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Stefan Grigorakis
University of Windsor

Sherri A. Mason
State University of New York at Fredonia

Ken G. Drouillard
University of Windsor

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1 **Title:** Determination of the gut retention of plastic microbeads and microfibers in goldfish

2 (*Carassius auratus*)

3

4 **Author Names and Affiliations**

5 Stefan Grigorakis[1], Sherri A. Mason[2], Ken G. Drouillard[1]

6 [1]Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON,

7 Canada, N9B 3P4

8 [2] State University of New York at Fredonia, Department of Chemistry and Biochemistry, 280

9 Central Avenue, Science Complex 340, Fredonia, NY 14063, USA

10

11 **Corresponding author**

12 Ken G. Drouillard

13 Great Lakes Institute for Environmental Research,

14 University of Windsor

15 401 Sunset Ave,

16 Windsor, ON, N9B 3P4

17 Tel: +1 (519)-253-3000 ext 4744 Mobile: +1 (519)-903-5422

18 Fax: +1 (519)-971-3616

19 Email: kgd@uwindsor.ca

20

21

22

Abstract

Microplastics are ubiquitous pollutants in aquatic habitats and commonly found in the gut contents of fish yet relatively little is known about the retention of these particles by fish. In this study, goldfish were fed a commercial fish food pellet amended with 50 particles of one of two microplastics types, microbeads and microfibers. Microbeads were obtained from a commercial facial cleanser while microfibers were obtained from washed synthetic textile. Following consumption of the amended pellet, fish were allowed to feed to satiation on non-amended food followed by fasting for periods ranging from 1.5 h to 6 days. Fish sacrificed at different time points were dissected to remove gut contents and the digesta contents retention and microplastic retention was determined. Although a small number of microplastic particles were retained in fish GI-tracts after 6 days (0-3 particles/50), the retention of microplastics was generally similar to the retention of bulk digesta contents. According to a breakpoint regression model fitted to digesta contents and microplastic particles, the 50% and 90% evacuation times were 10 h and 33.4 h, respectively. The results of this study indicate that neither microbeads nor microfibers are likely to accumulate within the gut contents of fish over successive meals.

Keywords: microplastics, bioaccumulation, gut retention, microbeads, microfibers

1.0 Introduction

Microplastics are a diverse array of synthetic polymer particles that vary in chemical composition, size (from low micrometre scale to an upper size range variously defined between 1 nm and 5 mm), density and shape (Andrady, 2011). They have been observed in most freshwater and marine environments (Eriksen et al., 2014; Corcoran, 2015; Eerkes-Medrano et al., 2015) to such an extent that they were included as sedimentary geochemical markers of the Anthropocene

46 (Waters et al., 2016). Microplastics are often distinguished between those that are synthesized at
47 the defined sizes for an intended application (primary microplastics) relative to particles derived
48 from the breakdown of macroplastics (secondary microplastics). Microbeads are defined as
49 primary microplastics that range in size between 0.1 μm to <5 mm (Environment Canada, 2015)
50 and are used in a wide variety of industrial and consumer applications including personal care
51 products (PCPs). Legislation banning the production of microbeads in PCPs comes into effect in
52 2017 as passed by the U.S. federal government and similar legislation is under review in Canada.
53 While much of the legislative focus has been on microbeads used in PCPs, other common
54 sources of microplastics to municipal wastewaters include abraded particles from synthetic
55 textiles such as nylon and acrylics, henceforth referred to as microfibers, used in clothing
56 (Browne, 2011).

57 Concerns have been raised about the ecotoxicology of microplastics in the environment,
58 including their potential to bioaccumulate in organisms and subsequent transfer through food
59 webs (Sánchez et al., 2014). Zooplankton are capable of ingesting microplastics, potentially
60 mistaking them for food, and can further transfer these to tertiary consumers (Frias et al., 2014;
61 Browne et al., 2013; Setala et al., 2014; Rehse et al., 2016). Mussels have been shown to
62 accumulate microplastics and transfer them to higher trophic levels (Browne et al., 2013; von
63 Moos et al., 2012; Collignon et al., 2012). In a study examining 504 fish from the English
64 Channel that included benthic and pelagic species, 36.5% of specimens had microplastics in their
65 gastrointestinal (GI-) tracts (Lusher et al., 2013). Microplastics in the gut contents of field
66 collected fish have subsequently been widely reported in coastal and freshwaters (Sanchez et al.,
67 2014; Neves et al., 2015; Avio et al, 2015; Phillips and Bonner, 2015; Biginagwa et al., 2016;

68 Bellas et al., 2016). Considering microplastics are being found in fish, there are relatively few
69 studies focussing on the potential of microplastics to bioaccumulate.

70 Exposure to microplastics in water and food can interfere with normal digestive processes
71 due to intestinal blockage, causing reductions in animal feeding rates and energy assimilation
72 (Besseling et al., 2012), lead to histopathological alteration to intestinal and hepatic tissues of
73 fish (Pedà et al. 2016; Lu et al., 2016) and lower hatching success of fish eggs (Lönnstedt and
74 Eklöv, 2016). Translocation of microplastics from gut to the circulatory system has been
75 demonstrated in mussels (Browne et al., 2008; von Moos et al., 2012; Avio et al. 2015a)
76 implying that retention of microplastics beyond entrainment in the GI-tract may be possible in
77 some animals. Avio et al., (2015b) and Lu et al., (2016) confirmed microplastics accumulation in
78 hepatic tissues of fish exposed to microplastics at elevated concentrations in water.

79 Although microplastics are commonly detected in the intestinal tracts of fish, there is
80 limited information characterizing the retention of microplastics by fish. Particle size and shape
81 are likely to influence factors such as GI-retention but limited information is available comparing
82 microplastic types. Neves et al. (2015) observed a higher frequency of fibers in commercial fish
83 gut contents compared to plastic fragments. The above study further reported differences in
84 plastic types in benthic fish, which tended to accumulate a greater proportion of fibers, compared
85 to pelagic fish which contained more fragments. It is not known whether these differences are
86 related to emission patterns and fate of different particle types or whether particle shape might
87 influence the gut retention characteristics of these microplastic types. In this study, the GI-tract
88 retention of two microplastic types, microbeads and microfibers, was determined in goldfish with
89 the objective to determine if i) retention of microplastics by fish exceeds that of food digesta, i.e.

90 exhibits net accumulation in the GI-tract of fish, and ii) to determine if microfibers are retained
91 to a greater or lesser degree than PCP derived microbeads.
92
93 **2.0 Methods**
94
95 *2.1. Microplastic source*
96 Microfibers were extracted from clothing (35cm x 12cm cut out of a commercial
97 polyester fleece scarf) by mechanical agitation in hot water. Following agitation, the water was
98 sieved through stacked 500 µm, 250 µm and 63 µm sieves. Fibers retained on the 63µm sieve
99 were removed by tweezer under magnification and size graded to between 50-500 µm fiber
100 lengths under a dissecting microscope. Microplastic beads were extracted from a commercial
101 cosmetic product (facial cleanser labelled with polyethylene). The contents of the product was
102 poured onto a 63 sieve and the soluble matrix associated with the product washed with water
103 until only microplastics remained. Microbeads were removed from the sieve under
104 magnification. **Figure 1** provides images of isolated microbeads and microfibers under 5x
105 magnification.
106 *2.2 Experimental*
107 Goldfish were selected as a model fish species because they have been routinely used in
108 many bioaccumulation/toxicokinetic studies owing to the ease of husbandry, tolerance to
109 handling and willingness to accept artificial diets. In their wild state, goldfish are benthic feeders
110 and thus might be expected to accumulate microplastics similar to those reported for other
111 benthic feeders. Fish were exposed to microplastics via food. Commercial fish pellets (0.18-
112 0.21g, ~3 mm size) were placed in warm water to soften them. Treatment pellets were amended

113 with 50 microbeads or 50 microfibers per pellet by manual insertion of macroplastic particles
114 into each pellet under microscope. Pellets were air dried after manipulation. Control pellets were
115 wetted and dried in an identical manner but not amended with microplastics. The food was
116 prepared in this manner to ensure that every experimental fish consumed exactly 50 microplastic
117 particles to increase precision of gut retention characterization.

118 Fifty three sexually mature goldfish were fasted for 48 h prior to exposing them to
119 prepared food in order to ensure complete evacuation of gut contents from previous meals and to
120 increase the likelihood that they would accept the microplastic amended pellet provided to them.
121 After fasting, fish were removed from their communal tank and placed in individual fish bowls.
122 Twenty four fish were allocated to the microbead and microfiber treatments, respectively. Five
123 fish were allocated as controls and fed non-amended pellets. Each fish was presented with a
124 single treatment pellet and observed until it was verified that the fish consumed the pellet. After
125 the fish consumed the treatment pellet, non-amended fish pellets were added to the bowl and the
126 fish was allowed to consume to satiation for up to 60 minutes. Any remaining fish food in the
127 bowl was subsequently removed. Fish were fasted for the remainder of the experimental period.
128 Control fish were sacrificed after 1.5 h from feeding the control pellets. Triplicate animals from
129 each treatment were sacrificed after 1.5, 4, 8, 16, 32, 48, 96, and 144 h. The mean ± SE of water
130 temperatures was 14.2 ± 0.21 ºC and exhibited no changes over the fasting duration. The mean ±
131 SE body weights of fish from the microbead and microfiber treatments were 24.80 ± 2.77 g and
132 27.07 ± 3.40 g and were not significantly different from one another ($p>0.4$; ANOVA). On
133 sacrifice, fish were euthanized by immersion in a solution of MS-222 (100 mg/L) and stored
134 frozen until subsequent analysis. This research was performed under ethics approval from the
135 University of Windsor's Animal Care Committee.

136

137 *2.3 Microplastic analysis*

138 On analysis, the gut tract of each fish was dissected and removed. The gut contents were

139 pushed thought the intestine using tweezers and a probe onto a pre-weighed aluminum weight

140 boat and the gut tract tissues were retained for further analysis. The weigh boat was dried at

141 110°C for 1 h and reweighed to determine dry food digesta weight. Subsequently, the dried

142 digesta and gut tissues were re-combined and placed into a 10% KOH solution on a hot plate set

143 at its lowest setting for 1 hour. The solution was taken off of the hot plate and after 2 additional

144 hours, 5mL of 30% H_2O_2 was added to the solution. The solution was poured through a vacuum

145 filtered Buchner funnel using Whatman^{TM} (55mm) filter papers (1 µm glass fiber filters). Fish

146 carcass samples were also digested in a similar manner. Filter papers from each digestion were

147 analyzed under a stereomicroscope to quantify the number of microplastics remaining in the GI-

148 tract/contents, fish carcass or digested food pellets. Quality control of the method was

149 established by measuring and verifying microbeads and microfibers in 5 amended pellets. The

150 mean ± standard deviation of recoveries of microplastic particles for the digested pellets was

151 98.8±1.8%.

152

153 *2.4 Data analysis*

154 Digesta contents weights were standardized to the mean body weight according to:

155 $$X_{DG(ss)} = X_{DG(s)} \cdot \frac{BW_{(mean)}}{BW_{(s)}} \quad (1)$$

156 where $X_{DG(ss)}$ is the size standardized digesta weight (g), $X_{DG(s)}$ is the digesta weight measured in

157 an individual fish, $BW_{(mean)}$ is the mean body weight of fish from the treatment and $BW_{(s)}$ is the

158 body weight of the individual fish. The % remaining of digesta contents was calculating by

159 dividing $X_{DG(ss)}$ by the mean $X_{DG(ss)}$ generated for fish sampled at the first time point (1.5h) and
160 multiplying by 100. For microbeads and microfibers, %remaining was calculated by dividing the
161 number of microplastics measured in a fish's digestive tract by 50 and multiplying by 100.

162 Statistical analysis was performed using a general linear model (GLM) according to:

163 $$\text{Model} = \text{Time} + \text{Group} + \text{Time} * \text{Group} + \text{Constant} \quad (1)$$

164 Where time is the time since feeding (h), group represents a categorical variable specified as
165 digesta retention treatment 1, digesta retention treatment 2, microfibers and microbeads. Under
166 cases where the interaction term (Time * Group) was non-significant, analysis of covariance
167 (ANCOVA) was performed to adjust for time as a covariate and increase the statistical power of
168 the group comparison test. Where the interaction term was found to be significant, GLMs were
169 performed on subsets of the data to test for differences between selected group comparisons.
170 GLM(1) tested for differences in digesta retention time between treatment 1 and treatment 2.
171 GLM(2) tested for differences in digesta retention time and microfiber retention from
172 measurements taken in treatment 1. GLM(3) tested for differences in digesta retention time and
173 microbead retention from measurements taken in treatment 2. Finally, GLM(4) tested for
174 differences in microfiber and microbead retention. Data transformation was necessary owing to
175 failure of normality of the % retention data on the combined data (digesta, microbeads and
176 microfibers). However, when the fist time point (1.5 h) was removed, transformation of %
177 retention data by natural log transformation yielded a normal data set ($p>0.05$; Lillefor's test).
178 Thus, statistical comparisons by GLM were performed with the 1.5 h time point removed and
179 applying a ln transformation. Non-transformed digesta retention data (inclusive of the 1.5 h time
180 point) for individual fish were subsequently fit to an exponential model using non-linear least
181 squares regression according to:

182 $$\%Retained = 100 \cdot e^{-B \cdot time} \quad (2)$$

183 Where 100 is constant forcing 100% of gut contents retention at time 0, b is the fitted coefficient
184 and time is time since feeding (h). The ability of Eq. 2 calibrated independently to gut contents
185 to predict microplastic retention was evaluated using goodness of fit tests by performing a linear
186 regression on observed (microplastic) vs model (Eq. 2) predicted digesta retention. The
187 goodness of fit result was evaluated by determining if the slope was significantly different from
188 1, the constant was significantly different from 0 and by evaluating the magnitude of the
189 coefficient of determination. All statistics were performed using Systat 13 statistical software.
190 Except where otherwise noted, measures of central tendency and variation are expressed as mean
191 and standard error (SE).

192 **3.0 Results and Discussion**

193 *3.1 Digesta retention*

194 During experimental trials, all fish were observed to consume the microplastic amended
195 treatment pellet. No fish mortalities occurred nor were there apparent signs of distress following
196 exposure to the amended food pellet. Fish sacrificed at the 1.5 h time point had a mean $X_{DG(ss)}$
197 weight of 0.60±0.04 g. This corresponds to a food consumption of 2.32% body weight across
198 the treatments and is consistent with expected food consumption rates in fasted fish.

199 A general linear model (GLM(1) as described in methods) was performed to compare %
200 retention of digesta between the two treatments. The GLM and ANCOVA revealed a non-
201 significant ($F_{1,39}$ = 0.92; p>0.3; ANCOVA) difference in digesta retention between the
202 treatments after adjusting for time as a covariate. Given that digesta retention did not
203 significantly differ between the two treatments, the data were combined and fit to the exponential
204 model yielding the following solution:

205 $$\%Retained = 100 \cdot e^{-0.069 \cdot time}; R^2 = 0.69 \quad (3)$$

206 Based on Eq. 3, the time to evacuate 50% and 90% of digesta was 10.0 and 33.4 h, respectively.
207 Overall, the exponential model fit described the temporal trends of digesta contents well during
208 the first 24 h but tended to underestimate observed digesta contents at longer time points (Figure
209 2). This may be related to the method of separating gut contents from the intestinal tissues which
210 could have included residual gut secretions and/or sloughed cells/tissues generated from the GI-
211 tract processing method itself. However, the fitted model produced retention estimates that were
212 generally consistent with other studies on digesta retention in fish of similar size and
213 temperature. Stehlik et al., (2014) reported full clearance of gut contents from clearnose skate
214 (*Raja eglanteria*) by 48 h when held at 15°C. Yellow perch held at 17.1°C exhibited a gut
215 evacuation coefficient of 0.035·time (h^{-1}) based on a log linear model which implies a 50%
216 digest retention of 19.8 h and 90% retention of 65 h (Gringas and Boisclair, 2000).
217
218 *3.2 Microplastic retention in GI-tracts*
219 Control fish sacrificed after 1.5 h were examined for evidence of microplastics in gut
220 contents and carcass samples. No microplastics were found in control fish or within their gut
221 contents. In addition, 10 control fish pellets were examined for presence of microplastics.
222 Similar to control fish, microplastics were not observed in non-ammended food pellets.
223 During the first sampling point (1.5 h), there was good recovery of microplastics within
224 the gut contents of treatment fish. For microfibers, 2 fish had 50 microfibers recovered (100%
225 recovery) and the third fish had 48 fibers recovered in the GI-tract. For the microbeads, 40 to 44
226 particles (80-84% recovery) were recovered from fish during the first time point. Small numbers

227 of microplastics were recovered at the 144 h time point (1 to 3 microfibers in replicate 144h
228 sampled fish and 0 to 3 microbeads in triplicate fish).

229 A general linear model (GLM) was applied to test percent retention of all treatments
230 (digesta from each treatment, microbeads and microfibers) within the study. Both Time ($F_{1,76}$ =
231 88.1; $p<0.001$) and the Group x Time ($F_{3,76}=3.09$; $p<0.05$) interaction terms were significant but
232 group was not significant ($F_{3,76}=0.212$; $p>0.8$) in the overall GLM. Due to the significant
233 interaction terms, additional GLMs were applied to subsets of the data to evaluate for differences
234 in retention on selected measurements. GLM(2) and the ANCOVA revealed no significant
235 differences (F1,39=0.959; $p>0.5$; ANCOVA) in microfiber and digesta retention. Similarly,
236 GLM(3) and ANCOVA revealed non-significant (F1,39=4.00; $p>0.05$; ANCOVA) differences in
237 microbead retention from gut digesta retention. Finally, a comparison of microfiber and
238 microbead retention yielded non-significant differences (F1,39=0.678; $p>0.4$; ANCOVA) from
239 one another. Microplastic and microfiber retention with time along with digesta contents trends
240 are presented in **Figure 2**.

241 For microfibers, the linear regression between %microfiber retention and gut digesta
242 model (Eq. 3) prediction yielded a slope of 0.96±0.09, constant of (7.33±4.07) and coefficient of
243 determination (R^2) of 0.85. The above slope was not significantly different from unity
244 ($t_{1,22}=0.042$; $p>0.5$; t-test) and the constant was not different from zero ($t_{1,22}=1.80$; $p>0.05$; t-
245 test). For microbeads, the goodness of fit test produced a similar slope (0.94±0.04) that was not
246 significantly different from unity ($t_{1,22} = 1.44$; $p>0.1$; t-test) and constant (3.67±2.06) not
247 significantly different from zero ($t_{1,22}=1.79$; $p>0.05$; t-test) with an R^2 of 0.95. It is perhaps
248 notable that the digesta retention model (Eq. 3) which was calibrated only to digesta retention
249 data explained even more variation in microplastic retention than digesta contents itself. This

250 was mainly related to the better fit of model predictions to microplastic retention at the later time
251 points (Figure 2). The reason for the differences in model fit across measurements is attributed
252 to the fact that microplastic exposure was controlled with a high degree of precision compared to
253 gut contents. Although each fish was given exactly 50 microplastic particles, they were provided
254 with food ad libitum after verifying their consumption of the microplastic amended pellet. Thus,
255 digesta contents would have varied to a greater extent between fish compared to microplastic
256 exposures. Overall the goodness of fit tests indicates that the gut digesta retention model
257 adequately described the retention of both microplastic types.

258 Similar observations were generated for the marine isopod *Idotea emerginata* fed a diet
259 spiked with microplastic particles and fibers (Hämer et al,. 2014). In the study by Hämer et al.,
260 (2014), microplastic particles appeared in the stomach and gut contents of isopods but were also
261 readily egested with the feces. Mazurais et al. (2015) examined microplastic retention in
262 European sea bass (*Dicentrarchus labrax*) larvae when exposed to microplastics added to food.
263 The above authors observed a correlation between microbeads in the gut of larvae with
264 concentration of microbeads added to the diet. However, the authors noted that microbeads were
265 fully cleared from the gut of larvae after 2 days post exposure and could be identified in feces
266 suggesting passive retention in the gut contents of fish.

267 Microplastics were also examined in carcass samples of treatment fish but were not
268 observed apart from the gut tissue and gut contents analyzed separately and discussed above.
269 This differs from the results of Avio et al., (2015b) who observed translocation of polyethylene
270 and polystyrene microplastics to liver of laboratory held mullet (*Mugil cephalus*) exposed to
271 microplastics in water (nominal microplastic dose was $2.5x10^3$ particles/L of polyethylene or
272 polystyrene particles sized from 100 to 1000 μm) for 7 days. Between 1-2 microplastic particles

273 per individual were detected in liver of exposed fish, although the presence of microplastics in
274 liver was two orders of magnitude lower than what was observed in gut contents of fish.
275 Similarly, Lu et al. (2016) exposed zebrafish (*Danio rerio*) to solutions containing 5 or 20 µm
276 diameter polystyrene microplastics at concentrations of between 4.5×10^6 to 2.9×10^8 particles/L
277 for 7 days. The above authors reported that 5 µm microplastics accumulated in fish gills, liver
278 and gut, whereas larger microplastics (20 µm in diameter) accumulated only in fish gills and gut
279 but not in liver. Time to steady state of microplastics in zebra fish was reported to be 48 h,
280 implying rapid clearance from animals consistent with the gut retention data presented here (Lu
281 et al., 2016). While the present study failed to identify microplastic translocation in fish tissues
282 apart from their detection in the GI tract, this could be a function of exposures to different
283 microplastic types, different dosing strategies, levels of exposures and differences in the method
284 of detection of microplastics in exposed animals. The lack of translocation of larger (20 µm
285 plus) sized microbeads to liver in zebra fish as reported by Lu et al., (2016) is consistent with the
286 present work given that particles greater than 63 µm were utilized but is not consistent with Avio
287 et al. (2015b) who exposed fish to microplastics of comparable size to this research. Avio et al.,
288 (2015b) and Lu et al., (2016) provided continuous exposures of fish to microplastic contaminated
289 water for up to 7 d days compared to a single dose from a microplastic amended meal applied in
290 the present study. The above authors also used nominal microplastic concentrations in water that
291 were considerably higher than what is present in natural waters. Avio et al., (2015b) used a more
292 sensitive microplastic extraction/detection technique that employed a combination of density
293 gradient separation and oxidant treatment which was shown to yield higher recoveries of
294 microplastics from animal tissues then the oxidation treatment alone. Lu et al., (2016) utilized
295 microplastic particles with encapsulated fluorescent dies to facilitate their detection in tissues

which potentially yielded much lower detection limits then the visual method employed here. Thus, even though microplastics had very good recovery in pellets and gut contents of early time point sacrificed fish from the present work, translocation of smaller microplastic particles when exposed at higher concentrations or under long term exposures cannot be ruled out based on the results of this study.

4.0 Conclusions

Microplastics of two distinct particle shapes (microbeads and microfibers) exhibited similar retention in the GI-tract of goldfish compared to bulk food and digesta. Although a small number of particles were retained in fish after 6 days of fasting, there was no evidence for net bioaccumulation of microplastics in the GI-tract or internal translocation to tissues of fish post exposure. This implies that the potential for long term entrainment and retention of textile derived microfibers or PCP-derived microbeads in fish is relatively low and the detection of microplastics in fish gut contents in the environment most likely represents recent exposures to microplastics in the diet as opposed to cumulative retention across multiple meals. However, this study was limited to evaluation of only two microplastic types and one species of fish. As such, further research to characterize microplastic retention by fish species over different plastic types, shapes and dietary concentrations may be warranted.

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320

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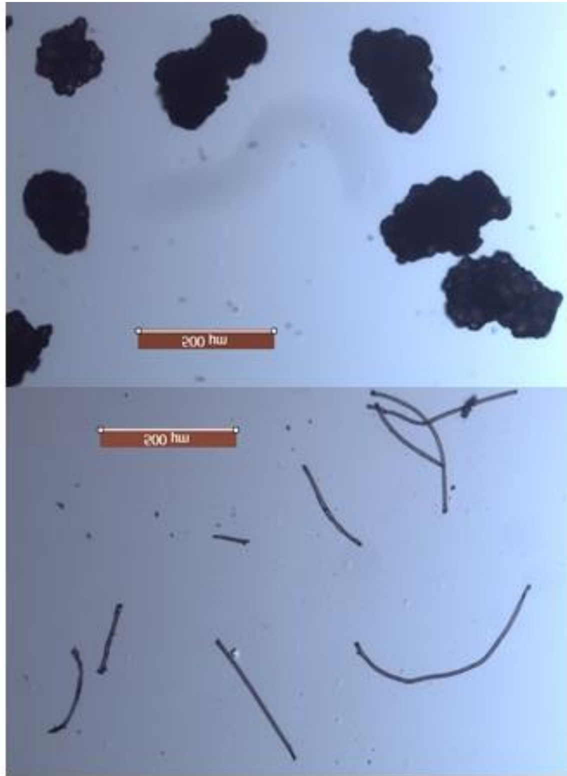

Figure 1. Image of microbeads (left) and microfibers (right) used for feeding trials (5 x magnification).

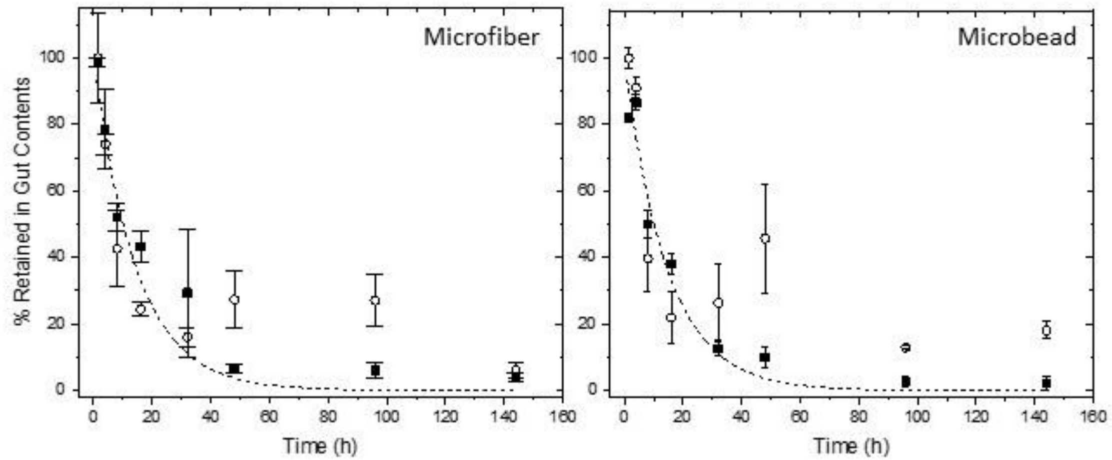

Figure 2. Gut retention of digesta and microplastics in gold fish post feeding. Left graphic presents mean microfiber (■) retention compared to digesta (O). Right graphic presents mean microbeads (■) retention compared to digesta (O). Dashed line is the exponential fit to the combined digesta retention data (Eq. 3). Error bars are standard error.