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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.**

3

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16

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*.

21

22



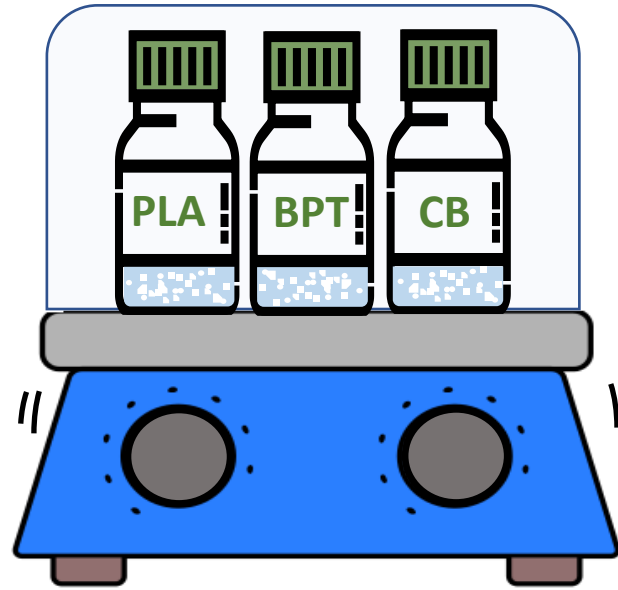
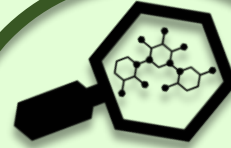
Polylactic acid (PLA)



Bio PET (bioPET)



Compostable bags (CB)

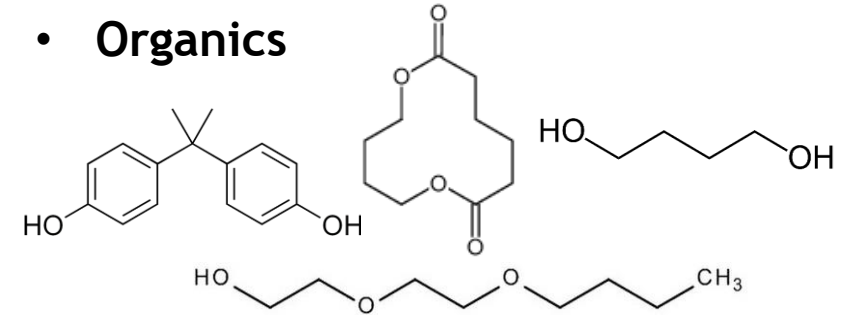


LEACHATE PREPARATION



CHEMICAL ANALYSIS

- Organics

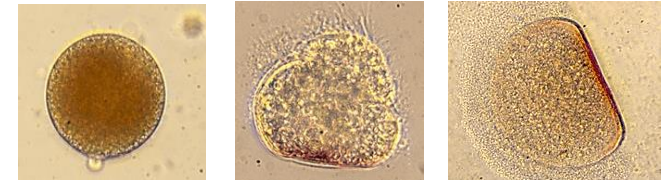


- Metals

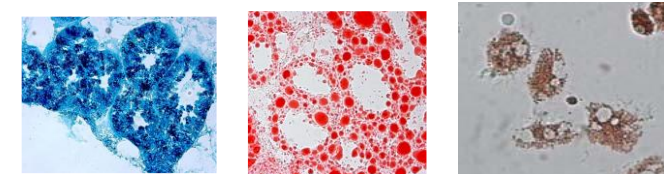
Cu, Zn, Pb, Cr, Ni, (...)

BIOLOGICAL ANALYSIS

↑ Early-stage effects



↑ Biomarker alterations



23 **ABSTRACT**

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

49 1. Introduction

50

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

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

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

74 (Capolupo et al., 2021a, 2020), providing evidence that plastic additive leachates are
75 chemically complex, and can affect marine organisms' growth, development and survival.
76 To tackle the long-term persistence of conventional plastics, bioplastics are promoted as
77 safer alternatives. Bioplastics commonly encompass a diverse family of polymeric materials
78 that originates from biomass and/or are biodegradable. Confusion usually occurs among
79 bio-, bio-based and bio-degradable plastics (Lambert and Wagner, 2017; Wang et al., 2022).
80 Biobased plastics are those containing organic carbon of renewable origin from the natural
81 environment, while biodegradable plastics are made of polymers susceptible to
82 mineralization into CO₂, biomass and water by biological activity (Kjeldsen et al., 2019;
83 Razza and Degli Innocenti, 2012). Synthetic polyesters are commonly employed in the
84 production of biodegradable plastics based on fossil resources, such as poly (butylene
85 adipate-co-terephthalate) (PBAT), polymers that are both bio-based and biodegradable
86 such as polylactic acid (PLA), and further polymers partially biobased such as poly(ethylene
87 terephthalate) (PET) from biobased ethylene. Bioplastics made of these polyesters can be
88 manufactured in forms of composites, as for instance PBAT/PLA and PBAT/starch.
89 The market share of bioplastics is relatively low compared to conventional thermoplastics;
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
92 from degradation of biodegradable polymers (Haider et al., 2019), very little is known on the
93 bioplastic chemical safety, the chemical nature of compounds included in the items, and the
94 potential toxicity of the leachates for ecosystem and human health. It has been reported that
95 bioplastics contain similar additives as their conventional counterparts (Lehtiniemi et al.,
96 2021) and may be similarly toxic (Uribe-Echeverría and Beiras, 2022; Zimmermann et al.,
97 2020a). Bioplastics undergo ageing processes mediated by abiotic and biotic agents
98 (mechanical abrasion, thermal degradation, hydrolysis, photo-oxidation, biodegradation)
99 that can deeply affect the properties of the polymer matrix, with consequent additive release.

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

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

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

119

120 **2. MATERIALS AND METHODS**

121 **2.1. Bioplastic leachate preparation**

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

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 ≥3 h.

130 Leachates were produced in artificial seawater (ASW), prepared according to ASTM (2004)
131 as previously described (Capolupo et al., 2020). Briefly, selected materials were ground into
132 <5 mm pieces and individually added to ASW at a final concentration of 80 g plastic / L.
133 Samples were placed in a rotating incubator (125 rpm) at RT (~20 °C) for 14 d in the dark
134 to allow for chemical leaching. Leachates were then passed through a sterile filter (0.2 mm
135 Nalgene®) to eliminate particles and kept in darkness at 4 °C until use.

136

137 **2.2. Chemical analysis**

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

149

150 **2.2.1 Analysis of bioplastic materials**

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

162 **2.2.2 Analysis of leachates**

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

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

178 **2.3 Mussel early life stages endpoints**

179 Early life stages endpoints investigated in this work encompassed mussel gamete
180 fertilization, embryotoxicity, larvae motility and survival. Biological effects of 11 different
181 leachate concentrations were assessed *in vitro*, ranging from 100% (no dilution) to 0.6%
182 (167 times dilution) leachate concentrations in seawater. All experiments were carried out
183 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
186 reported by Capolupo et al. (2020), by exposing sperms to the leachates (100% to 0.6%
187 concentration) for 1 h prior to add eggs in 1:5 proportion as in ASTM (2004). The reaction
188 was blocked after 30 min by adding calcium buffered formalin (4%). The acute
189 embryotoxicity test (ASTM, 2004) was adapted to 96 microwell plates (Fabbri et al., 2014)
190 to screen the impact of bioplastic leachates on *M. galloprovincialis* embryo-larval
191 development. Prior to leachate exposure, mussel oocytes were fertilized by mixing eggs and
192 spermatozoa at a 1:5 ratio in 96-well plates. Fifty eggs/well were used for fertilization test.
193 After microscopical verification of (> 90%) fertilization success, embryos (50 embryos/well)
194 were exposed for 48 h to different dilutions of bioplastic leachates; the test was blocked as
195 above described, and samples examined at 40 x magnification using an inverted
196 microscope.

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

200 **2.3.2. Larvae motility and survival**

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

206

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)
210 and acclimated in controlled laboratory conditions (filtered seawater, 16 °C, 14 h:10 h
211 light/dark conditions) for four days before experimental treatment. Ten mussels were then
212 placed in aquaria (3 per experimental condition) each containing 10 L of filtered seawater,
213 and exposed to 0.6% concentration of leachate (167 x dilution of the original leachates) for
214 seven days in line with previous experimental exposure using thermoplastic leachates
215 (Capolupo et al., 2021a). All leachates were tested in triplicate, each aquarium representing
216 a single experimental replicate (n=3). Aquaria for control condition (CTR) with only filtered
217 seawater were run in parallel (n=3). The exposure was performed in controlled conditions of
218 temperature (16-18 °C), photoperiod 14 h: 10 h light/dark) and feeding (1,200 cells/mL of
219 the green alga *Nannochloropsis oculata*), as previously described (Capolupo et al., 2021a).
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
223 tested, frozen in Liquid N₂ then stored at -80 °C, or immediately used for analysis. A battery
224 of nine biomarkers was assessed following the OSPAR 2013 protocol (OSPAR Commission,
225 2013), namely lysosome membrane stability (LMS), lysosome/cytosol ration (LYS/CYT),
226 neutral lipid (NL), malondialdehyde (MDA), and lipofuscin (LF) accumulation, and lysozyme
227 (LYZ), catalase (CAT), glutathione S-transferase (GST), and acetylcholinesterase (AChE)
228 activities. Haemocytes were collected from 4 mussels per vessel and LMS evaluated by the
229 Neutral Red Retention Assay (NRRA) (Martínez-Gomez et al., 2015). LYS/CYT, NL and LF
230 accumulation were assessed on 10 micron cryo-sections of mussel digestive glands as
231 published by Capolupo et al. (2021a). Enzymatic assays were performed in pools of
232 digestive glands and/or gills taken from 6 mussels per vessel (18 mussels per experimental
233 condition). After homogenization and centrifugation, specific assays were conducted
234 spectrophotometrically (Capolupo et al., 2021a). Gills homogenates were used for
235 determination of AChE activity; after incubation with 0.5 mM acetylthiocholine iodide and
236 0.33 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) changes in absorbance were followed
237 at 405 nm for 10 min (Valbonesi et al., 2003). Serum LYZ activity was measured as
238 previously described (Capolupo et al., 2021b), following for 10 min the decrease in
239 absorbance due to the LYZ effect on *Micrococcus lysodeikticus*. Details on methods for
240 biomarker measurements are reported in Supplemental materials.

241 **2.5. Statistical Analysis**

242 The statistical software packages 'R' and SigmaPlot 12 (Systat Software Inc. San Jose, CA,
243 USA) were employed. All data were tested for normality using the Shapiro-wilk test and for
244 variance equality using the Levene's test. On these bases, One-way Analysis of Variance
245 (ANOVA), followed by the Bonferroni post hoc test, was applied to assess statistically

246 significant variations. Differences were considered significant for $p < 0.05$. When applicable,
247 EC50 was calculated on data for early life stages bioassays using the Log- model LL.3
248 included in the 'R' statistical package.

249

250 **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
253 plastics (Venâncio et al., 2022; Zimmermann et al., 2020b). Effect-based approaches are
254 needed to assess the overall toxicity of plastic items, which consider known and unknown
255 additives released by plastic items, including NIAS, and their effects as mixtures. To the best
256 of our knowledge only two investigations are available that report on bioplastic leachates
257 composition and their biological effects on marine organisms, i.e. marine bacteria
258 (Zimmermann et al., 2020c) and sea urchin larvae (Uribe-Echeverría and Beiras, 2022).
259 Zimmermann et al. (2020b) applied methanol extraction and 1 hour- sonication in order to
260 obtain leachates from several bioplastics; a large number of different compounds ($> 1,000$
261 chemicals each in 80% of the samples) including toxic chemicals were found in the
262 bioplastics and plant-based items examined, including erucamide, Irganox 1076, tris(2-
263 nonylphenyl) phosphate etc. which also occurred in petroleum-based plastics. The Authors
264 also showed that commercial bio-based and/or biodegradable items may cause toxicity
265 similar to the conventional ones.

266 Uribe-Echeverría and Beiras (2022) investigated the chemical composition of leachates
267 obtained in seawater (24 hours) from 3 different bioplastics, i.e. polyhydroxybutyrate resin
268 (PHB), polylactic acid cups (PLA) and polylactic acid/polyhydroxyalkanoate items
269 (PLA/PHA). Unexpectedly, a wide range of additives was found in PHB including chlorinated
270 (1-chloro-tetradecane), brominated (dodecyltrimethylammonium bromide) and iodinated (1-

271 iodo-hexadecane) biocides. A few chemicals were released from PLA (oxo-methanol
272 benzoate, 1,5-dimethyl-1H-Pyrazole-3,4-diamine) and PLA/PHA (including Isocrotonic and
273 crotonic acid, and 2-Pentenoic acid) items. The mixture toxicity for sea urchin larvae
274 fertilization and development was observed after exposure to PHB leachates, while PLA and
275 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**

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

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

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

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

311

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

313 **3.2.1 Organic compounds**

314 The compounds tentatively identified in seawater leachates that were extracted with ethyl
315 acetate are reported in **Table 1** along with their GC-MS data. Quantitation was not
316 performed because not all the compounds were commercially available for calibration. TR
317 was utilised as positive control for the several studies on the toxicity of leachates to water
318 organisms (see Capolupo et al., 2020). In fact, several compounds were released into the
319 sea water (**Table 1**). Among them benzothiazole and dicyclohexylamine were found in

320 particle tire leachates in citrate buffered water where aniline was also tentatively identified
321 (Seiwert et al., 2020). Aniline was also reported among the potential toxicants for aquatic
322 organisms in the elutriates of sediments containing tire and road wear particles (Marwood
323 et al., 2011). Benzothiazole and *N*-cyclohexylformamide were found in the water leachates
324 of car tire rubber obtained under similar conditions by Capolupo et al. (2020).

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

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

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

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

363 In the case of target additives, the attention was focused on BPA, worldwide exploited as a
364 plastic monomer and plasticizer. It occurs in many commercial items, including bottles, cans,
365 medical equipment, etc. (Prins et al., 2019). BPA occurred in all leachates analysed, from
366 0.3 to 4.8 µg/L concentrations (**Table 2**). BPA was found in the aquatic environment from
367 0.5 ng to 12 µg/L (Flint et al., 2012) and at these concentrations it has been reported to
368 cause significant damages on exposed mussel embryo-larval stages (e.g Balbi et al., 2016).

369 BPA exposure has clearly been correlated with endocrine disorders in humans (Valbonesi
370 et al., 2021 and reference therein).

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

385 Overall, the leachates composition is different from different bioplastics and medium of
386 extraction (Uribe-Echeverría and Beiras, 2022; Zimmermann et al., 2020a; present work)
387 however no shared protocols are available for plastic leachate preparation (Gunaalan et al.,
388 2020), yet.

389

390 **3.3 Biological impact of leachates**

391 ***3.3.1 Mussel early life stages endpoints***

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

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

409 Several NIAS associated to the chemical structure of the polyester (monomers and
410 oligomers of PLA and PBAT) were found in the leachates, as described in section 3.2.1. As
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

417 situation, not extendible to environmental conditions (Klein et al., 2021a). It is worth
418 underlining the ubiquity of the cyclic ester 1,6-dioxacyclododecane-7,12-dione in the mixture
419 of compounds prone to be released from bioplastics containing PBAT. This cyclic ester was
420 a major compound in the leachates of CB, but no information on its toxicity to marine
421 organisms is available in published studies. Significantly reduced motility (at 48h) was
422 observed in larvae exposed to TR leachates within a concentration range of 20-100% (**Fig.**
423 **4S**); significantly reduced larvae survival (at 216 h) was caused by TR leachates in the range
424 10-100% (**Fig. 5S**), with EC50 values of 17.3 and 11.9%, respectively. Other leachates were
425 ineffective on survival, and PLA and CB only reduced motility at the highest concentrations
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
430 mussels for 7 days (**Fig. 3**), and a significant LMS reduction was found in haemocytes
431 withdrawn from mussels exposed CB and TR leachates (**Fig. 3** panel A). LMS decreased
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)

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

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

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

492 significant. Such a low MDA production after exposure to CB and TR leachates could instead
493 be ascribed to its depletion to support LF production. Increase of LF contents is correlated
494 to the LMS decrease found in haemocytes; such a correlation was previously reported
495 (Donnini et al., 2007; Franzellitti et al., 2014) and confirms the relationship between oxidative
496 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
501 and GST activities, in gills and digestive gland of exposed animals (**Fig. 4**). The different
502 role of the enzymes in the two tissues was confirmed, with CAT having higher basal activity
503 in digestive glands, where peroxidation reactions are mostly performed, and GST showing
504 higher activity in gills, i.e. the first tissue exposed to environmental xenobiotics (Capolupo et
505 al., 2021a). CAT activity was significantly reduced by leachates from bioPET in gills, and
506 enhanced by leachates from CB in digestive glands (**Fig. 4** panel A); GST was significantly
507 enhanced by leachates from PLA in gills, and from CB in digestive glands (**Fig. 4** panel B).

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

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

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

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

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

557

558 **4.0 CONCLUSION**

559 This investigation increases the knowledge gained from two previous studies carried out by
560 other laboratories which addressed the contents of polyester-based bioplastic leachates and
561 their biological effects on marine organisms, challenging bacteria and sea-urchin larvae. The
562 originality and strength of the present work is that both early-larval stages and adult mussels
563 were exposed through *in vitro* (at 11 different dilutions) and *in vivo* approaches to leachates
564 obtained after 14 days in seawater and chemically characterized. The study confirms that
565 leachates from bio-plastics do contain organic compounds (additives, non-intentionally

566 added compounds such as oligomers) and trace metals, and exposure to leachates does
567 affect *M. galloprovincialis* early embryo stages development and adult physiological
568 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.

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

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

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

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604

605 **6. Bibliography**

606 Agamuthu, P., Mehran, S.B., Norkhairah, A., Norkhairiyah, A., 2019. Marine debris: A
607 review of impacts and global initiatives. *Waste Manag. Res.* 37, 987–1002.
608 <https://doi.org/10.1177/0734242X19845041>

609 Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T., Narbonne, J.F., 2000.
610 Enzymatic biomarker measurement and study of DNA adduct formation in
611 benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. *Aquat. Toxicol.* 49,
612 269–287. [https://doi.org/10.1016/S0166-445X\(99\)00082-X](https://doi.org/10.1016/S0166-445X(99)00082-X)

613

614 ASTM, 2004. Standard Guide for Conducting Acute Toxicity Test Starting with Embryos of
615 Four Species of Saltwater Bivalve Molluscs, Marine Environmental Research. Elsevier
616 Ltd. <https://doi.org/E724e98>

617 Balbi, T., Ciacci, C., Canesi, L., 2019. Estrogenic compounds as exogenous modulators of
618 physiological functions in molluscs: Signaling pathways and biological responses.
619 Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 222, 135–144.
620 <https://doi.org/10.1016/j.cbpc.2019.05.004>

621 Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E., Canesi, L., 2016. Impact of
622 bisphenol A (BPA) on early embryo development in the marine mussel *Mytilus*
623 *galloprovincialis*: Effects on gene transcription. Environ. Pollut. 218, 996–1004.
624 <https://doi.org/10.1016/j.envpol.2016.08.050>

625 Balestri, E., Menicagli, V., Ligorini, V., Fulignati, S., Raspolli Galletti, A.M., Lardicci, C.,
626 2019. Phytotoxicity assessment of conventional and biodegradable plastic bags using
627 seed germination test. Ecol. Indic. 102, 569–580.
628 <https://doi.org/10.1016/j.ecolind.2019.03.005>

629 Bejgarn, S., MacLeod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from
630 weathering plastics: An exploratory screening study with *Nitocra spinipes*.
631 Chemosphere 132, 114–119. <https://doi.org/10.1016/j.chemosphere.2015.03.010>

632 Brooks, S.J., Escudero-Oñate, C., Gomes, T., Ferrando-Climent, L., 2018. An integrative
633 biological effects assessment of a mine discharge into a Norwegian fjord using field
634 transplanted mussels. Sci. Total Environ. 644, 1056–1069.
635 <https://doi.org/10.1016/j.scitotenv.2018.07.058>

636 Canellas, E., Vera, P., Nerín, C., 2015. UPLC–ESI-Q-TOF-MSE and GC–MS identification
637 and quantification of non-intentionally added substances coming from biodegradable

638 food packaging. *Anal. Bioanal. Chem.* 407, 6781–6790.
639 <https://doi.org/10.1007/s00216-015-8848-2>

640 Canesi, L., Ciacci, C., Betti, M., Malatesta, M., Gazzanelli, G., Gallo, G., 1999. Growth
641 factors stimulate the activity of key glycolytic enzymes in isolated digestive gland cells
642 from mussels (*Mytilus galloprovincialis* Lam.) through tyrosine kinase mediated signal
643 transduction. *Gen. Comp. Endocrinol.* 116, 241–248.
644 <https://doi.org/10.1006/gcen.1999.7366>

645 Canesi, L., Ciacci, C., Lorusso, L.C., Betti, M., Guarnieri, T., Tavolari, S., Gallo, G., 2006.
646 Immunomodulation by 17 β -estradiol in bivalve hemocytes. *Am. J. Physiol. - Regul.*
647 *Integr. Comp. Physiol.* 291, 664–673. <https://doi.org/10.1152/ajpregu.00139.2006>

648 Canesi, L., Fabbri, E., 2015. Environmental effects of BPA: Focus on aquatic species.
649 *Dose-Response* 13, 1–14. <https://doi.org/10.1177/1559325815598304>

650 Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A.,
651 2007. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals
652 and environmentally relevant mixtures of estrogens: In vitro and in vivo studies. *Aquat.*
653 *Toxicol.* 81, 36–44. <https://doi.org/10.1016/j.aquatox.2006.10.010>

654 Capolupo, M., Franzellitti, S., Kiwan, A., Valbonesi, P., Dinelli, E., Pignotti, E., Birke, M.,
655 Fabbri, E., 2017. A comprehensive evaluation of the environmental quality of a coastal
656 lagoon (Ravenna, Italy): Integrating chemical and physiological analyses in mussels
657 as a biomonitoring strategy. *Sci. Total Environ.* 598, 146–159.
658 <https://doi.org/10.1016/j.scitotenv.2017.04.119>

659 Capolupo, M., Gunaalan, K., Booth, A.M., Sørensen, L., Valbonesi, P., Fabbri, E., 2021a.
660 The sub-lethal impact of plastic and tire rubber leachates on the Mediterranean
661 mussel *Mytilus galloprovincialis*. *Environ. Pollut.* 283, 117081.

662 <https://doi.org/10.1016/j.envpol.2021.117081>

663 Capolupo, M., Sørensen, L., Jayasena, K.D.R., Booth, A.M., Fabbri, E., 2020. Chemical
664 composition and ecotoxicity of plastic and car tire rubber leachates to aquatic
665 organisms. *Water Res.* 169, 115270. <https://doi.org/10.1016/j.watres.2019.115270>

666 Capolupo, M., Valbonesi, P., Fabbri, E., 2021b. A comparative assessment of the chronic
667 effects of micro-and nano-plastics on the physiology of the mediterranean mussel
668 *Mytilus galloprovincialis*. *Nanomaterials* 11, 1–17.
669 <https://doi.org/10.3390/nano11030649>

670 Cheung, S.G., Tong, P.Y., Yip, K.M., Shin, P.K.S., 2004. Chemical cues from predators
671 and damaged conspecifics affect byssus production in the green-lipped mussel *Perna*
672 *viridis*. *Mar. Freshw. Behav. Physiol.* 37, 127–135.
673 <https://doi.org/10.1080/10236240410001705798>

674 Dailianis, S., 2011. Environmental impact of anthropogenic activities: the use of mussels
675 as a reliable tool for monitoring marine pollution, in: *Mussels: Anatomy. Habitat and*
676 *Environmental Impact*. McGevin, L.E. Nova Science Publishers, Inc, pp. 1–30.

677 Donnini, F., Dinelli, E., Sangiorgi, F., Fabbri, E., 2007. A biological and geochemical
678 integrated approach to assess the environmental quality of a coastal lagoon
679 (Ravenna, Italy). *Environ. Int.* 33, 919–928.
680 <https://doi.org/10.1016/j.envint.2007.05.002>

681 Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., Canesi, L., 2014. Adaptation of
682 the bivalve embryotoxicity assay for the high throughput screening of emerging
683 contaminants in *Mytilus galloprovincialis*. *Mar. Environ. Res.* 99, 1–8.
684 <https://doi.org/10.1016/j.marenvres.2014.05.007>

685 Félix, J.S., Isella, F., Bosetti, O., Nerín, C., 2012. Analytical tools for identification of non-

686 intentionally added substances (NIAS) coming from polyurethane adhesives in
687 multilayer packaging materials and their migration into food simulants. *Anal. Bioanal.*
688 *Chem.* 403, 2869–2882. <https://doi.org/10.1007/s00216-012-5965-z>

689 Fernández, B., Albentosa, M., 2019. Insights into the uptake, elimination and accumulation
690 of microplastics in mussel. *Environ. Pollut.* 249, 321–329.
691 <https://doi.org/10.1016/j.envpol.2019.03.037>

692 Flint, S., Markle, T., Thompson, S., Wallace, E., 2012. Bisphenol A exposure, effects, and
693 policy: A wildlife perspective. *J. Environ. Manage.* 104, 19–34.
694 <https://doi.org/10.1016/j.jenvman.2012.03.021>

695 Fokina, N.N., Ruokolainen, T.R., Nemova, N.N., Bakhmet, I.N., 2013. Changes of blue
696 mussels *Mytilus edulis* L. lipid composition under cadmium and copper toxic effect.
697 *Biol. Trace Elem. Res.* 154, 217–225. <https://doi.org/10.1007/s12011-013-9727-3>

698 Franzellitti, S., Buratti, S., Capolupo, M., Du, B., Haddad, S.P., Chambliss, C.K., Brooks,
699 B.W., Fabbri, E., 2014. An exploratory investigation of various modes of action and
700 potential adverse outcomes of fluoxetine in marine mussels. *Aquat. Toxicol.* 151, 14–
701 26. <https://doi.org/10.1016/j.aquatox.2013.11.016>

702 Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit
703 acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE
704 activity as a biomarker of metal toxicity. *Biomarkers* 10, 360–375.
705 <https://doi.org/10.1080/13547500500264660>

706 Gallo, F., Fossi, C., Weber, R., Santillo, D., Sousa, J., Ingram, I., Nadal, A., Romano, D.,
707 2018. Marine litter plastics and microplastics and their toxic chemicals components:
708 the need for urgent preventive measures. *Environ. Sci. Eur.* 30.
709 <https://doi.org/10.1186/s12302-018-0139-z>

710 Gerdol, M., Gomez-Chiarri, M., G. Castillo, M., Figueras, A., Fiorito, G., Moreira, R.,
711 Novoa, B., Pallavicini, A., Ponte, G., Roubledakis, K., Venier, P., R. Vasta, G., 2018.
712 Immunity in Molluscs: Recognition and Effector Mechanisms, with a Focus on
713 Bivalvia, in: *Advances in Comparative Immunology* . Springer Verlag, pp. 225–341.
714 https://doi.org/10.1007/978-3-319-76768-0_11

715 Giamberini, L., Pihan, J.C., 1997. Lysosomal changes in the hemocytes of the freshwater
716 mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. *Dis. Aquat.*
717 *Org.* 28, 221–227. <https://doi.org/10.3354/dao028221>

718 Gomes, T., Pereira, C.G., Cardoso, C., Sousa, V.S., Teixeira, M.R., Pinheiro, J.P.,
719 Bebianno, M.J., 2014. Effects of silver nanoparticles exposure in the mussel *Mytilus*
720 *galloprovincialis*. *Mar. Environ. Res.* 101, 208–214.
721 <https://doi.org/10.1016/j.marenvres.2014.07.004>

722 Gowland, B.T.G., McIntosh, A.D., Davies, I.M., Moffat, C.F., Webster, L., 2002.
723 Implications from a field study regarding the relationship between polycyclic aromatic
724 hydrocarbons and glutathione S-transferase activity in mussels. *Mar. Environ. Res.*
725 54, 231–235. [https://doi.org/10.1016/S0141-1136\(02\)00129-0](https://doi.org/10.1016/S0141-1136(02)00129-0)

726 Groh, K.J., Backhaus, T., Carney-Almroth, B., Geueke, B., Inostroza, P.A., Lennquist, A.,
727 Leslie, H.A., Maffini, M., Slunge, D., Trasande, L., Warhurst, A.M., Muncke, J., 2019.
728 Overview of known plastic packaging-associated chemicals and their hazards. *Sci.*
729 *Total Environ.* 651, 3253–3268. <https://doi.org/10.1016/j.scitotenv.2018.10.015>

730 Gualtieri, M., Andrioletti, M., Vismara, C., Milani, M., Camatini, M., 2005. Toxicity of tire
731 debris leachates. *Environ. Int.* 31, 723–730.
732 <https://doi.org/10.1016/j.envint.2005.02.001>

733 Gunaalan, K., Fabbri, E., Capolupo, M., 2020. The hidden threat of plastic leachates: A

734 critical review on their impacts on aquatic organisms. *Water Res.* 184, 116170.
735 <https://doi.org/10.1016/j.watres.2020.116170>

736 Hahladakis, J.N., Velis, C.A., Weber, R., Iacovidou, E., Purnell, P., 2018. An overview of
737 chemical additives present in plastics: Migration, release, fate and environmental
738 impact during their use, disposal and recycling. *J. Hazard. Mater.* 344, 179–199.
739 <https://doi.org/10.1016/j.jhazmat.2017.10.014>

740 Haider, T.P., Völker, C., Kramm, J., Landfester, K., Wurm, F.R., 2019. Plastics of the
741 Future? The Impact of Biodegradable Polymers on the Environment and on Society.
742 *Angew. Chemie - Int. Ed.* 58, 50–62. <https://doi.org/10.1002/anie.201805766>

743 Jia, C., Luo, J., Zhang, S., Zhu, X., 2020. N-rich hydrochar derived from organic solvent as
744 reaction medium generates toxic N-containing mineral in its pyrochar. *Sci. Total*
745 *Environ.* 729, 138970. <https://doi.org/10.1016/j.scitotenv.2020.138970>

746 Jian, J., Xiangbin, Z., Xianbo, H., 2020. An overview on synthesis, properties and
747 applications of poly(butylene-adipate-co-terephthalate)–PBAT. *Adv. Ind. Eng. Polym.*
748 *Res.* 3, 19–26. <https://doi.org/10.1016/j.aiepr.2020.01.001>

749 Juhel, G., Bayen, S., Goh, C., Lee, W.K., Kelly, B.C., 2017. Use of a suite of biomarkers to
750 assess the effects of carbamazepine, bisphenol A, atrazine, and their mixtures on
751 green mussels, *Perna viridis*. *Environ. Toxicol. Chem.* 36, 429–441.
752 <https://doi.org/10.1002/etc.3556>

753 Kjeldsen, A., Price, M., Lilley, C., Guzniczak, E., Archer, I., 2019. A review of standards for
754 biodegradable plastics, Industrial Biotechnology Innovation Centre, IBioIC.
755 [https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachm
756 ent_data/file/817684/review-standards-for-biodegradable-plastics-IBioIC.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/817684/review-standards-for-biodegradable-plastics-IBioIC.pdf).

757 Klein, K., Hof, D., Dombrowski, A., Schweyen, P., Dierkes, G., Ternes, T., Schulte-

758 Oehlmann, U., Oehlmann, J., 2021a. Enhanced *in vitro* toxicity of plastic leachates
759 after UV irradiation. *Water Res.* 199. <https://doi.org/10.1016/j.watres.2021.117203>

760 Klein, K., Piana, T., Lauschke, T., Schweyen, P., Dierkes, G., Ternes, T., Schulte-
761 Oehlmann, U., Oehlmann, J., 2021b. Chemicals associated with biodegradable
762 microplastic drive the toxicity to the freshwater oligochaete *Lumbriculus variegatus*.
763 *Aquat. Toxicol.* 231. <https://doi.org/10.1016/j.aquatox.2020.105723>

764 Ko, E., Ku, S.I., Kim, D.Y., Shin, S., Choi, M., 2018. Effects of heavy metals and albumin
765 on lysozyme activity. *J. Appl. Biol. Chem.* 61, 367–370.
766 <https://doi.org/10.3839/jabc.2018.051>

767 Konti, A., Mamma, D., Scarlat, N., Damigos, D., 2022. The Determinants of the Growth of
768 the European Bioplastics Sector—A Fuzzy Cognitive Maps Approach. *Sustain.* 14, 1–
769 17. <https://doi.org/10.3390/su14106035>

770 Lambert, S., Wagner, M., 2017. Environmental performance of bio-based and
771 biodegradable plastics: The road ahead. *Chem. Soc. Rev.* 46, 6855–6871.
772 <https://doi.org/10.1039/c7cs00149e>

773 Lehtiniemi, M., Hartikainen, S., Turja, R., Lehtonen, K.K., Vepsäläinen, J., Peräniemi, S.,
774 Leskinen, J., Setälä, O., 2021. Exposure to leachates from post-consumer plastic and
775 recycled rubber causes stress responses and mortality in a copepod *Limnocalanus*
776 *macrurus*. *Mar. Pollut. Bull.* 173. <https://doi.org/10.1016/j.marpolbul.2021.113103>

777 Liu, Y., Tong, L., Si, N., Xing, J., Zhang, Q., Ma, Q., Lv, Q., 2021. Non-targeted
778 identification of unknown chemical hazardous substances in infant teether toys by gas
779 chromatography-Orbitrap high resolution mass spectrometry. *Ecotoxicol. Environ. Saf.*
780 224, 112676. <https://doi.org/10.1016/j.ecoenv.2021.112676>

781 Lowe, D.M., Moore, M.N., Clarke, K.R., 1981. Effects of oil on digestive cells in mussels:

782 Quantitative alterations in cellular and lysosomal structure. *Aquat. Toxicol.* 1, 213–
783 226. [https://doi.org/10.1016/0166-445X\(81\)90016-3](https://doi.org/10.1016/0166-445X(81)90016-3)

784 Luo, Y., Zhang, Z., Naidu, R., Zhang, X., Fang, C., 2022. Raman imaging of microplastics
785 and nanoplastics released from the printed toner powders burned by a mimicked
786 bushfire. *Sci. Total Environ.* 849, 157686.
787 <https://doi.org/10.1016/j.scitotenv.2022.157686>

788 Marchi, B., Burlando, B., Moore, M.N., Viarengo, A., 2004. Mercury- and copper-induced
789 lysosomal membrane destabilisation depends on $[Ca^{2+}]_i$ dependent phospholipase
790 A2 activation. *Aquat. Toxicol.* 66, 197–204.
791 <https://doi.org/10.1016/j.aquatox.2003.09.003>

792 Maria, V.L., Bebianno, M.J., 2011. Antioxidant and lipid peroxidation responses in *Mytilus*
793 *galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. *Comp. Biochem.*
794 *Physiol. - C Toxicol. Pharmacol.* 154, 56–63.
795 <https://doi.org/10.1016/j.cbpc.2011.02.004>

796 Martínez-Gomez, C., Bignell, J., Lowe, D., 2015. Lysosomal membrane stability in
797 mussels. *ICES Tech. Mar. Environ. Sci.* 56, 41–41.
798 <https://doi.org/doi.org/10.17895/ices.pub.5084>. Copenhagen

799 Marwood, C., McAtee, B., Kreider, M., Ogle, R.S., Finley, B., Sweet, L., Panko, J., 2011.
800 Acute aquatic toxicity of tire and road wear particles to alga, daphnid, and fish.
801 *Ecotoxicology* 20, 2079–2089. <https://doi.org/10.1007/s10646-011-0750-x>

802 Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C., 2008. Vitellogenin as a
803 biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review.
804 *Environ. Int.* 34, 531–545. <https://doi.org/10.1016/j.envint.2007.09.008>

805 Matozzo, V., Marin, M.G., 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the

806 clam *Tapes philippinarum*? Environ. Res. 97, 43–49.
807 <https://doi.org/10.1016/j.envres.2004.03.002>

808 Miglioli, A., Balbi, T., Besnardeau, L., Dumollard, R., Canesi, L., 2021. Bisphenol A
809 interferes with first shell formation and development of the serotonergic system in
810 early larval stages of *Mytilus galloprovincialis*. Sci. Total Environ. 758, 144003.
811 <https://doi.org/10.1016/j.scitotenv.2020.144003>

812 Moore, M.N., 2008. Autophagy as a second level protective process in conferring
813 resistance to environmentally-induced oxidative stress. Autophagy 4, 254–256.
814 <https://doi.org/10.4161/auto.5528>

815 Moore, M.N., Allen, J.I., McVeigh, A., Shaw, J., 2006. Lysosomal and autophagic reactions
816 as predictive indicators of environmental impact in aquatic animals. Autophagy 2,
817 217–220. <https://doi.org/10.4161/auto.2663>

818 Moore, M.N., Depledge, M.H., Readman, J.W., Paul Leonard, D.R., 2004. An integrated
819 biomarker-based strategy for ecotoxicological evaluation of risk in environmental
820 management. Mutat. Res. - Fundam. Mol. Mech. Mutagen. 552, 247–268.
821 <https://doi.org/10.1016/j.mrfmmm.2004.06.028>

822 Muncke, J., 2009. Exposure to endocrine disrupting compounds via the food chain: Is
823 packaging a relevant source? Sci. Total Environ. 407, 4549–4559.
824 <https://doi.org/10.1016/j.scitotenv.2009.05.006>

825 Myrnes, B., Johansen, A., 1994. Recovery of Lysozyme From Scallop Waste. Prep.
826 Biochem. 24, 69–80. <https://doi.org/10.1080/10826069408010083>

827 Nazareth, M., Marques, M.R.C., Leite, M.C.A., Castro, Í.B., 2019. Commercial plastics
828 claiming biodegradable status: Is this also accurate for marine environments? J.
829 Hazard. Mater. 366, 714–722. <https://doi.org/10.1016/j.jhazmat.2018.12.052>

830 Orbea, A., Garmendia, L., Marigómez, I., Cajaraville, M.P., 2006. Effects of the “Prestige”
831 oil spill on cellular biomarkers in intertidal mussels: Results of the first year of studies.
832 *Mar. Ecol. Prog. Ser.* 306, 177–189. <https://doi.org/10.3354/meps306177>

833 OSPAR Commission, 2013. Background document and technical annexes for biological
834 effects monitoring, Update 2013, Monitoring and Assessment Series, Publication
835 Number: 589/2013. <https://www.ospar.org/documents?v=7316>.

836 Pazmiño, M., Monica, S., James, W., Juan, G., Luis, A.J., Wilman, C., 2018. Heavy metal-
837 induced inhibition of enzymatic activity in hen egg white lysozyme. *Asian J. Pharm.*
838 *Clin. Res.* 11, 212–217. <https://doi.org/10.22159/ajpcr.2018.v11i11.22892>

839 Peng, L., Fu, D., Qi, H., Lan, C.Q., Yu, H., Ge, C., 2020. Micro- and nano-plastics in
840 marine environment: Source, distribution and threats — A review. *Sci. Total Environ.*
841 698, 134254. <https://doi.org/10.1016/j.scitotenv.2019.134254>

842 Petushok, N., Gabryelak, T., Palecz, D., Zavodnik, L., Szollosi Varga, I., Deér, K.A., 2002.
843 Comparative study of the xenobiotic metabolising system in the digestive gland of the
844 bivalve molluscs in different aquatic ecosystems and in aquaria experiments. *Aquat.*
845 *Toxicol.* 61, 65–72. [https://doi.org/10.1016/S0166-445X\(02\)00030-9](https://doi.org/10.1016/S0166-445X(02)00030-9)

846 Prins, G.S., Patisaul, H.B., Belcher, S.M., Vandenberg, L.N., 2019. CLARITY-BPA
847 academic laboratory studies identify consistent low-dose Bisphenol A effects on
848 multiple organ systems. *Basic Clin. Pharmacol. Toxicol.* 125, 14–31.
849 <https://doi.org/10.1111/bcpt.13125>

850 Razza, F., Degli Innocenti, F., 2012. Bioplastics from renewable resources: The benefits of
851 biodegradability. *Asia-Pacific J. Chem. Eng.* 7. <https://doi.org/10.1002/apj.1648>

852 Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., Nigro, M.,
853 2004. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal

854 stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment.
855 Aquat. Toxicol. 68, 167–178. <https://doi.org/10.1016/j.aquatox.2004.03.011>

856 Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative
857 stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117.
858 <https://doi.org/10.1016/j.marenvres.2013.07.006>

859 Robillard, S., Beauchamp, G., Laulier, M., 2003. The role of abiotic factors and pesticide
860 levels on enzymatic activity in the freshwater mussel *Anodonta cygnea* at three
861 different exposure sites. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 135, 49–
862 59. [https://doi.org/10.1016/S1532-0456\(03\)00049-8](https://doi.org/10.1016/S1532-0456(03)00049-8)

863 Seiwert, B., Klöckner, P., Wagner, S., Reemtsma, T., 2020. Source-related smart suspect
864 screening in the aqueous environment: search for tire-derived persistent and mobile
865 trace organic contaminants in surface waters. Anal. Bioanal. Chem. 412, 4909–4919.
866 <https://doi.org/10.1007/s00216-020-02653-1>

867 Serrano-Ruíz, H., Eras, J., Martín-Closas, L., Pelacho, A.M., 2020. Compounds released
868 from unused biodegradable mulch materials after contact with water. Polym. Degrad.
869 Stab. 178, 109202. <https://doi.org/10.1016/j.polymdegradstab.2020.109202>

870 Sharifinia, M., Bahmanbeigloo, Z.A., Keshavarzifard, M., Khanjani, M.H., Lyons, B.P.,
871 2020. Microplastic pollution as a grand challenge in marine research: A closer look at
872 their adverse impacts on the immune and reproductive systems. Ecotoxicol. Environ.
873 Saf. 204, 111109. <https://doi.org/10.1016/j.ecoenv.2020.111109>

874 Shaw, J.P., Moore, M.N., Readman, J.W., Mou, Z., Langston, W.J., Lowe, D.M., Frickers,
875 P.E., Al-Moosawi, L., Pascoe, C., Beesley, A., 2019. Oxidative stress, lysosomal
876 damage and dysfunctional autophagy in molluscan hepatopancreas (digestive gland)
877 induced by chemical contaminants. Mar. Environ. Res. 152, 104825.

878 <https://doi.org/10.1016/j.marenvres.2019.104825>

879 Signa, G., Di Leonardo, R., Vaccaro, A., Tramati, C.D., Mazzola, A., Vizzini, S., 2015. Lipid
880 and fatty acid biomarkers as proxies for environmental contamination in caged
881 mussels *Mytilus galloprovincialis*. *Ecol. Indic.* 57, 384–394.
882 <https://doi.org/10.1016/j.ecolind.2015.05.002>

883 Sprung, M., 1984. Physiological energetics of mussel larvae (*Mytilus edulis*). II Food
884 uptake. *Mar. Ecol. Prog. Ser.* 17, 295–305. <https://doi.org/10.3354/meps017295>

885 Stabili, L., Pagliara, P., 2009. Effect of zinc on lysozyme-like activity of the seastar
886 *Marthasterias glacialis* (Echinodermata, Asteroidea) mucus. *J. Invertebr. Pathol.* 100,
887 189–192. <https://doi.org/10.1016/j.jip.2009.01.005>

888 Steinert, S.A., Pickwell, G. V., 1984. Quantitative determination of lysozyme in the
889 hemolymph of *Mytilus edulis* by the enzyme-linked immunosorbent assay (ELISA).
890 *Mar. Environ. Res.* 14, 229–243. [https://doi.org/10.1016/0141-1136\(84\)90080-1](https://doi.org/10.1016/0141-1136(84)90080-1)

891 Tang, Y., Han, Y., Zhang, W., Yu, Y., Huang, L., Zhou, W., Shi, W., Tian, D., Liu, G., 2022.
892 Bisphenol A and microplastics weaken the antimicrobial ability of blood clams by
893 disrupting humoral immune responses and suppressing hemocyte chemotactic
894 activity. *Environ. Pollut.* 307, 119497. <https://doi.org/10.1016/j.envpol.2022.119497>

895 Terman, A., Brunk, U.T., 2006. Oxidative Stress, Accumulation of Biological “Garbage”,
896 and Aging. *Antioxid. Redox Signal.* 8, 20–30.
897 <https://doi.org/https://doi.org/10.1089/ars.2006.8.197>

898 Thiébaud, B., Lattuati-Derieux, A., Hocevar, M., Vilmont, L.B., 2007. Application of
899 headspace SPME-GC-MS in characterisation of odorous volatile organic compounds
900 emitted from magnetic tape coatings based on poly(urethane-ester) after natural and
901 artificial ageing. *Polym. Test.* 26, 243–256.

902 <https://doi.org/10.1016/j.polymeresting.2006.10.006>

903 Tsuge, S., Ohtani, H., Watanabe, C., 2012. Pyrolysis–GC/MS Data Book of Synthetic
904 Polymers - Pyrograms, Thermograms and MS of Pyrolyzates, Elsevier. ed.

905 Ubeda, S., Aznar, M., Nerín, C., Kabir, A., 2021. Fabric phase sorptive extraction for
906 specific migration analysis of oligomers from biopolymers. *Talanta* 233, 122603.
907 <https://doi.org/10.1016/j.talanta.2021.122603>

908 Uribe-Echeverría, T., Beiras, R., 2022. Acute toxicity of bioplastic leachates to
909 *Paracentrotus lividus* sea urchin larvae. *Mar. Environ. Res.* 176, 105605.
910 <https://doi.org/10.1016/j.marenvres.2022.105605>

911 Valbonesi, P., Profita, M., Vasumini, I., Fabbri, E., 2021. Contaminants of emerging
912 concern in drinking water: Quality assessment by combining chemical and biological
913 analysis. *Sci. Total Environ.* 758. <https://doi.org/10.1016/j.scitotenv.2020.143624>

914 Valbonesi, P., Sartor, G., Fabbri, E., 2003. Characterization of cholinesterase activity in
915 three bivalves inhabiting the North Adriatic sea and their possible use as sentinel
916 organisms for biosurveillance programmes. *Sci. Total Environ.* 312, 79–88.
917 [https://doi.org/10.1016/S0048-9697\(03\)00227-4](https://doi.org/10.1016/S0048-9697(03)00227-4)

918 Venâncio, C., Lopes, I., Oliveira, M., 2022. Bioplastics: known effects and potential
919 consequences to marine and estuarine ecosystem services. *Chemosphere* 309.
920 <https://doi.org/10.1016/j.chemosphere.2022.136810>

921 Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of
922 biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-
923 induced stress syndrome in sentinel organisms. *Comp. Biochem. Physiol. - C Toxicol.*
924 *Pharmacol.* 146, 281–300. <https://doi.org/10.1016/j.cbpc.2007.04.011>

- 925 Walpitagama, M., Carve, M., Douek, A.M., Trestrail, C., Bai, Y., Kaslin, J., Wlodkovic, D.,
926 2019. Additives migrating from 3D-printed plastic induce developmental toxicity and
927 neuro-behavioural alterations in early life zebrafish (*Danio rerio*). *Aquat. Toxicol.* 213,
928 105227. <https://doi.org/10.1016/j.aquatox.2019.105227>
- 929 Wang, X., Zhu, Q., Yan, X., Wang, Y., Liao, C., Jiang, G., 2020. A review of
930 organophosphate flame retardants and plasticizers in the environment: Analysis,
931 occurrence and risk assessment. *Sci. Total Environ.* 731, 139071.
932 <https://doi.org/10.1016/j.scitotenv.2020.139071>
- 933 Wang, Y., Ding, K., Ren, L., Peng, A., Zhou, S., 2022. Biodegradable Microplastics: A
934 Review on the Interaction with Pollutants and Influence to Organisms. *Bull. Environ.*
935 *Contam. Toxicol.* 108, 1006–1012. <https://doi.org/10.1007/s00128-022-03486-7>
- 936 Watanabe, M., Nakata, C., Wu, W., Kawamoto, K., Noma, Y., 2007. Characterization of
937 semi-volatile organic compounds emitted during heating of nitrogen-containing
938 plastics at low temperature. *Chemosphere* 68, 2063–2072.
939 <https://doi.org/10.1016/j.chemosphere.2007.02.022>
- 940 Wik, A., Dave, G., 2006. Acute toxicity of leachates of tire wear material to *Daphnia*
941 *magna*-Variability and toxic components. *Chemosphere* 64, 1777–1784.
942 <https://doi.org/10.1016/j.chemosphere.2005.12.045>
- 943 Xia, C., Lam, S.S., Zhong, H., Fabbri, E., Sonne, C., 2022. Assess and reduce toxic
944 chemicals in bioplastics. *Science (80-.)*. 378. <https://doi.org/10.1126/science.ade9069>
- 945 Zhang, C., Zhang, E., Guo, Z., Ouyang, C., Lu, C., Zeng, X., Li, J., 2022. Detection and
946 control of cyclic esters in biodegradable polyesters. *Adv. Ind. Eng. Polym. Res.* 1–10.
947 <https://doi.org/10.1016/j.aiepr.2022.07.001>
- 948 Zimmermann, L., Dombrowski, A., Völker, C., Wagner, M., 2020a. Are bioplastics and

949 plant-based materials safer than conventional plastics? *In vitro* toxicity and chemical
950 composition. *Environ. Int.* 145, 106066. <https://doi.org/10.1016/j.envint.2020.106066>

951 Zimmermann, L., Dombrowski, A., Völker, C., Wagner, M., 2020b. Are bioplastics and
952 plant-based materials safer than conventional plastics? *In vitro* toxicity and chemical
953 composition. *Environ. Int.* 145, 106066. <https://doi.org/10.1016/j.envint.2020.106066>

954 Zimmermann, L., Göttlich, S., Oehlmann, J., Wagner, M., Völker, C., 2020c. What are the
955 drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and
956 particles to *Daphnia magna*. *Environ. Pollut.* 267.
957 <https://doi.org/10.1016/j.envpol.2020.115392>

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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
	<i>N</i> -cyclohexyl formamide	12.0	45 , 56, 82, 84, 127
	<i>N</i> -cyclohexyl acetamide	12.5	56, 60 , 98, 141
	dicyclohexylamine	14.3	82, 56, 138
CB	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 (µg/L) in leachates from bioplastics and tire rubber.

CTR	CB	BioPET	PLA	TR
<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 $\mu\text{g/L}$ and represent the mean media \pm expanded uncertainty ($K=2$; $df= 10$) of measured levels for each treatment.

$\mu\text{g/L}$	Al	Fe	Cu	Zn	Pb	Hg	Cr	Ni	Cd	As	Sb	Co	Sr	Mn
ASW	<5	<5	3.1 \pm 0.5	150 \pm 46	<1	<0.5	<1	<1	<1	<1	<1	<1	5100	12.0
CB	<5	11.1 \pm 1.4	36.5 \pm 5.6	110 \pm 35	2.1 \pm 0.3	<0.5	<1	5.0 \pm 1,5	<1	1.9 \pm 0.5	<1	<1	4900	12.9
Bio PET	<5	<1	16.8 \pm 2.6	150 \pm 46	<1	<0.5	<1	2.7 \pm 0,8	<1	<1	<1	<1	4800	18.5
PLA	<5	<5	12.9 \pm 2.0	82 \pm 25	<1	<0.5	<1	<1	<1	<1	<1	<1	4900	18.5
TR	6.5 \pm 0.9	13.8 \pm 1.8	2.8 \pm 0.4	220 \pm 69	1.6 \pm 0.3	<0.5	1.3 \pm 0.5	<1	<1	1.3 \pm 0.4	<1	<1	5400	26.5
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

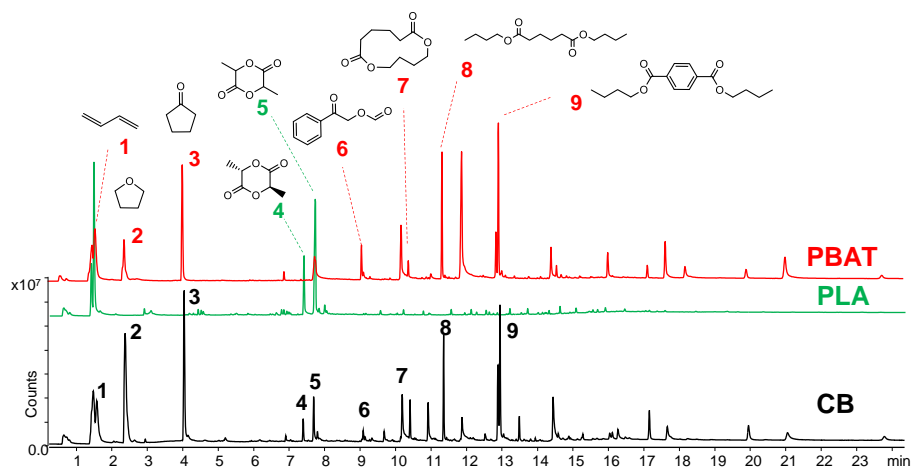


Figure 1. Total ion chromatogram (pyrogram) obtained from Py-GC-MS of CB (full line) compared with the pyrograms of PLA and PBAT. Tentative product identification: (1) 1,3 butadiene; (2) tetrahydrofuran; (3) cyclopentanone; (4) meso lactide; (5) D,L-lactide; (6) 2-(formyloxy)-1-phenyl- ethenone; (7)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

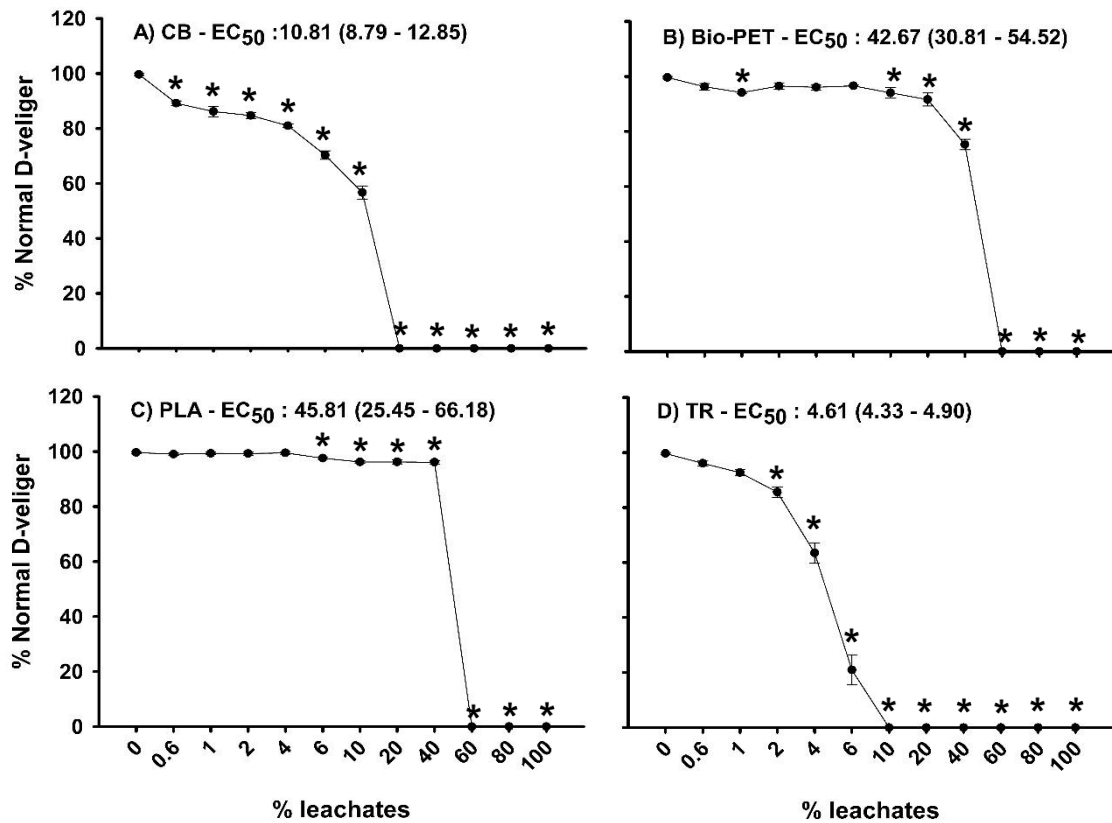


Figure 2. Mean percentage of *M. galloprovincialis* normal D-veliger larvae after 48h exposure to leachates from bio-plastics (CB, Bio-PET, PLA) and conventional tire rubber (TR). Data are expressed as mean \pm SEM (N = 5). Asterisks indicate significant differences compared to the control ($p < 0.05$,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

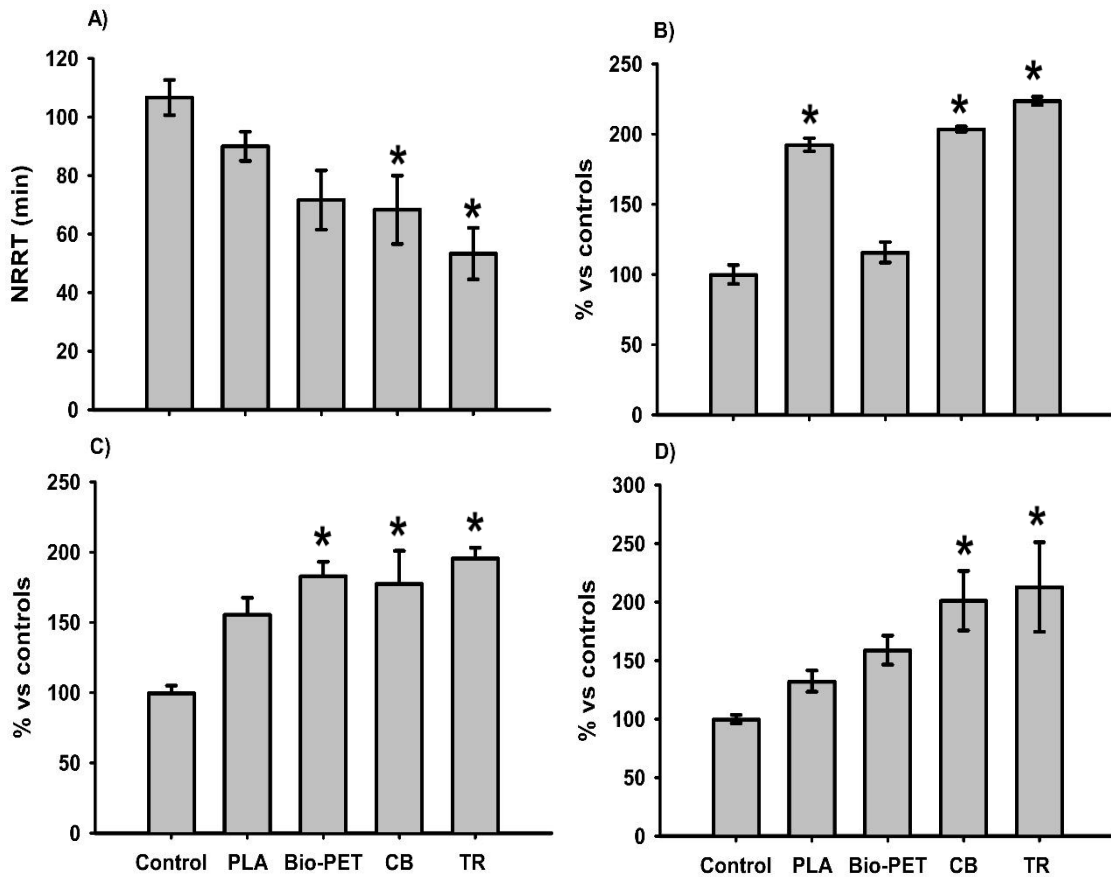


Figure 3. Lysosomal parameters evaluated in adult mussels after in vivo 7-day exposure to 0.6% concentrations bio-leachates (PLA, BioPET, CB) and tire rubber (TR) leachate. A) Lysosome membrane stability; B) Lysosome/Cytosol ratio; C) unsaturated neutral lipid accumulation; D) lipofuscin accumulation. 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).

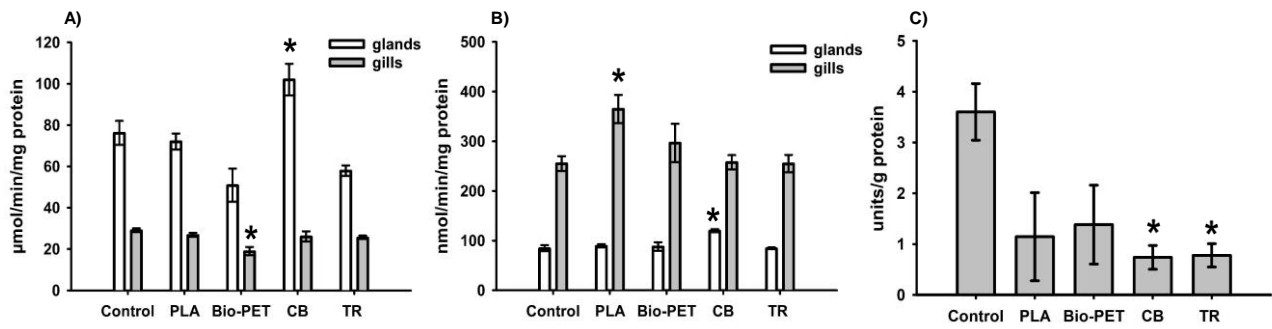


Figure 4. Enzymatic activities evaluated in adult mussels after in vivo 7-day exposure to 0.6% concentrations bio-leachates (SB, BPT, PLA) and tire rubber (TR) leachate. A) Catalase activity in digestive glands and gills; B) Glutathione-S transferase activity in digestive glands and gills; C) Lysozyme 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 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: