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Additives in polypropylene and polylactic acid food packaging: Chemical analysis and bioassays provide complementary tools for risk assessment

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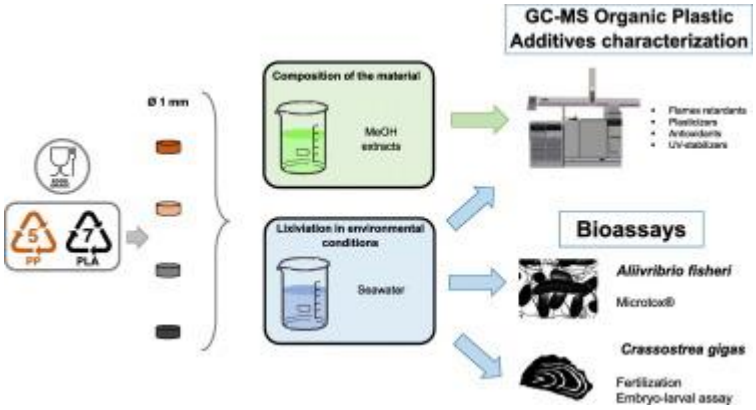
Abstract :

Plastic food packaging represents 40 % of the plastic production worldwide and belongs to the 10 most commonly found items in aquatic environments. They are characterized by high additives contents with >4000 formulations available on the market. Thus they can release their constitutive chemicals (i.e. additives) into the surrounding environment, contributing to chemical pollution in aquatic systems and to contamination of marine organism up to the point of questioning the health of the consumer. In this context, the chemical and toxicological profiles of two types of polypropylene (PP) and polylactic acid (PLA) food packaging were investigated, using in vitro bioassays and target gas chromatography mass spectrometry analyses. Plastic additives quantification was performed both on the raw materials, and on the material leachates after 5 days of lixiviation in filtered natural seawater. The results showed that all samples (raw materials and leachates) contained additive compounds (e.g. phthalates plasticizers, phosphorous flame retardants, antioxidants and UV-stabilizers). Differences in the number and concentration of additives between polymers and suppliers were also pointed out, indicating that the chemical signature cannot be generalized to a polymer and is rather product dependent. Nevertheless, no significant toxic effects was observed upon exposure to the leachates in two short-term bioassays targeting baseline toxicity (Microtox® test) and Pacific oyster Crassostrea gigas fertilization success and embryo-larval development. Overall, this study demonstrates that both petrochemical and bio-based food containers contain harmful additives and that it is not possible to predict material toxicity solely based on chemical analysis. Additionally, it highlights the complexity to assess and comprehend the additive content of plastic packaging due to the variability of their composition, suggesting that more transparency in polymer formulations is required to properly address the risk associated with such materials during their use and end of life.

Graphical abstract



Highlights

► Petro- and bio-based plastics packaging contains organic plastic additives ► Leaching of organic plastic additives was observed with all the tested protocols ► Differences in chemical signature between polymers and suppliers were observed ► Leachates from PP and PLA food containers did not show any in vitro toxicity

Keywords : Food containers, Plastic, Additives, Leachates, Bioassays

1. Introduction

Plastic debris is the major fraction of solid waste pollution in the marine environment. It is estimated that 75% of all marine litter is plastic (Napper and Thompson, 2020). Among this plastic pollution, approximately 50% of the items are food packaging materials (de Kock et al., 2020; Gerigny et al., 2018; OSPAR et al., 2010), of which polypropylene (PP) is one of the most employed resin. Its production accounts for 80-90% of the global plastic demand (PlasticEurope, 2021; Zimmermann et al., 2019), along with expanded polystyrenes (EPS) and polyethylene terephtalate (PET) (Iñiguez et al., 2016; Zhou et al., 2011). More than 4000 chemicals are known to be in the composition of plastic packaging (Groh et al., 2019), including additives which are intentionally used to improve the properties of the material. A few review papers have highlighted the most common groups of additives in plastics, such as plasticizers, flames retardants, antioxidants and stabilizers (Fred-Ahmadu et al., 2020; Hahladakis et al., 2018). Many of the additives (e.g. bisphenols, phthalates, nonylphenols) are known to be hazardous, even at low concentrations, posing a risk for marine organisms (Hahladakis et al., 2018; Oehlmann et al., 2009) as a main driver of plastic toxicity (Beiras et al., 2021). The majority of plastic additives are not chemically bound to the plastic polymers and have the potential to leach out from the plastic to the surrounding environment (Andrady, 2011; Koelmans et al., 2014), causing various types of damages to organisms (e.g., embryo development, immobility, physical activity, mortality, endocrine disruption, gene mutation). The ecotoxicity of some plastic leachates has been characterized on diverse aquatic organisms such as copepods, barnacles, oysters, mussels, urchins, lugworms, fish and photosynthetic bacteria (Gardon et al., 2020; Hamlin et al., 2015; Huang et al., 2021; Oliviero et al., 2019; Tallec et al., 2022; Tetu et al., 2019), without, however, identifying the compound(s) responsible for the observed toxicity. Just one single study (Tian et al., 2021) managed this demonstrating the link between the high concentration of a rubber additive subproduct and acute toxicity events in coho salmon.

The use of petro-based food packaging being controversial, bio-based plastics are more and more promoted as an alternative to conventional plastics, with a production volume which is expected to increase in the future (EuropeanBioplastics, 2021; Geueke et al., 2014). The polylactic acid (PLA) is nowadays the most produced bio-based plastic, especially for food container (FC) products (Ncube et al., 2020). Either way, whether the materials are derived from a natural or petrochemical resource, they are both produced to fulfill the same function and are therefore similarly formulated. There are now some studies that gave first indications of the toxicity of bio-based and biodegradable products and that also demonstrated that bio-based materials are not necessarily safer than conventional plastics (Chagas et al., 2021a; Chagas et al., 2021b; de Oliveira et al., 2021; Klein et al., 2021; Lambert and Wagner, 2017; Malafaia et al., 2021; Zimmermann et al., 2020a).

Moreover, concerns have arisen concerning the safety of FCs (Groh and Muncke, 2017) especially in regards to the migration of a wide variety of chemicals for which there is a lack of hazard information (Muncke, 2011). Therefore, it is important to explore the threats of complex and diverse chemical mixtures emitted by plastic products. However, although non-target screening analyses have previously been applied on plastics leachates, most of the chemicals remained unidentified (Muncke, 2011; Zimmermann et al., 2021). As a result, a better and more specified understanding of the composition of plastics is required to relate their chemical content and their toxicity.

The migration of compounds that are allowed to be included in FCs are only tested in regards to food contact application. However, plastic FCs are widely found in the environment and are therefore present as microplastics (MPs). Hence, it's important to question the impacts of these particles once in the environment. Thus, this study aims to assess and compare the chemical additive contents and the ecotoxicity of the chemicals leaching from plastic marketed FCs made of PP and PLA. Target chemical analyses were carried out by a stir bar sorptive extraction (SBSE) followed by a thermal desorption gas chromatography–mass spectrometry in tandem (TD-GC-MS/MS) to characterize chemicals

present in the plastics and the ones released in sea water leachates. In addition, we also assessed the leachates ecotoxicity through sensitive short-term bioassays: (i) the base line toxicity with the Microtox® test on the bioluminescent *Aliivibrio fischeri* bacteria, chosen as a rapid assay which is reproducible, cost effective and more sensitive than other end points for nonspecific toxicity (Neale et al., 2012), and (ii), two bivalve sensitive endpoints (His et al., 1999), fertilization and embryo-larval developmental success of the Pacific oyster *Crassostrea gigas*. This specie was targeted as a key organism for coastal ecosystems and because of its ecological and economical roles (FAO, 2020).

2. Materials and methods

2.1. Plastic sample selection and production

In this study, four samples of food packaging items, available on the French market, made of polypropylene (PP) and polylactic acid (PLA) were used. Two items were selected per polymer resin, produced by two different suppliers, tagged A and B.

Small punches, i.e. cylinders with a diameter of 1 mm, were cut into the food packaging items using biopsy punches from Kai-la-Medical (Antwerpen, Belgium). Homogeneity of punches was assessed by measuring the thickness and the volume of n=20 punches per polymer type using an Olympus SZX16 stereomicroscope (France) equipped with a UC90 camera and treated using OLYMPYS CellSens Dimension 3.2 software. Data are presented as a mean value ± standard deviation (s.d).

2.2. Extraction of potential additives

To avoid sample contamination, all glassware was burnt for 6h at 500°C in a Nabertherm LT40/11/B410 muffle furnace (Lilienthal, Germany) prior to the experiments. Additionally, the preparation of the material's leachates was conducted under a laminar flow hood.

2.2.1. Extraction of additives from the punch surface (methanol extracts)

200 ± 0.51 mg of plastic punches were weighed for each plastic (n=4). 10 mL of methanol (MeOH) (Sigma-Aldrich Co., Saint-Quentin-Fallavier, France) were added to the punches in order to obtain a concentration of 20 $mg.mL^{-1}$. The mixtures were placed on an orbital shaker at 100 rpm for 24h, in dark conditions at room temperature (20 °C). A control experiment, i.e. MeOH treatment without any punch was also carried out following the same conditions. At the end of the extractions, the plastics were removed from the MeOH and the extracts were transferred into clean glass bottles and immediately submitted to chemical analysis (part 2.3).

2.2.2. Preparation of leachates in seawater

Leachates were prepared adding 2g of FC punch samples into 1L of filtered natural seawater (FSW) for each product. The FSW used for the leachate preparation was collected in Hardelot (France) autoclaved and filtered on Whatman™ 0.22 µm Millipore filters (Maidstone, United Kingdom). Each leachate was placed on an orbital shaker at 100 rpm, allowing the plastic pieces to move freely in the water. Leaching was performed for 24h and 5 days in dark conditions at room temperature (20°C) in order to be in accordance with previous studies mentioning a fast release of organic plastic additives (OPAs) within the first 120h (Gardon et al., 2020; Jang et al., 2021; Paluselli et al., 2019). Nine leaching treatments were prepared: PLA-A (n=2), PLA-B (n=2), PP-A (n=2) and PP-B (n=2) samples at 24h and 5 days, and a control seawater treatment without plastic. At the end of the leaching period, leachates were filtered through Whatman™ 1.6 µm GF/A filters (to remove punches), transferred to clean bottles and used as a stock solution for preparation of the six leachates concentrations levels obtained by serial dilutions: 0.02, 0.2, 2, 20, 200 and 2000 $mg.L^{-1}$. The middle range concentration 0,2 and 2 $mg.L^{-1}$ were chosen to be in similar range as to MPs concentration found in the marine environment, respectively medium and worst case scenario (Paul-Pont et al., 2018). All the leachate solutions were conserved at -20 °C during

one week prior to the chemical characterization and the toxicity assays. For each treatment, 100 mL of the initial leachate (2000 $mg.L^{-1}$) were sampled for chemical analysis.

2.3. Target chemical analyses

The OPAs were quantified in the methanolic extracts (cf. 2.2.1) and in the FSW leachates (cf. 2.2.2) in duplicates by SBSE-TD-GC-MS/MS following the methodology described by Lacroix et al. (2014).

Regarding the MeOH extracts, 1mL of the samples solutions were transferred to a clean glass bottle and supplemented by 9 mL of MeOH and 100 mL of FSW. For the seawater leachates, an aliquot of 100 mL was transferred to a clean glass bottle and 10 mL of MeOH was added. For both MeOH extract and seawater leachate, the prepared samples were doped with 10 ng of each deuterated standards, i.e. deuterated phthalates, deuterated polybromodiphényléters (PBDEs), deuterated polycyclic aromatic hydrocarbons (PAHs) and deuterated nonylphenol (NPd8).

Gerstel Twister, 20 mm x 0.5 mm polydimethylsiloxane stir bar, (Mülheim an der Ruhr, Germany) were then placed in each sample on a MIX15 magnetic stirrer (Munich, Germany) and stirred at 700 rpm for 16 h of extraction in the dark at room temperature (20 °C). At the end of the extraction time, stir bars were removed from the solutions, rinsed with distilled water, dried over a blot paper, and directly analyzed by TD-GC-MS/MS.

OPAs were analyzed using an Agilent 7890A gas chromatography system coupled to an Agilent 7000 triple quadrupole mass spectrometer (Little Falls, USA). GC-MS/MS device was equipped with a Gerstel thermal desorption unit (TDU) and a MultiPurpose Sampler in order to automatically introduce stir bars into the system. Thermodesorption were carried out at 280 °C for 6 min and samples were then cryofocused at -10 °C via a Gerstel cooled injection system (CIS). Analytes were injected in splitless mode into an Agilent HP-5MS GC column (Agilent Technologies) (30 m x 0.25 mm x 0.25 µm) and the CIS was heated to 310 °C at 12 $°C.s^{-1}$. The detailed analytical condition of the GC temperature program and the MS are

presented in Table S1. A stir bar conditioning was performed on each bar prior to re-use in order to eliminate any compounds not completely desorbed.

In total, 57 OPA's, i.e. 18 plasticizers, 19 flames retardants, 5 antioxidants and 15 stabilizers, were targeted and quantified (Table S2) based on criteria of use, toxicity, concentration in plastics and feasibility of GC-MS analysis. The quantitative analysis of plastic additives was performed by external calibration using a multiple reaction monitoring (MRM) method divided into 4 groups containing a maximum of 20 transitions (Table S3) with two transitions for each compound. Several levels of calibration (i.e. 0, 0.5, 1, 5, 10, 50, 100, 500 $ng.L^{-1}$), in duplicate, were prepared. Data analysis was performed using MassHunter software from Agilent (10.2.733.8). Analytes were quantified by calculating the target additive/deuterated analyte ratio, and corrected by subtracting the blank (i.e. MeOH or FSW control without FC materials).

2.4. Toxicity assessment on a unicellular organism - Microtox® assay

The Microtox® assay is an acute test measuring the baseline toxicity of a substance based on the decrease or inhibition of the bioluminescence of the bacteria *Aliivibrio fischeri* (Wadhia and Thompson, 2007). Here, this acute test was performed on each FSW leachate at the highest concentration (i.e. 2000 $mg.L^{-1}$) and a control treatment (FSW with no addition of plastic), if no effect was observed, the lowest concentrations were not tested.

The bioluminescence level was measured by Modern Water Ltd Microtox® FX Analyser (New Castle, DE, USA), following the B-Tox Test procedure of the manufacturer's manual. Briefly, the lyophilized *A.fisheri* were rehydrated with 300 µL of the reconstituted solution (RS), the bacteria and the RS were gently mixed with a micropipette and 100 µL were immediately transferred into a clean test glass vial. After 15 min of exposure, 900 µL of working solutions, i.e. either FSW control or leachates, were added into the test vials. Measurements of the luminescence were recorded prior and 15 min after sample addition. The bioluminescence results were automatically compared and corrected with the light

output of the control sample, resulting in a relative luminescence inhibition (%). Each assay was performed in triplicate.

2.5. Toxicity assessment on an eukaryote organism - Oysters

2.5.1. Biological material (animal and gamete collection)

Mature Pacific oysters were produced as described in Petton et al. (2015) and held in the Ifremer nursery in Bouin (France). In January 2022, a stock of 120 oysters (36 month old, average weight: 47.6 ± 7.2 g) was transferred from the Ifremer nursery to the Ifremer experimental facilities in Argenton (France) at stage 0 (i.e. the undifferentiated stage) and conditioned for 6 weeks with suitable conditions for germ cell maturation. Briefly, oysters were placed in an experimental raceway, using a flow-through system with 20 µm-filtered running seawater at 18 ± 1.0 °C and fed with a mixed diet of two microalgae at a daily ration equal to 8% dry mass algae/ dry mass oyster. At ripeness (stage 3), oysters were randomly sampled to perform gametes and embryo-larval assays. Oyster sex was determined under an EVOS™ XL Core Imaging System microscope (ThermoFisher Scientific Waltham, Massachusetts, USA), × 10–20 magnification, on a 50-µL subsample from the gonad of each individual. Gametes from 3 males and 3 females were collected by stripping the gonad as described by Steele and Mulcahy (1999). This step was repeated in four replicates, with a total of 12 males and 12 females per condition. Sperm and oocytes solutions were then sieved at 60 µm in order to eliminate debris. Spermatozoa and oocytes were diluted with respectively 100 mL and 1 L of FSW at 20 °C, and left for 1 h prior to use to ensure gamete quality, i.e. spermatozoon mobility and round shape of oocytes, which were checked by microscopy (Tallec et al., 2018).

2.5.2. Fertilization success assay

After collecting the gametes, their concentrations were assessed by flow cytometry using a EasyCyte Plus cytometer from Guava Merck Millipore (Burlington, Massachusetts, USA). Gametes were placed at the same time in glass vials with a spermatozoa-to-oocyte ratio of

100:1 and a final concentration of 1,000 oocytes.mL^{-1}. Vials were filled with the different solutions of leachate to a final volume of 5 mL (4 leachates from FC, 6 concentrations: 0 (control FSW), 0.02, 0.2, 2, 20 and 200 mg.L^{-1}, and 4 replicates per condition, leading to 96 vials). After 1.5 h of exposition to FC leachates, samples were fixed with a formaldehyde-seawater solution (0.1% final) to estimate the fertilization yield under a Zeiss Axio Observer Z1 microscope with ×10-40 magnification. Per vial, 100 oocytes were observed. An oocyte was considered fertilized when polar bodies or cell divisions were observable. The fertilization yield (%) was estimated as: number of fertilized oocytes / total of oocytes × 100. (Martinez-Gomez et al., 2017).

2.5.3. Embryo-larval assay

The standardized ISO 17244:2015 assay (ISO, 2015) was used to determine the embryo-toxicity of FC leachates. Fertilization was carried out following the procedure described above, in 4 replicates, with gametes collected from 3 males and 3 females per replicates (total: 12 males and 12 females) in 2-L glass beakers filled with 1.5 L of FSW. Once fertilization was achieved with high fertilization yields (>90%) and embryos were at the 2-cell stage (verified using a Zeiss Axio Observer Z1; ×10-40 magnification), embryos were collected and placed in 25 mL of the different leachate treatments (control FSW, 0.02, 0.2, 2, 20, 200 and 2000 mg.L^{-1}) to achieve 60 embryos.mL^{-1}. After 48h of exposure in dark conditions, all samples were fixed with a formaldehyde-seawater solution (0.1% final) to estimate the normal D-larval yield. For each vial, 100 larvae were observed using a Zeiss Axiostar Observer Z1 microscope, with ×10-40 magnification. The normal D-larval yield (%) was defined as: number of normal D-larvae ÷ (number of normal + abnormal D-larvae) × 100. Abnormal D-larvae were identified based on morphological malformations (Mottier et al., 2013) such as shell, mantle or hinge malformations, developmental arrest during embryogenesis or evidence of larvae death, e.g. D-stage larvae with an empty shell.

2.6. Statistical analysis

Statistical analyses were performed using R-Studio software (1.4.1106) (R Core Team). Concerning the bioassays, i.e. fertilization success, embryo-larval and Microtox® assay, all data expressed in percentages were normalized using $\sin^{-1}(\sqrt{X})$ transformation. Normality and homoscedasticity were verified before carrying out two-way parametric ANOVA to test the differences in variables between factors, i.e. polymers and leachate concentration. When necessary a Tuckey's post hoc test was carried out using the *car* package (3.0-12) (Fox et al., 2022) was used to determine the significant differences between each group. Assuming that one of the hypotheses was not verified, a non-parametric Kruskal-Wallis test was performed. Kruskal Wallis tests were followed by a Nemenyi's post hoc test using *agricolae* (1.3-5) (De Mendiburu, 2021) and *PMCMR* (4.4) (Pohlert, 2021) packages. Mean differences were considered as significant when p-value < 0.05. Data presented onto the figures are not square root transformed. Target chemical analyses were performed on leachates from all products, statistical significance of differences could not be carried out as only n = 2 leachate solution were analyzed per item. Data were compared based on mix – max of these n = 2 values.

3. Results

3.1. Characterization of FC punch

The thickness (µm) of each sample punches was measured, and the surface areas (mm²) and masses (µg) were calculated (Table S4).

The thickness, surface area and mass for each resin sample were, respectively: 277 ± 10 µm, 2.44 ± 0.01 mm² and 272.00 ± 0.01 µg for PLA-A, 353 ± 18 µm, 2.68 ± 0.06 mm² and 346.00 ± 0.02 µg for PLA-B, 451 ± 13 µm, 2.99 ± 0.04 mm² and 340.00 ± 0.01 µg for PP-A, and 245 ± 15 µm , 2.34 ± 0.05 mm² and 185.00 ± 0.01 µg for PP-B.

Significant differences in mass were observed between all samples varying from 25 to 46%, except between PLA-B and PP-A (ANOVA followed by Tuckey post Hoc test, p-value <

0.05). Similarly, significant differences were observed in surface area between all samples (ANOVA followed by Tuckey post Hoc test, p-value < 0.05). However, within a polymer the punches metrics were homogeneous (Table S4 and Fig. S1).

3.2. Target OPAs TD-GC-MS/MS analyses into plastic food packaging materials (MeOH extracts)

A total of 21 compounds: 8 plasticizers, 3 phosphorous flame retardants, 5 antioxidants and 5 UV-stabilizers were quantified in all MeOH extracts (Fig. 1D).

The bio-based PLA samples contained the highest number of chemicals: 17 additives were identified in both PLA samples and only 8 to 9 additives were identified in PP samples. PLA and PP samples both contained a majority of plasticizers (respectively 7 compounds out of 17, and 6 compounds out of 8 to 9) and 3 UV-stabilizers. 6 compounds were common to both PP and PLA samples, i.e. plasticizers: Bis(2-Ethylhexyl) Adipate (DEHA), Diisoheptyl phthalate (DIHP), Tributyl Acetyl Citrate (ATBC) and Tri(2-ethylhexyl) phosphate (TEHPA), UV stabilizers: UV-328 and UV-327 (Fig. 1A). However PFRs and nonylphenol antioxidants were exclusively identified in PLA extracts and absent from PP extracts.

Overall, the number of OPAs within samples made of the same polymer, A and B, was equivalent: 17 OPAs were identified in both PLA-A and PLA-B, with 16 compounds in common plus one specific for each product (Fig. 1B). Concerning PP samples, 9 and 8 OPAs were detected in PP-A and PP-B respectively, with 8 compounds in common and one specific to PP-A (Fig. 1C).

The detected OPAs were quantified in the $ng.mg^{-1}$ range (i.e. between 0.04 to 7.5 $ng.mg^{-1}$). For the 6 compounds common to both PP and PLA extracts, all the concentrations were higher in PLA extracts in comparison to PP samples (e.g. x5 for UV-328 and ATBC, x9 for UV-327, x2 for TEHPA and x12 for DEHA). The concentrations were considered higher when the factor was > x1.5.

Out of the 16 additives common to all PLA extracts, 7 compounds (Triphenyl Phosphate (TPhP), UV-327, Dicylcohexyl phthalate (DCHP), Nonylphenol Monoethoxylate (NP1OE), 4-Tert-Octylphenol (4-t-OP), 4-Nonylphenol Monoethoxylate (4-NP1OE) and 4-nonylphenol (4-NP)) were measured in higher concentrations (x12 for TPhP and x2 for the other OPAs) in PLA-A extracts than in extracts from PLA-B, and 4 compounds (UV-326, TEHPA, Tris(1,3-Dichloro-2-Propyl)Phosphate (TDCPP) and DEHA) were measured in higher concentrations (x3 for UV-326 and x2 for the other OPAs) in PLA-B extracts compared to PLA-A (Fig. 1D). For OPAs that are common in PP extracts, one compound out of 8 (UV-327) was measured in higher concentrations (x5) in PP-A extracts than in PP-B , and 3 (DEHA, Diisononyl hexahydrophthalate (DINCH) and DIHP) were measured in higher concentrations (all x2) in PP-B extracts compared to PP-A (Fig. 1D).

Figure 1 goes here

3.3. Target OPAs analyses into plastic food packaging leachates

3.3.1. Impacts of the lixiviation duration on OPAs release

OPAs have been detected in all the leachate samples. Overall, the number of additives identified is slightly higher in the 5 days (5d) leachates than in the 24h leachates. A leaching time of 5 days permitted to retrieve the Tricresyl phosphate (TCrP), Nonylphenols (NPs), NP1OE, 4-NP1OE and UV-328. For PLA-A, 10 and 11 compounds were identified in the 24h and 5d leachates respectively, with 10 compounds in common and one specific to the 5d leachates (Fig. 2A and 3A). For PLA-B, 8 and 9 compounds were identified in the 24h and 5d leachates respectively, with 8 compounds in common and one specific to the 5d leachates (Fig. 2B and 3B). Concerning PP leachates, 9 and 12 compounds were identified in the 24h and 5d PP-A leachates respectively, with 8 compounds in common, one in the 24h leachates only, and 4 specific to the 5d leachates (Fig. 2C and 3C). Finally, 10 and 12 compounds were identified in the 24h and 5d PP-B leachates respectively, with 9 compounds in common, one in the 24h leachates exclusively, and 3 specific to the 5d leachates (Fig. 2D and 3D).

The quantitative results do not show any clear pattern in 24h *vs*. 5d leachates. Concerning PLA-A leachates, 2 compounds (ATBC and NPs) were present in higher concentration in the 24h leachates, as well as two compounds (TDCPP and TCrP) that showed higher concentrations in the 5d leachates (Fig. 2E.1.). Similarly, the analysis of PLA-B leachates showed higher concentration for 3 compounds (ATBC, NPs and NP1OE) in 24h leachates, as well as 3 compounds (Dimethyl phthalate (DMP), Tripropyl phosphate (TPP) and TDCPP) in 5d leachates (Fig. 2E.2.).

The PP-A 5d leachates presented higher concentrations for 5 compounds out of 13 (DMP, TCrP, NPs, 4-NP and NP1OE), when the PP-A 24h leachate had higher concentrations for only 2 OPAs (ATBC and TPP) (Fig. 2E.3.). The PP-B leachate showed 4 compounds out of 13 with a higher concentration in the 24h leachates (DMP, DINCH, TPP and TDCPP). In contrast, the 5d leachate had higher concentrations for 3 compounds (ATBC, 4-NP1OE and UV-327) (Fig. 2E.4.).

Considering the results presented above, the 5 days leaching time was chosen for the further chemical and ecotoxicological experiments.

Figure 2 goes here

3.3.2. OPAs in food packaging's 5 days leachates

In 5d leachates from PP and PLA samples, a total 16 OPAs were detected. PLA leachate samples contained plasticizers (3 compounds out of 9 and 12 for A and B suppliers respectively), phosphorous flames retardants (3 and 2 compounds), antioxidants (3 compounds each) and UV-stabilizers (2 and 1 compounds). PP leachates samples contained a majority of plasticizers (4 and 5 compounds out of 12, for A and B suppliers respectively), followed by phosphorous flames retardants and antioxidants (3 and 2 compounds each), and UV-stabilizers (2 to 3 compounds). (Fig. 3C).

Beyond that, the number of OPAs between the PP and the PLA leachates was equivalent, with 11 and 9 OPAs identified in PLA-A and PLA-B respectively (8 common compounds, 3

compounds specific to the supplier A and one specific to the supplier B) (Fig. 3A), and 12 OPAs identified in both PP-A and PP-B leachates (9 common OPAs and 3 specific to each suppliers) (Fig. 3B).

OPA concentrations in 5d FC leachates ranged between 0.02 and 135.82 $ng.L^{-1}$. The quantitative results of OPAs do not show any clear patterns between the different leachate samples. Detailed results are presented in Figure 4C, and some tendencies, that illustrate differences between polymer leachates or suppliers, are given below: 2 plasticizers (DEHA and DIHP) were quantified exclusively in PP leachates (at concentrations ranging from 1.55 to 52.28 $ng.L^{-1}$). DINCH was only found in PLA-A at a concentration of 16.66 $ng.L^{-1}$. ATBC was quantified in PP leachates from the supplier B only (135.8 $ng.L^{-1}$) at a concentration higher (x3) than in PLA-A and B leachates (46.9 ± 1.42). TCrP was only present in PP-A and PLA-A, both at a concentration of 2.22 $ng.L^{-1}$. All the leachates contained UV-327 at similar concentrations (0.29 $ng.L^{-1}$) except in PP-B where this additive was measured at higher (x21) concentration (6.12 $ng.L^{-1}$) (Fig. 3C).

Figure 3 goes here

3.3.3. Comparison of the additive contents between raw materials and seawater leachates

Some additives were only detected in PLA and PP MeOH extracts (Fig. 1D) but not in their respective leachates (Fig. 3C) (i.e. UV-328, UV-326, TPhP and DEHA for PLA and DINCH and ATBC for PP-A only; Fig S2). Conversely, some additives detected in leachates were absent from MeOH extract (i.e. TDCPP and 4-NP in PP samples, TPP in PP samples and PLA-B, DMP in PLA-B, NPs and NP1OE in PP-A sample) (Fig. S2).

3.4. Evaluation of the ecotoxicity of plastic food packaging leachates

3.4.1. Baseline toxicity using Microtox® assay

No significant effect of leachate exposure was observed on the bioluminescence of the bacteria *Aliivibrio fischeri* (Fig. S3). The results showed less than 10% of bioluminescence inhibition regardless of the material and concentration used.

3.4.2. Early life stages of Pacific oyster

3.4.2.1. Effects of FC leachates on fertilization

No significant differences (ANOVA, p-values > 0.05) were observed on the fertilization yield following the exposure of the oyster gametes to the different concentrations of plastic packaging leachates compared to the control treatment (i.e. FSW) (86.5 ± 6.5%). Only the highest concentration (Ii.e.200 mg.L^{-1}) of PLA-B significantly reduced the fertilization yield in comparison to the FSW control treatment (-12%; ANOVA followed by Tuckey post Hoc test, p-value < 0.05) (Fig. 4). Overall, the fecundation rate remained high (>70%) regardless of the treatment (except for PLA-B at 200mg.L^{-1}).

Figure 4 goes here

3.4.2.2. Effects of FC leachates on oyster embryo-larval development

The percentage of normal D shaped larvae in controls was >80% (Fig. 5). None of the leachate concentrations induced embryo-toxicity (ANOVA or Kruskal-Wallis, p-values > 0.05) compared to the control treatment (mean D-larvae yield = 86 ± 9.2%) (Fig. 5).

Figure 5 goes here

4. Discussion

4.1. Characterization of OPAs and their release from FCs

Material MeOH extracts and leachate analyses provided information on the chemicals associated with plastic packaging and those able to desorb into seawater. Despite some additives that were identified below the detection limit, 22 additives (i.e. phthalates, PFRs, antioxidants and UV-stabilizers) were successfully identified and quantified among the selected compounds. Only 7 of the identified chemicals are included in the permitted starting material of EU No 10/2011 (EuropeanCommission, 2011) (i.e. Uvinul 3008; UV 327; UV 326; DEHA; DINCH; Di-allyl phthalate (DAlP); ATBC), and 9 are included in the list established by Oltmanns et al. (2020) compiling 2336 potential emerging toxic chemicals used in FCs, based on a previous EFSA study compiling substances registered under the REACH Regulation (i.e. Uvinul 3008, UV 328,UV 327, TPP, TDCPP, DEHA, DINCH, ATBC and 4-NP) (Fig. 1 and 3). It means that for some of the additives not included in those regulatory lists, the sanitary risks remain unknown since no toxicological or migratory test has been performed.

It is noteworthy that in this study, the number of detected phthalates are underestimated since some phthalate compounds such as DEHP, DEP, DBP and DIDP could not be properly characterized because they were ubiquitous contaminants in the laboratory and instruments. Additionally, quantities of some additives, e.g. DMP and ATBC, recorded in the controls (MeOH and seawater without plastic) indicated presence of these compounds in the reagents employed in this experiment or contamination during sample preparation. Such results underline the difficulties and the challenge of studying additive composition of plastic in the laboratory (Zimmermann et al., 2019). Indeed, they are omnipresent (e.g. found in indoor air, solvents, water, experimental apparatus, protection equipment, glassware) and may prevent their studies (Hermabessiere et al., 2020; Ye et al., 2013).

The higher additive occurrence and concentration (e.g. TDCPP in PLA-A, DMP in PP-A) observed in 5d leachates in comparison to 24h leachates was the basis for choosing a 5 days leaching time for further experiments. This was in agreement with other studies that used a leaching time of 5 days (CEN, 2002; Tetu et al., 2019), and studies that also demonstrated higher additive concentrations in 5d leachates than in 24h leachates (Gardon et al., 2020). It also permitted a great chemical desorption while avoiding the readsorption of the leached chemicals onto the surface of the plastic particle as noticed by Romera-Castillo et al. (2018). However, it is a complicated task to choose an appropriate leaching duration. Indeed, results published in the literature highlight the dependence of additives' desorption processes on many parameters including the polymer type and the additive. For instance, León et al. (2019) mentioned higher additive desorption rates for PP in comparison to PE. Additionally, the leaching dynamics differ according to the nature of additives. For example, the time needed to reach the desorption equilibrium concentration was estimated to be 3 days for BPA, while it was 80 days for phthalates compounds (Suhrhoff and Scholz-Böttcher, 2016).

Our results showed the presence of OPAs such as phosphorous flame retardants (PFRs), antioxidants and UV-stabilizers and with a dominance of plasticizer compounds (Fig. 1 and 3) both in MeOH extracts and leachates. Similar compounds have already been identified and quantified in diverse polymer FC items in the literature. For instance, ATBC and Uvinul 3008 (i.e. Octabenzone) were identified in PP samples (Lahimer et al., 2017; Zimmermann et al., 2019) and in plastic-based candy wrappers (Galmán Graíño et al., 2018). Several PAEs, ATBC and DINCH were detected in PVC FC (Carlos et al., 2018), and, Lahimer et al. (2017) identified UV 326 (i.e. Bumetrizole) in PLA samples.

Discrepancies in the chemical signature of MeOH extracts and leachates suggest that not all OPAs are leaching or that the concentration of the leachable additives was below the detection limit (Zimmermann et al., 2021). The presence of additives in leachates that were not detected in the MeOH extract (e.g. UV 326, TPhP, Di-n-hexyl phthalate (DHP), DCHP,

DAlP and 4-OP) suggests a preferential migration or dissolution into water over methanol (Zimmermann et al., 2019; 2021) .

On the one hand, differences in chemical composition and concentration of MeOH extracts between the two types of polymers selected were observed. A greater number of additives and higher concentrations were measured in bio-based PLA MeOH extracts in comparison to PP MeOH extracts, which was also observed in the study of Zimmermann et al. (2019) study. Moreover, the presence of PFRs and nonylphenols antioxidants, exclusively identified in PLA extracts and absent from PP extracts, suggest that the bio-based PLA material contains more hazardous additives than the PP material. On the other hand, the number of additives between PLA and PP leachates was more or less equivalent and were only differentiated by the signatures and concentrations of additives which was not in accordance with other studies. For instance, using high performance liquid chromatography (HPLC) coupled to HR-MS, Klein et al. (2021) detected the highest number of chemicals and intensities in bio-based plastic leachates (PBAT + PLA) in comparison to other polymers including PP. The amount of additives in bio-based samples was even comparable to PVC, known to be a polymer containing larger amounts of plasticizers and stabilizers (Groh et al., 2019; Hahladakis et al., 2018). Gewert et al. (2018) also detected similarly low amounts of OPAs in PP using LC-HRMS, while Bradley and Coulter (2007) identified more chemicals but using a wide variety of analytical techniques. Additionally, Zimmermann et al. (2021) showed that products made of PLA leached relatively few products compared to PP. Evidentially, it is complex to draw conclusions about each type of plastic material, as their recipes and individual properties can be major factors in desorption.

Results also pointed out leaching differences between polymers. For instance, DEHA plasticizer, which is present in all the materials' MeOH extract samples (Fig 1D), leached in the SW only for PP samples. This may highlight a difference in leaching properties of additives between the two polymers used in this study, which could be explained by the

nature of the polymer (Li et al., 2016) and notably their differences in physicochemical properties (i.e. surface and porosity) (Barrick et al., 2021).

The surface, known to significantly affect desorption (Sun et al., 2021; Van de Ven, 1994) , also differs between the sample resin and between the suppliers, but was considered homogeneous within each replicate of the same FC. However, despite the surfaces' disparities no relationship could be established with among leaching concentrations. As an example, the lower surface area of PP-B (2.34 ± 0.05 mm^2), compared to the other PP and PLA samples, is not related to lower quantities of additives.

4.2. Complexity of plastic products' chemical composition

This study highlighted differences in the chemical composition and concentration between manufacturers. Diversity in chemical signatures and high variability of OPAs migration between polymers and suppliers have also been observed in a few studies (Hamlin et al., 2015; Zimmermann et al., 2019 and 2021). Beyond differences in the formulation of each plastic product (Groh et al. 2019), highlighted by different chemical signatures in the MeOH extracts within a polymer type, the release of additives from plastic materials in leachates is also modulated by the permeability of the polymeric matrix, gaps between polymer molecules, physicochemical properties of the additives and properties of the surrounding medium (e.g. salinity, temperature, pH) and time (Kwan and Takada, 2016). It reinforces the challenge to assess the exhaustive chemical composition of plastic materials and leachates by current analytical methodologies (Bolgar et al., 2007; Muncke et al., 2020).

Given the diversity of plastic associated chemicals (Groh et al., 2019) the target analysis based on 57 targeted additives (Table S1) is certainly not representative. Several studies have lead a non-target screening of compounds in plastic food packaging, revealing more than 1000 chemical features in petro- and bio-based FC materials, including PP and PLA (Zimmermann et al., 2020a; Zimmermann et al., 2020b). Nonetheless, compounds identification with non-target screening approaches are approximate and care should be taken when interpreting the results. Zimmerman et al., (2020b; 2021) and von Eyken et al.

(2020) demonstrates that most plastic chemicals remain unknown due to incorrect identification by databases. But this approach can however help to highlight patterns and emerging compounds. Additionally, targeting molecules of interest may help to show the presence of potentially toxic compounds, which will, in combination with ecotoxicological studies, be complementary to gain a global insight of the material risk.

Overall, this work provides information on the chemical composition of FC samples made out of PP and PLA, along with the identification of 21 additives in these materials and 16 that leached into SW, in particular phthalates, followed by flame retardants, antioxidants and UV stabilizers. Once released from the polymer matrix into the environment, those molecules can become available for organisms and could cause diverse effects such as endocrine disruption, reproductive, development, mutagenic or behavioral effects (Gunaalan et al., 2020; Muncke, 2011).

4.3. Bioassays

The previous chemical analysis showed the leaching of some additive compounds known to be toxic to marine organisms (e.g. UVFs and phthalates) (Hamlin et al., 2015; Hermabessiere et al., 2017; Schrank et al., 2019). However, no effects were observed in the study for any of the carried out bioassay, i.e. *Microtox*® base line toxicity test, fertilization and embryo-larval development of *C.gigas* for short leachate exposure times (a few minutes in *Microtox*® to 1.5 h for the oyster embryo-larval test).

Previous *in vitro* experiments conducted on plastic FC leachates (including PP and PLA) reported baseline toxicity migrating from the products (Szczepannska et al., 2018; Zimmermann et al. 2019; 2020b). Nonetheless, Zimmermann et al. (2020b) pointed out that toxicity was less prevalent in FCs than in plastic not intended to be in contact with food. Disparities of additive numbers and concentrations between manufacturers, as well as variation in base line toxicity depending on the products have also been reported (Klein et al.,

2021; Zimmermann et al., 2019). However, the leached additives that were toxic *in vitro* remained mostly unidentified (Zimmermann et al., 2019).

Additionally, previous studies have demonstrated toxic impacts of various plastic leachates (not labelled FC) on fertilization or embryo development of diverse aquatic species such as oysters (Gardon et al., 2020; Tallec et al., 2022), mussels (Capolupo et al., 2020; Gandara et al., 2016) and urchins (Oliviero et al., 2019). However, it is important to highlight that most studies conducted their experiments with a worst-case scenario approach, i.e. with high concentrations of plastics (5 to 50 times higher than ours). In addition, some studies enhance migration with a polar solvent (dimethyl sulfoxyde, dichloromethane, MeOH (Capolupo et al., 2020; Pannetier et al., 2019)), instead of testing migration using more realistic and softer solvents (e.g. seawater). Although the latter example aims to mimic the desorption of polar organic contaminants, it do not represent the conditions occurring in digestive guts of animals which are characterized by specific pH, digestive enzyme contents, and organic matter (Hermabessiere et al., 2020). Besides, in the case of FC studies, the leaching tests of additives are often perform according to the protocol set by the EU regulation for plastic FCs (i.e. during 10 days at 40 °C in the dark) (EuropeanCommission, 2011; Zimmermann et al., 2021) which was not selected in this case as this work aimed at studying the impact of chemical release in the marine environment. Moreover, to the best of our knowledge, no study conducted analyses of plastic FC leachate in regards to their effects on fertilization and embryo toxicity.. Thus, this present study could be a first attempt to evaluate the effects of plastic FC leached chemicals on the gamete fertilization and embryo-larval development of an aquatic species.

As no toxic effects were observed, the estimation of the half maximal effective concentration (EC50) (i.e. indicating the concentration of a compound when 50% of its maximal effect is observe, that require a wider range of tested concentrations, , was not possible, or, was higher than 2000 mg/L for all the polymer leachates and for all bioassays performed (i.e. embryo toxicity and Microtox®). Furthermore, the absence of toxicity can also be explained

by an incomplete lixiviation of additives from the materials due to the low diffusivities of certain additives, like NPs, from certain rigid plastics (Berens, 1997; Koelmans et al., 2014), resulting in a low exposure of the test organisms to OPAs. In addition, in this study, leachates were produced in seawater in the dark. However, different environmental conditions such as water movement, salinity, UV irradiance, and environmental degradation processes, influence the leaching behavior of additives from plastic items. These environmental conditions can also facilitate the release of plastic chemicals and/or generate active compounds and, thus, can affect their toxicity to organisms (Huang et al., 2021; Klein et al., 2021). Likewise, in a human health sanitary safety approach, or in the case of OPAs release in the digestive tract after ingestion of micro particles, different and enhanced mechanisms of lixiviation may occur. For instance, NPs being lipophilic could be expected to more readily migrate into fatty foods over food with lower lipid content or seawater (Hamlin et al., 2015).

In any case, it should be kept in mind that "the absence of evidence is not evidence of absence" (Leslie and Depledge, 2020). Even if short-term acute bioassays are useful tools, they neither allow the observation of long term and transgenerational effects, nor the assessment of reproductive disruption effects, that are both widely suspected consequences of plastic additives.

5. Conclusion

The results demonstrate that all the tested products (PP and PLA polymers) contained and released OPAs into seawater under the tested conditions. The chemical content and the leachate composition differed from one polymer to another and, most importantly, variations were found among the same polymer type from one supplier to another. As a result, it was not possible to generalize and attribute a chemical pattern to a specific polymer type since variations were recorded at product level. Evidently, this part highlights the importance of the characterization of the "additivome", i.e the additive's content, of the microplastics used for toxicological tests.

Even if the results demonstrate that the tested petro- and biobased samples both leached additives compounds, none of the *in vitro* bioassay showed any acute toxicity of the leachates at relevant or high environmental concentrations, with the selected experimental conditions. However, although three different bioassays were tested, it is only possible to draw a conclusion for the perimeter of the conditions tested. As a result, beyond the standard tests applied for food contact packaging which imply that these materials do not transfer compounds to food, results showed that once in the environment the tested FC might not induce acute toxic effects. In future work, modifications of environmental parameters (e.g. temperature, microbial activity, UVs, weathering), organisms tested, and duration of exposure, may provide additional understanding of the toxicology associated with the leachates of these FCs.

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7. Authors contribution

F.Akoueson: Conceptualization, Investigation, Methodology, Formal analysis, Software, Validation, Visualization, Writing - original draft, review & editing. **K. Tallec**: Methodology, Writing - review & editing. **A. Huvet**: Methodology, Writing - review & editing. **I. Paul-Pont**:

Methodology, Writing - review & editing. **P. Doyen**: Writing - review & editing. **A. Dehaut**: Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **G. Duflos**: Resources, Conceptualization, Funding acquisition, Supervision, Project administration, Writing - review & editing.

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9. Figures Captions

Figure 1: Distribution of the chemical compounds identified by SBSE-TD-GC/MS in MeOH extracts of (A) PLA (A and B), (B) PP (A and B), (C) comparison of PLA and PP samples. (D) Heat map of the chemical compounds quantified in MeOH extracts. (n=2). Values were adjusted according to the chemicals found in the control (MeOH). The white color indicate that the quantitative value of the detected compounds was above the quantitation limit (<LQ). *: additives included in the positive list of the European Commission regulation EU No 10/2011. △: additives included in the Emerging toxic cemical list of Olmans et al., 2020.

With 4-NP: 4-nonylphenol; 4-NP1OE : 4-Nonylphenol Monoethoxylate; 4-tOP: 4-Tert-Octylphenol; ATBC: Tributyl Acetyl Citrate; DAlP: Di-allyl phthalate; DCHP: Dicylcohexyl phthalate; DEHA: Bis-2-Ethylhexyl Adipate; DHP: Di-n-hexyl phthalate; DIHP: Diisoheptyl phthalate; DINCH: Diisononyl hexahydrophthalate; DMP: Dimethyl phthalate; NPs: Nonylphenols isomer; NP1OE: Nonylphenol Monoethoxylate; TDCPP: Tris(1,3-Dichloro-2-Propyl)Phosphate; TEHPA: Tri(2-ethylhexyl) phosphate; TPhP: Triphenyl Phosphate and TPP: Tripropyl Phosphate.

Figure 2: Distribution of the chemical compounds identified by SBSE-TD-GC/MS in 24h and 5 days leachates of (A) PLA A, (B) PLA-B, (C) PP-A and (D) PP-B, at 2000 mg/L. (E) Heat map of the chemical compounds quantified in 24h and 5 days leachates of (E.1.) PLA A, (E.2.) PLA-B, (E.3.) PP-A and (E.4.) PP-B, at 2000 mg/L. (n=2). Values were adjusted according to the chemicals found in the control (seawater). The white color indicate that the quantitative value of the detected compounds was above the quantitation limit (<LQ). *: additives included in the positive list of the European Commission regulation EU No 10/2011. △: additives included in the Emerging toxic chemical list of Olmans et al., 2020. *With 4-NP: 4-nonylphenol; 4-NP1OE : 4-Nonylphenol Monoethoxylate; ATBC: Tributyl Acetyl Citrate; DEHA: Bis-2-Ethylhexyl Adipate; DIHP: Diisoheptyl phthalate; DINCH: Diisononyl hexahydrophthalate; DMP: Dimethyl phthalate; NPs: Nonylphenols isomer; NP1OE:*

Nonylphenol Monoethoxylate; TCrP: Tricresyl phosphate; TDCPP: Tris(1,3-Dichloro-2-Propyl)Phosphate; TEHPA: Tri(2-ethylhexyl) phosphate and TPP: Tripropyl Phosphate.

Figure 3: Distribution of the chemical compounds identified by SBSE-TD-GC/MS in 5 days leachates of (A) PLA (A and B), (B) PP (A and B), at 2000 mg/L. (C) Heat map of the chemical compounds quantified in leachates. (n=2). Values were adjusted according to the chemicals found in the control (seawater). The white color indicate that the quantitative value of the detected compounds was above the quantitation limit (<LQ). "*": additives included in the positive list of the European Commission regulation EU No 10/2011. "△": additives included in the Emerging toxic chemical list of Olmans et al., 2020. *With 4-NP: 4-nonylphenol; 4-NP1OE : 4-Nonylphenol Monoethoxylate; ATBC: Tributyl Acetyl Citrate; DEHA: Bis-2-Ethylhexyl Adipate; DIHP: Diisoheptyl phthalate; DINCH: Diisononyl hexahydrophthalate; DMP: Dimethyl phthalate; NPs: Nonylphenols isomer; NP1OE: Nonylphenol Monoethoxylate; TCrP: Tricresyl phosphate; TDCPP: Tris(1,3-Dichloro-2-Propyl)Phosphate; TEHPA: Tri(2-ethylhexyl) phosphate and TPP: Tripropyl Phosphate*

Figure 4: Fertilization yield (%) after exposure (1.5h) of oyster gametes (oocytes + spermatozoa) to leachates of several food containers: PLA-A (yellow), PLA-B (green), PP-A (light blue) and PP-B (blue), at five concentrations: 0.02, 0.2, 2, 20 and 200 mg/L, compared to the FSW control (Red). Homogeneous groups are indicated by the same letter, after statistical tests using ANOVA followed by Tuckey post Hoc test. (n=4)

Figure 5: Normal D-larval yield (%) after exposure (48h) of fertilized oyster oocytes to leachates issued from (A) PLA-A, (B) PLA-B, (C) PP-A and (D) PP-B, food plastic packaging at five concentrations: 0.2, 2, 20, 200 and 2000 mg/L, compared to the FSW control. Values are expresses as mean± 95% confidence interval. Homogeneous groups are indicated by the same letter, after statistical tests using ANOVA or Kruskal-Wallis tests. (n=3)

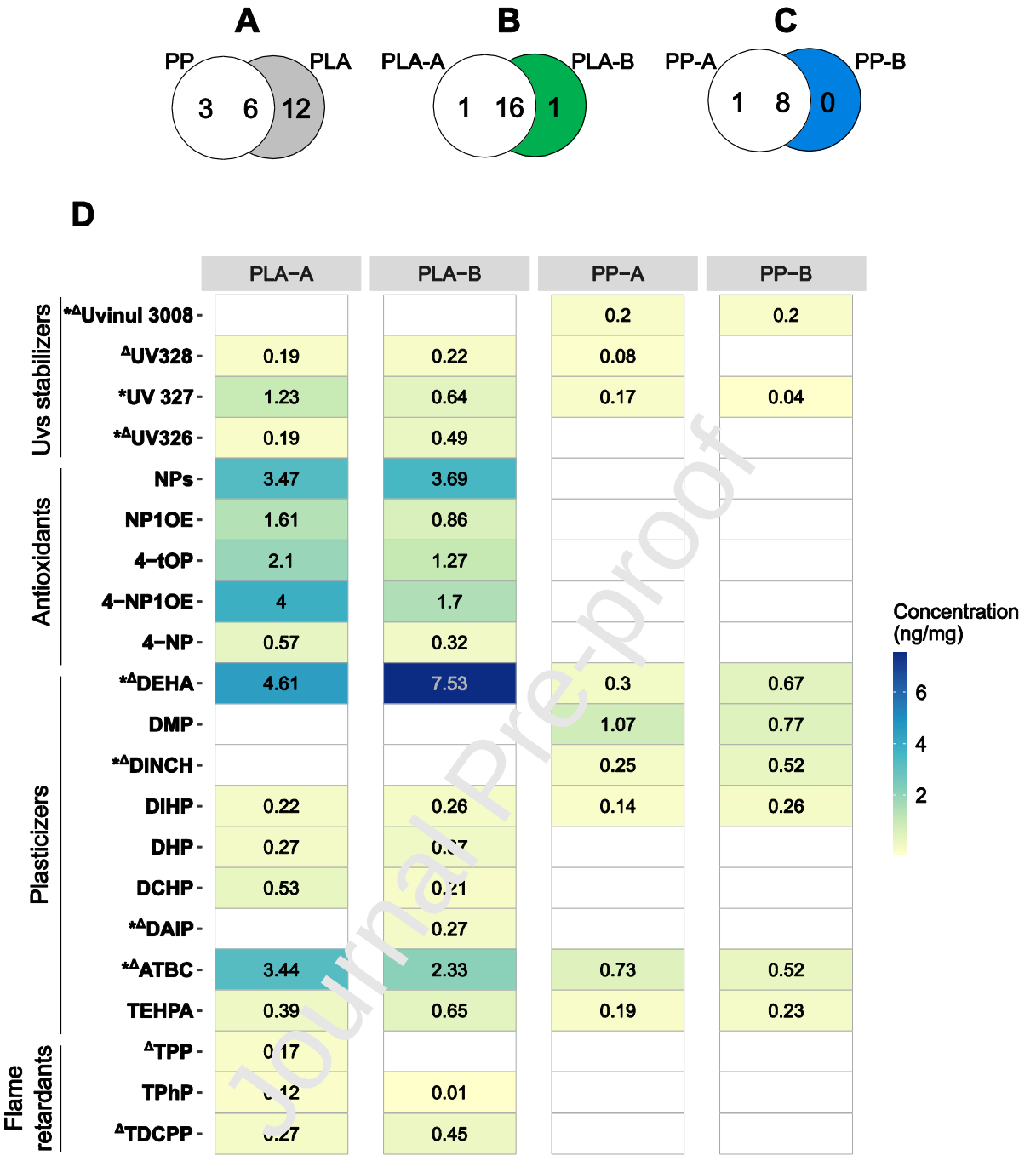


Fig. 1

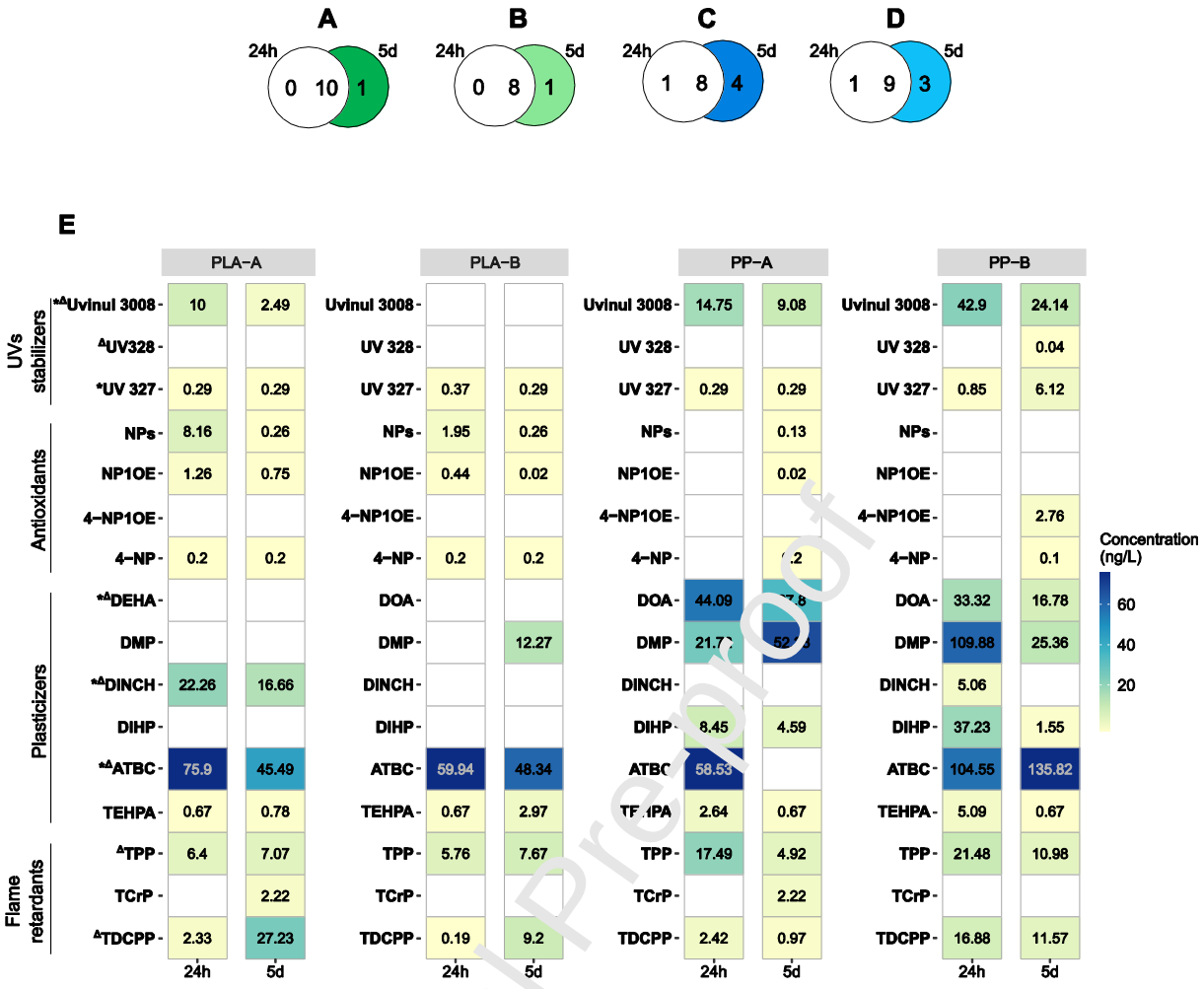


Fig. 2

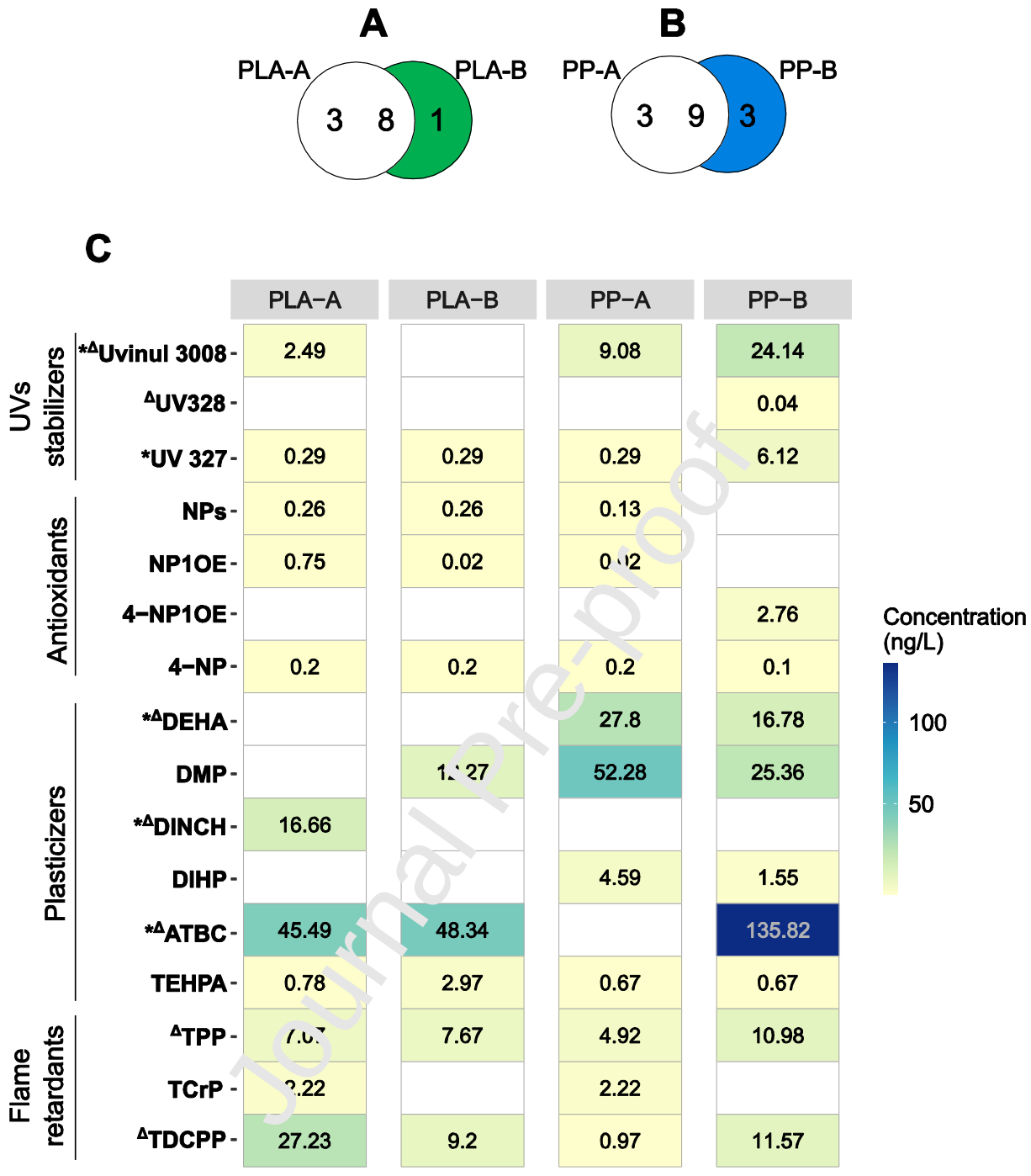


Fig. 3

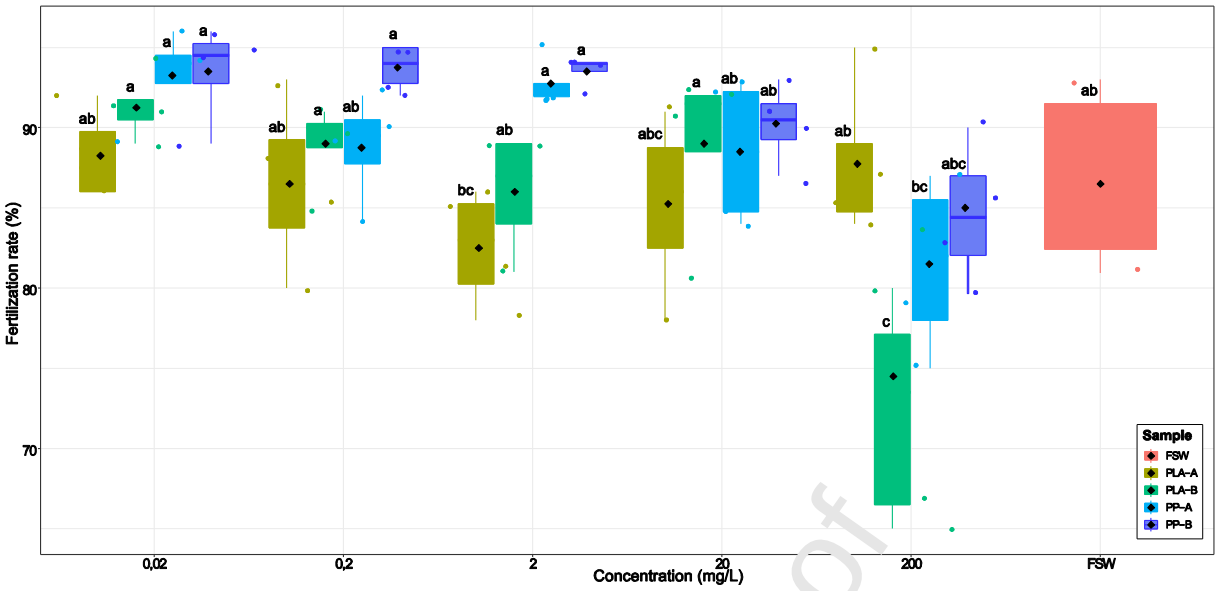


Fig. 4

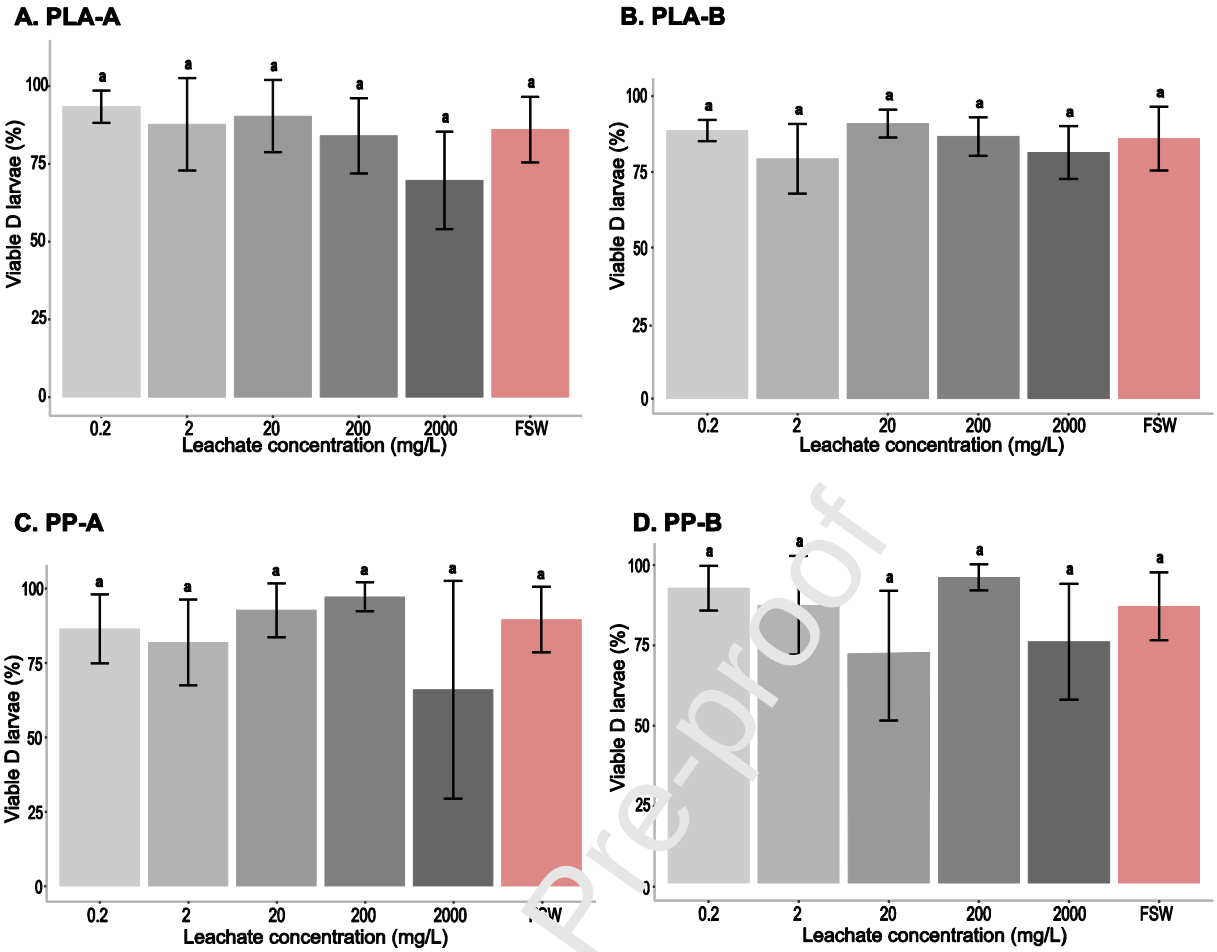


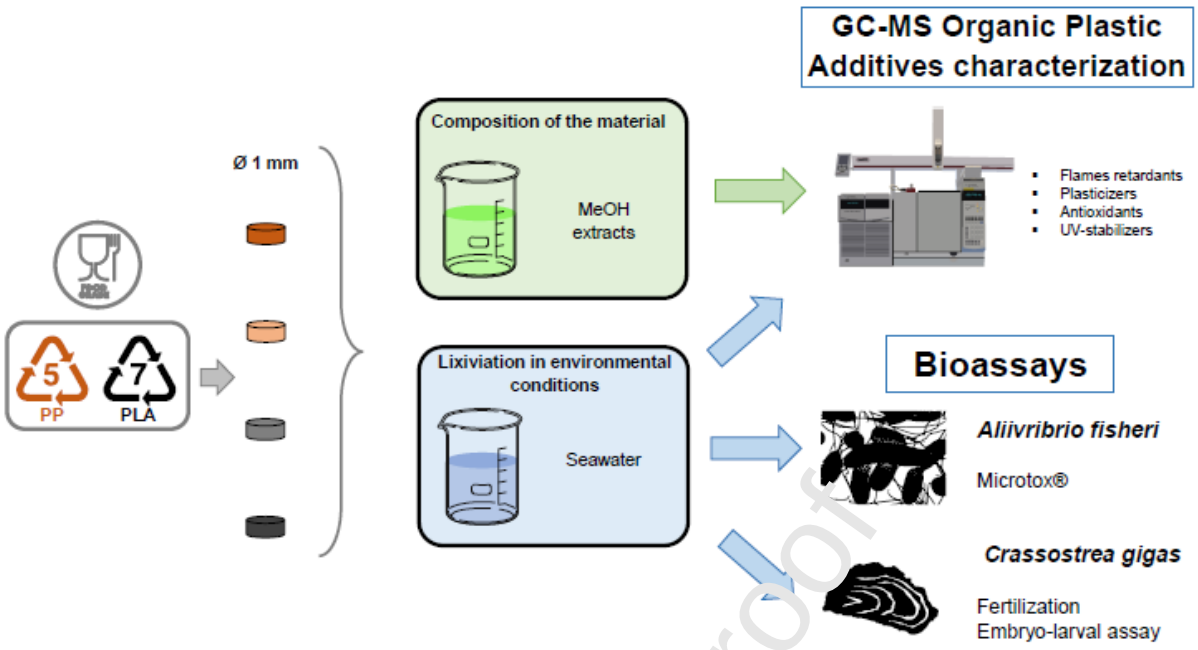
Fig. 5

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Fleurine Akoueson reports financial support was provided by National Centre for Scientific Research.



Graphical abstract

Highlights

- Petro- and bio- based plastics packaging contains organic plastic additives
- Leaching of organic plastic additives was observed with all the tested protocols
- Differences in chemical signature between polymers and suppliers were observed
- Leachates from PP and PLA food containers did not show any *in vitro* toxicity