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1 **Motion behavior and metabolic response to microplastic leachates in the benthic foraminifera**  
2 ***Haynesina germanica***

3

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34

35 **Abstract**

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

45

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

47

## 48 **1 Introduction**

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

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

73 To date and to the best of our knowledge, there is still a critical lack of information available on effect  
74 of microplastic on protists, despite a recent urge to fill this knowledge gap (Rillig and Bonkowski, 2018).  
75 However, MP ingestion is likely to be common in protists (Setälä et al., 2014), including foraminifera  
76 (Ciacci et al., 2019), and subsequently negatively impact their metabolic activity (Ciacci et al., 2019; Su  
77 et al., 2020). Benthic foraminifera were targeted in this work due to their importance in the structure and  
78 function of benthic ecosystems (Geslin et al., 2011; Gooday et al., 1992), their ability to respond to  
79 various types of pollutant both under laboratory conditions (Denoyelle et al., 2012; Ernst et al., 2006;  
80 Nigam et al., 2009) and *in situ* (see Alve, 1995 for a review). Like any benthic organisms, they are  
81 directly exposed to the range of pollutants, including microplastics (Schwarz et al., 2019) which cannot  
82 be degraded by bacteria (Nauendorf et al., 2016) and therefore accumulate in coastal sediments (Galgani  
83 et al., 1996). In this context, the present study assessed the potential short-term effects of the leachates  
84 from virgin polypropylene pellets considered at both environmentally realistic and chronic

85 concentrations on the stress level of the benthic foraminifera *Haynesina germanica*. Specifically,  
86 movement behavior (Seuront, 2018) and respiration rate (Su et al., 2020) were considered as proxies of  
87 the stress level of *H. germanica* following an exposure to polypropylene leachates. This foraminiferal  
88 species and this plastic polymer were specifically chosen for their high abundances along the French  
89 coast of the eastern English Channel (Armynot du Châtelet et al., 2018; Francescangeli et al., 2017;  
90 Hermabessiere et al., 2019).

## 91 **2 Material and methods**

### 92 2.1 *Haynesina germanica* collection

93 Surface sediment (0-1cm) from Boulogne-sur-Mer harbor mudflat (eastern English Channel,  
94 50°43'06.4"N 1°34'22.0"E) was sampled in June 2019 and stored in 100 ml polypropylene containers.  
95 Sediment was kept at ambient temperature during transportation and placed within one hour in English  
96 Channel seawater aquarium (12°C and 35 PSU) under a natural day-light cycle conditions until the  
97 experiment took place. Sediment was sieved over a 125 µm stainless-steel mesh and colored-cytoplasm  
98 *Haynesina germanica* were subsequently sorted. Only the active specimens (i.e. leaving a displacement  
99 track on a thin layer of sediment) were considered as living and selected for the experiment. Living  
100 individuals were transferred in artificial seawater (ASW) prepared with 35 grams of sea salt (RedSea  
101 Fish Farm, Israel) per liter of Milli-Q water (Merck Millipore, Germany) and gently cleaned with a  
102 brush to remove any surrounding particles.

103

### 104 2.2 Experimental conditions

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

114

### 115 2.3 Behavioral experiment

116 For each experimental condition (i.e. control seawater and the two leachate treatments PP20 and PP200),  
117 15 living *Haynesina germanica* (maximum diameter range: 300-440 µm) were spread randomly on the  
118 bottom of 15-cm wide glass-Petri dishes filled with ASW, PP20 or PP200 (Figure 1). Petri dishes were  
119 placed in a light and temperature-controlled incubator (MIR-154, Panasonic, Japan) set at 12°C. The  
120 movements of *H. germanica* were recorded every 10 minutes using a digital camera (V1 with a 10-30

121 mm lens, Nikon, Japan) under homogenous dim light conditions (photosynthetically active radiation  
122  $<100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ; SA-190 quantum sensor, LI-COR, USA) provided by a horizontal array of  
123 LEDs (YN-160 III, Yongnuo, China). Each experiment lasted 10 hours.

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

133

#### 134 2.4 Respiration measurements

135 Five *H. germanica* specimens were randomly selected from the individuals used in behavioral  
136 experiments and transferred from the Petri dish to a 1-mm wide glass microtube containing the three  
137 tested seawater (ASW for control and PP20 and PP200 to test the effect of polypropylene leachates).  
138 Steady-state oxygen consumption gradient ( $dC/dz$ , in  $\text{pmol cm}^{-4}$ ) in the millimeter above the organisms  
139 were measured using a 50- $\mu\text{m}$  Clark-type oxygen microelectrode (Unisense, Denmark). Oxygen fluxes  
140 ( $J$ ,  $\text{pmol cm}^{-1} \text{ s}^{-1}$ ) in the microtube were calculated using Fick's first law of free diffusion as  $J = D \times dC/dz$   
141 (Li and Gregory, 1974) with  $D$  being the free diffusion coefficient for oxygen ( $D = 1.6 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  at  
142  $12^\circ\text{C}$  and 35PSU). Individual respiration rate ( $R$ ,  $\text{pmol ind}^{-1} \text{ day}^{-1}$ ) was then calculated as  $R = J \times S/n$   
143 (considering the microtube inner section  $S = 7.9 \cdot 10^{-3} \text{ cm}^2$  and the number of individuals  $n = 5$ ). Note  
144 that our measurements were conducted on groups of 5 individuals both to take into account the low  
145 individual respiration rate of benthic foraminifera and to overpass the sensor detection limit (Geslin et  
146 al., 2011). Respiration rate measurements were replicated 6 times in control seawater and triplicated in  
147 both P20 and PP200 leachate treatments. Since respiration is influenced by individual size, specimens  
148 were measured to normalize the respiration rates by the foraminiferal biovolume ( $8 \cdot 10^6 \mu\text{m}^3$  in average;  
149 estimated following Geslin et al., 2011). All respiration measurements were carried out in the dark in a  
150  $12^\circ\text{C}$  temperature-controlled water bath (Huber CC-K12, Germany).

151

#### 152 2.5 Data analysis

153 Due to our small size samples, the effect of the 3 experimental conditions on movement speed, fractal  
154 dimension and foraminiferal respiration rate was tested using Kruskal-Wallis test (Hollander and Wolfe,  
155 1999) in R v.3.5.3 (R Core Team, 2019).

156

### 157 **3 Results**

158 Image analysis show that 100% of the individuals tested were moving throughout the experiments and  
159 were still alive after being exposed to PP20 and PP200 for 10 hours. *Haynesina germanica* moved over  
160 distances ranging from 7 to 32 mm, at locomotion speed ranging from 0.7 to 3.2 mm h<sup>-1</sup>, 1.6 to 2.8 mm  
161 h<sup>-1</sup> and 1.1 to 3.1 mm h<sup>-1</sup> for ASW, PP20 and PP200 respectively (Figure 2A). All the trajectories  
162 considered in this work were significantly described in terms of fractal dimensions that ranged between  
163 1.02 and 1.13 with average values of 1.07, 1.06 and 1.06 in ASW, PP20 and PP200 respectively (Figure  
164 2B). Finally, respiration rate ranged from 41 to 114 10<sup>-6</sup> pmol μm<sup>-3</sup> day<sup>-1</sup> in the ASW control, from 66  
165 to 164 10<sup>-6</sup> pmol μm<sup>-3</sup> day<sup>-1</sup> in PP20 and from 84 to 98 10<sup>-6</sup> pmol μm<sup>-3</sup> day<sup>-1</sup> in PP200 (Figure 2C).  
166 Neither locomotion speed, fractal dimensions nor respiration rates exhibited any significant differences  
167 between the three experimental conditions (Kruskall-Wallis-test: p>0.05; Table 1).

168

### 169 **4 Discussion**

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

175 Similarly, no sublethal effect were perceptible through *H. germanica* locomotion and metabolism.  
176 Specifically, locomotion speed was nearly 2-fold lower than those reported previously on the same  
177 species (here ~2 mm h<sup>-1</sup> vs. ~4 mm h<sup>-1</sup> in Seuront and Bouchet, 2015) probably due to the lower  
178 experimental temperature (12°C here vs. 22°C in Seuront and Bouchet, 2015) since decreasing  
179 temperature is known to reduce foraminiferal activity (Bradshaw, 1961). Our results nevertheless clearly  
180 indicated that PP leachates did not affect foraminiferal behavior (Figure 2A, B). This is consistent with  
181 the observed lack of behavioral impairment in the intertidal gastropod *Littorina littorea*; as PP20  
182 leachates impaired their chemosensory ability without impacting their neuromuscular abilities (Seuront,  
183 2018). In turn, our results contrast with previous evidence that PP-plasticizers reduce fish larvae velocity  
184 in the first days after hatching (Inagaki et al., 2016; Wang et al., 2013) and negatively impact adult-fish  
185 locomotion and reproductive behavior after at least 2 months of exposure (Gray et al., 1999; Xia et al.,  
186 2010). Note that the apparent discrepancy observed between the aforementioned studies and our  
187 experiment might be due to differences in exposure duration as reported in Table 2.

188 Plasticizers have previously been reported to lead to an immediate increase followed by a decrease in  
189 respiration rates with rising phenols concentration in mollusks (Levine and Cheney, 2000). They can  
190 also induce energetical impairments in crustaceans anaerobic metabolism in less than 2 days (Nagato et  
191 al., 2016). In contrast, we did not find any significant effects of PP leachates on foraminiferal respiration,  
192 even under very high leachates concentrations, i.e. PP200 (Figure 2C). To the best of our knowledge,  
193 the only other study that investigated the effect of PP leachates on a unicellular organism found a

194 decrease in dinoflagellate photosynthesis (M'Rabet et al., 2018) after 1 day of exposure, in accordance  
195 with the reduced growth and oxygen production observed in the marine cyanobacteria *Prochlorococcus*  
196 following a 24h-long exposure to leachates of common plastic items (i.e. HDPE shopping bags and PVC  
197 matting; Tetu et al., 2019). Note that, conversely to M'Rabet et al. (2018) who specifically worked with  
198 bisphenol A (Table 2), we did not have any control on the composition of the PP leachates. This is a  
199 clear limitation of our study that will need to be improved in future works.

200 Overall, both the behavioral and metabolic activity data gathered in this preliminary study indicate that  
201 the benthic foraminifera *Haynesina germanica* do not respond to MP unlike other unicellular and  
202 metazoan organisms. Though this is highly speculative, this observation may suggest that their  
203 resistance to leachates from virgin PP might induce a competitive advantage for benthic foraminifera.  
204 Such a competitive advantage for foraminifera has previously been observed in relation to some  
205 anthropo-natural phenomena such as organic-matter enrichment and anoxia (Langlet et al., 2013;  
206 Stachowitsch, 2014). Note, however, that the observed lack of effect of MP on foraminiferal activity  
207 may also be due to the relatively short-term exposure used in our experiments. More fundamentally, the  
208 diversity of methods reported in the literature related to the type of polymer considered, the use of  
209 unidentified leachates or a specific plasticizer, the pollutant concentrations, the duration of exposure as  
210 well as the biology of the organisms considered (Table 2) dramatically prevent to reach a general  
211 consensus when comparing the effect of MP on foraminifera with other organisms in our study. In this  
212 context, future experiments aiming to assess the effects of MP leachates on benthic foraminifera should  
213 benefit from (i) being more specific about the acute or chronic nature of their exposure, and (ii)  
214 identifying and quantifying the plasticizers used or present in the leachates. Finally, further work is also  
215 needed to assess the potential effects of leachates from (i) weathered PP in particular as they have shown  
216 to have significantly stronger effects than virgin plastics (Bejgarn et al., 2015; Gandara e Silva et al.,  
217 2016; Kedzierski et al., 2018; Nobre et al., 2015; Seuront, 2018), (ii) different plastic polymers (H.-X.  
218 Li et al., 2016; Lithner et al., 2012, 2009; Tetu et al., 2019) and (iii) ingested plastic particles (Ciacci et  
219 al., 2019).

220

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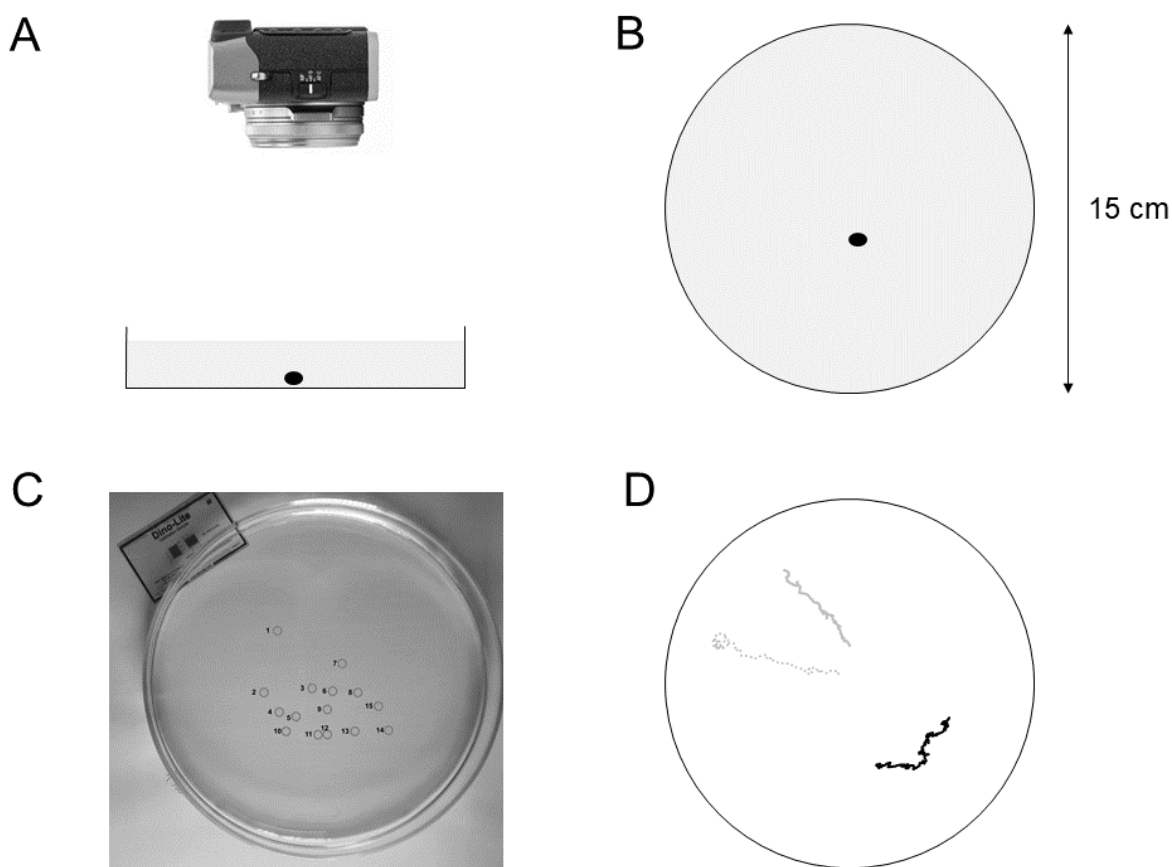
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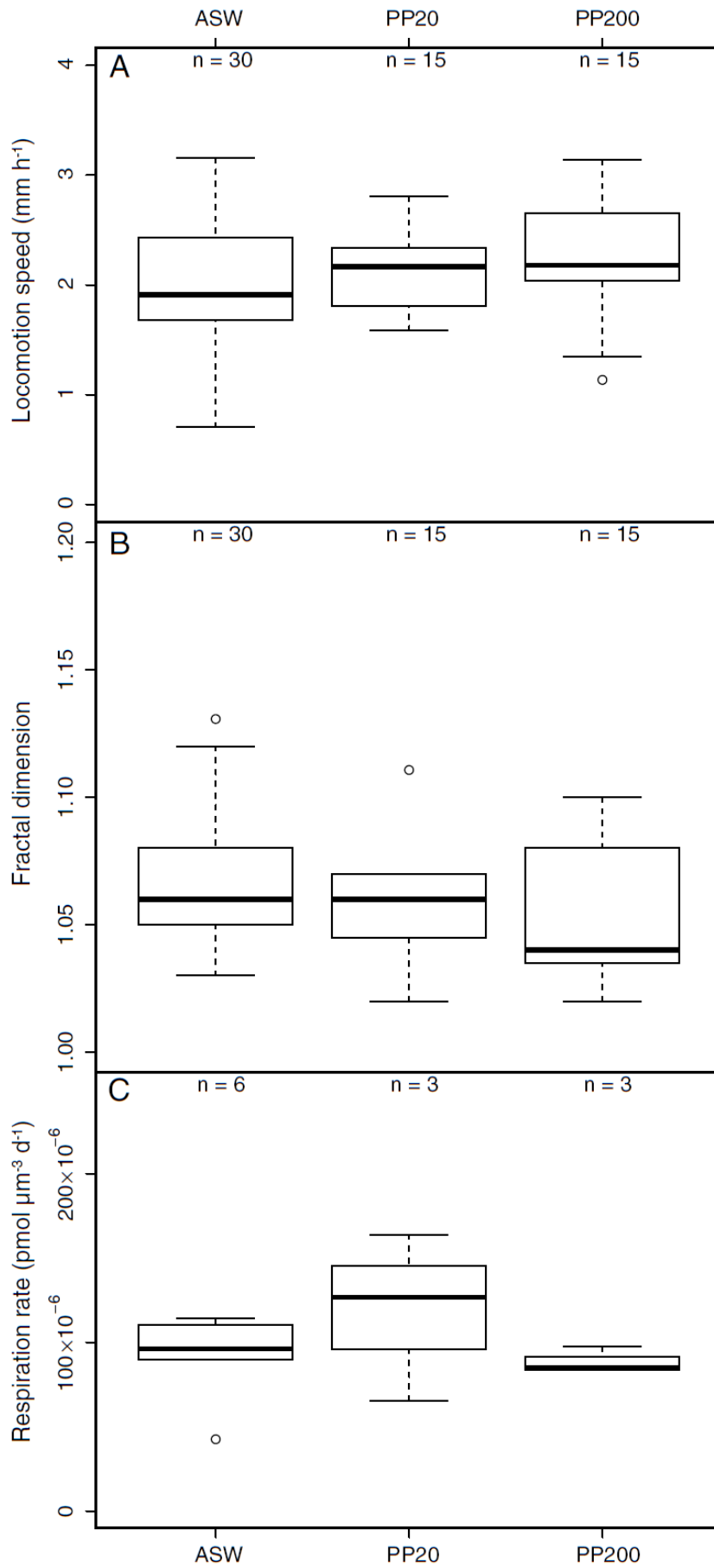
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412 Figure 1 caption: A and B: schematic representation of the experimental setup with lateral view  
 413 (A) and top view (B) of the position of the foraminifera (black ovoid shape) placed on the petri-  
 414 dish. C: photograph of the initial position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



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

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

Response variable	Kruskal-Wallis X <sup>2</sup>	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

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430 Table 2 caption: organisms, response observed, type of pollutant, concentration, equivalent concentration in the present study and exposure duration  
 431 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 <sup>2</sup> /L	PP200 ~ 0.17m <sup>2</sup> /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

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