Chemosphere 333 (2023) 138894

Contents lists available at ScienceDirect

Chemosphere

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

A protocol for lixiviation of micronized plastics for aquatic toxicity testing

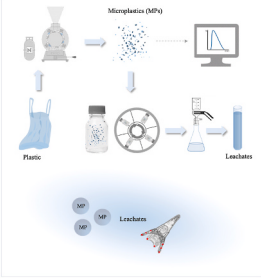
Rodrigo Almeda [a,*], Kuddithamby Gunaalan [b], Olalla Alonso-López [a,c], Alejandro Vilas [c], Christelle Clérandeau [d], Tara Loisel [d], Torkel Gissel Nielsen [b], Jérôme Cachot [d], Ricardo Beiras [c]

[a] EOMAR Group, ECOAQUA, University of Las Palmas de Gran Canaria, Spain
[b] DTU AQUA, Technical University of Denmark, Denmark
[c] ECOTOX Group, ECIMAT-CIM, University of Vigo, Spain
[d] EPOC UMR 5805, University of Bordeaux, CNRS and INP Bordeaux, France

HIGHLIGHTS

- A first attempt to standardize the methodology for lixiviation of microplastics.
- Recommendations for particle size, solid-to-liquid ratio, lixiviation time, and mixing conditions are provided.
- Leachates from the studied micronized plastics were toxic to marine biota.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Michael Bank

Keywords:
Plastic leachates
Microplastics
Toxicity testing
Methodology
Micronization
Lixiviation

ABSTRACT

Plastics contain various types and amounts of additives that can leach into the water column when entering aquatic ecosystems. Some leached plastic additives are hazardous to marine biota at environmentally relevant concentrations. Disparate methodological approaches have been adopted for toxicity testing of plastic leachates, making comparison difficult. Here we propose a protocol to standardize the methodology to obtain leachates from microplastics (MPs) for aquatic toxicity testing. Literature reviewing and toxicity tests using marine model organisms and different types of MPs were conducted to define the main methodological aspects of the protocol. Acute exposure to leachates from the studied plastics caused negative effects on the early life stages of sea urchins and marine bacteria. We provide recommendations of key factors influencing lixiviation of MPs , such as particle size (<250 μm), solid-to-liquid ratio (1–10 g/L), mixing conditions (1–60 rpm), and lixiviation time (72 h). The proposed methodology was successful to determine the toxicity of leachates from different micronized plastics on marine biota. Our recommendations balance sensitivity, feasibility and environmental relevance, and their use would help ensure comparability amongst studies for a better assessment of the toxicity of plastic leachates on aquatic biota.

Abbreviations: MPs, Microplastics.

* Corresponding author. Campus Universitario de Tafira, Edificio de Ciencias Básicas Las Palmas de Gran Canaria, CP35017, Canary Islands, Spain.
E-mail address: rodrigo.almeda@ulpgc.es (R. Almeda).

https://doi.org/10.1016/j.chemosphere.2023.138894
Received 2 January 2023; Received in revised form 25 April 2023; Accepted 7 May 2023
Available online 8 May 2023

1. Introduction

At least eight million tons of plastic litter enter the ocean every year (Jambeck et al., 2015) and the amount of plastic in the aquatic ecosystems is projected to triple by 2040 (UNEP, 2021). Microplastics (MPs) have been found in all aquatic systems and, globally, ocean waters are polluted with tens of thousands of tons of microplastics (Cózar et al., 2014, ISO, 2021). Understanding the consequences of plastic pollution in aquatic ecosystems is, therefore, a major societal and scientific concern. The overall assessment of the environmental impacts of plastic pollution is still under study. Current knowledge generally points to a lack of strong acute toxicity at the organism level from MPs derived from pure synthetic polymers, called "virgin MPs" (Beiras et al., 2018; Gambardella et al., 2019; Cormier et al., 2021; Rodríguez-Torres et al., 2020; JPI Oceans, 2020). However industrial and commercial plastic items also contain chemical additives (e.g., plasticizers, antioxidants, flame-retardants, antimicrobial coatings, and dyes) added to the polymer matrix during the manufacturing process. Plastic additives are generally not intrinsically bound to the synthetic polymers, and certain additives or their degradation products readily leach into liquid matrices (e.g. Brede et al., 2003; Kim et al., 2006; Fernandes et al., 2008; Lithner et al., 2012). Plastic debris can continue leaching chemicals for months in the marine environment (Paluselli et al., 2019). Fragmentation of marine plastic debris into MPs increases particle surface area and may accelerate leaching into the ocean. Some of these industrial plastic additives (e.g., polybrominated flame-retardants, *ortho*-phthalates, bisphenol A, nonylphenol, biocides) or their transformation products (e.g., 6 PPD-quinone) are hazardous to marine biota (Gunaalan et al., 2020; Tian et al., 2021; Page et al., 2022) as well as human health (Erni--Cassola et al., 2019). Knowledge of the impacts of plastic leachates and specific additives on marine organisms is therefore essential to evaluate the consequences of plastic pollution in marine ecosystems and to provide information to the plastic industry to encourage both the reduction of harmful additives and their replacement with environmentally safer additives.

There is solid evidence that leachates from plastics can have toxic effects on aquatic organisms (as summarized in S.I. Table S1) but disparate methodological approaches have been adopted (S·I., Table S1; Bridson et al., 2021). The lack of a standard methodology for MP lixiviation prevents comparison amongst studies, already hampered by the complexity of weathering processes in field conditions and the wide heterogeneity of plastic particles in terms of size, degradation phase, and levels of contained/adsorbed chemicals. Additionally, more studies on the toxicity of leachates from small plastic particles are needed since up to 80–90% of the microplastics in marine surface waters are smaller 300 μm (e.g., Rist et al., 2020; Gunaalan et al., 2023).

Sorption/desorption kinetics of plastic chemicals depend on the characteristics of both the polymeric matrix (polymer type, particle size, volume, crystallinity) and the associated chemicals (hydrophobicity, diffusion coefficient, molecular volume), as well as the specific leaching conditions (e.g., S/L ratio, temperature, extraction solvent, time of extraction, and mixing speed) (Town and van Leeuwen, 2020; Suhrhoff and Scholz-Böttcher, 2016). Multiple environmental factors can affect the leaching of plastic additives, including temperature, salinity, and UV radiation (Suhrhoff and Scholz-Böttcher, 2016;Dhavamani et al., 2022). Temperature and UV radiation can increase the leaching of plastic additives whereas salinity causes a minor effect with positive or negative effects depending on the additives (Suhrhoff and Scholz-Böttcher, 2016; Dhavamani et al., 2022). To keep the environmental relevance of ecotoxicity tests, these factors need to be adapted to the test species and local environmental conditions, particularly for species from different latitudes. This makes it difficult to standardize certain environmental parameters for the lixiviation of MPs for aquatic ecotoxicity. However, some key factors related to the intrinsic particle properties (size) or methodological aspects (solid-to-liquid ratio, mixing conditions, and lixiviation time) allow some degree of standardization for obtaining leachates for toxicity testing.

The general aim of this study is to propose a protocol to standardize certain key methodological factors to obtain leachates from MPs for aquatic ecotoxicity testing. This study does not intend to resolve all the complexity on the topic of plastic leaching such as the interactions between parameters, the chemical composition of leachates, or their effects on biota but it aims to provide some relevant methodological recommendations for obtaining leachates for toxicity testing, allowing better comparability among studies. Although it is not the main scope of this study, we also provide some suggestions for acute toxicity testing with leachates and chemical analyses. We first reviewed the current literature to evaluate whether key methodological aspects of plastic leaching can be standardized. Second, we conducted experimental research using different types of plastics and biological models to assess some of the recommendations indicated in the protocol to balance feasibility and environmental relevance.

2. Methodology

2.1. General approach

We considered previous research/literature (S.I. Table S1) and conducted new laboratory studies to define the protocol. The proposed protocol takes into consideration previous methodological efforts intended to test the potential toxicity of lixiviates from several solid matrices such as residues (U.S. EPA, 1992; CEN, 2002) and medical devices (ISO, 2021).

The proposed protocol is divided into 5 main steps: micronization, size fractionation and particle size characterization, lixiviation, leachate filtration, and toxicity testing (Fig. 1). These general steps were used in our bioassays to investigate the following critical aspects of the lixiviation protocol: Solid-to-Liquid (S/L) ratio, particle size, contact/lixiviation time and mixing conditions.

We used a variety of plastic materials including conventional petroleum-based plastics and bioplastics. Beach-collected plastics were selected due to environmental relevance, polyvinyl chloride (PVC) because it is well-known for its high concentration of additives, and a biodegradable bag because bioplastics are expected to largely replace conventional plastics in the future and therefore, their potential toxicity on the marine environment need to be evaluated.

To test the influence of particle size and lixiviation time on leachate toxicity (S/L ratio = 10 g/L), we used two types of plastic (a commercial compostable carrier bag made of Mater-Bi® biopolymer and beach-collected plastics), and two types of standard toxicity tests, the sea urchin embryo test ("SET") (Beiras et al., 2012; Alonso-López et al., 2021) and the bacterial bioluminescence toxicity assay (Microtox®, Modern Water, Acute toxicity 100% Test) (S·I Table S2). To investigate the effect of mixing conditions, we used a toy made of soft polyvinyl chloride (PVC) material to assess the influence of rotation speed (1 and 60 rpm) on leachate toxicity (S/L ratio = 1 and 10 g/L) (S·I., Table S2).

In the next subsections, we present key aspects of the proposed methodology based on previous studies and the description of the specific methods used in the bioassays conducted here to support the protocol guidelines. In the Results and Discussion section, we present the results of the bioassays, the general guidelines of the protocol, and the rationale that justifies the main recommendations for critical aspects of the protocol.

2.2. Micronization

Micronization of plastic allows for obtaining micro-size particles of irregular shapes that can mimic microplastics for aquatic toxicity testing (Oliviero et al., 2019; Beiras et al., 2019). Cut plastic and large MPs (1–5 mm) can be ground in ultracentrifuge mills or other types of laboratory-grinding mills after embrittlement using liquid nitrogen (LN) (Fig. 1A–C). Adding dry ice (or LN) in the grinding chamber retards

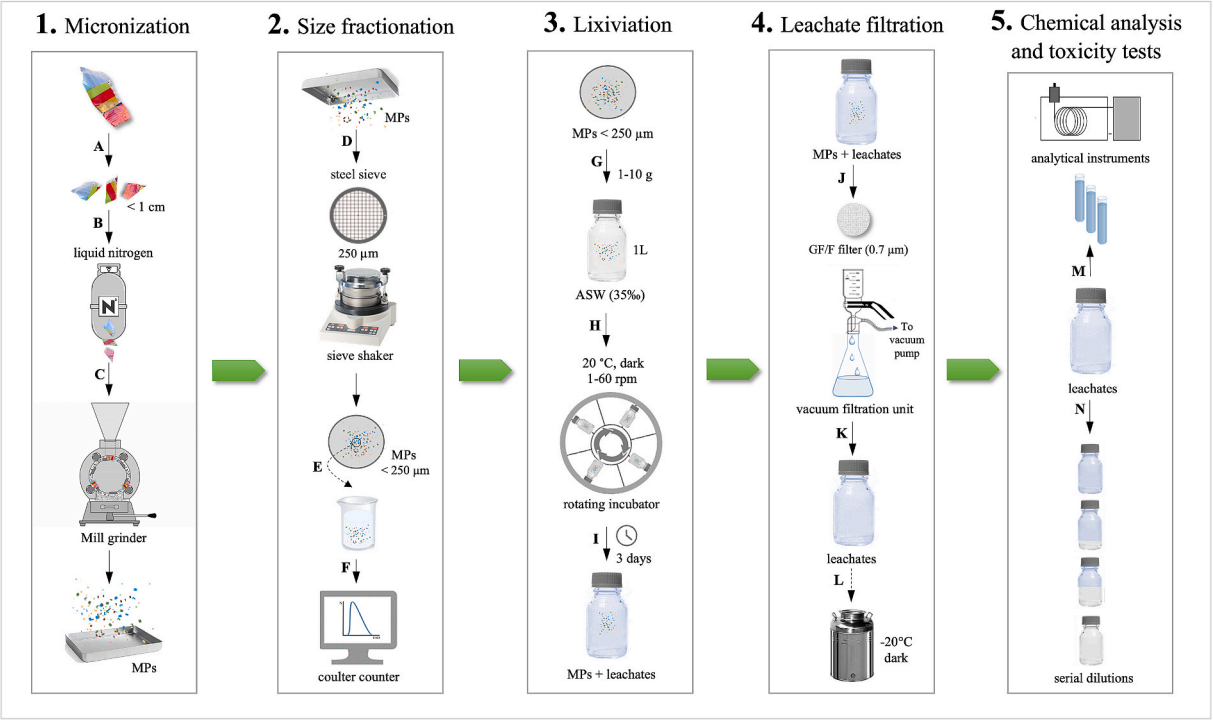


Fig. 1. Scheme of the proposed methodology for lixiviation of microplastics for toxicity testing and chemical analysis of leachates. A: cutting, B: freezing; C: grinding, D: dry sieving, E: suspension of subsample, F: particle sizing, G: loading (1 g L-1), H:mixing, I: lixiviation, J: filtration; K: leachate obtained, L: leachate preservation (if needed), M: chemical characterization of leachates, N: toxicity testing.

plastic melting and aids in the grinding process. For soft materials (e.g., tire rubber), a stainless-steel milling cutter can be used for micronization (Page et al. 2022). Smaller particle sizes can be obtained by using cryo-mills, although the overall mass that can be ground is lower.

The plastics used in the bioassays were first embrittled using LN and second ground using an ultracentrifuge mill (ZM200, Retsch) using dry ice during the process. Field macroplastics (mainly bottles, caps, hard containers, bags, and ropes.) were collected from three different beaches of the French Atlantic coast: Tarnos (43.540°N, 1.510°W), Le Porge (44.900°N, 1.217°W) and Les Sables d'Olonne (43.567°N, 1.483°W) in March and April 2021. Field plastics were stored at 4 °C in dark before being used. Each plastic item was processed separately. The polymer composition was identified by ATR- FT-IR. Then macroplastics were cut into 5 cm squares and ground separately using an ultracentrifuge mill with a ring sieve of 1 mm aperture size (ZM200, Retsch) with dry ice. Based on previous field data (e.g., Erni-Cassola et al., 2019), an environmentally realistic mixture of MPs was prepared by mixing <1 mm particles from the different polymers. The final composition of the MPs mixture (in dry weight) was polyethylene (PE) = 46%, polystyrene (PS) = 40%, polypropylene (PP) = 12%, and polyethylene terephthalate (PET) = 2%.

2.3. Size fractionation and particle size characterization

Ground plastics can be dry sieved through metallic/steel meshes to obtain homogeneous size fractions (Fig. 1D), a key aspect for standardization. The size of plastics used for toxicity testing of leachates varies notably among studies (S.I. Table 1). After reviewing the literature on plastic leachate toxicity tests and the size of microplastics commonly found in seawater in the environment, we found two relevant size fractions to be tested: (1) MPs <250 µm since it is a size fraction that has been used in several bioassays and up to 80% of marine microplastics are smaller than 250 µm (Rist et al., 2020; Gunaalan et al., 2023) and (2) MPs <1 mm, since this fraction covers a major variety of particle sizes and it is more feasible to obtain in the laboratory since obtaining this size fraction is less time-consuming than for small-size fractions that can have a low yield depending on the plastic material.

In the bioassays, each type of ground plastic was dry-sieved through ISO-certified stainless-steel sieves to obtain homogeneous size fractions to be tested: <250 µm and <1000 µm. The size distribution of the particles between 0.4 and 2000 µm in each MP fraction was measured using a laser diffraction particle analyzer (LS I3 320, Beckman Coulter) after dispersion with the aid of a surfactant (Tween 80) and 60″ sonication (Fig. 1*E*–F).

2.4. Lixiviation

2.4.1. Solid to liquid (S/L) ratio

The plastic particles chosen for lixiviation must be mixed with seawater at a given S/L ratio. European standards intended to test for leaching of landfilled waste materials with particle size below 4 mm (CEN, 2002) use an S/L ratio of 1:10 (i.e., 100 g/L), whereas American standards (U.S. EPA, 1992) use 1:20 (i.e., 50 g/L), and Dutch standards (NEN, 1995) proposed 1:100 (i.e. 10 g/L). The CEN (2002) terrestrial protocol was likewise adopted for aquatic toxicity testing of plastic leachates (Bejgarn et al., 2015; Lithner et al., 2012). The effect of plastic load on lixiviate toxicity assessment in a marine context was previously investigated by Beiras et al. (2019), who used the sea-urchin embryo and *Acartia* nauplius tests to compare the toxicity of leachates obtained from micronized (<250 µm) PVC resin and a commercial product made of soft PVC using S/L ratios ranging from 1:10 (100 g/L) down to 1:100,000 (0.01 g/L). In that study, PVC resin used as reference material showed no toxicity (TU < 1) (Beiras et al., 2019). The soft PVC leachates obtained at 0.01 and 0.1 g/L did not cause larval growth inhibition even undiluted, and thus these S/L ratios were considered too low for detecting any effect. In contrast, 1, 10, and 100 g/L leachates did produce dose-response data suitable for the calculation of EC50s and toxic units (TU) (Beiras et al., 2019). Based on these previous results, a S/L ratio of 1 and 10 g/L was used in our toxicity tests (Fig. 1G).

Table 1
Toxicity of lixiviates obtained from MPs <250 μm and <1 mm from the biodegradable and beached plastic materials on sea urchin embryo at different lixiviation times. No observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) are indicated as the dilutions of the leachates obtained using an S/L ratio of 10 g/L. EC50: leachate dilution that produced a 50% decrease in larval growth with respect to the control C.I. confidence interval. EC50 × expressed in g L^{-1} based on the S/L ratio used to produce the leachates. n.c.: not calculable.

Type	Size	Time	NOEC	LOEC	EC50 (95% C·I.)	EC50* (g/L)
Biodegradable Plastic	<250 μm	24 Hours	1/30	1/10	1/5.8 (1/4.7–1/7.1)	1.73
		72 Hours	1/10	1/3	1/5.8 (1/3.6–1/14.2)	1.72
		7 Days	1/10	1/3	1/4.4 (1/3.0–1/8.4)	2.28
	<1 mm	24 Hours	1/30	1/10	1/4.9 (1/3.2–1/10.9)	2.04
		72 Hours	1/10	1/3	1/5.5 (1/3.5–1/14.5)	1.82
		7 Days	1/10	1/3	1/4.0 (1/3.0–1/5.4)	2.51
Beached Plastic	<250 μm	24 Hours	1/10	1/3	1/1.2 (1/1.0–1/1.3)	8.3
		72 Hours	1/10	1/3	n.c.	n.c.
		7 Days	1/30	1/10	1/1.3 (1/0.8–1/2.0)	7.86
	<1 mm	24 Hours	1/3	1	1/2.1 (1/1.5–1/2.6)	4.66
		72 Hours	1/3	1	n.c.	n.c.
		7 Days	1/30	1/10	1/1.6 (1/1.3–1/2.0)	6.27

2.4.2. Lixiviation medium

Artificial seawater (ASW) and natural seawater (NSW) are ecologically relevant media for ecotoxicity tests with marine organisms (U.S. EPA, 1998 ;2002; Volpi Ghirardini et al., 2009) Lorenzo et al. (2002) provided a common formulation of ASW. Filtered (0.2 μm), autoclaved, and/or UV-sterilized natural seawater can also be used as media, and in this case, we advise using seawater of oceanic characteristics collected in non-polluted areas. The salinity of the seawater used for leaching can be adjusted to specific test species and local environmental conditions. For freshwater toxicity tests, standard synthetic water (e.g., ISO, 1999; US U. S. EPA, 2002) prepared with ultrapure water (e.g. MilliQ®) is recommended as a lixiviation medium.

In the bioassays, chemically defined ASW was used as the lixiviation medium for all sea urchin embryo tests (salinity = 35.1 ppt ± 0.3). Previous research showed that the size (length) of *P. lividus* pluteus larvae was higher in the controls with ASW than with 0.22 μm filtered NSW (details about the composition and preparation of ASW can be found in Saco-Álvarez et al., 2010). 0.22 μm-filtered NSW (32 PSU, pH 8.25) collected in a reference non-polluted site outside Arcachon Bay (Gamain et al., 2016) was used as the lixiviation medium for the bacterial bioluminescent toxicity test. Previous tests indicated that toxicity in this test with ASW and NSW is similar (Volpi Ghirardini et al., 2009).

2.4.3. Mixing conditions

Mixing of the solid and liquid phases is recommended for the lixiviation of microplastics since the aquatic systems are turbulent. Agitation of the solid and liquid phases can be provided by rotatory mixing (U.S. EPA, 1992). Overhead rotators, laboratory rollers, or plankton wheels of different dimensions can be used for rotatory mixing to obtain plastic leachates. Glass bottles with screw caps with polytetrafluoroethylene (PTFE) protected seal should be used; this seal is chemically inert and allows the bottles to be closed without air/headspace. The volume of the bottles can vary according to the amount of leachate needed. In our bioassays, an experiment was conducted to evaluate the influence of mixing conditions (1 and 60 rpm) on the toxicity of the leachates at two S/L ratios (1 and 10 g/L). Based on these results (see section 2.6), lixiviation for the SET was conducted at 1 rpm by placing the glass bottles in a rotatory wheel at 20 °C in the dark (Fig. 1H–I).

2.4.4. Lixiviation/contact time

Standard procedures for medical devices recommend 24 or 72 h (ISO 10993–12), whereas those for residues use 18 ± 2 h (U.S. EPA, 1992) and 24 ± 0.5 h (CEN, 2002) as contact time. According to the literature, toxic leachates have been obtained from microplastics after 1 to 336-h mixing, although the most frequent contact times range from 24 to 72 h (S·I., Table S1).

In our bioassay to evaluate the influence of contact time on the toxicity of leachates, we compared the toxicity of leachates obtained after 24 h, 72 h, and 1 week of mixing using both the SET and the Microtox® tests. We used micronized (<250 μm) MaterBi biopolymer bags and beach-collected plastics both at S/L ratio of 10 g/L.

2.5. Leachate filtration

After mixing, microplastics can be removed from the leachate by filtration (Fig. 1J), using glass-fiber filters to minimize the retention of organic molecules leaching from the polymer matrix, for example by using Whatman GF/F filters (nominal pore size = 0.7 μm) (U.S. EPA, 1992). Glass or metal funnel in individual vacuum filtration unit is recommend (Fig. 1J). This filtration system does not remove nanoplastics (<1 μm), which can be present in the obtained leachates (Fig. 2K). For ecotoxicological bioassays, filtered leachates should be tested immediately after preparation, or frozen (−20 °C) to allow time-deferred studies and prevent changes in composition. ISO (2021) prescribes stability verification when leachates are stored refrigerated (2 °C to 8 °C) for longer than 24 h.

In our bioassays, leachates were filtered through pre-combusted (450 °C, 2 h) Whatman GF/F filters before toxicity testing. Temperature, salinity, pH, and dissolved oxygen were measured in the obtained leachates to ensure these values complied with test requirements, i.e., 20 ±1 °C, salinity >30 PSU, pH between 7.5 and 8,5, and dissolved oxygen >5 mg/L (Beiras et al., 2012). The test requirements for the Microtox® assay are 15 ± 0.5 °C, salinity = 20 PSU, and pH between 6.0 and 8.5 (Canada Environment, 1992). In our experiments, the pH in the leachates was around 8–8.2, and the leachates were tested immediately after filtration.

2.6. Toxicity testing

The *Paracentrotus lividus* sea urchin embryo test was carried out as described by Beiras et al. (2012) using 4 mL glass vials and four replicates per treatment. Serial dilutions of the leachates in control ASW, 1 (undiluted), 1/3, 1/10, and 1/30 were tested (S.I. Fig. S2). Briefly, fertilized eggs were exposed for 48 h to the different dilutions at 20 °C in the dark. The maximum length of 35 larvae (n = 35) per vial was measured under an inverted microscope (Leica DMI 4000 B) using Leica Application Suite LAS image analysis software version 4.12.0 (Leica Microsystems, Germany). The acceptability criteria for this test were the percentage of fertilized eggs >98% and size increase in controls >218 μm after 48 h incubation at 20 °C (Saco-Álvarez et al., 2010).

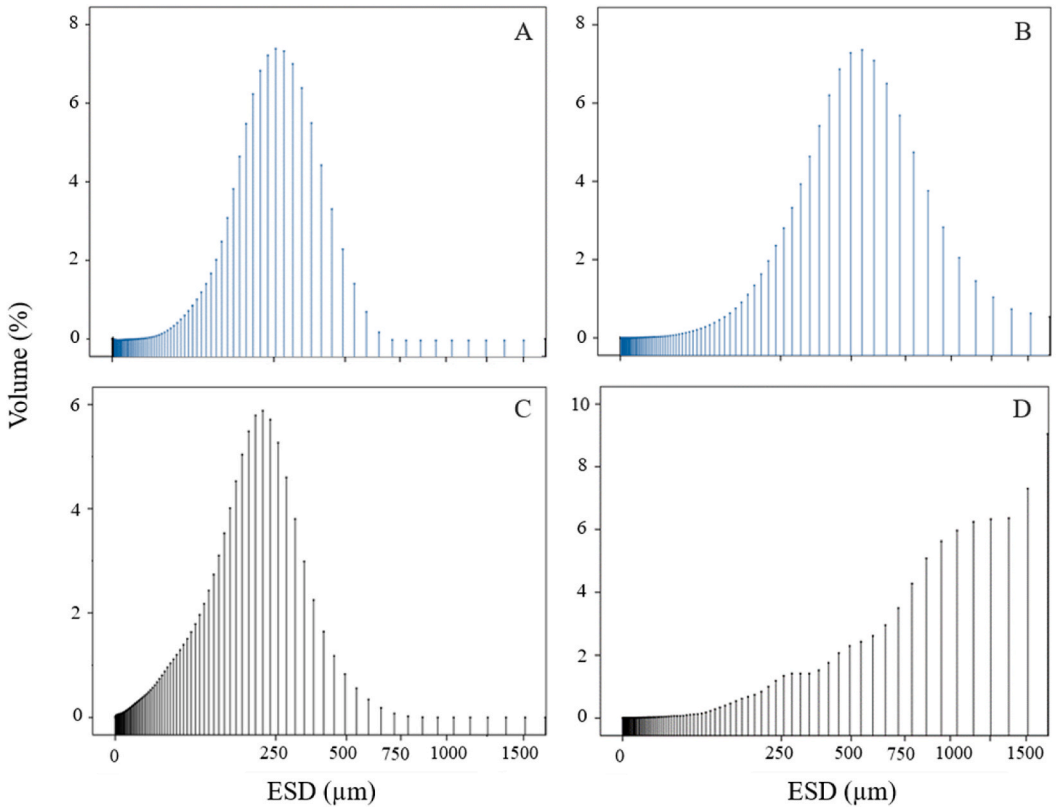


Fig. 2. Particle-size distribution shown as relative volume percentage frequency of micronized plastics: A) 250 μm-sieved biopolymer; B) 1 mm-sieved biopolymer; C) 250 μm-sieved beach plastics; D) 1 mm-sieved beach plastics. ESD: equivalent spherical diameter.

The influence of particle size and lixiviation on the toxicity of leachates from the beached plastics and the biopolymer was also investigated using the Microtox® assay. The Microtox® is a standardized acute toxicity assay using the marine bacteria *Vibrio fisheri* (ISO, 1999). The bottles were incubated in an orbital shaker (KS 501 digital IKA WERKE) at 175 rpm and 20 °C in the dark. The bioassay was carried out on four serial dilutions of lixiviates from 1/1.6 to 1/48. Each assay was run in triplicate. Bioluminescence was measured after 30 min of exposure and compared with a blank (FSW, 20 PSU) and a positive control (200 mg/L Potassium dichromate) using a Microtox 500 analyzer (Azur environmental).

The statistical analyses were performed with IBM SPSS (v. 24). ANOVA was executed to test significant differences ($p < 0.05$) between each treatment and the control in the sea urchin embryo bioassays, using Dunnett's post hoc test or Dunnets T3 when variances were not homogeneous. The lowest observed adverse effect concentration (LOEC) and the highest no observed adverse effects concentration (NOEC) were obtained. A probit dose-response model was conducted to calculate the leachate dilution that produced a 50% decrease in larval growth compared to the control (EC50) and their 95% confidence intervals (CIs). EC50 was also expressed in g L^{-1} by multiplying the dilution corresponding to a 50% effect times the S/L ratio used to produce the leachates. In the Microtox test, we used ANOVA or Kruskal-Wallis test and Tukey posthoc test to determine statistically significant differences among treatments ($p < 0.05$).

3. Results and discussion

3.1. Micronization, size fractionation, and particle size characterization

The proposed methodology for plastic micronization and size fractionating was successful to obtain microplastics from the three types of plastic materials. Size distribution of the plastic fractions used in our experiments are shown in Fig. 2; S·I.: Table S2 and Fig. S2). Sieving with a 250 μm-sieve provided more uniform particle size distributions for both plastic types than sieving with a 1000 μm sieve (Fig. 2). Therefore, using small size fractions (MPs <250 μm) ensures better comparability among different types of micronized plastics.

Large plastic items or plastic litter (macro and mesoplastics) must be initially cut down manually by using stainless steel scissors or other tools to a maximum dimension not larger than 1 cm to facilitate subsequent grinding. Characteristics of the collected plastics, such as type of item/product, shape, size, colors, and polymer composition by Fourier-transform infrared spectroscopy (FTIR) should be determined in advance if lixiviates results have to be precisely related to the different typologies of investigated plastics. For field-collected plastics, it is recommended to store the samples in dark and at 4 °C to avoid the degradation of additives and the growth of microorganism on the plastic surfaces. There should be no washing of plastic before fragmentation to avoid leaching before bioassays. Once airdried, the surface of the plastics can be cleaned with a natural paintbrush before the micronization.

Standard sieves with certified mesh size must be used, and sieving may be conducted by hand with the help of a natural (no plastic) paintbrush or automatically by using a sieve shaker (Fig. 1D). The efficiency of sieving depends on shaking intensity, time, and properties of the material such as static electricity charge capacity. Therefore, it is advisable to check actual particle size distribution by using a particle size analyzer (e.g., laser diffraction particle analyzer, Multisizer coulter counter, Flow Imaging Microscopy "FlowCam") (1 E, 1 F) or at least, by microscopy and image analyses. For micronized MPs (irregular fragments), we recommend using the equivalent spherical diameter (ESD, μm) to define the MP size and ensure comparability among studies. To measure the size (ESD) and concentration of MPs using a coulter counter, a subsample of the MPs <250 μm (1 mg) should be suspended in 100 mL of 0.2 μm-filtered autoclaved seawater with a non-ionic surfactant (e.g., Tween-80, 0.1% solution) (1 E), and sonicated. For the

preparation of the surfactant solution, it is recommended to boil the 0.2 μm-filtered seawater (FSW) to sterilize the solution and to facilitate the dispersion of the surfactant uniformly (Cospheric, 2018).

3.2. Effects of particle size on the toxicity of plastic leachates

Particle size has a strong influence on the uptake and release kinetics of chemicals associated with plastic (Town and van Leeuwen, 2020). Since surface to volume ratio increases with decreasing size, it is expected that the desorption rate of chemicals increases as particle size decreases. In a previous study, the toxicity of lixiviates from ground-stranded fishing nets on sea-urchin embryos increased significantly as particle size decreased (Beiras et al., 2019). However, we did not find statistically significant differences in toxicity between the <250 μm and <1 mm size fractions in the sea urchin embryo toxicity tests for the tested material (Figs. 3 and 4). Similarly, we did not observe

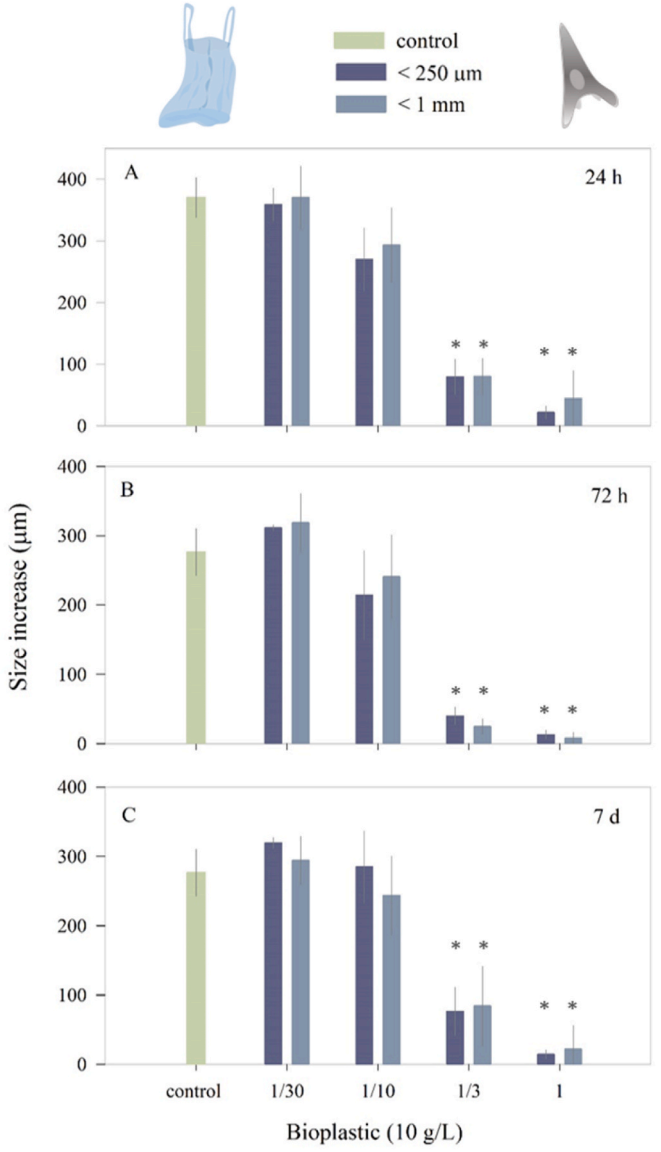


Fig. 3. Effect of plastic particle size (<250 μm and <1 mm) and lixiviation time (A: 24 h, B: 72 h, C: 1 week) on leachate toxicity of a micronized commercial biopolymer ("bioplastic") at different dilutions (1/3, 1/10; 1/30) of a leachate prepared at a S/L ratio = 10 g/L. Size increase from sea urchin embryo to pluteus larvae was used as the endpoint in the toxicity test. Values are expressed in mean ± SD, n = 10. Asterisk indicates statistically significant difference compared to the control ($p < 0.05$).

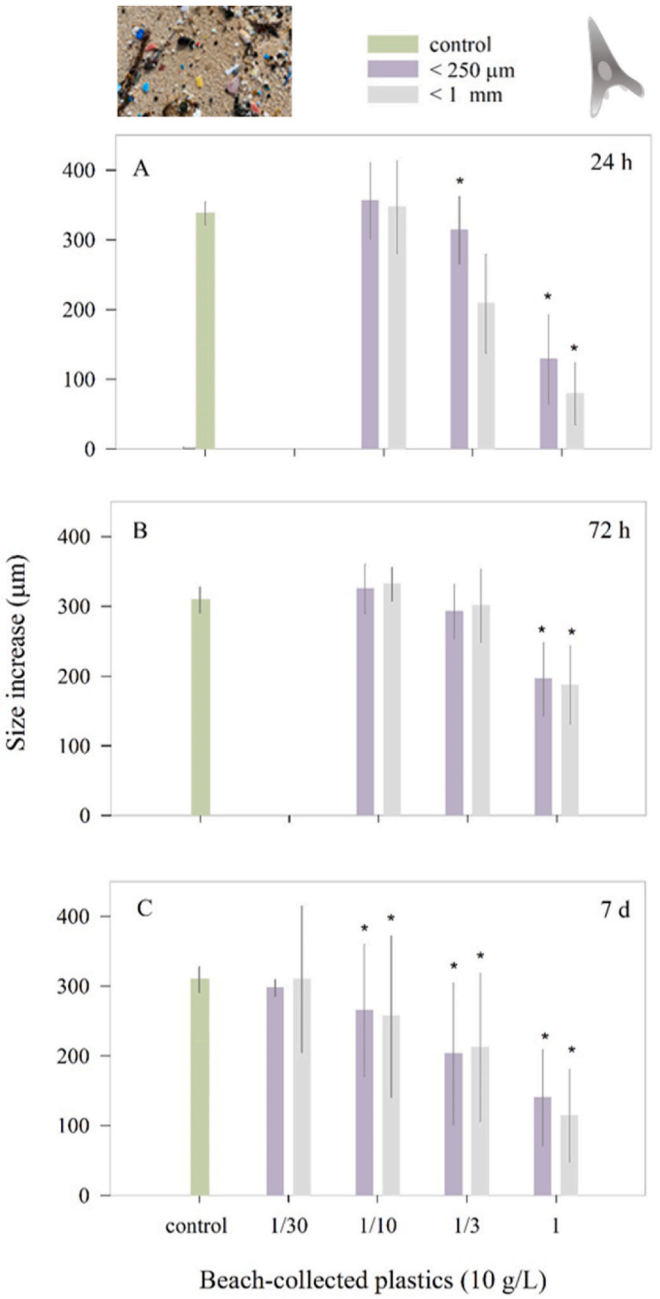


Fig. 4. Effect of particle size (<250 μm and <1 mm) and lixiviation time (A: 24 h, B: 72 h, C: 1 week) on leachate toxicity of a micronized beach-collected plastics. (S/L ratio = 10 g/L). Size increase from sea urchin embryos to pluteus larvae was used as endpoint in the toxicity test. Note that in A and B, we did not used the dilution 1/30. Asterisk indicates statistically significant difference compared to the control ($p < 0.05$).

significant differences in toxicity between MP sizes in the Microtox® test, except at a lixiviation/contact time of 72 h where the smaller fraction (<250 μm) was more toxic than the larger fraction (<1 mm) (Fig. 5). Therefore, the correlation of plastic particle size on leachate toxicity can vary depending on the plastic item and organisms.

Although MPs <1 mm can have similar toxicity than MPs <250 μm, we recommend using the MP size fraction <250 μm for toxicity testing of leachates from micronized plastics since most of the MPs found in the aquatic environments are in that size fraction (Rist et al., 2020; Kooi et al., 2021; Gunaalan et al., 2023). Also, tire wear particles, a dominant source of MPs in aquatic systems (Boucher and Friot, 2017;Kole et al., 2017; Baensch-Baltruschat et al., 2020) with toxic plastic leached

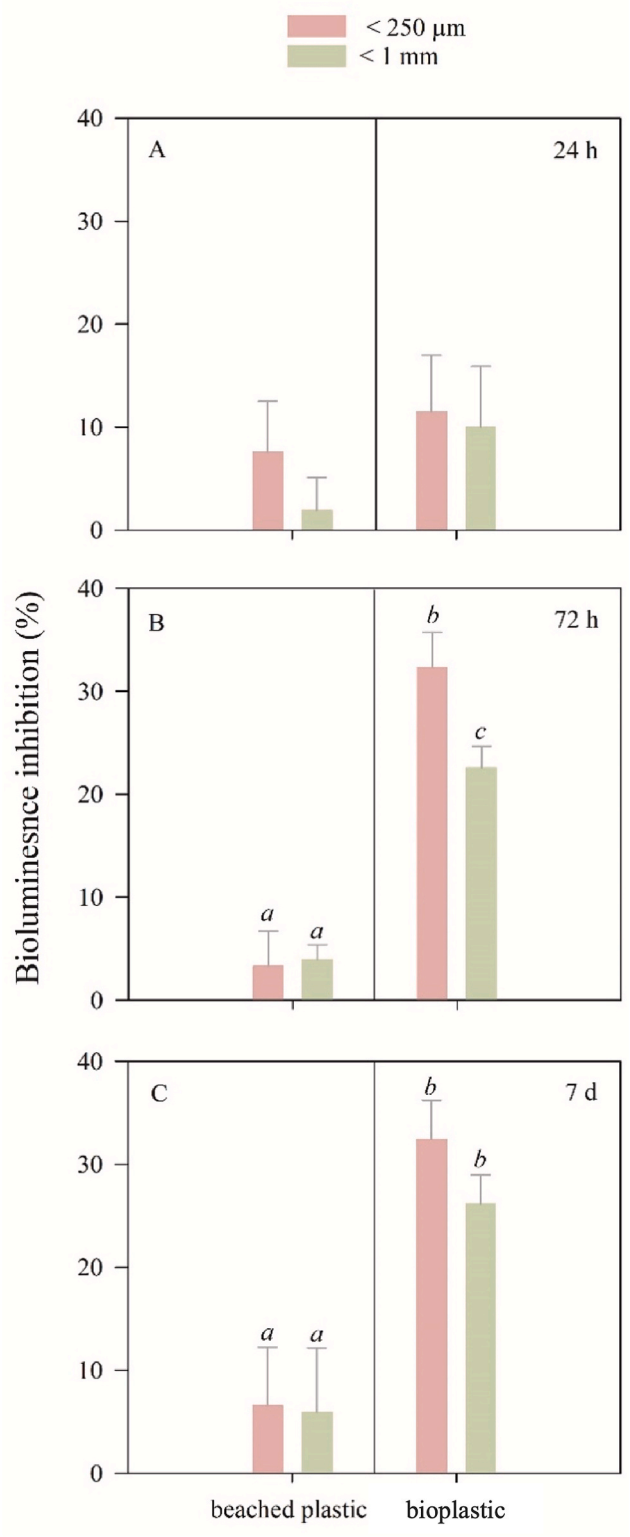


Fig. 5. Effect of particle size (<250 µm and <1 mm) and lixiviation time (A: 24 h, B: 72 h, C: 1 week) on leachate toxicity of a micronized beach-collected plastics and commercial biopolymer (S/L ratio = 10 g/L) using the Microtox® test. % of bacterial bioluminescence inhibition compared to the control was used as endpoint. Bioluminescence was measured after 30 min of exposure. Values are expressed in mean ± SD, n = 3. Letters indicate statistically significant differences among treatments ($p < 0.05$, Anova (B and C), Kruskal-Wallis (A) and Tukey post-hoc test).

additives (Tian et al., 2021) are typically smaller than 250 µm.

If the micronization step is skipped, then remarkably longer lixiviation times may be needed for leached plastic additives to reach equilibrium (up to 60 days (Suhrhoff and Scholz-Böttcher, 2016; Do et al., 2022). On the other hand, the increase in sensitivity and robustness obtained by using the micronization step may result in high time consumption, increased costs for relevant equipment, and limitations in the number of samples that can be tested. If those requirements cannot be met, an alternative time cost-effective approach can be carried out to obtain lixiviates from particles fragmented by other mechanical methods and/or sieved to a maximum standard size of 1 mm (small microplastics) or 5 mm (all microplastics).

3.3. Solid-to-liquid ratio

Leachates (PVC, <250 µm) from a S/L ratio of 10 g/L were more toxic than those from a S/L ratio = 1 g/L to the sea urchin embryo (Fig. 6, Table 1). However, an increase of ten times in the S/L ratio of micronized PVC (<250 µm) resulted in only a 3-fold increase in toxicity (EC50, Table 1). This indicates the increase in toxicity is not of the same magnitude that the increase in plastic load. Similar results were found by Beiras et al. (2019), where a 10-fold increase in the load of particles used to make up the leachate from 1 to 10 g/L produced a 3.4-fold increase in TUs only.

Very high plastic loads lack environmental relevance in aquatic ecosystems. Based on data from Manta net samplings, plastic concentrations in surface open sea waters frequently range within the µg L^{-1} level and never exceed the mg L^{-1} (Beiras and Schönemann, 2020). However, although mass estimates from field studies are lacking, concentrations of MPs can be higher in estuaries (Hu et al., 2018) and coastal areas when accumulations of floating plastics are formed as "marine litter windrows" (Cózar et al., 2021) or after runoff events (e.g., predicted concentrations of tire wear particles in surface waters can be in the mg L^{-1} level, particularly in coastal waters, Wik and Dave, 2009). However, very high S/L ratios allow for maximizing the sensitivity in detecting any potential toxicant released from the plastic. Therefore, a solid-to-liquid ratio of a maximum of 1–10 g/L is recommended. The dilutions from the leachates obtained at these solid-to-liquid ratios

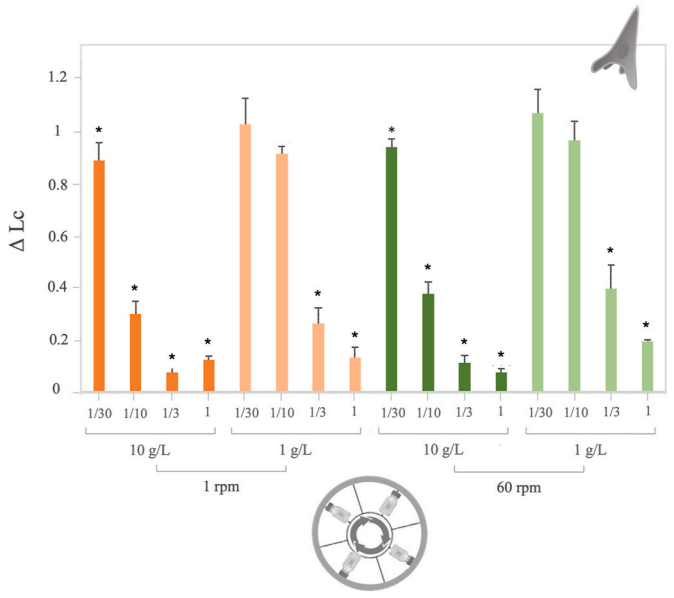


Fig. 6. Effect of rotation speed (rpm) and solid to liquid ratio (S/L) on the toxicity of leachates from micronized PVC. Size increase ratio compared to the control treatment (ΔLc) of *Paracentrotus lividus* larvae exposed to different leachate dilutions from soft PVC MPs (<250 µm). Asterisk indicates statistically significant difference compared to the control ($p < 0.05$).

should cover concentrations equivalent to mg L^{-1} in the for the exposure test to increase the environmental relevance.

3.4. Lixiviation medium

For ecologically relevant studies, water is recommended as the extraction vehicle (U.S. EPA, 1998; 2002; Volpi Ghirardini et al., 2009). When the focus is placed on *a priori* hazard assessments with industrial products, or identification of chemicals in field-collected samples, we may be interested in maximizing desorption and increasing the sensitivity of the method by increasing plastic load, temperature, extraction time, decreasing particle size, or even using non-toxic organic solvents such as dimethyl sulfoxide (DMSO) (Pannetier et al., 2019b, 2019a). The different protocol steps can be followed with a certain level of flexibility depending on the investigation: in environmental studies, the intrinsic variability will always prevent full comparability of data, while for regulatory and industry-related studies, standardization of all the below phases would be strongly recommended.

3.5. Mixing conditions

Leaching of organic plastic additives under agitation/turbulence is higher than in static conditions (Suhrhoff and Scholz-Böttcher, 2016). We found that increasing rotatory speed from 1 to 60 rpm did not significantly change the toxicity parameters of the lixiviate at either 1 g/L or 10 g/L (Fig. 6, Table 2). Therefore, rotation speeds from 1 to 60 rpm can be used as mixing conditions for obtaining leachates from microplastics (Fig. 1H).

Temperature and light conditions must also be standardized when possible. Typical standard conditions of T = 20 °C and in dark are highly advisable. Considering environmental relevance, additional experiments with different temperatures can be conducted to evaluate plastic leaching and toxicity in aquatic systems with extreme thermal conditions, e.g., polar regions. We advise lixiviation in the dark since organic compounds may be photosensitive and different light intensities and photoperiods may affect comparability amongst studies. The influence of light/UV radiation in leaching and the potential photo-modification and degradation of plastic additives should be further investigated and requires a proper experimental setup (e.g., use of quartz bottles).

3.6. Lixiviation time

We found that toxicity did not increase with lixiviation/contact time in any of the plastic materials and size fractions in the sea urchin embryo test (Figs. 3 and 4). In the Microtox test, we did not find significant differences in toxicity among lixiviation times for the beach microplastics. However, the toxicity of leachates from bioplastics increased from 24 h to 72 h, but not from 72 h to 1-week treatment (Fig. 5). This indicates that desorption of additives from small MPs fractions occurs fast. This result agrees with Seidensticker et al. (2017) semi-analytical model that predicts short partition equilibrium times (1–10 h) under typical laboratory experimental conditions (limited water volume).

Table 2
Toxicity of lixiviates obtained by mixing the MP fraction <250 μm from a soft PVC material at different rotatory speeds (1 and 60 rpm) and solid to liquid ratios (S/L) (1 and 10 g/L) on sea urchin embryo. No observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) are indicated as the dilutions of the leachates obtained using plastic loads of 1 and 10 g/L. EC50: leachate dilution that produced a 50% decrease in larval growth with respect to the control C.I. confidence interval.

Rotatory speed (rpm)	S/L (g/L)	NOEC	LOEC	EC50 (95% C·I.)
1	1	1/10	1/3	1/3.9 (1/3.0–1/5.0)
60	1	1/10	1/3	1/2.9 (1/2.3–1/3.6)
1	10	n.c.	1/30	1/11.6 (1/7.7–1/19.9)
60	10	n.c.	1/30	1/9.7 (1/7.3–1/13.2)

Also, as reviewed by Bridson et al., 2021, several studies indicate that rapid leaching of plastic additives in water occurs over hours to days followed by an extended period of slower release of additives over weeks to months.

An increase in plastic leachate toxicity to invertebrate larvae has been observed in previous studies when large particles are used. For instance, Rendell-Bhatti et al., 2021 found that toxicity of leachates from plastic pellets and granulates to sea urchin larvae increased with lixiviation time from 24 to 72 h. Similarly, Gardon et al., 2020 found the toxicity of leachates from several millimeter-sized MPs to bivalve larvae increased from 24 to 48 h in new (unweathered) plastics, but not in aged MPs. This indicates that the effect of lixiviation time on the toxicity can also vary depending on the plastic material characteristics (e.g., unweathered vs weathered).

The amount of plastic additive leached at any given time will depend on multiples factors such as the type of polymer, the size of the particles, the polymer/water partition coefficient, any intrinsic affinity between the additive and the polymer backbone, and the solid to liquid ratio (Town and van Leeuwen, 2020). If the lixiviation time is long enough, leached additives reach a steady state (partition equilibrium between the plastic phase and the aqueous medium), thus the amounts of additives extracted will reflect their relative concentrations in the polymers, weighted by their partition coefficients (Town and van Leeuwen, 2020). The lixiviation time of a plastic item needed to reach this equilibrium will depend on the specific polymer and its additives, and the conditions mentioned above. The smaller the plastic particles, the shorter the time needed to reach this equilibrium (Koelmans et al., 2013; Town and van Leeuwen, 2020).

Taking into consideration our results and previous studies, we advise a lixiviation time of 72 h for both field-collected and non-weathered micronized plastics since the toxicity of leachates did not increase when lixiviation time was extended to 1 week in any of the studied micronized plastics and sizes (<250 μm, < 1 mm) and because in previous studies with leachates from large plastic particles show an increase in toxicity from 24 h to 48–72 h.

The leaching kinetics of plastic additives has been commonly investigated in static batch systems without media exchange to establish the equilibrium partitioning between the plastics and the aqueous (Bridson et al., 2021). In dynamic and semi-dynamic approaches, where plastics are exposed to a water continuous flow or renewed media (Suhrhoff and Scholz-Böttcher, 2016; Bridson et al., 2021), the leaching of plastic additives is not constrained by their solubility or the attainment of partition equilibrium, allowing to provide sink conditions. These conditions represent a situation closer to the open ocean, where near-infinite dilution occurs (Bridson et al., 2021). Therefore, to increase environmental relevance, dynamic and semi-approaches should be also considered to better understand the leaching kinetics of plastic additives in the marine system and the assessment of their environmental risks.

3.7. Acute toxicity testing

In our bioassays, when considering a similar size (<250 μm) and lixiviation conditions (S/L = 10 g/L, 1 rpm), soft PVC was the most toxic plastic/polymer type to sea urchin embryos (Tables 1 and 2). Leachates from a non-weathered commercial biopolymer and soft PVC were more toxic than leachates from beached/weathered micronized plastics to marine biota (Tables 1 and 2), suggesting that industrial chemical additives are the main drivers of chemical toxicity from plastics as supported also by previous studies (Gardon et al., 2020; Beiras et al., 2021). These results emphasize the need for research on chemical analysis and toxicity of industrial plastic additives on marine biota to better evaluate the toxicity of conventional plastic and alternative polymers.

Characteristics of the leachates (pH, temperature, salinity, dissolved oxygen) must be recorded before each toxicity test, and values must be checked according to the requirements of the test species, and then

adjusted if needed, to avoid false positives. In the case of the absence of an oximeter, both the leachates and control seawater can be saturated with oxygen by air bubbling for 15 min for example by using a glass pipette as an "air outlet" connected with a silicon tube and a sterile syringe filter (0.22 μm) to an aquarium pump.

For dose: response toxicity testing, geometric serial dilutions of the leachate in control seawater should be tested. Dilutions should cover the range from no effect to >50% effect to allow precise calculation of EC10 and EC50 for each biological endpoint. These two parameters allow a quantitative comparison of the toxicity among different stocks of microplastics. The use of negative controls or blanks (seawater not containing the test material but otherwise exposed to identical vessels and filters) is required for toxicity testing. A positive control of reference materials (e.g., commercial, or customized materials from a homogeneous stock of previously known composition and toxicity) is strongly advisable. We recommend a minimum of 5 doses in toxicity testing of leachates including the control.

As mentioned in the method section, the leachates obtained with the proposed methodology can also include nanoplastics (NPs, <1 μm). Micronization is a useful tool to obtain particles that mimic environmental microplastics (fragments) for relevant ecotoxicity tests, but the process of micronization also produces NPs (Gardon et al., 2022). Likely, NPs can also be released during the lixiviation time. All the leachates from the different micronized plastics used in the study are expected to have NPs but they show contrasting toxicity, which suggests that leached chemicals/additives are the key players in the observed toxic effects in our protocol. However, to avoid overestimating the effects of leachates on biota, future research should include efforts for the quantification of NPs in the obtained leachates and to separate the effects of leached chemicals from nanoparticles. For instance, the concentration and sizes of NPs in the leachates could be characterized by laser diffraction, Dynamic Light Scattering, or Secondary ion mass spectrometry after the filtration with a glass fiber filter. Additional bioassays could also be performed to evaluate the toxicity of leachates after removing NPs (e.g., centrifugation at 10,000 rpm for 10 min, Murray and Örmeci, 2020).

Chemical analyses of leachates and the seawater used as the lixiviation medium are recommended. Leachates can be preserved and stored in glass containers at −20 °C in the dark for analyses of organic and inorganic compounds or in metal containers for analyses of organic compounds (Fig. 1L). The chemical analyses of the leachates should include measurements of metals and organic additives, including those of emerging concern or/and ecotoxicological relevance such as brominated flame retardants (BFR) (e.g., polybrominated diphenyl ethers, PBDE), Organophosphate ester (OPE) flame retardants; Phthalic acid esters (PAE) used as plasticizers, Nonylphenols (NP), Bisphenols and Antioxidants (e.g. Irgafos® 168, phenylenediamine (PPD) additives). For field-collected MPs other organic pollutants are also relevant, e.g., PAHs, PCBs, and pesticides (Camacho et al., 2019). Bridson et al., 2021 provide a summary of the extraction protocols and analytical techniques used in the identification and quantification of additives in plastic debris and test materials used in environmental studies. A strategy to identify toxic chemicals leaching from the polymers combines microscale toxicity testing and analytical chemistry and consists of testing extracts obtained with different solvents and effect-directed analysis (EDA) (Hecker and Hollert, 2009). We recommend using EDA as a useful approach to identify toxicity-driving compounds in plastic leachates and their potential mode of action.

4.Conclusions

The proposed methodology was successful to determine the toxicity of leachates from different micronized plastics on marine biota and it balances sensitivity, feasibility, and environmental relevance. The main recommendations for both field-collected and non-weathered micronized plastics are the use of small size fractions (<250 μm-sieved MPs), an S/L ratio of 1-10 g/L, a rotatory speed of 1-60 rpm, and a lixiviation/contact time of 72 h. Following these recommendations would allow comparability amongst studies to better assess the toxicity of leached plastic additives and their risk for aquatic environments.

Author contributions statement

Rodrigo Almeda: conceptualization, writing - original draft, graphical representations, supervision, writing - review & editing, funding acquisition. Kuddithamby Gunaalan: laboratory and data analysis methodological application, writing - review & editing writing, graphical representations. Olalla Alonso-López: laboratory and data analysis methodological application, writing - review & editing writing. Alejandro Vilas: laboratory and data analysis methodological application, writing - review & editing writing. Clérandeau Christelle: laboratory and data analysis methodological application, writing - review & editing writing. Tara Loisel: laboratory and data analysis methodological application, writing - review & editing writing. Torkel Nielsen: writing - review & editing, funding acquisition. Jérôme Cachot: supervision, writing - review & editing. Ricardo Beiras: conceptualization, supervision, writing - review & editing, funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

The authors thank Edgar Dusacre, Camille Llech, Bettie Cormier, and Bénédicte Morin for their contribution to the sampling and sorting of macroplastics. We are also thankful to Clara Mendoza for their helpful technical assistance and to Raewyn Town, Maura Benedetti, Francesco Regoli and Xavier Cousin for their comments on previous drafts of this manuscript. This study was supported by The RESPONSE project, founded by the "Joint Programming Initiative Healthy and Productive Seas and Oceans, "JPI Oceans" (FCT JPI OCEANS MICROPLAST/0005/2018), through the national funding agencies of Spain (Spanish National Research Agency) and Denmark (Innovation Fund-Denmark). This study was also supported by the The Canarian Science and Technology Park Foundation of the University of Las Palmas de Gran Canaria (FCPCT), (C2020/65 DTU-ULPGC agreement), the Spanish Ministry of Science and Innovation and Spanish Agency of Research through a Ramón y Cajal Program grant (RYC 2018-025770-I) to RA, the MICROPLEACH project (PID 2020-120479 GA-I00) to RA, the RisBioPlas Project to RB (AEI: 10.13039/501100011033). ECOTOX is supported by grants for the consolidation and structuring of competitive research units of the Galician University System ED431C 2021/42, and belongs to CIM, funded by the Galician Government (Xunta de Galicia).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138894.

References

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

Beiras, R., Schönemann, A.M., 2020. Currently monitored microplastics pose negligible ecological risk to the global ocean. Sci. Rep. 10, 22281.

Beiras, R., Durán, I., Bellas, J., Sánchez-Marín, P., 2012. Biological Effects of Contaminants: Paracentrotus lividus Sea Urchin Embryo Test with Marine Sediment Elutriates. International Council for the Exploration of the Sea (ICES) (Report).

Baensch-Baltruschat, B., Kocher, B., Stock, F., Reifferscheid, G., 2020. Tyre and road wear particles (TRWP) - A review of generation, properties, emissions, human health risk, ecotoxicity, and fate in the environment. Sci. Total Environ. 733, 137823. https://doi.org/10.1016/j.scitotenv.2020.137823.

Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., Gambardella, C., Garaventa, F., Keiter, S., Le Bihanic, F., López-Ibáñez, S., Piazza, V., Rial, D., Tato, T., Vidal-Liñán, L., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. J. Hazard Mater. 360, 452–460.

Beiras, R., Tato, T., López-Ibáñez, S., 2019. A 2-Tier standard method to test the toxicity of microplastics in marine water using *Paracentrotus lividus* and *Acartia clausi* larvae. Environ. Toxicol. Chem. 38, 630–637.

Beiras, R., Verdejo, E., Campoy-López, P., Vidal-Liñán, L., 2021. Aquatic toxicity of chemically defined microplastics can be explained by functional additives. J. Hazard Mater. 406, 124338.

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.

Boucher, J., Friot, D., 2017. Primary Microplastics in the Oceans: A Global Evaluation of Sources. IUCN, Gland, Switzerland. https://doi.org/10.2305/IUCN.CH.2017.01.en.

Brede, C., Fjeldal, P., Skjevrak, I., Herikstad, H., 2003. Increased migration levels of bisphenol A from polycarbonate baby bottles after dishwashing, boiling and brushing. Food Addit. Contam. 20, 684–689.

Bridson, J.H., Gaugler, E.C., Smith, D.A., Northcott, G.L., Gaw, S., 2021. Leaching and extraction of additives from plastic pollution to inform environmental risk: a multidisciplinary review of analytical approaches. J. Hazard Mater. 414, 125571.

Camacho, M., Herrera, A., Gómez, M., Acosta-Dacal, A., Martínez, I., Henríquez-Hernández, L.A., Luzardo, O.P., 2019. Organic pollutants in marine plastic debris from Canary Islands beaches. Sci. Total Environ. 662, 22–31.

Canada Environment, 1992. Biological Test Method: Acute Toxicity Test Using Luminescent Bacteria (Photobacterium Phosphoreum), Report EPS 1/RM/24. Environment Canada, Ottawa, Ontario.

CEN, 2002. European committee for standardization, characterization of waste –leaching – compliance test for leaching of granular waste materials and sludges. Part 2: One Stage Batch Test at a Liquid to Solid Ratio of 10 L/kg for Materials with Particle Size Below 4 mm (without or with size reduction) CEN/TC292/WG2,EN 12457-2:2002.

Suspension of Hydrophobic Particles in Aqueous Solution. Technical Information Sheet, 2018. Cospheric. MFG-WI-88-rev1 Issue. Date: 04/23/2018. https://www.cospheric.com/images/PDFs/Suspension-of-Hydrophobic-Particles-in-Aqueous-Solution-1.pdf.

Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Úbeda, B., Hernández-León, S., Palma, Á.T., Navarro, S., García-de-Lomas, J., Ruiz, A., Fernández-de-Puelles, M.L., Duarte, C.M., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. USA 111, 10239–10244.

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

Cózar, A., Aliani, S., Basurko, O.C., Arias, M., Isobe, A., Topouzelis, K., Rubio, A., Morales-Caselles, C., 2021. Marine litter windrows: a strategic target to understand and manage the ocean plastic pollution. Front. Mar. Sci. 8, 571796.

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

Do, A.T.N., Ha, Y., Kang, H.J., Kim, J.M., Kwon, J.H., 2022. Equilibrium leaching of selected ultraviolet stabilizers from plastic products. J. Hazard Mater. 427, 128144.

Erni-Cassola, G., Zadjelovic, V., Gibson, M.I., Christie-Oleza, J.A., 2019. Distribution of plastic polymer types in the marine environment; A meta-analysis. J. Hazard Mater. 369, 691–698.

Fernandes, A.R., Rose, M., Charlton, C., 2008. 4-Nonylphenol (NP) in food-contact materials: analytical methodology and occurrence. Food Addit. Contam. 25, 364–372.

Gamain, P., Gonzalez, P., Cachot, J., Pardon, P., Tapie, N., Gourves, P.Y., Budzinski, H., Morin, B., 2016. Combined effects of pollutants and salinity on embryo-larval development of the pacific oyster, Crassostrea gigas. Mar. Environ. Res. 113, 31–33.

Gambardella, C., Piazza, V., Albentosa, M., Bebianno, M.J., Cardoso, C., Faimali, M., Garaventa, F., Garrido, S., González, S., Pérez, S., Sendra, M., Beiras, R., 2019. Microplastics do not affect standard ecotoxicological endpoints in marine unicellular organisms. Mar. Pollut. Bull. 143, 140–143.

Gardon, T., Huvet, A., Paul-Pont, I., Cassone, A.-L., Sham Koua, M., Soyez, C., Jezequel, R., Receveur, J., Le Moullac, G., 2020. Toxic effects of leachates from plastic pearl-farming gear on embryo-larval development in the pearl oyster *Pinctada margaritifera*. Water Res. 179, 115890.

Gardon, T., Paul-Pont, I., Le Moullac, G., Soyez, C., Lagarde, F., Huvet, A., 2022. Cryogrinding and sieving techniques as challenges towards producing controlled size range microplastics for relevant ecotoxicological tests. Environ. Pollut. 315, 120383.

Gunaalan, K., Fabbri, E., Capolupo, M., 2020. The Hidden Threat of Plastic Leachates: A Critical Review on Their Impacts on Aquatic Organisms, vol. 184. Water Research.

Gunaalan, K., Almeda, R., Lorenz, C., Vianello, A., Iordachescu, L., Konstantinos Papacharalampos, K., Rohde Kiær, C.M., Vollertsen, J., Nielsen, T.G., 2023. Abundance and distribution of microplastics in surface waters of the Kattegat/Skagerrak (Denmark). Environ. Pollut. 318, 120853.

Hecker, M., Hollert, H., 2009. Effect-directed analysis (EDA) in aquatic ecotoxicology: state of the art and future challenges. Environ. Sci. Pollut. Res. Int. 16, 607–613.

Hu, L.L., Chernick, M., Hinton, D.E., Shi, H.H., 2018. Microplastics in small waterbodies and tadpoles from yangtze river delta. China. Environ. Sci. Technol. 52, 8885–8893.

ISO, 1999. Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio Fischeri* (Luminescent Bacteria Test). Part 3: Method Using Freeze Dried Bacteria. European Committee for Standardization, Bruxelles. European Standard ISO 11348-3:1998.

ISO, 2021. Biological Evaluation of Medical Devices — Part 12: Sample Preparation and Reference Materials. ISO 10993-12:2021.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771. https://doi.org/10.1126/science.1260352.

Kim, Y.-J., Osako, M., Sakai, S., 2006. Leaching characteristics of polybrominated diphenyl ethers (PBDEs) from flame-retardant plastics. Chemosphere 65, 506–513.

Koelmans, A.A., Besseling, E., Wegner, A., Foekem, E.M., 2013. Plastic as a carrier of POPs to aquatic organisms: a model analysis. Environ. Sci. Technol. 47, 7812–7820.

Kole, P.J., Löhr, A.J., Van Belleghem, F.G.A.J., Ragas, A.M.J., 2017. Wear and Tear of Tyres: A Stealthy Source of Microplastics in the Environment. Inter. J. Environ. Res. Pub. He. 14, 1265. https://doi.org/10.3390/ijerph14101265.

Kooi, M., Primpke, S., Mintenig, S.M., Lorenz, C., Gerdts, G., Koelmans, A.A., 2021. Characterizing the multidimensionality of microplastics across environmental compartments. Water Res. 202, 117429.

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.

Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquat. Toxicol. 58, 27–41.

Murray, A., Örmeci, B., 2020. Removal effectiveness of nanoplastics (<400 nm) with separation processes used for water and wastewater treatment. Water 12, 635.

NEN:7341 Leaching Characteristics of Solid Earthy and Stony Building and Waste Materials - Leaching Tests - Determination of the Availability of Inorganic Components for Leaching, 1995. NEN, The Netherlands (Withdrawn).

Key Results JPI Oceans Microplastics Projects 2016-2019, 2020. JPI Oceans, Brussels, Belgium. https://doi.org/10.13140/RG.2.2.11658.00960.

Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S., Beiras, R., 2019. Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin *Paracentrotus lividus*. Environ. Pollut. 247, 706–715.

Page, T.S., Almeda, R., Koski, M., Bournaka, E., Nielsen, T.G., 2022. Toxicity of tyre wear particle leachates to marine phytoplankton. Aquat. Toxicol. 252, 106299.

Paluselli, A., Fauvelle, V., Galgani, F., Sempéré, R., 2019. Phthalate release from plastic fragments and degradation in seawater. Environ. Sci. Technol. 53, 166–175.

Pannetier, P., Cachot, J., Clérandeau, C., Faure, F., Van Arkel, K., de Alencastro, L.F., Levasseur, C., Sciacca, F., Bourgeois, J.-P., Morin, B., 2019a. Toxicity assessment of pollutants sorbed on environmental sample microplastics collected on beaches: Part I-adverse effects on fish cell line. Environ. Pollut. 248, 1088–1097.

Pannetier, P., Clérandeau, C., Laurent, J., Chapelle, C., Morin, B., Cachot, J., 2019b. Toxicity assessment of pollutants sorbed on environmental microplastics collected on beaches. Part II-adverse effects on Japanese medaka first life stage. Environ. Pollut. 248, 1098–1107.

Rendell-Bhatti, F., Paganos, P., Pouch, A., Mitchell, C., D'Aniello, S., Godley, B.J., Pazdro, K., Arnone, M.I., Jimenez-Guri, E., 2021. Developmental toxicity of plastic leachates on the sea urchin *Paracentrotus lividus*. Environ. Pollut. 269, 115744.

Rist, S., Vianello, A., Winding, M.H.S., Nielsen, T.G., Almeda, R., Rodriguez-Torres, R., Vollertsen, J., 2020. Quantification of plankton-sized microplastics in a productive Arctic marine ecosystem. Environ. Pollut. 266, 115248.

Rodríguez-Torres, R., Almeda, R., Kristiansen, M., Rist, S., Winding, M.S., Nielsen, T.G., 2020. Ingestion and impact of microplastics on arctic Calanus copepods. Aquat. Toxicol. 228, 105631.

Saco-Álvarez, L., Durán, I., Ignacio Lorenzo, J., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicol. Environ. Saf. 73, 491–499.

Seidensticker, S., Zarfl, C., Cirpka, O.A., Fellenberg, G., Grathwohl, P., 2017. Transfer of wastewater contaminants from microplastics in the presence of dissolved substances. Environ. Sci. Technol. 51, 12254–12263.

Suhrhoff, T.J., Scholz-Böttcher, B.M., 2016. Qualitative impact of salinity, UV radiation and turbulence on leaching of organic plastic additives from four common plastics — a lab experiment. Mar. Pollut. Bull. 102, 84–94.

Tian, Z., Zhao, H., Peter, K.T., others, 2021. A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon. Science 371, 185–189.

Town, R.M., van Leeuwen, H.P., 2020. Uptake and release kinetics of organic contaminants associated with micro- and nanoplastic particles. Environ. Sci. Technol. 54, 10057–10067.

From Pollution to Solution: a Global Assessment of Marine Litter and Plastic Pollution, 2021. UNEP, Nairobi.

U.S. EPA, 1992. Code of Federal Regulations (CFR) 40. Toxicity Characteristic Leaching Procedure (TCLP). U.S. Environmental Protection Agency. Federal Register 40:261(1 July 1991): Appendix II.

U.S.EPA, 1998. Evaluation of Dredged Material Proposed for Discharge in Waters of the U.S.—Inland Testing Manual, 1998. U.S. EPA, Washington, DC. EPA-823-B-98-00.

U.S. EPA, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, fith ed. United States Environmental Protection Agency, Washington, DC. EPA-821-R-02-012.

Volpi Ghirardini, A., Girardini, M., Marchetto, D., Pantani, C., 2009. Microtox solid phase test: effect of diluent used in toxicity test. Ecotoxicol. Environ. Saf. 72, 851–861.

Wik, A., Dave, G., 2009. Occurrence and effects of tire wear particles in the environment - a critical review and an initial risk assessment. Environ. Pollut. 157, 1–11.