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Chemical effects of different types of rubber-based products on early life stages of Pacific oyster, *Crassostrea gigas*

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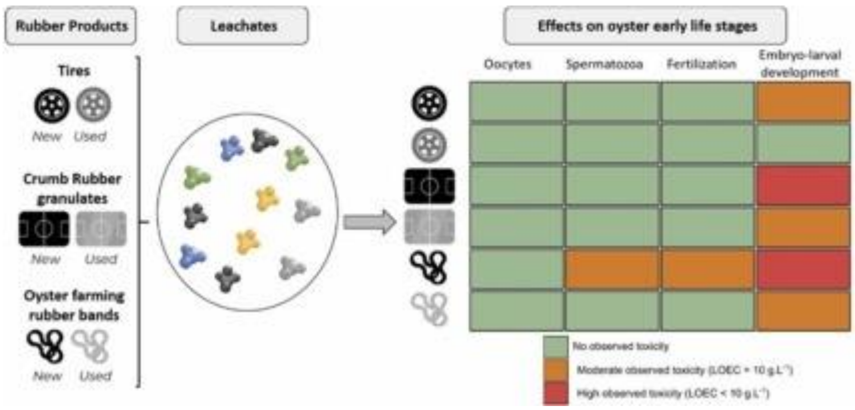
Abstract :

Rubber products and debris with specific chemical signatures can release their constitutive compounds into the surrounding environment. We investigated the chemical toxicity of different types of new and used rubber products (tires, crumb rubber granulates, aquaculture rubber bands) on early life stages of a model marine organism, Pacific oyster Crassostrea gigas. Leachates obtained from used products were generally less toxic than those from new ones. Leachates from new products induced embryotoxicity at different concentrations: oyster-farming rubber bands (lowest observed effect concentration, LOEC = 1 g L-1) and crumb rubber granulates (LOEC = 1 g L-1) > tires (LOEC = 10 g L-1). Moreover, new oyster-farming rubber bands induced spermiotoxicity at 10 g L-1 (-29% survival) resulting in decreased oyster reproductive output (-17% fertilization yield). Targeted chemical analyses revealed some compounds (2 mineral contaminants, 15 PAHs, 2 PCBs) in leachates, which may have played a role. Rubber used in marine aquaculture (rubber bands) or present at sea as waste (tire, crumb rubber granulates) therefore release hazardous chemical molecules under realistic conditions, which may affect oyster development. Aquaculture development work is necessary to improve practices for eco-safety, as are efforts to limit the contamination of marine environments by terrestrial rubber debris.

Graphical abstract



Highlights

► Leachates of rubber-based products can affect early life stages of Pacific oysters. ► Toxicity differed according to the type of rubber-based products and stade of use (new *vs.* used). ► Oyster-farming rubber bands induced the highest toxicity compared to tires and crumb rubber granulates.

Keywords : Oyster, Rubber, Tire, Aquaculture gears, Leachate, Early life stages

Introduction

Contamination by plastic waste is a continuous, pervasive, and global problem, making assessment of the risks a major issue (Borrelle et al., 2020; Galloway et al., 2017; Lau et al., 2020). Among the vast diversity of plastic equipment and polymers found in the environment, rubber materials make up a major part of the waste released (e.g., Boucher and Friot, 2020; Evangeliou et al., 2020; Werbowski et al., 2021). The use of rubber-based products (e.g., tires, aquaculture gear), or their weathering after intentional or accidental disposal in the environment, can induce "rubber-contamination" by the release of micro-rubber (MR) or/and constitutive compounds added during manufacturing processes and subsequently leached out by the action of water (Halle et al., 2020; Wagner et al., 2018). Marine coastal waters, which are already the marine area the most impacted by human activities (Halpern et al., 2008), can be a sink for MR and leachates released by rubber-based products and debris via urban runoff or direct weathering in marine environments.

Each year, driving generates 2.9 Mt of tire particles (Evangeliou et al., 2020), which represent 28% of the primary microplastics released into the global ocean (Boucher and Friot, 2020). Road runoff is presently considered to pose an environmental risk due to tire particles and some constitutive compounds (Tamis et al., 2021). Mismanagement of end-of-life tires (ELT; annual production = 17 Mt; Sienkiewicz et al., 2012) is likely to cause environmental contamination in two main ways: (i) ELT directly dumped in the environment, and (ii) release of crumb rubber granulates (CRG) issued from ELT recycling processes. Crumb rubber granulates are notably used for playgrounds or synthetic turf pitches (ECHA, 2017). In Europe, 80,000–130,000 tons of ELT are used annually for synthetic sport fields (ECHA, 2017). This amount is expected to increase in the coming years due to growing demand, e.g., 1,200–1,400 new synthetic sports fields are created in Europe per year (ANSES, 2018). However, this recycling method is problematic owing to a release of CRG in environment: 1.5–5 tons of CRG are estimated to be lost from a full-sized artificial soccer field per year (Lassen et al., 2015; Magnusson et al., 2016). Based on these data, considering only artificial soccer fields built in Europe (≈21,000), we estimate an annual emission of 31,500–105,000 tons. In addition to the tire industry and recycling processes, rubber-based debris can be released into the environment from other sources using rubber as a raw material (Parker-Jurd et al., 2019). These other rubber products found in the environment can be linked to specific activities, such as the plastic gear used in aquaculture that could lead to local contamination (Andréfouët et al., 2014; Bringer et al., 2021a; Gardon et al., 2021). For instance, a pilot study revealed that plastic aquaculture gear constituted 70% of the plastic debris collected on a beach close to an aquaculture farming area (Bringer et al., 2021a). Such data reveal the importance of assessing the toxic effects of this plastic gear on the farmed animals and associated biodiversity in areas close to these structures and the need for new data to support improvement of aquaculture practices.

More than half the mass of rubber-based products can consist of complex chemicals. For instance, a tire is made up on average of: (1) rubber (40–60 wt%), (2) fillers (20–35 wt%; e.g., carbon black, silica), (3) process oils (12–15 wt%), (4) textile and metal nets (5–10 wt%), (5) additives (5–10 wt%; e.g., plasticizers, anti-oxidants), and (6) vulcanization agents (1–2 wt%; zinc oxide, tellurium) (e.g., Wagner et al., 2018). Many of these compounds are considered hazardous (Halle et al., 2020; Wagner et al., 2018). Various types of damage have been demonstrated by laboratory exposures to rubber leachates, mainly focusing on new-tire leachates, including disruption of developmental processes (*Rana sylvatica*, Camponelli et al., 2009; *Mytilus galloprovincialis*, Capolupo et al., 2020; *Xenopus laevis*, Gualtieri et al., 2005; *Pimephales promelas*, Kolomijeca et al., 2020), decrease in growth or survival (*Daphnia magna*, Gualtieri et al., 2005; *Aedes triseriatus*, Villena et al., 2017; *D. magma*, Wik et al., 2009; Wik and Dave, 2006), alteration of reproductive output (*Ceriodaphnia dubia*, Wik et al., 2009). Furthermore, recent studies highlighted a specific relationship between the chemical signature of tires in urban runoff and freshwater samples from western North America and abnormal mortalities of coho salmon (*Oncorhynchus kisutch*) over decades (Peter et al., 2018). The major environmental results obtained from the "coho salmon case" underline the need to explore the threat posed by the complex chemical mixture emitted by new and used rubber products in order to consider transformation products that can affect the toxicity (Tian et al., 2020).

Here, we chose the oyster *Crassostrea gigas* as a marine model organism to investigate rubber-based product toxicity based on its ecological (e.g., control of phytoplankton biomass, biodiversity richness, water quality) and economic roles (global production: 644 000 tons in 2018) in coastal waters (Grabowski and Peterson, 2007; FAO, 2020). We assessed the sensitivity of oyster early life stages (ELS; spermatozoa, oocytes, embryos) to the chemical compounds released into seawater by three types of new or used rubber-based products (tires, crumb rubber granulates, oyster-farming rubber bands) using several endpoints: (1) gamete viability (survival, intracellular reactive oxygen species production), (2) fertilization success, and (3) embryo-larval developmental success. In addition, we performed targeted chemical analyses on all leachates to compare their chemical signatures with regard to the observed toxicity. We hypothesize that rubber products can induce fluctuating chemical toxicity depending on their usage and that the use of the products changes their toxicological signature, notably a higher content of additives expected for new products versus higher contents of adsorbed waterborne contaminants and transformation products for aged materials.

1. Material and Methods

1.1. Rubber products

Three types of rubber-based products were used in this study: (i) tires, (ii) crumb rubber granulates, and (iii) oyster-farming rubber bands. The latter was chosen in order to examine potential local contamination by oyster farming. For each type of rubber-based product (Table 1), we used new and used products to explore potential modifications in their toxicity signatures. Although the exact duration of use is unknown for each material, it is expected to exceed several months. The outer rings of the tires (first 2 cm) and the oyster-farming rubber bands were cut into small fragments using stainless steel scissors to produce similar sized fragments to the crumb rubber granulates in order to perform leachate extractions on the same masses and surface areas (area= 2.1 ± 1.6 mm²; length = 2.3 ± 0.9 mm; morphological analysis were performed using the ImageJ software on 261 items according to da Costa Araújo et al. (2020)).

1.2. Leachate preparation

Prior to the experiments, all glassware was rinsed with acetone and, whenever feasible, burnt in a muffle furnace for 6 h at 450°C. Leachates were prepared by adding the rubber fragments to sterile, 0.2-µm filtered natural seawater (SW; pH 7.92, 35 PSU) at a concentration of 100 g L^{-1} of each rubber-based product, and stirring the mixture continuously using an orbital shaker at 250 rpm for 14 days in dark conditions at room temperature (Capolupo et al., 2020; Halsband et al., 2020). The stock concentration was chosen to be in similar range than those employed in the literature exploring the chemical risk of plastic debris (e.g., Gardon et al., 2020; Halsband et al., 2020; Cormier et al., 2021; Oliviero et al., 2019). After this incubation, leachates were obtained by filtering off the rubber particles using a glass-fiber filter (GF/F; porosity 1.2 µm). For toxicity assays, the 6 stock leachates (3 types of rubber-based products × 2 states, new and used) were diluted in SW to reach final concentrations of 0.1, 1, and 10 g L^{-1} in addition to a control condition composed of SW (no leachate added; Ctl). Due to the technical limitations allowing the assessment of environmental concentrations of rubber debris and their chemicals, the tested concentrations were chosen based on the results available in the literature tires and CRG toxicity (e.g., Gualtieri et al., 2005; McIntyre et al., 2021; Capolupo et al., 2020; Wik and Dave, 2006). The addition of leachates had no effect on the solutions' characteristics (pH, color) compared to the control.

1.3. Animals and gamete collection

Mature oysters (*Crassostrea gigas*; 24 months old, total weight = 44.3 ± 6.3 g) were collected in September 2020 from an oyster-farming area located at Aber Benoît (France; 48°34'30''N, 4°36'18''W) and were directly used for experiments upon their return to the lab. Oyster sex was determined under a microscope (EVOS™ XL Core Imaging System; ×10–20 magnification) on a 50-µL sub-sample from the gonad of each individual. Gametes were collected by stripping as described in Steele and Mulcahy (1999). Spermatozoa and oocytes were diluted in 100 mL and 1 L of SW (20°C), respectively, and left for 1 h prior to use to ensure gamete quality (spermatozoon mobility and round shape of oocytes), which was checked by microscopy (EVOS™ XL Core Imaging System; ×10–20 magnification). Gamete concentrations were assessed by flow cytometry (EasyCyte Plus cytometer; Guava Merck Millipore).

1.4. Gamete assays

1.4.1. Exposure of spermatozoa

Spermatozoa concentration was set at 10^6 cell mL^{-1} in glass tubes filled with the different concentrations of each leachate (Ctl, 0.1, 1, and 10 g L^{-1}; final volume = 5 mL). Exposures lasted 1 h and were performed on four replicates for each treatment, each replicate corresponding to a pool of spermatozoa from three males. The exposure duration was chosen to be in the same range of duration used for other pollutants (e.g., nanomaterials, Tallec et al., 2020; toxic microalgae, Castrec et al., 2021; pesticides, Akcha et al., 2012).

1.4.2. Exposure of oocytes

Oocyte concentration was set at 10^5 cell mL^{-1} in glass tubes filled with the different concentrations of each leachate (Ctl, 0.1, 1, and 10 g L^{-1}; final volume = 5 mL). Exposures lasted 1 h and were performed on four replicates for each treatment, each replicate corresponding to a pool of oocytes from three females. In the same way as for spermatozoa, the exposure duration was chosen to be in the same range of duration used for other pollutants (e.g., nanomaterials, Gonzalez-Fernandez al., 2018; toxic microalgae, Castrec et al., 2021; dispersed oil, Vignier et al., 2017).

1.4.3. Flow cytometry analyses

All cellular measurements were performed by flow cytometry at the end of the exposures using two detectors (green 525/30nm and red 680/30 nm) of an EasyCyte Plus cytometer equipped with a 488-nm argon laser. A minimum of 4000 spermatozoa and 600 oocytes per replicate and treatment were analysed for each measure.

Spermatozoa and oocyte viabilities were assessed by dual-staining using propidium iodide (final concentration: 10 µg mL^{-1}) and SYBR-14 (final concentration: 1 µM) for spermatozoa or SYBR-Green (final concentration: 1% commercial solution) for oocytes, according to Le Goïc et al. (2013, 2014). Cells were incubated for 10 min (dark conditions, room temperature). Propidium iodide only penetrates cells that have lost membrane integrity (considered as dead cells) while SYBR-14 enters into intact spermatozoa (considered as live spermatozoa) and SYBR-Green stains both intact oocytes and oocytes displaying membrane breakage (Le Goïc et al., 2014; Garner et al.,1994). Data were expressed as the percentage of live spermatozoa or oocytes.

Intracellular reactive oxygen species (ROS) production by spermatozoa and oocytes was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA; final concentration 10 mM; Sigma-Aldrich) (Gonzalez-Fernandez al., 2018). Cells were incubated for 50 min (dark conditions, room temperature). DCFH-DA penetrates cells before being transformed by esterase activity into DCFH, which emits green fluorescence after its oxidation by ROS to dichlorofluorescein (DCF) (Lambert et al., 2003). Therefore, the relative ROS production in the cells was expressed as the mean level of green fluorescence (Arbitrary Units, A.U.).

1.5. Fertilization assay

Control gametes from 3 males and 3 females with a spermatozoa-to-oocyte ratio of 100:1 and a final concentration of 1,000 oocytes mL^{-1} were placed in glass tubes filled with the different concentrations of each leachate (Ctl, 0.1, 1, and 10 g L^{-1}; final volume = 5 mL). This step was repeated in four replicates. After 1.5 h, samples were fixed with a formaldehyde-seawater solution (0.1% final) to estimate the fertilization yield under a microscope (Zeiss Axio Observer Z1; ×4−10 magnification; observation of 100 oocytes $tube^{-1}$). The fertilization yield (%) was estimated as: number of fertilized oocytes / (number of fertilized + unfertilized oocytes) × 100 (Tallec et al., 2018).

1.6. Embryo-larval development assay

A standardized assay (ISO 17244:2015) was used to assess embryotoxicity of rubber leachates. To produce embryos, control gametes from 3 males and 3 females were placed in a glass beaker filled with 1.5 L SW at a spermatozoa-to-oocyte ratio of 100:1 and a final concentration of 1,000 oocytes mL^{-1}. This step was repeated in three replicates. Once fertilization was achieved (fertilization yields > 90%) and embryos at the 2-cell stage, tubes were set up with 60 embryos mL^{-1} in the different leachate treatments (Ctl: only SW, 0.1, 1, and 10 g L^{-1} of each rubber type; final volume = 25 mL). Exposures lasted 36 h in dark conditions, then samples were fixed in a formaldehyde-seawater solution (0.1% final) to estimate the normal D-larval yield under a microscope (Zeiss Axio Observer Z1; ×4−10 magnification; observation of 100 cells tube^{-1}). The normal D-larval yield (%) was estimated as: number of normal D-larvae ÷ (number of normal + abnormal D-larvae) × 100. Abnormal D-larvae are those with morphological (e.g., shell, mantle) malformations or those which have experienced developmental arrest during embryogenesis (Tallec et al., 2018).

1.7. Chemical analyses

All chemical analyses were performed on leachates obtained from the products at 100 g L^{-1}. Mineral contaminant concentrations (Zn, S, Al, Cu, Fe) were assessed by inductively coupled plasma optical emission spectrometry using an ICP-OES 5110 Agilent spectrophotometer and an SPS 4 Agilent automatic sample changer according to the standardized protocol NF ISO 11885. Samples were diluted (1/10) with acidified water and 0.5% HNO_3 to reduce matrix effects. An internal standard (Yttrium) was added to the sample. Control solutions were injected every 10 samples and compared with control charts to validate the results obtained. Organic micropollutant concentrations were assessed by gas chromatography–mass spectrometry (GC-MS/MS) using a 6890 N Network GC System equipped with a 7683 B Series Injector autosampler and a 5973 Network Mass Selective Detector. Internal standards (PCB 29 D5, PCB 103, pyrene D10, benzo[*a*]pyrene D12, naphthalene D8, benzanthracene D12, dibenzanthracene D14, benzo[*ghi*]perylene D12, and fluorene D10) were added to the sample before extraction with CH_2Cl_2 and 20 min agitation. For the second extraction, the leachate was adjusted to pH 2 with H_2SO_4 50/50. After adding ethyl acetate, glacial acetic acid and CH_2Cl_2, the sample was agitated for 20 min. The organic phase was added to the first fraction. For the last extraction, CH_2Cl_2 was added, followed by 5 min agitation. Then, the extracts were purified on a fluorisil column. The evaporation of the solvent is done with a Zymark evaporator at 35°C before the injection into the GC-MS. In total, 20 polycyclic aromatic hydrocarbons (PAH) and 9 polychlorinated biphenyls (PCB) were screened for using the selected ion monitoring (SIM) method based on the data available in the literature (Supplementary Table 1; e.g., Aatmeeyata and Sharma, 2010; Capolupo et al., 2020; Chen et al., 2007; Kolomijeca et al., 2020).

1.8. Statistical analyses

Statistical analyses and graphs were made using R software (R Core Team, 2016). Percentages were analyzed after angular transformations. Normality and homoscedasticity were screened with Shapiro-Wilk and Levene tests, respectively. Toxicity assays, flow cytometry analyses, fertilization success, and embryo-larval development success among treatments were analyzed by one-way ANOVAs followed by Tukey *post hoc* tests to make pairwise comparisons. Targeted chemical analyses were performed on leachates from all products, but quantitative comparisons among leachates could not be statistically evaluated as these chemical analyses were done on n = 1 leachate solution per new or used rubber item. The significance threshold was set at 0.05. Data are expressed as the mean ± standard deviation (SD).

2. Results

2.1. Effects of rubber leachates on oyster spermatozoa

The viability (Figure 1) and ROS production (Figure 2) of oyster spermatozoa were not significantly (p-values > 0.05) affected by exposure to the leachates issued from new tires (Figures 1A and 2A), used tires (Figures 1B and 2B), new crumb rubber granulates (Figures 1C and 2C), used crumb rubber granulates (Figures 1D and 2D), and used oyster-farming rubber bands (Figures 1F and 2F) at any of the concentrations tested compared with the control treatment (live spermatozoa = 97 ± 2%; ROS production = 30 ± 11 A.U.). Conversely, the most concentrated leachate (10 g L^{-1}) issued from new oyster-farming rubber bands (Figures 1E and 2E) significantly reduced the percentage of live spermatozoa (-29%; p-value < 0.001) and significantly increased ROS production (+83%; p-value = 0.041).

2.2. Effects of rubber leachates on oyster oocytes

The viability (Supplementary Figure 1) and ROS production (Supplementary Figure 2) of oyster oocytes were not significantly affected by exposures to any of the leachates, regardless of their nature or concentration, comparison with the control treatment (p-values > 0.05; live oocytes = 98 ± 2%; ROS production = 229 ± 105 A.U.).

2.3. Effects of rubber leachates on oyster fertilization

No significant effect (*p*-values > 0.05) on the fertilization yield was observed following co-exposure of oyster gametes (oocytes + spermatozoa) to the different concentrations of leachates issued from new tires (Figure 3A), used tires (Figure 3B), new crumb rubber granulates (Figure 3C), used crumb rubber granulates (Figure 3D), or used oyster-farming rubber bands (Figure 3F) compared with the control treatment (93 ± 3%). Conversely, the most concentrated leachate (10 g L^{-1}) issued from new oyster-farming rubber bands (Figure 3E) significantly reduced the fertilization yield by a mean of 17% (*p*-value < 0.01).

2.4. Effects of rubber leachates on oyster embryo-larval development

The new-tire leachate (Figure 4A) significantly reduced embryo-larval development success at 10 g L^{-1} by 53% (*p*-value < 0.001) compared with the control treatment (mean D-larval yield = 79 ± 1%). Regarding the new crumb rubber granulates (Figure 4C), used crumb rubber granulates (Figure 4D), and used oyster-farming rubber bands (Figure 4F), the highest leachate concentration (10 g L^{-1}) completely inhibited embryo-larval development (*p*-value < 0.001). The same result was observed from 1 g L^{-1} in the case of new oyster-farming rubber bands (*p*-value < 0.001; Figure 4E). Lastly, any of the concentrations of the leachate issued from used tires induced embryotoxicity (Figure 4B; *p*-value > 0.05).

2.1. Chemical analyses

2.1.1. New and used tires

In leachates from new and used tires, 2 mineral contaminants and 13 chemicals (11 PAHs and 2 PCBs) were detected (Table 2). Similar concentrations of sulfur (S) were found between the two types of leachates (931 ± 1 mg L^{-1}). A higher concentration (×1.6) of zinc (Zn) was measured in leachate from new tires (519 µg L^{-1}) than in leachate from used tires (319 µg L^{-1}). Concentrations of the other measured metals (Al, Cu, Fe) were below detection limits. Leachates from new tires and used tires contained 1 (benz[*a*]anthracene) and 4 (anthraquinone, benzo[*1,2*]fluorene, 2-methylfluoranthene, PCB 118) specific compounds, respectively, while 8 were detected in both leachates (acenaphthene, acenaphthylene, benz[*e*]acephenanthrylene, indenopyrene, naphthalene, phenanthrene, pyrene, PCB 101; Figure 5A&D and Table 2). New-tire leachate contained higher concentrations of acenaphthylene (×3.9), naphthalene (×9.2), phenanthrene (×12.0), and pyrene (×4.3) compared with used-tire leachate. In contrast, new-tire leachate had lower concentrations of benz[*e*]acephenanthrylene (×4.9) and PCB 101 (×2.3) compared with leachate from used tires.

Similar concentrations of acenaphthene (average = 25.8 ± 2.4 ng L^{-1}) and indenopyrene (average = 0.8 ± 0.4 ng L^{-1}) were found in both new- and used-tire leachates.

2.1.2. New and used crumb rubber granulates

In leachates from new and used crumb rubber granulates, 2 mineral contaminants and 13 PAHs were detected (Table 2). Similar concentrations of sulfur (S) were found between leachates (average = 933 ± 18 mg L^{-1}). A higher amount of zinc (Zn) was measured in leachate from new crumb rubber granulates compared with leachates from used crumb rubber granulates (×1.4). Amounts of other measured metals (Al, Cu, Fe) were below detection limits. Leachates from new crumb rubber granulates and used crumb rubber granulates contained one (anthraquinone) and 3 (anthracene, fluoranthene, 2-methylfluoranthene) specific compounds, respectively, while 9 were detected in both these leachates (acenaphthylene, benzo[*1,2*]fluorene, benzo[*a*]pyrene, benz[*e*]acephenanthrylene, benzo[*ghi*]perylene, indenopyrene, naphthalene, phenanthrene, pyrene; Figure 5B&D and Table 2). Leachate from new crumb rubber granulates had lower concentrations of phenanthrene (×2.6), benzo[*ghi*]perylene (×1.5), naphthalene (×1.9) and pyrene (×8.9) compared with leachate from used crumb rubber granulates. Similar concentrations of acenaphthylene (average = 18.4 ± 4.7 ng L^{-1}), benzo[*1,2*]fluorene (average = 2.1 ± 0.6 ng L^{-1}), benzo[*a*]pyrene (average = 1.6 ± 0.1 ng L^{-1}), benz[*e*]acephenanthrylene (average = 3.0 ± 0.8 ng L^{-1}), indenopyrene (average = 3.0 ± 0.6 ng L^{-1}) were found in the 2 types of leachate.

2.1.3. New- and used oyster-farming rubber bands

In leachates from new and used oyster-farming rubber bands, 2 mineral contaminants and 8 chemicals (7 PAHs and one PCB) were detected (Table 2). Similar concentrations of S were found between leachates (average = 933 ± 3 mg L^{-1}). A lower amount of Zn was measured in leachate from new oyster-farming rubber bands compared with used oyster-farming rubber bands (×3.1). Amounts of other measured metals (Al, Cu, Fe) were below detection limits.

Leachates from new and used oyster-farming rubber bands contained 1 (benzo[*ghi*]perylene) and 2 (2-methylfluoranthene, pyrene) specific compounds, respectively, while 5 were detected in both leachates (acenaphthylene, benz[*e*]acephenanthrylene, naphthalene, phenanthrene, PCB 101; Figure 5C&D and Table 2). Leachate from new oyster-farming rubber bands showed higher concentrations of acenaphthylene (×8.7) and naphthalene (×58.5), compared with that from used oyster-farming rubber bands. Similar concentrations of benz[*e*]acephenanthrylene (average = 0.6 ± 0.5 ng L^{-1}), phenanthrene (average = 10.7 ± 2.3 ng L^{-1}) and PCB 101 (average = 4.9 ± 1.0 ng L^{-1}) were found in the two types of leachate.

3. Discussion

Leachates from rubber products affect oyster ELS. Healthy early life stages (ELS) are critical for species sustainability. External fertilization exposes oyster gametes and embryos to waterborne stressors as constitutive compounds released by plastic waste, including rubber debris, which accounts for a significant part of the anthropogenic waste emitted from terrestrial to aquatic systems (Boucher and Friot, 2020; Evangeliou et al., 2020; Werbowski et al., 2021). In the present study, spermiotoxicity and embryotoxicity were observed in response to rubber-based product leachates, with a higher sensitivity of embryos compared with gametes (summarized in Figure 6). Due to the current absence of *in situ* data about the environmental concentrations of chemicals released by rubber products, this toxicological data cannot be extrapolated to an *in situ* threat. Experimentally, oyster spermatozoa appear less sensitive than embryos as their functioning is exclusively based on the motile tail driven by the energy supplied by the metabolic machinery (Salisbury et al., 1976). In addition, the longer exposition of the embryos (36 h) compared with the gametes (1.5 h maximum) can exacerbate the observed higher sensitivity of embryos. The data obtained in the present study agrees with previous results showing the high sensitivity of embryonic development to rubber leachates, regardless of the biological model (e.g., Fish, Kolomijeca et al., 2020; Chicken, Xu et al., 2019; Frog, Gualtieri et al., 2005). Due to the crucial role of embryonic development (e.g., organogenesis, germline differentiation) for downstream development, it is relevant to explore potential “developmental domino effects” after early exposures to rubber leachates (Hamdoun and Epel, 2007; Byrne, 2012).

Toxic concentrations of tire and crumb rubber granulate leachates found in this study are consistent with EC_{50} values determined using microalgae (*Raphidocelis subcapitata*, EC_{50} = 0.4 g L^{-1}; *Skeletonema costatum*, EC_{50} = 15.2 g L^{-1}; Capolupo et al., 2020), copepods (*Calanus* sp., EC_{50} = 35 g L^{-1}; *Acartia* sp., EC_{50} < 5 g L^{-1}; Halsband et al., 2020), daphnid (*D. magma*, EC_{50} = 0.5–>10 g L^{-1}; Wik and Dave, 2006), and mussel fertilization (*Mytilus galloprovincialis*, EC_{50} = 29.1 g L^{-1}; Capolupo et al., 2020) and embryogenesis (EC_{50} = 1.8 g L^{-1}; Capolupo et al., 2020). Regarding the toxicity of oyster-farming rubber bands, no comparison can be made because, to our knowledge, no data have been previously published on this specific plastic item. However, compared with other aquaculture gear, oyster-farming rubber bands appear more toxic than spat collectors or synthetic rope according to data obtained on pearl oyster embryos (Gardon et al., 2020).

Potential toxic pathways. Deleterious effects observed on oyster spermatozoa and/or embryos could be linked to oxidative stress, as suggested by the higher level of ROS production in spermatozoa exposed to leachates from new oyster-farming rubber bands. Although ROS are involved in cell defenses, their continuous production at a higher level than in control spermatozoa/embryos can lead to major cell disruptions. An increase in ROS concentration corresponds, according to Trestrail et al. (2020), to Stage 1 of a “pollutant × antioxidant system” interaction. Subsequently, the spermatozoon/embryo antioxidant system responses can be altered (Stage 2) resulting in oxidative stress and cytotoxicity (DNA damage, lipid peroxidation; Stage 3) (Trestrail et al., 2020). This cytotoxicity can lead to apoptosis pathways, potentially explaining the decrease in live spermatozoa and embryos/D-larvae. Tire additives disrupted the antioxidant system in rainbow trout (*Oncorhynchus mykiss*; Stephensen et al., 2005) and provoked lipid peroxidation in mussel (*Mytilus galloprovincialis*; Capolupo et al., 2021). Leachate from crumb rubber granulates (80 g L^{-1}) drastically reduced chicken embryonic development and molecular investigations suggested oxidative stress and apoptosis (Xu et al., 2019). Functional analyses considering the state of antioxidant enzymes, DNA damage, lipid peroxidation and apoptotic markers may be of great help to decipher toxic pathways and demonstrate the role of oxidative stress in the observed spermiotoxicity and embryotoxicity.

Leachates from new rubber products are more toxic than their used counterparts. Three main hypotheses exist regarding the differing chemical toxicity between new and used items: (i) new products induce stronger deleterious effects owing to a higher amount of additives in their leachates compared with their used counterparts that have already released these additives during their lifetime; (ii) adsorption of environmental contaminants (e.g., metals, PAHs) and/or product transformation occurring throughout the item's lifetime increase the toxicity of used products compared with new ones; and (iii) weathering alters the surface layers of plastic products, exacerbating the release of additives (e.g., Simon et al., 2021; Gardon et al., 2020; Tian et al., 2020; Nobre et al., 2015).

Here, our results are consistent with the first hypothesis, while previous work suggested a higher toxicity of used tires compared with new ones (Day et al., 1993). It is impractical to extrapolate these results on the overall greater toxicity of new tires to oyster ELS because each tire type displays specific chemical signatures and, therefore, different toxicity potential according to brand (Wik and Dave, 2006). In a comparison of 25 types of tires, toxicity assays on *D. magma* revealed EC_{50} between 0.5 and >10 g L^{-1} (Wik and Dave, 2006). To be more environmentally relevant, future studies should, in connection with the local watersheds, produce leachates from a mix of a large number of new and used tires corresponding to the type of tires purchased in the province/state of interest.

The toxicity of rubber-based product leachates is commonly attributed to the release of metals (Zn in particular) and PAHs incorporated during the manufacture of these products (Halle et al., 2020; Halsband et al., 2020; Kolomijeca et al., 2020). The levels of metals in leachates revealed different concentrations of Zn according to the products, in the order of abundance: crumb rubber granulates > oyster-farming rubber bands > tires. Because the leachates from crumb rubber granulates were not the highest toxic, whether new or used, the release of Zn seems unlikely to be the main cause of the observed effects. Regarding all leachates studied here, 15 PAHs and 2 PCBs were detected and quantified in total. These compounds had already been detected in leachates of rubber debris (especially tire debris) (e.g., Aatmeeyata and Sharma, 2010; Capolupo et al., 2020; Chen et al., 2007; Halsband et al., 2020; Kolomijeca et al., 2020). As highlighted in the results, leachates from used items can have compounds distinguishable from those of new products (e.g., anthraquinone, benzo[*1*,*2*]fluorene, 2-methylfluoranthene, PCB 118 in leachate from used tires). Because used items are exactly the same reference as new ones, these distinguishable compounds were likely adsorbed on items during their usage. The ability of plastic debris to act as pollutant carriers due to their surface hydrophobicity is well known and was extensively studied by León et al. (2018). Hence, it is highly likely that rubber debris manufactured and used on land could transfer terrestrial contaminants adsorbed during the lifetime of its usage and journey to the ocean.

Targeted chemical analyses are not sufficient to identify the compound(s) responsible for toxicity. Despite our efforts to quantify a wide range of potential toxic compounds (5 mineral contaminants, 16 PAHs, 7 PCBs) released by rubber items in seawater, we cannot clearly identify those responsible for the observed effects although some compounds are known to be toxic to oyster young stages (e.g. Lyons et al., 2002; Nogueira et al., 2017). For instance, leachates from new oyster-farming rubber bands were more toxic than those from other items, but the targeted chemical analyses did not reveal compounds with higher concentrations than in leachates from tires or crumb rubber granulates. Overall, this identification is particularly challenging because nontargeted chemical analyses of rubber items can reveal hundreds of constitutive compounds (Halle et al., 2020). The “coho-salmon case” demonstrated that high-throughput nontargeted chemical analyses can allow the detection of specific and uncommon compounds responsible for harmful effects (Tian et al., 2020). This approach should be prioritized in future studies whose primary objective is to identify and experimentally test toxic compounds. However, chemical toxicity is not necessarily linked to a single compound. In certain cases a “cocktail effect” can be responsible for the observed harmful effects, making the identification of the compounds directly involved especially laborious (Svingen and Vinggaard, 2016; D’Almeida et al., 2020). In a decision-making context, therefore, the use of bio-assays, as performed in the present study, allowing a large screening (high number of products and concentrations) and integrating potential interactions and cocktail effects among contaminants appears to be a relevant and viable compromise providing useful data.

Promoting eco-friendly aquaculture practices. Due to the high concentration of chemicals in plastic products, it is important to test their toxicological potential to support the development of safer products (for humans and biodiversity) by manufacturers. Reduced survival of spermatozoa and embryos in response to leachates from rubber oyster-farming gear suggests that aquaculture practices may have direct impacts on oyster reproductive output. It is currently difficult to determine the use of oyster-farming rubber bands at global (basins, regions) and local (farm) scales. It would require a social sciences approach to evaluate the most common practices and an in-depth field survey on product usage and end of life management as well as important and deep chemical analyses to measure the environmental concentrations for a better risk assessment by comparative studies with our toxicological data. Nonetheless, the data provided here, compiled with the recent works on pearl oyster embryos (Gardon et al., 2020) and Pacific oyster larvae and spat (Bringer et al., 2021b), constitute a starting point to engage discussion with the aquaculture sector in order to improve *in situ* practices to control and avoid environmental contaminations. In particular, it would be interesting to implement “safe-by-design” approaches (i.e., optimization of industrial processes to minimize the health and environmental risks of materials) to develop new aquaculture gears in the context of eco-friendly practices (van de Poel and Robaey, 2017).

4. Conclusion

This study highlights detrimental effects on oyster early life stages provoked by all tested rubber products with a higher toxicity of aquaculture rubber bands compared to tires and CRG. Overall, higher toxicity was observed for new items in comparison with their used counterparts. Such variability in chemical toxicity is likely linked to the initial chemical composition (chemical compounds added during manufacture including non-intentionally added substances), the age of the product (e.g. release of transformation and degradation products, monomers) and its environment of use (air vs water leading to differences in adsorbed contaminants during their use). High-throughput untargeted chemical analyses are crucial to better understand the potential toxicity of plastic materials over their lifetime and as they age and degrade in the environment. Moreover, this approach could help to constrain the uncertainties on the environmental concentrations of rubber debris and their chemicals leached in the environment. To conclude, this study brings background data useful from a toxicological point of view aiming to test the safety of materials but cannot be considered as a proof of *in situ* toxicity of tires, CRG or aquaculture rubber bands on oyster gametes and embryos.

CRediT authorship contirbution statement

Kevin Tallec: Conceptualization, Methodology, Investigation, Formal Analyses, Visualization, Writing – Original Draft, Writing – Review & Editing; **Nelly Le Goïc**: Investigation, Writing – Original draft preparation. **Valerie Yeuc'h**: Investigation, Writing – Original draft. Arnaud Huvet*:* Supervision, Conceptualization, Writing – Original Draft, Writing – Review & Editing, Project administration, Funding acquisition; **Ika Paul-Pont:** Supervision, Conceptualization, Investigation, Writing – Original Draft, Writing – Review & Editing, Project administration, Funding acquisition

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.

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

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The authors report no conflicts of interest.

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Tables

Table 1. Types and sources of the rubber-based products used in this study.

Rubber-based products	Sources
New Tires	New Dunlop SP Sport Maxx (Dot code: DM75 JA2R 3916) purchased from an automobile repair garage located in Brest (France).
Used Tires	Same reference as above, but discarded after full use for a passenger car (i.e. ELT). The used tire was recovered from the same garage that the new one.
New crumb rubber granulates	Items provided by the local intermunicipal body “Brest Metropole” from the stock used for sport fields in the city of Brest (France).
Used crumb rubber granulates	Items directly sampled at a sports field located in Brest. The CRG were used for an artificial soccer field. The exact duration of the deployment is unknown but exceeds several months.

New oyster-farming rubber bands	New rubber bands purchased from a maritime cooperative (Auray, France)
Used oyster-farming rubber bands	Same reference as above, but recovered from an active oyster-farming area. The rubber bands were used to close oyster bags commonly used in aquaculture practices. The exact duration of the deployment is unknown but exceeds several months.

Table 2. Summary of chemical analyses performed on leachates from tires, crumb rubber granulates (CRG), and oyster-farming rubber bands (ORB) for both new and used products, obtained after 14 days in sterile, 0.2-µm filtered seawater in dark conditions at 100 g L^{-1}. Values were adjusted according to the amount of chemicals found in the control (seawater).

Compounds	New Tire	Used Tire	New CRG	Used CRG	New ORB	Used ORB
S (mg L^{-1})	930	932	920	946	930	936
Al (mg L^{-1})	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
Cu (µg L^{-1})	<100	<100	<100	<100	<100	<100
Fe (µg L^{-1})	<200	<200	<200	<200	<200	<200
Zn (µg L^{-1})	519	319	9,416	6,581	1,228	3,762
Acenaphthene (ng L^{-1})	24.1	27.5	0	0	0	0
Acenaphthylene (ng L^{-1})	222.6	57.4	15.1	21.7	108.3	12.4
Anthracene (ng L^{-1})	0	0	0	0,3	0	0
Anthraquinone (ng L^{-1})	0	29.8	77	0	0	0
Benz[*a*]anthracene (ng L^{-1})	2.1	0	0	0	0	0
Benzo[*1,2*]fluorene (ng L^{-1})	0	226.9	1.6	2.5	0	0
Benzo[*a*]pyrene (ng L^{-1})	0	0	1.5	1.7	0	0
Benz[*e*]acephenanthrylene (ng L^{-1})	0.8	3.9	3.5	2.4	0.2	0.9
Benzo[*ghi*]perylene (ng L^{-1})	0	0	11.8	17.2	1.1	0
Fluoranthene (ng L^{-1})	0	0	0	11.9	0	0
Indenopyrene (ng L^{-1})	0.5	1.1	2.5	3.4	0	0
2-Methylfluoranthene (ng L^{-1})	0	6.7	0	15.8	0	6.2
Naphthalene (ng L^{-1})	438.5	47.7	3.8	7.3	99.4	1.7
Phenanthrene (ng L^{-1})	49.4	4.1	8.3	21.8	12.3	9.1
Pyrene (ng L^{-1})	5.1	1.2	8.6	76.9	0	3.2
PCB 101 (ng L^{-1})	8.4	19	0	0	5.6	4.2
PCB 118 (ng L^{-1})	0	0.3	0	0	0	0

Figures

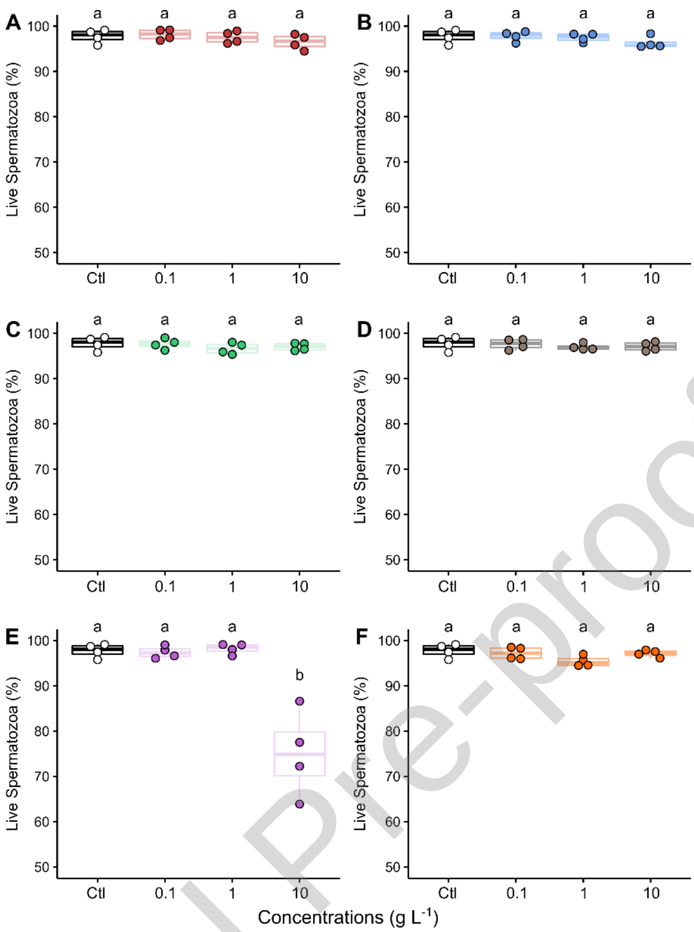


Figure 1. Percentage of live spermatozoa after 1 h exposure to leachates issued from (A) new tires, (B) used tires, (C) new crumb rubber granulates, (D) used crumb rubber granulates, (E) new oyster-farming rubber bands, and (F) used oyster-farming rubber bands at four concentrations: 0 (Ctl), 0.1, 1, and 10 g L^{-1}. The assay was replicated four times. ANOVAs were used to compare treatments with Tukey HSD for pairwise comparisons at the 5% level; homogeneous groups are indicated by the same letter.

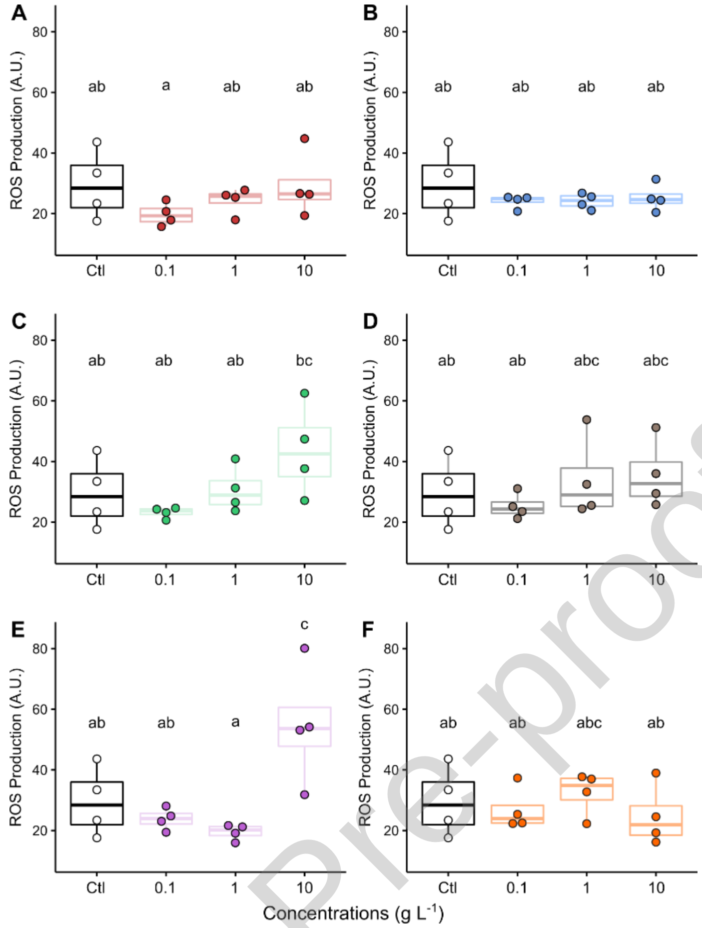


Figure 2. ROS production (A.U.) of oyster spermatozoa after 1 h exposure to leachates issued from (A) new tires, (B) used tires, (C) new crumb rubber granulates, (D) used crumb rubber granulates, (E) new oyster-farming rubber bands, and (F) used oyster-farming rubber bands at four concentrations: 0 (Ctl), 0.1, 1, and 10 g L^{-1}. The assay was replicated four times. ANOVAs were used to compare treatments with Tukey HSD for pairwise comparisons at the 5% level; homogeneous groups are indicated by the same letter.

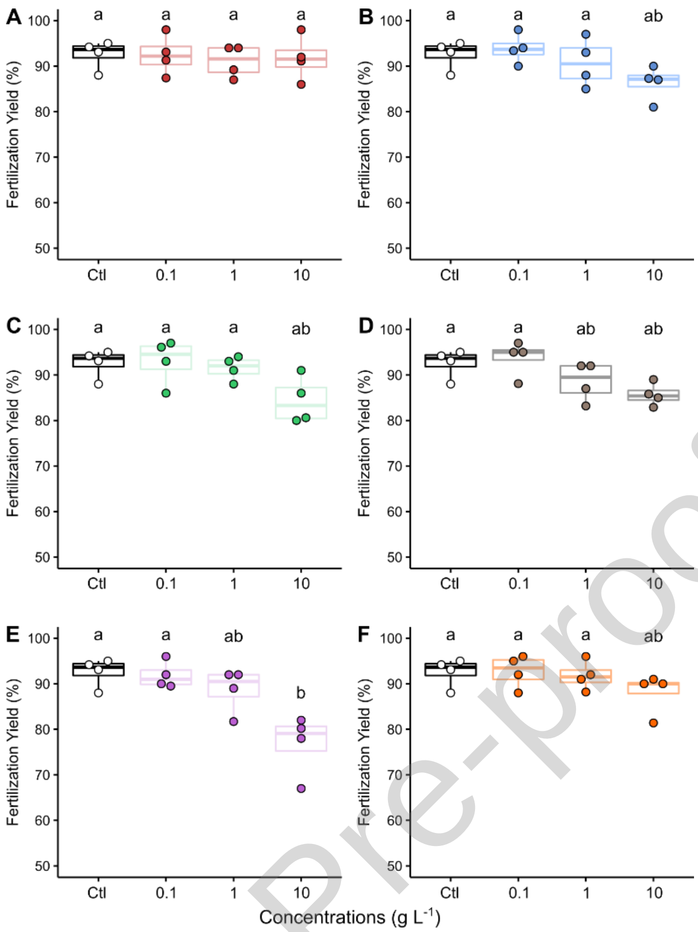


Figure 3. Fertilization yield (%) determined after simultaneous exposure (1.5 h) of oyster gametes (oocytes + spermatozoa) to leachates issued from (A) new tires, (B) used tires, (C) new crumb rubber granulates, (D) used crumb rubber granulates, (E) new oyster-farming rubber bands, and (F) used oyster-farming rubber bands at four concentrations: 0 (Ctl), 0.1, 1, and 10 g L^{-1}. The assay was replicated four times. ANOVAs were used to compare treatments, with Tukey HSD for pairwise comparisons at the 5% level; homogeneous groups are indicated by the same letter.

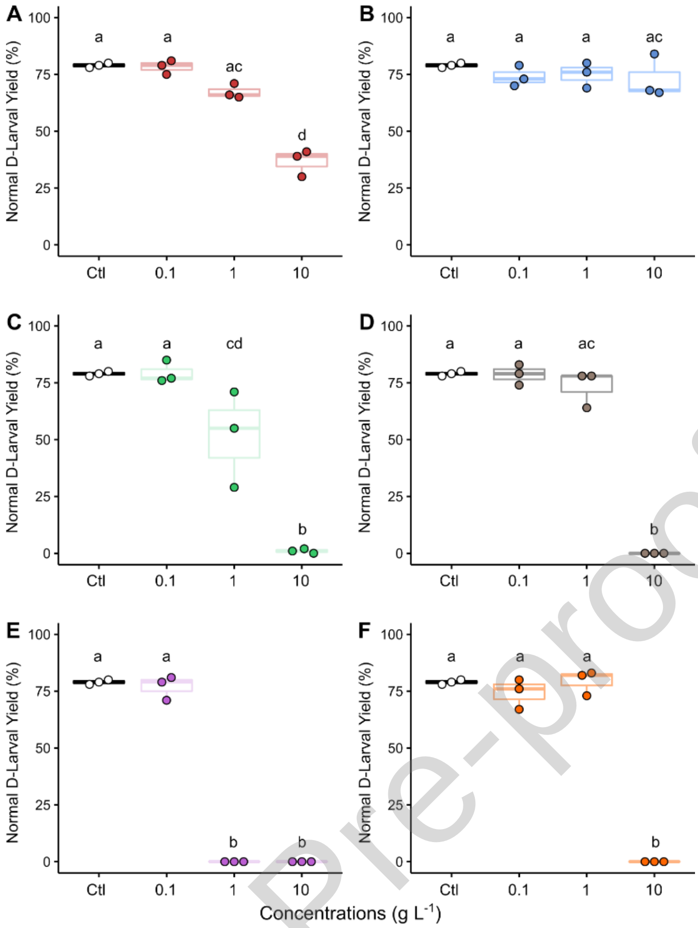


Figure 4. Normal D-larval yields (%) after 36 h exposure of fertilized oocytes to leachates issued from (A) new tires, (B) used tires, (C) new crumb rubber granulates, (D) used crumb rubber granulates, (E) new oyster-farming rubber bands, and (F) used oyster-farming rubber bands at four concentrations: 0 (Ctl), 0.1, 1, and 10 g L^{-1}. The assay was replicated three times. ANOVAs were used to compare treatments, with Tukey HSD for pairwise comparisons at the 5% level; homogeneous groups share the same letter.

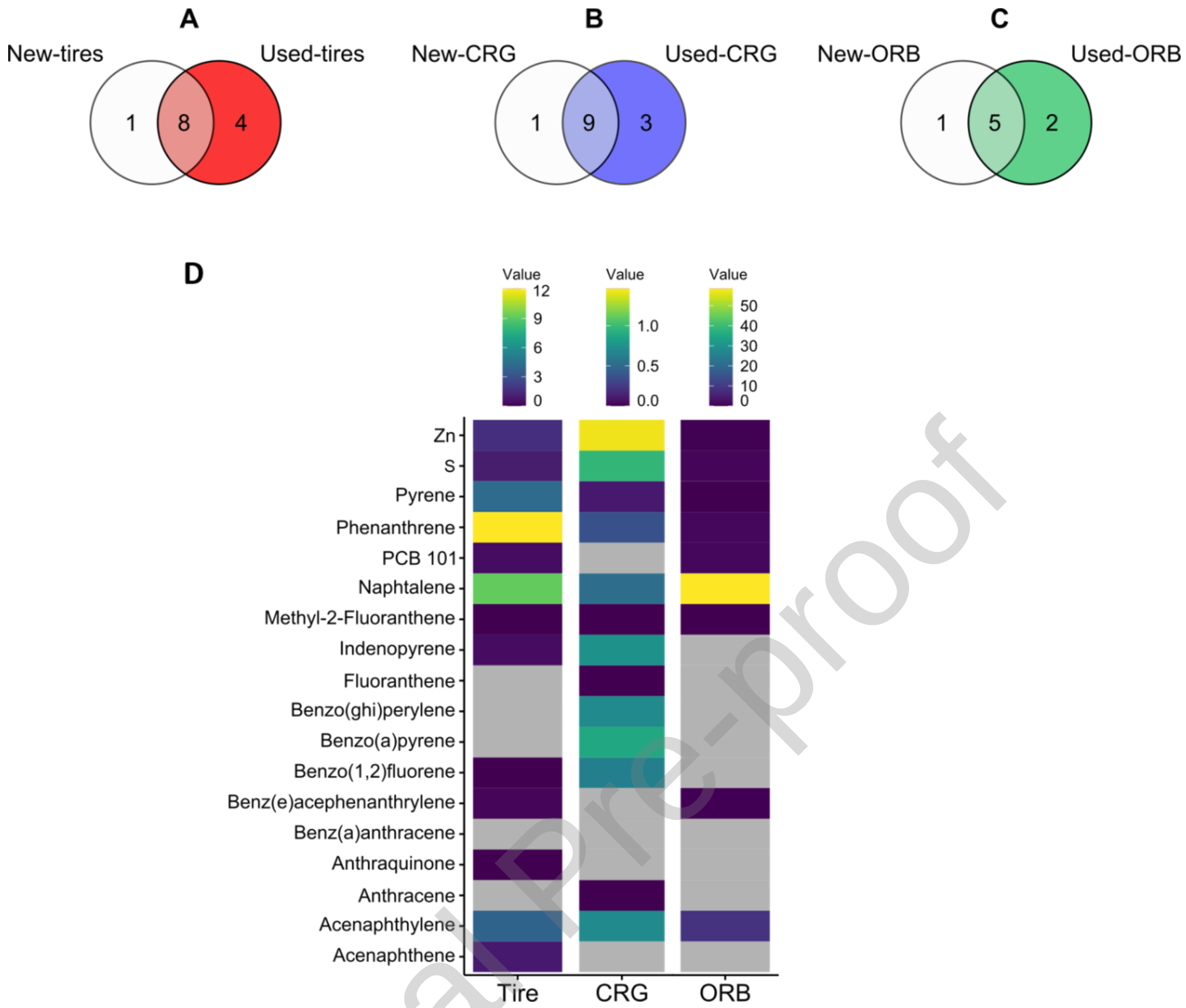


Figure 5. Distribution of the chemical compounds (PAHs and PCB) assayed in leachates issued from (A) new tires vs. used tires, (B) new crumb rubber granulates (CGR) vs. used crumb rubber granulates, (C) new oyster-farming rubber bands (ORB) vs. used oyster-farming rubber bands; (D) Heatmap of chemical compounds detected in leachates using the ratio of new : used products with a specific scale for each rubber item as indicated on the figure. Dark blues indicate negligible difference between new and used products in terms of chemical concentrations while yellow indicates higher concentrations from new items compared with used ones. Grey indicates that the specific compounds were not detected in the corresponding rubber item.

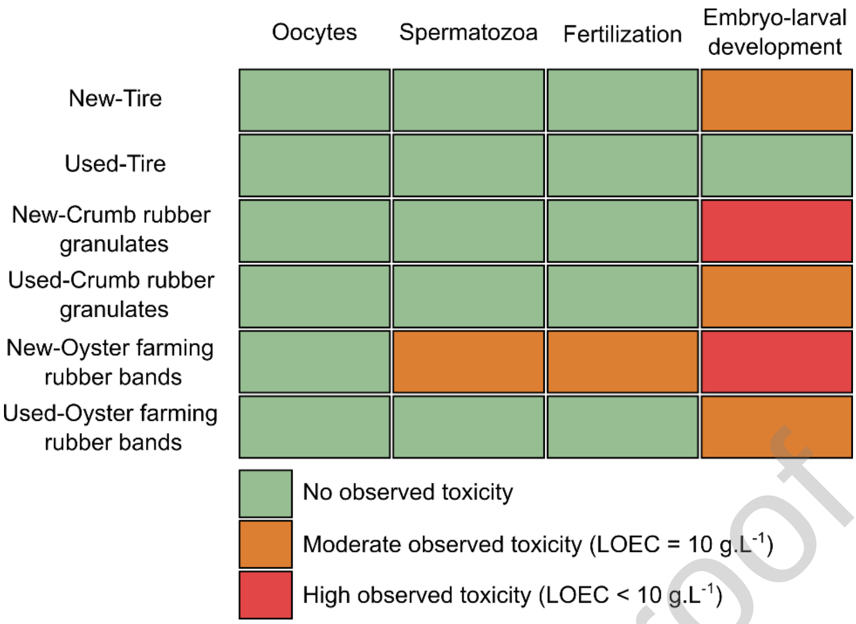
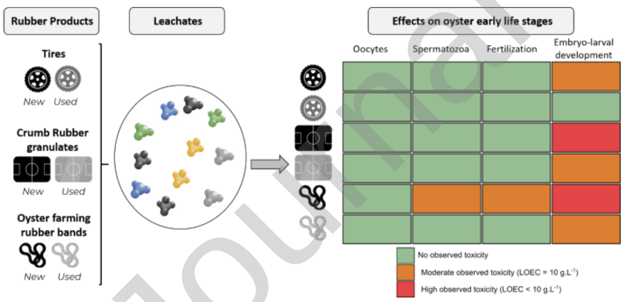


Figure 6. Summary of the toxicity assays performed on oyster early life stages and levels of toxicity observed. LOEC = the lowest observed effect concentration.

Graphical abstract



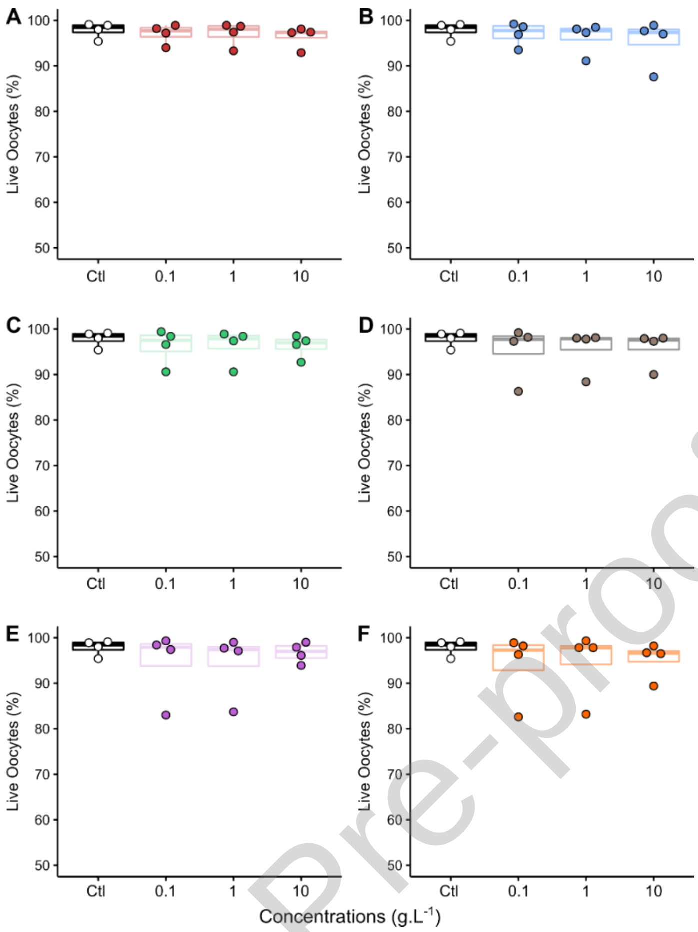
Highlights:

- Leachates of rubber-based products can affect early life stages of Pacific oysters.
- Toxicity differed according to the type of rubber-based products and stade of use (new *vs.* used).
- Oyster-farming rubber bands induced the highest toxicity compared to tires and crumb rubber granulates.

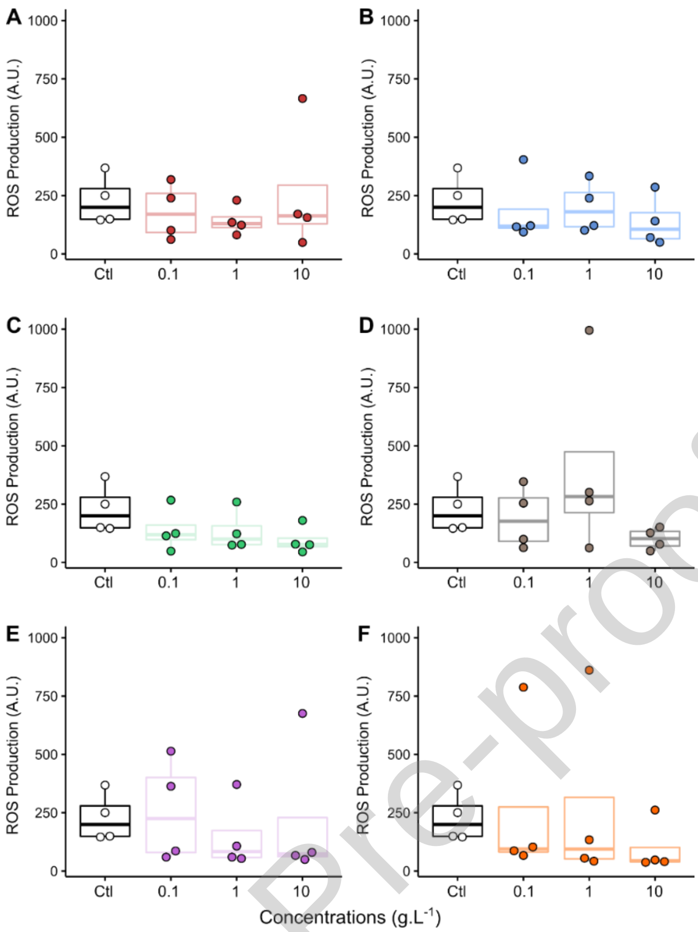
Supplementary Information

Supplementary Table 1. List of targeted compounds analysed in leachates.

Family	Compounds
HAP	Acenaphthene
	Acenaphthylene
	Anthracene
	Anthraquinone
	Benz(a)anthracene
	Benzo(1,2)fluorene
	Benzo(a)pyrene
	Benz(e)acephenanthrylene
	Benzo(ghi)perylene
	Benzo(k)fluoranthene
	Chrysene
	Dibenzanthracene
	Fluoranthene
	Fluorene
	Indenopyrene
	Methyl-2-Fluoranthene
	Methyl-2-Naphtalene
	Naphtalene
	Phenanthrene
	Pyrene
PCB	PCB 101
	PCB 105
	PCB 118
	PCB 138
	PCB 153
	PCB 156
	PCB 180
	PCB 28
	PCB 52



Supplementary figure 1. Percentage of live oocytes after 1 h exposure to leachates issued from (A) new tires, (B) used tires, (C) new crumb rubber granulates, (D) used crumb rubber granulates, (E) new oyster-farming rubber bands, and (F) used oyster-farming rubber bands at four concentrations: 0 (Ctl), 0.1, 1, and 10 g L^{-1}. The assay was replicated four times. ANOVAs were used to compare treatments at the 5% level.



Supplementary Figure 2. ROS production (A.U.) of oyster oocytes after 1 h exposure to leachates issued from (A) new tires, (B) used tires, (C) new crumb rubber granulates, (D) used crumb rubber granulates, (E) new oyster-farming rubber bands, and (F) used oyster-farming rubber bands at four concentrations: 0 (Ctl), 0.1, 1, and 10 g L^{-1}. The assay was replicated four times. ANOVAs were used to compare treatments at the 5% level.