

## LETTER

**Microbial iron reduction does not release microplastics from organo-metallic aggregates**Rico Leiser  \* Katrin Wendt-Potthoff 

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**Scientific Significance Statement**

Sediments of freshwater lakes and reservoirs are major sinks for microplastics. This is partly caused by sinking aggregates, consisting of iron and organics formed during the stratification of the water body. These aggregates can enclose and transport initially buoyant microplastics down to the sediments. The long-term deposition of microplastics in the sediments is expected to be affected by the stability of the aggregates against degradation and reductive dissolution once reaching the profundal. This study shows that such microplastic-bearing iron-organo aggregates are resistant to degradation by sediment-dwelling microorganisms. Hence, microplastics are retained in the aggregates, which is a prerequisite for stable deposition in freshwater sediments.

**Abstract**

Iron flocculants play a major role in the remediation of water bodies, removing particulate pollutants such as microplastics through floc formation. Such flocs are prone to microbial iron reduction while lying on top of anoxic sediments, which possibly leads the release of bound microplastics. In this study, *Shewanella oneidensis* was employed to simulate the impact of microbial iron reduction on the release of polyethylene spheres from sunken flocs in 120 d batch experiments. Most of the flocs iron (oxyhydr)oxides were reduced (70–90%), but this did not affect their integrity. Only a negligible proportion (0.2–2.7%) of polyethylene spheres was released, while the majority remained bound inside the floc matrix. This study exemplifies that flocs are quite stable, even when experiencing microbial iron reduction under anoxic conditions. Thereby incorporation into such aggregates may display a potential mode of long-term microplastics storage in freshwater sediments.

Iron-based flocculants naturally occurring in freshwaters (Oliver et al. 1985) or employed in water treatment (Deppe and Benndorf 2002) are known to effectively bind dissolved and particulate pollutants. They form large flocs comprised of

precipitated iron (oxyhydr)oxides and organics collected from the surrounding water (henceforth iron-organo flocs), which settle in aqueous media through gravitational force. By this, iron-organo flocs may transport initially buoyant particulate

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matter such as cyanobacterial colonies or the emerging pollutants microplastics (plastic particles < 5 mm) from the water phase into the sediments (Oliver et al. 1985).

Microplastics are ubiquitously detected in the sediments of lakes and freshwater reservoirs (Di and Wang 2018), with low-density polymer types ( $\rho < 1.0 \text{ g cm}^{-3}$ ) such as polyethylene (PE) usually comprising the highest proportion of detected particles (Lin et al. 2021). Ongoing research efforts elucidated mechanisms governing transport of initially buoyant microplastics to the profundal zone of standing freshwater bodies. Biofilm formation with associated mineral entrapment (Leiser et al. 2021a) and aggregation with natural particles such as organics or iron-organic flocs (Leiser et al. 2020) have been recognized as important processes leading to sinking of PE microplastics in freshwater bodies. However, the fate of microplastic particles once they reach the sediments is not well understood (Rogers et al. 2020).

Iron-organic flocs are a relevant aggregate type in freshwater bodies, forming during or after summer stratification (Mortimer 1942). Many freshwater lakes and reservoirs are stratified over summer, with an oxycline separating the oxic epilimnion from the anoxic hypolimnion (Mortimer 1942). The oxycline is a region with sharp oxygen and density gradients, at which intense iron cycling takes place (Bravidor et al. 2015). Ferrous iron diffuses upward from the hypolimnion into the epilimnion and precipitates in the form of positively charged iron (oxyhydr)oxides (Ma et al. 2019) right above the oxycline (Bravidor et al. 2015). These minerals aggregate with dissolved and particulate net-negatively charged organic material, forming large (60–370  $\mu\text{m}$ ), sinking aggregates (Reiche et al. 2011; Elliott et al. 2014). These iron-organo flocs sink downward from the oxycline back into the hypolimnion and subsequently to the sediments. They were shown to aggregate cyanobacterial colonies (Oliver et al. 1985) and negatively charged PE particles (Ma et al. 2019; Leiser et al. 2020), by this facilitating their transport into the sediments of standing freshwater bodies.

Once reaching the sediments, iron-organo flocs will experience anoxic conditions as long as the overlying water column remains stratified and anoxic. Given their high Fe oxide content (35–50%), iron-organo flocs are prone to iron reduction (Reiche et al. 2011; Leiser et al. 2021b), mediated by specialized sediment-dwelling bacteria such as freshwater *Shewanella* spp. and *Geobacter* spp. (Kappler et al. 2021). Microbial reduction of iron oxides has been attributed to the release of nutrients (Mortimer 1942), pollutants (Revesz et al. 2016), and organic matter (Patzner et al. 2020), initially bound to or within the mineral matrix. Whether this also leads to the disintegration of flocs and subsequently to the release of incorporated PE microplastics, was investigated in laboratory batch experiments employing the model organism *Shewanella oneidensis*. Furthermore, the role of iron (oxyhydr)oxides in stabilizing the floc aggregates was studied using acid treatment and microscopic techniques.

## Materials and methods

### Floc preparation and microplastics

PE spheres (d:  $118 \pm 6 \mu\text{m}$ ,  $\rho$ :  $0.98 \text{ g cm}^{-3}$ ) spiked with fluorescent red Rhodamine B (RHBPMs-0.98 106–125  $\mu\text{m}$ ) were bought from Cospheric. Surface water from eutrophic Bautzen reservoir (Saxony, Germany) was retrieved on 4<sup>th</sup> of September 2020, filtered (10  $\mu\text{m}$ ), and stored in the dark until use. Iron-organo flocs were formed by addition of 300  $\mu\text{L}$   $\text{FeSO}_4$  (500 mM, pH 1.8) and 10 mg PE spheres ( $2.4 \times 10^4$  particles  $\text{L}^{-1}$ ) to 500 mL surface water (pH 9.5) followed by incubation on a tumbling roller incubator (3–4 rpm, 24 h, RM5, M. Zipperer GmbH). To test the effect of iron (oxyhydr)oxides removal on floc stability, several flocs were immersed in 1 M HCl (24 h, room temperature) until visible decolorization of the flocs. Flocs were removed from the acid using inverted Pasteur pipettes, transferred into water filled tubes, followed by vigorous shaking on a vortexer until disintegration. The resulting suspension was filtered over stainless steel sieves (10  $\mu\text{m}$ ) and retained microplastic spheres were counted under a light microscope (Zeiss Axioplan). The 1 M HCl solution was also filtered and checked for its plastic content to count spheres released through acid treatment of the flocs.

### Microcosm experiment

The potential to release microplastics from iron-organo flocs via microbial iron reduction was tested using pure cultures of bacteria *S. oneidensis* LMG19005 (BCCM, Belgium). *S. oneidensis* was grown under oxic conditions for 16 h until late log phase ( $\text{OD}_{600}$ : 0.8852, cell concentration:  $3.54 \times 10^{10} \pm 1 \times 10^{10}$ ,  $n$ : 10) in soy broth media (Medium 14, BCCM, Belgium) at 28°C (Xiao et al. 2018). Cells were centrifuged (3 $\times$ , 1500 rpm, 20 min) and washed with mineral media for iron-reducing *Shewanella* spp. (Burlage et al. 1998) to remove the soy broth media. Anoxic microcosms ( $n$ : 20) containing mineral media (V: 20 mL), five iron-organo flocs with PE (3 $\times$  washed with tap water) and Na-lactate (10 mM), were inoculated with 5 mL of washed *S. oneidensis* cultures (cell concentration in microcosm:  $\sim 7 \times 10^9$  cells  $\text{mL}^{-1}$ ). Microcosms were bubbled with nitrogen (1 h) to remove the oxygen, incubated at 28°C in the dark and sampled right after inoculation, and after 20, 40, 60, and 120 d. On each sampling date, the Fe(II) release into the water phase of three microcosms was measured using ferrozine assay (Stookey 1970; Leiser et al. 2020). Afterward the flocs were carefully removed from the microcosms using an inverted Pasteur pipette and transferred into 5 mL of 1 M HCl (4 h, room temperature). Their Fe(II)/Fe(III) content was analyzed by reduction with hydroxylammonium chloride—HCl (0.5/1 M) and ferrozine assay (Stookey 1970). The solution was then filtered onto stainless steel sieves (10  $\mu\text{m}$ ) and the PE spheres formerly bound to the flocs were counted under a light microscope (Zeiss Axioplan). In order to quantify the PE spheres released from the flocs during incubation, the remaining liquids inside of the microcosms were filtered and checked for PE spheres as well. On each sampling date, two additional microcosms were

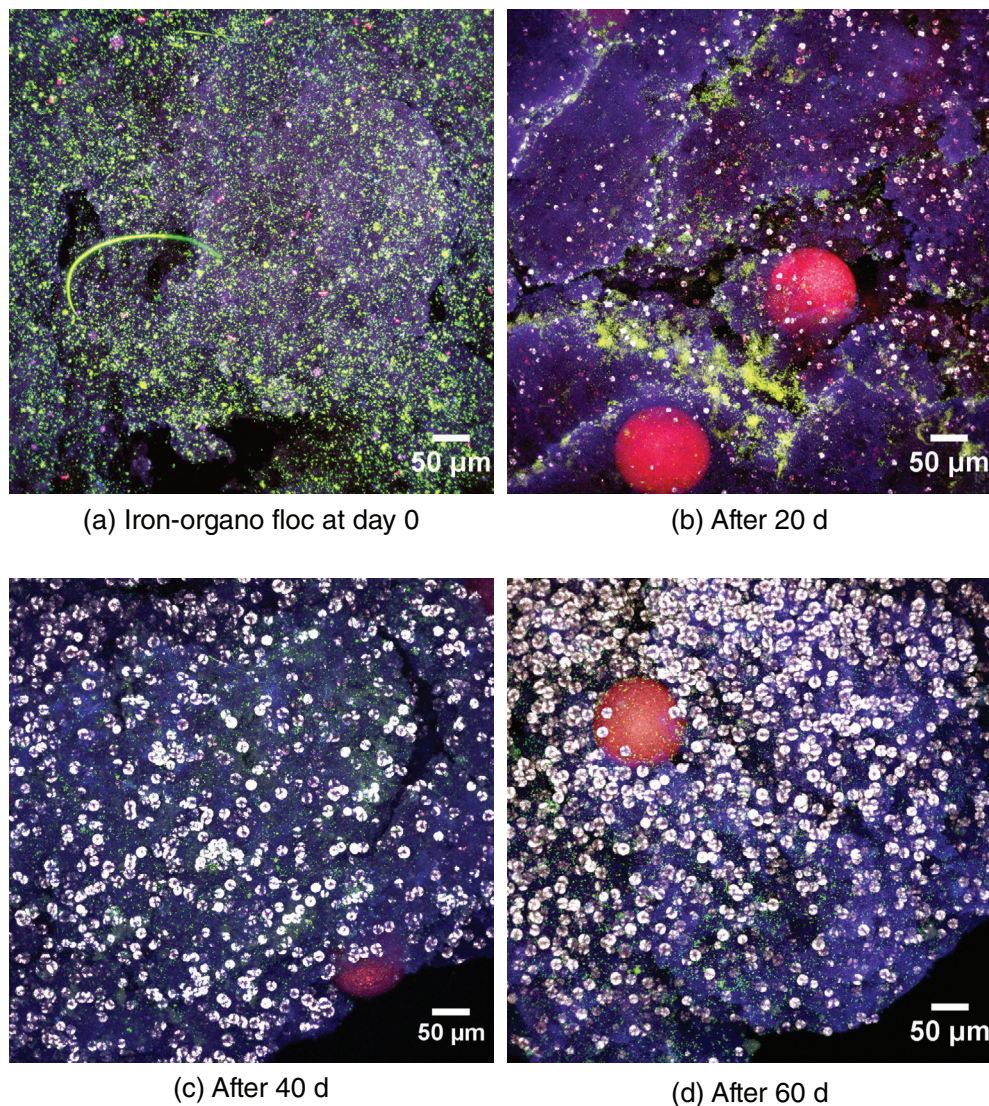
used to retrieve six individual iron-organo flocs, which were examined on five randomly chosen spots each using confocal laser scanning microscopy (CLSM). Control microcosms ( $n$ : 5) without additional *S. oneidensis* were run in parallel and sampled as described above after 120 d.

### Microscopic techniques

Flocs were visualized using CLSM as described elsewhere (Neu et al. 2001; Leiser et al. 2020, 2021a,b). Biovolumes of bacteria, algae, cyanobacteria, and extracellular polymeric substance (EPS) inside the flocs were estimated from CLSM datasets using a customized version of ImageJ (Staudt et al. 2004).

### Data analysis

Statistical testing was conducted for datasets with a minimum sample size of six individual replicates. Q-Q plots were examined to check for data normality. Variance homogeneity was tested with Bartlett's test prior to ANOVA (two-sided, type II) followed by examination of residual plots to verify the reliability of the ANOVA. Null hypothesis of equal group means was discarded for  $p < 0.05$ . Software R (R Core Team 2020) was used for all statistical analysis and data visualizations. Mean values with standard deviations or median values with 5–95% confidence intervals (CIs) are reported where applicable. Data and metadata are available in the UFZ Data Investigation Portal repository at <https://doi.org/10.48758/ufz.11330>.



**Fig. 1.** Iron-organo flocs recorded via CLSM right at the beginning (a), after 20 d (b), 40 d (c), and 60 d (d) of anaerobic incubation with *S. oneidensis*. False color coding refers to bacteria (green), algae (blue), cyanobacteria (pink), EPS (purple), PE spheres (bright red), and reflection by secondary minerals (white). (a) Shows an iron-organo floc before the iron-reducing bacteria *S. oneidensis* attached to its surface. Bacteria in this image originate from the natural microbial community of the lake water used to form the flocs. (b–d) Show iron-organo flocs incubated and colonized with *S. oneidensis*. Scale bars refer to 50  $\mu\text{m}$ .

## Results and discussion

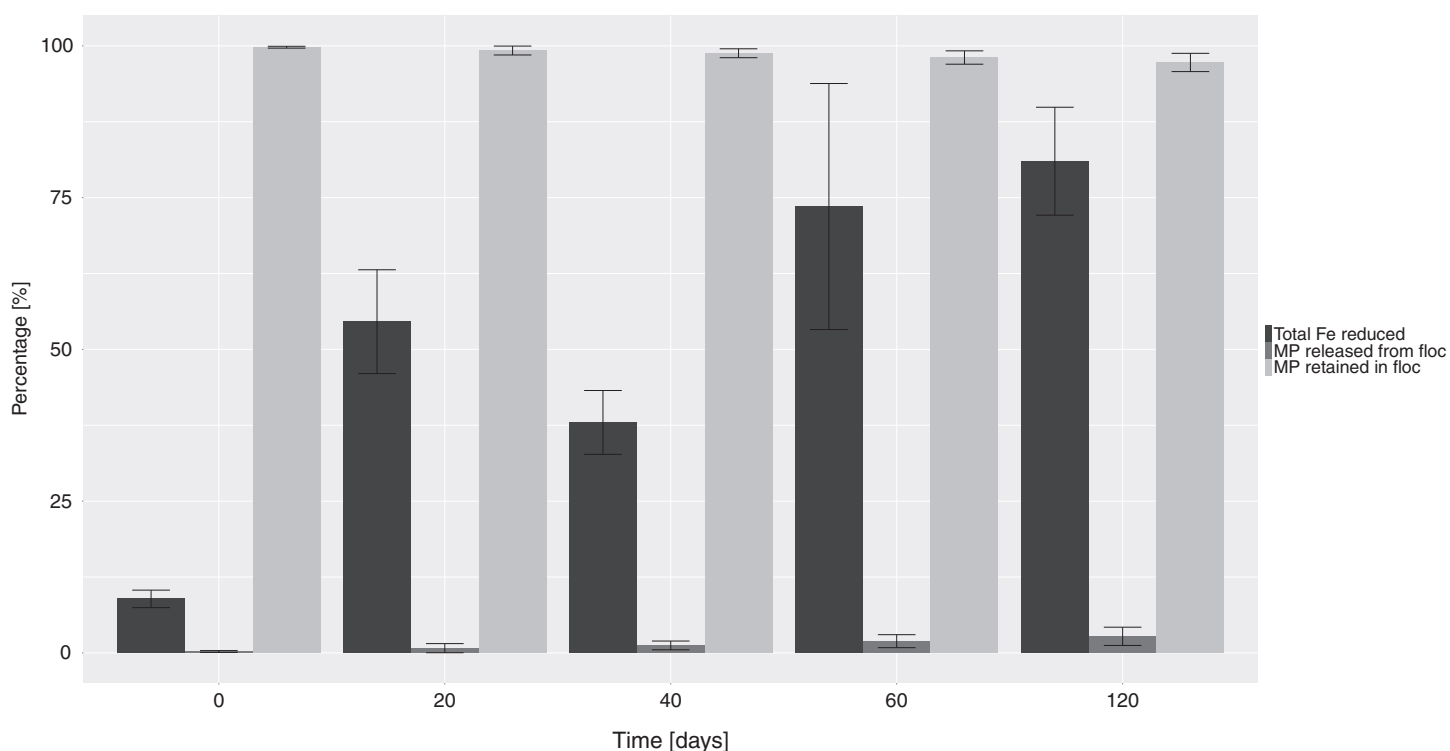
Iron-organo flocs readily formed in surface water from Bautzen reservoir; incorporating PE spheres (median: 650 particles per 5 flocs, 5–95% CI: 543–1265,  $n$ : 12) in their matrix (see Fig. 1). The flocs sedimented to the bottom of the bottles and thereby mediated the sinking of the initially buoyant PE spheres. In a complementary study, it was shown that this mechanism is capable of aggregating and transporting approximately 99% of added PE microplastics into the sediments of Bautzen reservoir (Leiser et al. 2021b).

The iron-organo flocs were incubated for 120 d to simulate the potentially destabilizing effect of microbial iron reduction, which likely takes place in the sediments of freshwater Bautzen reservoir. Iron reduction by *S. oneidensis* changed the appearance of the flocs. From day 20 onward, flocs were covered by black spots, ultimately turning entirely black until day 60 (Fig. S1). The color change indicated formation of secondary minerals, which was supported by the increasing number of rounded, reflecting mineral spherules covering the surface of the flocs with extended incubation time (Fig. 1b–d).

*S. oneidensis* is well known for transforming ferrihydrite into magnetite via iron reduction, whose presence was supported by the black color of the flocs (Dippon et al. 2015). However, the rounded spherules (Fig. 1) closely resembled pyrite framboids in shape and size (Ohfuji et al. 2005).

Furthermore, the black flocs exhibited a sulfidic smell when treated with acid, indicating the presence of secondary sulfides. *S. oneidensis* is able to couple sulfite and thiosulfate reduction to lactate oxidation, but cannot reduce sulfate (Dippon et al. 2015). Hence, sulfide mineral formation might have rather been promoted by sulfate-reducing bacteria enclosed inside the nonsterile flocs. Sulfate-reducing bacteria such as *Desulfovibrio* are common in lake water (Berg et al. 2020) and could have been present inside the iron-organo flocs, precipitated from Bautzen reservoir surface water. Consortia of iron- and sulfate-reducing bacteria are known to precipitate iron sulfides onto the surface of iron (oxyhydr)oxides (Berg et al. 2020). We hypothesize that the observed color change and the formation of spherules might be attributed to the combined activity of *S. oneidensis* and unknown sulfate-reducing bacteria. Although no data can be provided to support this hypothesis, it is notable that no floc color change was observed in the control treatment without additionally added *S. oneidensis*.

*S. oneidensis* was observed to bind to the surface of the iron-organo flocs, accumulating mostly in cracks and on the edges of the flocs (Fig. 1b). This is in line with previous findings, reporting that iron reduction of *S. oneidensis* relies on direct contact of the cells with the iron mineral surface or by close range electron shuttling with endogenous flavins or exogenous humic substance (Dippon et al. 2015).



**Fig. 2.** Iron reduction and release of PE spheres from iron-organo flocs incubated with *S. oneidensis* under iron-reducing conditions. Bars and arrows display the means and standard deviation of three individual replicate microcosms ( $n$ : 3).

At the end of the experiment, most of the iron (up to 90%; Fig. 2) was reduced and either bound to the flocs in form of reduced minerals or released in form of dissolved ferrous iron (Table S1). The transformation of iron minerals did not affect the integrity of the flocs, as indicated by only a minor fraction of the bound PE microplastics being released during the 120 d incubation (Fig. 2; Table S1). Although significantly more plastics (ANOVA,  $p < 0.05$ ) were released on day 60 and day 120 ( $20 \pm 19$  particles, mean  $\pm$  SD,  $n$ : 6) compared to the previous sampling dates ( $4 \pm 3$ , mean  $\pm$  SD,  $n$ : 9), the proportion of released (median: 8,  $n$ : 12) compared to retained microplastics (median: 650,  $n$ : 12) remained negligible (Fig. 2). The finding that iron (oxyhydr)oxide presence exerted a minor role for the floc integrity was further supported by the treatment of flocs with hydrochloric acid. This led to the removal of iron (oxyhydr)oxides from the flocs (Fig. 3) but did not visibly destabilize the flocs.

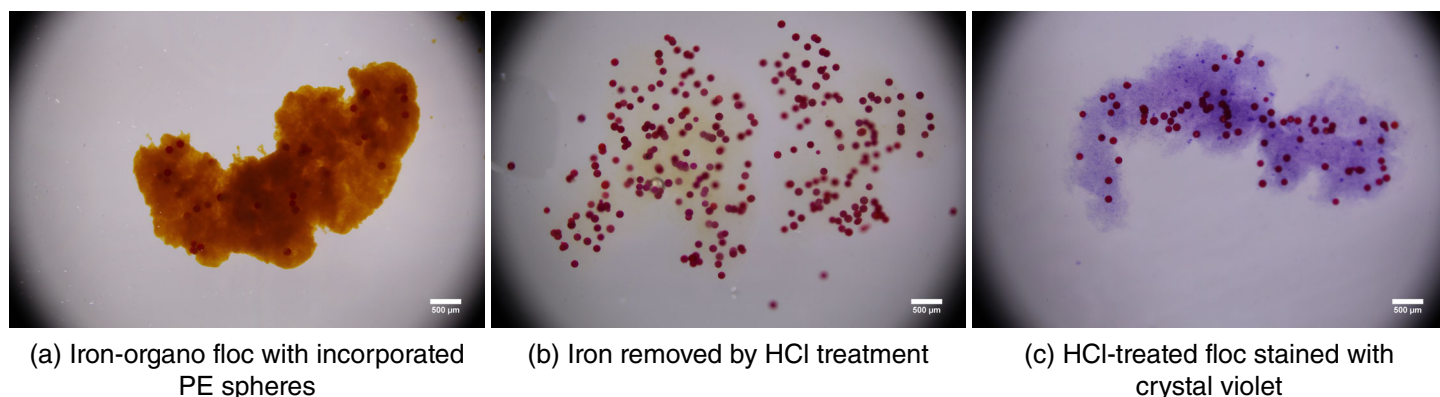
In addition, this treatment released only small quantities of PE microplastics from the flocs (Table S1). Hence, it can be assumed that iron (oxyhydr)oxides precipitation is a prerequisite for the formation of flocs, but iron is not necessary to assure floc stability after formation. Further PE microplastics are expected to stick rather within the organic matrix than being covered by iron (oxyhydr)oxides.

*S. oneidensis* is able to use a wide range of different organic compounds for their energy conservation (Venkateswaran et al. 1999), possibly enabling them to couple oxidation of the organics bound to the flocs to iron reduction. However, no substantial degradation of the organic floc matrix was observed over time (Fig. S2), as indicated by fucosylate glycan-binding *Aleuria aurantia* lectin (Neu et al. 2001). This does not exclude degradation of other organic moieties possibly bound inside the flocs and not stained by the lectin. But it supports the observation that no visible change in floc size or shape occurred during the 120 d of incubation. Association with iron minerals is considered as an effective way of carbon preservation in soils and sediments, with organics being protected

from microbial degradation by steric hindrance or highly energetic binding to the mineral surfaces (Lalonde et al. 2012). It has been shown that reduction of the preserving iron (oxyhydr)oxides minerals leads to the liberation and subsequent degradation of the associated organic matter (Patzner et al. 2020). However, if the reduction of iron (oxyhydr)oxides is coupled to the simultaneous formation of iron sulfide minerals, these secondary minerals can also effectively bind and preserve organic matter from microbial degradation (Picard et al. 2019). The observed iron sulfide formation in our study supports this phenomenon and indicates the stability of the iron-organic flocs under anoxic conditions.

An artificial system with lactate as favorable electron donor was used in this study. It cannot be ruled out that *S. oneidensis* preferably utilized the easily degradable lactate instead of the flocs' recalcitrant organic matter (Vidal-Melgosa et al. 2021). The preferential usage of lactate might have artificially prevented or retarded the microbial degradation of flocs. Under natural conditions in freshwater sediments, easily degradable substrates are scarce and microbial communities might be specialized to degrade deposited flocs, as it was shown for fresh phytoplankton aggregates mineralized after 12–20 weeks in anoxic lake sediments (Schulz and Conrad 1995). However, given their more recalcitrant nature compared to fresh phytoplankton aggregates and following the results of the presented study, it can be expected that iron-organic floc degradation will be considerably slow (several weeks to months) within the cold profundal of lakes or reservoirs. We cannot exclude that trace amounts of ethylene monomers or fluorescence label leached from the PE spheres also influenced the metabolism of *S. oneidensis*. However, given their low toxicity (Augimeri and Strap 2015; Skjolding et al. 2021), a significant effect of these chemicals is unlikely in the microcosm incubations.

In highly productive Bautzen reservoir, several millimeters of sediments (average deposition rate from June to August  $\sim 12$  g dry weight  $m^{-2} d^{-1}$ ) are deposited during the



**Fig. 3.** Iron-organic floc recorded via light microscope (10 $\times$  magnification), presented in its natural state (a), after treatment with HCl (1 M, 24 h) (b), and after staining the HCl-treated floc with crystal violet (0.1%, 30 min) (c). Scale bars refer to 500  $\mu$ m.

approximately 3 months of summer stratification (unpublished data). Hence, it is likely that iron-organo flocs lying on the sediment surfaces will become covered by freshly deposited material before being completely mineralized (Schulz and Conrad 1995). Once microplastic bearing flocs are covered by fresh sediments, the escape of microplastics from the sediment matrix becomes unlikely, regardless of the flocs being intact or mineralized. However, this might only hold true for undisturbed sediments which are not affected by physical (Hurley et al. 2018) or biological (Zdeněk and Aršálek 2013) driven resuspension processes.

Still, this work shows that organo-metallic flocs might not readily be degraded once reaching the sediments of freshwater bodies. Outgoing from the presented results, the observed transformation of the iron (oxyhydr)oxides into secondary sulfides may contribute to floc stability. This prevents the release of bound PE spheres from their matrix once deposited in the lake profundal. Hence, microplastic aggregation by iron-organo flocs might govern the long-term storage of this contaminant in freshwater sediments.

## Conclusions

Iron-organo flocs precipitated from reservoir water effectively sequester PE microplastics and are stable under anoxic conditions for up to 120 d, even when the flocs experience microbial iron reduction. Hence, it can be assumed that iron-organo flocs lying on top of anoxic sediments will not readily release incorporated microplastics into the water phase. Thereby incorporation into such aggregates may display a potential mode of long-term microplastic storage in freshwater reservoirs. Consequently, the use of iron flocculants and the natural process of Fe(III) precipitation could contribute to the removal of microplastics from surface waters and to stable storage in the sediments of freshwater reservoirs. This long-term deposition may decrease mobile microplastics and would mitigate adverse effects of this contaminant for the ecosystems of the downstream rivers, estuaries, and subsequently the oceans.

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