# HAZARD EVALUATION OF PLASTIC MIXTURES FROM FOUR ITALIAN SUBALPINE GREAT LAKES ON THE BASIS OF LABORATORY EXPOSURES OF ZEBRA MUSSELS

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## ABSTRACT

Studies related to the evaluation of plastics in freshwaters have been increasing in recent years because approximately 80% of plastic items found in the sea are from inland waters. Despite the ecological relevance of these surveys, no information has been available until now about the hazard related to plastic mixtures in freshwaters. To fill this knowledge gap, we carried out a study aimed to assess the environmental risk associated with the "cocktail" of plastics and environmental pollutants adsorbed on their surface in one of the larger European freshwater basins. Plastic debris was collected by a manta trawl along one transect each in four of the Italian subalpine great lakes (Lake Maggiore, Como, Iseo and Garda) and

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administered to zebra mussels (*Dreissena polymorpha*), a useful freshwater biological model present in all these lakes. We estimated a plastic density from 4908 MPs/km$^2$ (Lake Iseo) to 272261 MPs/km$^2$ (Lake Maggiore), while the most common polymers found were polyethylene and polypropylene, with percentages varying between 73% and 100%. A biomarkers suite consisting of 10 different endpoints was performed after 7 days of exposure to investigate the molecular and cellular effects of plastics and related adsorbed pollutants. The main results highlighted a diffuse but different toxicity due to plastics for each lake, and there were significant changes in the antioxidant and detoxifying enzyme activities in Lake Maggiore, Iseo and Garda, an increase in protein carbonylation in L. Como, and a cellular viability decrease of approximately 30% for zebra mussels from L. Iseo and Garda. Despite this variability in the endpoints' responses, the application of the biomarker response index showed a similar environmental hazard due to plastics for all the sampled lakes.

Keywords: microplastics, biomarkers, water pollution, risk assessment, lakes

## 1. INTRODUCTION

There are many examples of the benefits of plastics for our lifestyle, such as their use in packaging, transportation, construction and the creation of new, high-technological polymers that allow the production of low-cost objects. The most recent report (PlasticEurope, 2018) revealed an increasing trend in plastic production worldwide, reaching approximately 350 million tons in 2017, of which more than 46 million tons were from Europe. Unfortunately, a growth in plastic production increases their release to the environment, as shown by the influx

of plastics to seas, which has reached 4.4-12.7 million tons/year (Jambeck et al., 2015). Plastics have an estimated lifetime of up to hundreds of years or even longer (Moore; 2008) but, once in the environment, the larger plastic items are also degraded into smaller debris by UV radiation from sunlight, mechanical abrasion, biodegradation and environmental erosion. This fragmentation originates the so-called microplastics (MPs) and nanoplastics (NPs), which can also directly derive from consumer products containing plastic microbeads, such as toothpastes, scrubs and cosmetic products.

Historically, studies on the presence and impact of plastics began in the oceans, but the pollution related to plastics in freshwaters has been an increasing concern in recent years since 80% of plastic items found in marine ecosystems derive from land-based sources (Andrady, 2011; GESAMP, 2015). Schmidt and collaborators (2017) calculated that 88-95% of the total plastic flux ending in the seas derives from only ten rivers, eight of which are located in the Asian continent and two in Africa. The major sources of plastic pollution in freshwaters are thought to be littering, dumping of plastic waste, loss from inappropriately managed landfill sites and waste collection (Lambert et al., 2014).

Although the number of studies focused on the presence of plastics in marine ecosystems is much higher than that currently found in freshwaters, the limited information suggests a similar pollution due to plastics (Blettler et al., 2018). The highest density of plastic items ranges from 1000 to 100000 items/$m^3$ in surface waters (Eerkes-Medrano et al., 2015), while the average density of the MPs alone varies from almost none to millions of MPs/$m^3$ (Li et al., 2018). The few studies carried out to evaluate the impacts of MPs and NPs in freshwater organisms showed similar effects to those found in marine fauna and in their capability to induce adverse effects along the entire aquatic trophic chain. For instance, an increase in mortality and a slight reproductive decrease were highlighted in daphnids of *Daphnia magna*

71 exposed to a concentration of $10^5$ polyethylene (PE) spherical beads/mL (Ogonowsky et al.,
72 2016), while no effects were observed in the amphipod *Gammarus pulex* exposed to irregular
73 polyethylene terephthalate (PET) fragments at concentrations ranging from 0.4-4,000
74 particles/mL with a size of 10-150 μm (Weber et al., 2018). An inflammatory response,
75 oxidative stress and lipid accumulation in the liver were found in *Danio rerio* exposed to high
76 concentrations (2 mg/L) of 0.07 and 5 μm polystyrene (PS) beads (Lu et al., 2016). Moreover,
77 exposure to 5 mg/$m^2$ microplastics for 2 days inhibited survival rates, body length and
78 reproduction of *Caenorhabditis elegans* (Lei et al., 2018). Our previous studies demonstrated
79 a variation in catalase and glutathione peroxidase activities, an increase in dopamine
80 concentration and a modulation of 78 different proteins mainly related to the response against
81 oxidative stress in zebra mussels (*D. polymorpha*) exposed to 2 mixtures of 1 and 10 μm
82 polystyrene microbeads (Magni et al., 2018; 2019a). Almost all of these studies exemplify
83 one of the main problems related to current studies on plastics carried out under laboratory
84 conditions in which virgin spherical beads are generally used, that certainly do not represent
85 the plethora of irregularly shaped polymers (lines, films, fragments, pellets and fibres)
86 collected in aquatic ecosystems. Furthermore, plastic debris adsorbs or contain many
87 environmental contaminants (e.g. heavy metals, pesticides, hydrocarbons, flame retardants,
88 dioxins and plasticizers; Bakir et al., 2012) and are colonized by several microorganisms and
89 proteins that can alter not only the uptake, but also infiltration, accumulation in tissues and
90 toxicity. The complexity of these aggregates is poorly considered in laboratory studies, as is
91 the weathering process and the selection of plastic concentrations, generally much higher than
92 the levels estimated in freshwater ecosystems. This is due to both the current scarcity of
93 available environmental data and the need to first evaluate the potential effects and
94 mechanisms of action of plastics. This means that exposures to plastics administered under

95 laboratory conditions currently lack the ecological realism caused by the enormous
96 complexity of potential exposure scenarios. On the other hand, the surveys carried out in
97 many surface waters have begun to shed light on the ubiquitous presence of plastics
98 worldwide (Eerkes-Medrano et al., 2015), but they do not provide information regarding the
99 toxicity of the "cocktails" of plastics and chemicals adsorbed on their surface.
100 To evaluate the presence and potential danger of plastic mixtures in freshwater ecosystems,
101 we conducted a survey in a major Italian reservoir aimed at measuring the toxicity of plastic
102 mixtures collected in four of the Italian subalpine great lakes (L. Maggiore, Como, Iseo and
103 Garda). To do this, specimens of zebra mussels were exposed for 7 days under laboratory
104 conditions to the four plastic mixtures collected along one transect in each lake. We used a
105 biomarker suite composed of 10 different endpoints covering both cellular and molecular
106 effects to evaluate the primary effect of plastics. The entire biomarker dataset was
107 subsequently ranked in the biomarker response index (BRI) to compare the effects revealed
108 in the four lakes. To our knowledge, this is the first study aimed at evaluating the pollution
109 due to plastic cocktails sampled in the field and simultaneously investigating its potential
110 ecotoxicological risk.
111
112 ## 2. MATERIALS AND METHODS
113
114 ### *2.1 Study area*
115 L. Maggiore (MA), Como (CO), Iseo (IS) and Garda (GA) are four of the six Italian subalpine
116 great lakes (Fig. 1) and represent the greatest freshwater resource of Italy, with more than
117 70% of the available total volume (Tartari et al., 2004). They are located in the central-eastern
118 hydrographic basin of the River Po (15299 $km^2$), which is one of the most populated and

119 industrialized European districts, characterized also by agriculture and intensive farming
120 (Mosello et al., 2010). In addition to their importance as water resources, these lakes are also
121 valuable due to their naturalistic, touristic and environmental values. In the recent decades,
122 they have been affected by several environmental problems, such as eutrophication and
123 cyanobacterial blooms (Salmaso, 2000), contamination due to persistent organic pollutants
124 (POPs; Binelli et al., 2005), water warming caused by climate change (Mosello et al., 2010)
125 and MP pollution (Sighicelli et al., 2018).
126
127 *2.2 Water sampling*
128 Samplings were carried out by one of the greater Italian environmental nonprofit associations
129 (Legambiente) during the 2018 edition of the 12$^{th}$ monitoring survey "Goletta dei Laghi". In
130 June and July 2018, the crew of the boat collected floating plastics in 24 transects in the study
131 area: 6 transects in L. Maggiore, 6 in L. Como, 5 in L. Iseo and 7 in L. Garda.
132 To determine the environmental risk, plastics were sampled two consecutive times by a manta
133 trawl (60x20 cm opening and 330 μm mesh size) along the transects which showed the higher
134 amount of plastics found in the previous 2016 and 2017 surveys (Sighicelli et al., 2018; Tab.
135 1). The manta trawl was dropped and dragged by the boat at an average trawling speed of 3
136 kn and maintained along the windward side of the boat (Hidalgo-Ruiz et al., 2012). It was
137 immersed 20 cm below the surface and filtered a mean of 80 m$^3$ of water, depending on the
138 transect length.
139 The first fraction was gathered from the manta net through many water washes, and samples
140 were immediately stored in glass vials with 30% hydrogen peroxide at 4 °C until observations
141 began (Sighicelli et al., 2018). To collect plastics from the manta trawl for the
142 ecotoxicological analyses, 1 L of NaCl hypersaline solution (1.2 g/cm$^3$; Magni et al., 2019b)

143 was used for each sample to separate suspended particulate matter from plastic debris, which
144 was then stored in glass jars at 4 °C pending the following exposures. No information is
145 available about the possible changes of chemicals adsorbed by MPs by the use of NaCl
146 hypersaline solution and until now alternative methods to separate MPs from the enormous
147 amount of organic and inorganic matter collected in natural samples did not exist.
148 Furthermore, unlike $H_2O_2$ (not used in the pools dedicated to ecotoxicological assays), NaCl
149 does not produce oxidant conditions able to interfere with the adsorbed contaminants.
150
151 *2.3 Plastics analyses*
152 Samples of plastics collected were washed, separated from organic matter, dried at 50 °C for
153 24 h in an oven and stored in a desiccator. The particles were counted and sorted into
154 categories based on shape and possible source: fragments of larger plastic debris, filaments
155 from fishing lines and textile fibres, sheets from thin items such as bags or packaging films,
156 pellets from industrial raw material and Styrofoam balls. Due to the abundance and visual
157 identification, the PS balls were assigned to a special category.
158 The mass and abundance of microplastics was determined in all trawl samples and estimated
159 in particles/km$^2$ on the basis of the area covered by the trawl (Tab. 1). According to Hidalgo-
160 Ruz et al. (2012), Fourier transform infrared spectroscopy (FT-IR) was applied to each MP
161 sample to identify the polymer. FT-IR spectra were collected in attenuated total reflectance
162 (ATR) mode using Nicolette 6700 spectrophotometer (Thermo Scientific, Rodano, Italy). The
163 chemical composition of the polymer particles was identified by comparison with reference
164 spectra (from the instrument library and http://www.ftir-polymers.com/soon.htm) and
165 according to the spectra collected during other degraded polymers characterizations
166 (matching factor ≥ 0.7).

167 *2.4 Exposures with zebra mussels*

168 Before the exposures, many separation steps were needed because all samples contained many
169 interfering materials, such as leaves, branches, dead insects and some macroplastics. Thus,
170 we preliminarily sieved the supernatant of each sample with 5 mm and then 63 μm steel sieves
171 (ISO 3310-1:2000) and immediately eliminated the material retained by the coarsest one.
172 Materials remaining on the 63 μm sieve were rinsed with Milli-Q water and then recovered
173 in a glass bottle by subsequent washes with the NaCl hypersaline solution and decanted
174 overnight. Despite these pretreatment steps, there was still a fair amount of sludge on the
175 bottom of the bottles, which was eliminated by a siphoning, while the plastics remained on
176 the surface. Finally, to be sure to expose the biological model to the plastics only, each sample
177 was filtered by the 63 μm sieve again, via the remaining NaCl hypersaline solution. Plastics
178 and the few small, inert materials remaining on the sieve were washed with tap water and
179 added to the glass exposure tanks. These sample treatments allowed the exposure only to
180 plastics and their adsorbed contaminants, which, being lipophilic, tend to remain tied to
181 organic substances rather than pass to the aqueous matrix.

182 Adult zebra mussels (length>1.5 cm), which are typical representatives of the fauna in the
183 sampled lakes, were collected in L. Iseo at a depth of 2-3 m and maintained for two weeks
184 under laboratory conditions to eliminate the inherent contaminants accumulated in their
185 tissues. The mussels were fed daily with a water suspension of dried blue-green alga
186 (*Spirulina spp.*; Magni et al., 2016; 2017; 2018). We randomly measured the diameter of the
187 inhalant siphon, which was approximately 1 mm in size.

188 Exposures were carried out in 4 L of a mixture of tap and deionized water (1:1) by placing 40
189 zebra mussel specimens on a metallic net in the middle of 5 tanks (1 tank for control and 4
190 tanks for exposures). A stirrer was placed at the bottom, which allowed the plastics to mix,

and an aerator supplied the oxygenation. We carried out the exposures in static conditions, having only one pool of plastics from each lake. This limited the exposure time to 7 days, which represented the maximum period in which hydro-chemical conditions in the tanks remained constant and did not represent a stress condition for the zebra mussels. During the exposure, the mussels were fed two times (t=1 and 5 days) with a water suspension of dried *Spirulina spp*.

*2.5 Biomarker measurements*

The biomarker suite consisted of 10 different endpoints: three antioxidant enzymes (superoxide dismutase-SOD, catalase-CAT and glutathione peroxidase-GPx), a phase II detoxifying enzyme (glutathione-S-transferase-GST) and the measures of reactive oxygen species (ROS) concentration and protein carbonylation content (PCC). Moreover, we measured some cyto- and genotoxicity endpoints: the percentage of apoptotic and necrotic cells, frequency of micronuclei (MN test) and percentage of cell viability.

At the end of exposures, the haemolymph was sampled from 9 mussels *per* tank, using a hypodermic syringe with 100 µL of PBS-EDTA 10 mM solution (1:1), to immediately measure the cell viability, while the remaining haemolymph was used for the biomarkers of genotoxicity. The leftover soft tissues, as well as the soft tissues of another 9 mussels *per* treatment, were stored at −80 °C to measure the PCC and the antioxidant/detoxifying enzyme activity. Finally, we stored soft tissues from 9 other mussels *per* tank to measure the ROS amount. The abovementioned endpoints were also measured at t = 0 for 2 pools of 5 mussels, which were directly collected from the acclimatization tank, to evaluate the baseline levels of the considered biomarkers.

214 We briefly described the methods used because they were described by previous studies
215 (Magni et al., 2016; 2017; 2018).
216
217 *2.5.1 Biomarkers of cellular stress*
218 Nine mussels *per* treatment were pooled in three different samples (*n*=3 pools of three mussels
219 *per* treatment) to evaluate the enzymatic activity of SOD, CAT and GPx, as well as GST.
220 First, we separately homogenized the 3 pools *per* treatment in 100 mM phosphate buffer (pH
221 = 7.4; 1:10 W/V ratio), with 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and
222 protease inhibitors (1:100 v/v). Then, we centrifuged the homogenates at 15,000 g for 30 min
223 at 4 °C to produce the S15 fraction, from which we quantified the proteins by the Bradford
224 (1976) method to normalize the enzymatic kinetics which were then measured by a 6715
225 UV/Vis spectrophotometer (Jenway, Staffordshire, UK) as reported in Magni et al. (2016,
226 2017, 2018 and citations therein).
227 For the ROS quantification, we used 10 mg/mL dichlorofluorescein-diacetate (DCFH-DA) in
228 DMSO. We added 20 µL of S15 fraction to a 96-well plate and incubated for 5 min at 37 °C.
229 Subsequently, we added 100 µL of PBS and 8.3 µL of DCFH-DA to each well and incubated
230 the plate at 37 °C for 30 min. We measured the fluorescence at ex. 485 nm and em. 530 nm
231 using the EnSight™ multimode plate reader (PerkinElmer), as reported in Parenti et al.
232 (2019). All endpoints were measured in triplicate.
233
234 *2.5.2 Biomarker of oxidative damage*
235 The PCC was measured in triplicate on the homogenate of 3 mussels collected from each tank
236 (*n*=3 pools of three mussels *per* treatment; 9 mussels *per* treatment) as performed for the
237 biomarkers of cellular stress. We homogenized the mussels' soft tissues in 100 mM

238 phosphate buffer (pH = 7.4; 1:10 W/V ratio), with 100 mM KCl, 1 mM EDTA, 1 mM DTT
239 and protease inhibitors (1:100 v/v). After the protein quantification (Bradford, 1976), we
240 processed the samples and measured the absorbance as reported in Magni et al. (2016, 2017,
241 2018 and citations therein).
242
243 *2.5.2 Biomarkers of genotoxicity*
244 In contrast to the previous assays, the measurement of genotoxicity was carried out for each
245 individual mussel (*n*=9) sampled from the tanks.
246 The haemocyte viability was measured by the trypan blue exclusion method: 10 µL of
247 haemocyte suspension was added to 10 µL of trypan blue dye (0.4%), gently mixed and
248 allowed to stand for 3-5 min. Then, 10 µL of the suspension was put in a Burker chamber,
249 and the live cell count was performed by a light microscope (10x). The haemocyte viability
250 was expressed as the percentage of live cells.
251 We followed the method of Singh (2000), adapted for zebra mussel, for the evaluation of
252 apoptotic and necrotic frequencies. Cell suspensions (10 µL) were added onto coated slides
253 in an agarose multilayer composed of low- and normal-melting agarose. Then, they were
254 treated with a lysing solution (NaCl 2.5 M, $Na_2EDTA$ 100 mM, Tris-HCl 8 mM, pH 10) in
255 the dark at 4 °C for 1 h. The slides were then washed with a neutralizing buffer and fixed in
256 absolute ethanol. We observed 300 cells *per* slide (9 slides *per* treatment) by a fluorescence
257 microscope (DMR, Leitz, Germany) and counted the apoptotic and necrotic cells.
258 To determine the micronucleus frequency, the haemolymph was placed on slides and then
259 fixed with glutaraldehyde solution (1% in PBS) for 5 min. Then, the cells were dyed with
260 Hoechst 33258, washed and mounted in glycerol-McIlvane buffer (1:1). The slides were
261 observed by a fluorescence microscope (DMR, Leitz, Germany), with 400 cells *per* slide

262 counted. Micronuclei were identified using the criteria suggested by Kirsch-Volders et al.
263 (2000).
264
265 *2.6 Evaluation of plastics' intake in zebra mussels*
266 To evaluate the plastic intake in the exposed mussels, we collected 5 specimens from each
267 tank, then pooled and homogenized them, using a ceramic pestle, in 10 mL of NaCl
268 hypersaline solution (1.2 g/cm$^3$; Avio et al., 2015). We filtered the supernatant on 8 µm
269 cellulose nitrate membrane filters (Sartorius$^{TM}$ 50 mm), using a vacuum pump, and then
270 digested the samples with 15% $H_2O_2$ overnight under a laminar flow hood. To monitor the
271 eventual atmospheric contamination by plastics (especially fibres), a nitrate cellulose
272 membrane filter was processed as a blank (Magni et al., 2019b). All debris (natural and
273 synthetic) extracted by the mussels was quantified and characterized in terms of shape,
274 dimension, colour and polymer composition using a Fourier Transform Infrared Microscope
275 System (µFT-IR; Spotlight 200i equipped with Spectrum Two, PerkinElmer). Spectra of
276 debris was acquired in attenuated total reflectance (ATR) mode, compared with library
277 standard spectra and accepted (matching factor $\geq 0.7$) after visual examinations (Magni et al.,
278 2019b). This detection was not possible for specimens from L. Garda due to the lack of
279 mussels.
280
281 *2.7 Statistical analyses and data exploration*
282 Data normality/homoscedasticity were evaluated using the Shapiro-Wilk and Levene tests
283 respectively. The significant differences (*$p < 0.05$; **$p < 0.01$), between treated samples
284 (those with plastics from the subalpine great lakes) and the control at $t = 21$ days were
285 evaluated using a one-way analysis of variance (one-way ANOVA) followed by the Fisher

LSD post hoc test through STATISTICA 7.0 software. To compare the toxicity of plastics from the subalpine great lakes on zebra mussels, we integrated each endpoint into the BRI modified from Hagger et al. (2008), as described in detail by Magni et al. (2017). First, we calculated the percentage of alteration level (AL) for each biomarker in comparison with the controls. Then, we ascribed a different score from 1 (≤ 20% of AL) to 4 (AL>100%) to each endpoint, multiplying this value by the biological weight of the measured biomarker (score=1 for molecular endpoints; score=2 for cellular endpoints). Finally, the BRI was calculated according to the following algorithm:

BRI: $\Sigma$ (AL biomarker$_x$ score) x (biomarker$_x$ weight)/ $\Sigma$ (biomarker$_x$ weight)

where AL=alteration level and x=measured endpoint.

## 3. RESULTS AND DISCUSSION

### *3.1 Quali-quantification of plastics*

Many factors can influence the diffusion of MPs in freshwater systems. Parameters such as the size of the waterbody, number of wastewater treatment plants, urban waste management system, wind and proximity of urban agglomerates and/or industrial activities strongly influence the quantity, and often typology, of MPs. Table 1 summarizes the density and polymer characteristics of the collected MPs from each lake. The abundances of MPs varied widely not only between the monitored lakes but also within each lake (data not shown). The highest mean abundance of MPs in surface water was found in L. Maggiore (100036 MPs/km$^2$, min = 23202, max = 272261), where the transect applied to collect the MPs utilized for the ecotoxicology tests showed the highest abundance of MPs (272261 MPs/ km$^2$).

309 Most of the plastic debris characterized in our samples was PE and PP (73-100%). This result
310 is not surprising as plastics are a large family of different polymers, and according to
311 PlasticEurope (2018), they are the best-selling polymers especially to produce large-
312 consumer objects. Within the collected samples no primary MPs were found, while secondary
313 MPs, which arise from disintegration and fragmentation of larger plastic items were
314 recovered; in particular, almost all of the PE MPs seem to be degraded from films/foils
315 traceable to old LDPE shopping bags.
316
317 *3.2 Ecotoxicity of plastic mixtures*
318 The evaluation of the intake and adverse effects due to plastics is hard to obtain due to the
319 many variables involved, such as size, shape, polymer type and even colour, as well as their
320 role as carriers for many environmental contaminants. The size of plastic debris is a crucial
321 factor not only for uptake in organisms, but also mainly for the capability to pass through
322 biological barriers (Rist and Hartmann, 2018), which creates many effects at the cellular level.
323 In general, smaller particles are more prone to toxic effects due to their easier entry into tissues
324 (Koelmans et al., 2015) and, in the case of natural samples, their higher capability to be a
325 vector for the adsorbed chemicals due to the higher surface/volume ratio. The size and shape
326 also play a fundamental role in relation to the different feeding strategies of organisms. For
327 instance, filter-feeders are more likely to ingest MPs, because they feed on the suspended
328 particulate matter, while aquatic vertebrates have different feeding strategies, which vary
329 depending on life stages, complicating the prediction of the capability of ingesting plastics
330 (Scherer et al., 2018). Food selection can imply a lack of correspondence between monitoring
331 and ecotoxicological data, because manta nets normally collect plastic debris larger than 300

μm, although the presence of many floating organic and inorganic materials decreases the mesh size and allows the sampling of smaller plastic debris.

*3.2.1 Characterization of plastics filtered by zebra mussels*

To ensure that zebra mussels filtered at least a fraction of the collected plastics from the four Italian subalpine great lakes, we checked for the presence of plastic debris in the tissues of some bivalves at the end of the exposures (Tab. 2). We found natural and synthetic debris in the mussels ranging from 50 μm to 3 mm in length. While the shortest length is comparable to data obtained by Winkel and Davids (1982), who showed zebra mussel filtration and ingestion of suspended particulate matter up to 40 μm, the presence of larger particles was less predictable. The debris in the mm order could probably pass across the mussel inhalant siphon, which was approximately 1 mm in size, as described above, and accumulate in the pallial cavity but could not be ingested. The data shown in table 2 clearly highlight that only a few MPs were filtered by zebra mussels, ranging from 1 fibre found in specimens from L. Maggiore and Iseo to 2 fibres in mussels from L. Como. Note that this check was not possible for L. Garda because of the high mortality (29%) observed after the exposure. We were not able to detect many pieces of debris with confidence because ageing greatly modifies the particles' structure, and the adsorption of many other natural compounds interferes with spectral identification. The identification of azlon (Tab. 2) deserves a separate discussion because it can be overestimated despite the high identification score obtained. Azlon is a regenerated fibre formed by proteins derived from natural sources, such as casein which has been used in several fabrics (Erinoid, Aralac), whose IR spectrum is very similar to that of azlon. Thus, since they can be confused with natural and weathered debris, we decided to consider only the azlon fibres as possible MPs, while fragments and lines revealed by the

instrument to be azlon, which were probably debris soiled with casein, were included in the uncharacterized debris (Tab. 2). In this context, an interesting debate, which deserves a formal position from scientific community, arises regarding the inclusion of regenerated materials derived from cellulose or animal proteins in MP categorization after FT-IR analysis, considering the complexity of discriminating between parental and regenerated compounds through IR spectra (Hartmann et al., 2019a, 2019b; Stark, 2019).

Surprisingly, we observed many natural fibres of cotton and cellulose in the exposed mussels, mainly in L. Maggiore and Como (Tab. 2), which suggests a possible high impact of washing clothes and/or atmospheric transport in these two basins. The lack of the presence of MPs in the controls (Fig. 2) excluded the possible contamination of samples during the analytical phases. This suggests that the quali-quantification of plastics, collected mainly in natural ecosystems, is not easy and requires an in-depth knowledge of data interpretation to avoid an overestimation of their environmental presence.

*3.2.2 Cyto-genotoxicity of the plastics' mixtures*

Among the plethora of possible adverse effects from plastics in an organism, we selected a biomarker suite, covering many molecular and cellular endpoints, to investigate the primary effect of plastic debris ingested by zebra mussels. The whole dataset highlighted a negative effect of plastics and related adsorbed pollutants in the zebra mussels exposed to all the samples from the four subalpine Italian great lakes (Figs. 2, 3, 4). The details of the results show that we measured the effect of the treatments on GPx ($p < 0.01$, $F_{4,10} = 6.7$) and GST activities ($p < 0.01$, $F_{4,10} = 62.5$) and found a significant decrease in GPx ($p < 0.05$) in zebra mussels exposed to plastics from L. Iseo, and of GST ($p < 0.01$) in zebra mussels exposed to plastic debris from L. Maggiore and L. Iseo (Fig. 2). A significant ($p < 0.01$) increase in GST

activity was observed only in specimens from L. Garda. Although no effects were observed for the other antioxidant enzyme (SOD and CAT) activities, we suggest a possible decrease in total glutathione at the cellular level. The thiolate anion ($GS^-$) is the typical cofactor for conjugation with electrophilic groups mediated by the detoxifying enzyme GST and represents the substrate reacting with $H_2O_2$ in the antioxidant response due to GPx. The lack of changes in ROS production (Fig. 2) seemed to confirm this hypothesis, as did the absence of the oxidative stress increase as a result of the tested plastic mixtures.

Since the GST was the most affected biomarker after exposure to MPs, another possible explanation of its variation could be related to the role played by the pollutants adsorbed on MPs, bearing in mind that this biomarker is implicated in phase II detoxification.

Protein carbonylation is a typical endpoint for measuring oxidative stress-related disorders and represents one of the most dangerous irreversible oxidative protein modifications. We found a significant effect of treatments ($p < 0.01$, $F_{4,10} = 17.7$) on PCC, with a significant ($p < 0.01$) doubling in specimens exposed to plastics from L. Como in comparison to baseline levels (Fig. 3), which showed a clear accumulation of these protein adducts in the stressed mussels' cells. This result seems to be inconsistent with the lack of ROS increase, but the relationship between oxidative stress and its consequences at the molecular and cellular level is very complex. Indeed, a very recent study demonstrated both the presence of different pools of functionally distinct ROS in the cells, and that low levels of ROS may still be harmful for some cellular functions (Hauck et al., 2018). Furthermore, Dukan et al. (2000) showed that protein carbonylation is limited not only by available ROS but also by the levels of aberrant proteins produced after drug administrations. Our results are comparable to those of Schirinzi et al. (2017) who found no significant ROS generation in human cells exposed to 0.05-10 mg/L polyethylene microspheres (3-16 μm). These results indicate a great complication in

tying together the results of the different biomarkers besides those measured in exposures carried out with natural plastic mixtures. In this problematic context, it is probably better to use the measured biomarkers only as prognostic tools instead of diagnostic tools, aimed to point out only a potential environmental hazard without any expectation to evaluate the mechanism of action.

As for the genotoxic effects, no variations were measured in the micronucleus frequencies and percentages of necrotic cells, while a significant effect of treatments ($p < 0.01$, $F_{4,36} = 12.7$) with significant ($p < 0.01$) increases in the apoptosis percentages were evaluated in mussels exposed to plastics from L. Maggiore, Como, Iseo and Garda (Fig. 4). This cannot be considered a harmful result because the measured levels of apoptotic cells have a statistical significance, but not a biological one, because similar values have been detected in previous studies as baseline levels for zebra mussels (Parolini et al., 2013; Magni et al., 2016; 2017). Much more harmful could be the significant effect of treatments ($p < 0.01$, $F_{4,39} = 3.3$) on haemocyte viability. A significant ($p < 0.05$) decrease of approximately 30% of the haemocyte viability was observed in mussels from L. Iseo and Garda (Fig. 4) which suggests an impact on survival, especially considering the high mortality observed for the bivalves from L. Garda, as mentioned above. This seems in contrast to the recent *in vitro* study of Espinosa et al. (2018), which found no effect on the cell viability of head-kidney leucocytes of gilthead seabream (*Sparus aurata*) exposed to 10 and 100 mg/mL PVC and PE particles with sizes of 40-150 μm. However, the comparison between these two studies is only virtual due to the different approach (*in vivo vs in vitro*), polymers tested (environmental plastic mixtures *vs* PVC and PE), targets (entire organism *vs* leucocytes) and biological models (zebra mussels *vs* gilthead seabream), which heavily influence the uptake, accumulation and effects of pollutants.

428 *3.2.3 Toxicity comparison by BRI*

429 The whole dataset of biomarkers was summarized by the BRI, a useful tool that allows the
430 production of a unique value for each lake and makes easier the comparison of the
431 environmental hazard due to plastic mixtures and associated contaminants easier. As
432 described in the 2.6 paragraph, the BRI assigns an increasing score to the increasing
433 difference percentages for each measured endpoint in comparison with the relative controls
434 and gives a higher importance (x2) to biomarkers to have a cellular effect than those with
435 molecular effects (x1). The BRI revealed a quite homogeneous hazard due to plastic mixtures
436 (Fig. 1S), which varied between 1.73 in L. Maggiore and Garda to 1.80 in L. Como and Iseo,
437 notwithstanding the different endpoints for each lake (Figs. 2, 3, 4). Furthermore, the
438 associated environmental risk reflects neither the evident differences in plastic concentrations
439 observed, which ranged from over 11000 MPs/km$^2$ in L. Iseo up to approximately
440 100000/km$^2$ MPs in L. Maggiore, nor the diverse polymer composition found in the lakes
441 (Tab. 1), which can act on different targets. This might be due to the very low number of
442 plastic debris items found in the mussels (Tab. 2), which makes it difficult to distinguish
443 between the impact on the organisms and the potential lack of correspondence between the
444 monitoring and ecotoxicological data, as explained above.

445

446 *3.2.4 Final remarks*

447 Our results raise several questions about the future need to evaluate not only the presence of
448 plastics in aquatic ecosystems but also their potential environmental hazard. For instance, the
449 ecological realism in our study has been only partially reached because we were forced to
450 concentrate the collected plastic debris, considering the mean volume of water filtered by the
451 manta net and the volume (4 L) of the exposure tanks. From the ecological point of view, it

452 would be better to conduct the experiments at a lower concentration of plastics for longer
453 exposure times to simulate the actual exposure conditions. Mirroring the real world is not
454 easy, especially for this kind of pollutant whose physical nature greatly complicates the
455 assessment of their environmental hazard compared with that of chemical contaminants.
456 Thus, in the next survey, we will try to partially solve these drawbacks, extending the
457 exposure period and administering less plastics through specific expedients.
458
459 ## 4. CONCLUSION
460 This study aimed to evaluate the potential hazard from the plastic mixtures collected in one
461 of the main European aquatic reservoirs and revealed only a moderate impact of these
462 physical contaminants and related adsorbed contaminants on the selected biological model.
463 The biomarker suite used highlights of direct effects on biological pathways instead of
464 defensive responses of organisms to the uptake of plastics. Indeed, the antioxidant machinery
465 was not activated against the possible increase in oxidative stress, which is one of the main
466 effects of plastics, and we observed a reduction in GPx and GST activities, which are possible
467 direct targets of plastics. The decrease in cell viability found in L. Iseo and Garda also seems
468 to show that the organisms suffer an impact from the plastics rather than having a defensive
469 response.
470 The lack of relationship between the concentration of plastics and data from the effect-based
471 tools clearly underlines that it is not possible to predict the environmental hazard of plastics
472 through simple quali-quantitative evaluation. Instead, it is necessary to integrate
473 ecotoxicological experiments that are able to also consider the potential magnification of toxic
474 effects due to the associated environmental pollutants. This should be the next challenge,

475 confronted in the near future, related to the impact of these physical contaminants on
476 organisms and human health.
477
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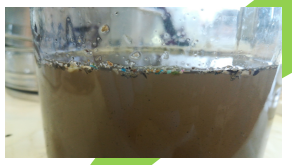


NaCl hypersaline solution

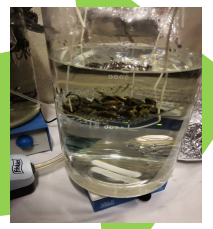
collection

sieving
63 μm

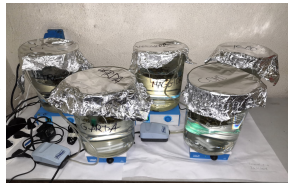
interfering materials

removal

plastic debris recovering



adaptation



exposures for 7 days

BIOMARKERS OF CYTO- AND GENOTOXICITY

biological model: *Dreissena polymorpha*

Experimental design

HIGHLIGHTS

1) Plastics was from 11000 MPs/km$^2$ (L. Iseo) up to 100000/km$^2$ (L. Maggiore)
2) A similar environmental hazard due to plastics was found for all the sampled lakes
3) Some enzymatic activities, protein carbonylation and cell viability were changed
4) No relation between sampled plastic amount and biomarkers data was found

Table 1. Density and polymeric composition of MPs sampled in the lakes.

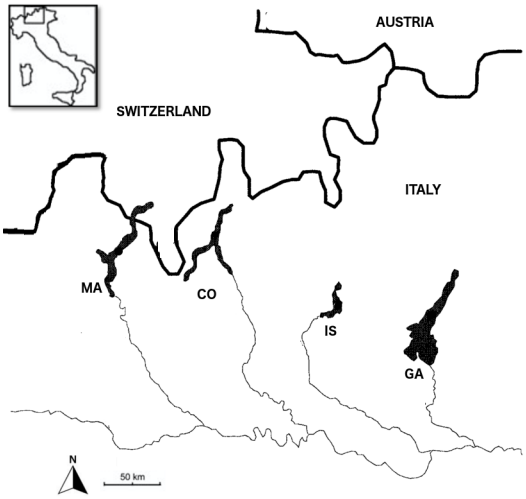
| | Maggiore | Como | Iseo | Garda |
|---|---|---|---|---|
| Surface ($km^2$) | 212.2 | 145.9 | 65.3 | 370 |
| Transect MPs/$km^2$ | 272261 | 15914 | 4908 | 6640 |
| Average MPs/$km^2$ | 100036 | 28675 | 11479 | 36063 |
| Transect MPs/$m^3$ | 1.361 | 0.080 | 0.025 | 0.033 |
| Average MPs/$m^3$ | 0.500 | 0.143 | 0.057 | 0.180 |
| PE (%) | 60.3 | 33.3 | 100.0 | 50.0 |
| PP (%) | 23.5 | 44.4 | | 50.0 |
| PS (%) | 7.4 | | | |
| PSE (%) | 1.5 | | | |
| CELL (%) | 1.5 | | | |
| PVC (%) | | 11.1 | | |
| PEST (%) | 1.5 | | | |
| PA (%) | | | | |
| PAK (%) | | 11.1 | | |
| Other (%) | 2.94 | | | |

PE=Polyethylene, PP=Polypropylene, PS=Polystyrene, PVC=Polyvinyl chloride, PEST=Polyester, PA=Polyamide, PAK=Polyacrylate, Other=unidentified polymers

Table 2 Number and characteristics of plastic debris found in the zebra mussels after the experiments.

| | | total number | Shape | Size in mm (lenght) | Color | Polymer |
|---|---|---|---|---|---|---|
| **MPs** | Control | 0 | - | - | - | - |
| | L. Maggiore | 1 | fibre | 1.48 | transparent | azlon |
| | L. Como | 2 | fibre | 0.60 | transparent | polyamide |
| | | | fibre | 0.90 | transparent | azlon |
| | L. Iseo | 1 | fibre | 1.51 | black | azlon |
| **Naturals** | Control | 1 | fibre | 1.20 | blue | cellulose |
| | L. Maggiore | 6 | fibre | 0.27 | violet | cotton |
| | | | fibre | 1.07 | blue | cotton |
| | | | fibre | 0.71 | transparent | cotton |
| | | | fibre | 0.79 | black | cotton |
| | | | fragment | 0.05 | blue | cellulose |
| | | | fibre | 0.15 | black | cellulose |
| | L. Como | 11 | fibre | 0.51 | transparent | cotton |
| | | | fibre | 0.50 | blue | cotton |
| | | | fibre | 0.67 | blue | cotton |
| | | | fibre | 0.59 | black | cotton |
| | | | fibre | 0.28 | blue | cotton |
| | | | fibre | 1.45 | blue | cotton |
| | | | fibre | 0.44 | black | cellulose |
| | | | fibre | 0.61 | transparent | cotton |
| | | | fibre | 0.63 | black | cotton |
| | | | fibre | 1.32 | blue | cotton |
| | | | fibre | 1.16 | transparent | cotton |
| | L. Iseo | 2 | fragment | 0.39 | white | aragonite |
| | | | fragment | 2.55 | white | aragonite |
| **Uncharacterized debris** | Control | 0 | - | - | - | - |
| | L. Maggiore | 9 | fibre | 0.53 | violet | - |
| | | | fibre | 0.70 | blue | - |

| | | | | | |
|---|---|---|---|---|---|
| | | fibre | 0.31 | violet | - |
| | | fibre | 1.44 | violet | - |
| | | fibre | 0.63 | blue | - |
| | | fibre | 0.48 | black | - |
| | | fibre | 0.19 | violet | - |
| | | line | 0.90 | brown | (azlon) |
| | | fibre | 0.53 | violet | - |
| L. Como | 8 | fragment | 0.37 | white | - |
| | | fragment | 0.25 | white | - |
| | | fibre | 0.93 | transparent | - |
| | | fibre | 0.55 | blue | - |
| | | fragment | 0.29 | brown | (azlon) |
| | | line | 2.97 | brown | (azlon) |
| | | line | 0.93 | brown | (azlon) |
| | | line | 1.42 | brown | - |
| L. Iseo | 3 | fragment | 0.15 | white | - |
| | | fibre | 0.79 | transparent | - |
| | | fibre | 0.66 | blue | - |


AUSTRIA
SWITZERLAND
ITALY
MA
CO
IS
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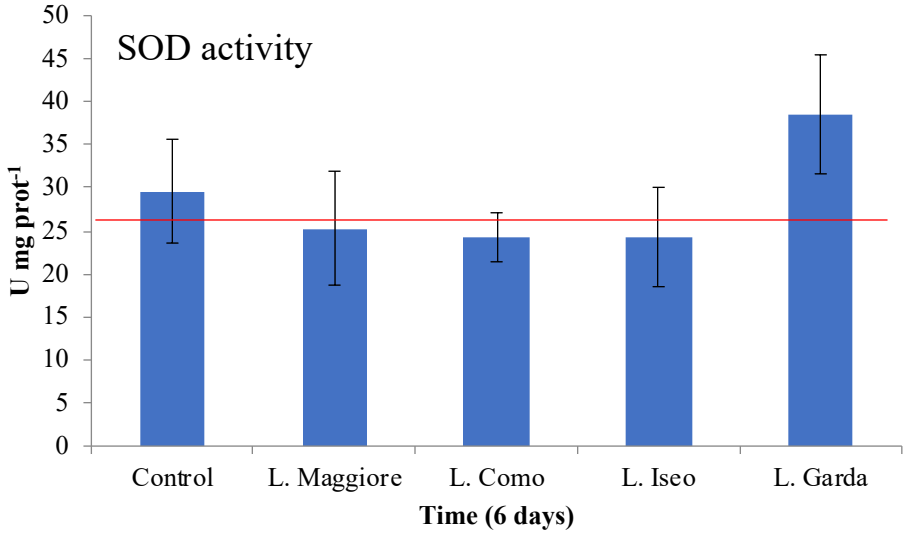
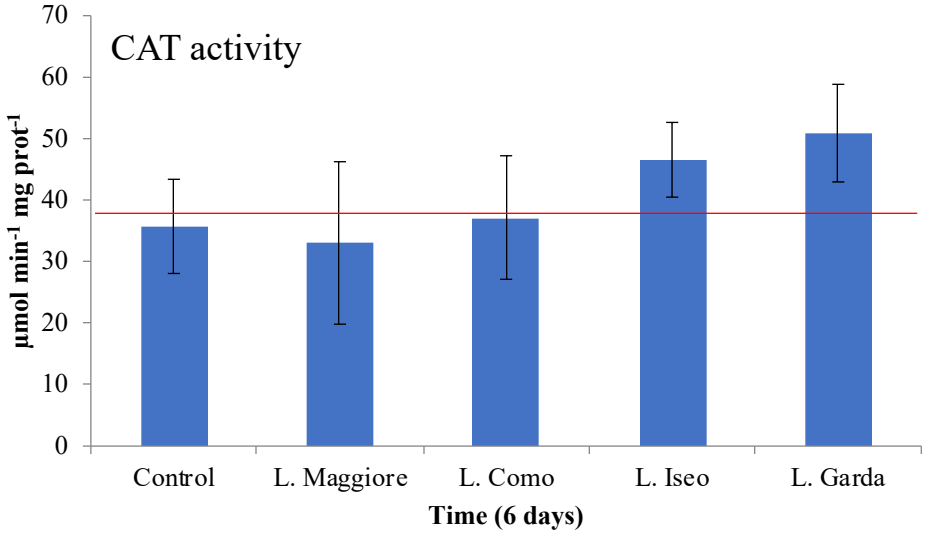
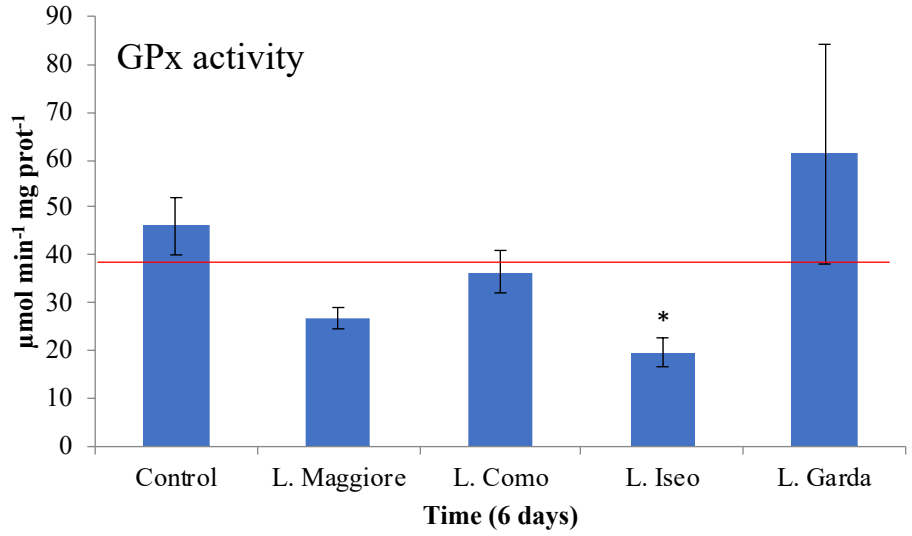
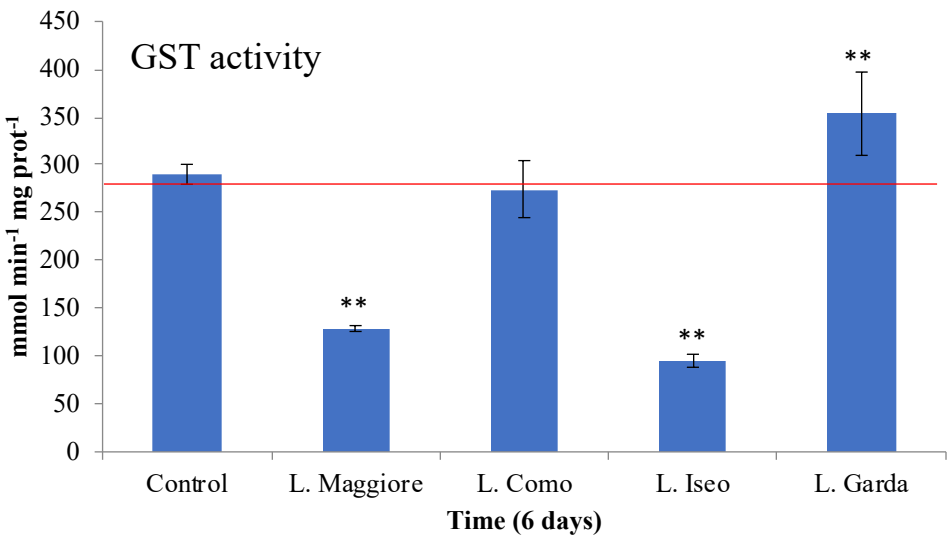
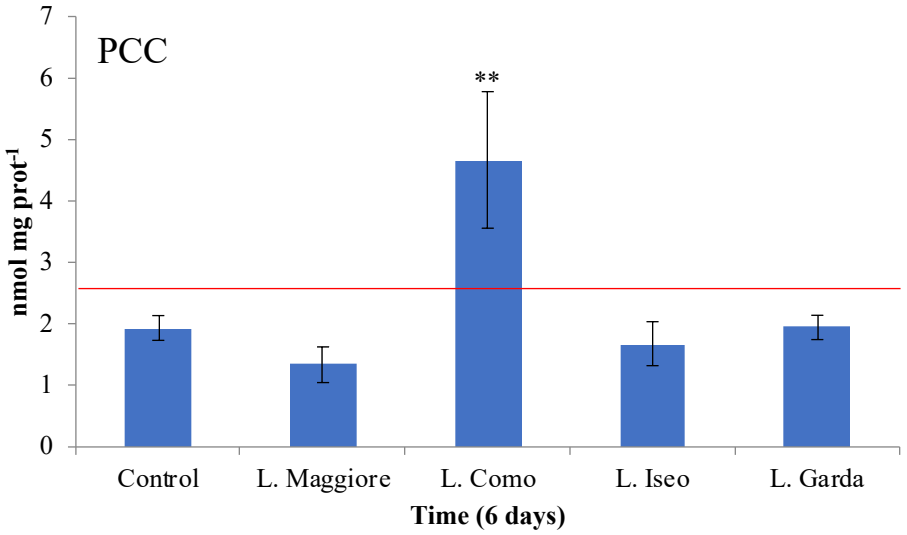
N
50 km

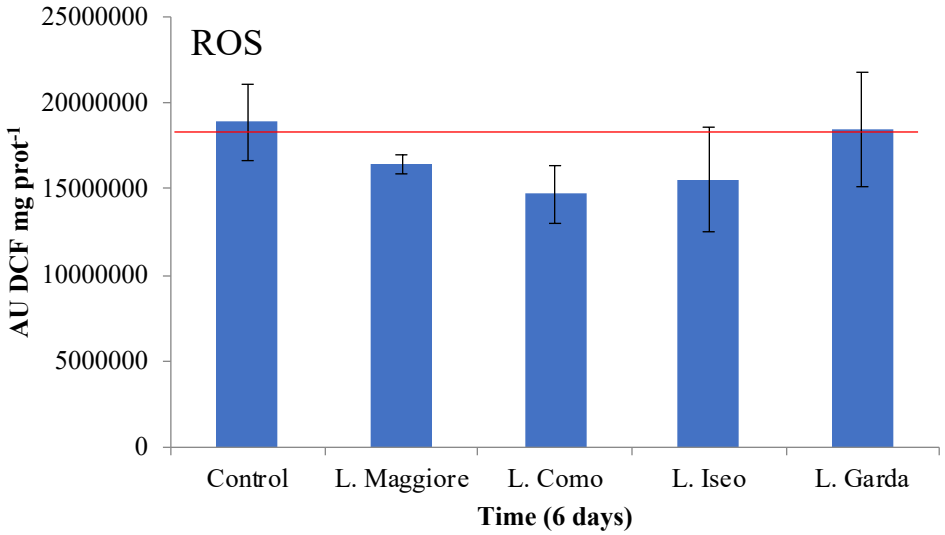

SOD activity
U mg prot-1
50
45
40
35
30
25
20
15
10
5
0
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)

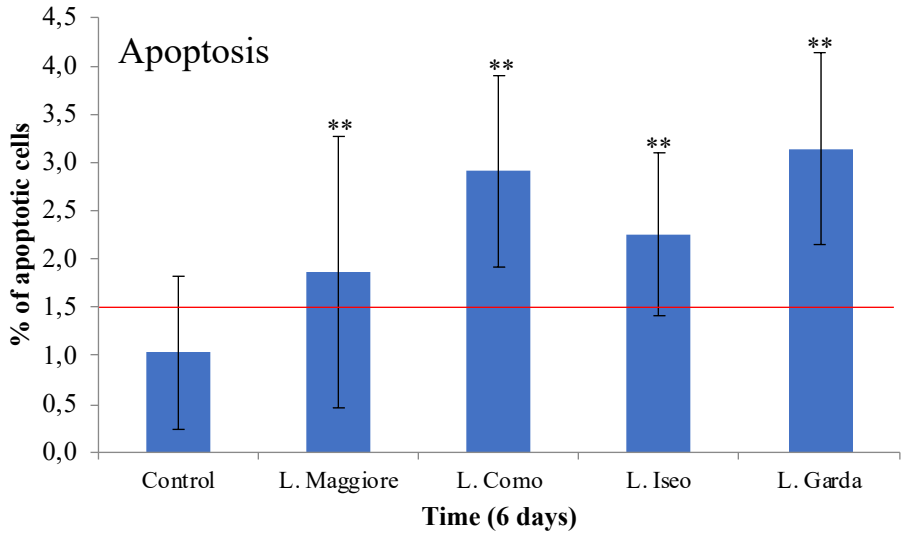

CAT activity
µmol min-1 mg prot-1
70
60
50
40
30
20
10
0
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)

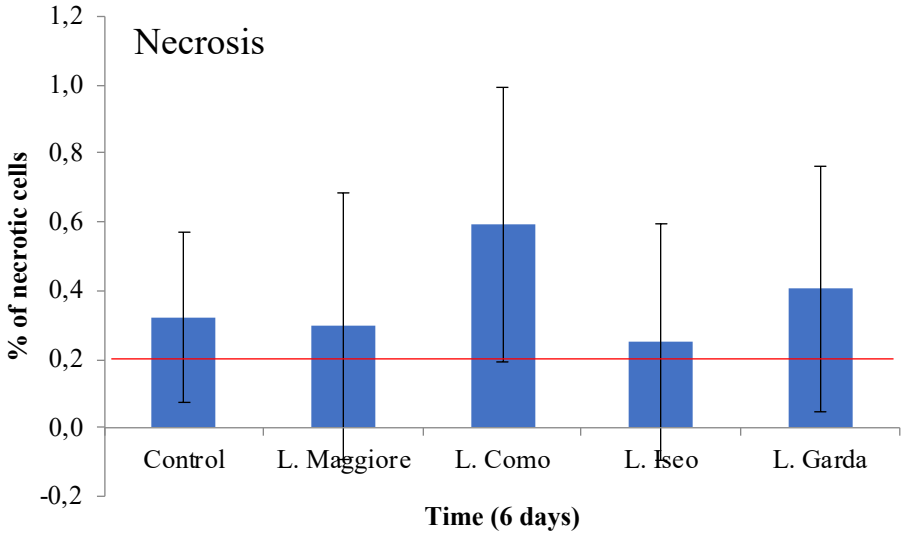

GPx activity
µmol min-1 mg prot-1
90
80
70
60
50
40
30
20
10
0
*
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)


GST activity
mmol min-1 mg prot-1
450
400
350
300
250
200
150
100
50
0
**
**
**
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)


PCC
**
nmol mg prot-1
0
1
2
3
4
5
6
7
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)


ROS
AU DCF mg prot-1
0
5000000
10000000
15000000
20000000
25000000
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)


Apoptosis
% of apoptotic cells
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
**
**
**
**
Control
L. Maggiore
L. Como
L. Iseo
L. Garda
Time (6 days)


Necrosis
% of necrotic cells
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
Control
L. Maggiore
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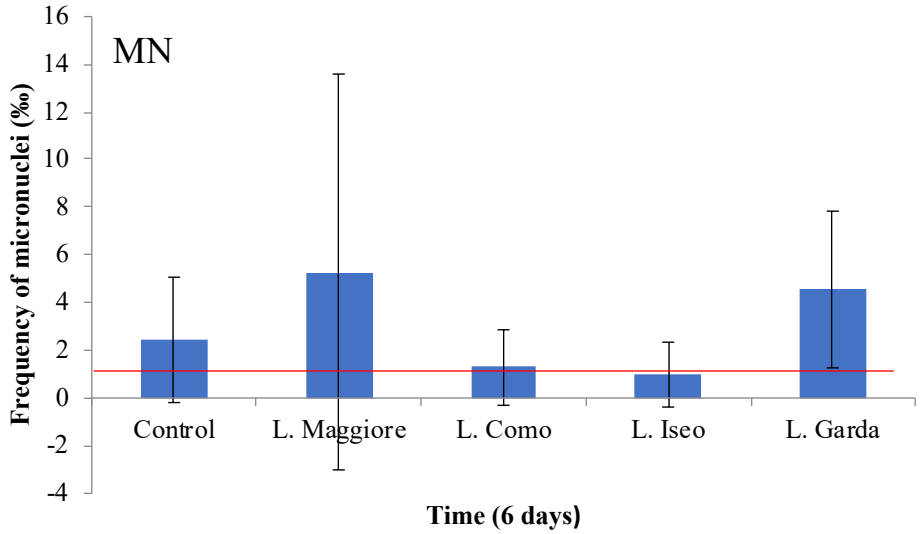
L. Garda
Time (6 days)


MN
Frequency of micronuclei (‰)
16
14
12
10
8
6
4
2
0
-2
-4
Control
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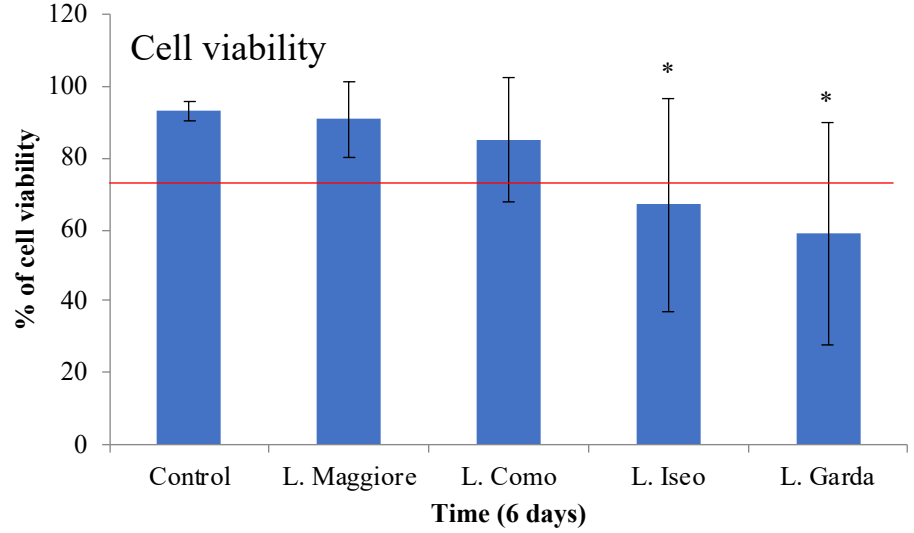
L. Iseo
L. Garda
Time (6 days)


Cell viability
% of cell viability
120
100
80
60
40
20
0
*
*
Control
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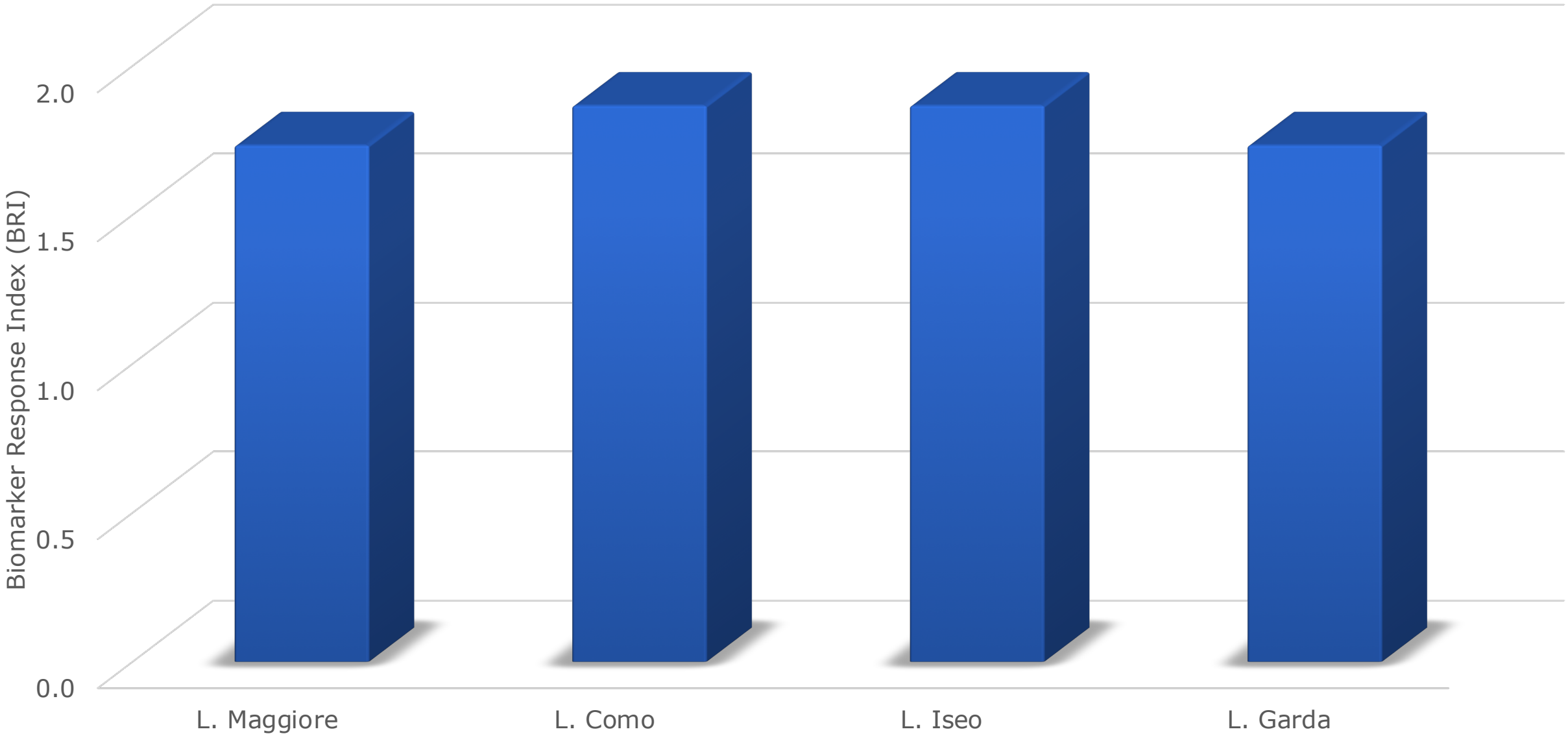
Time (6 days)


Biomarker Response Index (BRI)
2.0
1.5
1.0
0.5
0.0
L. Maggiore
L. Como
L. Iseo
L. Garda