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Tire rubber chemicals reduce juvenile oyster (*Crassostrea gigas*) filtration and respiration under experimental conditions

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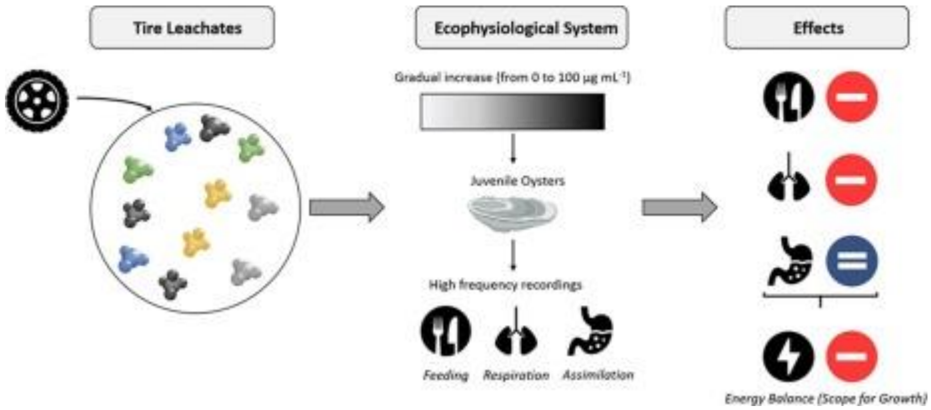
Abstract :

Tires can release a large number of chemical compounds that are potentially hazardous for aquatic organisms. An ecophysiological system was used to do high-frequency monitoring of individual clearance, respiration rates, and absorption efficiency of juvenile oysters (8 months old) gradually exposed to four concentrations of tire leachates (equivalent masses: 0, 1, 10, and 100 μg tire mL−1). Leachates significantly reduced clearance (52 %) and respiration (16 %) rates from 1 μg mL−1, while no effect was observed on the absorption efficiency. These results suggest that tire leachates affect oyster gills, which are the organ of respiration and food retention as well as the first barrier against contaminants. Calculations of scope for growth suggested a disruption of the energy balance with a significant reduction of 57 %. Because energy balance directs whole-organism functions (e.g., growth, reproductive outputs), the present study calls for an investigation of the long-term consequences of chemicals released by tires.

Graphical abstract



Highlights

► Leachates of tires affected clearance and respirations rates of juvenile oysters. ► Decreases in Scope For Growth (SFG) calculations suggest energy balance impairment. ► Leachates did not affect the absorption efficiency suggesting no effect on the digestive functions.

Keywords : Oyster, Tire leachates, Ecophysiology, Scope for growth

1. Introduction

Exponential growth in the use of plastics, leading to continuous release of waste into environment, is a crucial issue (Borrelle et al., 2020; Lau et al., 2020). Each year, 50–70 % of annual rubber production (28.7 Mt in 2019) is used for the tire industry. Road traffic inevitably generates tire particles (TP) by mechanical abrasion. These TP display a large range of sizes, from a few nanometers to particles larger than 100 µm (Kole et al., 2017; Wagner et al., 2018). Emissions of 0.23–4.7 (average = 0.81) kg TP $year^{-1}$ $capita^{-1}$ were estimated across the world (Kole et al., 2017). Annually, global TP emissions could represent 2.9 million tons (Mt) (Evangeliou et al., 2020). Hence, they constitute a significant part of microplastic (MP; particles <5 mm) emissions, notably in the oceans, where TP represent emissions of 0.2–0.7 Mt $year^{-1}$ (Boucher and Friot, 2017). Overall, TP are expected to constitute 93% of the MP contamination in aquatic systems in terms of mass by 2040 if no major improvements are made in plastic waste management (Lau et al., 2020). In addition, tires and TP can release chemical compounds potentially hazardous for aquatic organisms (e.g., Halle et al., 2020). Indeed, tires contain chemical compounds that represent up to 50% of their mass, while this value is on average 7% for other conventional plastic products (Geyer et al., 2017; Wagner et al., 2018). Leachate from tires is made up of a wide diversity compounds of varying complexity. For instance, no less than 2,000 chemical signatures were found in leachates obtained from 250 mg TP L^{-1} (Tian et al., 2020). They contain additives (e.g., antioxidants), transformation products, and other hazardous compounds such as metals (e.g., Zn) or extender oils and softeners (i.e., polycyclic aromatic hydrocarbons, PAHs) (Kolomijeca et al., 2020; Sadiktsis et al., 2012; Wagner et al., 2018). For instance, leachate analyses revealed the presence various compounds including zinc (519 µg L^{-1}), acenaphthene (24.1 ng L^{-1}), acenaphthylene (222.6 ng L^{-1}), benz[a]anthracene (2.1 ng L^{-1}), naphthalene (438.5 ng L^{-1}), phenanthrene (49.4 ng L^{-1}), pyrene (5.1 ng L^{-1}) or PCB101 (8.4 ng L^{-1}) after 14 days of leaching in seawater using a concentration of 100 g tires L^{-1} (Tallec et al., 2022).

Although TP ingestion could induce physical damage (Halle et al., 2020), the toxicological risk posed by tires has been suggested to be mainly associated with the release of chemical compounds (McIntyre et al., 2021). Tire leachate has been reported to induce detrimental effects on survival, reproductive outputs, and developmental processes under experimental conditions (e.g., *Mytilus galloprovincialis*, Capolupo et al., 2020; *Crassostrea gigas*, Tallec et al., 2022; *Daphnia magna* and *Xenopus laevis*, Gualtieri et al., 2005; *Pimephales promelas*, Kolomijeca et al., 2020; *Ceriodaphnia dubia* and *Daphnia magma*, Wik et al., 2009). In addition, it has recently been demonstrated that mortality events affecting coho salmon (*Oncorhynchus kisutch*) for decades in western North America are related to tire leachate released by road traffic (Tian et al., 2020). Commonly, the toxicity of tire leachates is associated to the release of metals (especially the zinc; e.g., Gualtieri et al., 2005), PAHs (e.g., Kolomijeca et al., 2020) even though in some cases it is unknown products (e.g., transformation products) that are responsible for the toxic effects (Tian et al., 2020). Investigating the “tire chemical risk”, therefore, appears to be an important issue that can provide additional data for risk assessments (McIntyre et al., 2021).

Monitoring the ecophysiological response of marine mollusks provides valuable data to estimate their bioenergetics and explore the effects of contaminants on their life history traits and population sustainability (Sokolova, 2013). Energy balance is based on controlled and integrated processes enabling the organism to regulate energy intake, expenditure, storage and excretion (Nadal et al., 2017). Alterations of energy budget can have long-term and irreversible consequences for individuals and populations. The automatic ecophysiological system used in the present study, based on flow-through chambers, is a powerful, non-invasive method to obtain reliable data on the individual physiological response of bivalves and to estimate their energy budget before, during, and after an exposure to environmental stressors. Here, we used the Pacific oyster (*Crassostrea gigas*) as a marine model organism. Oysters are engineer species regulating essential ecological functions (e.g., water quality, phytoplankton biomass, biodiversity) in coastal environments, and are commonly used as bioindicators of ecosystem health (e.g., Bayne, 2017). We assessed the effects of tire leachates on individual ecophysiological performances (clearance and respiration rates, absorption efficiency) of juvenile oysters using gradual concentration exposures (from 0 to 100 µg tires mL^{-1}). A flow-through system was used and provided a constant flow of phytoplankton enriched seawater at controlled temperature, equipped with a tracking system consisting of continuous recorders of hydrobiological parameters taking high-frequency measurements. It provides a life-cycle approach as a complement to the standardized embryo-larval development tests available for marine bivalves and already shows to be disrupted in oyster when facing tires chemical compounds (Tallec et al., 2022).

2. Materials and Methods

2.1. Animals

Juvenile oysters (8 months old; length: 31.6 ± 2.9 mm; dry mass: 0.11 ± 0.04 g; produced as described in Petton et al., 2015) were transferred from the Ifremer nursery in Bouin, France, to the experimental facility in Argenton, France, in September 2020. The oysters were acclimatized for 10 days in a 50-L tank supplied with UV-treated 1-µm filtered seawater (17°C, pH 8.1) and fed *ad libitum* with a balanced mixture of two microalgae, *Tisochrysis lutea* (T-iso CCAP927/14, cell volume = 40 µm^3) and *Chaetoceros* sp. (CCAP 1010/3, cell volume = 80 µm^3), so that the algae concentration in the tank outflow was kept constant at 1500 µm^3 mL^{-1}. Between each trial, oysters were maintained under similar conditions to those applied in the ecophysiological system (see section 2.3).

2.2. Tires and leachate preparation

A mixture of three tires (one new and two secondhand; Table 1) was used to produce leachates in order to incorporate the variability in tire chemical composition resulting from usage (new/used) and brand (Redondo-Hasselerharm et al., 2018; Sadiktsis et al., 2012; Wik et al., 2009). The outer rings of the tires (first 2 cm) were cut into 1–5 mm pieces using stainless steel scissors, then mixed (taking equal weights of each tire) to produce leachates. Leachates (concentrations detailed in section 2.3) were produced as described in Capolupo et al. (2020), i.e., in sterile, 0.2-µm filtered seawater for 14 days using an orbital shaker (240 rpm) in dark conditions at room temperature (~20°C). At the end of this incubation, stock solutions were filtered on glass-fiber filters (GF/F; porosity 1.2 µm) to remove particles. To avoid a degradation of chemicals in the leachate during the exposures, a new and fresh leachate solution is used every day.

Table 1. Type of tires used in the present study.

Tires	Sources
New Tire	Dunlop SP Sport Maxx (Dot code: DM75 JA2R 3916) purchased from an automobile repair garage located in Brest (France).
Used Tire 1	Dunlop SP Sport Maxx (Dot code: DM75 JA2R 3916) purchased from an automobile repair garage located in Brest (France).
Used Tire 2	Michelin Radial X (Dot code: HDWC KUBX 2013) obtained from personal usage.

2.3. Exposures and ecophysiological measurements

The ecophysiological system consists of nine individual flow-through chambers (0.54 L) supplied with seawater (flow rate = 27 ± 3 mL min^{-1}) containing a mixed diet of *T. lutea / Chaetoceros sp.* (50/50, v/v). A schema of the system is shown in Figure 1. It is described in detail in Pousse et al. (2018). This system has been designed to be as respectful as possible of the rearing conditions and not to cause stress to the animals, which is shown by the absence of a time effect in several series of experiments (*e.g.* Pousse et al., 2018; Tallec et al., 2021). Briefly, all chambers are managed by a programmable controller allowing high-frequency automatic recordings of fluorescence (used as a proxy for phytoplankton concentration), oxygen concentration, and water flow in the seawater outflow using a WETStar fluorimeter (WSCHL-1400 WETLABS; USA), a WTW multiparameter probe (WTW Multi 3430), and a SONOFLOW CO.55 ultrasonic flow meter (Sonotec; Germany), respectively. Temperature and pH were continuously recorded using the multiparameter probe (Supplementary Table 1 to see the data of all trials). In each chamber, measures were performed for 12 minutes every 3.5 h. Eight oysters were placed in the system, one animal per chamber, while a "fake oyster" (a clean empty shell) was put in the remaining chamber (control chamber – CC). Upstream of the nine chambers, a mixing tank (0.94 L) was used to inject leachates into running seawater, using a peristaltic pump to ensure a continuous exposure. Over one trial, oysters were exposed gradually to four concentrations of tire leachate corresponding to 0, 1, 10, and 100 µg tire mL^{-1}. These gradual exposures were used to check the capacity of oysters to modify rapidly their responses to increasing concentrations of tire leachates based on tipping points approach very used in OA (e.g. Lutier et al., 2022). For each concentration, exposures were conducted for 40.5 h. By targeting exposure duration of a mean of 2 days, a recording of 40.5 h was obtain taking into account the time required to set up and clean up the system plus the time needed for the oysters to start their breathing and feeding. One trial, therefore, lasted 7 days (40.5h × 4 concentrations = 162h). Three successive trials were run leading to individual data for 22 oysters (2 oysters remained completely closed during the trials and were, therefore, removed from the dataset).

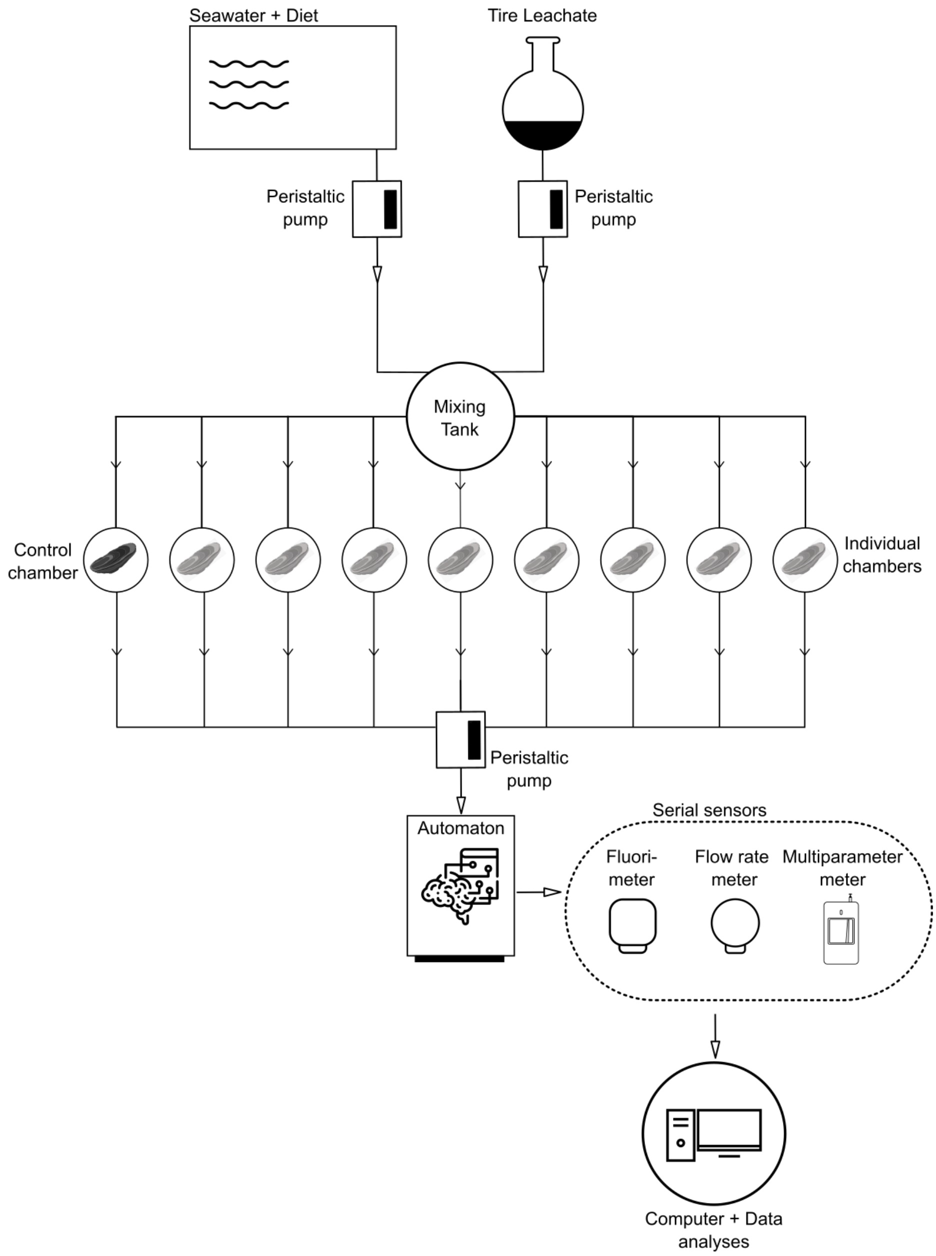


Figure 1. Schema of the ecophysiological system.

2.3.1. Clearance and respirations rates

The individual clearance rate (CR), an indicator of feeding activity, of each oyster was calculated as: CR = (fl × ($C_{CC} - C_N$)/C_{CC}), where fl is the flow rate through the chamber (L h^{-1}), C_{CC} is the fluorescence in the control chamber, and C_N is the fluorescence in a chamber housing one oyster (e.g., Bayne, 2017). The individual respiration rate was calculated as: RR = (fl × ($O_{CC} - O_N$)), where O_{CC} and O_N are the O_2 concentrations (mg O_2 L^{-1}) in the control chamber and in a chamber housing one oyster, respectively (Savina and Pouvreau, 2004). To standardize CR and RR for 1 g of dry tissue, oysters were sacrificed at the end of the experiment and stored at -20°C, then used to estimate the individual dry mass after 24-h lyophilization of the soft tissues (Bayne et al., 1987).

2.3.2. *Absorption efficiency*

The absorption efficiency (AE) of organic matter from the diet was estimated using Conover's method, which consists of collecting feces over 24 hours and 50 mL of the diet. No pseudo-feces were produced by oysters in any of the chambers. Feces and microalgae were filtered and rinsed three times with isotonic ammonium formate. All filters were frozen at -20°C. The total weight and organic fraction were calculated after measurement of the filter weight of each sample after 24 h at 60°C and then 4 h at 450°C (Pousse et al., 2020). Absorption efficiency was calculated as: $AE = (f - e)/((1 - e) \times f)$, where f corresponds to the organic fraction of the microalgae ration and e is the organic fraction of the feces (Conover, 1966).

2.3.3. *Scope for growth (SFG)*

The scope for growth ($J\ h^{-1}\ g^{-1}$) was estimated as: $SFG = (C \times AE) - R$, where C is the rate of energy ingestion ($J\ h^{-1}\ g^{-1}$), AE the absorption efficiency, and R the rate of energy catabolized through respiration ($J\ h^{-1}\ g^{-1}$). The rate of energy ingestion was calculated as: $C = CR \times POM$ where POM corresponds to the particular organic matter measured in the water ($mg\ L^{-1}$). For energy conversion, values of 20.3 J and 14.1 J were used for 1 mg of particulate organic matter and 1 mg of O_2, respectively (Bayne et al., 1987; Bayne and Newell, 1983; Gnaiger, 1983).

2.3.4. *Histology*

In addition to the ecophysiological parameters, 10 control oysters were sampled in the oyster batch (see section 2.1) just before the beginning of the exposure (T0) and 6 oysters (2 oysters randomly selected per trial) were sampled at the end of the exposure to the highest leachate concentrations to conduct histology analyses. A 3-mm cross-section was cut and fixed in modified Davidson's solution (4°C for 48 h) and further processed as described in Fabioux et al. (2005). Finally, sections were observed under a microscope to identify potential tissue damage (Zeiss AxioObserver Z1).

2.4. Chemical analyses

Chemical analyses were conducted on: (1) the seawater not supplemented with leachate, i.e. the control treatment, (2) the tire leachate at concentration 1 ($1\ \mu g\ mL^{-1}$), (3) the tire leachate at concentration 2 ($10\ \mu g\ mL^{-1}$), and (4) the tire leachate at concentration 3 ($100\ \mu g\ mL^{-1}$). The samples (1L per treatment) were stored at -20°C until chemical analyses. Targeted chemicals were selected according to the data available in the literature (e.g., Aatmeeyata and Sharma, 2010; Kolomijeca et al., 2020; Tallec et al., 2022). Metal concentrations (Zn, Al, Cu, Fe; LOQ and LOD described in the Supplementary Table 2) were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES) according to the standardized protocol NF ISO 11885. Analyses were performed using an Agilent ICP-OES 5110 coupled with an Agilent SPS 4 autosampler. To reduce matrix effects, samples were diluted (1:10) with acidified water and HNO_3 (0.5%). An internal standard (Yttrium) was added to all samples. To verify results, control solutions were injected every 10 samples and compared with control charts. Organic micropollutant concentrations were measured by gas chromatography–mass spectrometry (GC-MS/MS). Analyses were performed using a 6890 N Network GC System coupled with an autosampler 7683 B Series Injector 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. We performed a first extraction with CH_2Cl_2. For the second extraction, the leachate was adjusted to pH 2 using H_2SO_4 50/50. Then, we added ethyl acetate, glacial acetic acid and CH_2Cl_2 and the sample was agitated for 20 min. The organic phase was added to the first fraction. Finally, a last extraction was performed using CH_2Cl_2. The extracts were purified by passing them through a fluorisil column. Before injection into the GC-MS, we completed an evaporation step at 35°C in an evaporator (Zymark). For the four treatments, 20 polycyclic aromatic hydrocarbons (PAHs; 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-naphthalene, naphthalene, phenanthrene, pyrene; LOQ and LOD described in the Supplementary Table 2) and 9 polychlorinated biphenyls (PCBs; PCB 101, PCB 105, PCB 118, PCB 138, PCB 153, PCB 156, PCB 180, PCB 28, PCB 52; LOQ and LOD described in the Supplementary Table 2) were screened using the selected ion monitoring (SIM) method.

2.5. Statistical analyses

Statistical analyses and graphical representations were conducted using R software (R version 4.0.2). Normality and homogeneity of variance were screened for using Shapiro–Wilk and Levene tests, respectively. Clearance and respiration rates estimated from data recorded by the ecophysiological system were analyzed using repeated measures ANOVAs with pairwise comparisons using Tukey's method when necessary. Absorption efficiency and SFG were compared using one-way ANOVAs and Tukey tests. Differences were considered significant when p-values < 0.05. Data are reported as mean ± standard deviation.

3. Results and discussion

Tire leachates impair juvenile oyster physiology with repercussions for the energy budget. Investigating changes in ecophysiological parameters of mollusks in response to plastic debris, specifically tire leachates, is a novel approach. From the lowest leachate concentration (1 µg mL^{-1}), both clearance rate (CR) and respiration rate (RR) were significantly altered compared with the control (Figures 2A & 2B). At 1 µg mL^{-1}, the CR decreased significantly by 52% (p-value < 0.001), but no differences (p-value > 0.05) were observed among leachate concentrations. The RR was significantly reduced by 16% at 1 µg mL^{-1} and by 32% at the two higher concentrations, which remained statistically similar (10 and 100 µg mL^{-1}) (p-values < 0.001). No effects (p-value > 0.05) were recorded on the absorption efficiency whatever the leachate concentration (average AE = 41.8 ± 6.2%; Figure 2C). These energetic and metabolic losses lead to a direct incidence on the oyster bioenergetics, reflected by significant decreases in SFG for all leachate concentrations (-56%; p-value < 0.001; Figure 2D). No significant differences were observed among leachate concentrations, suggesting no cumulative impacts despite the use of gradual exposures (p-value > 0.05; Figure 2D). The identification of tipping points beyond which adverse effects are observed would be a broadly generalizable approximation of the susceptibility of tire leachates, but this proved impossible as effects surprisingly appeared at the lowest concentration. Scope for growth is defined as the remaining energy after the maintenance costs necessary for individual survival, i.e., it is the energy available for growth, reproduction, and survival (Bayne and Newell, 1983; Stickle and Bayne, 1987). Overall, a positive SFG means that animals have enough energy for growth and reproduction, while a negative SFG means the animal loses energy, with drastic consequences potentially implying death (Stickle and Bayne, 1987). The SFG reduction from 1 µg mL^{-1} is noteworthy but may be inconsequential for oysters in such experimental conditions based on the concept of energy-limited tolerance to stress (Sokolova, 2013). Our lab-controlled "environmental" conditions, i.e., seawater temperature and oxygen concentration, trophic conditions, and absence of environmental stressors of any sort except tire leachates, can be considered to be optimal for *C. gigas* (e.g., Bayne 2017). The aerobic scope decreased upon leachate exposure but we cannot detect any potential shift from the optimum range to the pejus (moderate stress that still allows the animal to sustain itself) or the pessimum range (high stress implying whole-organism effects, e.g., decreases in growth and reproductive output) (Sokolova, 2013; Nadal et al., 2017). Based on the present ecophysiological data, we consider that more investigation is needed on the effects of long-term exposures on key oyster functions (e.g., growth, reproduction) in a more realistic context (harsher environmental conditions such as low food and water quality). In addition, because some chemicals commonly added to tires during their manufacture (e.g., bisphenol A; PCBs, PAHs) are considered as "metabolism-disrupting chemicals" (Nadal et al., 2017), assessing their impact on oyster maturity over generations is of interest to investigate the long-term toxicity to aquatic life of chemicals released by tires.

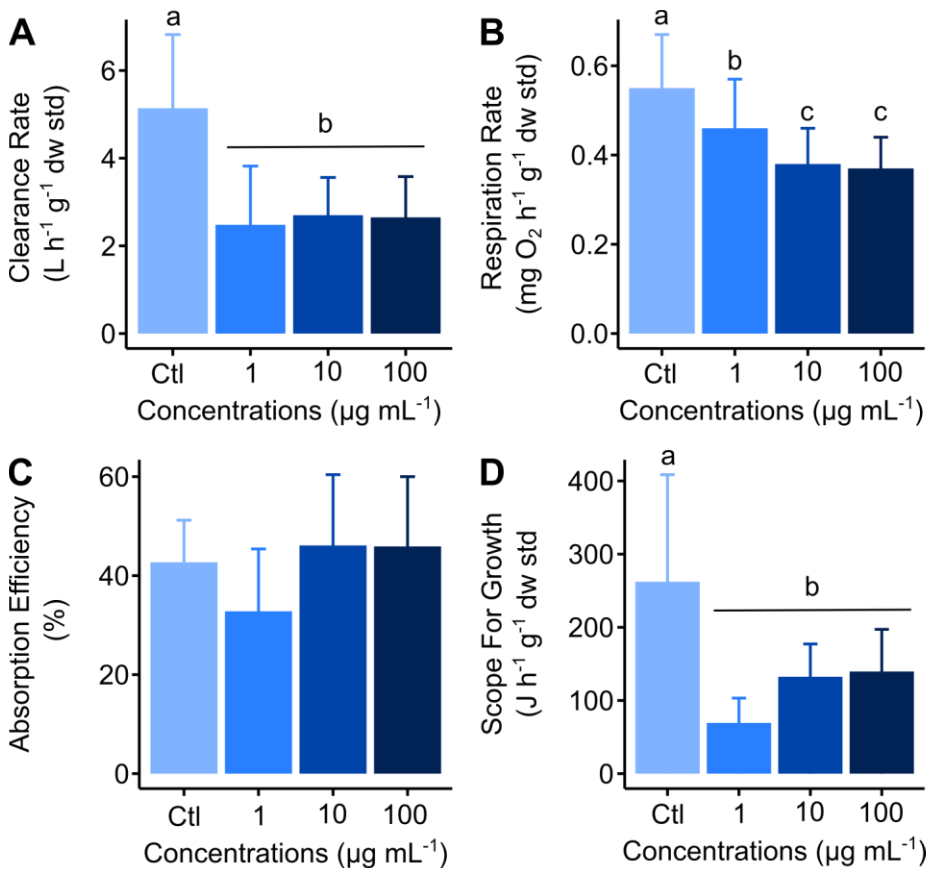


Figure 2. Individual clearance rate (A; L h^{-1} g^{-1} dw std), respiration rate (B; mg O_2 h^{-1} g^{-1} dw std), absorption efficiency (C; %) and scope for growth (D; J h^{-1} g^{-1} dw std) in juvenile oysters exposed to different concentrations of tire leachates (from 0 – Ctl to 100 µg mL^{-1}). Results are expressed as mean ± SD (n = 22 oysters). Repeated measures ANOVAs were conducted to compare treatments for the clearance and respiration rates while one-way ANOVAs were used for the absorption efficiency and SFG at the 5% level; homogeneous groups are indicated by the same letter.

Potential toxic pathways. The decreases in clearance and respiration rates with exposure to tire leachates suggest detrimental effects on the gills, which are a major barrier oysters have against any physical particles and chemical molecules they come into contact with in the surrounding environment, including contaminants. Indeed, gills have several crucial roles in bivalves as they are responsible for oxygen and food intake. Initially, we thought that, in an extreme case, tire leachates could induce gill tissue damage, but histology revealed no effect of this kind on any of the analyzed oysters (Supplementary Figure 1). The effect must, therefore, operate in another manner. As bivalves possess chemoreceptors on pallial organs that react to chemical cues (Newell and Jordan, 1983; Ward et al., 1992), the gills may act as sensors of chemical signals from the tire leachates. This could affect valve activity (e.g., valve gap, Jørgensen, 1996) as previously suggested in response to various contaminants (e.g., MP, Bringer et al., 2021; algal toxins, Mat et al., 2013, Castrec et al., 2018). Valve activity modifications can lead to changes in energy budget that result in whole-organism effects. For instance, in juvenile oysters, MP alter valve activities, reducing the daily growth, a sign of change in food intake (Bringer et al., 2021). Furthermore, the “chemicals × gills” interaction could also affect the production and quality of mucus. In oysters, mucus on pallial organs participates in many functions, including particle capture, defenses, and depuration processes (Pales Espinosa et al., 2016; Sze and Lee, 1995). Chemical modifications in the surrounding environment lead to modifications in mucus secretion in marine bivalves and affect nutrition, notably particle ingestion and rejection processes (Meseck et al., 2020; Pales Espinosa et al., 2016). It would therefore be interesting to study the mucus to test the relationship between mucus production and gill functioning during exposure to tire leachates. At the cellular level, the reduction in oxygen uptake could be linked to a disruption of mitochondria functioning in gills following an oxidative stress, i.e., a modified balance between reactive oxygen species (ROS) and antioxidant production in the presence of stressors (Sokolova and Lannig, 2008; Bhatti et al., 2017). Overproduction of ROS in gills can result in them attacking mitochondria, altering membrane permeability, and inducing mitochondrial DNA damage and homeostasis disruption, resulting in overall mitochondria damage, notably to oxidative phosphorylation (the mechanism that uses O_2 to generate ATP) (Bhatti et al., 2017). In line with this hypothesis, recent studies have suggested that oxidative stress resulting from tire leachate exposures can lead to increased ROS production in oyster spermatozoa (Tallec et al., 2022), increased lipid peroxidation in mussels (Capolupo et al., 2021), and disruption of the antioxidant system in rainbow trout (Stephensen et al., 2005).

Absorption efficiency (AE), i.e., the capability of oysters to convert the organic content of food into energy to supply key functions, represents a robust proxy of the overall functioning of the digestive system (concept developed by Conover, 1966; see Huvet et al., 2015 for an example). In the past, it was demonstrated that exposures to toxins or chemicals can potentially affect energy intake from food by alterations of the gut cells and microbiota (Nadal et al., 2017; Widdows et al., 1979). In our control treatment, the level is in line with the AE described in the literature for similar conditions (Hall et al., 2020; Sussarellu et al., 2016; Tallec et al., 2021). During the exposures, the AE remained unaltered, suggesting that our short-term exposures to tire leachates did not affect food assimilation or general digestive functions.

Chemical compounds beyond the scope of quantification. Our targeted chemical analyses performed on all leachate concentrations did not permit us to detect or quantify chemical compounds (metals, PAHs and PCBs) released into the seawater during the 14-day leaching period. Detection of chemicals in tire leachates are usually performed on much higher concentrations (> 1 g L^{-1}; e.g., Capolupo et al., 2020, Kolomijeca et al., 2020) than those of the present study (the highest concentration = 0.1 g L^{-1}). In a previous study using the same tires but a leachate concentration of 100 g L^{-1}, a wide range of hazardous chemicals were identified, including zinc, acenaphthene, acenaphthylene, benz(*a*)anthracene, naphthalene, phenanthrene, and pyrene (Tallec et al., 2022). However, as seen in our study, chemicals present in quantities below the detection limit, acting alone or in combination with others (cocktail effect), can induce deleterious effects (Escher et al., 2020). Using *in situ* tire fragments, smaller than those used in the present study, would be interesting to carry out, in particular testing whether smaller particles with a higher surface/volume ratio lead to higher leaching and toxicity. A first study has suggested a low incidence of particles size in the leaching potential (Halsband et al., 2020).

While the numerous compounds listed above can be responsible for toxicity (Dey et al., 2021; Sun et al., 2020), we cannot demonstrate their implication in the observed impacts. Yet understanding the modes of action and decision support need to know which compound alone or in a mixture is responsible for the toxicity even for low or ultra-low chronic exposures. The SIM mode was used to target specific compounds commonly identified in tire leachates (e.g., Kolomijeca et al., 2020). However, it is known that tires can harbor hundreds of chemicals, including unknown compounds (e.g., transformation products) in a similar way to NIAS (non-intentionally added substances (such as solvent, reaction, and degradation by-products) in the context of food or article contact materials (FCM or FCA) (Halle et al., 2020; Tian et al., 2020; Wiesinger et al., 2021; Zimmermann et al., 2021). Transformation products are hardly detectable or measurable without specific analytical developments (Tian et al., 2020) characterized by low detection limits, which requires significant methodological work that is time and budget intensive. High-throughput nontarget chemical analyses to identify chemicals released by tires (and plastics more generally), such as UPLC-QTOF-MS (Zimmermann et al. 2021), are critical in the context of regulation as well as for developing methods to spotlight potential tracers of tire debris (Klöckner et al., 2021; Tian et al., 2020; Wiesinger et al., 2021; Zimmermann et al., 2021). Because toxic effects may be related to a cocktail effect rather than to individual compounds, chemical analyses are not always enough for risk assessment and decision support (Svingen and Vinggaard, 2016; D'Almeida et al., 2020; Escher et al., 2020). Furthermore, not all chemicals can be detected and quantified owing to current methodological limits (Escher et al., 2020). Toxicological tools are, therefore, an alternative to gain an overall view of chemical risks (Kristiansson et al., 2021). Considering that bio-assays are sometimes more powerful than chemical quantification methods to detect presence and effect of compounds often present below detection limit (Kiyama and Wada-Kiyama, 2015), the ecophysiological system constitute a usefool tool to assess the whole risks related to tire (and other plastic products) leachates pending fine characterization of the unknown toxic compounds in order to set up specific regulations.

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Disclosure Statement

The authors report no conflicts of interest.

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