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Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms

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ABSTRACT

Synthetic polymer-based materials are ubiquitous in aquatic environments, where weathering processes lead to their progressive fragmentation and the leaching of additive chemicals. The current study assessed the chemical content of freshwater and marine leachates produced from car tire rubber (CTR), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS) and polyvinyl chloride (PVC) microplastics, and their adverse effects on the microalgae *Raphidocelis subcapitata* (freshwater) and *Skeletonema costatum* (marine) and the Mediterranean mussel *Mytilus galloprovincialis*. A combination of non-target and target chemical analysis revealed a number of organic and metal compounds in the leachates, including representing plasticizers, antioxidants, antimicrobials, lubricants, and vulcanizers. CTR and PVC materials and their corresponding leachates had the highest content of tentatively identified organic additives, while PET had the lowest. The metal content varied both between polymer leachates and between freshwater and seawater. Notable additives identified in high concentrations were benzothiazole (CTR), phthalide (PVC), acetophenone (PP), cobalt (CTR, PET), zinc (CTR, PVC), lead (PP) and antimony (PET). All leachates, except PET, inhibited algal growth with EC_{50} values ranging from 0.5% (CTR) and 64% (PP) of the total leachate concentration. Leachates also affected mussel endpoints, including the lysosomal membrane stability and early stages endpoints as gamete fertilization, embryonic development and larvae motility and survival. Embryonic development was the most sensitive parameter in mussels, with EC_{50} values ranging from 0.8% (CTR) to 65% (PET) of the total leachate. The lowest impacts were induced on D-shell larvae survival, reflecting their ability to down-regulate motility and filtration in the presence of chemical stressors. This study provides evidence of the relationship between chemical composition and toxicity of plastic/rubber leachates. Consistent with increasing contamination by organic and inorganic additives, the leachates ranged from slightly to highly toxic to mussels and algae, highlighting the need for a better understanding of the overall impact of plastic-associated chemicals on aquatic ecosystems.

1. Introduction

Contamination of the oceans by plastic has been estimated to cause an annual loss of 1–5% in ecosystem services, resulting in economic damages of approximately US$ 2.5 billion per year (Beaumont et al., 2019). Owing to the increasing rate of discharge (now estimated at >8 million tons per year) and very slow degradation rates under typical environmental conditions, plastic litter is expected to persist in the environment over geological timescales (Beaumont et al., 2019). Macroplastic (<1 m), mesoplastic (2.5 cm–5 mm), microplastic (5 mm–1 μm) and nanoplastic (<1 μm), have the potential to interact with aquatic organisms from all trophic levels, resulting in a range of deleterious effects (Kühn et al., 2015; Barboza et al., 2019; GESAMP, 2016). In addition to the risks associated to conventional thermoplastics (e.g. PVC, PET, polyethylene or polystyrene), concerns are emerging about the possible impacts of other polymer-based materials, including microparticles derived from car tire rubber (CTR) (Wagner et al., 2018). CTR microparticles generated during use or through

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processing end-of-life tires (ELTs) are regarded as one of the greatest contributors to 'microplastic' pollution. These fragments, which fall within the definition of microplastics, are increasingly employed in a range of products and in sport facilities, and are estimated to reach the marine environment at a rate of half million tons per year (Hann et al., 2018).

Most thermoplastics are made by mixing the basic polymer(s) with a variety of chemicals that either aid the production process or impart specific properties that render the final product commercially more desirable or more appropriate for its intended application (Hermabessiere et al., 2017). These chemicals include, amongst others, low molecular-weight oligomers, catalyst materials, solvents, and a wide range of organic and inorganic compounds (Turner, 2016). The latter group, referred to as additives, may represent a high percentage of the final plastic materials (in some cases up to 50%) and are used to meet safety standards in electronic materials and prevent ignition (e.g. flame retardants, including polybrominated diphenyl ether), make plastics more flexible (e.g. polyesters, phthalates), reduce degradation from UV rays and heat (e.g. phenols, arylamines), protect from chemical degradation, or impart a desired color (Thompson et al., 2009; Turner, 2016). CTR also contains a broad range of components, including fillers (carbon black, clay, silica, calcium carbonate), stabilizers (antioxidants, antiozonants, waxes), cross-linking agents (sulfur, accelerators, activators), and secondary components such as pigments, oils, resins, and short fibers potentially harmful to health and environment (Canepari et al., 2018; Rodgers and Waddell, 2013).

Most additives are not covalently bound to the plastic polymer and so they can migrate to the material surface, potentially being released into the environment (Hermabessiere et al., 2017; Paluselli et al., 2019; Schmidt et al., 2019). Indeed, additives have been found in effluents from waste water treatment plants, surface and marine waters (Al-Odaini et al., 2015; Hirai et al., 2011; Schmidt et al., 2019). Additives such as bisphenol A (BPA) and phthalates are listed as potential endocrine disrupting chemicals, as they are able to impair hormone regulation in wildlife and humans (ECHA, 2018; UNEP, 2017). BPA is widely used to obtain clear and tough plastics and has been detected in food and beverages following release from packaging (Vandermeersch et al., 2015). High molecular weight phthalates, which allow production of flexible vinyl plastics (e.g. PVC), and organophosphate esters have widely been found at μg/L concentrations in surface waters (Golshan et al., 2015; Schmidt et al., 2019). In addition, brominated flame retardants (BFRs) are known to leach from plastics into the surrounding matrix (Engler, 2012; Meeker et al., 2009). Compounds leaching from CTR include benzothiazoles, phthalates, and phenols, where benzothiazole is typically observed in the highest quantities (Li et al., 2010; Llompart et al., 2013).

Several additives have been shown to adversely affect aquatic species, including BPA (Canesi and Fabbri, 2015), phthalates (Net et al., 2015) and BFRs (Ezechiáš et al., 2014). However, exposure to leached plastic additive mixtures has received far less attention, and knowledge regarding fate and effects is limited. The leaching of additives from plastic materials is differently modulated by the permeability of the polymer matrix, gaps between polymer molecules, physicochemical properties of the additives, properties of the surrounding medium (e.g. salinity, temperature, pH) and time (Kwan and Takada, 2016). Although a high number of chemical compounds may leach from plastics, these can be at concentrations lower than analytical detection limits or not included in routine analysis protocols. This reduces the potential for identifying and quantifying the chemical composition of leachates and subsequently establishing possible links between observed toxicity and specific leachate constituents. Nevertheless, adverse effects from leachate exposures have been documented on several organisms, including fish (He et al., 2011), photosynthetic bacteria (Tetu et al., 2019), *Daphnia* spp. (Lithner et al., 2009, 2012; Thaysen et al., 2018), barnacle nauplii (Li et al., 2016), and brown mussels (Gandara e Silva et al., 2016).

This study assesses the chemical composition of aqueous leachates from plastic materials and evaluates the effects of leachates on the growth of microalgae *Raphidocelis subcapitata* (freshwater) and *Skeletonema costatum* (marine), and on the Mediterranean mussel *Mytilus galloprovincialis.* Microalgae and marine mussels were selected as model organisms owing to their "key" ecological roles (i.e. primary producer and filter feeder, respectively) within aquatic ecosystems, and therefore increase the level of inference of the obtained results to 'real' environmental settings. Additionally, the selected species are widely distributed in aquatic environment, show a high responsiveness to chemical stressors (Fabbri et al., 2014) and can be readily grown or maintained in laboratory conditions. The study aimed to relate any observed toxicity to specific additives or additive groups. The organic and inorganic constituents of leachates generated from different types of synthetic thermoplastic and CTR were characterized following an initial non-target screening of each plastic reference material. Biological endpoints studied encompassed algal growth inhibition, impairment of mussel early stages processes, such as gamete fertilization, embryotoxicity and larvae survival and motility, and lysosomal biomarkers (i.e. lysosomal membrane stability). Toxicity tests were performed over a wide range of leachate dilutions to identify and quantify eventual dose-response relationships for each selected endpoint.

2. Materials and methods

2.1. Test materials and their preparation

All organic solvents and salts were of analytical grade. Solvent purity was verified in-house prior to use by pre-concentration (200x) followed by screening analysis using the same methods as applied for the samples. Deionized water was of MilliPore® MilliQ quality. Natural seawater was collected from 90 m in Trondheimsfjorden (Norway) and 20 m depth at Cesenatico (Italy), filtered to remove coarse particles, and subjected to sterile filtration (0.22 μm Sterivex®) prior to use in the experiments. Reference organic chemical standards were supplied by Chiron AS (Trondheim, Norway) and Sigma-Aldrich (Darmstadt, Germany). Reference inorganic chemical standards were supplied by Inorganic Ventures (VA, USA). A suite of PP, PET, PS, PVC (cryomilled, 1000 μm sieved fraction) and CTR-derived granulate (1–2 mm) reference materials was procured from CARAT GmbH (Germany) and Ragn-Sells (Norway) (SI, Table S1).

The materials were subjected to a non-target screening for organic additive chemicals by solvent extracting the polymers and analyzing the extracts by gas chromatography mass spectrometry (GC-MS), as previously reported (Cole et al., 2019). Details of the additive extraction method from the polymers and the analytical chemical conditions can be found in the Supplementary Information (SI, Section S1). Tentatively identified compounds were filtered based on their observed presence in at least 3 of 6 replicates for each polymer and >90% match to NIST 2017 library mass spectra. Biogenic compounds, or compounds of possible biogenic origin were removed from the data set. All compounds found in control samples were removed from the data set.

2.2. Leachate preparation

Leachates of each plastic and CTR material were generated in three different media; seawater, marine algae growth media (DM) and freshwater algae growth media (TG201). All media were sterilized and adjusted to 20 °C before use. The composition of DM and TG201 is presented in the SI (Tables S2 and S3). The plastic-water ratio was kept constant at 80 g/L, with a leachate volume of 100–250 mL. The samples were shaken in a rotating incubator (125 rpm) at room temperature (~25 °C) for 14 d in the dark. Upon sampling, the leachate was isolated by passing each sample through a sterile filter (0.2 μm Nalgene®). Subsamples for chemical analysis (3 x 2 mL for both organics and metals analysis) were acidified (15% HCl, pH < 2) and stored under dark and cool conditions until further processing. Additionally, the pH in the leachates was measured and compared to the pH in control media that had been shaking for the same length of time.

2.3. Leachate characterization and (semi)quantification

2.3.1. Extraction of organic additives

Samples (2 mL) of aqueous leachate were spiked with surrogate internal standards (25.08 ng naphthalene-*d*8, 4.86 ng chrysene-*d*12) and DCM:*n*-hexane (1:1 v/v, 1 mL) was added. Samples were then vortexed for 1 min followed by centrifugation for 2 min at 2,000 rpm. After centrifugation the upper organic phase removed and transferred to a glass vial. The addition of solvent, vortexing and centrifugation was repeated three times. The combined extract was dried with anhydrous Na_2SO_4 and evaporated under a gentle stream of N_2 to approximately 400 μL. Finally, a recovery internal standard (9.84 ng fluorene-*d*10) was added prior to GC-MS analysis.

2.3.2. GC-MS analysis of organic additive leachate extracts

The leachate extracts were first analyzed by GC-MS using the non-target screening method described above (SI, Section S1). After initial inspection of chromatograms, peaks were deconvoluted using Unknowns algorithms and best hits from NIST 2017 library were extracted. Acetophenone, benzothiazole, bisphenol A (BPA), *n*-cyclohexylformamide and phthalide were obtained as reference standards, and a 6-level calibration curve (0.01–1 ng/mL in DCM) was prepared for quantification purposes. Leachates were re-analyzed using a GC-MS selected ion monitoring (SIM) method (Tables S4 and SI), and analytes were quantified by linear regression after peak areas were normalized to fluorene-*d*10. Finally, recovery of surrogate standards of comparable volatility (chrysene-*d*10 for bisphenol A, otherwise naphthalene-*d*8) was used to correct for losses during extraction.

2.3.3. ICP-MS analysis for metals/inorganics

The leachates generated from each of the polymer materials were also subjected to an elemental screening for metal content. For metal determination, aqueous samples were first diluted in MilliQ water before ^{103}Rh and ^{115}In was added as internal standards. Analysis was performed using an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) fitted with a SPS 4 Autosampler. Quantification was achieved using standards obtained from Inorganic Ventures.

2.4. Toxicity to freshwater and marine algae

Toxicity of leachates towards freshwater (*Raphidocelis subcapitata*) and marine microalgae (*Skeletonema costatum*) was determined as inhibition of growth over 72 h at 21 ± 1 °C (OECD, 2011). An initial 72 h test was performed (without replicates) to determine the range of leachate dilutions that were suitable for the full test. In the full test, three replicates of each dilution were prepared, and growth measured after 0, 24, 48, and 72 h. Growth in control samples (media and algae culture alone) was determined with six replicates. Dilutions for each leachate and each species are described in the SI (Table S5). Algal growth was assessed by measurement of chlorophyll *a* fluorescence (TD-700 Fluorometer, excitation 340–500 nm, emission >665 nm).

2.5. Mussel toxicity test

2.5.1. Animal holding and experimental treatments

The toxicity of the prepared leachates was screened on the Mediterranean mussel *M. galloprovincialis* using a set of assays performed on different life stages (including spermatozoa, eggs, embryos, D-shaped veliger larvae and adult hemocytes) and biological levels (cellular to organism). Sexually mature mussels (5–6 cm length) were collected from a government certified farm (COPRALMO, Cesenatico, Italy), immediately transported to the laboratory and acclimated for 5 d in 0.22 μm filtered seawater at 16 °C (5 mussels L^{-1}). In all mussel tests, treatments encompassed a range of dilutions, including 0.6, 1, 2, 4, 6, 10, 20, 40, 60, 80 and 100% of the prepared leachates. Regardless of the bioassay performed, a control condition containing only 0.22 μm filtered seawater was run in parallel. All experiments were performed at 16 ± 1 °C, as previously reported (Capolupo et al., 2018a). Replicate numbers (N) employed in each bioassay are provided in the following sections.

2.5.2. Lysosomal membrane stability

The lysosomal membrane stability (LMS) of the mussel hemocytes was evaluated using the neutral red retention assay (NRRA) according to Martínez-Gómez et al. (2015). Aliquots of hemocytes from 10 randomly selected mussels were placed on microscope slides previously treated with 0.01% Poly-L-lysine and stained using 0.01% Neutral Red (NR) acidophile dye. After 15 min, hemocytes were spiked with selected dilutions of the plastic leachates and checked microscopically at 15 min intervals. The NRRA was performed in quadruplicate (N = 4) and the LMS assessed as the capability of lysosomes to retain the NR within lysosomes. Results were expressed as mean ± standard error of the mean (SEM) of the NR retention time (NRRT), which is the time (min) at which more than the 50% of the lysosomes released the dye into the cytosol.

2.5.3. Gamete fertilization and embryotoxicity tests

Adult mussels were experimentally induced to spawn according to the ASTM (2004) standard protocol, which consists of a 10 °C thermal shock (from 16 to 26 °C). Sperm and eggs were sieved through 50 μm and 100 μm nylon meshes to remove impurities and checked for quality (egg shape/size, spermatozoa motility) using an inverted light microscope (OPTECH, IB series; Munich, Germany; 40 × magnification). Pools of gametes from at least three specimens per sex were employed.

The effect of plastic leachates on mussel gamete fertilization was assessed according to the method reported by Capolupo et al. (2018b), with slight modifications. Briefly, an aliquot of spermatozoa was added to 96-well plates previously spiked with selected dilutions of the plastic leachates. At 60 min after spiking, oocytes were added at a 1:5 proportion with spermatozoa according to ASTM (2004). After 30 min, the test was blocked by adding calcium-buffered formalin (4%) to each well. The fertilization success was assessed by inverted microscopy (Carl Zeiss, PRIMOVERT series; Oberkochen, Germany; 40 × magnification); adopted criteria were the appearance of the polar lobe and/or the cell division initiation (Capolupo et al., 2018b). Data were expressed as the mean ± standard error of the mean (SEM) (N = 5) of the percentage of fertilization success attained at each tested condition. The tests validity

was set at a mean fertilization rate for controls of >60% and ≤98% (Environment Canada, 2011).

The embryotoxicity test was performed as described by Fabbri et al. (2014). Mussel eggs and spermatozoa were mixed in 96-well plates (1:5 egg to spermatozoa ratio) to allow for fertilization. After 30 min, the (≥80%) fertilization success was microscopically verified and leachate spiked at the chosen dilution. After a 48 h incubation period, samples were fixed with calcium-buffered formalin (4%) and examined by optical microscopy using an inverted microscope. A larva (veliger) was considered normal when it had reached the D-shelled veliger stage (straight hinge) and the velum did not protrude out of the shell, and malformed if had not reached the typical 48 h *post fertilization* (pf) stage (trocophore or earlier stages) or in the presence of developmental defects (concave, malformed or damaged shell, protruding velum). The recorded endpoint was the mean ± SEM (N = 5) of the percentage of normal larvae. The acceptability of test results was based on controls for a percentage of normal D-shaped veligers >70% (ASTM, 2004).

2.5.4. Survival/immobilization test

The effects of plastic leachates on the survival and motility of mussel early stages were tested using D-shaped veliger larvae obtained in laboratory rearing conditions. Oocyte fertilization was performed in 2 L glass beakers containing 1.5 L filtered seawater; starting from 48 h post fertilization (pf), larvae were fed daily with a suspension of microalgae (*Nannochloropsis oculata*; 1,200 cell mL^{-1}) until 5 d pf. At 6 d pf, larvae were collected by sieving the culture on a 20 μm pore nylon mesh and added to 96-well plates (50 individuals/well) previously spiked with selected leachate dilutions. All treatments were performed in quadruplicate (N = 4). Based on the results obtained in preliminary trials, the number of motile and viable larvae was recorded after 48 and 144 h of exposure, respectively, using an inverted optical microscope (40−100 × magnification). Criteria for mortality assessment included the clearly visible degradation/decomposition of the soft tissue and/or the absence of visceral movement and heart/cilia beating. Conversely, a larva was recorded as immobilized when it showed no swimming or spiral/circulatory movements typical of mussel D-shaped veligers (Sprung et al., 1984), although in the presence of visceral, heart and/or cilia beating. Data for survival were expressed as the mean ± SEM of the percentage of viable larvae, while immobilization was expressed as the mean ± SEM of the percentage of non-motile larvae over the proportion of viable larvae.

2.6. Statistical analysis

Chemical data were subjected to a *t*-test using the R software 'drc' package to evaluate the presence of statistically significant changes between the water concentrations of organic and inorganic compounds before and after the leaching process. Data obtained in marine mussel toxicity tests were analyzed for significant differences compared to controls using a non-parametric analysis of variance (Kruskal-Wallis test) followed by the Dunn's corrected test for multiple comparison using the statistical package SigmaPlot 13 (Systat Software Inc. San Jose, CA, USA). Deviations from normality and homoscedasticity were screened using the Shapiro-Wilk and Bartlett's test, respectively, and statistical significance was accepted for $p < 0.05$. Data from algae toxicity tests, mussel gamete fertilization, embryotoxicity, and survival/immobilization tests were subject to a non-linear regression analysis (four-parameter dose-response model; bottom plateau constrained to 0) and EC_{50} calculation (including the 95% confidence interval) using GraphPad Prism 7 (mussel tests) or R 'drc' package (algae tests) according to Ritz et al. (2015). To ensure precision and accuracy of the dose-response analysis, EC_{50} calculations were considered applicable only to data showing statistically significant differences compared to controls or, in the absence of significant treatments, a nonlinear regression coefficient (R squared) ≥ 0.70. According to the guidelines reported by Noel et al. (2018), EC_{50} values calculated as beyond the range of tested dosages (i.e. ≤ 100% leachate concentration) were reported as '>100%'.

3. Results and discussion

3.1. Additives in the polymer test materials and leaching into aqueous media

A broad range of organic additive chemicals were identified in the solvent extracts generated from the polymer test materials, representing plasticizers, antioxidants, antimicrobials, lubricants, and vulcanizers. A list of the organic compounds found in the polymer extracts that were tentatively identified by a >90% mass spectral match to reference compounds in the NIST 2017 mass spectral library is presented in the Supplementary Information (Table S6). This list was used as a basis for studying the additive compounds present in the leachates. The highest number of tentatively identified organic compounds in the polymer extracts was found in PP (19), followed by CTR (17), PVC (9), PS (9) and PET (1). With only one identifiable organic additive compound found in the original polymer material, the PET particles were the "purest" material used in the present study.

Of the tentatively identified compounds in the polymer extracts (SI, Table S6), only a few were found in detectable amounts in the full scan analyses of the corresponding leachate samples (freshwater and marine media). Acetophenone and phenoxyethanol were found in the PP particles and in both the PP leachates (freshwater and marine). Phthalide was found in PVC particles and in both PVC leachates. Benzothiazole, 2(3H)-benzothiazolone and phthalimide were found in the CTR particles and both CTR leachates. A number of compounds (including *n*-cyclohexylformamide in the case of the CTR particles) were identified only in the leachates and not in the polymer extracts. This could be due to the more complex nature of the polymer extracts compared to the leachate extracts, rendering the compound peaks unresolvable in the chromatograms from the polymer extracts. The presence of specific compounds in the leachates of specific polymers, together with their absence in control samples, suggests that these are not the result of contamination introduced during sample processing.

From the suite of organic compounds tentatively identified in the polymer extracts and leachates, four commercially available compounds were purchased as reference standards (acetophenone, benzothiazole, phthalide and *n*-cyclohexylformamide). In addition, BPA was included as a standard compound of interest in the target analyte study of the leachates (Table 1). For each respective target compound, the highest concentrations were found in leachates of polymers where the compound had already been identified as present in the polymer by non-target screening. The concentrations of the different additives varied from tens of ng to hundreds of μg/L. For example, benzothiazole was found in the highest concentrations in the freshwater and marine leachates of CTR (2,313 and 1,460 μg/L, respectively), but not present in any other leachate.

The sensitivity of the GC-MS SIM method allowed the quantification of some additives in polymer leachates where the compounds had not been observable under non-target screening analysis. For example, acetophenone was identified in the polymer extracts of PP and PS, but was present in the freshwater leachates of PP, PS and PVC, as well as the marine leachates of PP, PS, PVC and CTR; *n*-cyclohexylformamide was also found in high quantities in

Table 1
Organic compounds quantified in freshwater and marine leachates (μg/L). N = 3, except where standard deviation is missing, and one replicate sample was analyzed. Asterisks denote values statistically different from controls (*t*-test, $^{**}p < 0.005$, $^{*}p < 0.05$). ND = not detected.

	Acetophenone	Benzothiazole	*n*-cyclohexylformamide	Phthalide	Bisphenol A
Freshwater					
Control	5 ± 1	2.2 ± 1.6	0.6 ± 0.3	0.5 ± 0.2	0.13 ± 0.05
CTR	22	2,313	1,059	5.5	8.6
PET	4.9 ± 0.2	2.9 ± 0.3	0.39 ± 0.08	1.65 ± 0.01*	0.15 ± 0.03
PP	151 ± 3**	3.7 ± 0.2	0.92 ± 0.09	2.10 ± 0.02*	1.01 ± 0.05**
PS	16 ± 2**	1.9 ± 0.4	0.8 ± 0.2	4.1 ± 0.4**	3.6 ± 0.4**
PVC	22.3 ± 0.9**	2.7 ± 0.2	0.9 ± 0.3	387 ± 8**	0.25 ± 0.04
Marine					
Control	4.6 ± 0.7	3 ± 2	0.6 ± 0.4	0.2 ± 0.1	0.14 ± 0.05
CTR	13.2 ± 0.3**	1,460 ± 35**	788 ± 23**	2.4 ± 0.1**	6.4 ± 0.8*
PET	5.3 ± 0.5	2.2 ± 0.7	0.8 ± 0.2	0.23 ± 0.01	0.17 ± 0.03
PP	166 ± 3**	4 ± 1	3.4 ± 0.2**	2.3 ± 0.2*	2.83 ± 0.08**
PS	13.1 ± 0.6**	01.9 ± 0.5	0.5 ± 0.3	3.0 ± 0.1**	2.4 ± 0.2**
PVC	19.0 ± 0.8**	2.3 ± 0.3	ND	510 ± 218	0.28 ± 0.08*

the CTR freshwater and marine leachates (1,059 and 788 μg/L, respectively), despite not being observed in the polymer extract samples. This suggests *n*-cyclohexylformamide concentrations in CTR may be relatively low, but that the chemical leaches out from CTR far more readily than other additives. Finally, BPA was not observed in any of the polymer extracts or the leachate extracts subjected to non-target analysis, but was observed in the freshwater leachates of PP and PS (1.0 and 3.6 μg/L, respectively) and the marine leachates of CTR, PP, PS and PVC (6.4, 2.8, 2.4 and 0.3 μg/L, respectively) when analyzed using the SIM method. Levels of these compounds ranged from being significantly ($p < 0.05$) to highly significantly ($p < 0.005$) greater than their concentrations in the media control samples, indicating they were present due to polymer leaching. Based on these preliminary investigations of polymer leachates using a combination of target and non-target approaches, it is probable that the 'true' complexity is far greater. A larger number of organic additive chemicals are likely to be identified if several other analytical methods were also applied (i.e. liquid chromatography).

While the general trend in organic additive concentrations was similar between freshwater and marine media for organic compounds, this varied more for inorganics (Table 2). For example, freshwater PS leachates exhibited Al concentrations of 12,355 ± 347 μg/L while concentrations in the marine leachate were 755 ± 16 μg/L. A similar trend was observed for Sr in the PVC leachates. The highest metal concentrations in the freshwater and marine leachates were observed for Zn in CTR (12,130 ± 152 and 5,138 ± 128 μg/L, respectively) and PVC (2,806 ± 54 and 6,357 ± 140 μg/L, respectively). This is consistent with the high levels of Zn used in CTR and also the high Zn concentration in PVC leachates recently reported by Tetu et al., 2019. Furthermore, a 2.6 × increase of Pb levels (relatively to controls) was obtained in the PP seawater leachate, which may be noteworthy given its known toxicity to aquatic organisms (DeForest and Meyer, 2015). Interestingly, the presence of the polymer particles also modified the chemical composition of the media by sorbing some inorganic nutrients, thus significantly reducing their media concentration relative to controls. This was particularly evident in the PS particle leachates, which showed up to a 99% decrease in Mg concentration following the leaching process. A similar process was observed for Pb, which decreased in the seawater leachates of CTR, PET and PVC by 35%, 48% and 81%, respectively.

An increase in pH (relative to control samples without particles) was observed in both PS leachates and in the PET freshwater leachate (not marine). In contrast, CTR, PP and PVC leachates lowered the pH of both media (SI, Table S7). The pH change was considered an effect of leaching, and pH was therefore not adjusted prior to toxicity studies.

3.2. Acute leachate toxicity to freshwater and marine algae

Preliminary trials showed that PET leachates did not cause growth inhibition in either freshwater (*R. subcapitata*) or marine algae (*S. costatum*), even at the 100% leachate concentration. This is supported by the low additive chemical content determined in the PET extracts and leachates. Full toxicity tests were therefore performed only for leachates from CTR, PP, PS and PVC materials. The results, in form of 50% effect concentrations (EC_{50}), are summarized

Table 2
Metals quantified in freshwater and marine leachates (μg/L). N = 3, except where standard deviation is missing, and one replicate sample was analyzed. Asterisks denote values statistically different from controls (*t*-test, $^{**}p < 0.005$, $^{*}p < 0.05$). ND = not detected above detection limits. Values in bold indicate significant *decrease* compared to controls.

	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	Sr	Cd	Sb	Pb
Freshwater												
Control	684 ± 17	1.8 ± 0.1	114 ± 5	169 ± 5	0.31 ± 0.03	0.9 ± 0.1	17 ± 1	9 ± 1	1.7 ± 0.1	ND	0.14 ± 0.04	0.43 ± 0.04
CTR	439 ± 10	1.53 ± 0.08	162 ± 9**	130 ± 4	11.9 ± 0.6**	2.6 ± 0.3*	**6.4 ± 0.5***	12,130 ± 152**	16.2 ± 0.4**	ND	0.36 ± 0.08**	0.59 ± 0.06
PET	699 ± 33	5.8 ± 0.3	103 ± 5*	113 ± 9	5.8 ± 0.3**	4.1 ± 0.5	27 ± 1*	24 ± 3*	3.9 ± 0.4**	0.16	15.7 ± 0.7**	0.31 ± 0.03
PP	1002 ± 31	1.4 ± 0.1	114 ± 2	72 ± 4	0.38 ± 0.03*	1.8 ± 0.1	20.5 ± 0.5	111 ± 5**	15.8 ± 0.6**	0.6 ± 0.3	1.8 ± 0.1**	1.23 ± 0.05
PS	12,355 ± 347**	1.4 ± 0.1	**1.08 ± 0.05****	107 ± 3	ND	0.6 ± 0.1	**6.8 ± 0.4***	6 ± 2	16.7 ± 0.5**	0.16	0.52 ± 0.06**	0.63 ± 0.04
PVC	666 ± 13	1.7 ± 0.1	**86 ± 2****	63 ± 4	0.30 ± 0.02	2.1 ± 0.2	42 ± 1**	2,806 ± 54**	67 ± 2**	ND	0.21 ± 0.04*	0.29 ± 0.03
Marine												
Control	868 ± 10	2.7 ± 0.2	9.0 ± 0.5	198 ± 8	1.61 ± 0.07	27 ± 5	38 ± 5	15 ± 2	7,508 ± 141	0.48	0.27 ± 0.04	3.5 ± 0.1
CTR	884 ± 17	4.5 ± 0.2	34.1 ± 0.7**	187 ± 7	13.0 ± 0.3**	3.4 ± 0.3	44 ± 2	5,138 ± 128**	7,572 ± 181	0.2 ± 0.1	0.82 ± 0.07**	**2.28 ± 0.08****
PET	802 ± 9	4.3 ± 0.1	9.8 ± 0.2	233 ± 6	8.7 ± 0.2**	3.3 ± 0.3	53 ± 3	51 ± 3**	7,496 ± 172	0.16	12.9 ± 0.4**	**1.83 ± 0.08****
PP	1,216 ± 25	2.6 ± 0.1	11.7 ± 0.3**	220 ± 8	1.83 ± 0.09**	1.9 ± 0.2	260 ± 7**	180 ± 6**	7,460 ± 188	0.3 ± 0.2	1.8 ± 0.1**	9.0 ± 0.3**
PS	755 ± 16	4.9 ± 0.3	**0.98 ± 0.07****	161 ± 5	1.49 ± 0.09*	2.3 ± 0.2	36 ± 3	**7 ± 2***	7,383 ± 204	0.16	0.47 ± 0.04*	3.8 ± 0.1*
PVC	1,407 ± 30	3.0 ± 0.1	13.5 ± 0.2**	230 ± 10	1.81 ± 0.04**	2.8 ± 0.3	31 ± 3	6,357 ± 140**	7,638 ± 211	0.3 ± 0.3	0.33 ± 0.05	**0.68 ± 0.04****

in Table 3. The leachate toxicities varied between the different polymers and there were also observable differences in toxicity for some of the leachates between the two algal species. CTR produced the most toxic leachate, with EC_{50} values of 0.5% and 19% of total leachate towards the freshwater and marine algae, respectively. PP exhibited a similar toxicity towards marine algae to that observed for CRT (EC_{50} 18%), but showed the lowest toxicity towards freshwater algae (EC_{50} 64%). PVC had high toxicity towards freshwater algae (EC_{50} 1.6% of total leachate), but the second lowest toxicity towards marine algae (EC_{50} 35%). The PS leachate was toxic only at the highest concentrations towards the marine algae (EC_{50} >100%) and was the second least toxic (EC_{50} 26%) towards the freshwater algae (PP being the least toxic). The higher toxicity of CTR and PVC leachates could be directly linked to the higher additive chemical content of these leachates, both in terms of organics and metals (particularly Zn). A limitation to the current study is the non-quantifiable influence of the reduction in dissolved nutrients in exposure media due to sorption by the particles. However, this effect would be pronounced only at the highest exposure concentrations, as the media dilutions would make it negligible at lower concentrations.

3.3. Leachate toxicity to marine mussels

Data from this study provide clear evidence for the toxicity of polymer leachates on cellular and developmental parameters measured in the Mediterranean mussel *M. galloprovincialis*. The results of the LMS test performed on mussel hemocyte treated with increasing leachate concentrations are shown in Fig. 1. In agreement with safety standards reported in the UNEP RAMOGE manual (1999), LMS data from control treatments showed NRRTs >100 min, corresponding to the LMS physiological condition in the mussels (Fig. 1). The CTR leachate proved to be the most toxic with respect to LMS (Fig. 1A), where significant reductions relative to the control were observed in the range of 10–100%. Significantly lower LMS levels were also recorded for exposures representing 40–100% of the PVC leachate (Figs. 1D), 40–100% of PS leachate (Figs. 1C), 40 and 80 and 100% of PP leachate (Fig. 1B). Conversely, none of the PET leachate treatments affected the LMS in mussel hemocytes (Fig. 1E), which is consistent with the low levels of organic and inorganic additives present in this polymer material.

LMS reduction in mussel hemocytes is a well-known biomarker of cellular and general stress which has been recognized as prognostic of alterations to higher functions, such as growth and/or reproduction (Martinez-Gomez et al., 2017; Moore et al., 2006). Some of the chemicals detected in the tested leachates were previously found to induce lysosomal dysfunctions in bivalve hemocytes. In an *in vitro* investigation, Canesi et al. (2005) found that BPA affected LMS at concentrations one order of magnitude higher than those detected in the current study, suggesting that further additives may have contributed to the observed LMS modulation(s). For instance, benzothiazole, highly concentrated in the CTR leachate (146 μg/L), is known to display metal-chelating properties able to interfere with membrane-linked processes and lysosomal hydrolases activity (Wever and Verachtert, 1997). Zinc, which is essential to mussel cell metabolism at natural seawater levels (Olmedo et al., 2013), was found to affect LMS and induce hemocyte necrosis in the μg to mg/L range (as in CTR, PVC and PP leachates) or in combination with Pb, Cu and Cr (Giamberini and Pihan, 1997; Hietanen et al., 1988). In bivalves, hemocytes play a crucial role in the host-activated immune response and in modulating cell detoxification and degradation pathways (Viarengo et al., 2007). The LMS results in the current study highlight the need for a more comprehensive assessment of the effects of plastic leachates on the mussel adaptability to microbiological and/or physicochemical stressors.

Table 3
EC_{50} (% of total leachate) of algae growth inhibition. Values in parentheses represent 95% confidence intervals.

	Freshwater	Marine
CTR	0.50 (0.48–0.52)	19.0 (17.8–20.2)
PP	64.0 (59.6–68.4)	18.1 (7.5–28.8)
PS	23.5 (21.5–25.4)	>100
PVC	1.62 (1.50–1.74)	34.6 (30.3–34.6)

The effects induced by the leachates on mussel gamete fertilization are summarized in Fig. 2. In the control treatment, 84.4% of eggs were fertilized, thus falling within the standard thresholds for test validity (Environment Canada, 2011). Conversely, the 60 min exposure of mussel spermatozoa to increasing leachate concentrations significantly suppressed egg fertilization success. The PP leachate had the highest number of treatments that were significantly different to control values, with responses following exposure to the 6, 10, 40, 80 and 100% (Fig. 2B). A significant down-regulation was also triggered in CTR, PS and PVC leachates at 40–100% (Fig. 2A, C, D). The lowest effects were induced by PET, which exhibited a significant decrease only following exposure to the 80–100% of leachate (Fig. 2E). Dose-response curves of egg fertilization data, including R^2 and Hill-slope, are presented in the SI (Fig. S2, Table S8). Fertilization data for the CTR, PP and PVC leachates showed EC_{50} values in the range of 16.45–79.15% and were ranked in the order PVC < CTR < PP (Fig. 2). Conversely, the EC_{50} values for PS and PET leachates were >100% of the exposure concentrations (Fig. 2).

To our knowledge, this study represents the first attempt at evaluating the effects of leached chemicals on the gamete fertilization of an aquatic species. According to the results, leachates from CTR and PVC displayed a higher magnitude of effects and a stronger dose-responsiveness (in terms of EC_{50}) than the other leachates tested. However, a greater effect from the PP leachates was observed when analyzed in terms of the significance relative to the controls, likely reflecting the higher sensitivity of mussel gametes to the specific group of chemical additives leached by this polymer. In mussels, the inhibition of gamete fertilization has frequently been associated with toxicant-induced alterations of the cellular structure involved in the regulation of energetic metabolism and motility of spermatozoa. In the current study, Cu was present at the highest concentration in the PP leachates (260 μg/L). Previous studies have showed that *in vitro* exposure to 100 μg/L of Cu severely altered the mitochondrial activity in *Mytilus* spp. spermatozoa, leading a subsequent inhibition of motility and fertilization rate (Au et al., 2000; Fitzpatrick et al., 2008). Similarly, Riba et al. (2016) highlighted mechanistic links between the presence of 10–200 μg/L of Pb, Ni, Cu and Zn in marine sediment elutriates and decreased egg fertilization in mussels (*Mytilus edulis*), with an EC_{50} of 6.3% of the total elutriate. A synergistic effect might also be expected following co-exposure to acetophenone and BPA (both exhibited significant enrichment in the CTR and PVC leachates), as both chemicals are known to display a marked spermistatic activity in mammals and fish (Cariati et al., 2019; Canesi and Fabbri, 2015).

The results of the 48 h embryotoxicity test performed on *M. galloprovincialis* are shown in Fig. 3. The percentage of normal D-shaped veliger larvae in controls was >70%, which is consistent with the standard requirements for test validity (ASTM, 2004). All leachates induced a statistically significant ($p < 0.05$) inhibition of normal embryonic development with respect to controls. Notably, significant reductions were observed following exposure to 2–100% of the CTR leachate (Figs. 3A), 10–100% of the PP leachate (Figs. 3B), 40–100% of the PS leachate (Figs. 3C), 4–100% of the PVC

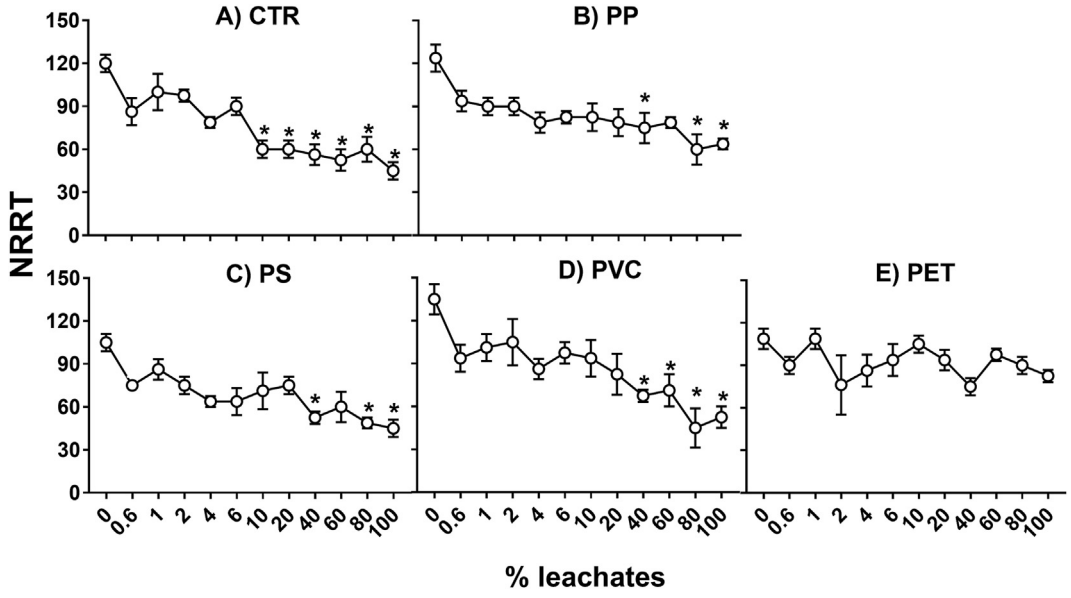

Fig. 1. Effects of leachates on the mussel lysosomal membrane stability. Mean ± SEM (N = 4) of the Neutral Red retention time assessed in the hemocyte of mussels *in vitro* exposed to plastic and CTR leachates. Asterisks show statistically significant differences *vs* control ($p < 0.05$).

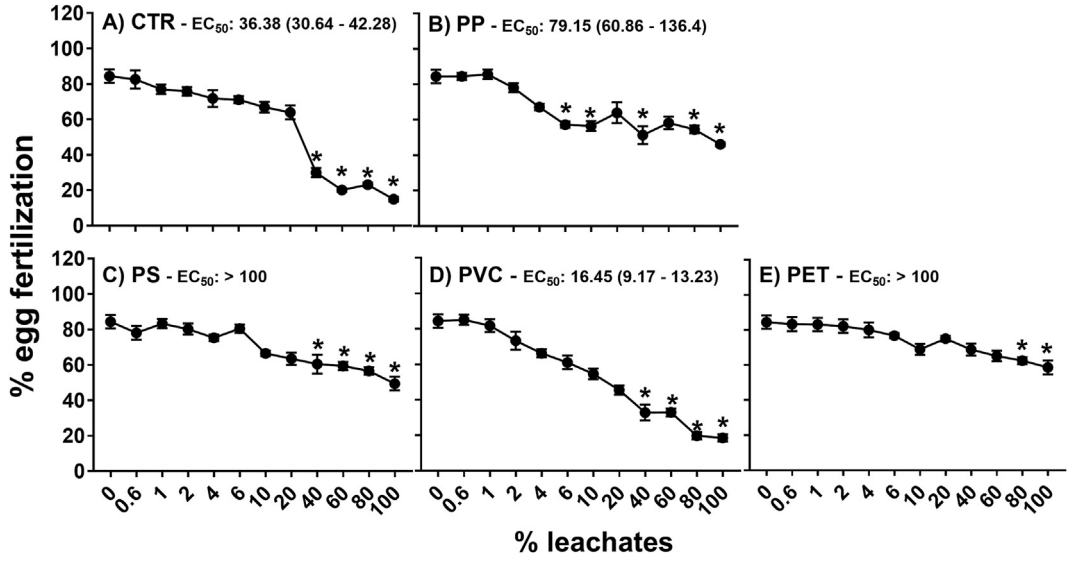

Fig. 2. Toxicity of leachates on the mussel gamete fertilization. Mean ± SEM (N = 5) of the percentage of fertilized mussel eggs measured following exposure to plastic and CTR leachates. Asterisks show statistically significant differences *vs* control ($p < 0.05$). EC_{50} values (including the 95% confidence intervals) are also reported.

leachate (Figs. 3D) and 60–100% of the PET leachate (Fig. 3E). Embryotoxicity data for all polymer leachates showed a $R^2 > 0.70$ (Fig. S3, Table S8). EC_{50} values were in the range of 2.22–96.84%, and were ranked in the order CTR < PVC < PP < PS < PET.

According to the obtained results, embryonic development was the most sensitive endpoint measured in the mussels. Based on existing literature, such an adverse outcome may reasonably be linked to the sensitivity of the embryos to some of the leached inorganic additives. Previous 48 h embryotoxicity testing of Cu, Zn and Pb on *M. galloprovincialis* showed EC_{50} values of 9.6, 99 and 63 μg/L, respectively (Nadella et al., 2009, 2013). These values are well below the levels of these elements detected in CTR, PVC and/or PP leachates in the current study. An alternative explanation may be the co-occurrence of BPA in the leachates, which is known to exert transcriptional and embryotoxic effects in *M. galloprovincialis* as a result of its endocrine disrupting activity (Fabbri et al., 2014; Balbi et al., 2016). In addition, heterocyclic aromatic compounds such as the benzothiazole and phthalide present in the CTR and PVC leachates are known to be embryotoxic in fish and/or mammal models (Sheftel, 2000). This suggests that the main alterations could involve early embryogenic processes (i.e. cleavage to gastrulation), which are phylogenetically more conserved across taxa. Interestingly, recent studies performed on sea urchins showed that a leachate from virgin PVC microplastics elicited significantly higher embryotoxicity than a direct particle administration through the same test medium (Nobre et al., 2015; Oliviero et al., 2019). Similarly, our previous study showed that exposure to 3 μm PS particles did not affect mussel embryonic development (Capolupo et al., 2018a), leading to the hypothesis that sole embryo exposure to waterborne plastics may not allow reliable estimation

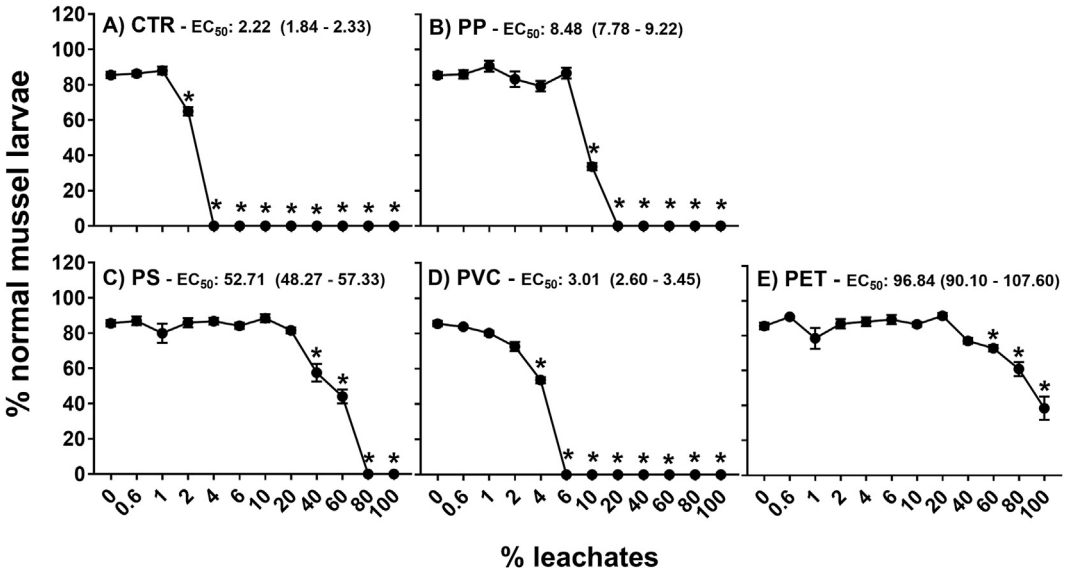

Fig. 3. Toxicity of leachates on the mussel embryo-larval development. Mean ± SEM (N = 5) of the percentage of normally developed mussel larvae (D-shaped veligers) following exposure to plastic and CTR leachates. Asterisks show statistically significant differences *vs* control ($p < 0.05$). EC_{50} values (including the 95% confidence intervals) are also reported.

of plastic additive effects.

The effects induced by leachates on the motility and survival of mussel D-shaped veligers are presented in Fig. 4. CTR leachates significantly reduced larvae motility (48 h) and survival (144 h) following exposure to leachates in the range of 10–100% and 40–100%, respectively (Fig. 4A). The PP leachate did not induce any effects on the motility of mussel larvae, but caused a significant decrease in survival at the 100% concentration (Fig. 4B). No effect on either motility or survival was induced by the PS and PET leachates (Fig. 4C, E), while a significant reduction of both parameters was induced by the PVC leachate in the range 20–100% (Fig. 4D).

According to the nonlinear regression analysis, an R squared value greater than 0.70, selected as a prerequisite for EC_{50} calculations, could only be calculated for survival data for the CTR, PP and PVC leachates and for motility data for the CTR and PVC leachates (Fig. S4, Table S8). The EC_{50} values for survival and motility for the CTR and PVC leachates were in the range of tested concentrations, while the effective concentration for survival after exposure to the PP leachate treatments was calculated to be >100% (Fig. 4). The lowest EC_{50} value for motility (18.75% of the total concentration) was observed for the CTR leachate, while the PVC leachates induced the lowest EC_{50} for survival at 54.04% (Fig. 4).

These results clearly indicate that leachates derived from different polymers vary considerably in their impacts on mussel larvae survival and motility. This is consistent with previous studies (Lithner et al. 2009, 2012) that showed leachates from 20 out of 58

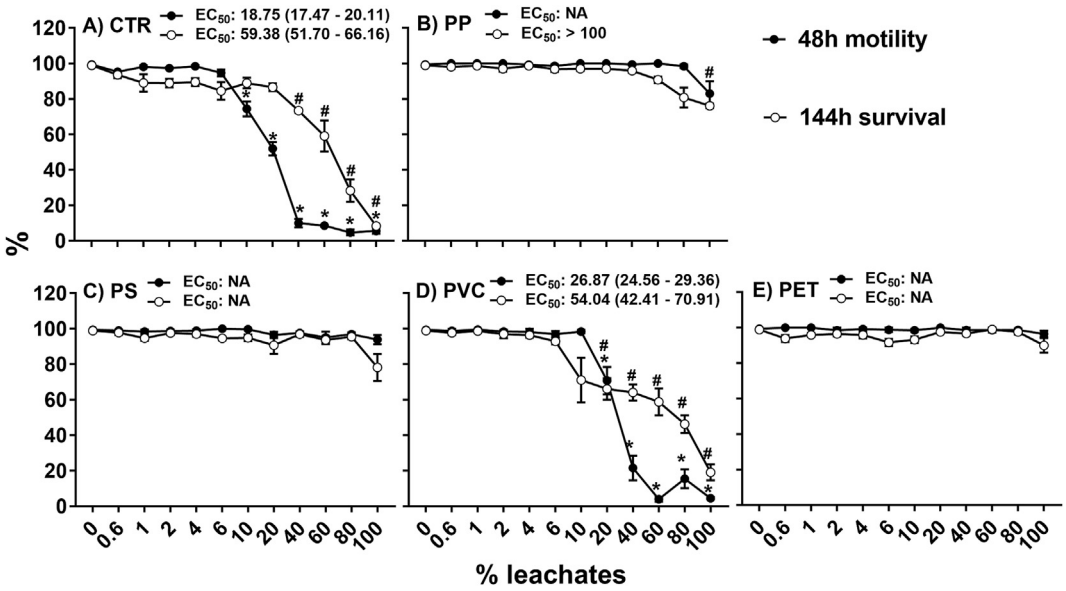

Fig. 4. Toxicity of leachates on the motility and survival of mussel larvae. Mean ± SEM (N = 4) of the percentage of motile (black dots) and viable (white dots) mussel following 48 h and 144 h exposure to prepared leachates, respectively. Asterisks and hash symbols show statistically significant differences (*vs* control) in motility and survival tests, respectively ($p < 0.05$). Unless not applicable (NA), EC_{50} values (including the 95% confidence intervals) are also reported.

commercial plastic products affected the mobility of the freshwater cladoceran *Daphnia magna.* While effects on the survival of barnacle (*Amphibalanus amphitrite)* nauplii were recorded in response to a range of plastic leachates (including PVC, PP, PS and PET) (Li et al., 2016), only PP, PVC and bioplastic-derived leachates caused mortality in the copepod *Nitocra spinipes* (Bejgarn et al., 2015). This suggests that species- or stage-specific traits may influence the overall toxicity of leached additives, although different additive chemical profiles in plastics comprised of the same polymer type may also play a role. Unlike embryos, mussel larvae possess D-shaped shells and well-developed chemoreceptors that allow them to respond to chemical stressors by closing their valves and reducing the filtration rate (Wong and Gerstenberger, 2015). Although these mechanisms would explain the absence of effects or the slight effects observed for exposure to the PP, PS and PET leachates in the current study, they contrast with the dose-related response induced by exposure to CTR and PVC leachates. It is possible that this might be related to the lower pH values (i.e. pH 7.5, Table S7) observed in both leachates relative to controls (pH 8.1). A recent study demonstrated that exposure to pH lower than 7.6 inhibits compensatory mechanisms in *Mytilus* larvae, leading to metabolic alterations in the short-term and increased mortality after prolonged exposure (Ventura et al., 2016).

4. Conclusions

This study provides new knowledge on the chemical characterization and ecotoxicological effects of chemical additive leachates from plastic and rubber materials under freshwater and marine conditions. The chemical composition of leachates was complex and varied considerably among tested polymers, with the main additives identified as plasticizers, antioxidants, antimicrobials, lubricants, vulcanizers and metals. In line with the relatively high content of organic and inorganic compounds in the parent materials, leachates from CTR and PVC exhibited the highest toxicity on algae growth and mussel early stages development, survival and motility. The lowest effects were generally observed for the PET leachates, which contained the lowest number and concentration of measured additives. Evidence of toxicity were also observed for mussel gamete fertilization and the LMS assessed in hemocytes, suggesting that leachate exposure may affect different stages of the life cycle of aquatic organisms.

The magnitude of effects observed in this study suggest that organic and inorganic additives leached from plastic/rubber polymers may cause substantially higher ecotoxicity than those previously reported following plastic ingestion. Although additives are subjected to a substantial dilution in natural environment, EC_{50} values determined in this study showed that leachate concentrations of less than 3% may because for concern in enclosed ecosystems (e.g. bays, lagoons or lakes) or oceanic 'hotspots' where plastics tend to accumulate (GESAMP, 2016). Current knowledge on ecotoxicological effects of plastic leachates is based upon a small number of studies conducted using acute toxicity tests and different leaching processes. Further efforts are therefore required to standardize methods for leachate preparation and sublethal effects testing to obtain a more detailed understanding on the long-term effects of plastic additive exposure to aquatic biota.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115270.

References

Al-Odaini, N.A., Shim, W.J., Han, G.M., Jang, M., Hong, S.H., 2015. Enrichment of hexabromocyclododecanes in coastal sediments near aquaculture areas and a wastewater treatment plant in a semi-enclosed bay in South Korea. Sci. Total Environ. 505, 290–298. https://doi.org/10.1016/j.scitotenv.2014.10.019.

ASTM, 2004. Standard Guide for Conducting Acute Toxicity Test Starting with Embryos of Four Species of Saltwater Bivalve Molluscs, p. 21. E 724e98.

Au, D.W.T., Chiang, M.W.L., Wu, R.S.S., 2000. Effects of cadmium and phenol on motility and ultrastructure of sea urchin and mussel spermatozoa. Arch. Environ. Contam. Toxicol. 38, 455–463. https://doi:10.1007/s002449910060.

Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E., Canesi, L., 2016. Impact of bisphenol A (BPA) on early embryo development in the marine mussel *Mytilus galloprovincialis*: effects on gene transcription. Environ. Pollut. 218, 996–1004. https://doi:10.1016/j.envpol.2016.08.050.

Barboza, L.G.A., Frias, J.P.G.L., Booth, A.M., Vieira, L.R., Masura, J., Baker, J., Foster, G., Guilhermino, L., 2019. Chapter 18 - microplastics pollution in the marine environment. In: Sheppard, C. (Ed.), World Seas: an Environmental Evaluation, second ed. Academic Press, pp. 329–351.

Beaumont, N.J., Aanesen, M., Austen, M.C., Börger, T., Clark, J.R., Cole, M., Hooper, T., Lindeque, P.K., Pascoe, C., Wyles, K.J., 2019. Global ecological, social and economic impacts of marine plastic. Mar. Pollut. Bull. 142, 189–195. https://doi:10.1016/j.marpolbul.2019.03.022.

Bejgarn, S., Macleod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from weathering plastics : an exploratory screening study with *Nitocra spinipes*. Chemosphere 132, 114–119. https://doi:10.1016/j.chemosphere.2015.03.010.

Canepari, S., Castellano, P., Astolfi, M.L., Materazzi, S., Ferrante, R., Fiorini, D., Curini, R., 2018. Release of particles, organic compounds, and metals from crumb rubber used in synthetic turf under chemical and physical stress. Environ. Sci. Pollut. Res. 25, 1448–1459. https://doi.org/10.1007/s11356-017-0377-4.

Canesi, L., Betti, M., Lorusso, L.C., Ciacci, C., Gallo, G., 2005. "In vivo" effects of bisphenol A in *Mytilus* hemocytes: modulation of kinase-mediated signalling pathways. Aquat. Toxicol. 71, 73–84. https://doi:10.1016/j.aquatox.2004.10.011.

Canesi, L., Fabbri, E., 2015. Environmental effects of BPA: focus on aquatic species. Dose-Response 13, 1–14. https://doi.org/10.1177/1559325815598304.

Capolupo, M., Díaz-Garduño, B., Martín-Díaz, M.L., 2018b. The impact of propranolol, 17α-ethinylestradiol, and gemfibrozil on early life stages of marine organisms: effects and risk assessment. Environ. Sci. Pollut. Res. 25, 32196–32209. https://doi.org/10.1007/s11356-018-3185-6.

Capolupo, M., Franzellitti, S., Valbonesi, P., Sanz, C., Fabbri, E., 2018a. Uptake and transcriptional effects of polystyrene microplastics in larval stages of the Mediterranean mussel *Mytilus galloprovincialis*. Environ. Pollut. 241, 1038–1047. https://doi.org/10.1016/j.envpol.2018.06.035.

Cariati, F., Uonno, N.D., Borrillo, F., Iervolino, S., Galdiero, G., Tomaiuolo, R., 2019. Bisphenol a: an emerging threat to male fertility. Reprod. Biol. Endocrinol. 6, 4–11. https://doi.org/10.1186/s12958-018-0447-6. Sci. Rep. 17/6.

Cole, M., Coppock, R., Lindeque, P.K., Altin, D., Reed, S., Pond, D.W., Sørensen, L., Galloway, T.S., Booth, A.M., 2019. Effects of nylon microplastic on feeding, lipid accumulation, and moulting in a coldwater copepod. Environ. Sci. Technol. 53, 7075–7082. https://doi.org/10.1021/acs.est.9b01853.

DeForest, D.K., Meyer, J.S., 2015. Critical review: toxicity of dietborne metals to aquatic organisms. Crit. Rev. Environ. Sci. Technol. 45 (11), 1176–1241. https://doi.org/10.1080/10643389.2014.955626.

ECHA, European Chemical Agency, 2018. Data on candidate list substances in articles, p. 209. Available at: https://echa.europa.eu/documents/10162/13642/data_candidate_list_substances_in_articles_en.pdf/d48a58e4-0d67-4c54-86a5-0b15877a8c93.

Engler, R.E., 2012. The complex interaction between marine debris and toxic chemicals in the ocean. Environ. Sci. Technol. 46, 12302–12315. https://doi.org/10.1021/es3027105.

Environment Canada, 2011. Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars), Method Development and Applications Unit, second ed. Science and Technology Brand, ISBN 978-1-100-17847-9, p. 140.

Ezechiáš, M., Covino, S., Cajthaml, T., 2014. Ecotoxicity and biodegradability of new brominated flame retardants: a review. Ecotoxicol. Environ. Saf. 110, 153–167. https://doi:10.1016/j.ecoenv.2014.08.030.

Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., Canesi, L., 2014. Adaptation of the bivalve embryotoxicity assay for the high throughput screening of emerging contaminants in *Mytilus galloprovincialis*. Mar. Environ. Res. 99, 1–8. https://doi:10.1016/j.marenvres.2014.05.007.

Fitzpatrick, J.L., Nadella, S., Bucking, C., Balshine, S., Wood, C.M., 2008. The relative sensitivity of sperm, eggs and embryos to copper in the blue mussel (*Mytilus trossulus*). Comp. Biochem. Physiol., C 147, 441–449. https://doi:10.1016/j.cbpc.2008.01.012.

Gandara e Silva, P.P., Nobre, C.R., Resaffe, P., Pereira, C.D.S., Gusmão, F., 2016. Leachate from microplastics impairs larval development in brown mussels. Water Res. 106, 364–370. https://doi.org/10.1016/j.watres.2016.10.016.

GESAMP, 2016. Sources, fate and effects of microplastics in the marine environment: part two of a global assessment. In: Kershaw, P.J., Rochman, C.M. (Eds.), (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection), vol. 93. Rep. Stud. GESAMP No., p. 220

Giamberini, L., Pihan, J.C., 1997. Lysosomal changes in the hemocytes of the freshwater mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. Dis. Aquat. Org. 28, 222–227. https://doi:10.3354/dao028221.

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

Hann, S., Darrah, C., Sherrington, C., Blacklaws, K., Horton, I., Thompson, A., 2018. Reducing household contributions to marine plastic pollution, report for "friends of the earth", p. 73 available at: https://cdn.friendsoftheearth.uk/sites/default/files/downloads/reducing-household-plastics_0.pdf.

He, G., Zhao, B., Denison, M.S., 2011. Identification of benzothiazole derivatives and polycyclic aromatic hydrocarbons as aryl hydrocarbon receptor agonists present in tire extracts. Environ. Toxicol. Chem. 30, 1915–1925. https://doi.org/10.1002/etc.581.

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., Duflos, G., 2017. Occurrence and effects of plastic additives on marine environments and organisms: a review. Chemosphere 182, 781–793. https://doi.org/10.1016/j.chemosphere.2017.05.09.

Hietanen, B., Sunila, I., Kristoffersonn, R., 1988. Toxic effects of zinc on the common mussel *Mytilus edulis* L. (Bivalvia) in brackish water. Physiological and histopathological studies. Ann. Zool. Fenn. 25, 341–347. http://www.jstor.org/stable/23734643.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H., Laursen, D., Zettler, E.R., Farrington, J.W., Reddy, C.M., Peacock, E.E., Ward, M.W., 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. Mar. Pollut. Bull. 62, 1683–1692. https://doi.org/10.1016/j.marpolbul.2011.06.004.

Kühn, S., Bravo Rebolledo, E.L., van Franeker, J.A., 2015. Deleterious effects of litter on marine life. In: Bergmann, M., Gutow, L., Klages, M. (Eds.), Marine Anthropogenic Litter. Springer International Publishing, pp. 75–116.

Li, H., Getzinger, G.J., Ferguson, P.L., Orihuela, B., Zhu, M., Rittschof, D., 2016. Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle *Amphibalanus amphitrite*. Environ. Sci. Technol. 50, 924–931. https://doi.org/10.1021/acs.est.5b02781.

Kwan, C.S., Takada, H., 2016. Release of additives and monomers from plastic wastes. In: Takada, H., Karapanagioti, H.K. (Eds.), Hazardous Chemicals Associated with Plastics in the Marine Environment. Springer Int. Publishing 51–70. https://doi.org/10.1007/698_2016_122.

Li, X., Berger, W., Musante, C., Mattina, M.I., 2010. Characterization of substances released from crumb rubber material used on artificial turf fields. Chemosphere 80, 279–285. https://doi.org/10.1016/j.chemosphere.2010.04.021.

Lithner, D., Damberg, J., Dave, G., Larsson, Å., 2009. Leachates from plastic consumer products – screening for toxicity with *Daphnia magna*. Chemosphere 74, 1195–1200. https://doi:10.1016/j.chemosphere.2008.11.022.

Lithner, D., Nordensvan, I., Dave, G., 2012. Comparative acute toxicity of leachates from plastic products made of polypropylene , polyethylene , PVC , acrylonitrile – butadiene – styrene , and epoxy to *Daphnia magna*. Environ. Sci. Pollut. Res. 19, 1763–1772. https://doi:10.1007/s11356-011-0663-5.

Llompart, M., Sanchez-Prado, L., Pablo Lamas, J., Garcia-Jares, C., Roca, E., Dagnac, T.I., 2013. Hazardous organic chemicals in rubber recycled tire playgrounds and pavers. Chemosphere 90, 423–431. https://doi.org/10.1016/j.chemosphere.2012.07.053.

Martínez-Gómez, C., Bignell, J., Lowe, D., 2015. Lysosomal membrane stability in mussels. ICES techniques in marine environmental sciences No. 56, p. 41. https://doi.org/10.17895/ices.pub.5084. Copenhagen.

Martinez-Gomez, C., Robinson, C.D., Burgeot, T., Gubbins, M., Halldorsson, H.P., Albentosa, M., Bignell, J.P., Hylland, K., Vethaak, A.D., 2017. Biomarkers of general stress in mussels as common indicators for marine biomonitoring programmes in Europe: the ICON experience. Mar. Environ. Res. 124, 70–80. https://doi.org/10.1016/j.marenvres.2015.10.012.

Meeker, J.D., Sathyanarayana, S., Swan, S.H., 2009. Phthalates and other additives in plastics: human exposure and associated health outcomes. Philos. Trans. R. Soc. Biol. Sci. 364, 2097–2113. https://doi.org/10.1098/rstb.2008.0268.

Moore, M.N., Allen, J.I., McVeigh, A., Shaw, J., 2006. Lysosomal and autophagic reactions as predictive indicators of environmental impact in aquatic animals. Autophagy 2, 217–220. https://doi:10.4161/auto.2663.

Nadella, S.R., Fitzpatrick, J.L., Franklin, N., Bucking, C., Smith, S., Wood, C.M., 2009. Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossolus*) and the protective effect of dissolved organic carbon. Comp. Biochem. Physiol., C 149, 340–348. https://doi.org/10.1016/j.cbpc.2008.09.00.

Nadella, S.R., Tellis, M., Diamond, R., Smith, S., Bianchini, A., Wood, C.M., 2013. Toxicity of lead and zinc to developing mussel and sea urchin embryos : critical tissue residues and effects of dissolved organic matter and salinity. Comp. Biochem. Physiol., C 158, 72–83. https://doi:10.1016/j.cbpc.2013.04.004.

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereira, C.D.S., Turra, A., 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus variegatus* (Echinodermata: echinoidea). Mar. Pollut. Bull. 92, 99–104. https://doi.org/10.1016/j.marpolbul.2014.12.050.

Noel, Z.A., Wang, J., Chilvers, M.I., 2018. Special Report - significant influence of EC_{50} estimation by model choice and EC_{50} type. Plant Dis. 102, 708–714. https://doi.org/10.1094/PDIS-06-17-0873-SR.

OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris.

Oliviero, M., Tato, T., Schiavo, S., Manzo, S., Beiras, R., 2019. Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin *Paracentrotus lividus*. Environ. Pollut. 247, 706–715. https://doi.org/10.1016/j.envpol.2019.01.098.

Olmedo, P., Hernández, A.F., Pla, A., Femia, P., Navas-acien, A., Gil, F., 2013. Determination of essential elements (copper , manganese , selenium and zinc) in fish and shellfish samples. Risk and nutritional assessment and mercury – selenium balance. Food Chem. Toxicol. 62, 299–307. https://doi:10.1016/j.fct.2013.08.076.

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

Riba, I., Gabrielyan, B., Khosrovyan, A., Luque, A., Valls, T.A. Del, 2016. The influence of pH and waterborne metals on egg fertilization of the blue mussel (*Mytilus edulis*), the oyster (*Crassostrea gigas*) and the sea urchin (*Paracentrotus lividus*). Environ. Sci. Pollut. Res. 14580–14588. https://doi:10.1007/s11356-016-6611-7.

Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLoS One 10 (12), e0146021. https://doi.org/10.1371/journal.pone.0146021.

Rodgers, B., Waddell, W., 2013. The science of rubber compounding. In: Mark, J.E., Herman, B., Roland, C.M. (Eds.), The Science and Technology of Rubber. Elsevier, Amsterdam, pp. 417–470.

Schmidt, N., Fauvelle, V., Ody, A., Castro-Jiménez, J., Jouanno, J., Changeux, T., Thibaut, T., Sempéré, R., 2019. The amazon river: a major source of organic plastic additives to the tropical north atlantic? Environ. Sci. Technol. 53, 7513–7521. https://10.1021/acs.est.9b01585.

Sheftel, V.O., 2000. Indirect Food Additives and Polymers: Migration and Toxicology. Food Science and Technology. CRC Press, p. 1320. https://doi.org/10.1201/9781482293821.

Sprung, M., 1984. Physiological energetics of mussel larvae (*Mytilus edulis*). Food uptake. Mar. Ecol. Prog. Ser. 17, 295–305. https://doi:10.3354/meps017295.

Tetu, S.G., Sarker, I., Moore, L.R., Paulsen, I.T., Pickford, R., Elbourne, L.D.H., 2019. Plastic leachates impair growth and oxygen production in *Prochlorococcus*, the ocean's most abundant photosynthetic bacteria. Commun. Biol. 2, 184 https://doi.org/10.1038/s42003-019-0410-x.

Thaysen, C., Stevack, K., Ruffolo, R., Poirer, D., De Frond, H., DeVera, J., Sheng, G., Rochman, C.M., 2018. Leachate from expanded polystyrene cups is toxic to aquatic invertebrates (*Ceriodaphnia dubia*). Front. Mar. Sci., 71 https://doi.org/10.3389/fmars.2018.00071.

Thompson, R.C., Moore, C.J., vom Saal, F.S., Swan, S.H., 2009. Plastics, the environment and human health: current consensus and future trends. Philos. Trans. R. Soc. Biol. Sci. 364, 2153–2166. https://doi.org/10.1098/rstb.2009.0053.

Turner, A., 2016. Heavy metals, metalloids and other hazardous elements in marine plastic litter. Mar. Pollut. Bull. 111, 136–142. https://doi.org/10.1016/j.marpolbul.2016.07.020.

UNEP, United Nation Environmental Programme, International Panel on Chemical Pollution (IPCP), 2017. Overview Report II: an overview of current scientific knowledge on the life cycles, environmental exposures, and environmental effects of select endocrine disrupting chemicals (EDCs) and potential EDCs, p. 208. Available at: http://hdl.handle.net/20.500.11822/25634.

Vandermeersch, G., Lourenço, H.M., Alvarez-Muñoz, D., Cunha, S., Diogène, J., Cano-Sancho, G., Sloth, J.J., Kwadijk, C., Barcelo, D., Allegaert, W., Bekaert, K., Fernandes, J.O., Marques, A., Robbens, J., 2015. Environmental contaminants of emerging concern in seafood – European database on contaminant levels. Environ. Res. 143, 29–45. https://doi.org/10.1016/j.envres.2015.06.011.

Ventura, A., Schulz, S., Dupont, S., 2016. Maintained larval growth in mussel larvae exposed to acidified under- saturated seawater. Nat. Publ. Gr. 1–10 https://doi.org/10.1038/srep23728.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-

induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol., C 146, 281–300. https://doi.org/10.1016/j.cbpc.2007.04.011.
Wagner, S., Hüffer, T., Kl, P., Wehrhahn, M., Klockner, P., Hofmann, T., Reemtsma, T., 2018. Tire wear particles in the aquatic environment - a review on generation, analysis, occurrence, fate and effects. Water Res. 139, 83–100. https://doi.org/10.1016/j.watres.2018.03.051.
Wever, H.D.E., Verachtert, H., Leuven, K.U., 1997. Review Paper. Biodegradation and toxicity of benzothiazoles. Water Res. 31, 2673–2684. https://doi.org/10.1016/S0043-1354(97)00138-3.
Wong, W., H, Gerstenberger, S.L., 2015. Biology and Management of Invasive Quagga and Zebra Mussels in the Western United States. CRC Press, ISBN 9781466595613, p. 566.