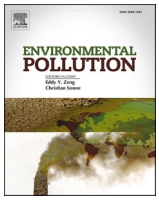
Environmental Pollution 347 (2024) 123717

Contents lists available at ScienceDirect

Environmental Pollution

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



Effects of primary leachates of conventional and alternative plastics in *Cyprinodon variegatus* fish larvae: Endocrine disruption and toxicological responses☆

Sandra Isabel Moreno Abril [a,b,*], Ana Olmos Pin [a,b], Ricardo Beiras [a,b]

[a] Marine Research Centre, University of Vigo (CIM-UVigo), 36310, Vigo, Galicia, Spain
[b] Department of Ecology and Animal Biology, University of Vigo, 36310, Vigo, Galicia, Spain

ARTICLE INFO

Keywords:
Resin
Biobased
Compostable
Bio-degradable
PLA
PBAT

ABSTRACT

The inclusion of hazardous substances in the formulation of plastics raises significant concerns, particularly, if those substances are released as primary leachates during plastic degradation and/or fragmentation. In this sense, the production of degradable plastics holding deleterious additives can increase the release of harmful substances into the environment. Additionally, the effects of primary leachates of "eco-friendly" materials remain unexplored. To address this, we performed exposures to primary leachates of alternative polymers, and commercial bags to verify possible responses associated with endocrine disruption and/or activation of the detoxification pathway in larvae of the marine fish model *Cyprinodon variegatus*.

The chemical characterization evidenced a great number of additives in the formulation of the materials analyzed in this study. Those include, except for the PLA sample, relevant levels of the hazardous phthalates DEHP and DiBP.

Regarding the effects on marine fish larvae, exposure to leachates from alternative polymers (10 g/L) PHB and PHBV produced remarkable mortality (100%). While the exposure to bag leachates of all tested materials (1 and 10 g/L) produced alterations in biomarkers for steroidogenic and detoxification pathways. To a lesser extent (10 g/L), three materials produced significant alterations in estrogenic biomarkers (Home-compostable bag 1, LDPE and Recycled PE bags). Although the alterations in gene expression were not directly correlated to the amount of DEHP or DiBP, we can conclude that primary leachates of "eco-friendly" bags are harmful to marine vertebrates.

1. Introduction

The problems associated with the broad use of plastics are becoming increasingly evident each day. With the aim to reduce the plastic pollution, numerous efforts in research and technology have been done in recent years. Due to the sensibilization of the consumers, the industry has rapidly turned to several alternatives to the conventional petro-based plastics loosely termed bioplastics and commercialized them as "eco-friendly". The market is now filled with products labeled as "bio-based", "compostable" or "bio-degradable" (Filiciotto and Rothenberg, 2021), which may pass a wrong idea to the consumer about their environmentally friendly characteristics and also the responsibility for the managent and impact of these plastics on the environment.

The most evident concern regarding plastic contamination is their persistence, consequently, the most suitable solution is the production of degradable plastics (Haider et al., 2019; Iwata, 2015). In order to transform the conventional non-biodegradable plastics into oxo- or hidro-degradable plastics (degraded by oxidation or hydrolysis, respectively), additives that induce time-controlled oxidation, can be added to alter their composition (Filiciotto and Rothenberg, 2021). On the other hand, bio-degradable alternatives have also been developed, which ideally mineralize into water, carbon dioxide, and biomass (Haider et al., 2019). Another category of alternative materials are the biobased plastics produced partially or totally from biomass, which are not necessarily biodegradable (Iwata, 2015).

The fragmentation and degradation of plastics in the environment produces micro and/or nano plastics (MNPs), which potentially release substances that could be toxic (Schiavo et al., 2020; Gunaalan et al.,

☆ This paper has been recommended for acceptance by Maria Cristina Fossi.

* Corresponding author. Department of Ecology and Animal Biology, University of Vigo, 36310, Vigo, Galicia, Spain.
E-mail address: pluieag@gmail.com (S.I. Moreno Abril).

https://doi.org/10.1016/j.envpol.2024.123717
Received 7 November 2023; Received in revised form 22 February 2024; Accepted 3 March 2024
Available online 4 March 2024

2020; Quade et al., 2022, 2023). Among the deleterious effects of MNPs in aquatic fauna, the more recognized ones are related to the obstruction caused by the uptake or absorption of plastic particles in the digestive and respiratory systems (Barría et al., 2020; Barrick et al., 2021). However, MNPs also can promote the transfer and mobilization of other contaminants through the absorption and adsorption on their surface, leading to the subsequent release of chemical additives by secondary leaching (Barrick et al., 2021; Kedzierski et al., 2018; Sridharan et al., 2022). Some studies have reported that additives with estrogenic endocrine disruptor activity are significant components of plastic leachates, among this Bisphenol A (BPA) and phthalates such as Bis (2-ethylhexyl) Phthalate (DEHP) and Diisobutyl Phthalate (DiBP) (Chen et al., 2019; Coffin et al., 2018; Kedzierski et al., 2018; Qiu et al., 2022) stand out.

For plastic production, independently of the origin or persistence (petro-based, bio-based, degradable, biodegradable, compostable, etc), functional additives and other substances are included in their formulations, to aid polymerization and processing or confer desired characteristics to the final material (Haider et al., 2019). Those substances can migrate and leach into the environment (primary leachates), depending on environmental factors such as temperature (Kedzierski et al., 2018), pH (Qiu et al., 2022), but also on the polymer-additive binding interactions (Gunaalan et al., 2020; Teuten et al., 2009; Barrick et al., 2021). The fragmentation and/or degradation process plays a pivotal role in releasing additives into the environment (Teuten et al., 2009), thereby aggravating the chemical contamination.

Additionally at the toxicological level, is fundamental to distinguish between the effects produced by the chemical composition of the plastic itself (primary leachate) or the effects generated by other contaminants adsorbed in the plastic surface (secondary leachates) (Delaeter et al., 2022). Recent studies have evidenced the adverse effects of plastic leachates in marine biota; in invertebrates the effects main addressed are mortality, cellular integrity and oxidative stress (Delaeter et al., 2022; Uribe-Echeverría and Beiras, 2022). *In-vivo* and *in-vitro* studies have evidenced in vertebrates effects related to endocrine disruption, cytotoxicity, and activation of the detoxificacion pathways (Chen et al., 2019; Coffin et al., 2018; Qiu et al., 2023). Given this, our experimental design focused on analyzing the effects of primary plastic leachates on biomarkers for endocrine disruption (estrogenic and steroidogenic) and also in a generalist biomarker for toxicity, in larvae of the marine fish model *Cyprinodon variegatus*. The acute responsiveness of these biomarkers (*vtgc, vtgab, zp2, zp3, and cyp19a2*) to the estrogenic stimulus in *C. variegatus* larvae has been recently demonstrated by Abril et al. (2022). The role of vitellogenins (VTGs) and zona pellucida (ZP) proteins in the egg formation, production and maintenance (Arukwe et al., 1997; Denslow et al., 1999); and aromatarse (CYP19A) in the autocrine production of estrogens (Abril et al., 2022) give an account of the physiological relevance of the studied biomarkers. Because of the lack of information regarding the toxicity of the resins compared to commercial plastics with chemical additives (Cormier et al., 2021), the first group of materials in this study addresses four resins: Polyhydroxybutyrate (PHB), Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) Polylactic acid (PLA) and Polybutylene adipate terephthalate (PBAT). Additionally, we tested primary leachates of commercial bags labeled as Home compostable, Industrial compostable, 100% degradable (made of polyethylene (PE)), recycled PE, and a traditional PE bag. This was done to investigate potential effects of "eco-friendly materials" on animal health. The chemical characterization of all the tested materials was made. Subsequently, the quantification was made of the more frequent leachable phthalates with endocrine disruptor activity DEHP, DiBP and tris(2-Ethylhexyl trimetilate) TOTM to verify possible links between the material composition and alterations in the physiological parameters (endocrine disruption and toxicity).

2. Materials and methods

2.1. Leachate preparation

The leachate preparation was done according to (Almeda et al., 2023). The materials (resins and commercial bags) were individually cut (except the PHB resin powder), and subsequently fractionated on a ZM200 ultracentrifuge mill (Retsch, Verder Scientific) with the aid of dry ice. After that, every material was sieved through a 250 μm metallic mesh, plastic particles of a diameter below 250 μm were used for the leaching preparation. (Alonso-López et al., 2021). The micronized material was stored (dry, darkness, 21 °C) until the preparation of the leachates.

To prepare the leachates (1 and 10 g/L), the sieved material was proportionally resuspend in 250 ml of seawater (28 ppm). Bottles were kept for 24 h at 1 rpm in an overhead rotator (GFL 3040) at 20 °C in darkness. Subsequently, the leachates were filtered with the aid of a vacuum pump, through glass fiber filters (Whatman, GF/F: 0.7 μm; 0.8 μm pore size) previously rinsed with 250 ml of distilled water, and 250 ml of diluted sea water. The sea water from filter rinsed was used to prepare the filtered control (FC) medium (Uribe-Echeverría and Beiras, 2022; Cormier et al., 2021).

2.2. Animal supply

C. variegatus 2 dph larvae were provided by the Toralla Marine Science Station (ECIMAT), an infrastructure of the Center for Marine Research of University of Vigo (CIM-UVigo). Maintenance and handling was done according to the plan approved by the Ethical Committee on the University of Vigo (REGA code: ES360570181401) and the method recommended by the international standard (ISO 7346–1:1996), considering the recommendations of the United States Environmental Protection Agency methods (US EPA 1004; 1005)

2.3. Exposure to leachates

Larvae of *C. variegatus* of 2 dph were exposed to 50 ml of leachate in glass flasks, in a density of 2 larve/10 ml, five replicates per treatment were done. No aeration or feed was supplied during exposure. Environmental conditions kept as photoperiod of 16:8 (light/dark, respectively), pH = 7.5, dissolved oxygen 6.5 mg/L, salinity 28 ppm and temperature 25 °C (measured at 0 and 24 h through multi-parameter sonde HACH HQ40D, pH: PHC10103; DO: LDO10103). For each material acute exposures (24 h) to leachates were done in isothermal rooms of ECIMAT; through individual experiments with different batches of larvae, over two years (from June 2021 to July 2023, Table 1).

To verify the larvae responsiveness to estrogenic substances according to Abril et al. (2022), solvent (Dimethyl sulfoxide (DMSO) < 0.005 % v/v. Scharlau) and positive (Ethinylestradiol (EE2) 100 ng/L Sigma-Aldrich, Switzerland) controls (SC and C+, respectively) were included in the exposures to: PHB, PBAT and the Stored

Table 1
Tested materials with the corresponding date of exposure.

			Expusure date
Tested materials	Resin	PHB	March 2022
		PHBv	May 2021
		PLA	February 2022
		PBAT	March 2022
	Bag	Industrial-compostable bag	June 2021
			June 2023
		Home-compostable bag 1	May 2021
		Home compostable bag 2	June 2021
		LDPE bag	May 2021
		100% degradable bag	June 2021
		Recycled PE bag	July 2021

Industrial-compostable bag (Table S3). In order to minimize the variability due to solvent, DMSO <0.005 % v/v was added to those experimental exposure medium. Each glass was sealed with parafilm to avoid water evaporation.

After 24 h of exposure, all the larvae were anesthetized and subsequently euthanized with tricaine methane sulfonate (MS-222; Sigma-Aldrich, GmhB) solutions of 0.1 and 0.2 g (respectively) in 100 ml of seawater. Every replicate was immediately stored in 205 μl of RNAlater (Thermo Fisher Scientific Baltic, Lithuania), according to manufacturer indications.

2.4. Gene expression analysis

All the procedures for gene expression analysis including whole RNA extraction (RNeasy® Plus mini kit Qiagen, Hilden, NRW, Germany), cDNA synthesis (RevertAid Reverse Transcriptase 200 U/μL), and qPCR (GoTaq® qPCR Master Mix enzyme Promega, Madison WI, USA, with a thermocycler standard program (initial denaturation 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s) in an Applied Biosystems QuantStudio 6 PRO system.) were done according to Abril et al. (2022). With the same set of primers being used for the estrogenic (*vtgc* Fw-TAGCCCTGACTCTGGCCTTT; Rv-GAAGGCCACCCAGGATTGAT, *vtgab* Fw-TTCCTCTTCACGCCGCAAAA; Rv-ACGTTGGCTAGAAGAGCTGC, *zp2* Fw-GATTGGGGGCTCCACAAAGA; Rv-TCAGTTGATTTTACGCACATCAAGA, *zp3* Fw-GATTGGGGGCTCCACAAAGA; Rv-TCAGTTGATTTTACGCACATCAAGA), steroid (*star* Fw-TTCCGCTCCAGCAGTTGAAT; Rv- CTTGGACGCTGAAAGGGGAT, *cyp19a2* Fw- GACCTTTCCTGTCCTACGGC; Rv-AGGACCTGCTGAAAATGATGGT), and detoxification pathways (*cyp1a1* Fw- TCTGAATGGCTACTTCATCCCC; Rv-CGAAGACGGGTCTTTCCACA) biomarkers, and also for the housekeeping genes (*tbp* Fw-AGAACCAGTGTGCGTCTCAA; Rv-GACCAAACAGCGAATGTCGG and *hprt* Fw-CCTTTTGCATCGTGTCACTTCA; Rv-TGCAGAACAGCTCAACTCAAAG).

2.5. Chemical characterization of plastic additives

The chemical characterization of plastic additives was done by the technical staff of the Center for Scientific-Technological Research Support (CACTI) of the University of Vigo. The preliminary no quantitative analysis (Table S2) and subsequent quantification of plasticizers (Table 2) in tested materials was done through Gas chromatography–mass spectrometry (GC-MS). The quantitative analysis was done by comparison with Proficiency Tested Materials: DEHP (Tokyo Chemical Industry Co, Japan), DiBP (Tokyo Chemical Industry Co, Japan), and TOTM (Tokyo Chemical Industry Co, Japan). 20 mg of each sample micronized (250 μm) were suspended on 1 ml of Dichloromethane (DCM) for extraction in an Ultrasonic bath for 30 min. After extraction, the samples were centrifuged for 10 min at 4 °C and 9500 rpm, and an aliquot was injected. The standardized parameters for the CG-MS were: injector temperature 300 °C, volume of injection 1 μL in Splitless, column HP-5MS (60 m × 0.25 mm; 0.25 μm), helium carrier gas at 1 mL/min. The temperature ramp was: 120 °C for 2 min increasing at 8 °C/min until 320 °C and holding for 10 min. The temperature for the transference line was 330 °C and the acquisition time was 37 min. The detection was made in SIM mode monitoring the main m/Z of each compound.

Table 2
Quantitative characterization of plastic additives DiBP, DEHP, and TOTM in the tested materials (mg/Kg).

Description	Diisobutyl phthalate DiBP	(mg/kg) Di (2-ethylhexyl) phthalate DEHP	Tris (2-ethylhexyl) trimellitate TOTM
PHB resin (ID_019_GLK_PHB_3000)	2.98	4.54	<0.60
PHBV resin (ID_021_GLK_PHBV_1000)	2.75	2.99	<0.59
PLA resin (ID_023_GLK_PLA)	<0.64	2.53	<0.55
PBAT (ID_068_RBP_ECOFLEX_1200)	1.2	2.12	<0.54
Industrial-compostable bag (ID_015_RBP_BagBeige)	0.78	5.72	<0.78
Home-compostable bag 1 (ID_016_RBP_BagGreen)	0.92	14.4	<0.80
Home compostable bag 2 (ID_045_RBP_BagMaterbi)	2.66	28.4	<1.39
LDPE bag (ID_017_RBP_BagWhite)	0.68	16.1	<0.80
100% degradable bag (ID_018_RBP_Bag100)	1.58	49.3	<0.80
Recycled PE bag (ID_046_RBP_BagRecycled)	2.38	40.3	<0.86

2.6. Statistical analysis

For gene expression the $2^{-\Delta Ct}$ analysis was developed according to Schmittgen and Livak (2008). Significant results ($P < 0.05$) were determined by Mann-Whitney paired tests, between the leachate exposed groups against the Filter control (FC) group (Graph Pad Prism 5). A fold change above 1.5 was taken as representative in treatments with statistical differences. The fold change is estimated as the mean values ratio of the Leached/FC for induction or −1/(Leached/FC) for inhibition (Schmittgen and Livak, 2008; Zhao et al., 2018). Fold change values are presented through Ballon plots (http://www.bioinformatics.com.cn/srplot), for the different concentrations of leachates tested (1 and 10 g/L, Fig. 1. A and B. respectively).

3. Results

The chemical characterization of the materials (Table 2), evidenced that all of them, including the resins, have quantifiable levels of DEHP and DiBP (except for the PLA resin DiBP <0.64 mg/kg) in their composition, being the DEHP levels significantly higher in bags. On other hand, the TOTM which is considered an alternative plasticizer could not be quantified in any of the analyzed materials. Remarkable differences in DEHP levels among materials were identified. First, all commercial products showed higher levels than the resins. Secondly, the recycled and the oxodegradable bags showed ca. 2-fold increased levels compared to conventional PE and home-compostable bags, with the Industrial-compostable brand showing the lowest concentrations.

3.1. Incubation conditions

Table S1 shows the incubation conditions and larval mortality data. Leachates of PHB and PHBV (10 g/L) generated 100 % of mortality in *C. variegatus* larvae after 24 h of exposure. The PHB resin leachate (10 g/L) also had a reduction of 2.4 mg/L O_2 after 24 h. Other leachates that also exhibit O_2 reductions (above 1 mg/L), but not associated mortality were: PLA resin (10 g/L), the Stored Industrial-compostable bag (1 and 10 g/L) and the Home-compostable bag 1 (10 g/L). It should be noted that even after filtering, both PHBV leachates presented particulate material floating and also was recoded a mortality (4 %) in the group exposed to PHBV 1 g/L.

3.2. Gene expression

3.2.1. Resins

The chemical quantification of phthalates (DiBP, DEHP, and TOTM, Table 2) evidenced that PHB and PHBV resins have quantifiable levels of DiBP and DEHP. Even so, at the gene expression level, the 1 g/L PHB leachate did not generate responses linked to endocrine disruption or activation of the detoxification pathways (Fig. 1).

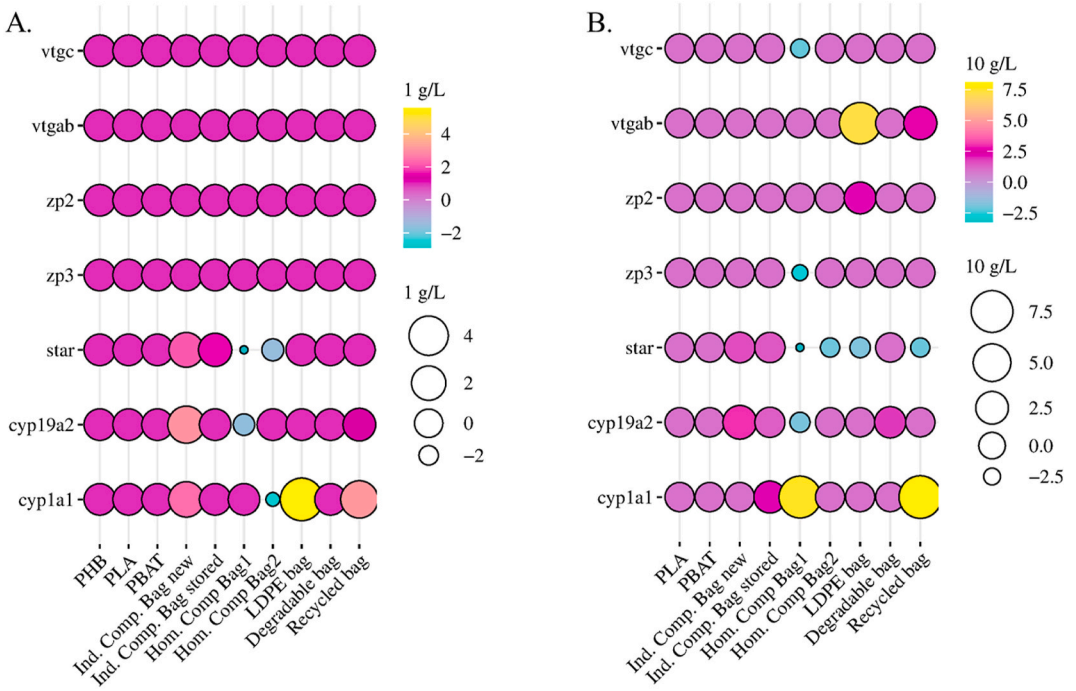


Fig. 1. Ballon plot presents the fold change values, estimated from the relative expression ($2^{-\Delta Ct}$) of biomarkers for endocrine disruption and detoxification in 2dph larvae of *Cyprinodon variegatus* exposed resins and plastic leachates A. 1 g/L, B. 10 g/L. Differences in color and circle size represent significant differences (fold change >1.5 or < -1.5) in the relative gene expression of tested biomarkers.

In the same way, PLA and PBAT resins also had quantifiable levels of phthalates in their constitution. DEHP could be quantified in both resins, whereas DiBP was found just in the PBAT resin. Even so, no significant alterations related to their estrogenic, steroid, or detoxification pathways were elicited in fish larvae exposed to PLA and PBAT resins (Fig. 1 A. and B.).

3.2.2. *Commercial bags*

The leachate obtained from the Industrial compostable bag shortly after purchase consistently induced the gene expression of steroidogenic biomarkers *star* (2.16 fold at 1 g/L and 1.62 fold at 10 g/L) and *cyp19a2* (3.08 fold at 1 g/L and 3.01 fold at 10 g/L) (Fig. 1 A. and B.). In the same way, at 1 g/L the response related to the activation of the detoxification pathway *cyp1a1* (2.42 fold) was induced (Fig. 1. A.).

In order to verify the reproducibility of the results, all the procedures were repeated in the same conditions with the Industrial compostable bag stored under "ideal" conditions (dry, darkness, 21 °C) for 21 months. A prominent reduction in the dissolved O_2 of both plastic leachates (1 and 10 g/L) obtained from these long-term stored bags was recorded (Table S1). However, the patterns of gene expression obtained were generally consistent with those recorded using brand-new bags (Fig. 1 A. and B.). The expression of *star* (1.6 fold at 1 g/L and 1.48 fold at 10 g/L) and *cyp19a2* (1.37 fold at 10 g/L) was again induced, although to a lesser extent than when using the brand new materials. On the other hand, the detoxification mechanism showed a strong differing response in the stored Industrial compostable bag. Here, an induction of *cyp1a1* (2,2 fold), at 10 g/L in contrast to a 1 g/L (2.42 fold) induction observed in the initial exposure.

At 1 g/l, the leachate obtained from the Home-compostable bag 1 inhibited the *star* (2.9 fold) and *cyp19a2* (1.62 fold) gene expression significantly (Fig. 1. A.). While at 10 g/L inhibited the gene expression of *vtgc* (2.15 fold), *zp3* (2.63 fold), *star* (3.22 fold), *cyp19a2* (1.86 fold), and induced prominently, the gene expression of *cyp1a1* (7.61 fold) (Fig. 1. B.).

The Home-compostable bag 2 leachate inhibited the mRNA gene expression of *star* at both tested concentrations (1.52 fold at 1 g/L and 2.03 fold at 10 g/L) (Fig. 1 A. and B). In the same way, the *cyp1a1* was significantly reduced at 1 g/L (2.66 fold).

At 1 g/L the larvae *C. variegatus* exposed to the leachate of Low-density polyethylene (LDPE) bag, did not exhibited any response linked to endocrine disruption, although they had a significant induction in the *cyp1a1* gene expression (5.59 fold) (Fig. 1. A.). Conversely, at 10 g/L there was quantified a significant induction of two biomarkers related to estrogenic activity *vtgab* (7.3 fold) and *zp2* (2.03 fold), joined by a significant inhibition (1.78 fold) of *star* (Fig. 1. B.). The chemical characterization of LDPE bag evidenced intermediate DEHP levels (16 mg/kg) and the lowest of DiBP (0.68 mg/kg), among all the analyzed bags (Table 2).

The 100% degradable PE bag was the one with the highest levels of DEHP in their composition (43,3 mg/kg, Table 2), surprisingly no alterations related to estrogenic endocrine disruption (EED) or detoxification pathways could be appreciated in fish larvae at any of the tested concentrations. Although a significant induction of *cyp19a2* (1.88 fold) was verified at 10 g/L.

The larvae exposed to Recycled PE bag at 10 g/L leachate (Fig. 1. B.), displayed a significant induction of *vtgab* (2.68 fold) joined with inhibition of *star* (2.05 fold). Both groups (1 and 10 g/L) exposed to Recycled PE bag leachate, had a significant induction of *cyp1a1* (3.19 fold 1 g/L and 8.12 fold 10 g/L, being the last one, the highest among all the exposures). The chemical characterization evidenced that his bag had 40.3 mg/kg of DEHP and 2.38 mg/kg DiBP in the composition, the second one with the higher levels of DEHP.

4. Discussion

Several studies have highlighted the importance of researching the effects of plastic leachates and identify their influence on animal reproduction (Kedzierski et al., 2018; Cormier et al., 2021; Delaeter et al., 2022; Gunaalan et al., 2020; Groh et al., 2019). Even so, characterize the effects and composition of plastic leachates is a hard work, due to the complexity of plastic formulations, the interactions among the constitutive substances, and also the factors influencing substances

leachability in the environment (Qiu et al., 2022; Gunaalan et al., 2020; Dhavamani et al., 2022).

Studies focused in the chemical characterization of plastic leachates have reported a high number of identifiable compounds (>240), but also a great amount of unidentified substances (Qiu et al., 2022). The qualitative (Table S2) and quantitative characterization (Table 2) of the materials in this study evidenced that hazardous substances such as DEHP, remain to be broadly used in plastic formulations, even to produce base polymers. The preliminary chemical characterization provided an insight into the substances to be addressed from a quantitative perspective, with well-known endocrine disruptors restricted in the EU such as DEHP and DiBP standing out as common across most tested materials. Other studies (Paluselli et al., 2019; Qiu et al., 2022), have remarked about the leachability of DEHP and DiBP in saltwater from different plastic devices.

4.1. Positive controls

Toxicity assessment in the early life stages of marine organisms is fundamental for understanding the effects of pollutants on the structure and functioning of marine ecosystems (Cormier et al., 2021) due to the importance of larvae for the establishment and success of the populations. The sensitivity of *C. variegatus* larvae to the estrogenic stimulus was verified previously by Abril et al. (2022); to validate this effect in our experiments, positive controls (C+ = EE2 100 ng/L) were incorporated in the PHB, PBAT and the Stored Industrial-compostable bag exposures. Our results (fold change inductions in the positive control group Table S3) are according to the ones reported by Abril et al. (2022) in which *vtgc*, *zps* and *cyp19a2* genes display significant inductions to the EE2 exposure, however, in our experiment *vtgab* was also significantly induced by EE2.

4.2. Leachates

Great efforts have been invested in research and innovation to propose solutions for plastic contamination. Even so, many "environmentally friendly" alternatives are commercialized without adequate prior testing, and in the end "*the cure is worse than the disease*" as evidenced by Quade et al. (2023) through sea urchin tests.

Due to the complexity in the formulation of plastics (resins, solvents, polymerization and processing aids, fillers, functional additives such as plasticizers, colorants, antioxidants, etc) (Barrick et al., 2021; Gunaalan et al., 2020; Qiu et al., 2022; Beiras et al., 2021), plastic contamination poses a huge challenge at ecotoxicological level. Furthermore, the leachability of plastic additives is influenced by chemical and environmental factors, including the ones related to the own nature of the plastic, such as the base polymer characteristics (polarity and porosity) (Barrick et al., 2021; Chen et al., 2019; Franzellitti et al., 2019), the additive-polymer chemical interactions, and the fragment size (Chen et al., 2019). Environmental factors such as UV irradiation (Chen et al., 2019), pH (Qiu et al., 2022), salinity (Gunaalan et al., 2020), and temperature (Kedzierski et al., 2018) also play an important role on plastic leachability (Dhavamani et al., 2022).

4.2.1. Resins

Beiras and coworkers (Alonso-López et al., 2021; Quade et al., 2022, 2023), postulated that the biopolymers themselves can induce additional toxicity compared to conventional PE. In this sense, our study aims to generate basic information about the biological effects of these alternative polymers and understand whether these effects are related with the levels of plastic additives in their composition (Delaeter et al., 2022). The chemical characterization evidenced quantifiable amounts of the restricted phthalates DEHP and DiBP (except for PLA resin) in the composition of all the commercial resins.

PHB and PHBV are polymers synthesized by microorganisms (Avella et al., 2000), the biodegradability of these polymers in marine water have been previously verified by López-Ibáñez & Beiras (2022). Because of the resistance to ultraviolet degradation and insolubility in water (González-Pleiter et al., 2019) PHB has been taken as a promising alternative to substitute synthetic plastics. Although the main objective of our study was to evaluate sublethal effects; PHB and PHBV polymers produced remarkable mortality at 10 g/L of both leachates and partial mortality (4 %) of larvae exposed to 1 g/L of PHBV resin. After filtering (0.8 μm filter) the presence of material in suspension was indicative of nano-particles in these leachates produced by the abiotic degradation of the resin. There is an inverse relationship between particles size and their toxicological risks (Beiras and Schönemann, 2020), where smaller particles can produce the mechanical obstruction of respiratory and digestive surfaces (Barrick et al., 2021). González-Pleiter et al. (2019), evidenced the toxicological effects (in planktonic organisms), related to the presence of nanoparticles (75–200 nm) by filtering and diluting PHB experimental medium. Other assays also have evidenced toxicological effects of PHB in invertebrates (Uribe-Echeverría and Beiras, 2022; Beiras et al., 2021).

PLA and PBAT are potentially biodegradable polymers, bio-based, and fossil-based, respectively (Iwata, 2015). Despite the quantified levels of DEHP in both resins, the larvae exposed to PLA or PBAT leachates did not exhibit any endocrine disruption or toxicity response at any tested concentration. Deleterious effects of PLA MPs have been reported in zebrafish behavior, related with the activity of Acetyl cholinesterase, and oxidative stress parameters (Chagas et al., 2021). However, those effects have been attributed to the interactions of the MPs particles with the cellular components rather than the chemical composition of PLA itself.

Aging plastic experiments have evidenced estrogen-like activities related to PBAT secondary leachates, with these effects attributed to the desorption of estrogenic contaminants adsorbed to the irregular surface of aged PBAT (Kedzierski et al., 2018).

4.2.2. Plastic bags

The second group of materials analyzed in this study is constituted by commercial plastic bags, including compostable, degradable, recycled and conventional brands. The leachability of additives from "eco-friendly" plastic materials remains poorly explored, and even less their effects related to endocrine disruption in marine vertebrates. Awuchi and Awuchi (2019) emphasized that endocrine disruption is a high health risk and a physiological effect produced by phthalates. The chemical characterization of the materials (Table S2) evidenced the presence of compounds previously reported with estrogenic effects such as the dibutyl phthalate (DBP) (Mu et al., 2018), the DEHP (Golshan et al., 2015; Moche et al., 2021; Mu et al., 2018) and the Phthalic Acid (Qiu et al., 2023). Although DiBP and DEHP are substances with concerning effects on reproduction, our results do not allow us to relate the effects in the estrogenic biomarkers with the quantified levels of these leachable phthalates. The PHB, PLA and PBAT resins all showed quantifiable levels of DEHP in their constitution. Even so, no significant alterations related to their estrogenic, steroid, or detoxification pathways were elicited in fish larvae exposed to those resins. Our study does evidence effects of primary leachates from commercial plastic bags on fish larvae. Significant alterations in four biomarkers for xenoestrogens (*vtga*, *vtgab*, *zp2*, and *zp3*) were produced by three of the six bags leachates, all of those at the concentration of 10 g/L. These changes include the induction of *vtgab* gene expression (LDPE and PE recycled bags) and *zp2* (LDPE bag). *vtg* and *zp* gene expression in *C. variegatus* larvae is rapidly (24 h) upregulated by the estrogenic stimulus (Abril et al., 2022). However these two bags that induced estrogenic biomarkers, showed in the analyses lower levels of DEHP than the 100% degradable bag, and similar to the Home-compostable 2 bag, and none of those two bags significantly induced any of the four xenoestrogen biomarkers here studied.

Similarly, among the materials with Phthalic Acid (PLA resin, Industrial compostable bag, Home compostable bag 2, Recycled PE bag)

the only response associated with an estrogenic effect was observed in the Recycled PE bag. Industrial organic chemicals such as those present in the materials tested are commonly manufactured as a mixture of isomers often termed technical mixtures. However, different isomers of the same molecule show different biological effects, since their affinity to molecular receptors may be very different. This is very well known for the differential endocrine disruption ability of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) isomers (Kelce et al., 1995) and nonylphenol isomers (De la Parra-Guerra and Acevedo-Barrios, 2023; Shioji et al., 2006). The unknown composition of the materials tested in the present study, and the isomerization changes that may take place during leaching and incubation may contribute to explain the variability of the biological responses here recorded.

Mechanistic studies focused on elucidating the regulation of the estrogenic process by different substances have concluded that whereas the magnitude of the induction of genes as *vtgs*, *chgs* or *ers* is linked to the affinity of the ligand; the estrogenic response of each gene/protein have a particular chronology (Kim et al., 2018), that depends on functional role of that molecule in the estrogenic process (Yost et al., 2014). Additionally, contaminants that mimic estrogens and/or mix of them will not exert the same affinity for linking domain, as the hormone. These facts reinforce the importance of explore more than one biomarker in ecotoxicological studies, and also explain the varied gene expression patterns that are not the same for all the biomarkers.

Transcriptomic studies have evidenced that contaminants identified as endocrine disruptors affect notably the lipidic metabolism (Kim et al., 2018; Schiller et al., 2013). Indeed, Franzellitti et al. (2019) have postulated that the effective concentrations of phthalates that generate molecular responses related to alterations in lipid homeostasis are lower than the ones required to induce estrogenic effects. Because of this, our study also includes two biomarkers to explore the effects in the steroidogenic pathway: *star*, and *cyp19a2*. StAR protein is the starting point for the production of steroid hormones by delivering cholesterol within the mitochondria (Stocco and Clark, 1996). On the other hand aromatase (CYP19A2) is the enzyme responsible for the local biosynthesis of estrogens in fish brain (Tchoudakova and Callard, 1998). Our results demonstrated that leachates of five of the six tested bags alter the *star* gene expression, even at concentrations of 1 g/L (New and stored Industrial compostable bag, and both home compostable bags). The Industrial compostable bag (new) leachate induced *star* and *cyp19a2* at both tested concentrations while the 100% degradable PE bag induced the gene expression of *cyp19a2* at 10 g/L. Conversely, 10 g/L leachates from both Home-compostable bags, the Recycled PE bag, and the conventional LDPE bag all down regulated the *star* gene expression, while in addition leachates form the Home compostable bag 1 inhibited the gene expression of *cyp19a2* at both tested concentrations. Previous studies in gold fish evidenced that "long term" exposures to DEHP (1 μg/L), have inhibited the gene expression of *star* and *cyp19a2*, and also have reduced sperm production and motility, without altering the vitellogenin production (Golshan et al., 2015). Although the regulatory process of *star* gene expression has not been completely elucidated, in the ovary of adult female largemouth bass, exposure to estrogenic substances produces the inhibition of the star mRNA (Prucha et al., 2020). Stocco and Clark (1996) postulated that the responses related to the availability of cholesterol are quickly regulated and argue that the main responsible for this process is the StAR protein. At this point it is necessary to highlight that *star* was the biomarker most responsive to the plastic leachates, and also alert about the possible effects of these by-products for the fish steroid metabolism.

The main mechanism for detoxification is initiated by the activity of the Cytochrome P450 (CYP) enzymes, with CYP1A1 standing out as a classic biomarker of exposure to aromatic organic pollutants in fish (Esteves et al., 2021; Stegeman, 1993). Our results evidenced that exposure to many of the tested leachates induced *cyp1A1* gene expression, but this induction did not show a response proportionally linked to the concentration tested. Surprisingly at 10 g/L, just three leachates (Home compostable, Stored industrial compostable and PE recycled bag), produced the induction of *cyp1a1*. At 1 g/L four of the six tested leachates produced alterations in the *cypa1a1* gene expression, being induced by the Industrial compostable (new), LDPE, and Recycled PE bags while inhibited by the Home compostable bag 2 leachate. Our results corroborate the ones reported by Qiu et al. (2023) in the medaka larvae (Oryzias melastigma) where exposures to plastic leachates alter the gene expression of *cyp1a*.

The 100 % degradable PE bag was the studied material with the highest levels of DEHP in the composition. DEHP is one of the most detected phthalates in seawater samples (Gunaalan et al., 2020) and also in leachates of virgin plastics (Coffin et al., 2018). No significant alterations in the gene expression of any of the studied biomarkers could be verified in larvae exposed to the leachate from the 100% Degradable PE bag, except for a slight induction of *cyp19a2* (1.88x).

Even if additives in plastic leachates can be under the analytic detection limits (Franzellitti et al., 2019), is necessary for future studies to increase the efforts for the chemical characterization of additives, in order to establish accurate cause-effect relationships.

There is little information regarding the effects generated by plastic leachates in marine vertebrates through standardized methods (Barrick et al., 2021; Qiu et al., 2023, 2022). In this sense, there is an urgency to produce standardized and comparative protocols to evaluate the effects of leachates, additives and MNPs in aquatic environments (Barrick et al., 2021; Franzellitti et al., 2019). Our experimental design seeks to produce comparative information related to primary leachates of materials that have been purposed as "environmentally friendly" alternatives using standardized protocols (Abril et al., 2022; Almeda et al., 2023). This approach aims to characterize the endocrine and toxicological implications of leachates in marine vertebrates.

5. Conclusions

The alternative plastic bags tested in this study showed restricted low-molecular weight phthalates and other toxic chemicals and thus they do not improve chemical safety compared to traditional LDPE bags. Most of these alternatives and their constitutive raw materials can leachate chemicals into seawater previously characterized as endocrine disruptors.

Because of the registered alterations in gene expression of genes responsive to xenoestrogens (*vtgc*, *vtgab*, *zp3*), alterations in steroidogenesis (*star* and *cyp19a2*) and indicative of xenobiotic biotransformation (*cyp1a1*); we can conclude that these alternatives are not less harmful for the marine fauna than the traditional LDPE bags.

The expression in 2-dph *C. variegatus* larvae of biomarker genes significantly altered upon exposure to leachates of several plastic materials is a rapid and sensitive molecular tool that may be part of an a priori scheme of assessment of plastic additives with similar functionality. This assessment would guide industry in the choice of the most innocuous materials with the lowest risk to the marine environment.

Funding

This work was funded by Spanish Government through Research Project RisBioPlas (PRDPID2019-108857RB-C32), and co-funded by the European Union through the European Regional Development Fund (ERDF), the GLAUKOS Project (this project has received funding from the Bio-based Industries Joint Undertaking (JU) under the European Union's Horizon 2020 research and innovation programme under grant agreement No. 887711. The JU receives support from the European Union's Horizon 2020 research and innovation programme and the Bio-based Industries Consortium).

CRediT authorship contribution statement

Sandra Isabel Moreno Abril: Writing – review & editing, Writing –

original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana Olmos Pin:** Methodology, Investigation. **Ricardo Beiras:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123717.

References

Abril, S.I.M., Pin, A.O., Schonemann, A.M., Bellot, M., Gómez-Canela, C., Beiras, R., 2022. Evaluating the alterations of the estrogen-responsive genes in Cyprinodon variegatus larvae for biomonitoring the impacts of estrogenic endocrine disruptors (EEDs). Environ. Toxicol. Pharmacol., 104042 https://doi.org/10.1016/j.etap.2022.104042.

Almeda, R., Gunaalan, K., Alonso-López, O., Vilas, A., Clérandeau, C., Loisel, T., Nielsen, T.G., Cachot, J., Beiras, R., 2023. A protocol for lixiviation of micronized plastics for aquatic toxicity testing. Chemosphere 333, 138894. https://doi.org/10.1016/j.chemosphere.2023.138894.

Alonso-López, O., López-Ibáñez, S., Beiras, R., 2021. Assessment of toxicity and biodegradability of poly(vinyl alcohol)-based materials in marine water. Polymers 13, 3742. https://doi.org/10.3390/polym13213742.

Arukwe, A., Knudsen, F.R., Goksøyr, A., 1997. Fish zona Radiata (Eggshell) protein: a sensitive biomarker for environmental estrogens. Environ. Health Perspect. 105, 418–422. https://doi.org/10.2307/3433339.

Avella, M., Martuscelli, E., Raimo, M., 2000. Review Properties of blends and composites based on poly(3-hydroxy)butyrate (PHB) and poly(3-hydroxybutyrate-hydroxyvalerate) (PHBV) copolymers. J. Mater. Sci. 35, 523–545. https://doi.org/10.1023/A:1004740522751.

Awuchi, Chinaza, Awuchi, Chibueze, 2019. Physiological effects of plastic Wastes on the endocrine system (Bisphenol A, phthalates, Bisphenol S, PBDEs. TBBPA) 4, 11–29.

Barría, C., Brandts, I., Tort, L., Oliveira, M., Teles, M., 2020. Effect of nanoplastics on fish health and performance: a review. Mar. Pollut. Bull. 151, 110791 https://doi.org/10.1016/j.marpolbul.2019.110791.

Barrick, A., Champeau, O., Chatel, A., Manier, N., Northcott, G., Tremblay, L.A., 2021. Plastic additives: challenges in ecotox hazard assessment. PeerJ 9, e11300. https://doi.org/10.7717/peerj.11300.

Beiras, R., Schönemann, A.M., 2020. Currently monitored microplastics pose negligible ecological risk to the global ocean. Sci. Rep. 10, 22281 https://doi.org/10.1038/s41598-020-79304-z.

Beiras, R., Verdejo, E., Campoy-López, P., Vidal-Liñán, L., 2021. Aquatic toxicity of chemically defined microplastics can be explained by functional additives. J. Hazard Mater. 406, 124338 https://doi.org/10.1016/j.jhazmat.2020.124338.

Chagas, T.Q., Freitas, Í.N., Montalvão, M.F., Nobrega, R.H., Machado, M.R.F., Charlie-Silva, I., Araújo, A.P. da C., Guimarães, A.T.B., Alvarez, T.G. da S., Malafaia, G., 2021. Multiple endpoints of polylactic acid biomicroplastic toxicity in adult zebrafish (Danio rerio). Chemosphere 277, 130279. https://doi.org/10.1016/j.chemosphere.2021.130279.

Chen, Q., Allgeier, A., Yin, D., Hollert, H., 2019. Leaching of endocrine disrupting chemicals from marine microplastics and mesoplastics under common life stress conditions. Environ. Int. 130, 104938 https://doi.org/10.1016/j.envint.2019.104938.

Coffin, S., Dudley, S., Taylor, A., Wolf, D., Wang, J., Lee, I., Schlenk, D., 2018. Comparisons of analytical chemistry and biological activities of extracts from North Pacific gyre plastics with UV-treated and untreated plastics using in vitro and in vivo models. Environ. Int. 121, 942–954. https://doi.org/10.1016/j.envint.2018.10.012.

Cormier, B., Gambardella, C., Tato, T., Perdriat, Q., Costa, E., Veclin, C., Le Bihanic, F., Grassl, B., Dubocq, F., Kärrman, A., Van Arkel, K., Lemoine, S., Lagarde, F., Morin, B., Garaventa, F., Faimali, M., Cousin, X., Bégout, M.-L., Beiras, R., Cachot, J., 2021. Chemicals sorbed to environmental microplastics are toxic to early life stages of aquatic organisms. Ecotoxicol. Environ. Saf. 208, 111665 https://doi.org/10.1016/j.ecoenv.2020.111665.

De la Parra-Guerra, A.C., Acevedo-Barrios, R., 2023. Studies of endocrine disruptors: nonylphenol and isomers in biological models. Environ. Toxicol. Chem. 42, 1439–1450. https://doi.org/10.1002/etc.5633.

Delaeter, C., Spilmont, N., Bouchet, V.M.P., Seuront, L., 2022. Plastic leachates: Bridging the gap between a conspicuous pollution and its pernicious effects on marine life. Sci. Total Environ. 826, 154091 https://doi.org/10.1016/j.scitotenv.2022.154091.

Denslow, N.D., Chow, M.C., Kroll, K.J., Green, L., 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8, 385–398. https://doi.org/10.1023/A:1008986522208.

Dhavamani, J., Beck, A.J., Gledhill, M., El-Shahawi, M.S., Kadi, M.W., Ismail, I.M.I., Achterberg, E.P., 2022. The effects of salinity, temperature, and UV irradiation on leaching and adsorption of phthalate esters from polyethylene in seawater. Sci. Total Environ. 838, 155461 https://doi.org/10.1016/j.scitotenv.2022.155461.

Esteves, F., Rueff, J., Kranendonk, M., 2021. The central role of Cytochrome P450 in xenobiotic metabolism—a Brief review on a Fascinating enzyme Family. J. Xenobiotics 11, 94–114. https://doi.org/10.3390/jox11030007.

Filiciotto, L., Rothenberg, G., 2021. Biodegradable plastics: Standards, Policies, and impacts. ChemSusChem 14, 56–72. https://doi.org/10.1002/cssc.202002044.

Franzellitti, S., Canesi, L., Auguste, M., Wathsala, R.H.G.R., Fabbri, E., 2019. Microplastic exposure and effects in aquatic organisms: a physiological perspective. Environ. Toxicol. Pharmacol. 68, 37–51. https://doi.org/10.1016/j.etap.2019.03.009.

Golshan, M., Hatef, A., Socha, M., Milla, S., Butts, I.A.E., Carnevali, O., Rodina, M., Sokołowska-Mikołajczyk, M., Fontaine, P., Linhart, O., Alavi, S.M.H., 2015. Di-(2-ethylhexyl)-phthalate disrupts pituitary and testicular hormonal functions to reduce sperm quality in mature goldfish. Aquat. Toxicol. 163, 16–26. https://doi.org/10.1016/j.aquatox.2015.03.017.

González-Pleiter, M., Tamayo-Belda, M., Pulido-Reyes, G., Amariei, G., Leganés, F., Rosal, R., Fernández-Piñas, F., 2019. Secondary nanoplastics released from a biodegradable microplastic severely impact freshwater environments. Environ. Sci.: Nano 6, 1382–1392. https://doi.org/10.1039/C8EN01427B.

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

Gunaalan, K., Fabbri, E., Capolupo, M., 2020. The hidden threat of plastic leachates: a critical review on their impacts on aquatic organisms. Water Res. 184, 116170 https://doi.org/10.1016/j.watres.2020.116170.

Haider, T.P., Völker, C., Kramm, J., Landfester, K., Wurm, F.R., 2019. Plastics of the future? The impact of biodegradable polymers on the environment and on Society. Angew. Chem. Int. Ed. 58, 50–62. https://doi.org/10.1002/anie.201805766.

ISO 7346-1:1996, n.d. ISO 7346-1:1996 [WWW Document]. EVS. URL https://www.evs.ee/en/iso-7346-1-1996 (accessed October.17.23).

Iwata, T., 2015. Biodegradable and bio-based polymers: future Prospects of eco-friendly plastics. Angew. Chem. Int. Ed. 54, 3210–3215. https://doi.org/10.1002/anie.201410770.

Kedzierski, M., D'Almeida, M., Magueresse, A., Le Grand, A., Duval, H., César, G., Sire, O., Bruzaud, S., Le Tilly, V., 2018. Threat of plastic ageing in marine environment. Adsorption/desorption of micropollutants. Mar. Pollut. Bull. 127, 684–694. https://doi.org/10.1016/j.marpolbul.2017.12.059.

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen, J.A., Wilson, E.M., 1995. Persistent DDT metabolite p,p'–DDE is a potent androgen receptor antagonist. Nature 375, 581–585. https://doi.org/10.1038/375581a0.

Kim, B.-M., Jo, Y.J., Lee, Nayun, Lee, Nayoung, Woo, S., Rhee, J.-S., Yum, S., 2018. Bisphenol A induces a Distinct Transcriptome Profile in the Male fish of the marine medaka Oryzias javanicus. BioChip J 12, 25–37. https://doi.org/10.1007/s13206-017-2104-0.

López-Ibáñez, S., Beiras, R., 2022. Is a compostable plastic biodegradable in the sea? A rapid standard protocol to test mineralization in marine conditions. Sci. Total Environ. 831, 154860 https://doi.org/10.1016/j.scitotenv.2022.154860.

Moche, H., Chentouf, A., Neves, S., Corpart, J.-M., Nesslany, F., 2021. Comparison of *in vitro* endocrine activity of phthalates and alternative plasticizers. J. Toxicol. 2021, e8815202 https://doi.org/10.1155/2021/8815202.

Mu, X., Huang, Y., Li, J., Yang, K., Yang, W., Shen, G., Li, Xuxing, Lei, Y., Pang, S., Wang, C., Li, Xuefeng, Li, Y., 2018. New insights into the mechanism of phthalate-induced developmental effects. Environ. Pollut. 241, 674–683. https://doi.org/10.1016/j.envpol.2018.05.095.

Paluselli, A., Fauvelle, V., Galgani, F., Sempéré, R., 2019. Phthalate release from plastic fragments and degradation in seawater. Environ. Sci. Technol. 53, 166–175. https://doi.org/10.1021/acs.est.8b05083.

Prucha, M.S., Martyniuk, C.J., Doperalski, N.J., Kroll, K.J., Barber, D.S., Denslow, N.D., 2020. Steroidogenic acute regulatory protein transcription is regulated by estrogen receptor signaling in largemouth bass ovary. Gen. Comp. Endocrinol. 286, 113300 https://doi.org/10.1016/j.ygcen.2019.113300.

Qiu, S.-Q., Huang, G.-Y., Fang, G.-Z., Li, X.-P., Lei, D.-Q., Shi, W.-J., Xie, L., Ying, G.-G., 2022. Chemical characteristics and toxicological effects of leachates from plastics under simulated seawater and fish digest. Water Res. 209, 117892 https://doi.org/10.1016/j.watres.2021.117892.

Qiu, S.-Q., Huang, G.-Y., Li, X.-P., Lei, D.-Q., Wang, C.-S., Ying, G.-G., 2023. A comparative study on endocrine disrupting effects of leachates from virgin and aged plastics under simulated media in marine medaka larvae (Oryzias melastigma). J. Hazard Mater. 446, 130700 https://doi.org/10.1016/j.jhazmat.2022.130700.

Quade, J., López-Ibáñez, S., Beiras, R., 2023. UV Dosage Unveils toxic Properties of Weathered commercial bioplastic bags. Environ. Sci. Technol. 57, 14807–14816. https://doi.org/10.1021/acs.est.3c02193.

Quade, J., López-Ibáñez, S., Beiras, R., 2022. Mesocosm trials reveal the potential toxic risk of degrading bioplastics to marine life. Mar. Pollut. Bull. 179, 113673 https://doi.org/10.1016/j.marpolbul.2022.113673.

Schiavo, S., Oliviero, M., Chiavarini, S., Manzo, S., 2020. Adverse effects of oxo-degradable plastic leachates in freshwater environment. Environ. Sci. Pollut. Res. 27, 8586–8595. https://doi.org/10.1007/s11356-019-07466-z.

Schiller, V., Wichmann, A., Kriehuber, R., Schäfers, C., Fischer, R., Fenske, M., 2013. Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption. Reprod. Toxicol. Elmsford N 42, 210–223. https://doi.org/10.1016/j.reprotox.2013.09.003.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Shioji, H., Tsunoi, S., Kobayashi, Y., Shigemori, T., Ike, M., Fujita, M., Miyaji, Y., Tanaka, M., 2006. Estrogenic activity of Branched 4-nonylphenol isomers Examined by Yeast two-Hybrid assay. J. Health Sci. 52, 132–141. https://doi.org/10.1248/jhs.52.132.

Sridharan, S., Kumar, M., Saha, M., Kirkham, M.B., Singh, L., Bolan, N.S., 2022. The polymers and their additives in particulate plastics: what makes them hazardous to the fauna? Sci. Total Environ. 824, 153828 https://doi.org/10.1016/j.scitotenv.2022.153828.

Stegeman, J.J., 1993. Cytochrome P450 forms in fish. In: Schenkman, J.B., Greim, H. (Eds.), Cytochrome P450, Handbook of Experimental Pharmacology. Springer, Berlin, Heidelberg, pp. 279–291. https://doi.org/10.1007/978-3-642-77763-9_18.

Stocco, D.M., Clark, B.J., 1996. Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. Biochem. Pharmacol. 51, 197–205. https://doi.org/10.1016/0006-2952(95)02093-4.

Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Björn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkhavong, K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., Takada, H., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2027–2045. https://doi.org/10.1098/rstb.2008.0284.

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

US EPA, 2002. Method 1004.0: Sheepshead Minnow, Cyprinodon variegatus, larval Survival and Growth test. Chronic Toxicity.

US EPA, n.d. Method 1005.0: Sheepshead Minnow, Cyprinodon variegatus, Embryo-Larval Survival and Teratogenicity Test; Chronic Toxicity.

Yost, E.E., Lee Pow, C., Hawkins, M.B., Kullman, S.W., 2014. Bridging the gap from Screening assays to estrogenic effects in fish: potential roles of Multiple estrogen receptor Subtypes. Environ. Sci. Technol. 48, 5211–5219. https://doi.org/10.1021/es404093n.

Zhao, B., Erwin, A., Xue, B., 2018. How many differentially expressed genes: a perspective from the comparison of genotypic and phenotypic distances. Genomics 110, 67–73. https://doi.org/10.1016/j.ygeno.2017.08.007.