Science of the Total Environment 946 (2024) 174403

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Comparative assessment of the acute toxicity of commercial bio-based polymer leachates on marine plankton

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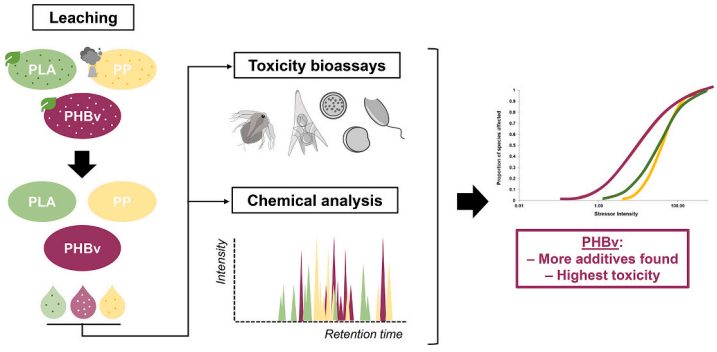
HIGHLIGHTS

- PHBv leachates caused acute toxic effects to all tested species.
- PLA and PP exhibits lower toxicity than PHBv to the tested species.
- A high number of harmful chemicals are identified in PHBv leachates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Julian Blasco

Keywords:
Microplastic
Leachate
Biopolymer
Bioplastic
Ecotoxicity
Marine species

ABSTRACT

Conventional plastics have become a major environmental concern due to their persistence and accumulation in marine ecosystems. The development of potential degradable polymers (PBP), such as polyhydroxyalkanoates (PHAs) and polylactic acid (PLA), has gained attention as an alternative to mitigate plastic pollution, since they have the potential to biodegrade under certain conditions, and their production is increasing as replacement of conventional polyolefins. This study aimed to assess and compare the toxicity of leachates of pre-compounding PBP (PLA and the PHA, polyhydroxybutyrate-covalerate (PHBv)) and polypropylene (PP) on five marine planktonic species. A battery of standard bioassays using bacteria, microalgae, sea urchin embryos, mussel embryos and copepod nauplii was conducted to assess the toxicity of leachates from those polymers. Additionally, the presence of chemical additives in the leachates was also verified through GC–MS and LC-HRMS analysis. Results showed that PHBv leachates exhibited higher toxicity compared to other polymers, with the microalgae *Rhodomonas salina*, being the most sensitive species to the tested leachates. On the other hand, PP and PLA generally displayed minimal to no toxicity in the studied species. Estimated species sensitivity distribution curves (SSD) show that PHBv leachates can be 10 times more hazardous to marine plankton than PP or PLA

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https://doi.org/10.1016/j.scitotenv.2024.174403
Received 20 October 2023; Received in revised form 23 June 2024; Accepted 29 June 2024
Available online 2 July 2024

leachates, as demonstrated by the calculated Hazardous Concentration for 5 % of species (HC_5). Qualitative chemical analysis supports the toxicological results, with 80 % of compounds being identified in PHBv leachates of which 2,4,6-trichlorophenol is worth mentioning due to the deleterious effects to aquatic biota described in literature. These findings underscore the fact that whereas environmental persistence can be targeted using PBP, the issue of chemical safety remains unsolved by some alternatives, such as PHBv. Gaining a comprehensive understanding of the toxicity profiles of PBP materials through a priori toxicological risk assessment is vital for their responsible application as alternatives to conventional plastics.

1. Introduction

Plastic has become increasingly ubiquitous in our everyday life due to its high functionality and relatively low cost. However, the extensive presence of conventional plastics, such as polyethylene (PE) or polypropylene (PP), poses significant environmental challenges. These usually petroleum-based synthetic polymers are not biodegradable in the natural environment, leading to their undesirable persistence. This persistence results in an estimated 11 % of global plastic production ending up in aquatic ecosystems (Borrelle et al., 2020), partly influenced by a substantial portion of plastic materials designed for single-use purposes. The accumulation of plastic litter in coastal and oceanic habitats has not only become an aesthetic nuisance (Galgani et al., 2019) but also poses threats to marine wildlife, including smothering and obstruction of the digestive system in marine fauna (Kühn et al., 2015).

To address these environmental concerns, the development of potentially biodegradable polymers (PBP) has gained significant attention. Although some PBP remains petroleum-based (e.g., PBAT), the most sustainable formulations are obtained from biomass (bio-based), such as chemosynthetic polylactic acid (PLA) and biosynthetic polyhydroxyalkanoates (PHA). Unlike conventional polyolefins (PP, PE), the polymeric chains of PBP are composed of C—O ester bonds instead of C—C bonds. Within the category of PBP, PLA and PHA account for 40 % and 8 % of the market, respectively, with the remaining global production consisting of petroleum-based PBAT and starch blends (European Bioplastics, 2022).

Polylactic acid (PLA) is a linear aliphatic polyester obtained through chemosynthesis from lactic acid, a natural substance. Industrial production of lactic acid involves the fermentation of carbohydrates by bacteria, with commonly used carbohydrate feedstocks being dextrose from corn or sugar cane. Polymerization of lactic acid into high molecular weight PLA (MW >100,000 Da) typically employs stannous octoate, an organotin chemical, as an initiator (Balla et al., 2021). Additional components like antioxidants (e.g., butylated hydroxytoluene BHT) and antimicrobial agents (e.g., Ag-zeolites, bacteriocins, enzymes, chitosan) are frequently added to PLA, especially for applications in food packaging. Although PLA is not biodegradable at environmental temperatures (Chamas et al., 2020; Martin et al., 2014; Weinstein et al., 2020), it can be hydrolyzed to reduce its molecular weight when exposed to temperatures above 58 °C. Consequently, PLA can be composted in industrial facilities but not in natural soil conditions. However, blending PLA with starch can enhance its degradability, although it may lead to a decrease in the composite's tensile strength when the starch content exceeds 20 % (Yew et al., 2005).

Polyhydroxyalkanoates (PHAs) are synthesized by bacteria, such as *Bacillus megaterium*, as carbon and energy reserves in the presence of excess carbon but limited nutrients. These reserves are stored as intracellular granules, varying in size and molecular weight depending on the microorganism and growth conditions (Lee, 1996). PHAs consist of fatty acid monomers, such as hydroxybutyrate (four carbon atoms) or hydroxyvalerate (five carbon atoms), connected by ester bonds during polymerization, resulting in polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), respectively. PHB has found applications in the medical field due to its biocompatibility, as hydroxybutyrate is a natural molecule present in blood plasma and does not typically cause immune reactions. PHB in its pure form, though, tends to be more brittle than petroleum-derived plastics, often necessitating copolymerization with hydroxyvalerate to produce polyhydroxybutyrate-covalerate (PHBv), which exhibits lower crystallinity, higher impact strength, and improved mechanical properties (Guho et al., 2020; Madison and Huisman, 1999). Industrial extraction of PHAs from bacteria involves the use of organic solvents, such as chloroform, propylene carbonate, and dichloroethane.

Unlike other PBPs, PHAs are truly biodegradable as they can be mineralized to CO_2 and water by bacteria, algae, and fungi in both aerobic and anaerobic natural environments, without the need for specific incubation conditions. Biodegradation of PHAs in seawater has been observed both in laboratory conditions (López-Ibáñez and Beiras, 2022; Tsuji and Suzuyoshi, 2002) and in situ (Brandl and Püchner, 1991; Sekiguchi et al., 2011). Furthermore, all PHAs exhibit thermoplastic properties that enable them to be processed using existing plastic manufacturing machinery. However, the adoption of PHAs as a replacement for traditional plastics is limited by their higher cost (Rodriguez-Perez et al., 2018).

PP is produced using different polymerization aids, including triethyl-aluminium, TiCl4 and nucleating agents such as for example trimethylallylsilane or vinylcyclohexane (De Rosa et al., 2017). Previous studies have examined the direct deleterious effects of plastic particles in marine environments (Burns and Boxall, 2018; Cole et al., 2015; Pannetier et al., 2020). Likewise, the indirect impact of plastic particles through chemical leaching to aquatic environments (Liu et al., 2020) or in digestive tract (Avio et al., 2015) was already proven to be an important source of toxicity. This toxicity is often linked to chemical additives (Beiras et al., 2021; Nobre et al., 2015) or sorbed environmental pollutants (Cormier et al., 2021; Gandara e Silva et al., 2016; Pannetier et al., 2019). Moreover, plastic leachates have also been implicated in altering behavioral responses in marine biota with possible implications for the ecosystem (Seuront, 2018; Seuront et al., 2021). In general, conventional olefin resins are rather innocuous (Beiras et al., 2018) but on the other hand, some PBP polymers, such as PHB, are suspected to impact aquatic organisms through different mechanisms associated with the emission of plastic particles within the nanometric range by these materials (González-Pleiter et al., 2019).

The aim of this study was to compare the toxicity of two pre-compounding PBP resins (PLA and PHBv) with a pre-compounding conventional polyolefin resin (PP) on marine plankton. These materials are transported by sea and spillages are not uncommon (Bourzac, 2023). With that aim, we used a standard protocol recently developed for ecotoxicological testing of plastic leachates (Almeda et al., 2023). We performed a battery of marine ecotoxicological bioassays with different taxa representing key groups in the marine plankton including both primary and secondary producers and different invertebrate planktonic larvae. Additionally, chemical analysis was performed to identify potentially hazardous additives in the plastic leachates responsible for the recorded effects.

2. Material and methods

2.1. Polymers and leachate

PLA was purchased from Naturplast (France), PHBv from Ningbo Tianan Biological Material Co. Ltd. (China), and Polypropylene from Sigma-Aldrich. The materials commercial codes and basic

physicochemical properties are shown in Table S1. In the three cases these materials are precompounding resins. This means that they are used as raw materials for compounding with functional additives. However, they are not necessarily pristine polymers since some chemicals are already added during polymerization and molding. To obtain the leachates we followed the standard protocol for plastic micronization and leaching published by Almeda et al. (2023). Briefly, PP and PLA pellets were ground through a 250 μm stainless-steel sieve on an ultracentrifuge mill (ZM200, Retsch) using dry ice during the process. PHBv was purchased as a powder so it was simply sieved through a 250 μm mesh. Particle size distributions of 250 μm-sieved materials were analyzed using a laser diffraction particle analyzer (LS I3 320, Beckman Coulter) (Table S9). Sieved resins were then shared with other participant laboratories to conduct the different bioassays. Leachates were obtained at a solid/liquid ratio of 1 or 10 g/L, and with different marine water preparations, depending on the test species (Table 1 and further details on specific section). Leaching was conducted in an overhead rotator at 1 rpm during 24 h in the darkness. Leachates were then filtered through glass microfiber filters (Whatman®, Grade GF/F 0.7 μm) and tested undiluted and after serial dilutions. Further information on specific leachate concentrations and dilutions is available in Table 1. Leachates pH range 7.7–8.4.

2.2. Toxicity assays

2.2.1. Microtox test

The Microtox® test is a standardized acute toxicity assay based on the bioluminescent marine bacteria *Aliivibrio fischeri*. The bioassay was carried out as described in the ISO 11348-3:2007 guidelines (ISO, 2007) with freeze-dried bacteria (Modern Water) but using serial dilutions of leachates (20, 40, 80 and 100 %) prepared with artificial seawater at 25 PSU (Instant Ocean salts). Each assay was run in duplicate on each triplicate of plastic leachate. After 30 min of exposure of the bacteria to the leachate at 15 °C, the bioluminescence was measured using the Microtox 500 analyzer (Modern Water) and compared to the negative control (artificial seawater at 25 PSU). A positive control (200 mg/L potassium dichromate) was run with each set of samples.

2.2.2. Microalgae test

The cryptophyte microalgae *Rhodomonas salina* was used as a model species of phytoplankton. Cultures were grown in B1 medium (Hansen, 1989) and maintained at 20 °C and on a 12: 12-h light/dark cycle in glass flasks. The microalgae were inoculated in 34 mL glass bottles and then made up to 25 mL with the leachate solutions prepared with autoclaved and UV-treated 0.1 μm filtered seawater. B1 stock solution was added to each bottle at a concentration of 1.1 mL/L (Hansen, 1989) to ensure algal growth. Three replicates were used for each tested leachate solution and the control. The initial concentration of cells in the solutions was 15,000 cells/mL. The bottles were incubated on static at 20 °C with an irradiance of 60 μE m^{-2} s^{-1} in a 12/12 h light/dark photoperiod for 72 h. Cell density and size (Equivalent spherical diameter, ESD) were determined using a Beckman Coulter Multisizer 4 particle counter with an aperture tube of 100 μm. Two measurements per sample were conducted to determine the mean concentration of cells in each replicate for all the treatments.

Table 1
Summary of the plastic leachate testing conditions for each bioassay.

Bioassay	Particle size	Leachate plastic load	Dilutions tested	Endpoint recorded
Microtox	<250 μm	10 g/L	100 % / 80 % / 40 % / 20 %	Bioluminescence inhibition
Algae test	<250 μm	1 g/L	100 % / 33.3 % / 10 % / 3.3 %	Growth inhibition
Sea Urchin embryo test	<250 μm	1 g/L	100 % / 33.3 % / 10 % / 3.3 %	Growth inhibition
Mussel embryo test	<250 μm	10 g/L	100 % / 50 % / 25 % / 12.5 %	Larval normality
Copepod nauplii test	<250 μm	1 g/L	100 % / 33.3 % / 10 % / 3.3 %	Survival

2.2.3. Sea urchin test

Adult sea urchins (*Paracentrotus lividus)* with mature gonads were obtained from the ECIMAT stock and the sea urchin embryo test (SET) was conducted using standard methods (Saco-Álvarez et al., 2010). The mature oocytes were fertilized, transferred to 5 mL glass vials with 4 mL of filtered leachates prepared with artificial seawater (ASW) according to the formulation by Lorenzo et al. (2002), and filtered ASW was used as control. After 48 h incubation in darkness at 20 ± 0.5 °C, the vials were fixed with a few drops of 40 % formaldehyde and the length (maximum linear dimension) of 35 larvae was measured under a Leica DMI 4000B inverted microscope. The mean larval length increase, from egg length at day 0, between each treatment and the control was used as endpoint.

2.2.4. Mussel test

Embryo-larval bioassays were performed using mussel embryos (*Mytilus galloprovincialis*). Gametes were obtained from mature adults in the lab and in vitro fertilization was conducted according to Beiras and Bellas (2008). Briefly, spawning was induced and, after fertilization, eggs were transferred to glass vials filled with leachates and fresh seawater (FSW) was used as control. The vials were incubated in darkness at 20 ± 1 °C. After 48 h incubation, the vials were fixed with a few drops of 40 % formaldehyde, and 100 individuals per vial were observed using an inverted microscope (Axiovert 40 CFL, Carl Zeiss AG, Germany). The individuals were classified as normal (D-veliger larvae) or abnormal (non-veliger, irregular shape, convex hinge, and/or protruding mantle, embryos) based on His et al.'s criteria (His et al., 1997). The percentage of normal larvae was used as the endpoint, being the response obtained by dividing the percentage of normal larvae in treatments by the percentage of normal larvae in the control group.

2.2.5. Copepods test

The acute lethal toxicity test with nauplius larvae of the copepod *Acartia tonsa* followed standard methods previously described (Beiras et al., 2019). Mature copepods were obtained from a laboratory stock maintained by ECIMAT from 48 to 72 h before the start of the test. From the initial stock, adults were collected by a 300 μm mesh and incubated in laboratory conditions to produce nauplii for ~24 h. A total of 10 nauplii were transferred to 20 mL glass vials containing exposure medium using a binocular stereoscope. Four vials for each dilution and eight vials of ASW control were used. Copepod nauplius survival was recorded after 48 h using a Leica S9 I binocular microscope.

2.3. Chemical analysis

In order to identify additives leached from plastic, a separate 650 mL leachate at 1 g/L was produced for each substance following the procedures previously described in the "Polymers and Leachate" section. The leachates, a seawater control (Seawater filtered by 0.22 μm and treated with UV light, also used to produce the leachates) and ultrapure water (Milli-Q) blank, were extracted followed by a Gas Chromatography-High Resolution Mass Spectrometry (GC-HRMS) and a Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) analysis. To cover a broader range of plastic additives in the leachates we have used both GC and LC work flows with the aim to detect the more apolar compounds with GC–HRMS and the polar compounds with LC-HRMS. Suspect additives were identified using the

HRMS spectral library combined with an in-house library (Price et al., 2021) as well as the NIST14 library. To keep this section brief, detailed methodology of chemical analysis can be found in supplementary material.

2.4. Statistical methods

Normal distribution and homoscedasticity of toxicity data was checked using the Shapiro–Wilk and Levene's tests, respectively. Whenever the data followed a normal distribution, statistically significant differences between treatments and control ($p < 0.05$) were established by one-way ANOVA followed by Dunnett's post hoc test or Dunnett's T3, when the variances were not homogeneous, to find the lowest observed adverse effect concentration (LOEC) and the highest concentration with no observed adverse effects (NOEC). Same endpoints were obtained for non-normal distribution of the data, using the Mann-Whitney non-parametric test. The dilutions that produced a 50 % and 10 % decrease in the endpoint (EC_{50} and EC_{10} respectively) and their 95 % confidence intervals (CIs) were also calculated by fitting a probit dose–response model to the data in Microsoft Excel, as described in Lei and Sun (2018). Toxic units (TU) were calculated as $TU = 1/EC_{50}$ and classified according to the assessment criteria previously determined (Alonso-López et al., 2021) for plastic leachates resulting from Solid/Liquid ratios of 1 and 10 g/L.

Species Sensitivity Distribution (SSD) generator, a Microsoft Excel Template from USEPA (https://www.epa.gov/caddis-vol4/download-software) was used to create SSDs. This fits a log-probit distribution (i.e., linearized log-normal) to data for concentrations at which different species exhibit a standard response to a stressor. As some of the bioassays did not attain a 50 % response (EC_{50} not calculated) we employed the inverse of EC_{10} as the bioassays calculated stressor. The hazardous concentration for 5 % of species (HC_5) was also calculated (van Straalen and Denneman, 1989).

3. Results

3.1. Toxicity assays

The summary of EC_{10}, EC_{50}, and TU values for the tested polymers can be found in Table 2, providing a comprehensive overview of their toxicity profiles, while Fig. 1 presents the comparative concentration-response curves for the tested polymers in each bioassay, allowing for visual comparisons of their toxic effects. The observed points and confidence intervals for each substance can be found in the Supplementary Material (Fig. S1), which complements Fig. 1.

PHBv leachates consistently exhibited higher toxicity compared to the other polymers (Fig. 1), with TUs indicating a slight to high toxicity across all bioassays. *R. salina* displayed high sensitivity to this polymer, with an estimated EC_{50} of 7 % dilution. For other species, PHBv turned out to be slightly toxic with EC_{50} ranging from 26 % to 93 % of the leachate. In fact, the microalgae *R. salina* is the most sensitive of all tested species, with PP being moderately toxic ($EC_{50} = 37$ % of leachate) and PLA slightly toxic ($EC_{50} = 54$ % of leachate) for this species. Apart from the slight toxicity to mussels caused by PLA ($EC_{50} = 46$ % of leachate), the EC_{50} of PP and PLA could not be calculated for all the other bioassays, due to the low toxicity observed.

Fig. 2 displays all estimated species sensitivity distributions (SSDs). The R^2 values for the probability distributions range from 0.87 for PHBv to 0.95 for PP, while the 95 % confidence intervals were up to a maximum of 10-fold wider in PHBv. It should be noted that the analysis was conducted with only five different taxa, which explains the relatively lower precision in the estimations. Nevertheless, once again, the higher impact of PHBv on the tested species is shown, with HC_5 calculated as 0.47 % dilution of the leachate, while HC_5 estimated for PLA and PP was around 7–18 times higher (3.20 % and 8.31 %, respectively).

3.2. Chemical analysis

The GC-HRMS analysis of the plastic leachates, the sea water control and the ultrapure water (Milli-Q) blank resulted in 33 high-confidence matches with the in-house library with confidence level 1, according to Koelmel et al. (2022). After discarding the matched substances that were present in all analyzed leachates, ultrapure blank (Table S6) and in FSW control (Table S7), only two substances were identified (2,4,6-trichlorophenol (level 1) and an estragole analogue (level 2)), both of which were solely present in the PHBv leachate (Figs. S2 and S3, Table 3).

Additionally, the remaining unmatched substances were explored using MS Dial to filter out the ones that were below 5-fold of blank levels and that displayed poor chromatography. This resulted in 103 remaining unidentified substances distributed over the three polymer leachates: PHBv (81 %), PLA (14 %), PP (3 %), and PLA and PP (2 %). Representative spectra from the aligned peak list of these 103 compounds were tentatively matched against the NIST14 library, which resulted in 18 hits above match factor 600 and RI < 1.5 % (Table 3). Of these, only one was present in the PLA leachate: bisphenol A diglycidyl

Table 2
Acute toxicity data obtained for each bioassay. n.c. = not calculable (estimation above tested concentrations). For EC_{10} and EC_{50} the 95 % confidence intervals are shown in parentheses.

Item	Species	EC_{10} (mg.L^{-1} / %)	EC_{50} (mg.L^{-1} / %)	TU
PP	*A. fischeri*	**4960.7** (3379.8 – 7281.27) / **49.6**%	**n.c.**	< 1
	R. salina	**102.3** (86.5-120.9) / **10.2**%	**376.9** (338.0-420.3) / **37.7**%	2.7
	P. lividus	**728.6** (556.3-954.1) / **72.9**%	**n.c.**	< 1
	M. galloprovincialis	**2637.3** (2201.1-3159.9) / **26.4**%	**n.c.**	< 1
	A. tonsa	**n.c.**	**n.c.**	< 1
PHBv	*A. fischeri*	**684.8** (467.1-1003.7) / **6.8**%	**3942.3** (3488.5-4455.0) / **39.4**%	2.5
	R. salina	**6.5** (4.1-10.4) / **0.7**%	**73.6** (60.6-89.2) / **7.4**%	13.6
	P. lividus	**392.0** (331.5-463.6) / **39.2**%	**931.8** (840.7-1032.6) / **93.2**%	1.1
	M. galloprovincialis	**1557.6** (1457.6-1664.6) / **15.6**%	**2647.0** (2536.0-2762.8) / **26.5**%	3.8
	A. tonsa	**421.6** (385.2-461.4) / **42.**%	**657.0** (618.0-698.5) / **65.7**%	1.5
PLA	*A. fischeri*	**4429.3** (2935.3 – 6683.7) / **44.3**%	**n.c.**	< 1
	R. salina	**42.1** (30.5-58.0) / **4.2**%	**538.2** (30.5-58.0) / **53.4**%	1.9
	P. lividus	**808.73** (723.8-903.6) / **80.9**%	**n.c.**	< 1
	M. galloprovincialis	**1888.89** (1720.7-2073.5) / **18.9**%	**4624.3** (4362.0-4902.3) / **46.2**%	2.1
	A. tonsa	**n.c.**	**n.c.**	< 1

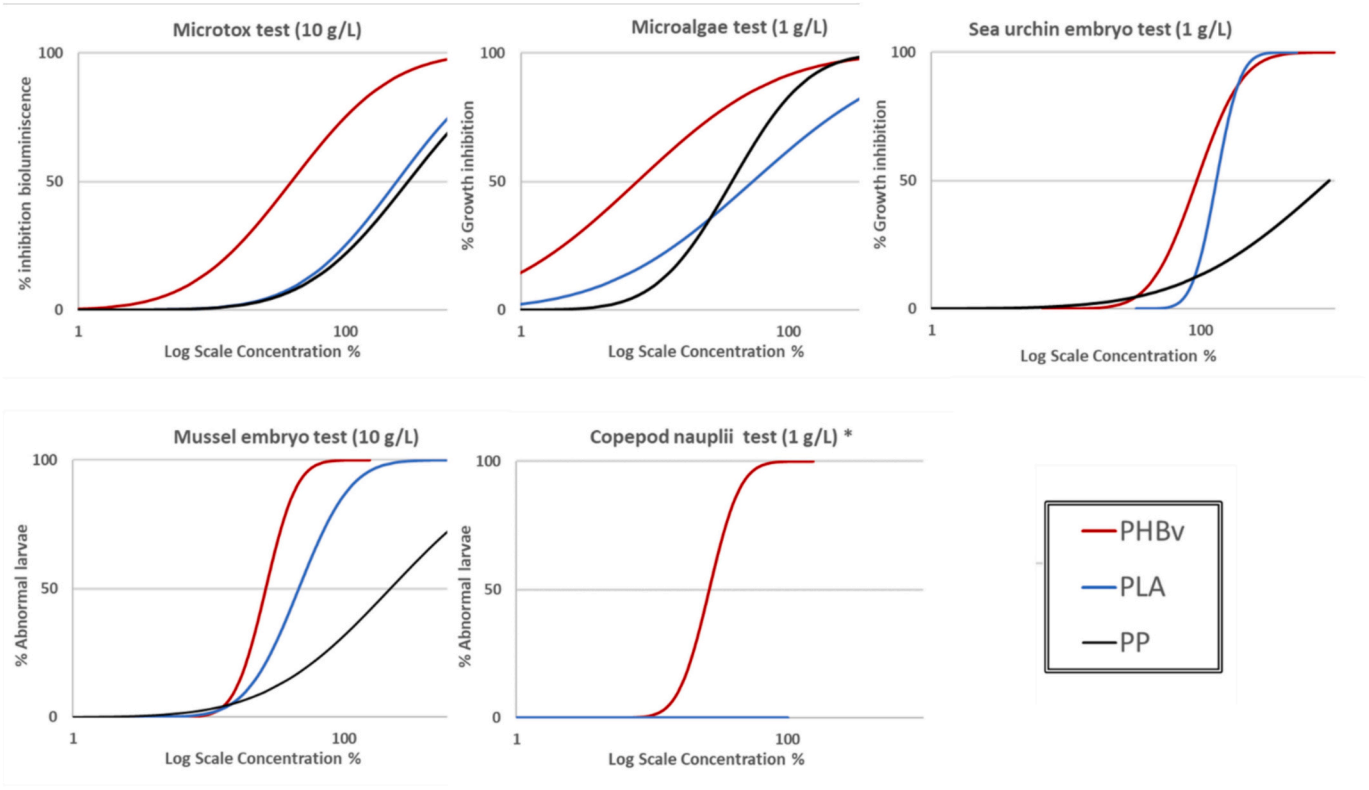

Fig. 1. Dose-response curves of PHBv, PLA and PP for each species. Log scale concentration is represented as percentage of leachate dilutions. *Only the dose-response curve for PHBv is shown as no effect was observed in any of the tested concentrations for the other two materials.

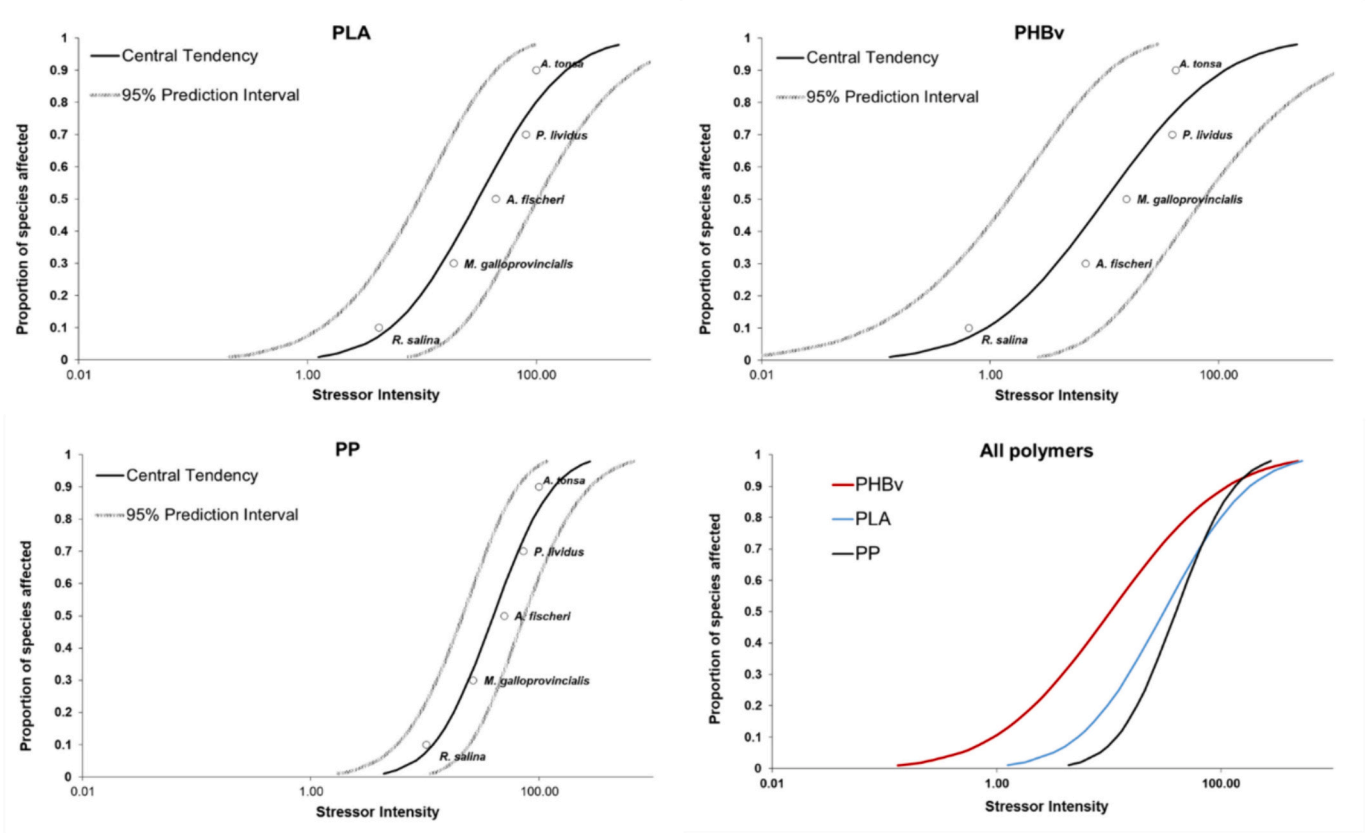

Fig. 2. Species sensitivity distributions and Confidence intervals for a set of five marine species exposed to PP, PLA and PHBv leachates. Comparative chart with 3 SSDs is shown for polymers comparison.

Table 3
Compounds above the blanks levels that were tentatively identified in PLA and PHBv leachates by GC-HRMS analysis. 2,4,6-Trichlorophenol was identified using an in-house library (level 1), an Estragole analogue was identified using an external HRMS library (Level 2, Price et al., 2021) while the remaining compounds were tentatively identified using the NIST14 mass spectral library. RT = Retention time, RI = Retention index, Qu mass = Quantification mass.

RT	RI	Qu mass	Compound	Formula	Match Factor	Reverse Match Factor	Identification method	RI library	Leachate
7.23	1214	147.0804	Estragole	C10H12O	0.80	0.88	External HRMS	1202	PHBv
9.61	1362	195.9244	2,4,6-Trichlorophenol	C6H3Cl3O	0.36	0.96	In-house HRMS	1349	PHBv
6.67	1178	73.0285	Octanoic acid	C8H16O2	659	734	NIST14	1180	PHBv
8.38	1285	142.9895	5-Chloro-guaiacol	C7H7ClO2	697	946	NIST14	1296[1]	PHBv
8.69	1305	117.0573	Indole	C8H7N	791	841	NIST14	1295	PHBv
10.44	1414	67.0542	1-Tetradecyne	C14H26	778	783	NIST14	1397	PHBv
11.41	1476	91.0309	Dodecane, 1-Chloro	C12H25Cl	773	773	NIST14	1469	PHBv
15.58	1767	87.0441	Tetradecanoic acid	C14H28O2	771	774	NIST14	1768	PHBv
16.24	1814	144.0444	1H-Indole-3-carboxaldehyde	C9H7NO	861	870	NIST14	1787	PHBv
33.36	2885	325.1433	Bisphenol A diglycidyl ether	C21H24O4	794	811	NIST14	2805	PLA
37.86	3168	285.2789	Octadecanoic acid, dodecyl ester	C30H60O2	671	674	NIST14	3150	PHBv
10.17	1397	69.0699	4-Tetradecene, (*Z*)-	C14H28	747	778	NIST14	1379	PHBv
10.29	1405	151.039	Vanillin	C8H8O3	794	825	NIST14	1404	PHBv
12.73	1564	136.9784	Dodecane, 1-bromo-	C12H25Br	658	660	NIST14	1549	PHBv
13.51	1617	67.0543	1-Hexadecyne	C16H30	840	843	NIST14	1664	PHBv
13.70	1630	193.0494	β-Asarone	C12H16O3	633	638	NIST14	1626	PHBv
16.58	1841	121.0284	isoAmyl-4-hydroxybenzoate	C12H16O3	709	836	NIST14	1822	PHBv
28.02	2570	201.1849	Dodecanoic acid, dodecyl ester	C24H48O2	592	594	NIST14	2554	PHBv
33.22	2878	105.0699	Cholesta-3,5-diene	C27H44	817	819	NIST14	2886	PHBv
34.71	2969	87.0441	Hexadecanoic acid, dodecyl ester	C28H56O2	607	620	NIST14	2951	PHBv

[1] RI for the structural analogue 2-Methoxy-4-chloro-phenol (same formula).

ether (Fig. S4), which has a high probability of being identified correctly (93 %), with the next hit in the hit list having a significantly lower match factor. All the other identified compounds on NIST14 library were present in the PHBv leachate (Table 3). Spectra properties of remaining unmatched substances are present in Table S8, where is possible to observe that the vast majority of substances were detected in PHBv leachate.

The results from the LC-Q-Tof analysis were in accordance with GC-HRMS, with more compounds present above blank levels in the PHBv leachate. Five compounds, among them four surfactants, were identified with the UNIFY software (Table 4). Bisphenol A and Bisphenol S were present in all leachates at blank levels.

4. Discussion

The effects of different plastics on the environment, as well as their leachates, have been widely discussed, prompting the search for environmentally sustainable alternatives (Andrady, 2011; Galgani et al., 2019). In this study, we explored the toxicity of PBP, PHBv and PLA, compared to a conventional resin, PP. The bioassays were conducted separately in four different laboratories, all using the same batches of resins. The results obtained for all species exhibited strong consistency, suggesting that the findings reflect real differences between the polymers. Our study suggests that PHBv leachate is more toxic than the other two materials, falling within the category of slightly toxic, using the classification proposed in a previous study (Alonso-López et al., 2021), except for the high toxicity found on *R. salina*. This research represents, to the best of our knowledge, the first ecotoxicological assessment of the impact of PHBv on marine species and provides novel insights into its potential harmful effects. While a previous study reported no ecotoxicity of PHBv film leachates to bacteria (Zembouai et al., 2016), the specific concentration of leachate used in that study remains unclear and therefore we are not able to properly compare the results. Pure PHBv is chemically inactive, shows low cytotoxicity and is used for biomedical applications because of its biocompatibility (Naser et al., 2021). Therefore, toxic effects in the leachates may be explained by release of small micro and nanoparticles or chemical additives. PHB-nanoparticles significantly decreased the growth of cyanobacteria and microalgae as well as induced immobilization in *Daphnia magna* (González-Pleiter et al., 2019). The analysis of the particle size distribution (Table S9) using laser diffraction showed that the percentage of particle volume corresponding to nanoplastics (<1 μm) was remarkably larger in PHBV (3.99 %) compared to PLA (0.37 %). Moreover, PHBV median particle size is 6 and 3-fold lower than PLA and PP, respectively, and toxicity of microplastics is inversely related to particle size (Beiras and Schönemann, 2020).

PHB leachates also caused growth impairment in sea-urchin that was associated with released chemicals (Uribe-Echeverría and Beiras, 2022). Furthermore, it is clear from our qualitative chemical analysis that PHBv released a larger number of compounds to water through leaching, which may be responsible for the higher toxicity found in all the bioassays. Two specific compounds, 2,4,6-trichlorophenol and estragole, were identified in PHBv leachates with high confidence. These compounds are harmful to aquatic life with long lasting effects, according to the European Chemical Agency classification (ECHA, 2017). There is no information in the scientific literature concerning the toxicity of estragole, but the acute aquatic toxicity of 2,4,6-Trichlorophenol is well known, with LC_{50} = 2.7 mg/L for the shrimp *Crangon septemspinosa*,

Table 4
Compounds tentatively identified in the PHBv leachate using LC-Q-Tof analysis. RT = Retention, Qu mass = Quantification mass.

RT	Precursor mass	Compound	Formula	Mass error	Confidence level[1]	Leachate
3.95	121.0304	4-hydroxybenzaldehyde/ salicylaldehyde	C7H6O2	−0.9	2a	PHBv
10.06	265.14017	Lauryl sulfate	C12H26O4S	−0.6	2a	PHBv
11.18	293.1927	Tetradecylsulfate	C14H30O4S		2a	PHBv
8.82	237.1159	Decylsulfate	C10H22O4S	−2.8	2b	PHBv
11.93	321.2099	Hexyldecylsulfate	C16H34O4S	−1.8	2b	PHBv

[1] Based on the system proposed by Schymanski et al., 2014.

LC_{50} = 3.9 mg/L for the clam *Mya arenaria* (McLeese et al., 1979), LC_{50} = 1.4 mg/L for the fish *Platichthys flesus* (Smith et al., 1994), LC_{50} = 0.69 mg/L for *Daphnia magna* (Kukkonen and Oikari, 1987), LC_{50} = 1.11 mg/L for the freshwater fish *Danio rerio* (Zhang et al., 2018), LC_{50} = 18.4 mg/L for *Aliivibrio fischeri,* or EC_{50} = 0.06 mg/L for the microalgae *Raphidocelis subcapitata* (Rosal et al., 2010). It is worth noting the high sensitivity of microalgae to this substance, which was also the most sensitive group to PHBv leachate. Also identified but with less confidence, 2-hexyldecanoic acid is considered toxic to aquatic life as per ECHA (ECHA, 2017). In addition, four surfactants were exclusively identified in the PHBv leachates (see Table 4). Surfactants are particularly toxic to naked embryo stages of bivalves and sea-urchins (Beiras and Bellas, 2008; Bellas et al., 2005). The exclusive presence of any of these substances may explain the higher toxicity of PHBv on plankton. However, the quantitative observed response is likely caused by the overall effect of the mixture of 83 compounds (56 of them unmatched) that leached into the water within 24 h.

Comparatively, our results indicate that PLA exhibits lower toxicity than PHBv, though it still poses slight toxicity to microalgae and mussels. Prior studies have presented mixed conclusions on the toxicity of PLA to aquatic organisms. Sublethal effects were reported in jellyfish (Di Giannantonio et al., 2022) and zebrafish (de Oliveira et al., 2021; Luan et al., 2023). While sublethal effects of PLA nanoparticles on hydras and fishes were also reported, they were also comparable to those imposed by PP and LDPE (Tamayo-Belda et al., 2023). This is also concluded in our study, with similar SSD curves estimated for PLA and PP. On the contrary, another study has shown no toxicity of leachates from PLA-based objects on sea-urchin larvae (Uribe-Echeverría and Beiras, 2022), which is confirmed by our results for this species. Also consistent with our findings, Zimmermann and co-workers reported no toxic effects from PLA resin with the Microtox test. Although the PLA resin showed no toxicity, certain PLA final products like food trays, coffee capsules, or bags exhibited toxic effects (Zimmermann et al., 2020). For PLA packaging applications, biocides (including silver-substituted zeolites, bacteriocins, enzymes and plant extracts) can be applied in the compounding post-polymerization phase to provide antimicrobial properties to the final commercial material (Jamshidian et al., 2010). Only one compound could be identified in PLA leachates, bisphenol A diglycidyl ether, a potential endocrine disrupting compound (Wang et al., 2021). The EU also considers this substance toxic to aquatic life with long-term effects (ECHA, 2017), with reported lethal concentrations of 0.13 mg/L for embryos of the amphibian *Rhinella arenarum* (Hutler Wolkowicz et al., 2016) or 6.03 mg/L for *Chironomus riparius* larvae (Ha and Choi, 2008).

Despite the limited number of available endpoints, leading to increased uncertainties, the SSD analysis revealed distinct differences in the estimated sensitivity of marine species to the studied polymers. Specifically, the estimated HC5, which aims to protect 95 % of the species, for PHBv is 0.47 % of the leachate, nearly 10 times lower than the estimated value for PLA. Even with the clear evidence provided by this analysis, SSD curves remain relatively uncommon in ecotoxicological studies of plastic leachates, possibly due to the lack of standardized methods, which makes it impossible to derive conclusive results. In the present study for example, due to specific requirements of each test species and logistic difficulties with mailing short-lived liquid samples, two different plastic loads had to be used (see Table 1). In this sense, to enhance the consistency of conclusions, it is recommended to employ standardized protocols for leaching of micronized plastics in aquatic toxicity testing, as proposed by Almeda et al. (2023).

It is difficult to understand the ecosystem implications of the observed effects of the polymers studied, especially with such a reduced number of tested species. Nevertheless, considering that microalgae act as primary producers and show sensitivity to the tested leachates, particularly PHBv, it is essential to acknowledge their pivotal role in marine trophic webs. Consequently, changes in microalgae populations can potentially exert significant impacts on marine ecosystems. This impact may extend further to zooplankton species, natural predators of microalgae, surpassing the direct effects of the polymers on zooplankton. Such dynamics could trigger a cascade response at subsequent trophic levels. Bacteria are also a relevant link in marine food webs. They are essential in transferring organic matter to higher trophic levels and contribute to nutrient cycling processes, gaining particular importance in oligotrophic areas. These processes could also be impacted by PHBv leaching. Recently, research efforts into understanding the impacts of plastic in the environment have started to consider the leaching of additives from plastic materials into the environment, which stems from the concern of potential adverse impacts on aquatic life and ecosystems (Capolupo et al., 2020; Gandara e Silva et al., 2016; Hermabessiere et al., 2017; Liu et al., 2020; Page et al., 2022). Studies reported by Beiras et al. (2021) and Harper et al. (2022) investigate the effect of different additives and plastic formulation, to enhance the environmental safety of plastics without compromising plastic product properties). This effort, assisted through ecotoxicological evaluations and the application of standardized leaching methods, holds paramount importance, not only for the environment but also for industry and its stakeholders. Furthermore, it is evident that certain PBP or bio-based options available in the market contain chemicals capable of inducing toxicity both in vivo and in vitro (Quade et al., 2022; Uribe-Echeverría and Beiras, 2022; Zimmermann et al., 2020), indicating that they might not yet meet the desired standards, although the environmental implications of these findings remain to be assessed. Considering this, the present study validates the negative impacts arising from the leaching of PHBv. We also propose a set of sensitive bioassays including species representative of different trophic levels and key taxonomic groups to assess a priori the ecotoxicity of plastic leachates on marine environments.

In conclusion, our study demonstrates unequivocally: 1) the deleterious effects caused by PHBv leachates to all tested species, with slight to no toxicity caused by PLA and PP leachates; 2) the presence of harmful chemicals in leachates, with the greater majority being present in PHBv. These findings emphasize the need for a priori risk assessment, particularly, of PHBv-based materials, and a stronger regulation and transparency on the use of chemical additives in plastics. Therefore, further studies focusing on a broader range of taxa and endpoints, and incorporating long-term exposure scenarios would provide a more comprehensive assessment of the ecological implications of the PBP in marine ecosystems.

Funding

This research was funded by the Ministry of Science and Innovation—Spanish Agency of Research through the RisBioPlas Project (AEI: 10.13039/501100011033) to RB, the MICROPLEACH project (PID2020-120479 GA-I00) to RA, the Joint Programming Initiative, Healthy and Productive Seas and Oceans, JPI Oceans-Spanish National Research funding Agency through the RESPONSE Project, (PCI2020-112110). S.L. I. was supported by the Ministry of Universities of Spain (FPU grant reference FPU19/02280). This study was also supported by the ULPGC-FPCT (subcontract DTU-ULPGC, C2020/65) and a Ramon y Cajal Program grant (RYC2018-025770-I) to RA. Universidade de Vigo/CISUG provides funding for open access charges.

CRediT authorship contribution statement

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174403.

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