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Biofouling, metal sorption and aggregation are related to sinking of microplastics in a stratified reservoir

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Abstract

Microplastic particles entering aquatic systems are rapidly colonized by microbial biofilms. The presence of microbial biomass may cause sinking of particles and as a consequence prevent their transport to the oceans. We studied microbial colonization of different polymer particles exposed in the epi-, meta- and hypolimnion of a freshwater reservoir during late summer for 47 days. Parameters measured included biofilm formation, metal sorption and sinking velocities. Microbial biofilms contained bacteria, cyanobacteria and algae as well as inorganic particles such as iron oxides. Regardless of biofilm thickness and biovolumes of different biofilm constituents, single polyethylene (PE) particles stayed buoyant, whereas the sinking velocity of single polystyrene (PS) and polyethylene terephthalate (PET) particles did not change significantly compared to initial values. During exposition, a mixing event occurred, by which anoxic, iron-rich water from the hypolimnion was mixed with water from upper layers. This induced aggregation and sinking of hypolimnetic PE particles together with organic matter, cyanobacteria colonies and iron minerals.

Keywords:

microplastics, reservoirs, biofouling, aggregation, microscopy, sinking

47 1. Introduction

48 Microplastics (MP) are one of the widely distributed man-made pollutants, found in nearly
49 any place of the earth. Zones with reduced flow velocities such as point bars, impoundments
50 and reservoirs allow particles to settle down to the sediment (Watkins et al., 2019), reducing
51 the plastic load of streams (Castañeda et al., 2014). As a consequence, high concentrations
52 of MP are found in sediments of natural lakes (Anderson et al., 2017) and man-made
53 reservoirs (Zhang et al., 2015), which may even act as permanent sink (Corcoran et al.,
54 2015). Elucidating the factors governing particle settling in zones with reduced flow, such as
55 reservoirs, is therefore crucial for a comprehensive understanding of MP transport in the
56 environment.

57 Microplastic particles are rapidly colonized by various microorganisms like bacteria,
58 cyanobacteria and microalgae when submerged in freshwater. This leads to the development
59 of a biofilm comprising microbial communities distinct from the surrounding water (Zettler et
60 al., 2013). Environmental conditions such as pH, temperature, light/oxygen availability or
61 dissolved nutrients mainly determine which organisms may occur within a biofilm; whereas
62 the surface properties seem to be less influential than these external factors (McCormick et
63 al., 2014; Oberbeckmann et al., 2016). However, if incubated under the same environmental
64 conditions, differences between microbial community composition and biofilm density can be
65 observed among different surfaces (Parrish and Fahrenfeld, 2019).

66 The settling of particles is influenced by their shape, size and density (Chubarenko et al.,
67 2016; Kowalski et al., 2016). Particles denser than water and above 5 µm in diameter will
68 eventually settle down by gravitational force within a few meters after being submerged in
69 water. Particles less dense than water or below 5 µm in size may stay buoyant or even float
70 at the surface if not altered by environmental processes (Besseling et al., 2017). For further
71 transport, aggregation with natural materials and colonization by organisms play a crucial
72 role by increasing the size and density of these particles. In oceans, the aggregation of
73 buoyant MP with algae (Long et al., 2015) and marine snow (Michels et al., 2018) leads to

74 rapid settling. Coverage of larger particles with calcareous macroorganisms, which is often
75 referred to as biofouling, also sinks buoyant plastic polymers (Kaiser et al., 2017).

76 Conditions in freshwater differ from the marine environment with regard to physical
77 (currents/waves, tides, wind, temperature), chemical (pH, salinity, oxygen availability,
78 nutrients/metals) and biological parameters. Additionally, the residence time in freshwater
79 reservoirs is by magnitudes lower than in the ocean, leading to the assumption that extensive
80 aging and biofouling by heavy calcareous organisms is unlikely to take place. Transfer of
81 buoyant MP to reservoir sediments may therefore rely rather on mechanisms distinct from
82 those observed in marine environments (Besseling et al., 2017). During summer, many
83 reservoirs become stratified and partly anoxic, leading to the reduction of iron oxides to
84 ferrous iron. Lake mixing leads to the autoxidation of ferrous iron and formation of iron oxide
85 colloids in the water column (Tipping et al., 1981). These colloids are known to sink together
86 with algae and cyanobacteria by forming sticky agglomerates with the cells (Oliver et al.,
87 1985).

88 We hypothesize that this aggregation may also be relevant for MP during mixing. Stratified
89 reservoirs, which exhibit gradients of redox potential and oxygen availability, may therefore
90 provide an ideal setting to study the factors governing biofilm development and plastic
91 sedimentation which are presently not well understood. We characterized biofilm formation
92 on PE, PET and PS in the stratified mesotrophic Malter reservoir during late summer. Other
93 factors such as aggregation with freshwater algae and cyanobacteria or sorption of metals
94 were considered as well. The following hypotheses were tested: i) incubation depth and time
95 influence biofilm composition; ii) biofilm covered MP sorb metal oxides; iii) aggregation or
96 biofouling influence MP settling in stratified reservoirs.

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100 2. Materials and methods

101 2.1 Location

102 The mesotrophic Malter reservoir (0.84 km^2, 335 m above sea level) is located in eastern
103 Germany (Dippoldiswalde, Saxony). Its maximum depth is 16-20 m and the maximum
104 storage capacity is 9.6 million m^3. Due to continuous discharge during summer, the water
105 level may drop by several meters. In summer the water column is stratified with an anoxic
106 hypolimnion separated from the oxic epilimnion by a thermocline at 8 m depth in 2018
107 (Figure S1 and Table S1). Autumnal mixing usually occurs during September restoring oxic
108 conditions near the bottom (Müller et al., 2000).

109 2.2 Plastic material and incubation conditions

110 Three different polymer foils in research quality and free of stabilizers, PE (ρ: 0.924 g cm^{-3},
111 contact angle: 99.2 ± 4.1°, ET311251), PS (ρ: 1.05 g cm^{-3}, contact angle: 91.7 ± 4.6°,
112 ST311125), and PET (ρ: 1.4 g cm^{-3}, contact angle: 84.3 ± 3.4°, ES301425) were purchased
113 from GoodFellow (Hamburg, Germany). Foil thicknesses were 0.125 mm for PS/PET and
114 0.15 mm for PE. Polymer squares (4 x 4 mm) were obtained employing a multiple puncher
115 (Pavo HD Wire Binder), using sterile techniques (ethanol-sterilized puncher and lab cloths,
116 laminar flow bench). PS particles exhibited a curved shape, whereas PE and PET particles
117 were even. The cutting edges of the particles were rougher compared to the uncut foil. The
118 squares and foils were stored at 20° C in a dry, dark place.

119 In order to incubate the particles in Malter reservoir, 500 particles of each polymer (PE, PS,
120 PET) were transferred to individual closed stainless steel cages (cylindrical shape, diameter
121 10 cm, length 25 cm, mesh width 3 mm) each containing just a single polymer type (Arias-
122 Andres et al., 2018). Particles were distributed to the cages as follows: 3 x 500 particles
123 (three individual cages containing PE, PET or PS) in the epilimnion, 3 x 500 in the
124 metalimnion and 9 x 500 (in 3 x 3 cages with PE, PET or PS) in the hypolimnion (Figure S2).
125 Cages were incubated from August 30 to October 16, 2018, with three samplings after 6

126 days (09/05), 22 days (09/21) and 47 days (10/16). Three incubation depths at 0.5 m
127 (epilimnion, O_2 saturation > 100 %, light), 8 m (metalimnion, O_2 saturation 80 %, no light) and
128 16 m (hypolimnion, O_2 saturation 0 %, no light) were chosen according to prevailing oxygen
129 concentrations (multi-parameter probe, Sea & Sun Technologies, Germany) and light
130 intensities (Licor 1400, Li-cor Biosciences, Germany) on August 30 (Figure S1a). Cages
131 were lifted to the surface for sampling and particles were gently transferred using tweezers or
132 flushing with reservoir water to pre-combusted (450° C, 4 h) glass Petri dishes filled with
133 water from the incubation depth. The hypolimnion was anoxic during the initial stage of the
134 experiment (Figure S1). To avoid repeated oxygen exposure of anaerobic organisms during
135 lifting of the cages for sampling, additional cages on separate ropes were deployed in the
136 hypolimnion so that for each sampling previously un-sampled cages were used. Some PS
137 particles were flushed out of the cages, leading to missing values of crystal violet staining in
138 the epilimnion and metalimnion for day 47. All samples were stored in the dark at 4° C until
139 processing in the laboratory. Samples for confocal laser scanning microscopy (CLSM) were
140 preserved in 4 % formalin solution, and particles for iron/manganese measurement were
141 directly placed in glass vials with hydroxyl ammonium chloride-hydrochloric acid (0.5 M / 1
142 M). Particles used to determine the sinking velocity and for conducting crystal violet staining
143 were transported in reservoir water.

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145 2.3 CLSM imaging

146 At every sampling date 10 randomly chosen particles per polymer were taken from each
147 incubation depth, and 5 random locations on each of them were examined via CLSM.
148 Samples were washed in tap water and mounted in a 5 cm Petri dish. For this purpose the
149 plastic squares were glued with silicone adhesive to the bottom of the dish. Nucleic acid
150 staining was done with SybrGreen (dilution 1:1000). After staining for 5 min the Petri dish
151 was flooded with tap water. For CLSM a TCS SP5X with upright microscope and super
152 continuum light source was available. The system was controlled by LAS AF version 2.4.1.

153 Samples were examined by using a long working distance 63x NA 0.9 water immersible
154 objective lens. Excitation was at 490, 561 and 633 nm. Emission was recorded sequentially
155 from 480-500 nm (reflection), 510-580 nm (SybrGreen) together with 650-720 nm
156 (autofluorescence of chlorophyll *a*) and separately from 575-650 nm (autofluorescence of
157 phycobilins). Datasets were recorded without average and a step size of 1 µm. For
158 visualisation Imaris (Bitplane) version 9.3 was employed. Projected image data sets were
159 printed from photoshop (Adobe). Calculations of cell biovolumes contained in the digital
160 images were done with an adapted version of ImageJ (Staudt et al., 2004). Extracellular
161 polymeric substances (EPS) were visualized by the glycoconjugate binding lectin AAL-A568
162 (Vector Laboratories, Burlingame, USA).

163

164 2.4 Iron and manganese analysis

165 Iron and manganese were determined by extracting 15 pooled particles per sampling date,
166 depth, and polymer type with 2 ml hydroxyl ammonium chloride-hydrochloric acid solution
167 (0.5 / 1 M) for 24 h in an overhead shaker (120 rpm). Iron was subsequently measured by
168 ferrozine assay (Stookey, 1970) at 562 nm using a spectrophotometer (Agilent Cary 60 UV-
169 VIS). Briefly, 50 µl of acidic, centrifuged sample (10 min, 15000 rpm) was added to 950 µl
170 ferrozine solution (50 mM HEPES, 1 mg ml^{-1} ferrozine, pH 7.0) and incubated for 10 min prior
171 to the measurement. If optical density exceeded 1.0 the remaining sample was diluted with
172 acidified ultra-pure H_2O prior to the addition of ferrozine. Manganese was measured using
173 the formaldoxime method adapted from Burlage et al. (1998), Goto et al. (1962) and Brewer
174 and Spencer (1971). The following solutions were prepared: (1) TRIS-Buffer (5 M TRIS-HCl,
175 pH 9.0 adjusted with H_2SO_4), (2) formaldoxime (20 g hydroxyl ammonium chloride in 450 ml
176 ultrapure H_2O, addition of 10 ml 10 % formaldehyde, filled up to 500 ml), (3) 0.1 M EDTA in
177 water and (4) 10 % hydroxyl ammonium chloride in water. One ml of TRIS solution was
178 added to an Eppendorf tube followed by addition of 100 µl supernatant from the centrifuged
179 sample. Then 100 µl of formaldoxime was added, followed by 2 min of incubation at room

180 temperature. To remove dissolved ferrous iron, 100 µl EDTA and 200 µl hydroxyl ammonium
181 chloride solution were added separately, subsequently the solution was incubated at room
182 temperature for 10 min and measured at 450 nm (Agilent Cary 60 UV-VIS).

183 2.5 Crystal violet staining

184 Crystal violet staining was conducted to quantify total biofilm mass (Arias-Andres et al.,
185 2018), as the dye stains both cells and the biofilm EPS (Xu et al., 2016). On each sampling
186 date 8 particles from each depth and of every polymer type were stained. During the
187 procedure some particles were lost leading to lower sample numbers (Figure 2). Briefly,
188 particles were dried (60° C, 24 h), stained with 250 µl crystal violet (0.3 % in ultrapure water)
189 for 15 min, washed 4 times with ultrapure water and de-stained with 200 µl ethanol (97 %).
190 The ethanol-crystal violet solution was measured at 595 nm (OD_{595}) using a multiplate reader
191 (Thermo Fisher Multiscan RC). Samples showing optical densities higher than 1.00 were
192 diluted with 97 % ethanol.

193 2.6 Measurement of sinking velocities

194 Sinking velocities were determined within 12 h after sampling. A sinking column (0.15 m x 2
195 m) filled with deionized water and placed in a climate chamber (20° C) was used. The water
196 was filled 2-3 days in advance to avoid currents and temperature differences in the column.
197 The time needed to settle 50 cm through the column was measured using a stop watch
198 (Kaiser et al., 2017). Particles were placed carefully beneath the water surface using
199 tweezers and then allowed to settle for 30 cm in order to reach their terminal velocity before
200 measurement was started. Particles settling close to the tube walls were excluded from data
201 analyses. Multicellular organisms attached to sinking polymers were identified via binocular
202 and a field guide (Streble and Krauter, 1988) to the family level.

203 2.7 Analysis of iron colloids

204 Iron colloids resulting from reservoir mixing were obtained by centrifuging two water samples
205 retrieved on September 21 (10 min, 15000 rpm). The resulting pellets were dried (60° C for

206 24 h) and iron and manganese were analysed according to methods described in 2.4. The
207 remaining solids were washed three times with acidified water (pH 1.8) and centrifuged/dried
208 again (60° C, 24 h) to determine the acid soluble fraction. Afterwards the ash content was
209 determined by combusting the sample at 450° C for 24 h. One aggregate formed by PE and
210 iron colloids was treated similarly and analysed for its iron and manganese content.

211 2.8 Data analysis

212 The critical thickness and mass of an attached fouling film in order to overcome a PE
213 squares buoyancy was calculated following Chubarenko et al., 2016:

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214 $d_{film} = \frac{h}{2} * \frac{\rho_w - \rho_0}{\rho_f - \rho_w}$ Equation 1

216 $m_f = a^2 * d * \rho_f$ Equation 2

217 with ρ_0: Density of unfouled PE particle (920 kg m^{-3}); ρ_f: Density of the fouling film (different
218 densities, Table S2); ρ_w: Density of water (1000 kg m^{-3}); h : Height of the particle (1.5 x 10^{-4}
219 m); d_{film}: Thickness of the fouling film; m_f: Mass of fouling film needed to sink PE; a: Length
220 of LDPE particle (4 x 10^{-3} m).

221 Results were compared among sampling dates and depths using non-parametric
222 bootstrapping (Efron and Tibshirani, 1986) because assumptions of the ANOVA were not
223 satisfied. For each comparison, we reported the median difference of
224 10000 bootstrapped samples with 95% confidence intervals. Differences in medians
225 containing values other than zero were regarded as significantly different from each other by
226 95 % chance. Software R (R Core Team, 2018) was used for all statistical analysis.

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230 3. Results

231 3.1 Biovolumes analyzed by CLSM

232 Biovolumes of three phylogenetic groups (bacteria, cyanobacteria and algae) were analyzed
233 for three different reservoir depths (epilimnion, metalimnion and hypolimnion) and time
234 intervals (day 6, day 22 and day 47). Cages from the epilimnion were covered with visible
235 biofilms after 47 days, whereas no visible biofilm formation was observed on cages from the
236 metalimnion and hypolimnion.

237 The cellular components of the biofilms on day 6 consisted predominantly of bacteria (Figure
238 1). PET had fewer bacteria compared to PS/PE in the metalimnion and to PE in the
239 epilimnion, while PS had significantly more bacteria than PE and PET in the hypolimnion.
240 Cyanobacteria and algae were scarce throughout, nonetheless small but statically significant
241 differences among the polymers were found (Figure 1b, c).

242 On day 22 (Figure 1) biofilms were again dominated by bacteria. PE showed the highest
243 bacterial biovolume of all polymers in each incubation depth (Figure 1a). PS had higher
244 values than PET in the epilimnion and metalimnion, but lower bacterial biovolumes in the
245 hypolimnion compared to PET. Cyanobacteria biovolumes were again negligible. Some
246 algae were found on PS exceeding PE and especially PET in the epi- and metalimnion. In
247 the hypolimnion PE had remarkably higher algae biovolumes than PET and PS.

248 Biovolumes on day 47 were distinct from the previous sampling dates, and characterized by
249 higher shares of phototrophic organisms in the epilimnion. Bacterial biovolume showed less
250 pronounced differences between the surfaces than before. Cyanobacteria in the epilimnion
251 made up a significant proportion of the phototrophic biovolume. Comparing the surfaces,
252 PET and PS showed the highest volumes, while PE only reached around 5 % of the
253 cyanobacteria biovolume found on PET or PS. In the metalimnion cyanobacteria were scarce
254 throughout. In the hypolimnion cyanobacteria biovolume was low but significantly different
255 between surfaces (Figure 1c). Substantial algae biovolumes were found on all polymers in

256 the epilimnion with PET/PS significantly exceeding PE. Few algae occurred in the
257 metalimnion and hypolimnion although some significant differences were found (Figure 1b).

258 The biovolumes of bacteria, cyanobacteria and algae increased over time on all polymers in
259 each of the incubation depths. Remarkably, algal biovolumes increased by at least one
260 order of magnitude between day 22 and day 47 in the epilimnion. No general differences
261 regarding the final bacterial biovolumes were found at day 47. However, PE tended to have
262 lower biovolumes of phototrophic organisms than the other surfaces.

263 3.2 Biofilms analysed by crystal violet staining

264 On day 6 the biofilm was not well established, with OD_{595} values being low for all polymers
265 (Figure 2), but still significantly higher than the pristine particles used as blanks. Differences
266 between the polymers were scarce with only PET exhibiting lower OD_{595} values than PE in all
267 samples on day 22 and day 47. Biofilm mass on PS on day 22 was equal to that on PE in the
268 epilimnion and metalimnion but lower than on PE in the hypolimnion. PS values were equal
269 to PET values on day 22 except for the epilimnion.OD_{595} was highest at day 47 for all
270 sampled polymers, which indicates an increase of stainable molecules over the incubation
271 time.

272 The OD_{595} values showed a positive linear correlation with their respective total biovolumes
273 (sum of algae, bacteria and cyanobacteria) derived from CLSM imaging. The increase of
274 OD_{595} per biovolume was higher for PE than for PET, as expressed by a steeper regression
275 line. Therefore higher OD_{595} values would result from similar or lower biovolume on PE
276 compared to PET (Figure S3, Table S3).

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281 3.3 Metal concentrations on MP

282 Iron and manganese (detection limit 0.3 µg mg^{-1}) were found on all tested biofilm-covered
283 MP. On day 6 and 22 no manganese was detected, but after 47 days manganese was found
284 on every polymer in most incubation depths. PE showed the highest manganese sorption,
285 exceeding 0.63 µg mg^{-1} in the epilimnion.

286 In contrast, iron was detected from day 6 on at all polymers. On day 22 substantial iron
287 sorption was observed in the hypolimnion (Figure 3). The highest iron concentration occurred
288 on PE on day 47 in the epilimnion (1.64 µg mg^{-1}).

289 At least for PE metal concentrations increased substantially over the incubation time.
290 Sorption of iron exceeded that of manganese on all polymers. As the data display an
291 integrated mean of 15 particles per measurement no statistical analysis was conducted.

292 3.4 Sinking velocity of particles

293 During the experiment no sinking of single PE particles regardless of incubation depth or
294 time occurred and so no sinking velocities were recorded.

295 The terminal sinking velocities of PET squares were roughly three times higher than those of
296 PS, with values of 0.028 m s^{-1} compared to 0.0075 m s^{-1} respectively (Figure 4). No
297 substantial differences between incubated particles and the control blank particles of PET
298 and PS were observed (Table S4). Small differences between some incubation depths or
299 sampling dates were occurring (Table S4). On day 47, larger organisms such as hydras and
300 cladocerans were attached to the surface of 9 % of the PS particles and 10 % of the PET
301 particles used in the sinking experiment. These organisms influenced the sinking speed of
302 PS slightly by accelerating the sinking speed by 4 % (median of particles with attached
303 multicellular organisms: 0.008 m/s, n: 4) and decreased the sinking speed of PET by 1 %
304 (median: 0.0276, n: 6) compared to particles with no attached larger organisms. On day 6
305 and day 22 no such organisms were observed on the particles.

3.5 Mixing event and aggregation of MP with iron colloids

Oxygen intruded into the anoxic hypolimnion of the stratified Malter reservoir between September 5 (day 6) and September 21 (day 22) (Figure S1b, c). Substantial amounts of brownish-red iron flocs (Figure 5a) floated freely in the whole water column on September 21. These reservoir-borne iron colloids consisted of approximately 12 % per weight iron, 40 % per weight organics, and 38 % per weight ash content referring to the dry mass. The acid soluble fraction was 26 % per weight which corresponds well to the mass of 27 % Fe_2O_3 x 0.5 H_2O stoichiometrically calculated from the measured iron content. The colloids were not observed at day 47 (October 16). Aggregation of four PE particles with iron colloids was observed in the hypolimnion (Figure 5a, b). The formed aggregate was stable enough for being transferred by tweezers without breaking apart. Its density was higher than water; subsequently it sank down to the bottom of a water filled storage container. Non-agglomerated PE particles retrieved from the hypolimnion on that day remained buoyant. The biofilm on the aggregate showed higher biovolumes of bacteria, algae and cyanobacteria compared to any other surface (Figure 5c). The agglomerate was of brownish color, indicating the presence of iron which made up approximately 3 % Fe (11 µg mg^{-1}) of the total dry mass. Small amounts of manganese reaching < 1 % (0.07 µg mg^{-1}) of the total dry mass were measured as well.

331 4. Discussion

332 In this study we measured biofilm formation and sorption of metals to different polymers in
333 MP size in order to investigate the effects on their sinking behavior. Employing CLSM, crystal
334 violet staining and spectrophotometric methods, we found microbial biofilm formation to be
335 not sufficient for promoting the sinking of single MP particles in Malter reservoir during late
336 summer. Sinking through aggregation with iron colloids and biomass (cells/EPS) was
337 observed after reservoir mixing in a single case.

338 Contrary to our findings, biofouling with cyanobacteria was found to sink polypropylene
339 particles in a eutrophic tropical lake (Chen et al., 2019). The authors hypothesized that the
340 binding of inorganic material contributed more significantly to the mass of the fouling film
341 than the phototrophic cells (Chen et al., 2019). Differences to our results can be explained by
342 distinct environmental conditions in Malter reservoir such as lower concentrations of
343 chlorophyll *a* (41 µg/l vs. 120 µg l^{-1}), phosphate (0.03 vs. 0.214 mg l^{-1}), ammonia (0.07 vs.
344 0.41 mg l^{-1}), and suspended solids (4 vs. 35 mg l^{-1}). Regarding the different conditions,
345 biofouling will be more intense in warm, nutrient-rich, shallow lakes compared to temperate,
346 nutrient-poor, deep reservoirs. The absence of calcareous macrofoulers which is considered
347 as a major factor promoting sinking of MP in marine enviroments (Kaiser et al. 2017) may
348 also explain low impact of biofouling on the particle densities in this study.

349 However it cannot be excluded that more extensive biofouling occurs during different
350 seasons and may facilitate MP sinking in Malter reservoir. Biofilm formation may strongly
351 influence aggregation dynamics and subsequently the sinking of MP by increasing the
352 stickiness, surface charge or altering the morphology (e.g. lobes, filaments) of the particles.
353 Aggregation with inorganic and organic particles is considered as another important process
354 that determines the environmental fate of MP (Besseling et al., 2017). Sticky organic material
355 such as marine snow (Porter et al., 2018), biogenic particles (Michels et al., 2018), EPS
356 (Summers et al., 2018), organo-mineral particulate matter (Möhlenkamp et al., 2018) and
357 marine (Long et al., 2015) or freshwater microalgae (Lagarde et al., 2016) can sink buoyant

358 MP through formation of large hetero-aggregates. Most studies used laboratory set-ups
359 employing conditions favoring aggregation such as low shear stress and high particle
360 concentrations (10^1-10^4 particles ml^{-1}) (Möhlenkamp et al., 2018) which may not reflect
361 natural conditions. Currents, grazing, microbial degradation and ingestion by larger
362 organisms could lead to rapid break-up or consumption of such aggregates in nature (Cole et
363 al., 2016). In this study aggregation of PE with organo-mineral matter was observed. The
364 formation right after mixing indicates that iron oxide containing colloids induced the
365 aggregation of PE with organic matter and cells of cyanobacteria, algae and bacteria. Iron
366 colloid formation is an important process capable to aggregate and sink buoyant
367 cyanobacteria in natural lakes after mixing (Oliver et al., 1985). Given that only 4 large
368 microplastic particles aggregated with such colloids; this study cannot provide sufficient data
369 on the importance and implications of this mechanisms for the fate of MP in reservoirs.
370 Furthermore it should be considered that reservoir mixing is no prerequisite for the
371 aggregation and subsequent settling of MP in the environment.

372

373 Considering the oxic conditions of the hypolimnion prevailing at least since day 22, the
374 majority of iron and manganese should have been present as metal oxides. Oxide minerals
375 such as ferrihydrite ($Fe_5HO_8 \times 4 H_2O$), magnetite (Fe_3O_4) or manganese (IV) oxide (MnO_2)
376 exhibit a high specific density and may influence the density of MP particles. The density
377 change of PE particles (4 x 4 x 0.15 mm) covered by a “fouling film” was calculated by using
378 the specific density of these minerals as input variable (Chubarenko et al., 2016). Afterwards
379 the mass of fouling films comprising ferrihydrite, magnetite, manganese oxide or bacteria
380 needed to sink the PE particle ($\rho_{PE} > \rho_{Water}$) were determined following equation 2.
381 Accordingly, the mass concentrations needed to sink PE are 54 µg mg^{-1} magnetite, 58 µg
382 mg^{-1} ferrihydrite, 55 µg mg^{-1} manganese oxide and 131, 156 or 218 µg mg^{-1} bacteria (with
383 different $\rho_{Bacteria}$, Table S2). The maximum iron concentration found on buoyant PE (1.64 µg
384 mg^{-1}) corresponded to 2.2 µg mg^{-1} ferrihydrite or 2.1 µg mg^{-1} magnetite whereas the

385 maximum manganese concentration (0.64 µg mg^{-1}) corresponded to 0.94 µg mg^{-1}
386 manganese oxide. Therefore, the mass concentration of metals bound to freely floating PE
387 particles was at least one order of magnitude too low to overcome their buoyancy. The
388 sinking aggregated PE particles showed iron concentrations of 11 µg mg^{-1} corresponding to
389 16.4 µg mg^{-1} ferrihydrite or 15.2 µg mg^{-1} magnetite. Biovolume (in $\mu m^3\,\mu m^{-2}$) of bacteria,
390 cyanobacteria and algae can be converted to cells mass per particle by multiplying with the
391 particle surface area ($1.6 \times 10^7\ \mu m^2$) and the specific density of microbial biomass (Table
392 S2). Buoyant PE particles contained approximately 8 µg mg^{-1} total biomass (for $\rho_{Biomass}$: 1500
393 kg m^{-3}) while the microbial biomass on the sunken PE agglomerate made up 149, 138 or 124
394 µg mg^{-1} depending on the specific density of microbial biomass applied. The biomass
395 calculated for the sunken agglomerated PE was in good agreement with the 131 µg mg^{-1} (for
396 $\rho_{Biomass}$: 1500 kg m^{-3}) (Chubarenko et al., 2018) theoretically needed to sink buoyant PE
397 particles. Assuming lower $\rho_{Bacteria}$ the results deviated slightly (156 µg mg^{-1} for $\rho_{Biomass}$: 1388
398 kg m^{-3}) (Besseling et al., 2017) or moderately (218 µg mg^{-1} for $\rho_{Biomass}$: 1250 kg m^{-3}) (Kooi et
399 al., 2017) from the biomass theoretically required for sinking. However it should be
400 considered that most of the aggregate organic mass was not quantified via CLSM as EPS
401 was not assessed with the used technique. Nonetheless ballasting effects of microorganisms
402 considerably exceeded the effect of metal oxides, comprising a likely reason for the observed
403 loss in buoyancy. This leads to the assumption that the aggregation of freshwater
404 microorganisms can sink MP if sufficient biomass is provided. However, under low bio-
405 productivity or low temperature conditions the critical mass to sink the MP may not be
406 reached within reasonable timescales.

407 The extent of EPS production within the biofilms seemed to differ between the polymers, as
408 shown via crystal violet assay. Algae and cyanobacteria cells were present in lower densities
409 on PE compared to PET and PS as seen from CLSM imaging. This may indicate that these
410 organisms had to put more effort into attachment to PE surfaces than to PET/PS. Low
411 attachment efficiency leads to environmental stress (Vosshage et al., 2018) and ultimately to

412 more EPS production by the stressed cells (Scott et al., 2014). Therefore the fewer but
413 stressed cells on PE could have produced more EPS than the cells on PET.

414 Regarding the occurrence of metals on biofilm covered MP, PE showed higher
415 concentrations of iron and manganese compared to the other polymers. Concentrations of
416 Fe and Mn exceeded up to 100 times (Fe) or 10 times (Mn) the concentrations found on
417 beached plastic pellets at British shores (Ashton et al., 2010; Holmes et al., 2012) and
418 plastics exposed to seawater for several months (Rochman et al., 2014). Lower ionic
419 strength and the higher abundance of Fe / Mn in freshwater water may explain the higher MP
420 metal concentrations found in this study. According to previous studies long-term metal
421 sorption to plastics does not differ between polymer types (Rochman et al., 2014). The metal
422 sorption is rather controlled by the biofilm thickness and the available binding places therein
423 (Rochman et al., 2014; van Hullebusch et al., 2003). In our study, PE showed the highest
424 crystal violet stainable biomass of all polymers. PE may therefore have provided more
425 binding places for metals within the EPS matrix, leading to the highest measured iron and
426 manganese concentrations.

427 The polymers were enclosed in steel cages during the experiment. For PS and PET this
428 produces artificial conditions, as particles of the used size will settle down to the sediment
429 within 10 (PET) to 30 minutes (PS) assuming a mean water depth of 16 m and no other
430 currents or mixing. However, polymers with mechanically changed properties such as PET
431 bottles with trapped air inside or expanded PS may stay afloat for a longer time-span than
432 the particles used in this experiment. This makes the description of biofilm formation on PS
433 and PET in the upper reservoir parts environmentally relevant even though the particles are
434 not buoyant. Conditions for biofilm formation may differ between the interior of the cages and
435 the open reservoir water, as larger grazing organisms were excluded by the mesh size.
436 Furthermore the cages had a shading effect reducing the light intensity by 33 %. Due to
437 biofilm formation on the cages this shading effect could have been even stronger than the 33
438 % measured for blank cages. Reservoirs and especially their hypolimnia experience very

low currents. For this reason, biofilms are only loosely bound to their carriers, making biofilm loss due to shear stress likely. This may partly explain the high variability of biovolumes on particles of the same material and exposure time. Formation of hetero-aggregates between PE and organo-mineral matter was only observed in the hypolimnion. Therefore, the possible sinking of PE floating at the water surface could not be proven directly. Only one aggregate with four PE particles has been observed hence coincidence cannot be excluded. The experiment was conducted from late summer to autumn which includes lowering of temperatures and light intensities during this time. As the extent of biofouling depends on season (Chen et al., 2019), it cannot be excluded that biofouling-induced sinking may occur during other times of the year.

5. Conclusions

- Late summer biofilm development within a temperate mesotrophic reservoir was not sufficient to facilitate sinking of buoyant MP or increasing the settling velocity of dense MP

- Biofilms grown on PET and PS microplastics in the mesotrophic reservoir contained more phototrophic microorganisms than those on PE microplastics

- Manganese and iron sorbed to biofilm covered microplastics in substantial amounts

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