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CHARACTERIZATION OF PLASTICS AND THEIR ECOTOXICOLOGICAL EFFECTS IN THE LAMBRO RIVER (N. ITALY)

--Manuscript Draft--

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Abstract:	This study had the dual objective of both the qualitative and quantitative assessment of plastic mixtures sampled along the River Lambro (Italy), and the contemporarily determination of the ecotoxicological effects of the same mixtures sampled, through a 21-day exposure of the freshwater bivalve Dreissena polymorpha . The monitoring survey was carried out by the vibrational microscopy, while the ecotoxicological assessment was performed by 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 near the inlet to Po 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.

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To the Editor of

Journal of Hazardous Materials

Dear Editor,

we re-submit for publication on Journal of Hazardous Materials the manuscript with the new title "CHARACTERIZATION OF PLASTICS AND THEIR ECOTOXICOLOGICAL EFFECTS IN THE LAMBRO RIVER (N. ITALY)".

We replied to review comments in the file "response to reviewers", point by point. We followed almost all the suggestions of reviewer n. 2 with the exception of those contradicting the requests of reviewer n. 1, to which we have already answered in the previous review and that confirmed the goodness of our changes. You can see our corrections in the version of manuscript with changes marked.

We hope that the changes made will allow a rapid publication of the manuscript.

Both my co-authors and I thank you in advance for your attention.

Best regards,

on behalf of all coauthors

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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.

Novelty Statement (maximum limit:100 words)

The environmental problem due to microplastics is basically tackled in two different ways, one based on the environmental monitoring, the other concerning the evaluation of adverse effects on different biological models. Often, these two approaches fail to support the environmental management because the ecotoxicological assessment carried out under laboratory conditions simplify too much the complexity of the plastic mixtures found in natural environments.

The novelty of this study is precisely the simultaneous qualitative and quantitative evaluation of plastic pollution and the assessment of possible negative effects conducted by using the same sampled plastic mixtures, increasing the ecological realism.

Graphical Abstract

Highlights:

-Plastic monitoring in Lambro River (Northern Italy) was performed
-Evaluation of toxicity of environmental plastics collected on Lambro River
-Lambro River releases more than 6 million plastics/day in its body receptor
-Plastics from Lambro River induce acute and chronic effects on exposed organisms

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Credit Author 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.

Reviewer #3: The manuscript "THE JOURNEY OF PLASTICS ALONG THE LAMBRO RIVER (N. ITALY) AND THEIR ECOTOXICOLOGICAL EFFECT" presents an interesting amount of data, despite the lack of replicates in toxicity assays, which is very poorly presented and reads more like a student report than a scientific paper. I do not believe this data has been analyzed in enough detail, meaning that some important conclusions have not been taken, nor that it has been properly discussed regarding current literature. The manuscript is too long and too confusing, which needs full rewriting and reorganization of its structure. This will help transform this data, which indeed seems interesting and worthy of publication, into a scientific paper worthy of publication. I have left detailed comments on what should be improved to help the authors through this dauting task.

The title does not clearly state what is presented in the manuscript. It would benefit from being more objective.

Response: The title has been changed in accordance with the suggestion.

In the abstract it is not clear if the mussels were exposed in situ or in the lab.

Response: This aspect has been added in the text (line 17).

The manuscript is long. Even though the introduction is well structured, it is too long, especially when giving such detailed information on the methods and results of the study. I believe that shortening it, almost to half, would improve readability. What is essential to stay is why there is a need to conduct this study, its objective, and only a sentence or two on what has been found.

Response: The introduction has been reduced, in particular the details concerning the methods have been deleted (lines 48-64, 104-110, 117-136).

I do not understand the steps in pre-treatment. Samples in glass bottles from the field sampling were first filtered through a mesh (63 μm) and then the solution passing though was filtered? What about microplastics that were in the mesh? I think this is not clear enough.

Response: We clarified this step in the manuscript (lines 177-195).

For toxicity assays, microplastics in meshes were used, but the mesh also retained organic matter (leaves, branches) which could interfere. How were these removed?

Response: In accordance with the previous comment, this part of the methods has been clarified in the paper (lines 177-195).

It is not clear in the toxicity assays what concentrations were used. Were these concentrations the same as found in each of the sampling points?

Response: Due to the very long procedure of characterization analyses (in the Table S1 are reported hundreds of particles analyzed one by one in the study) we quali-quantified only the plastics from one sampling net. Considering that we performed the sampling of plastics for the toxicity assay simultaneously and a few centimeters from the net for monitoring (please see the photos in the graphical abstract), we assumed that the plastic concentration of the two nets was the same. For this reason, the concentration in each exposure tank corresponded to those detected in each sampling point. Anyway, we better clarified this aspect in the paper (lines 246-250).

It is also worth noting that the toxicity assay has no replicates. Even though each tank has 75 bivalves, these are pseudoreplicates. This must be recognized in the discussion.

Response: Normally, with exposures of mussels to standards of contaminants, we performed a triplicate (see our previous works, Magni et al., 2016, 2017, 2018, 2020). In this study, where a more complex design was adopted, plastics for the exposure were collected directly in the environment. In this condition, it was not possible to perform an exposure in triplicate with the exact typology and concentration of plastics for each replicate, being the plastic mixtures much heterogeneous. For this reason, the exposures were conducted using only one tank for each experimental group, as proposed in our previous study (Binelli et al., 2020). However, the bivalves collected for the biomarkers and functional proteomics represent biological replicates and not pseudo-replicates. The pseudo-replicates were the technical ones derived, for instance, from the repeated measures of biomarkers in the same pool of mussels. In this context, the degrees of freedom reported in the text as subscript of the F value indicated that the pseudo-replicates were not considered in the statistical analyses.
As suggested by the reviewer, this aspect was added in the discussion (Lines 839-848).

"In detail, we sampled 5 mussels for the measurements of each biomarker class, for a total of 20 different animals," I do not understand what is 5 mussels for a total of 20 animals. Do you mean for each treatment since they are 4? Or 4 assessments per tank (since they are not true replicates)? Please clarify. Were these polled?

Response: this aspect was referred to the evaluation of basal level (t=0 day). As reported in the manuscript (lines 264-265) the animals were collected from the acclimation tanks and processed in the following manner: a pool of 5 mussels for the antioxidant/detoxifying enzyme and ROS evaluation, a pool of 5 animals for the oxidative damage evaluation, the hemolymphs of these specimens was used for cyto- and genotoxicity, gills from 5 animals for P-gp measurement and a pool of 5 animals for MAO assessment, for a total of 20 mussels. This aspect has been clarified in the text (lines 265-277).

There are chapters? I am confused? Will ecotoxicological effects not be discussed here despite being described in the materials and methods?

Response: The reviewer did not report the line numbers, without any other indication to understand this query. Anyway, the ecotoxicological effects were clearly discussed in the discussion section.

"5 analyzed filters 387 analyzed as controls, that contained only 15 fibers of cellulose." In total? Or per filter?

Response: Only 15 fibers in the total of 5 filters were observed. This information has been added more clearly in the text (lines 409-415).

I am still confused if the authors evaluated micro or mesoplastics.

Response: In the manuscript it is clearly reported in several points, including title and abstract, that the study was focused on the presence and effect evaluation of plastics. The generic term "plastics" was not used randomly in the manuscript. Indeed, as shown in the figure 2, all materials identified

with a plastic nature were subsequently classified, on the basis of their size, as micro, meso and macroplastics. Therefore, in the study, both micro-, meso- and macroplastics were considered.

"On the basis of filtered water volume, we calculated a concentration of 0.5±0.3 plastics/m3 397 in this sampling point (Figure 1), corresponding to about 215,000 plastics that pass daily through Merone, if we consider as 5 m3 398 /s the mean flow rate of Lambro River" This is the important part for each sampling point. The rest can go to the supplementary. Just as a showcase, the authors can mention the total amount of plastics sampled (a total of X particles were collected for the different sampling points).

Response: We eliminated some repetitive information, considering their presence also in the figures 2,3 and 4. Result and discussion sections were deeply re-written.

"which corresponded to the 395 quantity of plastics put in the 4 L tank of Merone group" This should be clarified in the methods for all, no need to repeat.

Response: Done. We clarified in the methods this aspect and deleted the repetitions in the results (Lines 246-250). In addition, we reported also the concentrations as plastics/L.

"In detail, the MPs were the main size of debris detected at Merone (63%), followed by meso403 (35%) and macroplastics (2%; Figure 2)," To do this type of description as in this paragraph for each sampling point will make the manuscript too long and tiring. This information for all points can be presented in a table or figure. Then in text differences or relevant information should be discussed. There is no need to produce such long description when what matters is the take-away message and while the same information can be much more effectively transmitted through a figure or table.

Response: We deleted this information from the manuscript. Completed details were already reported in the Figures 2, 3 and 4 (please, see Result section).

Comparisons between points must address the following questions: are there differences in concentrations between points? For each point between days? What about plastic types (size, shape, polymer, color)? What could be the sources (point or diffuse) in each location justifying this differences? As it is presented now it does not do a good point of highlighting this main finding, because there is so many information and so scattered that the reader cannot compare or memorize all that.

Response: The differences of concentration between the first 4 sampling stations and the last station of Graffignana were discussed in the paper. The same thing has been done for the other parameters (size, shape and polymer composition) where possible and avoiding speculations. We added and discussed only reasonable hypotheses, such as the possible contribution about plastic contamination by both Olona River (reporting the contamination of this river on the basis of our recent monitoring, data not published yet) and WWTPs (point sources). As suggested by the reviewer, we added also the diffuse sources (Impact of the City of Milan), but we must remember that this is the first monitoring study for plastics in this area and no other data are available to suggest possible diffuse and/or point sources without being too speculative. Anyway, considering also the other comments of reviewer the results and discussion have been re-write.

If WWTP is a main source in Merone, why are not fibers the prevalent shape?

Response:. We sampled few kilometers before the WWTP of Merone, to have as first point the effective outlet of Lambro River from Alserio and Pusiano Lakes (source). For this reason, the fibers were not the prevalent shape and we did not report that the WWTP is the main source at Merone, instead we hypothesized that the plastics found in Merone could derive from the upstream area of the Alserio and Pusiano Lakes. We invite the reviewer to see on google maps our sampling point in Merone on the basis of the sampling coordinates reported in the methods (we added more complete coordinates, Lines 152-158).

"Thus, other investigations are needed to clarify the origin of these pellets/beads, 438 mainly related to personal care product (PCP)" sizes of 300 um seems too big for microbeads from cosmetics.

Response: The reviewer reported that 300 μm seems to be a big range for microbeads in personal care products. However, recently Sun et al., 2020 (Incidence of microplastics in personal care products: An appreciable part of plastic pollution. Science of The Total Environment, 140218) reported the presence of microbeads with a mean size of 200, 300 and even 400 μm in face cleaners, toothpaste and shower gels. For this reason, our hypothesis regarding the origin of pellets/beads from PCPs is plausible and the related citation has been added in the text (Line 701).

Sizes (min, max, median) should also be addressed in the table and differences discussed. In the first paragraph of Results, it should say the smallest and largest size for all particles found.

Response: The sizes (min, max, median) of all detected particles were added in Table S1. On the basis of reviewer suggestion, we added also in the text the smallest and largest plastics (738-741).

The main results here are concentrations and daily amounts of plastics. Then all other information can be only discussed comparatively.

Response: On the basis of other review comments, we eliminated some repetitive parts about shape, size and polymer composition (see results).

"The fragments were the main shape of 448 detected plastics (69%), followed by pellets/beads (29%) and fibers (2%; Figure 3)," What shape is a "plastic"? Fragments? Spheres?

Response: The sentence has been re-written.

In the short discussion spread across all the data there is no consideration for sinks, only for sources. Some plastics could be lost along the way, depositing in certain areas. Also, diffuse sources are barely considered.

Response: This aspect was added in the discussion (Lines 733-738, 692-695).

"This surely represents a very important result that 480 can give a great contribution on the management of this kind of physical pollutants in this 481 environment." Useless. Please avoid this kind of sentences that are just making the manuscript too long and masking important results.

Response: The sentence has been deleted.

"it is possible to observe that the contamination of the Lambro River is 485 absolutely comparable with the plastic amounts monitored in European and American rivers," What is absolutely comparable? Discussion is still missing here. What concentrations were found for other rivers? There are a lot of works using manta nets that can be roughly compared, even though sample preparation is not exactly the same.

Response: Actually, there are many studies about marine environments (not comparable to our work), but much less regarding freshwater environments. Anyway, please note that the plastic concentrations detected in other rivers around the world were reported and compared in the text, as suggested, considering also different sampling methods used (Table 2; Lines 751-767).

"In particular, the plastic 487 contamination of Lambro River is completely superimposable to that of Ofanto River" where … was found? Why is it comparable?

Response: Please see our previous response and the relative comparison added in the discussion.

I do not remember all the information, but did the concentration increase downstream? Or some were lost/added during the course? If so why?

Response: Yes, the concentration increased downstream at Graffignana. This aspect could be due to the entrance of Olona River in the Lambro River, few kilometers before the Graffignana station. We clarified this part of discussion, considering also the diffuse sources and the sedimentation process, as suggested by the reviewer, to justify the constant amount of plastics in the first 4 sampling stations.

Section 3.1 can easily be summarized into 1 - 1.5 pages if only important information is shown and discussed. I do not agree so far with previous reviewers that this manuscript has too many results to be readable. It has a good amount of results, which is laudable, but not so many that cannot be presented in a clear way in a single manuscript if the writing is good enough. Here what applied is "less is more", since the authors should want to showcase their main results and discussion, instead of just adding "junk" which is hiding their efforts.

Response: The section 3.1 has been re-written and many aspects deleted.

Again, for biomarkers, a figure or table would present these results in a readable matter.

Response: Biomarkers were already presented in the Figures 6 and S1. Furthermore, these figures were modified in accordance with the suggestions of other reviewers.

Endpoints must be compared with statistical analysis

Response: Sorry, but we did not understand this comment. All endpoints were compared with statistical analyses. We performed a one-way ANOVA for biomarkers and a student T-test for the proteomic analysis. In addition, also a Pearson correlation was performed to correlate the filtered water volumes with the plastic amount. These analyses were clearly described in the paragraph 2.4.

"The southernmost sampling point of Graffignana showed the worst case with 31% of 525 mortality measured at the end of the plastic exposure" was this concentration the highest? Why not also present these results comparing concentration vs effect?

Response: Yes, Graffignana had the highest concentration of plastics, as well reported in several parts of paper. To clarify, the concentrations were reported in both method and result sections, also as plastics/L. The discussion was already performed with the comparison concentration/effect (more detail about concentrations of cited papers were added). Moreover, it is absolutely impossible to perform and compare dose/effect relationships on samples taken in the environment with this very high degree of heterogeneity and it is beyond the scope of this work.

"The trend observed for mussel mortality was confirmed by the percentage of hemocyte 528 viability aimed to investigate the cytotoxic effect of plastics" what is the relevance of this? Or will that be discussed later?

Response: Sure, it was discussed later in the discussion.

"The Venn's diagram chart revealed that only 2 proteins were in common among the 5 577 sampling sites (Figure S21), suggesting a different and specific effect due to plastic mixtures 578 for each station." Or effect of other concomitant contaminants that vary between locations.

Response: This aspect was added in the manuscript (Lines 635-636).

"We reported in Table 2 the plastic amount found in the pools of 10 mussels per treatment" was this related to treatment concentration? Or were some plastic types more frequently internalized than others?

Response: Please see our addition in the text about this aspect (Lines 979-992)

Discussion should also include discussion of the sampling effort and concentrations. Maybe it would be easier to join results and concentrations, in a first subsection discussing the sampling and the second discussing all data on toxicity concluding with a sub-subsection overarching toxicity results.

Response: We reduced the result section and the discussion of sampling was reported in the point 4.1 of discussion, as suggested. Both results and discussion were deeply re-written and re-checked.

"This means that mussels survived at the end of exposures, that can be considered 619 as the strongest organisms able to resist against the plastic injuries that killed the other 620 mussels" It is worth remembering that these organisms were not collected on site. Through selection, mussels on those sampling points could already be adapted to the contamination.

Response: As reported in the manuscript (Lines 237-238) the organisms were collected in the Lake Maggiore, with the same contamination history, and not in the Lambro River.

I am still waiting for the information on concentrations - effect to be discussed.

Response: Please see the discussion after the performed changes. I remember once again the impossibility of performing a dose/response relationship and comparison on physical contaminants that have heterogeneous characteristics regarding the type of polymer, size and shape. This is not a laboratory study with chemical pollutants administered to different concentrations.

There is no point in discussing ecotoxicity if concentrations are not taken into account. Here, for other studies, concentrations used must also be mentioned as well as plastic characteristics.

Response: The concentrations were already considered in the results and discussion. The obtained tested concentrations were clearly reported in the methods and results also. In addition, the aim of this study was the evaluation of environmental plastic mixture (as reported in the manuscript) and not of few plastic standards acquired by companies. This aspect makes the study much more complex. For this reason, to avoid speculations, it was not possible to consider the individual characteristics of the sampled plastics (size, shape, polymers) in the discussion of effects.

"Indeed, while Graffignana showed the highest average number of 648 sampled particles (99.7±67.3 plastic debris) that then ended into exposure tanks, we collected 649 at Melegnano a number of plastics (17.3±4.5) much lower than Milano (77.0±36.3) t" This means nothing, what is important is concentrations not numbers.

Response: As reported in our previous comments, we added in the text also the concentration expressed as plastics/L (each value of plastics/tank was divided for 4, considering the 4 L tanks used in the exposure). For instance, considering the mean amount of plastics collected at Graffignana and placed in the exposure tank (99.7 plastics/tank), this value was divided for 4 obtaining the concentration of 24.9 plastics/L for this group.

"whose ingestion, infiltration, accumulation and consequently 653 toxicity are largely dependent by size, shape, colour and polymer composition" These have not been addressed. First consider concentrations. Then different plastic characteristic between groups (qualitatively, since little data exists on toxicity due to the use of pseudoreplicates).

Response: Please see our responses to the previous comments, as well as the aspects considered in the new version of discussion.

The discussion mixes both sampling and effects, it is confusing. Also, discussion and comparison with other studies is still too light for a research paper.

Response: Sorry, but you encouraged the integration and discussion of concentrations with the effects, in your previous comments. Thus, this comment is not in accordance with the previous ones. Anyway, we re-written the discussion of monitoring and added in the paper the comparisons with other studies.

"Malafaia et al. (2020) recently found that MPs of PE were able to cause a 60% reduction in 665 the survival rate of zebrafish larvae after hatching, as well as Berber and Meral (2018) 666 demonstrated as the population growth of the rotifer Brachionus plicatilis significantly 667 decreased after 90 h exposure to 10-22 □m PE microspheres. Furthermore, exposures of 668 Chironomus tepperi carried out at relevant environmental concentrations of MPs of PE 669 revealed detrimental effects on the survival, growth and emergence of this freshwater benthic 670 organism (Ziajahromi et al., 2018)." Useless. What concentrations? Is this really relevant for your findings?

Response: Despite the comparisons among concentrations were very difficult, due to the differences in the units (particles/L or mass of particles/L), we added these information (Lines 830, 833, 835, 931-932, 935).

"Once it has been established that the toxicity of the plastic mixtures is not simply due to their 672 concentration" How do you know? This data has not been presented?

Response: Once again, the concentrations tested were already included in the manuscript and corresponded to the mean value of the quantity of plastics sampled in each stations in the three days of integrated sampling. This amount of plastics was then added to the tanks during the exposure. To clarify this aspect, we included some clarifications in the article and in the comments to reviewer, reported above.

So far, I feel that suggestions made by the previous reviewers were not taken seriously. Little changes have been made to the manuscript. They suggested deep restructuring but all I see is small changes. I hope that my comments are taken more seriously, since they are essential to turn this from a student's project to a scientific research paper. These comments are meant to help highlight the author's results and better present their work. I believe most problems rely on data analysis and presentation, which can be easily overcome by putting some effort in restructuring the manuscript. After all this, I believe it could be an important work, especially by combining sampling with ecotoxicity assays.

Response: This comment is offensive, such as the previous term “junk”. We remember the response of reviewer 1 to our corrections: “The authors adequately responded to my previous comments and implemented them into the new version of the manuscript accordingly”. Anyway, the result and discussion sections were re-structured, as well explained in other replies.

Why only CAT was activated here? Why not the rest of ROS defense?

Response: To clarify this aspect we added in the paper an additional explanation (lines 856-862).

Why were the mussels response like this? There is a need to propose some kind of explanation regarding what was seen here, besides just generally saying "different plastics produce different results" without showing that this is indeed true (significantly different polymer types, sizes) between toxicity treatments.

Response: We evaluated the effects of plastic mixtures, not of single plastic, as are done in laboratory studies in which you seem to report many of your observations. We cannot understand what the sense is to evaluate the significant single differences in polymers, shape and size. In this manner and in our opinion the discussion could get very speculative. It seems that the purpose of this study is not clear to reviewer.

There is no evidence for ROS response, for now it seems that mussels were able to deal with oxidative species with their machinery, meaning they must not have effect.

Response: Please see our response to one of the previous comments and the related addition in the text (Lines 856-862).

Is there any other explanation for the cytoskeleton increase besides ROS?

Response: As reported in previous works the alteration of the oxidative status affects the cytoskeleton dynamics (Caceres et al., 2012; Gonzalez-Billault, 2012; Wilson and Gonzalez-

Billault, 2015; Belcastro et al., 2017). Due to the complexity in the interpretation of these results, we preferred to avoid speculation, leaving in the text only this hypothesis.

"energy stock, alongside the increase in oxidative stress as the main effect at the cellular level." Maybe by increasing energy expenditure to deal with plastics? Or effects from their leachates?

Response: This aspect has been added in the article (Lines 918-919).

Maybe the pathways involved in mortality were not addressed in the biomarkers chosen…

Response: We applied a wide battery of biomarker in this study (in addition to proteomics), to cover the different levels of biological organization as biochemical level (CAT, SOD, GPx, GST and P-gp), cellular level (PCC, LPO, MN, Apoptosis, Necrosis, Cell viability) and organism level (MAO). In particular, apoptosis, necrosis, and cell viability are endpoints potentially linked to an extremely compromised state that could lead to mortality. Moreover, we remember that biomarkers measure some sub-lethal effects, sometimes not related to mortality ones.

"The double objective that this study had set highlighted rather interesting aspects both in 920 relation to the monitoring and environmental management of MPs" no management was discussed. The objective I see was determining environmental concentration and determining their effects on mussels. No management was discussed, and may should not be discussed in great detail in this manuscript.

Response: We are in accordance with the reviewer and this part was changed in the conclusions.

"Therefore, the protocol developed in this study turned out to be long and not easy but" Methods followed were not so complicated, nor the data, what is complicated is the way results are being presented and discussed.

Response: We deleted this aspect in the conclusions.

Why are protocol improvements being suggested in the conclusion if this was not the objective of the work nor was discussed in previous sections? These are not that helpful, nor all of them are really improvements to protocols… The only helpful suggestions that should be discussed in previous sections are those pertaining to toxicity.

Response: On the basis of the suggestion of reviewer 1 (What needs to be done differently and in addition to what has been done in this study? Will it be possible to use a biomarker approach for this complex group of contaminants?) we decide to re-write the conclusions including the aspects that need possible implementation in the future studies.

Fig 1. It turns out that only concentrations in Graffignana is significantly different from the previous points… Why was it presented as all were different in the manuscript? Also, there is no clear pattern of increasing concentrations downstream…

Response: In the manuscript we reported that the first 4 sampling stations had no significant differences in plastic amount. Please see our changes in result section. On the other hand, in the last sampling station there was a clear and significant increase of plastics (most likely due to the inlet of Olona River, as shown in the paper).

Graffignana receives water from Olona, maybe plastics are coming from there as well? IT seems a big change.

Response: Yes of course, this aspect was discussed in the paper.

Fig 2 would benefit from some pictograms to depict major cities or WWTP (sources in general).

Response: The pictogram of the Major cities is already reported in the Figure. We added the two main WWTPs as suggested by the reviewer.

There are still too many figures despite the suggestion made by previous reviewers. Why not combine some of them, such as Fig 2 - Fig 4.

Response: The suggestion of previous reviewer concerned the merge of pie charts or graphs related to biomarkers. We decide to merge the biomarker figures in a unique image (Figure S1), leaving in the manuscript only the graphs of significant endpoints (CAT, GST and PCC). The Venn Chart was placed in the supplementary materials. So, based on the comments of the other reviewers, the changes had already been made.

With so much data, why was it not interpreted more in-depth? For instance, were different particle sizes more dominated by a polymer type (e.g. fragments by PP, spheres by PS…)

Response: Please, see the new version of discussion regarding the plastic monitoring.

Both Melegnano and Graffignana present higher mortality and decrease in cell viability, it seems that both are impacted by the same factor despite having extremely different plastic concentrations. Could the authors explain what else could have gone through the test? Could there chemical contaminants also pass through?

Response: The potential role in the effects of plastics of chemicals has been added in the text (Lines 838-841).

Fig 6 does not support what has been discussed so far. Also, which are the letters for the control, meaning that there is no significant difference?

Response: Sorry, but we do not understand this comment, since these indication are always present in the caption of Figure 6: the asterisks indicate the significant differences between treated and control, the letters indicate the significant differences only between treated. Therefore, Figure 6 supports the discussion, since significant differences for CAT, PCC and GST between treated and control were reported.

Figure 7 does not make sense to present these results as pie charts… Why would they be a pie chart if they are not part of the same "total"? I do not even understand what I should take from here…

Response: Sorry, but we are not in accordance with the reviewer. The figure clearly reports the percentage of protein classes (on the total ones modulated by plastic mixtures) modulated at the end of exposure to plastics from the different river stations. This is absolutely the same percentage comparison performed for size, shape and polymer composition.

Table 1. There is no need to write "from X to Y" in a table, it just makes it difficult to read! Why not just X-Y?

Response: Done, as suggested.

Table 2. Has not been discussed and seems interesting. But I do not understand, this means that <1 plastic was found for each mussel? Were these the same plastics as found for each location?

Response: This aspect has been discussed (Lines 979-991).

Supplementary material can be combined in a single pdf file.

Response: Done.

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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 ~~River~~ Lambro River (northern Italy), and the contemporarily determination of the ecotoxicological effects of the same mixtures sampled, through ~~a~~ 21-day laboratory ~~—~~exposures ~~21-day exposure~~ 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.

Keywords: plastic monitoring; freshwaters; toxic effects; biomarkers; proteomics

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, ~~such as entanglement, strangulation, suffocation, drowning, starving and infections,~~ 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 (≥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. ~~In addition to this secondary origin, primary MPs are also contained in several large consumer products (e.g. toothpastes, cosmetics, scrubs) which are directly released in the aquatic environment.~~

~~There is not consensus on their definition since the most used categorization for MPs was proposed by the National Oceanic and Atmospheric Administration (NOAA; Arthur et al., 2009) as plastic particles <5 mm in diameter, while NPs are typically considered in the range 1-100 nm, following the definition of nanomaterials stated by the European Commission (EC, 2011). However, Hartmann et al. (2019) recently suggested to classify the plastic debris consistently with the International System of Units (SI), as macroplastics (≥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 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, colour, 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. ~~the~~ Po ~~River), the longest Italian river~~. The survey was conducted in 3 different days of a week, sampling each day the selected locations, for a total of 30 samples. ~~We used two twin plankton nets (mesh 300 µm), put contemporarily in water, that allowed to obtain homogeneous samples to be used both for the quali-quantitative recognition (15 samples from one net) and for the effect evaluation (15 samples from the other net) of plastic mixtures.~~

~~After several preliminary steps, t~~The plastic mixtures ~~collected in the 5 sites~~ 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 ~~some biochemical endpoints capable of~~ identify~~ing~~ many cellular and molecular effects. ~~were monitored, such as the quantification of reactive oxidative species (ROS) production, activation of the antioxidant machinery (activity of superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx) and the detoxification system of phase II (activity of glutathione S-transferase, GST), oxidative damage (protein carbonyl content, PCC; lipid peroxidation, LPO), neurotoxicity (activity of monoamine oxidase, MAO), multi xenobiotic transporter activation (activity of P-glycoprotein, P-gp) and cyto-genotoxicity (hemocytes' viability and identification of micronuclei, MN test; evaluation of apoptotic and necrotic cells).~~ 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. ~~Lastly, a check of the presence of plastic debris in the zebra mussel soft tissues after the exposure period was carried out by a preliminary visual sorting followed by the μFT-iR characterization.~~

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 study followed the design defined in our previous survey carried out in 4 of the Italian subalpine great lakes aimed to evaluate the hazard due to the sampled plastic mixtures (Binelli et al., 2020).~~ 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 behaviour 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 ~~Lake~~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 minutes: 40±6 m^3 for Merone, 86±2

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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~~s~~ and ~~the filtered~~the other interfering materials collected~~from Lambro River~~) ~~material present inside~~ were filtered on a steel sieve with a mesh of 63 µm to retain~~collect~~ 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 ~~once again~~ 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~~eliminated~~. 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).

~~The recovered plastics on the steel sieve, as well as the hypersaline solution filtered on the same sieve, were re-collected in the glass bottles to allow the density separation between the plastics and any finer suspended matter passed through the sieve mesh. The sludge sedimented to the bottom was then eliminated by siphoning in order to recover only the plastics.~~ 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, colour 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 4,000 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 ≥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 colour. 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) ~~enzymes~~ 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).

~~we sampled 5 mussels for the measurements of each biomarker class, for a total of 20 different animals, used for the activity of antioxidant/detoxifying enzymes and ROS quantification (measured in the same pool), P-gp evaluation, oxidative damage/cyto-genotoxicity and for neurotoxicity, respectively.~~

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 (~~superoxide dismutase;~~ SOD, ~~catalase;~~ CAT, ~~glutathione peroxidase;~~ GPx~~, and glutathione S-transferase~~; GST) and ~~Reactive Oxygen Species (~~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 method (1976), 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 hypoxantine. 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-glycoprotein (~~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 ~~lipid peroxidation (~~LPO~~)~~ and ~~protein carbonyl content (~~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 method (1976). We evaluated the LPO and PCC in accordance with Ohkawa, (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 1,000 g for 20 min at 4 °C (S1 fraction). Proteins were quantified using the Bradford method (1976) to normalize the neuro-enzyme kinetic. The activity of ~~monoamine oxidase (~~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 orthovandate (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 method (1976).

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 method (1976). 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 nano electrospray 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 (paragraph 2.2).

2.4 Statistical approach and data integration

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 par. 2.1), no significant

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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.

~~Due to the double objective of this study, based both on the qualitative and quantitative evaluation of the plastics sampled along the Lambro River and their ecotoxicological evaluation, it is not easy to show and discuss consistently the large amount of results obtained. Thus, to make clearer the illustration of this double approach, we decided to show and contemporarily discuss the results from the qualitative and quantitative evaluation of sampled plastic mixtures in this Chapter, postponing the wide discussion of the ecotoxicological effects and their possible relation to the monitoring data in the next Chapter 4.~~

3.1 Qualitative and quantitative assessment of sampled plastic mixtures

~~First of all, the analyses of blanks confirmed the absence of accidental contamination by plastics in our samples, considering that any plastics were detected on the 5 analyzed filters analyzed as controls, that contained only 15 fibers of cellulose.~~

~~Based on the volumes of water filtered in each sampling site and day (see par. 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.~~

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 (Figure 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 and Zuccato, 2011). ~~This plastic mixture detected in the upper course of Lambro River could directly derive from the upstream area of the Alserio and Pusiano Lakes, from which the Lambro River comes out.~~

In detail, the MPs were the main size of debris detected at Merone (63%; Figure 2), ~~followed by meso- (35%) and macroplastics (2%; Figure 2),~~ as well as fragments were the main shape (52%; Figure 3). About colour, white debris were the principal collected ones (54%), while the principal represented polymer~~ic~~ class~~es~~ ~~were~~ was polypropylene (PP - 58%; ~~), polyethylene (PE - 20%) and the co-polymer ethylene-vinylacetate (EVA - 5%;~~ Figure 4). ~~The presence of these polymers in the upper part of the river could be explained by their large use in packaging, in the production of bottle caps, labels and shoppers, as well as in adhesives and sealants, especially for EVA.~~

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

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amount of 0.4±0.2 plastics/m^3 in this sampling point, not significantly different to Merone (Figure 1), and represented by 57% MPs ~~and 43% mesoplastics~~ (Figure 2). As observed for Merone, fragments were the main plastic shape (57%~~;), followed by pellets/beads (27%;~~ Figure 3). The concentration of fibers increased in Brugherio, reaching the 14% and doubling that found in the northernmost station. ~~This growth could be associated with the entry of a WWTP effluent (650,000 inhabitant equivalent) just located few meters upstream this station, that may release the detected plastic fibers of polyester (PEST), polyamide (PA) and polyacrylate (PAK; Table S1), probably derived from synthetic cloth washing (Magni et al., 2019b).~~ Regarding the polymer~~ic~~ composition, the main detected polymer was~~s~~ ~~were~~ polyethylene (PE~~ - (~~36%; Figure 4)~~, polystyrene (PS; 25%), a plastic widely used in packaging, PP (14%) and PEST (7%; Figure 4)~~. The white was the colour 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~~a great~~ 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 (~~, which was double than the previous one~~ 19.2 plastics/L added in the exposure tank). In detail, we calculated 1.7±0.6 plastics/m^3 in this sampling point (Figure 1), represented by 75% MPs~~, 24% mesoplastics and 1% macroplastics~~ (Figure 2). Differently to the two northernmost sites, the pellets/beads were the main shape of plastics (55%; Figure 3)~~, followed by fragments (37%), fibers (6%) and lines (2%; Figure 3)~~. The white was confirmed as the main colour of synthetic debris, while polystyrene (PS) was the main detected polymer (48%; Figure 4)~~, followed by PE (29%) and PP (12%; Figure 4). 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 a specific inlet source of these particular debris between the two sampling stations. Thus, other investigations are needed to clarify the origin of these pellets/beads, mainly related to personal care product (PCP) use or to an involuntary release by plants of plastic production.~~ The daily amount of plastics that cross this point increased to about 730,000 debris~~, showing a clear higher release of plastics in Lambro River in comparison with the two previous sites~~.

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 ~~calculated~~ obtained a concentration of 1.3±0.7 plastics/m^3 (no significant differences in comparison with the other 3 sampling stations were reported; Figure 1), with 52% MPs and 48% mesoplastics (Figure 2). The main shape of plastics were fragments ~~were the main shape of detected plastics~~

(69%; Figure 3), ~~followed by pellets/beads (29%) and fibers (2%; Figure 3),~~ while transparent (56%) was the main observed colour.

~~Between Milano and Melegnano sampling stations, one of the main WWTP of Northern Italy (1,200,000 inhabitant equivalent) reverses 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 at Melegnano (Figure 1). Perhaps, the further entrance of waters from Naviglio Martesana, Seveso River and Addetta Canal just before Melegnano could dilute the plastic pollution. However, this hypothesis requires confirmations, considering that no evidences about the plastic contamination in the Lambro tributaries are available until now.~~ Lastly, we observed at Melegnano a high concentration of PE (42%; Figure 4), ~~followed by PS (21%) and PP (13%; Figure 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~~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 (Figure 1), with a similar percentage of MPs (49%) and mesoplastics (50%; Figure 2). ~~detected indicating that despite the mesh of 300 µm used for sampling, also smaller particles can be collected due to net occlusion.~~ The fragments were the main observed shape (73%; Figure 3)~~, followed by pellets/beads (15%), films (8%), fibers (3%) and lines (1%; Figure 3)~~. As for the colour, transparent synthetic materials were the main collected ones, while we sampled mainly plastic of PE (65%; Figure 4)~~, PS (12%) and PP (8%; Figure 4) as principal polymers. Our hypothesis for the great and significant increase ($F_{4,10}$=4.39; p<0.05) of plastics in this last sampling point (Figure 1) is associated to the inlet of Olona River (also known as Southern Lambro), that ends few kilometers upstream Graffignana. However, once again, no data are available regarding the plastic contamination of Olona River, even if other surveys are in progress to verify this hypothesis.~~

We observed a significant increase ($F_{4,10}$=4.39; p<0.05) of plastics in this last sampling point (Figure 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.

~~However, it is important to note that, while plastic pollution is fairly constant in the 4 previous sampling stations, with only an increase observed at Milano, the contribution to the Po River is mainly due to the plastic contamination detected in the final course of the Lambro River. This surely represents a very important result that can give a great contribution on the management of this kind of physical pollutants in this environment. Results obtained on Lambro River were compared with other available surveys on (micro)plastic contamination in several European, Asiatic and American water courses~~

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~~(Table 1). 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 the plastic amounts monitored in European and American rivers. In particular, the plastic contamination of Lambro River is completely superimposable to that of Ofanto River (134 km of length; Campanale et al., 2020), the only other Italian river in which the contamination of plastics has so far been evaluated.but generally lower than those measured in Asian water courses. In particular, the plastic contamination of Lambro River is completely superimposable to that of Ofanto River (134 km of length; Campanale et al., 2020), the only other Italian river in which the contamination of plastics has so far been evaluated.~~

~~Making a summary of the more general results obtained through this survey, we can emphasize how the monitoring showed that there is no clear trend in the increase in the quantity of MPs along the Lambro River. Indeed, albeit with fluctuating MP concentrations, the first 4 sampling stations showed a comparable pollution overall, while the worst case was observed for Graffignana, where we detected a concentration of MPs 6.5 times higher than that of Milano, the second most contaminated station monitored, and about 23 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. LambroRiver (both point sources and other natural/artificial tributaries and diffuse sources as the impact of the metropolitan area of Milan)also~~

~~We did not detect any clear trend even in the composition of the MPs sampled in the 5 different stations, as 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 the Milano station, where a high percentage of PS wastes was observed, a feature never found in the other 4 sampling stations. Lastly, the fact that in the 3 days of weekly sampling we observed a great variability in the quantity of plastics sampled (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.~~

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, 4,455,236±15,041 AU DCF mg $prot^{-1}$ for ROS, 99,073±28,606 fluorescence AU for P-gp, 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 tanks, 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 was died (Figure 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 (Figure 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 (Figure 6).

The enzymatic activities of the antioxidant machinery pointed out contrasting results (Figure 6): SOD and GPx did not show any significant variation against controls (Figure 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 ($p<0.05$; Figure 6).

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

3.6 Neurotoxicity and genotoxicity

The MAO kinetic revealed a constant and non-significant variation in comparison with controls (Figure S1), as well as all the measured endpoints of genotoxicity (Figure 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 (Figure 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 behaviour instead.

Very interestingly, the station with the greatest variability in the modified protein classes was that of Brugherio (Figure 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 (Figure 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 (Figure 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 the protein folding class, with 11-13% at Merone, Brugherio and Graffignana (Figure 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 (Figure 7).

3.8 Plastic uptake by mussels

We reported in Table 12 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 this context, it is important to take into account that other debris could be entered across the inhalant siphon of these filter feeder organisms, reaching the pallial cavity and tissues of mussels, also in the days upon the end of exposure and subsequently eliminated with faeces or pseudofaeces (Magni et al., 2020). For this reason, the presented results represent only a snapshot of plastic uptake at the end of exposures, which however confirmed the ability of the chosen biological model to allow the plastics' intake. 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.

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 (Figure 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; Figure 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%; Figure 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 (Figure 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%; Figure 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

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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; Figure 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 (Figure 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 (Figure 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 (Figures 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

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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 6,517 plastics/m^3 in the Qiantang River (Table 2; Zhao et al., 2020).

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4.2 *Effects of plastic mixtures*

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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 ~~dramatic~~ 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 (Figure 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 dramatic 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. On the other hand, it is important to consider that we tested very low concentrations in comparison with the abovementioned data and, probably, also the chemicals absorbed on the plastics surface could have an important role in the acute effects induction. HoweverIn this regard, theThe 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. We must underline another aspect related to this acute effect, since it is only partially explained by the number of plastic materials measured in the 5 sampling stations with which we carried out the experiments. Indeed, wWhile 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 debris) that then ended into exposure tanks, 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, colour 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 ~~microplastics~~ MPs (Figure 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 (Figure 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~~Meral~~ (2018) demonstrated as the population growth of the rotifer *Brachionus plicatilis* significantly decreased after 96~~90~~ 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 ~~and emergence~~ 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. ~~This finding is extremely important, also considering the very low tested concentrations, in comparison to those reported by other studies (Yurtsever et al., 2018; Malafaia et al., 2020).~~

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 (Figure 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.

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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 (Figure 6). All other biomarkers measured showed non-significant changes compared to controls (Figure 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 ~~6.5~~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 (Figure 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, 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 (Figure 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 ~~dramatic~~ 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 ~~MPs~~ 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 ($2x10^6$ MPs/L of 1 µm and $2x10^6$ 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.

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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 (Figure 7) and just related to the highest PCC levels measured in these two sites (Figure 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 behaviour 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 ~~microplastics~~ 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 faeces or pseudofaeces, 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 ~~open a new window~~suggest ~~in the ecotoxicological assessment of the environmental hazard due to plastic debris suggesting~~ the need ~~for~~ 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.

~~4.~~5. CONCLUSIONS

The double objective that this study had set highlighted rather interesting aspects ~~both~~ in relation to the monitoring ~~and environmental management of MPs,~~ 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 ~~MPs~~ plastics most likely located in the inlet of the Olona River certainly represents a fundamental indication for the ~~increase of knowledge management of this problem on~~ Lambro River pollution.

The evaluation of the ecotoxicological aspect due to the sampled ~~MPs~~ 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 ~~MPs~~plastics, which strictly depend not only on their concentration, but also on their chemical and physical characteristics.

Therefore, the protocol developed in this study ~~turned out to be long and not easy but, at the same time, capable~~allowed to~~of~~ obtain~~ing~~ a clear picture of both ~~the~~ contamination and ~~contemporarily the~~ 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~~s~~ 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.

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~~On the other hand, other studies with the same experimental design adopted in the present work are necessary to confirm these effects. Indeed, the exposure was conducted in single, since plastics were collected directly in the environment and it was not possible to perform an exposure in triplicate with the exactly typology and concentration of contaminants for each replicate.~~

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Captions

Figure 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).

Figure 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.

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

Figure 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).

Figure 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<0.05; one-way ANOVA) between treated and control.

Figure 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<0.05; one-way ANOVA) between treated and control.

Figure 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).

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.

Keywords: plastic monitoring; freshwaters; toxic effects; biomarkers; proteomics

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 (≥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 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, colour, 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 behaviour 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 minutes: 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, colour 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 4,000 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 colour. 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 method (1976), 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 hypoxantine. 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 method (1976). We evaluated the LPO and PCC in accordance with Ohkawa, (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 1,000 g for 20 min at 4 °C (S1 fraction). Proteins were quantified using the Bradford method (1976) 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 orthovandate (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 method (1976).

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 method (1976). 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 nano electrospray 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 (paragraph 2.2).

2.4 Statistical approach and data integration

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 par. 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 (Figure 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 and Zuccato, 2011).

In detail, the MPs were the main size of debris detected at Merone (63%; Figure 2), as well as fragments were the main shape (52%; Figure 3). About colour, white debris were the principal collected ones (54%), while the principal represented polymer class was polypropylene (PP - 58%; Figure 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 (Figure 1), and represented by 57% MPs (Figure 2). As observed for Merone, fragments were the main plastic shape (57%; Figure 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%; Figure 4). The white was the colour 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 (Figure 1), represented by 75% MPs (Figure 2). Differently to the two northernmost sites, the pellets/beads were the main shape of plastics (55%; Figure 3). The white was confirmed as

the main colour of synthetic debris, while polystyrene (PS) was the main detected polymer (48%; Figure 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; Figure 1), with 52% MPs and 48% mesoplastics (Figure 2). The main shape of plastics were fragments (69%; Figure 3), while transparent (56%) was the main observed colour.

Lastly, we observed at Melegnano a high concentration of PE (42%; Figure 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 (Figure 1), with a similar percentage of MPs (49%) and mesoplastics (50%; Figure 2). The fragments were the main observed shape (73%; Figure 3). As for the colour, transparent synthetic materials were the main collected ones, while we sampled mainly plastic of PE (65%; Figure 4)

We observed a significant increase ($F_{4,10}$=4.39; $p<0.05$) of plastics in this last sampling point (Figure 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, 4,455,236±15,041 AU DCF mg $prot^{-1}$ for ROS, 99,073±28,606 fluorescence AU for P-gp, 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 was died (Figure 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 (Figure 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 (Figure 6).

The enzymatic activities of the antioxidant machinery pointed out contrasting results (Figure 6): SOD and GPx did not show any significant variation against controls (Figure 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 ($p<0.05$; Figure 6).

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

3.6 Neurotoxicity and genotoxicity

The MAO kinetic revealed a constant and non-significant variation in comparison with controls (Figure S1), as well as all the measured endpoints of genotoxicity (Figure 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 (Figure 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 behaviour instead.

Very interestingly, the station with the greatest variability in the modified protein classes was that of Brugherio (Figure 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 (Figure 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 (Figure 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 the protein folding class, with 11-13% at Merone, Brugherio and Graffignana (Figure 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 (Figure 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.

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 (Figure 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; Figure 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%; Figure 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 (Figure 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%; Figure 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; Figure 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 (Figure 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 (Figure 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 (Figures 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 6,517 plastics/m^3 in the Qiantang River (Table 2; 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 (Figure 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, colour 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 (Figure 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 (Figure 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 (Figure 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 (Figure 6). All other biomarkers measured showed non-significant changes compared to controls (Figure 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 (Figure 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, 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 (Figure 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 ($2x10^6$ MPs/L of 1 μm and $2x10^6$ 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 (Figure 7) and just related to the highest PCC levels measured in these two sites (Figure 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 behaviour 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 faeces or pseudofaeces, 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.

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Captions

Figure 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).

Figure 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.

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

Figure 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).

Figure 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 (*$p<0.05$; **$p<0.05$; one-way ANOVA) between treated and control.

Figure 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 (*$p<0.05$; **$p<0.05$; one-way ANOVA) between treated and control.

Figure 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).

Figure 1

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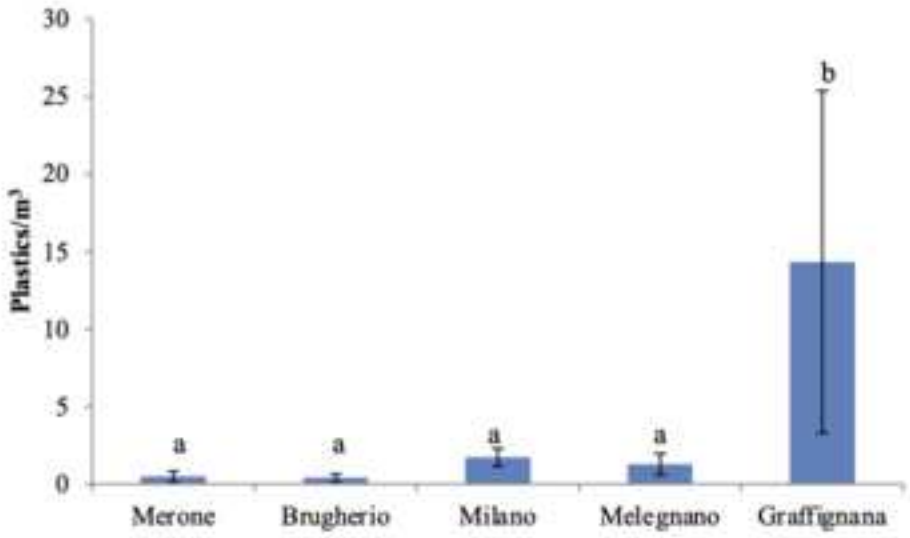

Figure 2

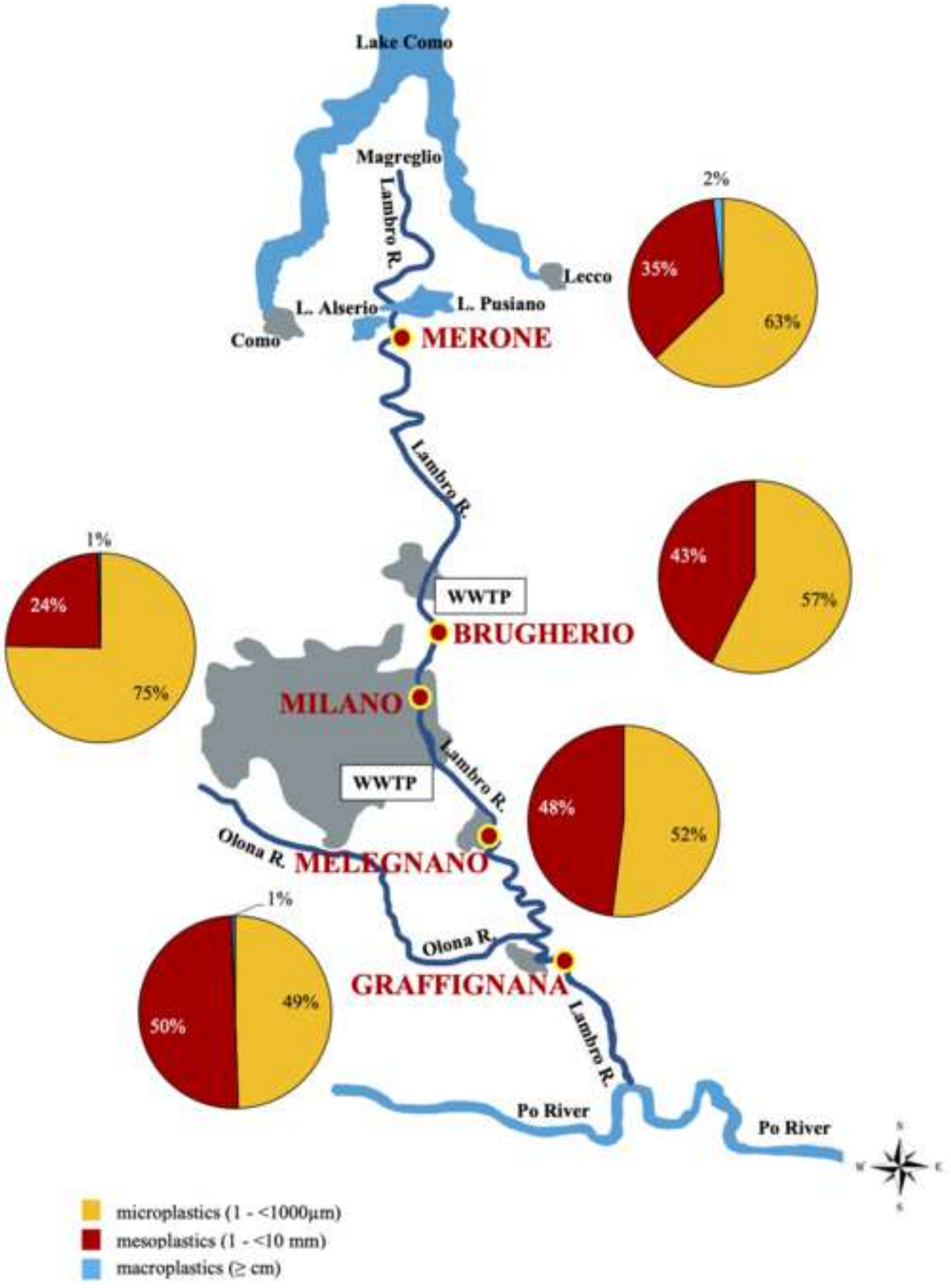

Figure 3

Click here to access/download;Figure;Figure 3.tiff

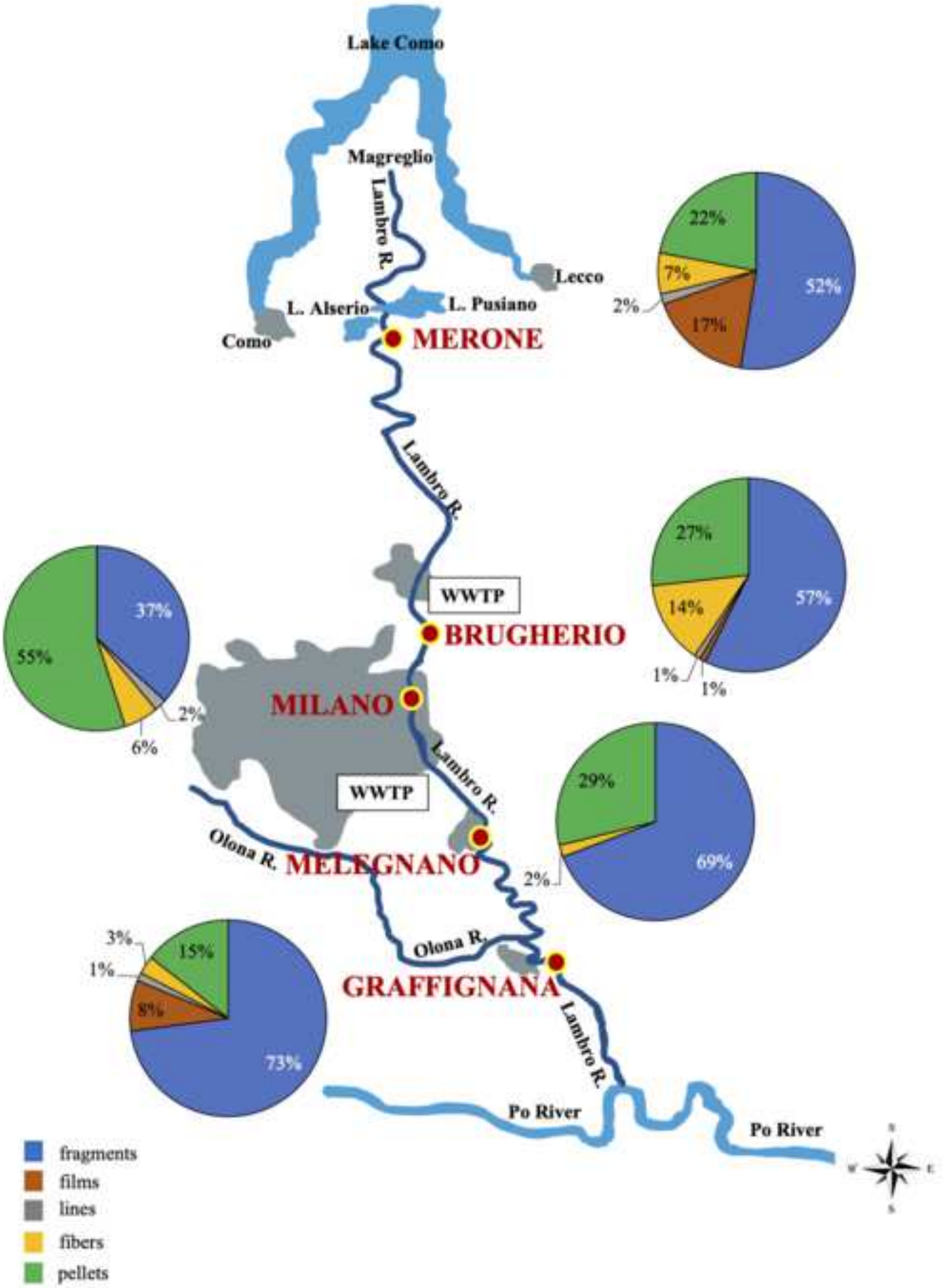

Figure 4

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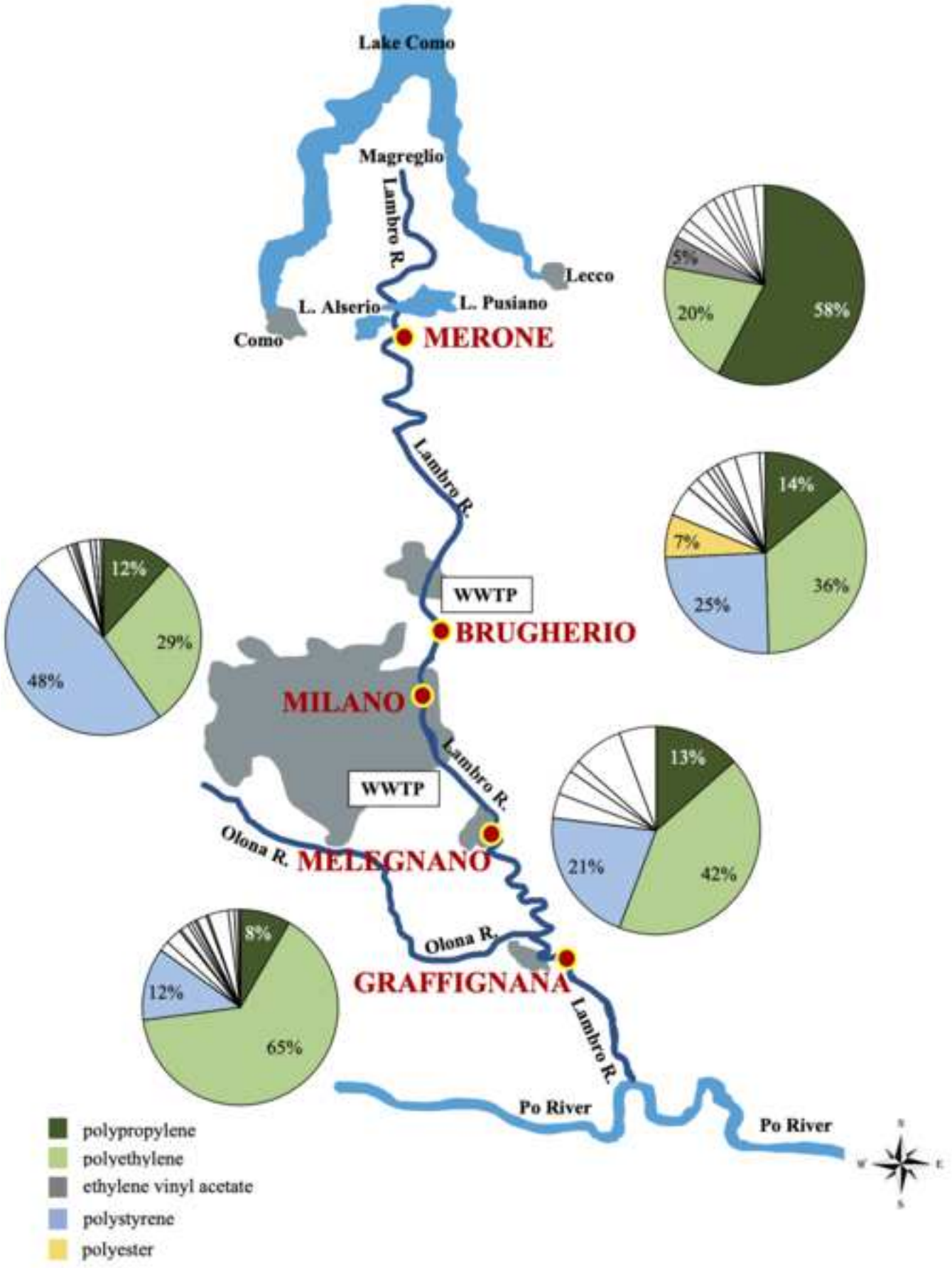


Figure 5

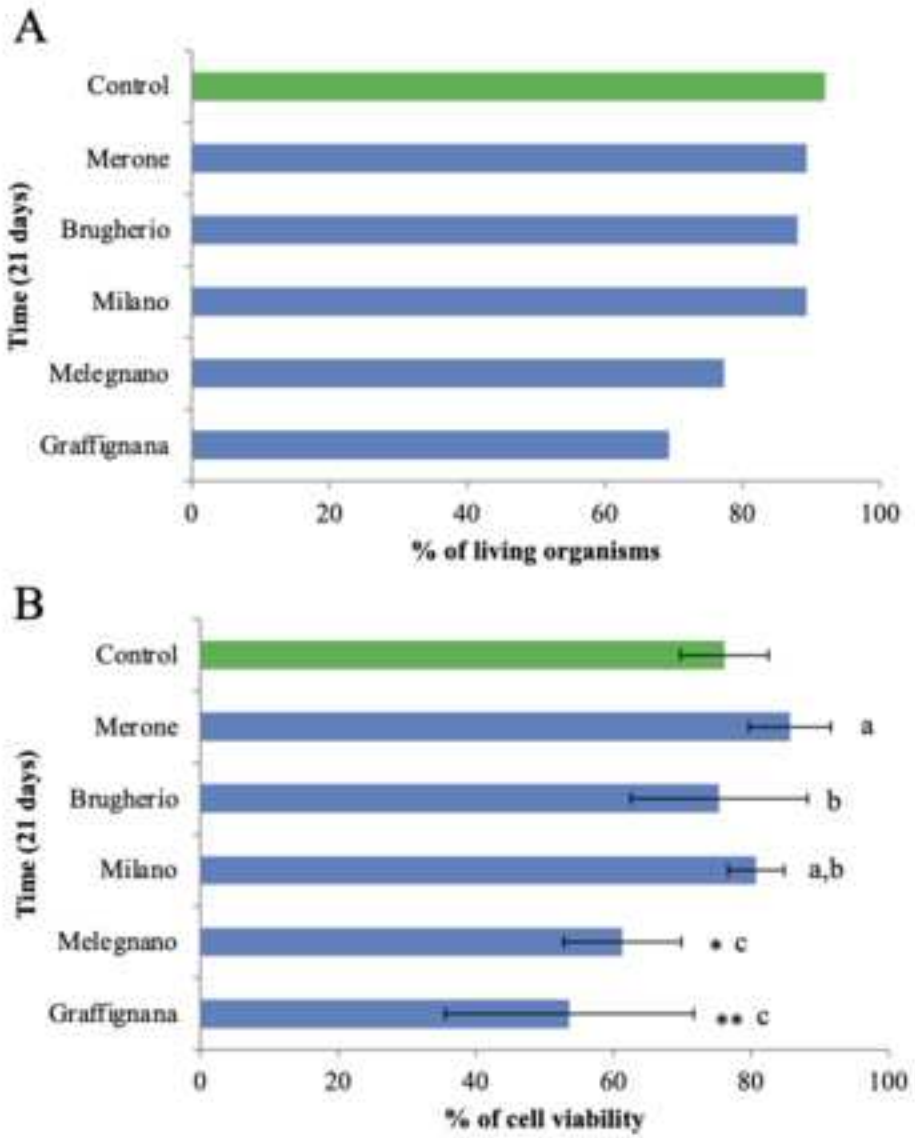


Figure 6

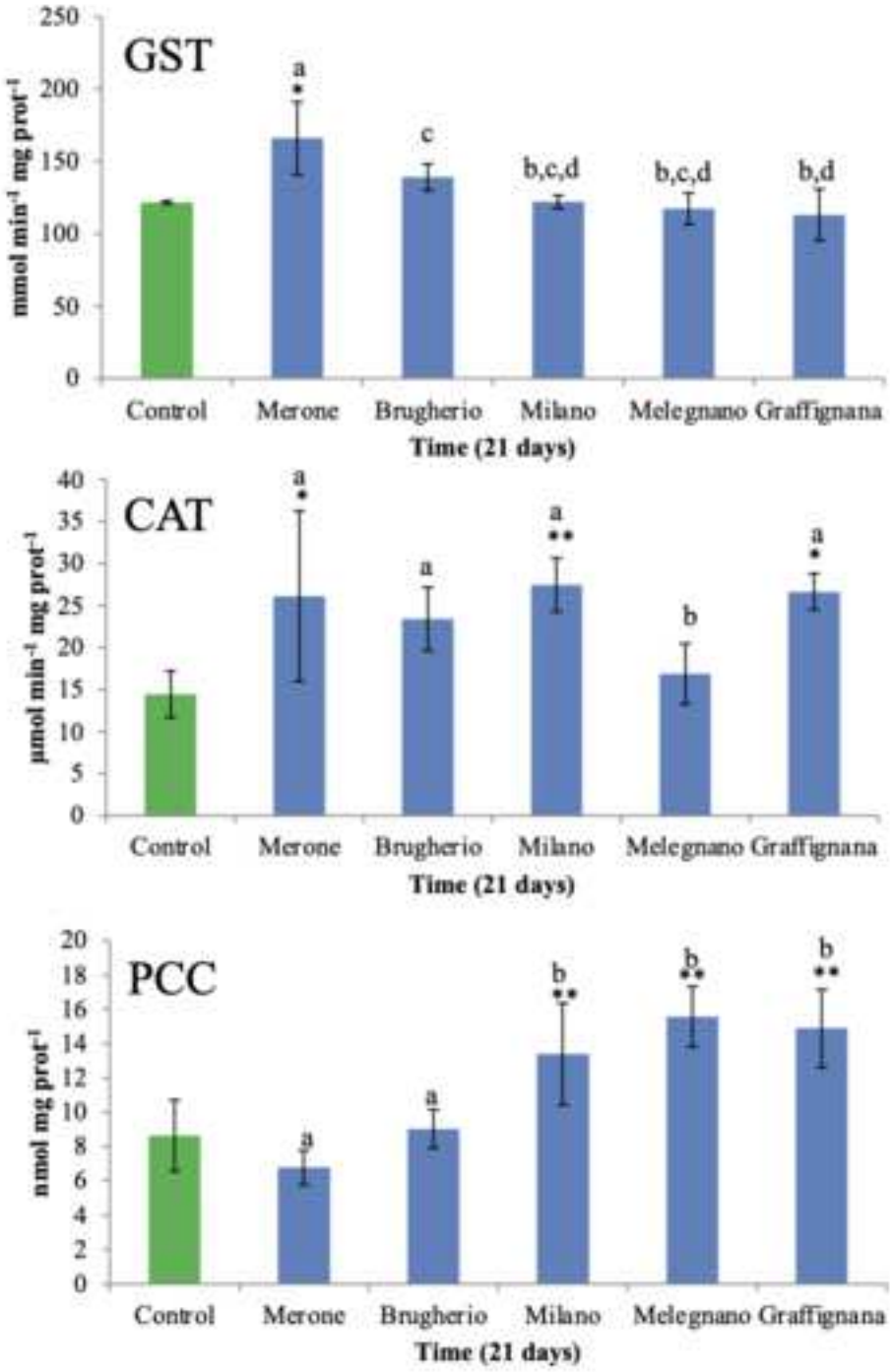
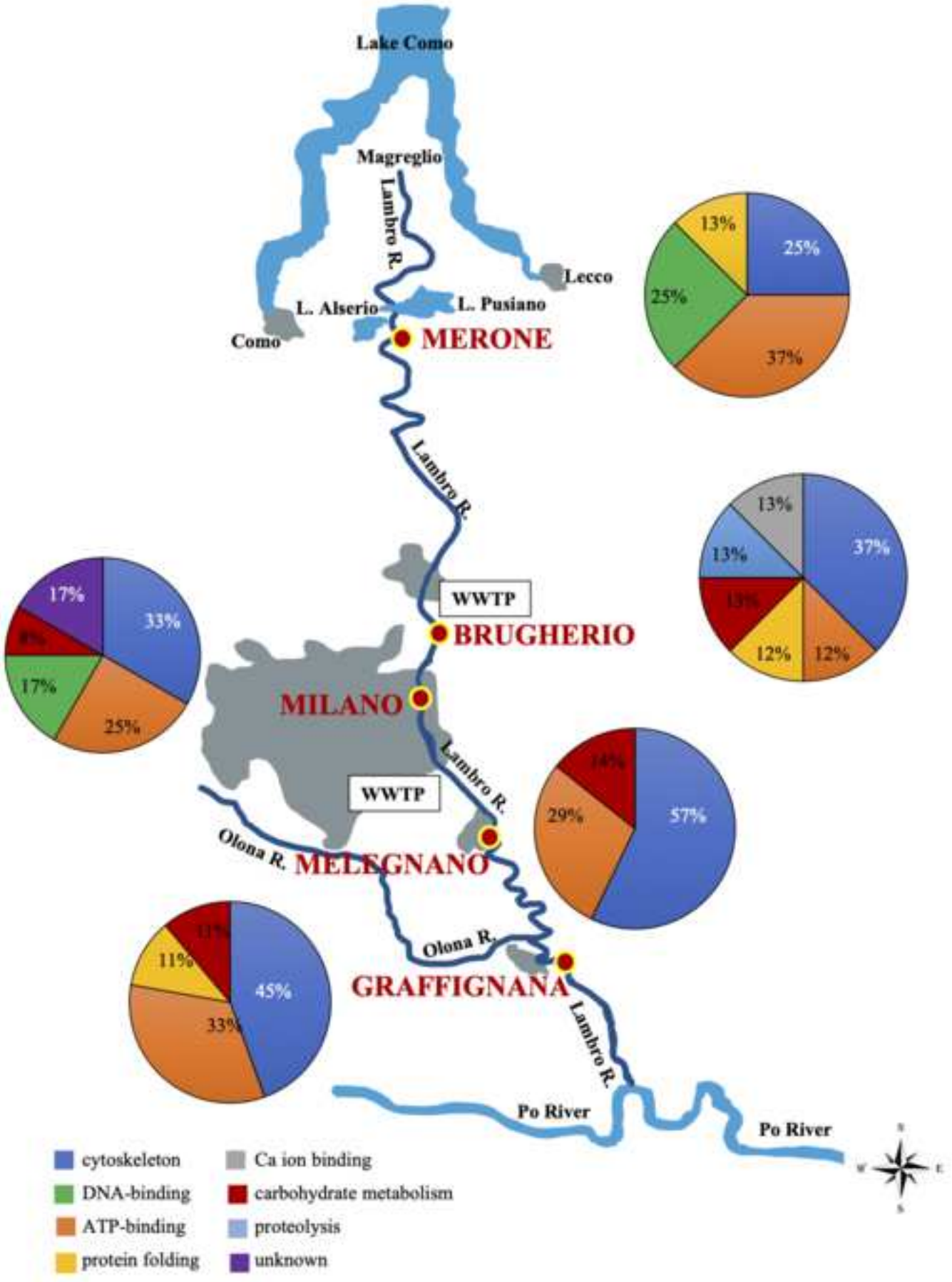


Figure 7

Click here to access/download;Figure;Figure 7.tiff



	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)

Table 1: Detected plastics in zebra mussels (1 pool of 10 mussels per treatment, 6 pools in total) at the end of exposure (t=21 days) to plastics from the 5 different sampling stations along the Lambro River.

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, Chicago (USA)	1.94±0.81 - 17.93±11.05	333	McCormik 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) and 50 - 3233 (dry season)	45	Zhao et al., 2020

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