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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Capolupo, M., Rafiq, A., Coralli, I., Alessandro, T., Valbonesi, P., Fabbri, D., et al. (2023). Bioplastic leachates characterization and impacts on early larval stages and adult mussel cellular, biochemical and physiological responses. ENVIRONMENTAL POLLUTION, 319, 120951-120962 [10.1016/j.envpol.2022.120951].

Availability:

This version is available at: https://hdl.handle.net/11585/983334 since: 2024-10-18

Published:

DOI: http://doi.org/10.1016/j.envpol.2022.120951

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(Article begins on next page)

1	Bioplastic leachates characterization and impacts on early larval stages
2	and adult mussel cellular, biochemical and physiological responses.
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17	Keywords: plastic additives, chemical mixtures, embryo development, biomarkers, Mytilus
18	galloprovincialis
19	Capsule sentence: Leachates from bioplastics cause embryotoxicity, alteration of
20	lysosomal parameters, and reduction of immune responses in Mytilus galloprovincialis.
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23 ABSTRACT

24 Bioplastics are promoted as safer alternatives to tackle the long-term persistence of conventional plastics. However, information on the potential release of additives and non-25 intentionally added substances (NIAS) in the surrounding environment is limited, and 26 biological effects of the leachates have been little studied. Leachates produced from three 27 bioplastics, i.e. compostable bags (CB), bio-polyethylene terephthalate bottles (bioPET) and 28 polylactic acid cups (PLA), and a control polymeric material, i.e. rubber tire (TR), were 29 examined. The chemical nature of bioplastic polyesters PET, PLA and poly(butylene 30 adipate-co-terephthalate) (PBAT) in CB, was confirmed by analytical pyrolysis. Fragments 31 were incubated in artificial sea water for 14 days at 20 °C in darkness and leachate contents 32 examined by GC-MS and HPLC-MS/MS. Catalysts and stabilizers represented the majority 33 of chemicals in TR, while NIAS (e.g. 1,6-dioxacyclododecane-7,12-dione) were the main 34 components of CB. Bisphenol A occurred in all leachates at a concentration range 0.3 - 4.8 35 µg/L. Trace metals at concentrations higher than control water were found in all leachates, 36 albeit more represented in leachates from CB and TR. A dose response to 11 dilutions of 37 leachates (in the range 0.6 -100%) was tested for biological effects on early embryo stages 38 of Mytilus galloprovincialis. Embryotoxicity was observed in the whole range of tested 39 concentrations, the magnitude of effect depending on the polymers. The highest 40 concentrations caused reduction of egg fertilization (CB, bioPET, TR) and of larvae motility 41 (CB, PLA, TR). TR leachates also provoked larvae mortality in the range 10-100%. Effects 42 on adult mussel physiology were evaluated after a 7-day in vivo exposure to the different 43 leachates at 0.6% concentration. Nine biomarkers concerning lysosomal functionality, 44 neurotransmission, antioxidant and immune responses were assessed. All lysosomal 45 parameters were affected, and serum lysozyme activity inhibited. Harmonized chemical and 46 biological approaches are recommended to assess bioplastic safety and support production 47 of sustainable bioplastics. 48

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49 1. Introduction

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The issue of ocean plastic pollution represents a global concern for its potential impact on 51 ecosystems' health (Agamuthu et al., 2019). Well before being degraded, plastics are 52 fragmented to micro and nano plastics, with higher potential to bioaccumulate and cause 53 detrimental health effects in marine species (Gallo et al., 2018; Peng et al., 2020). Plastic 54 fragmentation and weathering may facilitate the leaching of chemical additives, i.e. 55 compounds intentionally mixed with polymeric matrixes during manufacturing to confer the 56 final product specific requirements (Jia et al., 2020). Additives are polymer- and function-57 specific, and include plasticizers, flame retardants, stabilizers, antioxidants, pigments, 58 biocides, etc. Their presence can vary from relatively low to significant amounts (from 0.05 59 wt% for antioxidants up to 70 wt% for phthalate esters in flexible PVC), according to their 60 61 function (Gunaalan et al., 2020).

Among organic additives, bisphenols, phthalates, brominated flame retardants, organotin 62 compounds, alkylphenols, formaldehyde, antimicrobials and azocolorants are included 63 (Gunaalan et al., 2020; Luo et al., 2022). Many of the above chemicals are known or 64 suspected Endocrine Disrupting Chemicals (EDCs), i.e. compounds able to interfere at 65 66 different levels of the endocrine regulation inducing multiple adverse effects (Balbi et al., 2016; Canesi and Fabbri, 2015; Wang et al., 2020). Trace metals represent a prominent 67 group of inorganic additives, often demonstrated as hazardous to human and environmental 68 69 health (e.g. Capolupo et al., 2020). In addition, further intentionally and non-intentionally added substances including unreacted monomers and side or breakdown products do occur 70 in plastic items (Muncke, 2009). 71

Recent reports using experimentally produced plastic leachates have shown that plastics
 can release a variety of organic and inorganic additives into seawater within 1 to 14 days

(Capolupo et al., 2021a, 2020), providing evidence that plastic additive leachates are
 chemically complex, and can affect marine organisms' growth, development and survival.

To tackle the long-term persistence of conventional plastics, bioplastics are promoted as 76 safer alternatives. Bioplastics commonly encompass a diverse family of polymeric materials 77 that originates from biomass and/or are biodegradable. Confusion usually occurs among 78 bio-, bio-based and bio-degradable plastics (Lambert and Wagner, 2017; Wang et al., 2022). 79 Biobased plastics are those containing organic carbon of renewable origin from the natural 80 environment, while biodegradable plastics are made of polymers susceptible to 81 mineralization into CO₂, biomass and water by biological activity (Kjeldsen et al., 2019; 82 83 Razza and Degli Innocenti, 2012). Synthetic polyesters are commonly employed in the production of biodegradable plastics based on fossil resources, such as poly (butylene 84 adipate-co-terephthalate) (PBAT), polymers that are both bio-based and biodegradable 85 such as polylactic acid (PLA), and further polymers partially biobased such as poly(ethylene 86 terephthalate) (PET) from biobased ethylene. Bioplastics made of these polyesters can be 87 manufactured in forms of composites, as for instance PBAT/PLA and PBAT/starch. 88

The market share of bioplastics is relatively low compared to conventional thermoplastics; 89 90 however, it is steeply increasing worldwide after new law regulations have been approved 91 in many countries (Konti et al., 2022). Although most studies have shown no harmful effects from degradation of biodegradable polymers (Haider et al., 2019), very little is known on the 92 bioplastic chemical safety, the chemical nature of compounds included in the items, and the 93 potential toxicity of the leachates for ecosystem and human health. It has been reported that 94 bioplastics contain similar additives as their conventional counterparts (Lehtiniemi et al., 95 2021) and may be similarly toxic (Uribe-Echeverría and Beiras, 2022; Zimmermann et al., 96 2020a). Bioplastics undergo ageing processes mediated by abiotic and biotic agents 97 (mechanical abrasion, thermal degradation, hydrolysis, photo-oxidation, biodegradation) 98 that can deeply affect the properties of the polymer matrix, with consequent additive release. 99

Moreover, bioplastics are more vulnerable to degradation with respect to conventional 100 plastics, thus can produce microplastics or release associated toxic chemicals more readily 101 (Wang et al., 2022). 102

In general, hidden compromises and vagueness are found in the information provided by 103 the manufacturers both for bioplastic item composition as well as for their degradation due 104 to environmental agents (Haider et al., 2019; Nazareth et al., 2019), which hampers 105 establishing correlations between exposure and potential biological effects. To get this type 106 of information for bioplastics, a research effort focusing on both chemical and biological 107 aspects is needed (Xia et al., 2022). 108

109 The present work investigated the biological responses in mussels, *Mytilus galloprovincialis* exposed to seawater leachates from different types of bioplastics. Mussels of the genus 110 Mytilus are worldwide considered as a suitable sentinel organism for biomonitoring the 111 effects of contaminants in coastal waters (OSPAR Commission, 2013). Leachates were 112 experimentally produced and chemically characterized in terms of inorganic and organic 113 chemical content to identify possible relationships with biological effects. The screening of 114 a wide range of leachate concentrations was performed for the impairment of mussel 115 gamete fertilization, embryonic development, and larvae survival and motility. An array of 116 117 cellular, biochemical and physiological responses (biomarkers) was also investigated to evaluate adult mussel health status after in vivo exposure to the leachates. 118

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2. MATERIALS AND METHODS 120

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2.1. **Bioplastic leachate preparation**

122 Leachates were obtained from three commercial products representative of bioplastic materials made of aliphatic and aromatic polyesters: (1) PLA from commercial cups; (2) 123 bioPET from water bottles; and (3) PBAT mixed with PLA from compostable carrier bags 124

125 (CB). In addition, one conventional polymer was included, namely tire rubber (TR), used as 126 positive control (Bejgarn et al., 2015; Capolupo et al., 2020; Gualtieri et al., 2005; Wik and 127 Dave, 2006). Contamination was avoided by using glass or polytetrafluoroethylene materials 128 whenever feasible; laboratory items were rinsed with acetone (pico-grade, LGC Standards) 129 and glassware annealed at 200 °C for \geq 3 h.

Leachates were produced in artificial seawater (ASW), prepared according to ASTM (2004) as previously described (Capolupo et al., 2020). Briefly, selected materials were ground into <5 mm pieces and individually added to ASW at a final concentration of 80 g plastic / L. Samples were placed in a rotating incubator (125 rpm) at RT (~20 °C) for 14 d in the dark to allow for chemical leaching. Leachates were then passed through a sterile filter (0.2 mm Nalgene®) to eliminate particles and kept in darkness at 4 °C until use.

136

137 **2.2. Chemical analysis**

The procedure was carried out as previously reported by Capolupo et al. (2020) with a few 138 modifications. Seawater leachate samples (2 mL) were introduced into a 10 mL glass test 139 tube, added with internal standard (tri-tert-butyl benzene from Sigma-Aldrich) and extracted 140 141 3 times with ethyl acetate (1 mL) under vigorous magnetic stirring for 10 min. The organic extracts were collected and concentrated under nitrogen stream down to 0.5 mL; 1 µL was 142 then used for GC-MS analysis. A control seawater (ASW) leachate was analysed with the 143 same procedure, while procedural blank analyses with distilled water were performed in 144 between sample analyses. The overall procedure was run in triplicate for all samples. An 145 aliquot of the organic extracts (100 µL) of seawater samples, including control, was 146 subjected to trimethylsilylation with 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide for 2 147 hours at 60 °C. 148

149

150 **2.2.1 Analysis of bioplastic materials**

151 The chemical identity of the commercial plastic items was investigated by analytical pyrolysis (Py-GC-MS) and infrared spectroscopy. Specks of plastic materials (0.14 ± 0.02 mg) were 152 153 pyrolysed at 500 °C with a Multi-Shot Pyrolizer (EGA/PY-3030D Frontier Lab, Japan) interfaced to a gas chromatograph coupled with mass spectrometer (7890B and 5977B 154 Agilent Technology, USA). Thermally evolved products were injected in the GC at 280 °C 155 under 1:100 split ratio and separated with a HP-5ms Ultra Inert Agilent 19091S-433UI 156 column 30 m, 0.25 mm i.d., 0.25 µm film thickness operating from 40 °C (2 min) to 305 °C 157 at 20 °C/min. MS acquisition was performed under 70 eV electron ionization at *m/z* 35-600. 158 159 Plastic items cut in small fragments with the help of a scalpel were analysed by an ATR-FTIR spectrometer (Cary 630 FT-IR Spectrometer, Agilent, USA, with ATR diamond crystal) 160 in the $650 - 4000 \text{ cm}^{-1}$ wavenumber range. 161

162 **2.2.2 Analysis of leachates**

Gas chromatography-mass spectrometry (GC-MS) was performed on both underivatized 163 and silvlated organic extracts using a Shimadzu GC-2010 - GCMS-QP2010S system in 164 splitless mode at 250 °C under helium. Compounds were separated by a DB-5ms column 165 30 m, 0.250 mm i.d., 0.25 µm film thickness, with 1.1 mL min⁻¹ column flow working from 40 166 °C (2 min) to 320 °C at 10 °C min⁻¹, held at 320 °C for 6 min. The quadrupole mass 167 spectrometer operated under electron ionisation at 70 eV recording spectra in the 35 – 500 168 *m*/*z* interval. Temperature of MS source and quadrupole were set at 230 °C and 240 °C, 169 respectively. Chemical analyses of BPA were carried out with an HPLC system (Agilent 170 1.200 series, Agilent Technologies Italia S.p.A) coupled with a MS/MS spectrometer, 171 equipped with an electrospray ionization source (Quattro Premier XE Micromass, Waters 172 S.p.A.). Analytical details are reported in Supplemental material and in (Valbonesi et al., 173 2021). For trace metals assessment, samples were diluted in MilliQ water before internal 174

standards (¹⁰³Rh and ¹¹⁵In) were added. Analysis was performed using an Agilent 8800
 Triple Quadrupole ICP-MS (ICP-QQQ) equipped with a SPS 4 Autosampler, and
 quantification performed using standards from Inorganic Ventures.

178 **2.3 Mussel early life stages endpoints**

Early life stages endpoints investigated in this work encompassed mussel gamete fertilization, embryotoxicity, larvae motility and survival. Biological effects of 11 different leachate concentrations were assessed *in vitro*, ranging from 100% (no dilution) to 0.6% (167 times dilution) leachate concentrations in seawater. All experiments were carried out in quadruplicate (N = 4); parallel samples were run as controls (CTR, filtered seawater).

184 2.3.1 Fertilization and embryo-larval development

185 The effect of bioplastic leachates on gamete fertilization was evaluated as previously reported by Capolupo et al. (2020), by exposing sperms to the leachates (100% to 0.6% 186 concentration) for 1 h prior to add eggs in 1:5 proportion as in ASTM (2004). The reaction 187 was blocked after 30 min by adding calcium buffered formalin (4%). The acute 188 embryotoxicity test (ASTM, 2004) was adapted to 96 microwell plates (Fabbri et al., 2014) 189 to screen the impact of bioplastic leachates on *M. galloprovincialis* embryo-larval 190 development. Prior to leachate exposure, mussel oocytes were fertilized by mixing eggs and 191 spermatozoa at a 1:5 ratio in 96-well plates. Fifty eggs/well were used for fertilization test. 192 193 After microscopical verification of (> 90%) fertilization success, embryos (50 embryos/well) were exposed for 48 h to different dilutions of bioplastic leachates; the test was blocked as 194 above described, and samples examined at 40 x magnification using an inverted 195 196 microscope.

Normally developed larvae showing the typical "D-shaped" veliger stage in the absence of
 developmental failures (i.e. uncomplete shell, protruding velum) were identified. According
 to ASTM (2004), the test was considered acceptable if showing > 70% of normal D-veligers.

200 2.3.2. Larvae motility and survival

D-shaped larvae obtained by egg fertilization were reared until 5 days post fertilization (dpf) in laboratory conditions and then exposed to the leachates in 96-well microplates at a density of 50 larvae/well, as previously described (Capolupo et al., 2020). Results were recorded up to 48 h (motility) and 216 h (mortality) following the criteria previously reported by (Sprung, 1984).

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207 **2.4. Adult mussel exposure and biomarker evaluation**

208 2.4.1. Experimental design

209 Adult mussels (*M. galloprovincialis*) were purchased from a mussel farm (Cesenatico, Italy) and acclimated in controlled laboratory conditions (filtered seawater, 16 °C, 14 h:10 h 210 light/dark conditions) for four days before experimental treatment. Ten mussels were then 211 212 placed in aquaria (3 per experimental condition) each containing 10 L of filtered seawater, and exposed to 0.6% concentration of leachate (167 x dilution of the original leachates) for 213 seven days in line with previous experimental exposure using thermoplastic leachates 214 (Capolupo et al., 2021a). All leachates were tested in triplicate, each aquarium representing 215 a single experimental replicate (n=3). Aquaria for control condition (CTR) with only filtered 216 seawater were run in parallel (n=3). The exposure was performed in controlled conditions of 217 temperature (16-18 °C), photoperiod 14 h: 10 h light/dark) and feeding (1,200 cells/mL of 218 the green alga Nannochloropsis oculata), as previously described (Capolupo et al., 2021a). 219 220 Leachates and food were renewed daily after water change.

221 **2.4.2. Biomarker analysis**

222 After exposure, mussel tissues were dissected and, depending on the parameter to be tested, frozen in Liquid N₂ then stored at -80 °C, or immediately used for analysis. A battery 223 224 of nine biomarkers was assessed following the OSPAR 2013 protocol (OSPAR Commission, 2013), namely lysosome membrane stability (LMS), lysosome/cytosol ration (LYS/CYT), 225 neutral lipid (NL), malondialdehyde (MDA), and lipofuscin (LF) accumulation, and lysozyme 226 (LYZ), catalase (CAT), glutathione S-transferase (GST), and acethylcholinesterase (AChE) 227 activities. Haemocytes were collected from 4 mussels per vessel and LMS evaluated by the 228 Neutral Red Retention Assay (NRRA) (Martínez-Gomez et al., 2015). LYS/CYT, NL and LF 229 230 accumulation were assessed on 10 micron cryo-sections of mussel digestive glands as published by Capolupo et al. (2021a). Enzymatic assays were performed in pools of 231 digestive glands and/or gills taken from 6 mussels per vessel (18 mussels per experimental 232 condition). After homogenization and centrifugation, specific assays were conducted 233 spectrophotometrically (Capolupo et al., 2021a). Gills homogenates were used for 234 235 determination of AChE activity; after incubation with 0.5 mM acetylthiocholine iodide and 0.33 mM 5.50-dithiobis-2-nitrobenzoic acid (DTNB) changes in absorbance were followed 236 at 405 nm for 10 min (Valbonesi et al., 2003). Serum LYZ activity was measured as 237 previously described (Capolupo et al., 2021b), following for 10 min the decrease in 238 absorbance due to the LYZ effect on Micrococcus lysodeikticus. Details on methods for 239 biomarker measurements are reported in Supplemental materials. 240

241 **2.5. Statistical Analysis**

The statistical software packages 'R' and SigmaPlot 12 (Systat Software Inc. San Jose, CA, USA) were employed. All data were tested for normality using the Shapiro-wilk test and for variance equality using the Levene's test. On these bases, One-way Analysis of Variance (ANOVA), followed by the Bonferroni post hoc test, was applied to assess statistically significant variations. Differences were considered significant for p < 0.05. When applicable, EC50 was calculated on data for early life stages bioassays using the Log- model LL.3 included in the 'R' statistical package.

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3. RESULTS AND DISCUSSION

251 The need to obtain public information on the composition of plastics was stressed (Groh et 252 al., 2019), even more when proposing bioplastics as safer alternative to conventional plastics (Venâncio et al., 2022; Zimmermann et al., 2020b). Effect-based approaches are 253 254 needed to assess the overall toxicity of plastic items, which consider known and unknown additives released by plastic items, including NIAS, and their effects as mixtures. To the best 255 of our knowledge only two investigations are available that report on bioplastic leachates 256 composition and their biological effects on marine organisms, i.e. marine bacteria 257 (Zimmermann et al., 2020c) and sea urchin larvae (Uribe-Echeverría and Beiras, 2022). 258 259 Zimmermann et al. (2020b) applied methanol extraction and 1 hour- sonication in order to obtain leachates from several bioplastics; a large number of different compounds (> 1,000 260 chemicals each in 80% of the samples) including toxic chemicals were found in the 261 bioplastics and plant-based items examined, including erucamide, Irganox 1076, tris(2-262 nonylphenyl) phosphate etc. which also occurred in petroleum-based plastics. The Authors 263 also showed that commercial bio-based and/or biodegradable items may cause toxicity 264 similar to the conventional ones. 265

Uribe-Echeverría and Beiras (2022) investigated the chemical composition of leachates obtained in seawater (24 hours) from 3 different bioplastics, i.e. polyhydroxybutyrate resin (PHB), polylactic acid cups (PLA) and polylactic acid/polyhydroxyalkanoate items (PLA/PHA). Unexpectedly, a wide range of additives was found in PHB including chlorinated (1-chloro-tetradecane), brominated (dodecyltrimethylammonium bromide) and iodinated (1iodo-hexadecane) biocides. A few chemicals were released from PLA (oxo-methanol
benzoate, 1,5-dimethyl-1H-Pyrazole-3,4-diamine) and PLA/PHA (including Isocrotonic and
crotonic acid, and 2-Pentenoic acid) items. The mixture toxicity for sea urchin larvae
fertilization and development was observed after exposure to PHB leachates, while PLA and
PLA/PHA were ineffective.

276 Complementary to the above studies, the present experiments aimed to analyze the 277 chemicals released by bioplastic items after incubation for 14 days in seawater. Impairment 278 of biological endpoints were evaluated after exposure of both early-larval stages and adult 279 mussels to the leachates. Data were compared with control sample (seawater, incubated in 280 parallel) and a positive sample represented by tire rubber leachate.

281 **3.1 Characterisation of plastic materials**

Py-GC-MS and ATR-FTIR were used to confirm the chemical identity of the polymers composing the commercial materials. As an example of the importance of this analytical check, Klein et al. (2021b) found that bottles labelled as PLA resulted to contain other polymers after analysis by Py-GC-MS and ATR-FTIR.

The GC-MS traces (pyrograms) from Py-GC-MS of the investigated materials named as 286 PLA and bioPET (Fig. 1S) and the chemical composition of the pyrolysates (Table 1S) 287 confirmed the identity of the corresponding polymers (Tsuge et al., 2012). BioPET produced 288 a series of derivatives of terephthalic acid and benzoic acid. Lactides (meso and D,L forms) 289 were the main pyrolysis products of PLA along with their thermal degradation products 290 acetaldehyde, 2,3-pentadione and acrylic acid. Pyrogram of TR (Fig. 1S, Table 1S) was 291 featured by limonene and 2,4-dimethyl-4-vinylcyclohexene, typical marker of polyisoprene 292 rubber (Tsuge et al., 2012). Besides thermal degradation products of the rubber, two 293 additives could be identified that evolved by volatilisation, namely benzothiazole and 1,2-294 295 dihydro-2,2,4-trimethyl quinoline (Fig.1S).

Identification of materials composing CB was more challenging being a mixture of at least two polymers (**Fig. 1**). The presence of both *meso* and D,L-lactides in the pyrolysate indicated the presence of PLA. Several peaks not associated to PLA were tentatively identified as butyl esters of adipic acid (1,6-hexane dioic acid) and terephthalic acid. These pyrolysis products are consistent with building block of PBAT (**Fig. 1**) produced from the polycondensation of 1,4-butanediol with terephthalic and adipic acids (Jian et al., 2020).

Raw PBAT was pyrolyzed for confirmation and the resulting pyrogram (Fig. 1) presented 302 some of the products detected in the pyrogram of CB. In particular, a cyclic molecule, 303 virtually derived from the condensation of 1,4-butandiol and adipic acid, the 1,6-304 dioxacyclododecane-7,12-dione (Fig. 1, peak 7) was tentatively identified by NIST library 305 comparison. PBAT is used in packaging technology, in combination with other polyesters 306 combined with starch to improve properties and reduce costs while maintaining 307 biodegradability (Jian et al., 2020). Pyrolysis markers of starch were not detected indicating 308 that this compostable plastic bag was not based on starch. ATR-FTIR spectra of bioplastic 309 materials confirmed the polymeric species identified by Py-GC-MS (Fig. 2S). 310

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312 **3.2 Chemical composition of sea water leachates**

313 **3.2.1 Organic compounds**

The compounds tentatively identified in seawater leachates that were extracted with ethyl acetate are reported in **Table 1** along with their GC-MS data. Quantitation was not performed because not all the compounds were commercially available for calibration. TR was utilised as positive control for the several studies on the toxicity of leachates to water organisms (see Capolupo et al., 2020). In fact, several compounds were released into the sea water (**Table 1**). Among them benzothiazole and dicyclohexylamine were found in particle tire leachates in citrate buffered water where aniline was also tentatively identified (Seiwert et al., 2020). Aniline was also reported among the potential toxicants for aquatic organisms in the elutriates of sediments containing tire and road wear particles (Marwood et al., 2011). Benzothiazole and *N*-cyclohexylformamide were found in the water leachates of car tire rubber obtained under similar conditions by Capolupo et al. (2020).

In leachates from CB compounds that are structurally related to the PBAT backbone were 325 found, namely the monomer 1,4-butandiol and the cyclic ester 1,6-dioxacyclododecane-326 7,12-dione; this latter was a relevant pyrolysis product of CB (compound # 7 in Fig 1). In 327 accordance to our study, 1,6-dioxacyclododecane-7,12-dione was found in the leachates 328 obtained from weathered and original compostable plastic bags (Balestri et al., 2019). 329 Besides, 1,6-dioxacyclododecane-7,12-dione was found among the chemicals that migrated 330 into water from infant teether toys (Liu et al., 2021), as a potential migrant into isoctane from 331 polyurethane adhesives in laminates typical of food packaging (Félix et al., 2012) or into air 332 from volatilisation from materials based on polyurethanes (Thiébaut et al., 2007; Watanabe 333 et al., 2007). Moreover, 1,6-dioxacyclododecane-7,12-dione and other cyclic esters were 334 identified among the chemicals that migrated from adhesives of food packaging materials 335 into a solid food simulant (Canellas et al., 2015). These substances were presumed to be 336 NIAS associated to the aliphatic polyester-based portion consisting of adipic acid and 1,4-337 butanediol, as in PBAT. It is known that linear and cyclic oligomers of polyesters are 338 inevitably formed during manufacturing, probably through a "back-biting" mechanism of the 339 polymer backbone, and may occur in the final product as NIAS affecting its properties 340 (Zhang et al., 2022). This category of NIAS has the potential to migrate out the polymer. For 341 342 instance, linear and cyclic oligomers of PLA and PBAT with several repeating units were observed to migrate from a compostable PBAT/PLA into acidic water or pineapple juice fruit 343 (Ubeda et al., 2021). 344

One monomer of PBAT, 1,4-butanediol, was detected upon silylation of the sea water extracts. Similar to our finding, 1,4-butanediol was identified by Serrano-Ruíz et al. (2020) upon silylation among the compounds that migrated into a water mineral phase from bioplastic materials containing PBAT.

We cannot argue from the available data whether NIAS were originally present in the material or they have been formed by degradation during the leaching procedure. Canellas et al. (2015) proposed that cyclic esters can be neo-formed compounds derived from the cyclisation of 1,4-butandiol and adipic acid identified among the compounds prone to migrate from food packaging.

The occurrence of lactic acid in the leachate was in accordance with the presence of PLA in 354 the chemical composition of CB. Likewise, lactic acid was detected in the leachates of the 355 PLA material investigated in this study. In agreement with the available literature, lactic acid 356 was detected by GC-MS after silvlation of lyophilised mineral aqueous phase incubated with 357 biodegradable mulch blends containing PLA (Serrano-Ruíz et al., 2020). Lactic acid could 358 be formed by abiotic degradation of PLA or be originally present in the plastic material. As 359 discussed above, oligomers could be formed in the synthesis of polyesters and remain in 360 the final materials with a potential to migrate. Finally, no peaks were identified in the 361 chromatograms of the extracted samples of bioPET leachates with or without silvlation. 362

In the case of target additives, the attention was focused on BPA, worldwide exploited as a plastic monomer and plasticizer. It occurs in many commercial items, including bottles, cans, medical equipment, etc. (Prins et al., 2019). BPA occurred in all leachates analysed, from 0.3 to 4.8 μ g/L concentrations (**Table 2**). BPA was found in the aquatic environment from 0.5 ng to 12 μ g/L (Flint et al., 2012) and at these concentrations it has been reported to cause significant damages on exposed mussel embryo-larval stages (e.g Balbi et al., 2016). BPA exposure has clearly been correlated with endocrine disorders in humans (Valbonesi
et al., 2021 and reference therein).

The concentration of trace metals measured in the leachates is reported in Table 3. Zn 371 372 resulted to be the most abundant transition metal in the leachates. Zn is a metal utilised in a variety of additives as slip agents (stearate), fillers and pigments (e.g. oxides) (Hahladakis 373 et al., 2018). The relatively high content of Zn in polyester leachates could also be 374 associated to catalysts for polycondensation. As an example, organometallic compounds 375 based on Zn and Sn were reported in the synthesis of PBAT (Jian et al., 2020). Zn was 376 reported to be among the prominent trace metals in tires, and is considered a main 377 responsible for tire dust leachate toxicity (Marwood et al., 2011). However, Zn was present 378 at comparable concentrations in control samples (ASW), suggesting multiple sources. 379 Instead, copper was present at concentrations higher than ASW, in particular in CB. Salts 380 containing Cu could be utilised as plastic additives (biocides, pigments) and can act as pro-381 oxidants (Hahladakis et al., 2018). Iron, lead, nickel and arsenic were detected at very low 382 albeit detectable levels higher than ASW in the leachates of CB; among them Fe and Pb 383 could be components of some plastic additives (Hahladakis et al., 2018). 384

Overall, the leachates composition is different from different bioplastics and medium of extraction (Uribe-Echeverría and Beiras, 2022; Zimmermann et al., 2020a; present work) however no shared protocols are available for plastic leachate preparation (Gunaalan et al., 2020), yet.

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390 3.3 Biological impact of leachates

391 **3.3.1 Mussel early life stages endpoints**

In our experimental conditions, only leachates from TR significantly reduced the rate of egg 392 fertilization starting from 4% concentration, with an EC50 of 12.55% concentration (Fig. 3S). 393 Embryo development was the most affected endpoint (Fig. 2), in agreement with previous 394 results on embryos exposed to additives from conventional plastics (Capolupo et al., 2020). 395 Leachates from CB and TR induced significant effects already at 0.6% (CB) and 2% (TR) 396 leachate concentration. Leachates from PLA and bioPET also significantly impaired the 397 physiological larvae development with a consistent effect from 6% (PLA) and 10% (bioPET) 398 concentrations. The adverse outcome might be related to the sensitivity of embryos to the 399 metals found in the leachates. 400

The co-occurrence of BPA in the leachates may also contribute to the final outcomes, and 401 represents the predominant effect by PLA and bioPET leachates. Detrimental effects of BPA 402 on marine wildlife is well documented (Canesi and Fabbri, 2015) and embryotoxicity and 403 altered transcription effects on mussel embryo-larval stages were reported (Balbi et al., 404 2016). A specific action of BPA was demonstrated on mussel early larvae development, 405 where the xenobiotic affects both the deposition of the organic matrix as well as the 406 calcification of the shell, thus provoking altered phenotypes at 48 hour post-fertilization 407 (Miglioli et al., 2021). 408

Several NIAS associated to the chemical structure of the polyester (monomers and 409 oligomers of PLA and PBAT) were found in the leachates, as described in section 3.2.1. As 410 411 discussed above, this finding agreed with literature as far as the susceptibility of these 412 compounds to migrate out of the polymeric material into the surroundings medium is 413 concerned. Water leachates from microplastics of bioplastic, presumably containing 414 monomers/oligomers of different polyesters (among which PBAT) mixed with starch, 415 resulted to have almost no adverse effects on *L. variegatus* freshwater oligochaete; 416 however, toxicity was observed in the case of methanolic extracts, that represented a worst

situation, not extendible to environmental conditions (Klein et al., 2021a). It is worth 417 underlining the ubiquity of the cyclic ester 1.6-dioxacyclododecane-7.12-dione in the mixture 418 of compounds prone to be released from bioplastics containing PBAT. This cyclic ester was 419 a major compound in the leachates of CB, but no information on its toxicity to marine 420 organisms is available in published studies. Significantly reduced motility (at 48h) was 421 observed in larvae exposed to TR leachates within a concentration range of 20-100% (Fig. 422 **4S**); significantly reduced larvae survival (at 216 h) was caused by TR leachates in the range 423 10-100% (Fig. 5S), with EC50 values of 17.3 and 11.9%, respectively. Other leachates were 424 ineffective on survival, and PLA and CB only reduced motility at the highest concentrations 425 426 tested.

427

428 **3.3.1 Biomarker evaluation in adult mussels**

429 Biological effects of 0.6% leachate concentrations were assessed after in vivo exposure of mussels for 7 days (Fig. 3), and a significant LMS reduction was found in haemocytes 430 withdrawn from mussels exposed CB and TR leachates (Fig. 3 panel A). LMS decreased 431 432 also after PLA and bioPET leachate exposure, without reaching significance. LMS reduction 433 in haemocytes is the most sensitive biomarker of stress in mussels (Martínez-Gomez et al., 434 2015). Its reduction reflects the loss of membrane integrity and the impairment of lysosome 435 functionality, and is in fact an early warning signal for pathologies in Mytilus spp. (Moore et 436 al., 2006; Viarengo et al., 2007). LMS has been correlated with animal scope for growth and 437 total oxyradical scavenging capacity, and inversely correlated with protein catabolism, 438 lipofuscin and neutral lipid accumulation, lysosomal swelling, and DNA damage (Moore et 439 al., 2004). Significant reduction of LMS has previously been reported after mussel exposure 440 to leachates from conventional plastics (Capolupo et al., 2021a) and other chemical insults 441 such as polycyclic aromatic hydrocarbons, pesticides and metals (Shaw et al., 2019)

including conventional and emerging contaminants such as BPA (Canesi et al., 2007), which 442 occurs in all leachates presently tested. Trace metals such as copper, zinc and lead are 443 among the contaminants provoking destabilization of lysosome membranes (Giamberini and 444 Pihan, 1997), and these occur in TR and CB leachates. Copper is much higher than in 445 control water also in leachates from bioPET and PLA. Other compounds found in the 446 leachates could also affect LMS, alone or in combination. As previously shown, LMS is 447 reduced also after mussel exposure to microplastics (Canesi and Fabbri, 2015; Capolupo et 448 al., 2021a; Sharifinia et al., 2020). Whether the microplastics ingested do have time enough 449 to release additives along the digestive tract before elimination (Fernández and Albentosa, 450 2019) is not known yet, however it is a challenging issue in wildlife as in humans. Overall, 451 LMS is a useful biomarker to highlight the occurrence of low concentrations of organic and 452 inorganic contaminants in water either alone or in mixtures. Reduction of LMS often leads 453 to lysosome increase in size and fusions (Lowe et al., 1981; Moore, 2008). The increase of 454 the lysosomal volume is a condition predictive of impairment of viability and functionality of 455 digestive gland cells (Orbea et al., 2006). The increasing size of the lysosome compartment 456 (LYS) with respect to cytosol (CYT) has been measured in mussels exposed to leachates. 457 LYS/CYT ratios were significantly higher than in controls in mussels exposed to TR, CB and 458 459 PLA (Fig. 3 panel B). Metals occurring in the leachates could be in part responsible for this effect. In fact, Cu and other metals produced lysosome enlargement and organelle fusion, 460 with a mechanism at least in part mediated by a calcium dependent-phospholipase A2 461 stimulation (Marchi et al., 2004). High LYS/CYT ratio was measured in mussels which 462 accumulated Cu and Zn from coastal lagoon waters (Capolupo et al., 2017). These metals 463 occur in all tested leachates at concentrations that in some cases are significantly higher 464 than control water. Lysosomotropic effects could however be induced in mussels by further 465 chemical additives or NIAS, whose properties are not known, at present. 466

All leachates significantly enhanced the NL content in mussels with respect to the controls 467 (Fig. 3 panel C), except for PLA (p=0.054). NL increases in digestive gland indicate lipidosis, 468 a metabolic disorder consequence either of reduced lipid utilization or decrease in fatty acid 469 processing (Viarengo et al., 2007), (Dailianis, 2011). NL accumulation in mussels was also 470 provoked by exposure to leachates from conventional plastics (Capolupo et al., 2021a), and 471 by other water pollutants in laboratory (e.g. Canesi et al., 2007) as well as in field condition 472 (Capolupo et al., 2017; Signa et al., 2015). Similarly, field exposure to metals that are also 473 present in the leachates analyzed in the present work, were found to increase NL content in 474 mussel digestive gland lysosomes (Brooks et al., 2018; Donnini et al., 2007; Fokina et al., 475 476 2013).

Reactive oxygen species (ROS) production is a known effect of pollutants (Regoli and 477 Giuliani, 2014) able to induce lipid peroxidation (Moore, 2008). The products of lipid 478 peroxidation are effectively sequestered by lysosomes, among these MDA, as intermediate 479 compounds with respect to the final products represented by LF (Terman and Brunk, 2006). 480 In particular, LF are insoluble aggregates made of lipid peroxidation residues which bind to 481 food degradation by-products, oxidized proteins, carbohydrates, and metals (Terman and 482 Brunk, 2006). These compounds are observed in mussels in response to oxidative pollutants 483 484 including metals (Gomes et al., 2014; Maria and Bebianno, 2011). LF content in digestive glands was increased significantly after 1-week exposure to 0.6% leachates from TR and 485 CB (Fig. 3 panel D). Increases, although not significant, were noted also for PLA and 486 bioPET. It has to be underlined that 1 week is a minimum time for lipofuscin accumulation 487 (Viarengo et al., 2007), thus the one represented on Fig. 3 (panel D) may not be the 488 489 complete biomarker response. No significant change was instead noted for MDA content (Fig. 6S panel A). This response was different from what expected, especially in case of 490 moderate peroxidation as from PLA and bioPET, where the accumulation of LF is not 491

significant. Such a low MDA production after exposure to CB and TR leachates could instead
be ascribed to its depletion to support LF production. Increase of LF contents is correlated
to the LMS decrease found in haemocytes; such a correlation was previously reported
(Donnini et al., 2007; Franzellitti et al., 2014) and confirms the relationship between oxidative
stress and lysosomal disorders (Moore et al., 2006).

497 Overall, biomarkers of lysosomal dysfunction in mussels are predictors for pathology and 498 have ecosystem relevance (Moore, 2008; Moore et al., 2006), thus emphasising the 499 importance of considering (bio)plastic leachates among the environmental hazards.

500 Leachate potential to trigger mussel antioxidant system has been evaluated addressing CAT and GST activities, in gills and digestive gland of exposed animals (Fig. 4). The different 501 role of the enzymes in the two tissues was confirmed, with CAT having higher basal activity 502 in digestive glands, where peroxidation reactions are mostly performed, and GST showing 503 higher activity in gills, i.e. the first tissue exposed to environmental xenobiotics (Capolupo et 504 al., 2021a). CAT activity was significantly reduced by leachates from bioPET in gills, and 505 enhanced by leachates from CB in digestive glands (Fig. 4 panel A); GST was significantly 506 enhanced by leachates from PLA in gills, and from CB in digestive glands (Fig. 4 panel B). 507

Previous studies reported different responses of CAT and GST, increase, decrease or no 508 effect, after pollutant exposure (Akcha et al., 2000; Cheung et al., 2004; Gowland et al., 509 2002; Petushok et al., 2002; Regoli et al., 2004; Robillard et al., 2003). Cu and Zn were able 510 to activate GST in Mytilus spp (Canesi et al., 1999; Capolupo et al., 2017); differently, a 511 significant GST inhibition was reported in mussels after BPA exposure (Canesi et al., 2007). 512 513 The data presently observed might be the result of opposite effects by the compounds included in the leachate mixture, or the low concentrations were not sufficient to stimulate 514 bigger responses by the cellular defenses. 515

Bivalves, as all invertebrates, display an innate immune system to fight against pathogens 516 and xenobiotics. Lysozyme is a bacteriolytic enzyme concurring to the immune response 517 with the specific effect to hydrolyse the β -1,4-linked glycoside bonds of bacteria wall (Gerdol 518 et al., 2018). Lysozyme activity was found in bivalve hemolymph and tissues (e.g. Myrnes 519 and Johansen, 1994) and modulated by several contaminants (Matozzo et al., 2008; Stabili 520 and Pagliara, 2009). In our experimental trials, all leachates inhibited the lysozyme activity 521 in exposed mussels, although only the responses to CB and TR reached statistical 522 significance (Fig. 4 panel C). 523

A reduction of lysozyme activity is a marker of immunosuppression, thus of lower resistance 524 to bacterial insult. Lysozyme activity was also reduced by the estrogen-like compound 525 tributyltin (Matozzo and Marin, 2005), while increased by 17-β estradiol (Canesi et al., 2006). 526 527 suggesting that estrogen receptors may be involved in the control of enzyme activity. Although *Mytilus* spp. do not produce estradiol, they possess estrogen receptors that can 528 be involved in this function (Balbi et al., 2019). Recent studies have shown that blood clam 529 Tegillarca granosa lysozyme activity (together with some other innate immune effectors) 530 was inhibited by BPA, microplastics and BPA plus microplastics (Tang et al., 2022). The co-531 exposure of T. granosa to microplastics, B[a]P and E2, led to the highest reduction of 532 lysozyme release and activity (Tang et al., 2022). Furthermore, the leachates also contained 533 trace metals that may affect lysozyme activity. In fact, an effect of Cu on lysozyme molecular 534 configuration was suggested many years ago from investigations on *Mytilus* haemocytes 535 (Steinert and Pickwell, 1984). Copper was more recently reported to irreversibly inhibit 536 chicken egg white lysozyme activity up to 80% at 390 µM, as a consequence of binding to 537 538 specific aminoacidic residues at the catalytic site of the enzyme (Ko et al., 2018). Inhibition was also observed after exposure to Zn, Mn and Co, while Ca had no effect (Ko et al., 2018). 539 Cr, Ni, Cd, B, Hg, and Pb in the range 10-50 mM caused dose-dependent reduction of hen 540

egg white lysozyme activity already after 4 h of exposure, and the effect remained of similar extent at 12 and 24 h (Pazmiño et al., 2018). The strongest effects were obtained after exposure to CrVI or Cd, which reduced the lysozyme activity to about 25% of the control already within 4 hours at the lowest concentration tested. The combination of the different compounds found in the leachates may lead to the overall reduction of lysozyme activity shown in **Fig. 4** panel C.

AChE is an enzyme involved in nervous transmission useful as biomarker of neurotoxicity 547 (Valbonesi et al., 2003). Its inhibition, typically by organophosphates, induces a protraction 548 of the nervous stimulus (Valbonesi et al., 2003). In our experimental trials, however, no 549 change in AChE activity was detected of exposure to the different leachates at 0.6% 550 concentration (Fig. 6S panel B). In mussels AChE was found to be affected by the mixture 551 of BPA, carbamazepine and atrazine (Juhel et al., 2017), and inhibited by metals (Frasco et 552 al., 2005); moreover, AChE inhibition by plastic leachates has recently been reported in fish 553 (Walpitagama et al., 2019). The short exposure and/or the high dilution of the leachates may 554 555 be the reason of the lack of effect in our experimental system, although at least BPA and trace metals occur. 556

557

558 **4.0 CONCLUSION**

This investigation increases the knowledge gained from two previous studies carried out by other laboratories which addressed the contents of polyester-based bioplastic leachates and their biological effects on marine organisms, challenging bacteria and sea-urchin larvae. The originality and strength of the present work is that both early-larval stages and adult mussels were exposed through *in vitro* (at 11 different dilutions) and *in vivo* approaches to leachates obtained after 14 days in seawater and chemically characterized. The study confirms that leachates from bio-plastics do contain organic compounds (additives, non-intentionally added compounds such as oligomers) and trace metals, and exposure to leachates does affect *M. galloprovincialis* early embryo stages development and adult physiological parameters.

569 Overall, the magnitude of the effects was different depending on the polymer tested, possibly 570 reflecting the different chemical composition and/or concentration of the compounds in the 571 leachate mixtures. However, the effects of some bioplastic leachates were comparable to 572 (or even greater than) previously tested conventional polymers.

Among ontogenetic parameters, the embryo-larval development showed the greatest 573 574 adverse outcomes indicating the potential of all leachates to compromise the resilience of mussel populations in the long term. The use of a 96-microwell assay allowed to test 575 simultaneously a wide range of concentrations and demonstrate dose-dependent effects. 576 Its application helps understanding the relationship between xenobiotic exposure and 577 detrimental effects in marine organisms, and may be recommended for regulatory purposes. 578 Adult physiological parameters were also affected by leachates, with highest effects 579 observed for lysosomal and immunological biomarkers in mussels exposed to compostable 580 bag (mixture of PBAT/PLA) bioplastic and tire rubber. 581

582 Comparing with previous data, it can be observed that composition of leachates is different from different bioplastics and medium of extraction, however, no shared protocols are 583 available for plastic leachate preparation. Furthermore, different mixtures are expected to 584 induce different effects however full chemical characterization of (bio)plastics is not possible, 585 yet. Although the experimental conditions are not intended to mimic real environmental 586 situations and the investigated items are not necessarily representative of the entire class 587 of commercialised polyesters, the results presented in this study are important as a basis to 588 identify mechanisms of action and draw possible adverse outcome pathways that can result 589 in disturbances at the ecosystem level. They also support the use of effect-based tests for 590

591 designing new and less harmful additives in order to produce "sustainable" bioplastics. 592 Finally, they highlight that the leaching of additives and NIAS (e.g. monomers and oligomers) 593 and their effects on aquatic organisms need to be carefully considered when assessing the 594 environmental impacts of plastics.

595

596 **5. Acknowledgements**

This study was inspired in the context of PLASTOX (Direct and indirect ecotoxicological impacts of microplastics on marine organisms), grant agreement N° 495 696324 (<u>http://www.jpi-oceans.eu/plastox</u>). AR was supported by a grant funded by the European Commission under the Erasmus Mundus Master's Degree Programme in Water and Coastal Management (WACOMA; Project nr 586596-EPP-1-2017-1-IT-EPPKA1-JMD-MOB). The Authors declare that they have no known competing financial interests or personal relationships that may appear to have influenced the work reported in this paper.

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CAPOLUPO ET AL., FIGURES (4) AND TABLES (3)

Table 1. Tentative identification and GC-MS data (retention time in minutes, m/z of relevant ions in the mass spectrum with base peak in bold) of compounds detected in seawater leachates from polymeric materials. (*) Identified as pertrimethylsilyl derivative.

Material	Compound	min	m/z
TR	aniline	7.62	66, 93
	benzothiazole	11.6	69, 82, 108, 135
	N-cyclohexyl formamide	12.0	45 , 56, 82, 84, 127
	N-cyclohexyl acetamide	12.5	56, 60 , 98, 141
	dicyclohexylamine	14.3	82, 56, 138
СВ	Lactic acid*	9.17	73 , 117, 147
	1,4-butandiol*	10.6	73, 116, 147 , 177
	2-(2-butoxyethoxy) ethanol	11.0	45 , 57, 75, 87, 132
	2-(2-butoxyethoxy) ethanol*	13.1	57, 73 , 101, 117, 131
	1,6-dioxacyclododecane-7,12-dione	15.9	55 , 84, 100, 129
	oligomer of PBAT ?	25.6	55 , 71, 101, 127, 173
	oligomer of PBAT ?	27.1	55 , 71, 101, 127, 173
PLA	Lactic acid*	9.17	73 , 117, 147

Table 2. BPA concentration (μ g/L) in leachates from bioplastics and tire rubber.

CTR CB		BioPET	PLA	TR					
<loq< td=""><td>0.51</td><td>0.34</td><td>0.30</td><td>4.81</td></loq<>	0.51	0.34	0.30	4.81					
HPLC-MS/MS (LOQ 0.032 µg/L). See supplemental material for details.									

Table 3. Trace metals in leachates from bioplastics and tire rubber. Results are expressed in μ g/L and represent the mean media ± expanded uncertainty (K=2; df= 10) of measured levels for each treatment.

µg/L	AI	Fe	Cu	Zn	Pb	Hg	Cr	Ni	Cd	As	Sb	Со	Sr	Mn
ASW	<5	<5	3.1	150	<1	<0.5	<1	<1	<1	<1	<1	<1	5100	12.0
			±	±										
			0.5	46										
СВ	<5	11.1	36.5	110	2.1	<0.5	<1	5.0	<1	1.9	<1	<1	4900	12.9
		±	±	±	±			±		±				
		1.4	5.6	35	0.3			1,5		0.5				
Bio	<5	<1	16.8	150	<1	<0.5	<1	2.7	<1	<1	<1	<1	4800	18.5
PET			±	±				±						
			2.6	46				0,8						
PLA	<5	<5	12.9	82	<1	<0.5	<1	<1	<1	<1	<1	<1	4900	18.5
			±	±										
			2.0	25										
TR	6.5	13.8	2.8	220	1.6	<0.5	1.3	<1	<1	1.3	<1	<1	5400	26.5
	±	±	±	±	±		±			±				
	0.9	1.8	0.4	69	0.3		0.5			0.4				
LOQ	5	5	1	5	1	0.5	1	1	1	1	1	1	1	1

ASW: artificial sea water (Control) LOQ: limit of quantification



Figure 1. Total ion chromatogram (pyrogram) obtained from Py-GC-MS of CB (full line) compared with the pyrograms of PLA and PBAT. Tentative product identification: (1) 1,3 butadiene; (2) tetrahydrofuran; (3) cyclopentanone; (4) meso lactide; (5) D,L-lactide; (6) 2- (formyloxy)-1-phenyl- ethenone; (7) 1,6-dioxacyclododecane-7,12-dione; (8) dibutyl adipate; (9) unknown (m/z 54, 65, 121, 149, 166); (9) dibutylterephtalate.



Figure 2. Mean percentage of *M. galloprovincialis* normal D-veliger larvae after 48h exposure to leachates from bio-plastics (CB, Bio-PET, PLA) and conventional tire rubber (TR). Data are expressed as mean \pm SEM (N = 5). Asterisks indicate significant differences compared to the control (p < 0.05, One way ANOVA, Bonferroni post-hoc comparison). EC₅₀ values (95% C.I.) are also reported.







Figure 4. Enzymatic activities evaluated in adult mussels after in vivo 7-day exposure to 0.6% concentrations bio-leachates (SB, BPT, PLA) and tire rubber (TR) leachate. A) Catalase activity in digestive glands and gills; B) Glutathione-S transferase activity in digestive glands and gills; C) Lysozyme activity in serum. Data are expressed as mean \pm SEM (N = 3). Asterisks indicate significant differences compared to the control (p < 0.05, One-way ANOVA, Bonferroni post-hoc comparison).

Capolupo et al. HIGHLIGHTS

- 1. Polyesters composing commercial bioplastics identified by Py-GC-MS and ATR-FTIR
- 2. Leachates contained BPA, trace metals and NIAS
- 3. Leachates caused embryotoxicity in the tested concentration range (0.6-100%)
- 4. Adult mussel lysosomal and immune parameters were impaired at 0.6% leachates
- 5. Harmonized chemical/biological approaches are needed to assess bioplastic toxicity

AUTHORSHIP STATEMENT

Manuscript title: Bioplastic leachates characterization and impacts on early larval stages and adult mussel physiological parameters

All persons who meet authorship criteria are listed as authors, and all authors have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

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This material has not been and will not be submitted to any other publication.

This statement is signed by Elena Fabbri on behalf of all Authors

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: