)	0.005–0.7	330	Mai et al., 2019
Ottawa River (Canada)	1.35	100	Vermaire et al., 2017
Ebro River estuary (Spain)	3.5 ± 1.4	5	Simon-Sánchez et al., 2019
Rhine River (Germany)	0.05–8.3 (only spherical microplastics)	300	Mani et al., 2019
Elbe River (Germany)	0.88–13.24	150	Scherer et al., 2020
Ofanto River (Italy)	0.9 ± 0.4–13 ± 5	333	Campanale et al., 2020
Lambro River (Italy)	0.4 ± 0.2–14.3 ± 11.0	300	present study
North Shore Channel, (USA)	1.94 ± 0.81–17.93 ± 11.05	333	McCormick et al., 2014
Keelung River (Taiwan)	2.8 ± 1.2–64.4 ± 76.2	300	Wong et al., 2020
Xindian River (Taiwan)	2.5 ± 1.8–66.6 ± 58.0	300	Wong et al., 2020
Tamsui River (Taiwan)	10.1 ± 5.1–70.5 ± 30.6	300	Wong et al., 2020
Dahan River (Taiwan)	6.7 ± 2.4–83.7 ± 70.8	300	Wong et al., 2020
Seine River (France)	3–108	80	Dris et al., 2015
Zhangjiang River (China)	50–725	330	Pan et al., 2020
Qiantang River (China)	221–6517 (wet season) 50–3233 (dry season)	45	Zhao et al., 2020

4.2. Effects of plastic mixtures

The whole dataset pointed out as the exposure to the 5 plastic mixtures for 21 days caused an acute toxicity in the 2 southernmost sites, proven by the increase in mortality observed in zebra mussels exposed to plastics from Melegnano and Graffignana that clearly showed an overcoming of the homeostatic responses and the onset of adverse injuries so heavy as to lead to an extensive mortality, which reached up to a third of the mussels exposed to plastic mixture from Graffignana (Fig. 5A). This ecotoxicological profile was confirmed also by the hemocyte viability which decreased by 39% at Melegnano and even by 46% at Graffignana. This means that mussels survived at the end of exposures, that can be considered as the strongest organisms able to resist against the plastic injuries that killed the other mussels, were surely not in a satisfactory health condition, bearing in mind that a reduction in cell viability of over 30% leads to heavy cytotoxic effects that can be considered excessive also to carry out the genotoxicity tests (Tice et al., 2000).

This specific and worrisome effect was confirmed by results of the above-mentioned survey conducted on 4 of the subalpine Italian great lakes (Binelli et al. 2020), where actually a significant ($p < 0.05$) reduction of the hemocyte viability of about 30% was observed in zebra mussels exposed to plastic mixtures collected in L. Iseo and L. Garda, but not in L. Maggiore and L. Como (N. Italy). Another confirmation of this impact due to plastics is present in the recent study by Revel et al. (2020) in which a significant ($p < 0.05$) decrease in coelomocyte viability of the ragworm *Hediste diversicolor* exposed to a mixture of two types of PE and PP MPs (size distribution between 0.4 and 400 μm) was measured.

Turning to evidence on the increase in mortality of individuals attributed to plastics, the recent laboratory experiment by Eom et al. (2020) achieved similar effects to ours through the exposure of the brine shrimp (*Artemia francescana*) to different concentrations (1–1000 particles/mL) of 4 sizes (1, 3, 6, 10 μm) of PS microbeads. In detail, they found a mortality increase for the entire exposure period (30 days) at all sizes and especially a mortality rate in juvenile *A. francescana* exposed to 10 μm MPs at a concentration of 1000 particles/mL. Another proof about the acute effect of plastics was found by Aljaibachi and Callaghan (2018), who showed a significant ($p < 0.01$) increase of mortality in *Daphnia magna* specimens after only 7 days of exposure to different concentrations of 2 μm PS MPs administered alone and in mixture to an algal suspension of *Chlorella vulgaris*.

These are just few examples of the ecotoxicological role played by plastics in the acute effect on several target organisms, which confirmed our main results. The novelty of our study is linked to the fact that this adverse effect was found in organisms exposed to plastic mixtures collected in natural environments, greatly increasing the ecological realism. While Graffignana showed the highest average number of sampled particles and the highest value of these contaminants placed in the exposure tank (99.7 ± 67.3 plastics/tank; 24.9 plastics/L), we collected at Melegnano a number of plastics (17.3 ± 4.5 plastics/tank; 4.3 plastics/L) much lower than Milano (77.0 ± 36.3 plastics/tank; 19.2 plastics/L) that, on the contrary, showed neither an extensive mussel mortality nor significant cytotoxicity. This is another evidence of the complexity in the (eco)toxicological evaluation of the impacts made by these physical contaminants, whose ingestion, infiltration, accumulation and consequently toxicity are largely dependent by size, shape, color and polymer composition of the debris in the selected mixtures, showing once again that the simple quantification of plastics and the comparison among sampling sites is absolutely not sufficient to make a picture of the hazard caused by these pollutants on the community and ecosystem services. For instance, we can highlight that we measured a higher percentage of mesoplastics at Melegnano and Graffignana, which represented about the 50% of the total sampled plastics, compared to the other sites where we found a higher percentage of MPs (Fig. 2), suggesting as mesoplastics could represent the most dangerous size. Another characteristic of plastic mixtures that can influence their toxicity is the polymeric composition, since Melegnano and Graffignana showed a higher percentage of PE plastics in comparison with the other 3 sites (Fig. 4). Malafaia et al. (2020) recently found that MPs of PE (from 12.5 mg/L to 100 mg/L) were able to cause a 60% reduction in the survival rate of zebrafish larvae after hatching, as well as Berber and Yurtsever (2018) demonstrated as the population growth of the rotifer *Brachionus plicatilis* significantly decreased after 96 h exposure to 10–22 μm PE microspheres (from 0.1 to 0.4 mg/mL). Furthermore, exposures of *Chironomus tepperi* carried out at relevant environmental concentrations of MPs of PE (500 MPs/kg sediment) revealed detrimental effects on the survival and growth of this freshwater benthic organism (Ziajahromi et al., 2018). Another possible explanation about the acute effects observed in the experimental groups of Melegnano and Graffignana could be associated to the plethora of chemicals adsorbed on plastics surface. However, this hypothesis requires many confirmations about the characterization of the pollutants transported by these plastic mixtures that is beyond the scope of this first monitoring survey on the study area. However, it is important to underline how forced we were to carry out the exposures using a single tank per treatment, since plastic mixtures was very heterogeneous in the environment, making impossible to perform an exposure with the exact type and concentration of contaminants in each possible replicate. This does not exclude the "tank effect", potentially related to the high mortality levels in Melegnano and Graffignana experimental groups.

Once it has been established that the toxicity of the plastic mixtures is not simply due to their concentration, it would be important to understand their mechanism of action in determining this effect. The selected biochemical endpoints appear not to provide a conclusive answer as to the cause of the acute effects observed, since the measured biomarkers have shown low responses. Indeed, we highlighted only a slight activation of the antioxidant machinery, as pointed out by the significant ($p < 0.05$) increase of the CAT activity and the consequent rise in protein carbonylation ($p < 0.01$) observed after the zebra mussel exposure to plastics from Milano, Melegnano and Graffignana (Fig. 6). This aspect could be associated to an increase of H_2O_2 due to the exposure, which activated the CAT activity. At the same time, the method for ROS quantification, with DCFH-DA and used in this study, allows to detect mainly H_2O_2 in the plethora of ROS. Therefore, probably the CAT activity was able to neutralize the oxidizing activity of H_2O_2. No significant increase in ROS levels was measured and, consequently, the oxidative damage at the protein level could be associated to the activity of non-quantified ROS.

Furthermore, the main detoxification enzyme of phase II (GST) showed an interesting trend, starting with a significant ($p < 0.05$) increase at Merone and a low but constant non-significant decreasing trend along the Lambro River (Fig. 6). All other biomarkers measured showed non-significant changes compared to controls (Fig. S1) or did not possess a biological significance, as found for the micronucleus frequency measured for Milano (Magni et al., 2016; Binelli et al., 2020). Probably, considering also the tested concentrations, biomarkers are not enough sensitive tools to assess the toxicity of these pollutants.

In the attempt to shed light on the mechanism of action of the plastic mixtures collected along the Lambro River, proteomics can be a complementary or alternative approach to biomarkers' measurement. Actually, the analyses of the gill proteome carried out in zebra mussels seem to give more sensitive and clear results than biomarkers, as will be shown below.

First of all, the number of changed proteins in each site, which represented from 2.5% to 4% of the total quantified proteins, demonstrated once again the lack of correlation between the number of plastic debris and their effects. For instance, Graffignana was the site with the highest concentration of sampled plastics, but with an intermediate number of changed proteins, while we sampled at Milano about 8.4 times less plastics, which were however able to modulate the greatest number of proteins (12).

Overall, the modulation of many proteins involved in the structural and maintenance functions of cytoskeleton (Fig. 7) revealed much better than the measured biomarkers as plastics mainly act on the redox status imbalance, increasing the oxidative stress. Indeed, many previous studies showed as the redox balance regulates actin microfilaments and microtubules, affecting cytoskeleton dynamics (Caceres et al., 2012; Gonzalez-Billault et al., 2012; Wilson and Gonzalez-Billault, 2015; Belcastro et al., 2017). This is caused because of some amino acid residues contained in these cytoskeleton components are highly susceptible to oxidation, causing a reduction in the polymerization capability of microtubules and severing the actin microfilaments (Wilson and Gonzalez-Billault, 2015). The down-regulation of myosin observed at Graffignana can suggest not only eventual problem on muscle contraction (Yamada et al., 2000) in zebra mussels, but the deficiency in myosin may contribute also to less byssal threats secreted (Green et al., 2019), decreasing the byssus tenacity which is based on the number of threads or to their thickness (Carrington, 2002).

We can underline another crucial result obtained by proteomics, connected to the modulation of many ATP-binding proteins involved in functions related to energy pathways. Indeed, if we consider together the effects on carbohydrate metabolism and ATP-binding proteins, their percentages reached or overcame those of cytoskeleton proteins (Fig. 7). In detail, all the 4 modulated ATP-binding proteins (ABPs) were down-regulated (Table S2), suggesting a decrease in the energy storage and adverse effect on several pathways in which the release of energy is required. For instance, the modulation of the *Hsp 90* can be a negative event for many functions, such as the regulation of cell cycle, apoptosis, cell growth and survival (Park et al., 2015), also bearing in mind that the modulation of the Heath Shock Protein (HSP) family is a typical

response against environmental and physiological stress (Pirkkala et al., 2001).

Another interesting modulated protein belonging to this family was the *HSP 70* which contributes not only to the main function of the HSP family based on the recovery of stressed cells, but possesses also some house-keeping roles in non-stressed cells (Daugaard et al., 2007). This double function is extremely interesting because it confirms the hypothesis formulated in another our previous study (Magni et al., 2019a), in which we suggested that the down-regulation or even the block of the expression of *Hsps* noticed after the exposure to a mixture of plastics to zebra mussels could be a signal of the necessity of cells to save energy, by the no translation of mRNA relative to *Hsps*. This means that the effects due to plastic exposures drive the cells to consider the *Hsps* as house-keeping proteins, whose functions can be partially interrupted, instead of a direct response to oxidative stress. This must lead us to reflect on the toxicological role of plastics, which could heavily interfere with the cellular energy stock, growing the energy cost for their elimination after the organism intake, alongside the increase in oxidative stress as the main effect at the cellular level. In this way, the modulation of *Hsps* can also provide candidate markers for plastic exposures.

Moreover, the modulation of *Nsfb* could represent another confirmation about the redox status imbalance caused by plastic mixtures, since there are some evidences in the contribution of redox balance to vesicle trafficking (Grigoriev et al., 2011; Mackenzie et al., 2011; Villarroel-Campos et al., 2014) in which this protein is involved (Oho et al., 1995).

In summary, this high-throughput approach has highlighted several proteins, whose function has been modified by the action of plastic mixtures collected in a natural ecosystem, providing evidences that their main targets were related to the modification of cellular energy storage and the impairment of the redox balance. This latter effect was also found in our previous proteomic study (Magni et al., 2019a) carried out by two different sizes of PS microbeads, tested at high concentrations (2×10^6 MPs/L of 1 μm and 2×10^6 MPs/L of 10 μm). Also the recent paper by Green et al. (2019) showed a modulation of similar protein classes in blue mussels (*Mytilus edulis*) exposed for 52 days to polylactic acid and PE MPs (1296.3 ± 182.9 and 844.9 ± 138.7 particles/L) in an outdoor marine mesocosm. In addition to many proteins involved in some vital biological processes similar to ours, such as detoxification, metabolism and structural development, they highlighted also the changes of some haemocyte proteins engaged in the immune regulation, class not found in our work. To our knowledge, at this moment, these are the only 3 studies related to the application of proteomics to evaluate the effects of plastics on the proteome of freshwater and marine sentinel-organisms, and they clearly demonstrated as this approach could be a promising methodology to be applied in the ecotoxicological research aimed to investigate the impact, sometimes fleeing and not easy to evaluate, of the different type of plastics both in field and laboratory studies.

In conclusion, can the variation detected for some proteins and biochemical responses be sufficient to explain why we found the increase in mussel mortality and decrease in the viability of the hemocytes in mussels exposed to plastics from Melegnano and Graffignana? The answer is not easy, and we can only make some suggestions and hypotheses to be verified. The variation in the redox status, confirmed both by the oxidative damage noticed for PCC and by the modulation of several cytoskeleton proteins, as well as the possible interference in the cellular energy stock, are probably not sufficient to fully explain the acute effects produced by the exposures to plastics, but they surely represent a clear signal of the low health status of zebra mussels exposed to plastics, mainly in the two southernmost sampling stations. Indeed, we must underline that zebra mussels exposed to plastic mixtures collected at Melegnano and Graffignana suffered a modulation of proteins involved in cytoskeleton and energetic functions (100% and 89% of the total changed proteins, respectively) much higher than organisms exposed to the other mixtures (Fig. 7) and just related to the highest PCC levels measured in these two sites (Fig. 6). We must also remember that these molecular and cellular effects were measured in the surviving organisms, able to overcome or counteract the acute impact of the administered plastic mixtures. This may suggest that the growing oxidative damage, coupled with the modulation of proteins involved in fundamental energetic cellular pathways, may be a signal of a greater effect occurring at a higher biological level. One possible hypothesis of mortality and cytotoxicity observed in the two southernmost sites can be due to mechanical damage or blockage caused by plastics in the gastrointestinal tract and gills, interfering with digestive functions and respiration. There is a plethora of studies in which these effects have been found in many organisms: Bergami et al. (2016) observed a variation in feeding behavior due to 40 nm nano-sized PS in the gut lumen of the crustacean *A. francescana*, and abnormal ultra-structures of intestinal epithelial cells were found after only 24 h in *A. parthenogenetica* exposed to 10 μm PS microspheres (10–100 particles/mL; Wang et al., 2019), while Wright et al. (2013) suggested as MPs could potentially determine blockages through the digestive tract, suppressing feeding due to satiation. Different functionalized PS microspheres (8 μm) were proven to be able to accumulate in the gills of the shore crab *Carcinus maenas*, determining a significant, even if transient, effects on branchial functions, such as a change in the oxygen consumption and ion regulation (Watts et al., 2016). Unfortunately, since we did not carry out the evaluation of any eventual ultra-structural damage or physiological measurements able to identify possible acute injuries at gill and digestive tract, this hypothesis should be possibly tested in other future surveys. However, the plastic intake was confirmed in zebra mussels (Table 1), highlighting the presence of the same plastic polymers detected in the Lambro River. At the same time, also the polycarbonate (PC) was detected, despite its absence in the monitoring process. This evidence could be due to the heterogeneous dispersion of plastics in the water, which could justify the slight differences in the composition of plastic mixtures (for monitoring and toxicity assay) sampled with the two plankton nets. Regarding the number of detected particles in the exposed organisms, the amount of plastics was low (from 0.1 to 0.6 plastics/mussel; Table 1), but it is important to consider that other debris could be entered across the inhalant siphon of these filter feeder organisms also in the days upon the end of exposure and subsequently eliminated with feces or pseudofeces, as observed in a previous study on zebra mussel exposed to PVC and Mater-Bi® debris (Magni et al., 2020). For this reason, the presented results represent only a snapshot of plastic uptake at the end of plastic mixture exposures.

Our results suggest the need to apply a multi-step approach in the ecotoxicological assessment of plastic debris, covering different levels of the biological organization from the molecular one to the whole organism in order to understand the multiple effects caused by these physical contaminants.

5. Conclusions

The double objective that this study had set highlighted rather interesting aspects in relation to the monitoring as well as in the evaluation of the possible effects of plastics at the level of the aquatic wildlife. Indeed, the identification of a possible point source of contamination of plastics most likely located in the inlet of the Olona River certainly represents a fundamental indication for the Lambro River pollution.

The evaluation of the ecotoxicological aspect due to the sampled plastics has instead highlighted how it is absolutely necessary to use a multi-level approach, which is able to point out the different possible effects of plastics, which strictly depend not only on their concentration, but also on their chemical and physical characteristics.

Therefore, the protocol developed in this study allowed to obtain a clear picture of both contamination and ecotoxicological effects of complex plastic matrices taken directly from natural environments, greatly increasing the ecological realism. After the experience gained,

we are also able to suggest any changes and/or improvements to this protocol:

1) To collect even smaller plastics, which should be the most dangerous for aquatic organisms, it would be necessary to use nets with a mesh lower than that normally used. In this sense, we are carrying out other samplings with nets with 100 μm mesh.
2) Improvement and standardization of the plastic extraction protocol from such a complex matrix to obtain cleaner plastics, completely free by interfering substances, in the context of ecotoxicological effects.
3) To perform also microscopic analyses, at least in the gastrointestinal tract, to evaluate any mechanical effects or blockages that could be responsible for the observed macroscopic effects.
4) To evaluate any behavioral (e.g. total distance moved, turn angle) or physiological effects (e.g. filtration and feeding rate).
5) To measure other biomarkers for more specific assessment of inflammatory and energy budget related effects.

CRediT authorship contribution statement

Stefano Magni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Original Draft. **Camilla Della Torre**: Methodology. **Lara Nigro**: Investigation. **Andrea Binelli**: Conceptualization, Resources, Supervision, Writing-Review & Editing, Project administration, Funding acquisition.

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.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125204.

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