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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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18

19 **Abstract**

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

29

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

31

## 32 **1 Introduction**

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

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

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

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

## 75 **2 Material and methods**

### 76 2.1 *Haynesina germanica* collection

77 Surface sediment (0-1cm) from Boulogne-sur-Mer harbor mudflat (eastern English Channel,  
78 50°43'06.4"N 1°34'22.0"E) was sampled in June 2019 and stored in 100 ml polypropylene containers.  
79 Sediment was kept at ambient temperature during transportation and placed within one hour in English  
80 Channel seawater aquarium (12°C and 35 PSU) under a natural day-light cycle conditions until the  
81 experiment took place. Sediment was sieved over a 125 µm stainless-steel mesh and colored-cytoplasm  
82 *Haynesina germanica* were subsequently sorted. Only the active specimens (i.e. leaving a displacement  
83 track on a thin layer of sediment) were considered as living and selected for the experiment. Living  
84 individuals were transferred in artificial seawater (ASW) prepared with 35 grams of sea salt (RedSea  
85 Fish Farm, Israel) per liter of Milli-Q water (Merck Millipore, Germany) and gently cleaned with a  
86 brush to remove any surrounding particles.

87

### 88 2.2 Experimental conditions

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

98

### 99 2.3 Behavioral experiment

100 For each experimental condition (i.e. control seawater and the two leachate treatments PP20 and PP200),  
101 15 living *Haynesina germanica* (maximum diameter range: 300-440 µm) were spread randomly on the  
102 bottom of 15-cm wide glass-Petri dishes filled with ASW, PP20 or PP200 (Figure 1). Petri dishes were  
103 placed in a light and temperature-controlled incubator (MIR-154, Panasonic, Japan) set at 12°C. The  
104 movements of *H. germanica* were recorded every 10 minutes using a digital camera (V1 with a 10-30

105 mm lens, Nikon, Japan) under homogenous dim light conditions (photosynthetically active radiation  
106  $<100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ; SA-190 quantum sensor, LI-COR, USA) provided by a horizontal array of  
107 LEDs (YN-160 III, Yongnuo, China). Each experiment lasted 10 hours.

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

117

## 118 2.4 Respiration measurements

119 Five *H. germanica* specimens were randomly selected from the individuals used in behavioral  
120 experiments and transferred from the Petri dish to a 1-mm wide glass microtube containing the three  
121 tested seawater (ASW for control and PP20 and PP200 to test the effect of polypropylene leachates).  
122 Steady-state oxygen consumption gradient ( $dC/dz$ , in  $\text{pmol cm}^{-4}$ ) in the millimeter above the organisms  
123 were measured using a 50- $\mu\text{m}$  Clark-type oxygen microelectrode (Unisense, Denmark). Oxygen fluxes  
124 ( $J$ ,  $\text{pmol cm}^{-1} \text{ s}^{-1}$ ) in the microtube were calculated using Fick's first law of free diffusion as  $J = D \times dC/dz$   
125 (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  
126  $12^\circ\text{C}$  and 35PSU). Individual respiration rate ( $R$ ,  $\text{pmol ind}^{-1} \text{ day}^{-1}$ ) was then calculated as  $R = J \times S/n$   
127 (considering the microtube inner section  $S = 7.9 \cdot 10^{-3} \text{ cm}^2$  and the number of individuals  $n = 5$ ). Note  
128 that our measurements were conducted on groups of 5 individuals both to take into account the low  
129 individual respiration rate of benthic foraminifera and to overpass the sensor detection limit (Geslin et  
130 al., 2011). Respiration rate measurements were replicated 6 times in control seawater and triplicated in  
131 both P20 and PP200 leachate treatments. Since respiration is influenced by individual size, specimens  
132 were measured to normalize the respiration rates by the foraminiferal biovolume ( $8 \cdot 10^6 \mu\text{m}^3$  in average;  
133 estimated following Geslin et al., 2011). All respiration measurements were carried out in the dark in a  
134  $12^\circ\text{C}$  temperature-controlled water bath (Huber CC-K12, Germany).

135

## 136 2.5 Data analysis

137 Due to our small size samples, the effect of the 3 experimental conditions on movement speed, fractal  
138 dimension and foraminiferal respiration rate was tested using Kruskal-Wallis test (Hollander and Wolfe,  
139 1999) in R v.3.5.3 (R Core Team, 2019).

140

141 **3 Results**

142 Image analysis show that 100% of the individuals tested were moving throughout the experiments and  
143 were still alive after being exposed to PP20 and PP200 for 10 hours. *Haynesina germanica* moved over  
144 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  
145 h<sup>-1</sup> and 1.1 to 3.1 mm h<sup>-1</sup> for ASW, PP20 and PP200 respectively (Figure 1A). All the trajectories  
146 considered in this work were significantly described in terms of fractal dimensions that ranged between  
147 1.02 and 1.13 with average values of 1.07, 1.06 and 1.06 in ASW, PP20 and PP200 respectively (Figure  
148 1B). 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  
149 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 1C).  
150 Neither locomotion speed, fractal dimensions nor respiration rates exhibited any significant differences  
151 between the three experimental conditions (Kruskall-Wallis-test: p>0.05; Table 1).

152

153 **4 Discussion**

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

159 Similarly, no sublethal effect were perceptible through *H. germanica* locomotion and metabolism.  
160 Specifically, locomotion speed was nearly 2-fold lower than those reported previously on the same  
161 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  
162 experimental temperature (12°C here vs. 22°C in Seuront and Bouchet, 2015) since decreasing  
163 temperature is known to reduce foraminiferal activity (Bradshaw, 1961). Our results nevertheless clearly  
164 indicated that PP leachates did not affect foraminiferal behavior (Figure 1A, B). This is consistent with  
165 the observed lack of behavioral impairment in the intertidal gastropod *Littorina littorea*; as PP20  
166 leachates impaired their chemosensory ability without impacting their neuromuscular abilities (Seuront,  
167 2018). In turn, our results contrast with previous evidence that PP-plasticizers reduce fish larvae velocity  
168 in the first days after hatching (Inagaki et al., 2016; Wang et al., 2013) and negatively impact adult-fish  
169 locomotion and reproductive behavior after at least 2 months of exposure (Gray et al., 1999; Xia et al.,  
170 2010). Note that the apparent discrepancy observed between the aforementioned studies and our  
171 experiment might be due to differences in exposure duration as reported in Table 2.

172 Plasticizers have previously been reported to lead to an immediate increase followed by a decrease in  
173 respiration rates with rising phenols concentration in mollusks (Levine and Cheney, 2000). They can  
174 also induce energetical impairments in crustaceans anaerobic metabolism in less than 2 days (Nagato et  
175 al., 2016). In contrast, we did not find any significant effects of PP leachates on foraminiferal respiration,  
176 even under very high leachates concentrations, i.e. PP200 (Figure 1C). To the best of our knowledge,  
177 the only other study that investigated the effect of PP leachates on a unicellular organism found a

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

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

204

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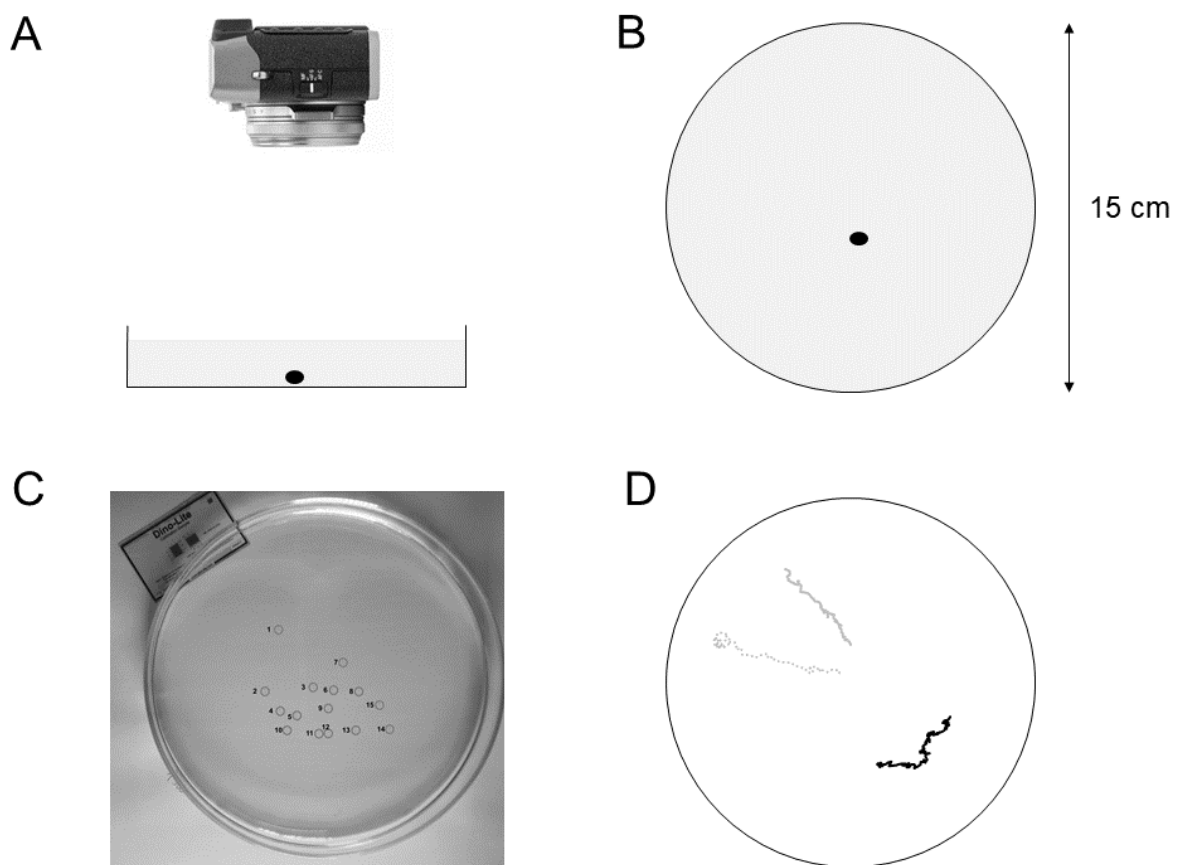
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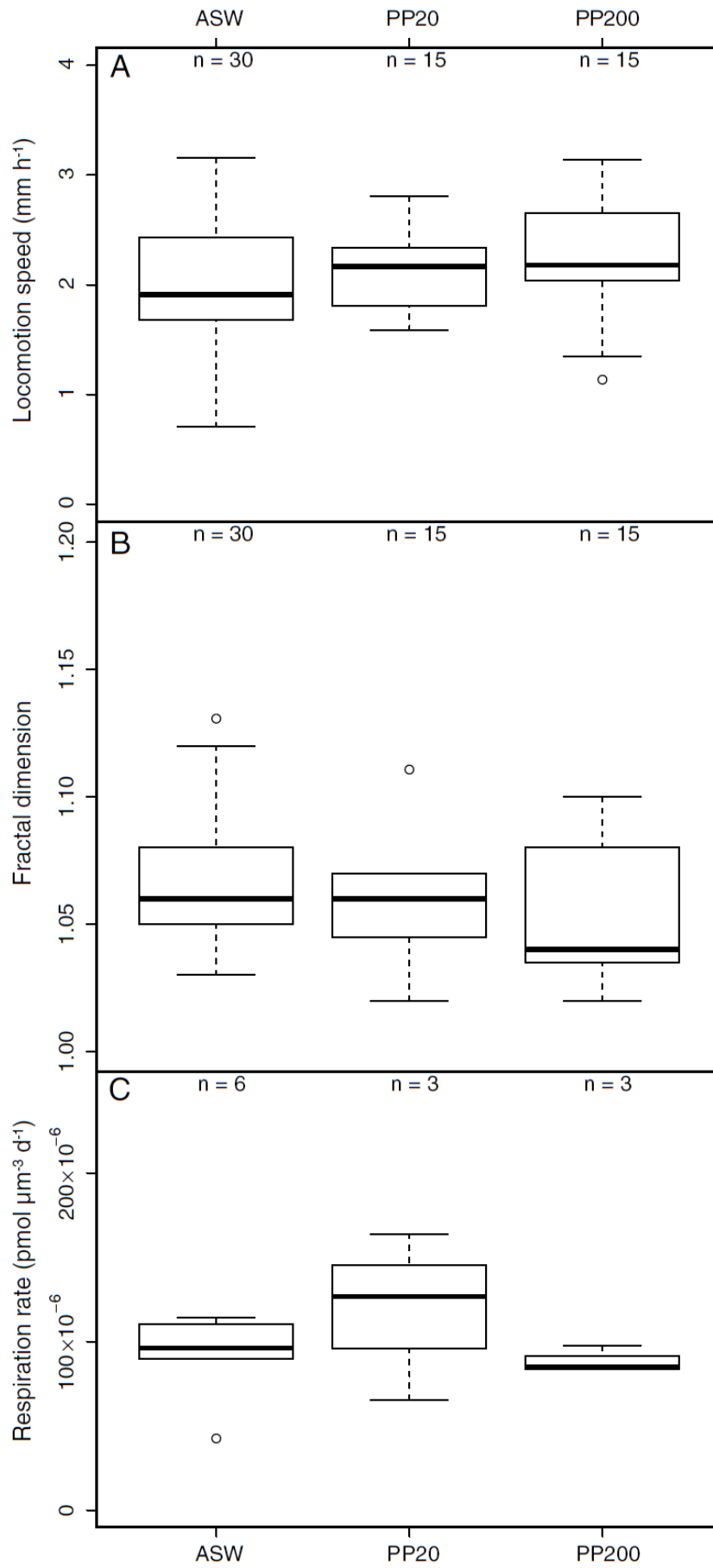
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396 Figure 1 caption: A and B: schematic representation of the experimental setup with lateral view  
 397 (A) and top view (B) of the position of the foraminifera (black ovoid shape) placed on the petri-  
 398 dish. C: photograph of the initial position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



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

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410 Table 1 caption: results of the Kruskal-Wallis statistical analyses testing the effect of the  
411 experimental conditions (ASW as a control, PP20 and PP200) on the three measured response  
412 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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414 Table 2 caption: organisms, response observed, type of pollutant, concentration, equivalent concentration in the present study and exposure duration  
 415 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
MRabet 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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