

What the fluff is this? - Gammarus pulex prefer food sources without plastic microfibers

Article

Accepted Version

Yardy, L. and Callaghan, A. (2020) What the fluff is this? - Gammarus pulex prefer food sources without plastic microfibers. *Science of the Total Environment*, 715. 136815. ISSN 0048-9697 doi: <https://doi.org/10.1016/j.scitotenv.2020.136815> Available at <http://centaur.reading.ac.uk/89509/>

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To link to this article DOI: <http://dx.doi.org/10.1016/j.scitotenv.2020.136815>

Publisher: Elsevier

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1 What the fluff is this? - *Gammarus pulex* prefer food sources without
2 plastic microfibers.

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6 Microplastics, Microfibres, Pollution, Amphipoda

7 Abstract

8

9 Investigations into the impact of micro plastics (MP) and microfibers (MFs) upon the
10 freshwater aquatic environment are still in their infancy despite our growing awareness of
11 their importance. *Gammarus pulex* have long been used as a study organism for
12 ecotoxicology and several studies have already used them to investigate the impact of MFs.
13 One area of research which has not been exploited is the extent to which *G.pulex* can detect
14 MFs and whether or not they avoid eating them. To answer this question we developed a
15 reliable and accurate method of exposing *Gammarus* to known amounts of MF embedded
16 in algal wafers. Here we show that when given the choice between control wafers and
17 those contaminated with 2% or 3% MF *Gammarus* ingest fewer MF than would be expected
18 if a random choice was made (2% W=7 P=0.01698, 3% W=13 P=0.03397). Their feeding
19 behaviour also changes, with a significant reduction in time feeding ($F_{1,18}=21.3$ P=0.0002) as
20 well as significantly fewer visits to contaminated wafers ($F_{1,18}= 5.312$ P=0.0333). This
21 suggests that *G.pulex* are able to detect MF in the 200-500 μ m range and are partially
22 repelled by them.

23

24 Introduction

25 Approximately 70% to 80% of microplastics (MPs) in marine environments are thought to
26 originate from inland sources and be transported out from rivers to the oceans (Andrady,
27 2011). Microplastics are defined as diverse plastics, including polyethylene and polystyrene,
28 whose fragments are smaller than 5 mm in size, they can be particles or fibres, fibres being
29 more than twice as long as they are thick and generally thinner than human hair (Cole,
30 Lindeque, Halsband, & Galloway, 2011). They can be produced by the degradation of larger
31 particles, for example through clothes washing (Browne et al., 2011; Napper & Thompson,
32 2016), or are manufactured as microbeads for use in personal care products including
33 toothpaste, sunscreen and facial scrubs (Duis & Coors, 2016; Fendall & Sewell, 2009;
34 Kalčíková, Alič, Skalar, Bundschuh, & Gotvajn, 2017; Leslie, 2014).

35 The highest volumes of MP pollution have been found in the Northern Hemisphere at water
36 fronts and in enclosed waters near to urban areas (Cózar et al., 2014; Barnes et al., 2009). As
37 well as accumulation in the environment (Cózar et al., 2014), MPs can accumulate in
38 individuals (Browne et al., 2008) and they have even been found in human stools (Schwabl et
39 al., 2018). Their size results in them being easily ingested by many aquatic organisms at
40 various trophic levels and stages of development, including freshwater invertebrates (Cole et
41 al., 2013; Scherer et al., 2017; Al-Jaibachi et al., 2018a, 2018b,; Aljaibachi and Callaghan,
42 2018). By entering the food chain MPs can be readily transferred between trophic levels (Chua
43 et al., 2014; Betts, 2008; Farrell and Nelson, 2013; Setälä et al., 2014; Davarpanah and
44 Guilhermino, 2015).

45 Studies to determine the impact of ingested MPs in smaller invertebrates such as copepods,
46 isopods and zooplankton have concluded that MPs have no detrimental effect following

47 ingestion, possibly because the MPs were too large to cross the midgut wall and were
48 eliminated in faeces (Cole et al., 2013; Cole, 2015). This was found in the isopod *Idotea*
49 *emarginata* (Hämer et al., 2014), cladoceran *Daphnia magna* (Al-jaibachi and Callaghan,
50 2017) and dipteran mosquito *Culex pipiens* (Al-Jaibachi et al., 2018a, 2018b,; Aljaibachi and
51 Callaghan, 2018). In studies using the larger *Gammarus fossarum*, the impact of MP
52 ingestion varied depending on the type of plastic (Straub et al. 2017). Petroleum-based MPs
53 significantly reduced the assimilation efficiency of MP contaminated food in the long-term,
54 whereas biodegradable plastic did not, although ingestion of both types of plastic led to
55 significantly reduced growth compared to the control (Straub et al. 2017). In other studies,
56 Irregular MP fragments of polyethylene terephthalate (PET) had no negative effects on
57 feeding in *Gammarus pulex* (Weber et al. 2019).

58 *A meta-analysis on the impact of MP on the aquatic environment revealed that most studies*
59 *had focussed on particles rather than fibres (Foley, Feiner, Malinich, & Höök, 2018).*

60 Microfibres (MFs) have been investigated in several marine crustaceans, including Sand
61 Hoppers (*Orchestia gammarellus*), Shore Crabs (*Carcinus maenas*, *Carcinus aestuarii*) and
62 Langoustine (*Nephrops norvegicus*) concluding that MF between 1-5mm were ingested
63 (Piarulli et al., 2019; Watts, Urbina, Corr, Lewis, & Galloway, 2015; Welden & Cowie, 2016).
64 Welden & Cowie (2016) found that the number and length of MF retained in the digestive
65 tract of *N. norvegicus* was related to the gastric mill, an organ used to grind food in the
66 upper gut, larger specimens had larger gaps and so more and larger fibres could pass
67 through the gut and be excreted. They found that the only way for these trapped fibres to
68 be lost was through moulting, where their gut lining and gastric mill was shed.

69 Most studies into MF have focussed on the marine environment and have found that the
70 majority of fibres from the deep sea benthos were of cellulose origin (80%) with the
71 remainder being polyester or acrylic. Degradation in the ocean is linked to UV action, so that
72 plastic MFs in the deep sea tend to persist for hundreds if not thousands of years (Browne
73 et al., 2011; Sanchez-Vidal, Thompson, Canals, & De Haan, 2018). As the UV absorbance of
74 freshwater is greater than saltwater, and there is likely to be turbidity, there is likely to be a
75 similar problem in deeper river and lakes (Markager & Vincent, 2000).

76 The freshwater shrimp *G. pulex* has been used as a model organism for investigating a range
77 of topics within ecotoxicology, for example hormonal responses (Gismondi, 2018),
78 metabolic responses (Lebrun, Perret, Geffard, & Gourlay-Francé, 2012), the effect of
79 pesticides (Auber, Roucaute, Togola, & Caquet, 2011), and heavy metals (Duddridge &
80 Wainwright, 1980). *Gammarus pulex* are especially useful for investigating the impact of MP
81 because of their variable diet (Bloor, 2010, 2011; Kunz, Kienle, & Gerhardt, 2010). While
82 predominantly shredders feeding on leafy detritus, they will predate several invertebrate
83 taxa as well as feed upon carrion. In addition they are an essential food source for many
84 small fish (Kunz et al., 2010; MacNeil, Dick, & Elwood, 1999) and represent a vector for
85 plastics to enter the vertebrate food chain. *Gammaridae* are a diverse family of amphipod
86 crustaceans with representatives in freshwater, brackish and marine environments.
87 Therefore conclusions drawn from studying them are applicable all over the globe (Costa,
88 Neuparth, Correia, & Helena Costa, 2005; Kunz et al., 2010).

89 No recent studies have investigated how MP may affect feeding behaviour and may cause
90 selective feeding in *G. pulex*, nor have *G. pulex* been exposed to MF. Previous studies have
91 shown that several macroinvertebrates, including *G. pulex*, will ingest MP in a variety of

92 presentations, from as a suspension that settles on food (Weber, Scherer, Brennholt,
93 Reifferscheid, & Wagner, 2018).

94 One difficulty in many studies into MFs has been that they are often studied without being
95 incorporated into food sources and in concentrations well above environmentally relevant
96 levels (Hanvey et al., 2017; Wagner et al., 2014). While some studies have produced a
97 method for exposing invertebrates to a reliable dose of MP alongside plant matter (Straub,
98 Hirsch, & Burkhardt-Holm, 2017), it is unknown how well they work with MF or larger MPs.
99 It has been shown that algae and grasses provide a vector for MP into taxa not obviously at
100 risk of MP ingestion (Goss, Jaskiel, & Rotjan, 2018; Gutow, Eckerlebe, Giménez, &
101 Saborowski, 2016), therefore this relationship must be thoroughly investigated.

102 In this study we have adapted a method for dosing food with MFs that was originally
103 developed to study plant litter decomposition and invertebrate consumption (Kampfraath
104 et al., 2012). Our new method permits a reliable quantifiable method for exposing benthic
105 macroinvertebrates to MFs. We used the method to identify whether *G.pulex* show any
106 preference or repellence towards MF when they are part of a food source. This
107 understanding is of utmost importance because it gives an idea as to the potential for
108 environmental MF to enter the food chain. In order to gain a greater understanding
109 behaviour must be investigated, previous studies have suggested that chronic exposure to
110 MP impacts growth (Straub, Hirsch, & Burkhardt-Holm, 2017), thus making it less nutritious
111 and could be a driver for food choice (Carrasco et al., 2019). However, if such avoidance is
112 detected during the first exposure to MP then avoidance cannot be due to the lower
113 nutritional value, as this has not yet been learned by individual organisms.

114

115 Materials and Methods

116

117 G. pulex Collection Site

118 The *G. pulex* were collected from Emm Brook, a tributary of the River Lodden, within Dinton
119 Country Park in Reading, between the points (Decimal Degrees 51.440494, -0.874373 to
120 51.442274, -0.874359). This site was chosen for its good population of *G. pulex*, ease of
121 access and because of its relatively shallow depth of <90cm. Animals over 12mm in length
122 were collected by kick sampling using a hessian net, placed in plastic bottles filled with
123 stream water and transported to the laboratory. The animals were briefly rinsed with
124 reverse osmosis (RO) water in the laboratory to remove silt and river water and then species
125 confirmed using a key (Eggers et al., 2016) .

126 In the laboratory *G. pulex* were placed in 45L plastic tanks (150 per tank) containing 40L
127 aerated Organisation for Economic Co-operation and Development (OECD) reconstituted
128 water (Hooper et al., 2006), maintained at 17°C with 12:12 light to dark ratio and fed algae
129 wafers (Wafer Algae Eater Fish Food, API).

130

131 Microfibre Preparation

132 Black 100% acrylic wool (Hayfield Bonus DK product code 5723101001, Hobbycraft,
133 Farnborough) was used to generate MFs. The wool was cut into pieces to generate lengths
134 of <5mm by wrapping a length 5 times around two nails placed into a piece of wood 10 cm
135 apart to generate ten parallel lengths. The wool was sprayed with RO water until it was
136 saturated and then frozen at -80°C for 1 hour. After an hour the wool was removed and the
137 first and last cm removed using a metal scalpel (Swann-Morton No 11 blade) and then cut

138 into 5cm lengths which were stored on ice until ready to be used. The wool lengths were
139 further sliced into <500µm lengths and dried on a hot plate.

140 Wafer Production

141 Algae wafers, were ground using a mortar and pestle for 1 min until they were powder and
142 stored in an airtight lidded glass beaker to prevent contamination. To make the wafers, 1g
143 of the algae powder was added to 0.5ml of RO water and mixed to form a paste. The paste
144 was shaped into a flat cake 5mm thick and placed on a hot plate at 70°C for 2 hours to dry.
145 Test wafers were prepared by adding 0.5%, 1%, 1.5%, 2%, 2.5% and 3% MF fibres by weight
146 to the powder and then homogenized by grinding for a further 1 min before adding the RO
147 water.

148 Once dried each cake was cut up into 0.05 g wafers with a scalpel and placed in a separate
149 lidded container to prevent contamination. To test the accuracy of this method for exposure
150 of animals to known amounts of fibre, ten of each nominal concentration of test wafer were
151 cut into quarters. Each quarter was crushed with a spatula and placed under a 10x binocular
152 microscope (Optech Microtech) for counting.

153 Execution of Tests

154 Eight individual *Gammarus* 12-20mm in length were placed in a 5L aquarium filled with
155 aerated 2L reconstituted water and starved for 24h. The *Gammarus* were then individually
156 placed into an aerated 5L aquarium filled with 2L reconstituted water along with one 0.05g
157 wafer (either control or treatment) and left for 4 hours to feed. After 4 hours each
158 *Gammarus* was removed from its tank, placed in a 5ml beaker and killed with 50°C water.
159 Eight tanks were used per day for 5 days, with concentrations distributed randomly across

160 the period, resulting in 10 replicates per treatment. Each day the aquariums were rotated in
161 order to ensure that there was no impact from position.

162 Guts were removed from dead *Gammarus* under a binocular dissection microscope at 10X
163 magnification. To remove the gut, the telson was removed with a second cut immediately
164 behind the eyes. The gut was then pulled whole from the body using fine point forceps and
165 picked through, counting the number of fibres.

166

167 Choice experiments were conducted using the same protocol, except each test aquarium
168 had one 0.05g control wafer as well as a 0.05g test concentration wafer. The amount of time
169 each *G.pulex* spent feeding on each wafer and the number of visits to each were recorded
170 over four hours, this was referred to as behavioural data.

171

172

173 Data Analysis

174 All data analysis was conducted using R and R Studio. Shapiro-Wilkes tests were used to test
175 for normality. The wafer data met the assumptions for normality and Two Way Analyses of
176 Variance were conducted to see if there was any significant difference between wafers or
177 wafer quadrants within each concentration. ANOVA was conducted between the
178 concentrations in order to confirm significant difference in the number of MF between the
179 concentrations.

180 The ingestion data met assumptions for normality therefore ANOVA was conducted to
181 identify the relationship between the number of MF ingested and the concentration of MF
182 in wafers.

183 The choice data did not meet the assumptions for normality, therefore Kruskal-Wallis tests
184 were used in place of ANOVAs to investigate MF ingestion between concentrations. It was
185 expected that the number of MF ingested would be half that of the non-choice experiment,
186 however it was found that approximately half *G.pulex* ingested no MF, these were ignored
187 and Wilcoxon Rank tests were used to investigate the difference between the treatments of
188 choice and no choice of those *G.pulex* which did ingest MF.

189 Behaviour data fit the assumptions for normality and so ANOVAs were used to identify the
190 functional response.

191

192

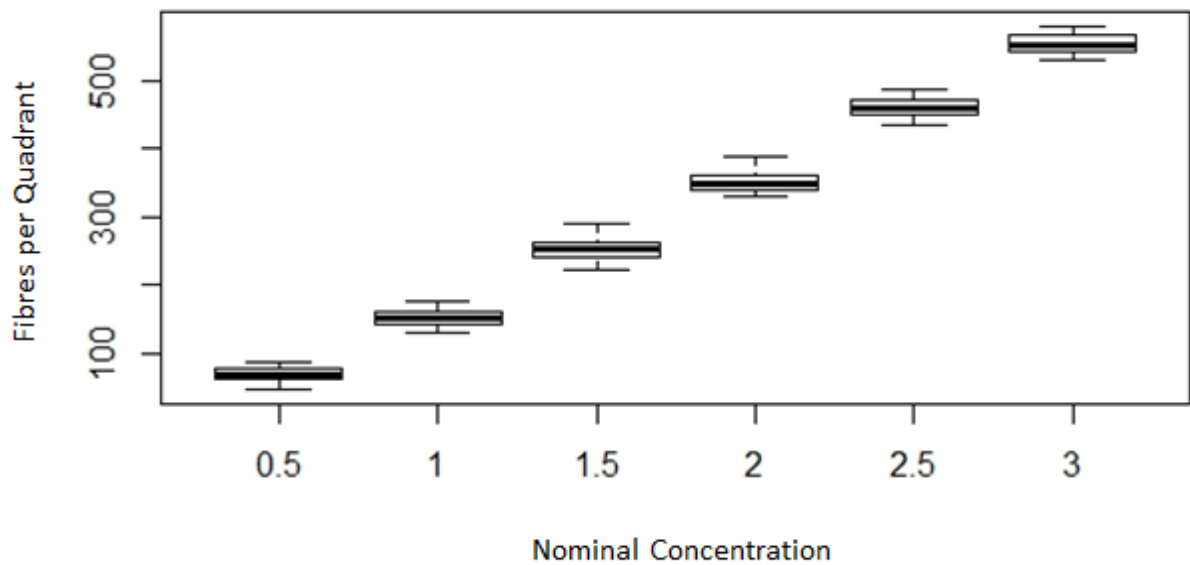
193 Results

194 Wafers

195 All wafers dried and set as expected and were easily dissected. There was no significant
196 difference in acrylic fibre counts between wafers or wafer quadrants within each
197 concentration (Table S1).

198 The number of fibres were directly proportional to the % of MF by mass added (Fig 1), and
199 significantly different between concentrations $F_{1,118}=14766$ $P<0.0001$.

200

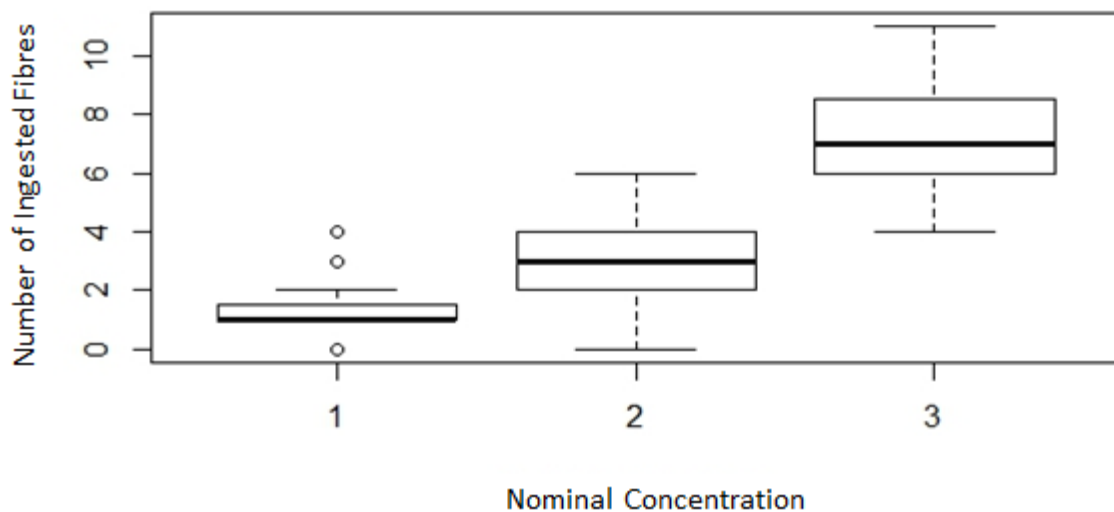


201

202 **Figure 1. The number of fibres per quadrant of algae wafers made using different percentages (by mass) of**
 203 **200-500µm Acrylic fibres, N at each concentration = 40.**

204 Ingestion

205 The *G.pulex* readily fed on the test wafers and ingested MFs. Thirty percent of the 1%
 206 treatment and 10% of the 2% treatment ingested no MF. There was a direct relationship
 207 between wafer concentration and the number of MF eaten (Fig 2), with a significant
 208 difference between test concentrations $F_{1,28}=54.21$ $P<0.0001$.



209 **Figure 2. The number of 200-500µm Acrylic fibres ingested by *G.pulex* in 4 hours at 3 test concentrations. N**
210 **for each concentration = 10.**

211

212 Choice experiments

213 *Gammarus* ingestion of MF approximately halved when animals were given a choice

214 between contaminated and uncontaminated food (Fig 3). There was no significant

215 difference in the number of MFs ingested between the concentrations when given a choice

216 of uncontaminated food $H(2)=3.028$ $P=0.22$. Of the 12 *G. pulex* at each concentration, 4 of

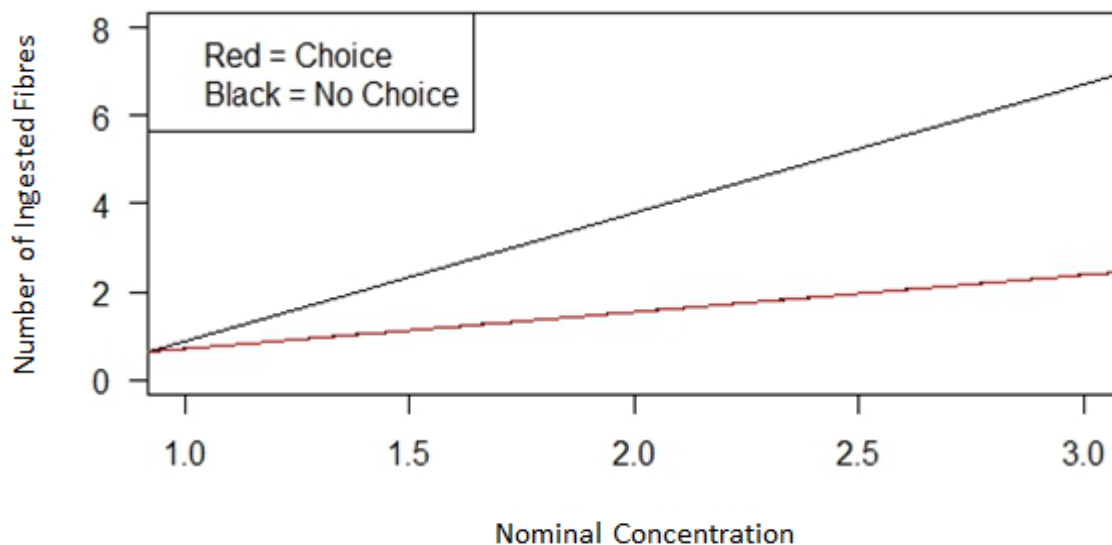
217 the 1%, 6 of the 2% and 5 of the 3% had ingested no MF, equating to approximately half of

218 each concentration. When those that had ingested no MF were removed from the data and

219 the remaining results were compared to the no-choice data, those *G. pulex* with a choice

220 ingested significantly fewer MF than those without a choice Fig 4 (2% $W=7$ $P=0.017$, 3%

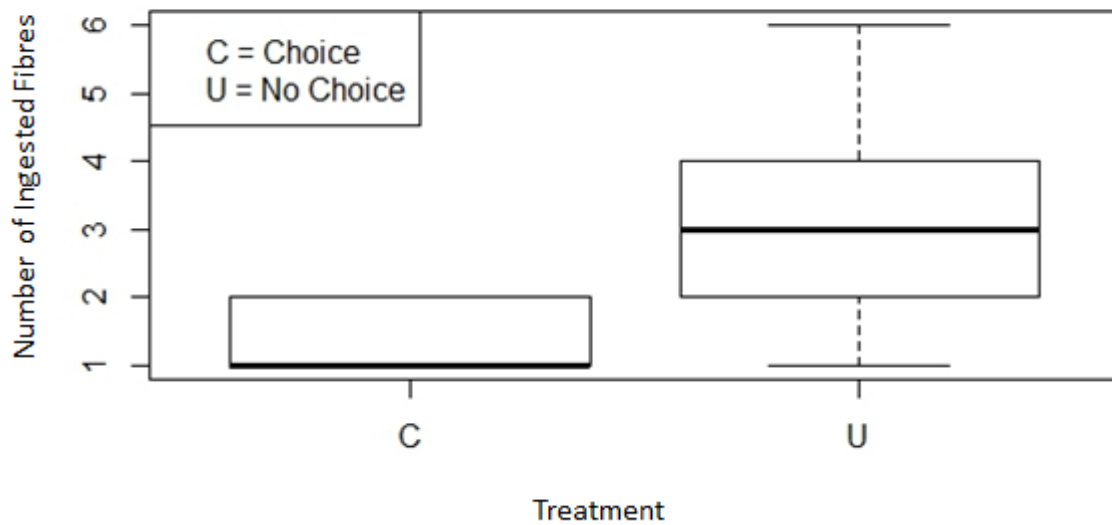
221 $W=13$ $P=0.034$).



222

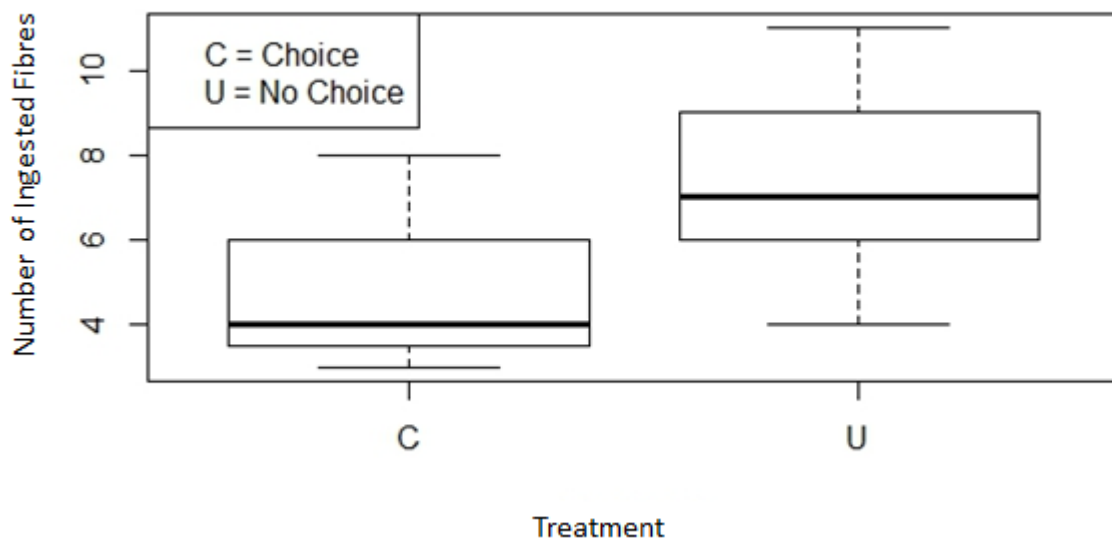
223 **Fig 3. Linear Regressions for the ingestion of 200-500µm Acrylic fibres by *G. pulex*, with and without the**
224 **choice of non-contaminated food. N for each concentration = 12.**

225 A



226

227 B



228

229 Fig 4. The ingestion of 200-500µm Acrylic fibres by *G. pulex* with and without the choice of uncontaminated
 230 food at fibre concentrations (by mass) of 2% (A) and 3% (B)

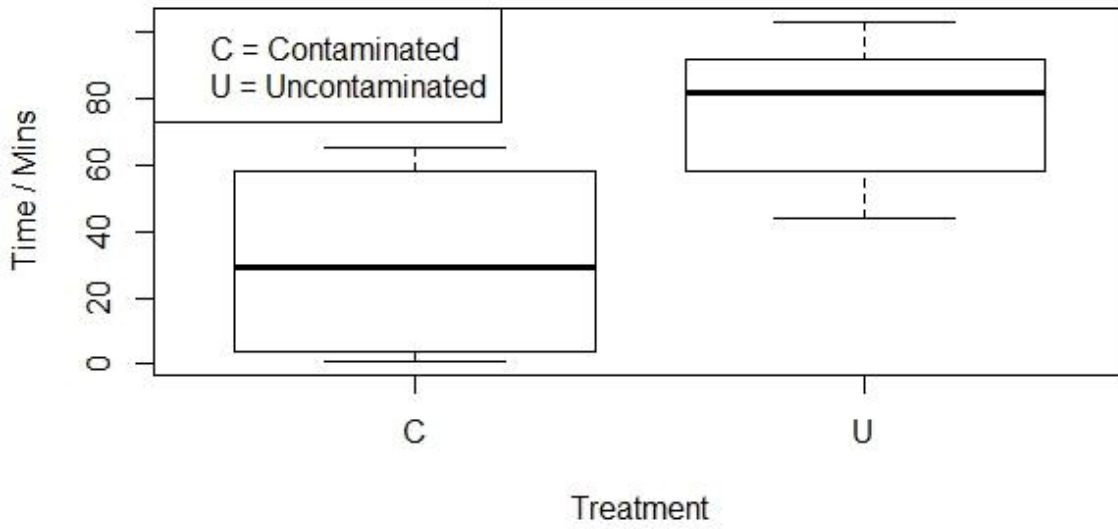
231

232 The observation tests revealed that *G. pulex* spent significantly less time feeding ($F_{1,18}=21.3$

233 $P=0.0002$) on and significantly fewer visits ($F_{1,18}= 5.312 P=0.0333$) to contaminated wafers

234 (Figure 5).

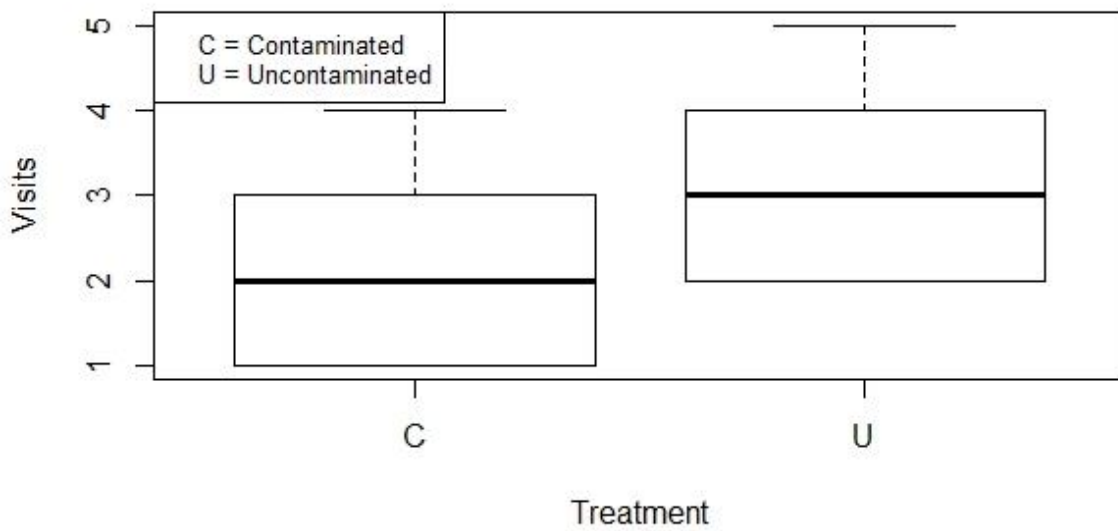
235 A



236

237

238 B



239

240 Fig 5. The amount of time in minutes *G.pulex* spend feeding from uncontaminated wafers and wafers
241 contaminated with 200-500µm Acrylic fibres (A) and the number of visits to each type of wafer (B).

242

243

244 Discussion

245 We have developed an accurate, cheap and easy method to produce wafers to investigate
246 the impact of MFs on aquatic invertebrates based on the method of Straub et al., (2017).
247 The wafers produced were homogenous within a concentration and MF counts were
248 directly proportional to the % of MF used to produce the wafer. Therefore we can be
249 confident that this method allows reliable dosing of MF which show a tendency to clump
250 together without a solid matrix. *G.pulex* ingest plastic MFs in lengths up to at least 500µm in
251 proportion to the concentration present.

252 This method allows researchers to instigate worst case scenarios where invertebrates may
253 be unable to avoid MF and can be used to study preference between different MFs. This
254 method would work for smaller MF and MP, and should be suitable for other organisms
255 which will feed upon algae wafers, enabling a standardised method for understanding the
256 impact of various MPs upon a range of environments.

257 There are several reasons why invertebrates may detect and avoid plastics in food, there
258 could be chemical cues (De Lange, Sperber, & Peeters, 2006) or it could be they can
259 physically feel their presence (Carrasco et al., 2019). If the main driving factor is the
260 difference in texture between food and MP then the main food media texture should match
261 the natural food texture as much as possible. An agar based gelatinous food source such as
262 is used by Straub et al., (2017) produces a greater contrast between the food and the MP
263 texture compared to this new method or natural food sources.

264 When given a choice of contaminated vs uncontaminated food, *Gammarus* significantly
265 avoided eating food with MFs, with fewer visits to the food and a reduction in time feeding.

266 These observations were supported by quantitative data demonstrating a significant
267 difference in MFs ingested. *Gammarus* have previously avoided eating contaminated food
268 including when chemical cues to bacteria and fungi are present (De Lange, Lürling, Van Den
269 Borne, & Peeters, 2005; De Lange, Sperber, & Peeters, 2006). Furthermore there is evidence
270 that animals can detect and avoid MPs. Carrasco et al(2019) exposed *Orchestoidea*
271 *tuberculata* to artificial food containing 8 µm particles of polystyrene MP spheres at 3
272 different concentrations (0%, 5% and 10%). The animals consumed significantly more food
273 when no MPs were present compared to food contaminated with 10% MPs. As this study
274 was a relatively short exposure (15 days) it is possible that the avoidance mechanism is
275 physical rather than biochemical.

276 In the current study contaminated wafers were eaten with no evident repulsion when no
277 uncontaminated food was available. This is in line with other studies which have recorded
278 MF ingestion of fibres of up to 5mm in length by taxa larger than *Gammarus*, including
279 crustaceans, molluscs, annelids and fish, (Farrell & Nelson, 2013; Foley et al., 2018; Straub et
280 al., 2017; Watts et al., 2015). Similar results have been found in the smaller *Daphnia magna*
281 with many studies showing that there is a positive relationship between concentration of
282 MP and the number ingested (Canniff & Hoang, 2018; Jemec, Horvat, Kunej, Bele, & Kržan,
283 2016; Rehse, Kloas, & Zarfl, 2016). However, Aljaibachi & Callaghan (2018) found that
284 *Daphnia* seemed to be able to selectively ingest algal cells and avoid 2µm MP particles.

285 These results are important in understanding the risk to the environment. It suggests that,
286 at least *Gammarus* is able to avoid MF contaminated food, meaning that as long as their
287 environment is not totally saturated with MF they could be ingested in rates lower than one
288 might assume given environmental concentrations. As macroinvertebrates are the main

289 vector for MP entering the higher trophic levels (Foley et al., 2018), including vertebrates
290 and ultimately humans, their ability to limit MP ingestion would in turn limit the amount
291 entering higher trophic levels. There is already a highlighted knowledge gap in this area
292 (Horton, Walton, Spurgeon, Lahive, & Svendsen, 2017) and its understanding would help
293 direct mitigation processes.

294

295 *Gammarus* produce copious amounts of faecal pellets which are eaten by other freshwater
296 macroinvertebrates and are important sources of organic matter for bacteria (Joyce,
297 Warren, & Wotton, 2007) . Microfibres were clearly observed in faecal pellets with no
298 evidence of being shortened which means that not only could *G.pulex* act as a vector for MP
299 to enter higher trophic levels if they are eaten by fish or other invertebrates, but their
300 faeces provide a source of MP to enter lower trophic levels through faecal ingestion (Kelly,
301 Dick, & Montgomery, 2002; Ladle & Griffiths, 1980) (Kelly, Dick, & Montgomery, 2002).

302 Despite their apparent ability to avoid ingesting MF contaminated wafers, it remains to be
303 seen whether *G. pulex* predation on differentially contaminated prey would vary.

304

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306

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308

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