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Bioplastic leachates characterization and impacts on early larval stages and adult mussel cellular, biochemical and physiological responses.

Marco Capolupo[1], Ayesha Rafiq[2], Irene Coralli[3], Tanya Alessandro[2,*] Paola Valbonesi[2], Daniele Fabbri[3], and Elena Fabbri[2,§].

1 Italian Institute for Environmental Protection and Research (ISPRA), Rome, Italy

2 Department of Biological, Geological and Environmental Sciences, Campus of Ravenna University of Bologna, Italy.

3 "Department of Chemistry "Giacomo Ciamician", Technopole of Rimini, University of Bologna, Italy

**Present affiliation Anton Dohrn Zoological Station, Naples, Italy*

§ Corresponding Author: Via S. Alberto 163, 48123 Ravenna, Italy.

E-mail: elena.fabbri@unibo.it (E. Fabbri)

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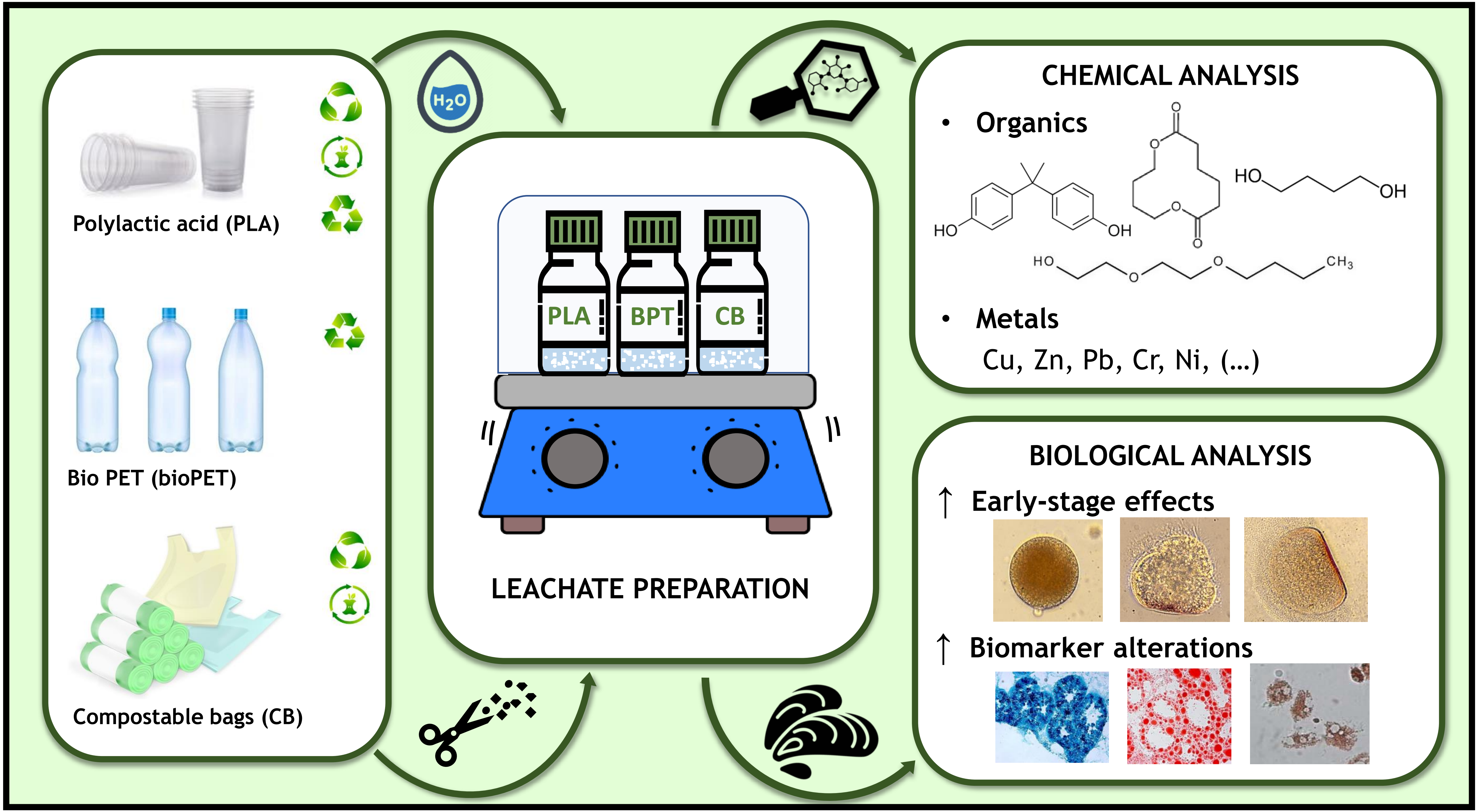
Capsule sentence: Leachates from bioplastics cause embryotoxicity, alteration of lysosomal parameters, and reduction of immune responses in *Mytilus galloprovincialis*.



Polylactic acid (PLA)
Bio PET (bioPET)
Compostable bags (CB)
H2O
PLA
BPT
CB
LEACHATE PREPARATION
CHEMICAL ANALYSIS
Organics
HO
OH
O
HO
OH
HO
O
O
CH3
Metals
Cu, Zn, Pb, Cr, Ni, (...)
BIOLOGICAL ANALYSIS
↑ Early-stage effects
↑ Biomarker alterations

ABSTRACT

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

1. **Introduction**

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

Among organic additives, bisphenols, phthalates, brominated flame retardants, organotin compounds, alkylphenols, formaldehyde, antimicrobials and azocolorants are included (Gunaalan et al., 2020; Luo et al., 2022). Many of the above chemicals are known or suspected Endocrine Disrupting Chemicals (EDCs), i.e. compounds able to interfere at 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 group of inorganic additives, often demonstrated as hazardous to human and environmental 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 in plastic items (Muncke, 2009).

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 safer alternatives. Bioplastics commonly encompass a diverse family of polymeric materials that originates from biomass and/or are biodegradable. Confusion usually occurs among bio-, bio-based and bio-degradable plastics (Lambert and Wagner, 2017; Wang et al., 2022). Biobased plastics are those containing organic carbon of renewable origin from the natural environment, while biodegradable plastics are made of polymers susceptible to mineralization into CO_2, biomass and water by biological activity (Kjeldsen et al., 2019; Razza and Degli Innocenti, 2012). Synthetic polyesters are commonly employed in the production of biodegradable plastics based on fossil resources, such as poly (butylene adipate-*co*-terephthalate) (PBAT), polymers that are both bio-based and biodegradable such as polylactic acid (PLA), and further polymers partially biobased such as poly(ethylene terephthalate) (PET) from biobased ethylene. Bioplastics made of these polyesters can be manufactured in forms of composites, as for instance PBAT/PLA and PBAT/starch.

The market share of bioplastics is relatively low compared to conventional thermoplastics; however, it is steeply increasing worldwide after new law regulations have been approved 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 bioplastic chemical safety, the chemical nature of compounds included in the items, and the potential toxicity of the leachates for ecosystem and human health. It has been reported that bioplastics contain similar additives as their conventional counterparts (Lehtiniemi et al., 2021) and may be similarly toxic (Uribe-Echeverría and Beiras, 2022; Zimmermann et al., 2020a). Bioplastics undergo ageing processes mediated by abiotic and biotic agents (mechanical abrasion, thermal degradation, hydrolysis, photo-oxidation, biodegradation) that can deeply affect the properties of the polymer matrix, with consequent additive release.

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

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

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

2. MATERIALS AND METHODS

2.1. Bioplastic leachate preparation

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

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

2.2. Chemical analysis

The procedure was carried out as previously reported by Capolupo et al. (2020) with a few modifications. Seawater leachate samples (2 mL) were introduced into a 10 mL glass test tube, added with internal standard (tri-*tert*-butyl benzene from Sigma-Aldrich) and extracted 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 then used for GC-MS analysis. A control seawater (ASW) leachate was analysed with the same procedure, while procedural blank analyses with distilled water were performed in between sample analyses. The overall procedure was run in triplicate for all samples. An aliquot of the organic extracts (100 µL) of seawater samples, including control, was subjected to trimethylsilylation with 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide for 2 hours at 60 °C.

2.2.1 Analysis of bioplastic materials

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 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 Agilent Technology, USA). Thermally evolved products were injected in the GC at 280 °C under 1:100 split ratio and separated with a HP-5ms Ultra Inert Agilent 19091S-433UI column 30 m, 0.25 mm i.d., 0.25 μm film thickness operating from 40 °C (2 min) to 305 °C at 20 °C/min. MS acquisition was performed under 70 eV electron ionization at *m/z* 35-600. 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) in the 650 – 4000 cm^{-1} wavenumber range.

2.2.2 Analysis of leachates

Gas chromatography-mass spectrometry (GC-MS) was performed on both underivatized and silylated organic extracts using a Shimadzu GC-2010 - GCMS-QP2010S system in splitless mode at 250 °C under helium. Compounds were separated by a DB-5ms column 30 m, 0.250 mm i.d., 0.25 μm film thickness, with 1.1 mL min^{-1} column flow working from 40 °C (2 min) to 320 °C at 10 °C min^{-1}, held at 320 °C for 6 min. The quadrupole mass spectrometer operated under electron ionisation at 70 eV recording spectra in the 35 – 500 *m/z* interval. Temperature of MS source and quadrupole were set at 230 °C and 240 °C, respectively. Chemical analyses of BPA were carried out with an HPLC system (Agilent 1.200 series, Agilent Technologies Italia S.p.A) coupled with a MS/MS spectrometer, equipped with an electrospray ionization source (Quattro Premier XE Micromass, Waters S.p.A.). Analytical details are reported in Supplemental material and in (Valbonesi et al., 2021). For trace metals assessment, samples were diluted in MilliQ water before internal standards (^{103}Rh and ^{115}In) 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.

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).

2.3.1 Fertilization and embryo-larval development

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% concentration) for 1 h prior to add eggs in 1:5 proportion as in ASTM (2004).The reaction was blocked after 30 min by adding calcium buffered formalin (4%). The acute embryotoxicity test (ASTM, 2004) was adapted to 96 microwell plates (Fabbri et al., 2014) to screen the impact of bioplastic leachates on *M. galloprovincialis* embryo-larval development. Prior to leachate exposure, mussel oocytes were fertilized by mixing eggs and spermatozoa at a 1:5 ratio in 96-well plates. Fifty eggs/well were used for fertilization test. 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 above described, and samples examined at 40 x magnification using an inverted 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.

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).

2.4. Adult mussel exposure and biomarker evaluation

2.4.1. Experimental design

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 light/dark conditions) for four days before experimental treatment. Ten mussels were then 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 seven days in line with previous experimental exposure using thermoplastic leachates (Capolupo et al., 2021a). All leachates were tested in triplicate, each aquarium representing a single experimental replicate (n=3). Aquaria for control condition (CTR) with only filtered seawater were run in parallel (n=3). The exposure was performed in controlled conditions of temperature (16-18 °C), photoperiod 14 h: 10 h light/dark) and feeding (1,200 cells/mL of the green alga *Nannochloropsis oculata*), as previously described (Capolupo et al., 2021a). Leachates and food were renewed daily after water change.

2.4.2. Biomarker analysis

After exposure, mussel tissues were dissected and, depending on the parameter to be tested, frozen in Liquid N_2 then stored at -80 °C, or immediately used for analysis. A battery of nine biomarkers was assessed following the OSPAR 2013 protocol (OSPAR Commission, 2013), namely lysosome membrane stability (LMS), lysosome/cytosol ration (LYS/CYT), neutral lipid (NL), malondialdehyde (MDA), and lipofuscin (LF) accumulation, and lysozyme (LYZ), catalase (CAT), glutathione S-transferase (GST), and acethylcholinesterase (AChE) activities. Haemocytes were collected from 4 mussels per vessel and LMS evaluated by the Neutral Red Retention Assay (NRRA) (Martínez-Gomez et al., 2015). LYS/CYT, NL and LF 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 digestive glands and/or gills taken from 6 mussels per vessel (18 mussels per experimental condition). After homogenization and centrifugation, specific assays were conducted spectrophotometrically (Capolupo et al., 2021a). Gills homogenates were used for 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 at 405 nm for 10 min (Valbonesi et al., 2003). Serum LYZ activity was measured as previously described (Capolupo et al., 2021b), following for 10 min the decrease in absorbance due to the LYZ effect on *Micrococcus lysodeikticus.* Details on methods for biomarker measurements are reported in Supplemental materials.

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.

3. RESULTS AND DISCUSSION

The need to obtain public information on the composition of plastics was stressed (Groh et 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 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 of our knowledge only two investigations are available that report on bioplastic leachates composition and their biological effects on marine organisms, i.e. marine bacteria (Zimmermann et al., 2020c) and sea urchin larvae (Uribe-Echeverría and Beiras, 2022). 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 chemicals each in 80% of the samples) including toxic chemicals were found in the bioplastics and plant-based items examined, including erucamide, Irganox 1076, tris(2-nonylphenyl) phosphate etc. which also occurred in petroleum-based plastics. The Authors also showed that commercial bio-based and/or biodegradable items may cause toxicity similar to the conventional ones.

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 (1-

iodo-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.

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

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 PLA and bioPET (**Fig. 1S**) and the chemical composition of the pyrolysates (**Table 1S**) confirmed the identity of the corresponding polymers (Tsuge et al., 2012). BioPET produced a series of derivatives of terephthalic acid and benzoic acid. Lactides (*meso* and D,L forms) were the main pyrolysis products of PLA along with their thermal degradation products acetaldehyde, 2,3-pentadione and acrylic acid. Pyrogram of TR (**Fig. 1S**, **Table 1S**) was featured by limonene and 2,4-dimethyl-4-vinylcyclohexene, typical marker of polyisoprene rubber (Tsuge et al., 2012). Besides thermal degradation products of the rubber, two additives could be identified that evolved by volatilisation, namely benzothiazole and 1,2-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 some of the products detected in the pyrogram of CB. In particular, a cyclic molecule, virtually derived from the condensation of 1,4-butandiol and adipic acid, the 1,6-dioxacyclododecane-7,12-dione (**Fig. 1**, peak 7) was tentatively identified by NIST library comparison. PBAT is used in packaging technology, in combination with other polyesters combined with starch to improve properties and reduce costs while maintaining biodegradability (Jian et al., 2020). Pyrolysis markers of starch were not detected indicating that this compostable plastic bag was not based on starch. ATR-FTIR spectra of bioplastic materials confirmed the polymeric species identified by Py-GC-MS (**Fig. 2S**).

3.2 Chemical composition of sea water leachates

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 found, namely the monomer 1,4-butandiol and the cyclic ester 1,6-dioxacyclododecane-7,12-dione; this latter was a relevant pyrolysis product of CB (compound # 7 in **Fig 1**). In accordance to our study, 1,6-dioxacyclododecane-7,12-dione was found in the leachates obtained from weathered and original compostable plastic bags (Balestri et al., 2019). Besides, 1,6-dioxacyclododecane-7,12-dione was found among the chemicals that migrated into water from infant teether toys (Liu et al., 2021), as a potential migrant into isoctane from polyurethane adhesives in laminates typical of food packaging (Félix et al., 2012) or into air from volatilisation from materials based on polyurethanes (Thiébaut et al., 2007; Watanabe et al., 2007). Moreover, 1,6-dioxacyclododecane-7,12-dione and other cyclic esters were identified among the chemicals that migrated from adhesives of food packaging materials into a solid food simulant (Canellas et al., 2015). These substances were presumed to be NIAS associated to the aliphatic polyester-based portion consisting of adipic acid and 1,4-butanediol, as in PBAT. It is known that linear and cyclic oligomers of polyesters are inevitably formed during manufacturing, probably through a “back-biting” mechanism of the polymer backbone, and may occur in the final product as NIAS affecting its properties (Zhang et al., 2022). This category of NIAS has the potential to migrate out the polymer. For 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 (Ubeda et al., 2021).

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

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 µg/L concentrations (**Table 2**). BPA was found in the aquatic environment from 0.5 ng to 12 µg/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 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 et al., 2018). The relatively high content of Zn in polyester leachates could also be associated to catalysts for polycondensation. As an example, organometallic compounds based on Zn and Sn were reported in the synthesis of PBAT (Jian et al., 2020). Zn was reported to be among the prominent trace metals in tires, and is considered a main responsible for tire dust leachate toxicity (Marwood et al., 2011). However, Zn was present at comparable concentrations in control samples (ASW), suggesting multiple sources. Instead, copper was present at concentrations higher than ASW, in particular in CB. Salts containing Cu could be utilised as plastic additives (biocides, pigments) and can act as pro-oxidants (Hahladakis et al., 2018). Iron, lead, nickel and arsenic were detected at very low albeit detectable levels higher than ASW in the leachates of CB; among them Fe and Pb could be components of some plastic additives (Hahladakis et al., 2018).

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.

3.3 Biological impact of leachates

3.3.1 Mussel early life stages endpoints

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

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

Several NIAS associated to the chemical structure of the polyester (monomers and oligomers of PLA and PBAT) were found in the leachates, as described in section 3.2.1. As discussed above, this finding agreed with literature as far as the susceptibility of these compounds to migrate out of the polymeric material into the surroundings medium is concerned. Water leachates from microplastics of bioplastic, presumably containing monomers/oligomers of different polyesters (among which PBAT) mixed with starch, resulted to have almost no adverse effects on *L. variegatus* freshwater oligochaete; 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 underlining the ubiquity of the cyclic ester 1,6-dioxacyclododecane-7,12-dione in the mixture of compounds prone to be released from bioplastics containing PBAT. This cyclic ester was a major compound in the leachates of CB, but no information on its toxicity to marine organisms is available in published studies. Significantly reduced motility (at 48h) was observed in larvae exposed to TR leachates within a concentration range of 20-100% (**Fig. 4S**); significantly reduced larvae survival (at 216 h) was caused by TR leachates in the range 10-100% (**Fig. 5S**), with EC50 values of 17.3 and 11.9%, respectively. Other leachates were ineffective on survival, and PLA and CB only reduced motility at the highest concentrations tested.

3.3.1 Biomarker evaluation in adult mussels

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 withdrawn from mussels exposed CB and TR leachates (**Fig. 3** panel A). LMS decreased also after PLA and bioPET leachate exposure, without reaching significance. LMS reduction in haemocytes is the most sensitive biomarker of stress in mussels (Martínez-Gomez et al., 2015). Its reduction reflects the loss of membrane integrity and the impairment of lysosome functionality, and is in fact an early warning signal for pathologies in Mytilus spp. (Moore et al., 2006; Viarengo et al., 2007). LMS has been correlated with animal scope for growth and total oxyradical scavenging capacity, and inversely correlated with protein catabolism, lipofuscin and neutral lipid accumulation, lysosomal swelling, and DNA damage (Moore et al., 2004). Significant reduction of LMS has previously been reported after mussel exposure to leachates from conventional plastics (Capolupo et al., 2021a) and other chemical insults 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 occurs in all leachates presently tested. Trace metals such as copper, zinc and lead are among the contaminants provoking destabilization of lysosome membranes (Giamberini and Pihan, 1997), and these occur in TR and CB leachates. Copper is much higher than in control water also in leachates from bioPET and PLA. Other compounds found in the leachates could also affect LMS, alone or in combination. As previously shown, LMS is reduced also after mussel exposure to microplastics (Canesi and Fabbri, 2015; Capolupo et al., 2021a; Sharifinia et al., 2020). Whether the microplastics ingested do have time enough to release additives along the digestive tract before elimination (Fernández and Albentosa, 2019) is not known yet, however it is a challenging issue in wildlife as in humans. Overall, LMS is a useful biomarker to highlight the occurrence of low concentrations of organic and inorganic contaminants in water either alone or in mixtures. Reduction of LMS often leads to lysosome increase in size and fusions (Lowe et al., 1981; Moore, 2008).The increase of the lysosomal volume is a condition predictive of impairment of viability and functionality of digestive gland cells (Orbea et al., 2006). The increasing size of the lysosome compartment (LYS) with respect to cytosol (CYT) has been measured in mussels exposed to leachates. LYS/CYT ratios were significantly higher than in controls in mussels exposed to TR, CB and 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, with a mechanism at least in part mediated by a calcium dependent-phospholipase A2 stimulation (Marchi et al., 2004). High LYS/CYT ratio was measured in mussels which accumulated Cu and Zn from coastal lagoon waters (Capolupo et al., 2017). These metals occur in all tested leachates at concentrations that in some cases are significantly higher than control water. Lysosomotropic effects could however be induced in mussels by further chemical additives or NIAS, whose properties are not known, at present.

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

Reactive oxygen species (ROS) production is a known effect of pollutants (Regoli and Giuliani, 2014) able to induce lipid peroxidation (Moore, 2008). The products of lipid peroxidation are effectively sequestered by lysosomes, among these MDA, as intermediate compounds with respect to the final products represented by LF (Terman and Brunk, 2006). In particular, LF are insoluble aggregates made of lipid peroxidation residues which bind to food degradation by-products, oxidized proteins, carbohydrates, and metals (Terman and Brunk, 2006). These compounds are observed in mussels in response to oxidative pollutants 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 CB (**Fig. 3** panel D). Increases, although not significant, were noted also for PLA and bioPET. It has to be underlined that 1 week is a minimum time for lipofuscin accumulation (Viarengo et al., 2007), thus the one represented on **Fig. 3 (**panel D) may not be the 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 moderate peroxidation as from PLA and bioPET, where the accumulation of LF is not 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).

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

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 role of the enzymes in the two tissues was confirmed, with CAT having higher basal activity in digestive glands, where peroxidation reactions are mostly performed, and GST showing higher activity in gills, i.e. the first tissue exposed to environmental xenobiotics (Capolupo et al., 2021a). CAT activity was significantly reduced by leachates from bioPET in gills, and enhanced by leachates from CB in digestive glands (**Fig. 4** panel A); GST was significantly enhanced by leachates from PLA in gills, and from CB in digestive glands (**Fig. 4** panel B).

Previous studies reported different responses of CAT and GST, increase, decrease or no effect, after pollutant exposure (Akcha et al., 2000; Cheung et al., 2004; Gowland et al., 2002; Petushok et al., 2002; Regoli et al., 2004; Robillard et al., 2003). Cu and Zn were able to activate GST in *Mytilus* spp (Canesi et al., 1999; Capolupo et al., 2017); differently, a significant GST inhibition was reported in mussels after BPA exposure (Canesi et al., 2007). 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 bigger responses by the cellular defenses.

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

A reduction of lysozyme activity is a marker of immunosuppression, thus of lower resistance to bacterial insult. Lysozyme activity was also reduced by the estrogen-like compound tributyltin (Matozzo and Marin, 2005), while increased by 17-β estradiol (Canesi et al., 2006). 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 be involved in this function (Balbi et al., 2019). Recent studies have shown that blood clam *Tegillarca granosa* lysozyme activity (together with some other innate immune effectors) was inhibited by BPA, microplastics and BPA plus microplastics (Tang et al., 2022). The co-exposure of *T. granosa* to microplastics, B[a]P and E2, led to the highest reduction of lysozyme release and activity (Tang et al., 2022). Furthermore, the leachates also contained trace metals that may affect lysozyme activity. In fact, an effect of Cu on lysozyme molecular configuration was suggested many years ago from investigations on *Mytilus* haemocytes (Steinert and Pickwell, 1984). Copper was more recently reported to irreversibly inhibit chicken egg white lysozyme activity up to 80% at 390 μM, as a consequence of binding to 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). Cr, Ni, Cd, B, Hg, and Pb in the range 10-50 mM caused dose-dependent reduction of hen 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 (Valbonesi et al., 2003). Its inhibition, typically by organophosphates, induces a protraction of the nervous stimulus (Valbonesi et al., 2003). In our experimental trials, however, no change in AChE activity was detected of exposure to the different leachates at 0.6% concentration (**Fig. 6S** panel B). In mussels AChE was found to be affected by the mixture of BPA, carbamazepine and atrazine (Juhel et al., 2017), and inhibited by metals (Frasco et al., 2005); moreover, AChE inhibition by plastic leachates has recently been reported in fish (Walpitagama et al., 2019). The short exposure and/or the high dilution of the leachates may be the reason of the lack of effect in our experimental system, although at least BPA and trace metals occur.

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.

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

Among ontogenetic parameters, the embryo-larval development showed the greatest 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 simultaneously a wide range of concentrations and demonstrate dose-dependent effects. Its application helps understanding the relationship between xenobiotic exposure and detrimental effects in marine organisms, and may be recommended for regulatory purposes. Adult physiological parameters were also affected by leachates, with highest effects observed for lysosomal and immunological biomarkers in mussels exposed to compostable bag (mixture of PBAT/PLA) bioplastic and tire rubber.

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 available for plastic leachate preparation. Furthermore, different mixtures are expected to induce different effects however full chemical characterization of (bio)plastics is not possible, yet. Although the experimental conditions are not intended to mimic real environmental situations and the investigated items are not necessarily representative of the entire class of commercialised polyesters, the results presented in this study are important as a basis to identify mechanisms of action and draw possible adverse outcome pathways that can result in disturbances at the ecosystem level. They also support the use of effect-based tests for designing new and less harmful additives in order to produce “sustainable” bioplastics. Finally, they highlight that the leaching of additives and NIAS (e.g. monomers and oligomers) and their effects on aquatic organisms need to be carefully considered when assessing the environmental impacts of plastics.

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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**
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 μg/L and represent the mean media ± expanded uncertainty (K=2; df= 10) of measured levels for each treatment.

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

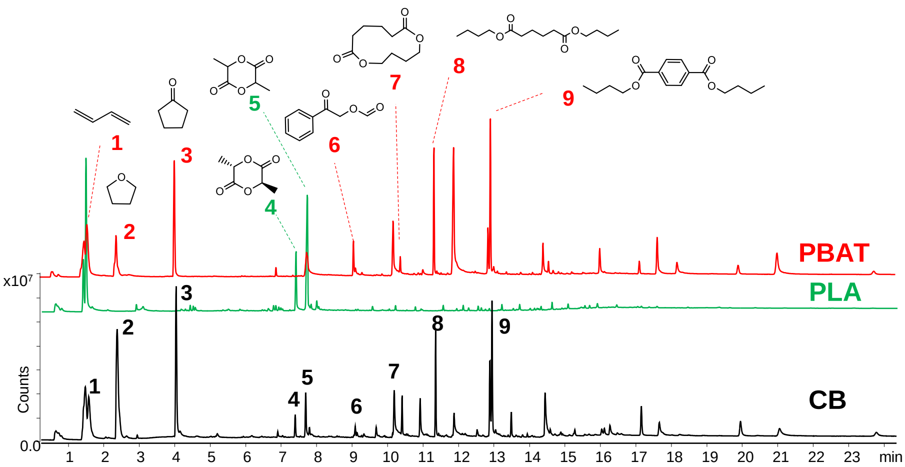

Figure 1. **Total ion chromatogram (pyrogram) obtained from Py-GC-MS of CB (full line) compared with the pyrograms of PLA and PBAT.** Tentative product identification: (1) 1,3 butadiene; (2) tetrahydrofuran; (3) cyclopentanone; (4) meso lactide; (5) D,L-lactide; (6) 2-(formyloxy)-1-phenyl- ethenone;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

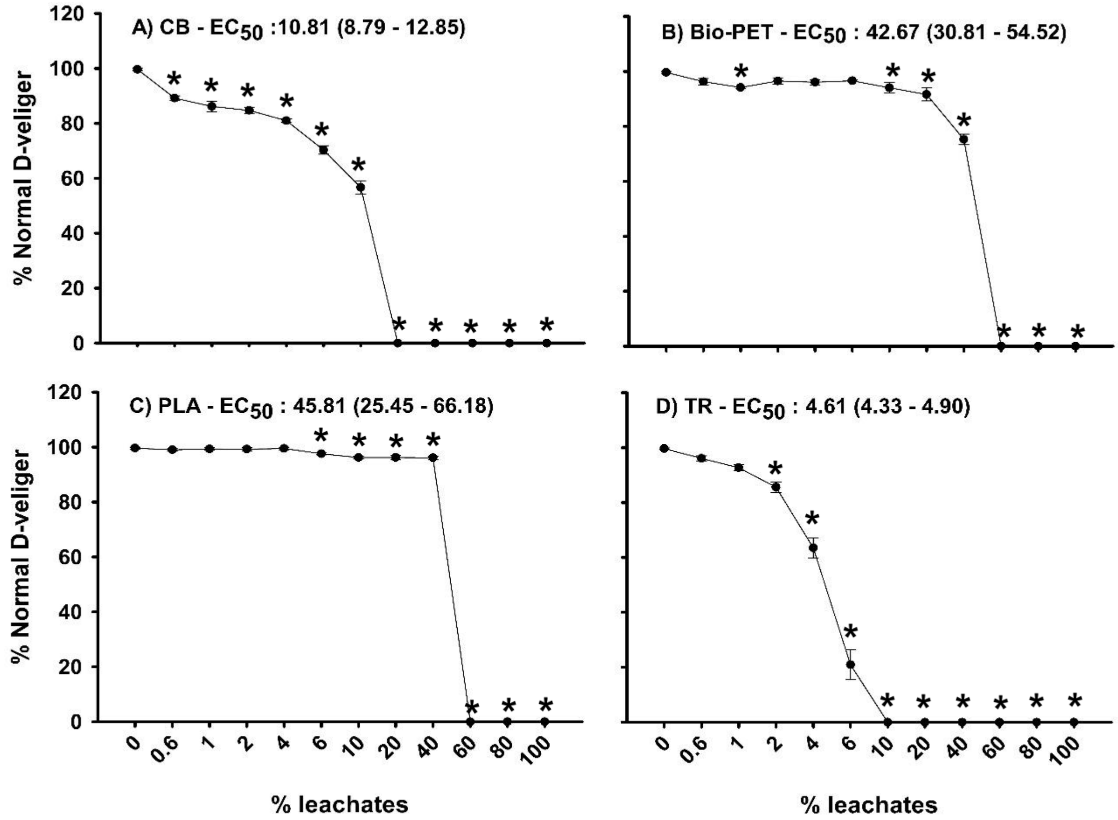

Figure 2. Mean percentage of *M. galloprovincialis* normal D-veliger larvae after 48h exposure to leachates from bio-plastics (CB, Bio-PET, PLA) and conventional tire rubber (TR). Data are expressed as mean ± SEM (N = 5). Asterisks indicate significant differences compared to the control ($p < 0.05$, One way ANOVA, Bonferroni post-hoc comparison). EC_{50} values (95% C.I.) are also reported.

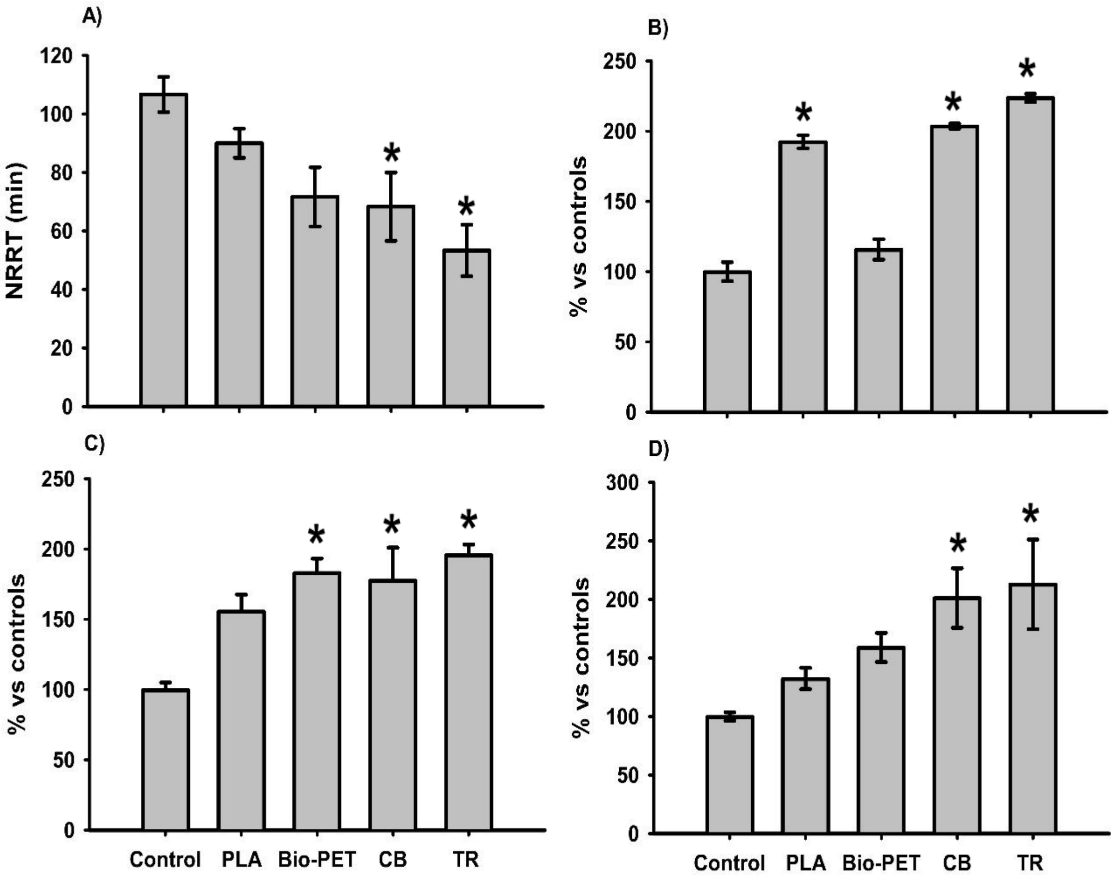

Figure 3. Lysosomal parameters evaluated in adult mussels after in vivo 7-day exposure to 0.6% concentrations bio-leachates (PLA, BioPET, CB) and tire rubber (TR) leachate. A) Lysosome membrane stability; B) Lysosome/Cytosol ratio; C) unsaturated neutral lipid accumulation; D) lipofuscin accumulation. Data are expressed as mean ± SEM (N = 3). Asterisks indicate significant differences compared to the control ($p < 0.05$, One-way ANOVA, Bonferroni post-hoc comparison).

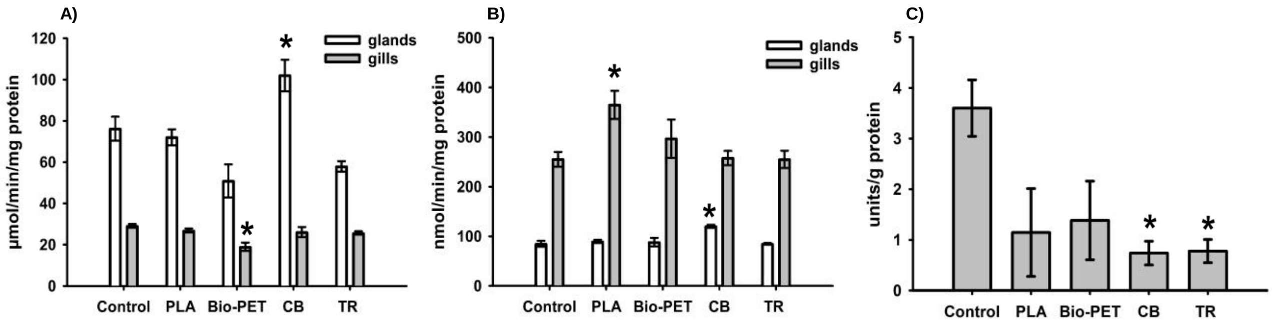

Figure 4. Enzymatic activities evaluated in adult mussels after in vivo 7-day exposure to 0.6% concentrations bio-leachates (SB, BPT, PLA) and tire rubber (TR) leachate. A) Catalase activity in digestive glands and gills; B) Glutathione-S transferase activity in digestive glands and gills; C) Lysozyme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

Authorship contributions

Conception and design of study: Capolupo M, Fabbri D, Fabbri E

Data aquisition: Rafiq A, Alessandro T, Valbonesi P, Coralli I

Analysis and/or interpretation of data: Capolupo M, Rafiq A, Coralli I, Valbonesi P

Drafting the manuscript: Capolupo M, Fabbri D, Fabbri E

Revising the manuscript critically for important intellectual content: Fabbri D, Fabbri E

Approval of the version of the manuscript to be published (the names of all authors must be listed): Marco Capolupo, Ayesha Rafiq, Irene Coralli, Tanya Alessandro, Paola Valbonesi, Daniele Fabbri, and Elena Fabbri

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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: