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Abstract

Plastic is one of the major sources of pollution in modern oceans. When in seawater, toxic plasticizers (the additives incorporated in plastic polymers during manufacturing processes) typically diffuse and accumulate in sediments and in benthic and pelagic organisms' tissues. These plastic leachates affect survival, behavior and metabolism of various marine metazoans, but little effort was placed in studying their effect on protists. In this contribution we monitored the short-term effect of polypropylene (PP) leachates at both environmentally realistic and chronic concentrations on *Haynesina germanica* locomotion and metabolism. We found that PP leachates has no lethal nor effects on this species activity. Taken together, these results suggest that benthic foraminifera may be more resistant than marine metazoans to plasticizers pollutants.

Keywords: Benthic foraminifera, Plastic leachates, Polypropylene, Survival, Behavior, Respiration

1 Introduction

Plastics are acknowledged as one of the most ubiquitous and conspicuous sources of pollution of the Anthropocene, especially in the marine environment (W. C. Li et al., 2016). Microplastics (MP) can either be small plastic particles (smaller than 5mm) released in the environment or result from the breakage and aging of macroplastics. They are now considered the most numerically abundant form of solid waste on the planet (Eriksen et al., 2014) and a potential threat to marine ecosystems globally (Galloway et al., 2017). Hence, they are widely observed from coastal waters to the deep-ocean floor and from tropical to polar regions (Barnes, 2005; Chiba et al., 2018).

Microplastics are also responsible for a range of sub-lethal effects related to their pernicious role as a vector of chemical pollutants. These pollutants leaching from MP to the marine environments originate from the additives compounds (e.g. plasticizers, flame retardant, UV stabilizers, antioxidant, and antistatic molecule) incorporated in plastics during the manufacturing process to modify the plastic polymers physical properties and durability, but also from the chemical compounds already present in the water (i.e. coming from another source of pollution) which are adsorbed at the MP's surface when aging in the environments. Plastic additives such as phthalates, bisphenol A, nonyphenols and brominated flame retardants can reach high concentrations in coastal waters (Hermabessiere et al., 2017; Sánchez-Avila et al., 2012) and accumulate in marine organisms tissues (Vered et al., 2019). This work specifically focuses on the toxicity of virgin MP leachates since they have recently been identified as one of the most critical threat related to the presence of plastics in the ocean (Hahladakis et al., 2018; Paluselli et al., 2019). The toxic effects of virgin microplastic leachates have been reported in various marine faunal taxa, such as barnacles (H.-X. Li et al., 2016), crustacean larvae (Lithner et al., 2009), gastropods (Seuront, 2018), bivalves (Ke et al., 2019) and sea urchins (Oliviero et al., 2019). Desorption of these chemicals in the surrounding environment causes a range of harmful effects on embryo development, reproduction, behavior or induce genetic aberrations (see Oehlmann et al. (2009) for a review).

To date and to the best of our knowledge, there is still a critical lack of information available on effect of microplastic on protists, despite a recent urge to fill this knowledge gap (Rillig and Bonkowski, 2018). However, MP ingestion is likely to be common in protists (Setälä et al., 2014), including foraminifera (Ciacci et al., 2019), and subsequently negatively impact their metabolic activity (Ciacci et al., 2019; Su et al., 2020). Benthic foraminifera were targeted in this work due to their importance in the structure and function of benthic ecosystems (Geslin et al., 2011; Gooday et al., 1992), their ability to respond to various types of pollutant both under laboratory conditions (Denoyelle et al., 2012; Ernst et al., 2006; Nigam et al., 2009) and *in situ* (see Alve, 1995 for a review). Like any benthic organisms, they are directly exposed to the range of pollutants, including microplastics (Schwarz et al., 2019) which cannot be degraded by bacteria (Nauendorf et al., 2016) and therefore accumulate in coastal sediments (Galgani et al., 1996). In this context, the present study assessed the potential short-term effects of the leachates from virgin polypropylene pellets considered at both environmentally realistic and chronic concentrations on the stress level of the benthic foraminifera *Haynesina germanica*. Specifically, movement behavior (Seuront, 2018) and respiration rate (Su et al., 2020) were considered as proxies of the stress level of *H. germanica* following an exposure to polypropylene leachates. This foraminiferal species and this plastic polymer were specifically chosen for their high abundances along the French coast of the eastern English Channel (Armynot du Châtelet et al., 2018; Francescangeli et al., 2017; Hermabessiere et al., 2019).

2 Material and methods

2.1 *Haynesina germanica* collection

Surface sediment (0-1cm) from Boulogne-sur-Mer harbor mudflat (eastern English Channel, 50°43'06.4"N 1°34'22.0"E) was sampled in June 2019 and stored in 100 ml polypropylene containers. Sediment was kept at ambient temperature during transportation and placed within one hour in English Channel seawater aquarium (12°C and 35 PSU) under a natural day-light cycle conditions until the experiment took place. Sediment was sieved over a 125 µm stainless-steel mesh and colored-cytoplasm *Haynesina germanica* were subsequently sorted. Only the active specimens (i.e. leaving a displacement track on a thin layer of sediment) were considered as living and selected for the experiment. Living individuals were transferred in artificial seawater (ASW) prepared with 35 grams of sea salt (RedSea Fish Farm, Israel) per liter of Milli-Q water (Merck Millipore, Germany) and gently cleaned with a brush to remove any surrounding particles.

2.2 Experimental conditions

Both behavioral experiments and metabolic measurements were conducted exposing *H. germanica* to artificial seawater as control and to microplastic leachates seawater. Microplastic leachates seawater was prepared from commercially available virgin polypropylene pellets (typically 3.3 to 4.7 mm in diameter; Pemmiproducts, Germany) mixed with artificial seawater at a concentration of 20 ml and 200 ml of pellets per liter (hereafter respectively referred to as PP20 and PP200) and aerated for 24 h before the beginning of the experiments following the protocol developed in Seuront (2018) to monitor the effect of plastic leachates on a marine gastropod. Although not quantified in this experiment, polypropylene leachates typically contain bisphenol A, octylphenol and nonylphenol (Hermabessiere et al., 2017).

2.3 Behavioral experiment

For each experimental condition (i.e. control seawater and the two leachate treatments PP20 and PP200), 15 living *Haynesina germanica* (maximum diameter range: 300-440 µm) were spread randomly on the bottom of 15-cm wide glass-Petri dishes filled with ASW, PP20 or PP200 (Figure 1). Petri dishes were placed in a light and temperature-controlled incubator (MIR-154, Panasonic, Japan) set at 12°C. The movements of *H. germanica* were recorded every 10 minutes using a digital camera (V1 with a 10-30 mm lens, Nikon, Japan) under homogenous dim light conditions (photosynthetically active radiation <100 µmol photon m^{-2} s^{-1}; SA-190 quantum sensor, LI-COR, USA) provided by a horizontal array of LEDs (YN-160 III, Yongnuo, China). Each experiment lasted 10 hours.

Images were compiled in the open-source image analysis software Fiji (Schindelin et al., 2012) and (x,y) coordinates were measured for each individual *H. germanica* using the *Manual Tracking* plugin (Figure 1). The distance travelled (D_t) by each individual between two images was calculated as: $D_t = \sqrt{[(x_t - x_{t+1})^2 + (y_t - y_{t+10})^2]}$ where (x_t, y_t) and (x_{t+10}, y_{t+10}) are the coordinates between two successive images taken at 10-minute intervals. The total distance travelled in 10 hours was calculated from the sum of all D_t and subsequently converted to locomotion speed (mm h^{-1}). These behavioral parameters were measured using *trajr* package (McLean and Skowron Volponi, 2018) in R v.3.5.3 (R Core Team, 2019). Trajectories complexity was assessed using fractal analysis. The fractal dimensions of foraminifera trajectories were estimated following the box dimension method (Seuront, 2015, 2010).

2.4 Respiration measurements

Five *H. germanica* specimens were randomly selected from the individuals used in behavioral experiments and transferred from the Petri dish to a 1-mm wide glass microtube containing the three tested seawater (ASW for control and PP20 and PP200 to test the effect of polypropylene leachates). Steady-state oxygen consumption gradient (dC/dz, in pmol cm^{-4}) in the millimeter above the organisms were measured using a 50-µm Clark-type oxygen microelectrode (Unisense, Denmark). Oxygen fluxes (J, pmol cm^{-1} s^{-1}) in the microtube were calculated using Fick's first law of free diffusion as $J = D\times dC/dz$ (Li and Gregory, 1974) with D being the free diffusion coefficient for oxygen (D = 1.6 10^{-5} cm^2 s^{-1} at 12°C and 35PSU). Individual respiration rate (R, pmol ind^{-1} day^{-1}) was then calculated as $R = J\times S/n$ (considering the microtube inner section S = 7.9 10^{-3} cm^2 and the number of individuals n = 5). Note that our measurements were conducted on groups of 5 individuals both to take into account the low individual respiration rate of benthic foraminifera and to overpass the sensor detection limit (Geslin et al., 2011). Respiration rate measurements were replicated 6 times in control seawater and triplicated in both P20 and PP200 leachate treatments. Since respiration is influenced by individual size, specimens were measured to normalize the respiration rates by the foraminiferal biovolume (8.10^6 $µm^3$ in average; estimated following Geslin et al., 2011). All respiration measurements were carried out in the dark in a 12°C temperature-controlled water bath (Huber CC-K12, Germany).

2.5 Data analysis

Due to our small size samples, the effect of the 3 experimental conditions on movement speed, fractal dimension and foraminiferal respiration rate was tested using Kruskal-Wallis test (Hollander and Wolfe, 1999) in R v.3.5.3 (R Core Team, 2019).

3 Results

Image analysis show that 100% of the individuals tested were moving throughout the experiments and were still alive after being exposed to PP20 and PP200 for 10 hours. *Haynesina germanica* moved over distances ranging from 7 to 32 mm, at locomotion speed ranging from 0.7 to 3.2 mm h^{-1}, 1.6 to 2.8 mm h^{-1} and 1.1 to 3.1 mm h^{-1} for ASW, PP20 and PP200 respectively (Figure 2A). All the trajectories considered in this work were significantly described in terms of fractal dimensions that ranged between 1.02 and 1.13 with average values of 1.07, 1.06 and 1.06 in ASW, PP20 and PP200 respectively (Figure 2B). Finally, respiration rate ranged from 41 to 114 10^{-6} pmol μm^{-3} day^{-1} in the ASW control, from 66 to 164 10^{-6} pmol μm^{-3} day^{-1} in PP20 and from 84 to 98 10^{-6} pmol μm^{-3} day^{-1} in PP200 (Figure 2C). Neither locomotion speed, fractal dimensions nor respiration rates exhibited any significant differences between the three experimental conditions (Kruskall-Wallis-test: $p>0.05$; Table 1).

4 Discussion

The additives leaching from polypropylene (i.e. essentially antioxidant additives such as bisphenol A, octylphenol and nonylphenol; Hermabessiere et al., 2017) have lethal effects on mollusks (Oehlmann et al., 2000), barnacle larvae (H.-X. Li et al., 2016), amphibians (Hogan et al., 2006), annelids and crustaceans (Staples et al., 2016). In contrast, the present work showed a lack of any lethal effect on *Haynesina germanica* of PP leachates.

Similarly, no sublethal effect were perceptible through *H. germanica* locomotion and metabolism. Specifically, locomotion speed was nearly 2-fold lower than those reported previously on the same species (here ~2 mm h^{-1} *vs.* ~4 mm h^{-1} in Seuront and Bouchet, 2015) probably due to the lower experimental temperature (12°C here *vs.* 22°C in Seuront and Bouchet, 2015) since decreasing temperature is known to reduce foraminiferal activity (Bradshaw, 1961). Our results nevertheless clearly indicated that PP leachates did not affect foraminiferal behavior (Figure 2A, B). This is consistent with the observed lack of behavioral impairment in the intertidal gastropod *Littorina littorea*; as PP20 leachates impaired their chemosensory ability without impacting their neuromuscular abilities (Seuront, 2018). In turn, our results contrast with previous evidence that PP-plasticizers reduce fish larvae velocity in the first days after hatching (Inagaki et al., 2016; Wang et al., 2013) and negatively impact adult-fish locomotion and reproductive behavior after at least 2 months of exposure (Gray et al., 1999; Xia et al., 2010). Note that the apparent discrepancy observed between the aforementioned studies and our experiment might be due to differences in exposure duration as reported in Table 2.

Plasticizers have previously been reported to lead to an immediate increase followed by a decrease in respiration rates with rising phenols concentration in mollusks (Levine and Cheney, 2000). They can also induce energetical impairments in crustaceans anaerobic metabolism in less than 2 days (Nagato et al., 2016). In contrast, we did not find any significant effects of PP leachates on foraminiferal respiration, even under very high leachates concentrations, i.e. PP200 (Figure 2C). To the best of our knowledge, the only other study that investigated the effect of PP leachates on a unicellular organism found a decrease in dinoflagellate photosynthesis (M'Rabet et al., 2018) after 1 day of exposure, in accordance with the reduced growth and oxygen production observed in the marine cyanobacteria *Prochlorococcus* following a 24h-long exposure to leachates of common plastic items (i.e. HDPE shopping bags and PVC matting; Tetu et al., 2019). Note that, conversely to M'Rabet et al. (2018) who specifically worked with bisphenol A (Table 2), we did not have any control on the composition of the PP leachates. This is a clear limitation of our study that will need to be improved in future works.

Overall, both the behavioral and metabolic activity data gathered in this preliminary study indicate that the benthic foraminifera *Haynesina germanica* do not respond to MP unlike other unicellular and metazoan organisms. Though this is highly speculative, this observation may suggest that their resistance to leachates from virgin PP might induce a competitive advantage for benthic foraminifera. Such a competitive advantage for foraminifera has previously been observed in relation to some anthropo-natural phenomena such as organic-matter enrichment and anoxia (Langlet et al., 2013; Stachowitsch, 2014). Note, however, that the observed lack of effect of MP on foraminiferal activity may also be due to the relatively short-term exposure used in our experiments. More fundamentally, the diversity of methods reported in the literature related to the type of polymer considered, the use of unidentified leachates or a specific plasticizer, the pollutant concentrations, the duration of exposure as well as the biology of the organisms considered (Table 2) dramatically prevent to reach a general consensus when comparing the effect of MP on foraminifera with other organisms in our study. In this context, future experiments aiming to assess the effects of MP leachates on benthic foraminifera should benefit from (i) being more specific about the acute or chronic nature of their exposure, and (ii) identifying and quantifying the plasticizers used or present in the leachates. Finally, further work is also needed to assess the potential effects of leachates from (i) weathered PP in particular as they have shown to have significantly stronger effects that virgin plastics (Bejgarn et al., 2015; Gandara e Silva et al., 2016; Kedzierski et al., 2018; Nobre et al., 2015; Seuront, 2018), (ii) different plastic polymers (H.-X. Li et al., 2016; Lithner et al., 2012, 2009; Tetu et al., 2019) and (iii) ingested plastic particles (Ciacci et al., 2019).

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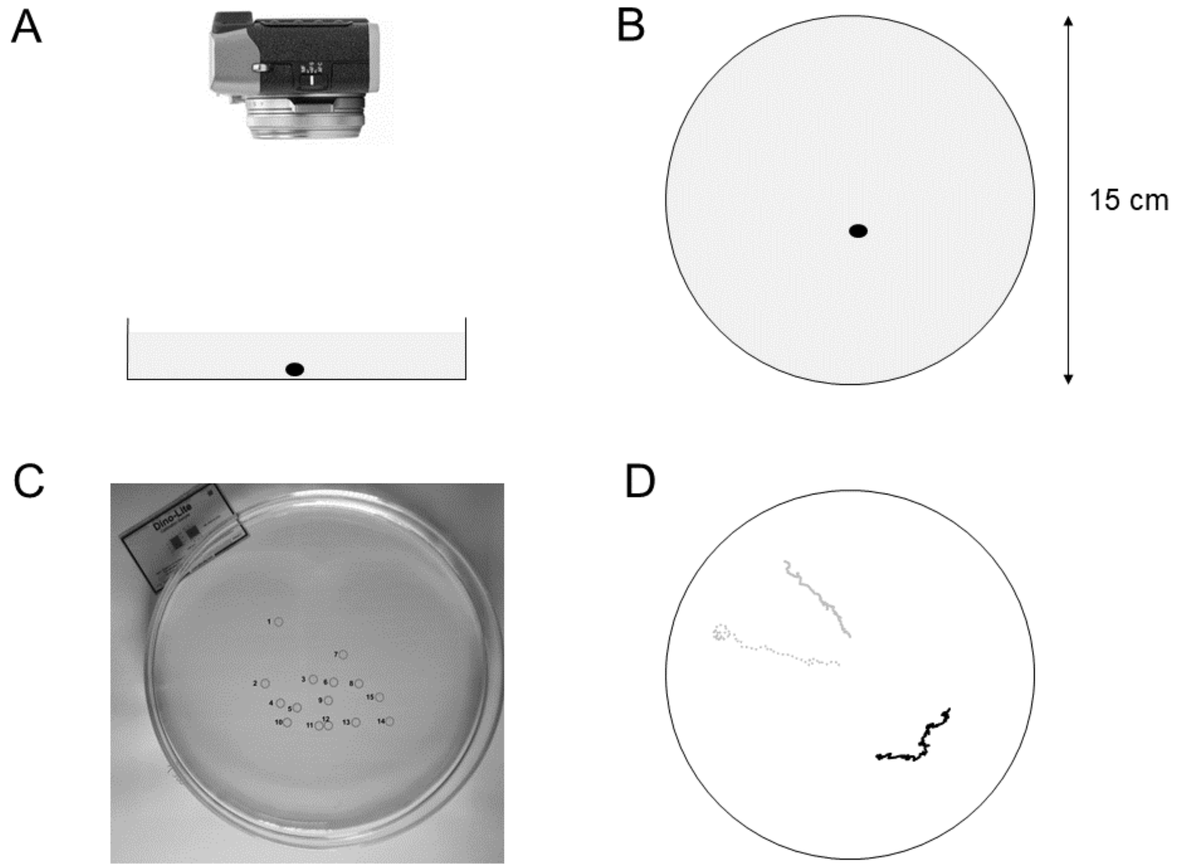

Figure 1 caption: A and B: schematic representation of the experimental setup with lateral view (A) and top view (B) of the position of the foraminifera (black ovoid shape) placed on the petri-dish. C: photograph of the initial position of the 15 individuals used in ASW control conditions. D: example of 3 extracted trajectories for ASW (full black line), PP20 (full grey line) and PP200 (dotted grey line).

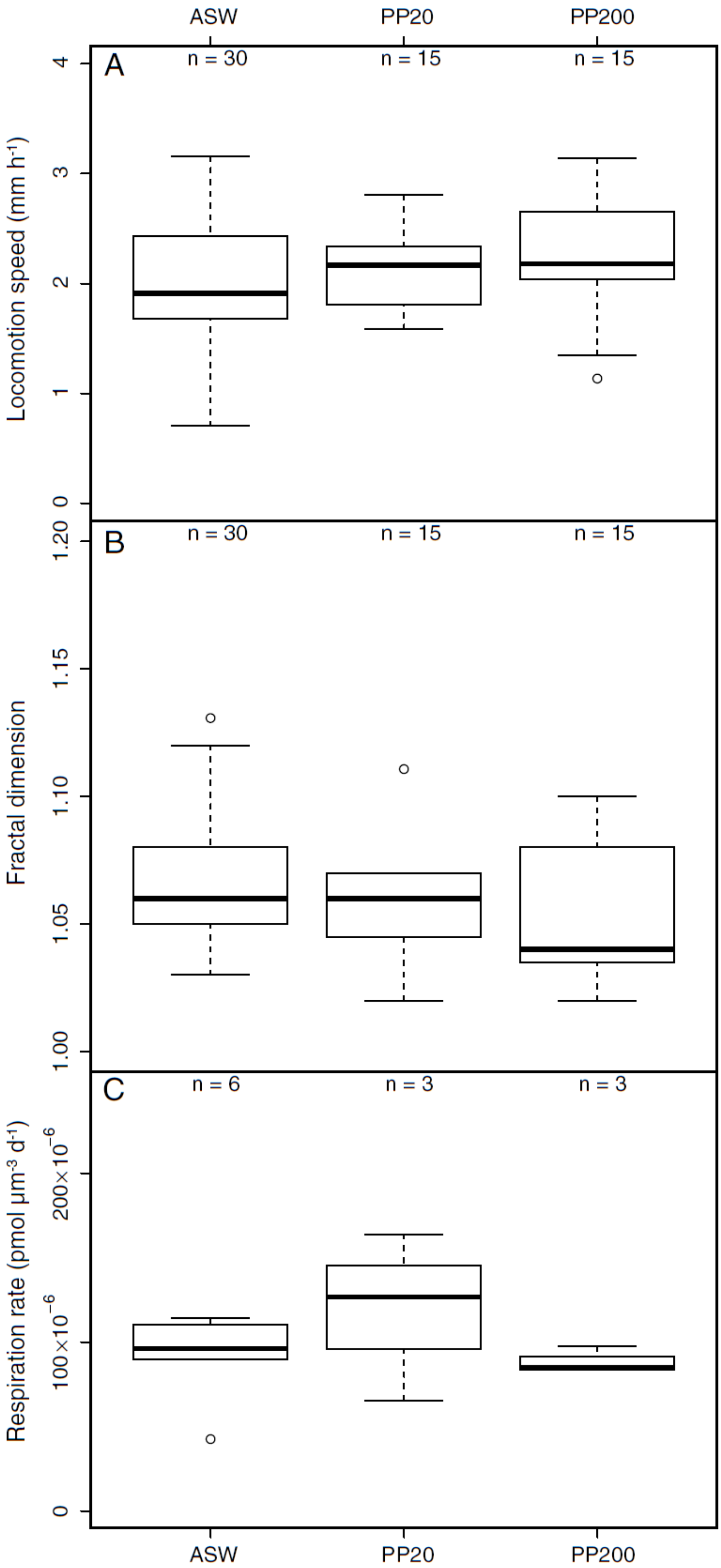
ASW
PP20
PP200
A
n = 30
n = 15
n = 15
Locomotion speed (mm h-1)
0
1
2
3
4
B
n = 30
n = 15
n = 15
Fractal dimension
1.00
1.05
1.10
1.15
1.20
C
n = 6
n = 3
n = 3
Respiration rate (pmol µm-3 d-1)
0
100×10−6
200×10−6
ASW
PP20
PP200

Figure 2: Locomotion speed (A), fractal dimension (B) and respiration rate (C) of *Haynesina germanica* under the three experimental conditions (ASW: artificial seawater, i.e. control conditions; PP20 and PP200: seawater prepared with 20 and 200 ml l^{-1} polypropylene pellets, respectively). The box represents the first, second and third quartiles and the whiskers extend to 1.5 times the interquartile range. Values outside of this range are represented by open circles.

Table 1 caption: results of the Kruskal-Wallis statistical analyses testing the effect of the experimental conditions (ASW as a control, PP20 and PP200) on the three measured response variables.

Response variable	Kruskal-Wallis X^2	degrees of freedom	p-value
Locomotion speed	1.6	2	0.44
Fractal dimension	3.9	2	0.14
Respiration rate	1.3	2	0.52

Table 2 caption: organisms, response observed, type of pollutant, concentration, equivalent concentration in the present study and exposure duration tested in the literature cited in this article’s discussion.

Reference	Organisms	Response observed	Pollutant type	Pollutant concentration	This study's equivalent	Exposure duration
Oehlmann et al. 2000	Mollusks	Mortality	Bisphenol A	1µg/L		5 months
		Mortality	Octylphenol	1µg/L		5 months
Li et al. 2016	Barnacle larvae	10% mortality	PP leachate	0.1 m^2/L	PP200 ~ 0.17m^2/L	1 day
Hogan et al. 2006	Amphibians	50% mortality	Octylphenol	1.4 µmol/L		2 weeks
Staples et al. 2016	Crustaceans	Mortality	Bisphenol A	78 mg/kg sedim dry weight		1 month
	Annelids	Mortality	Bisphenol A	60 mg/kg sedim dry weight		1 month
Seuront 2018	Gastropods	Behavior	PP leachate	20mL/L	PP20 = 20mL/L	3 hours
Inagaki et al. 2016	Fish larvae	Locomotion	Bisphenol A	200ng/mL		20 days
Wang et al. 2013	Fish larvae	Locomotion	Bisphenol A	15µmol/L		2 days
Gray et al. 1999	Adult fish	Reproductive behavior	Octylphenol	25µg/L		3 months
Xia et al. 2010	Adult fish	Locomotion	Nonylphenol	100µg/L		2 months
Levine and Cheney 2000	Mollusks	Respiration	Nonylphenol	10µmol/L		1 hour
Nagato et al. 2016	Crustaceans	Anaerobic metabolism	Bisphenol A	0.1mg/L		2 days
M'Rabet et al. 2018	Dinoflagellate	Respiration and photosynthesis	Bisphenol A	2µg/L		1 day
Tetu et al. 2019	Cyanobacteria	Photosynthesis	PVC leachate	1g/L	PP20 ~ 10g/L	3 hours