

Replies to the interactive comments on “Effects of extraction conditions on the redox properties of soil organic matter (SOM) and its ability to stimulate microbial iron(III) mineral reduction by electron shuttling”.

Comments by the Editor (hand-written comments on the pdf file of our manuscript)

Title: is “stimulated microbial iron (III) mineral reduction” demonstrated?

First we would like to thank the editor for spending time to go through our manuscript and helping us to improve the manuscript. We went through the manuscript thoroughly and made sure to carefully incorporate all suggestions and comments.

We believe that our results clearly demonstrated stimulating effects of soil organic matter extracts on both the rate and extent of microbial Fe(III) reduction. As shown in Figure 3 of the manuscript, after addition of only *Shewanella oneidensis* MR-1 (no soil organic matter added), after 16 days of incubation, about 3.87 mmol L⁻¹ Fe(II) was measured. However, after the amendment of soil organic matter (SOM) extracts as electron shuttle, the concentration of Fe(II) increased to 7.08-10.87 mmol L⁻¹. Regarding the rate of microbial Fe(III) reduction, as shown in Figure S3, the control sample without SOM extracts as electron shuttle showed a maximum reduction rate of 0.79±0.31 mmol Fe(II) d⁻¹. The rate of microbial Fe(III) reduction significantly increased with the addition of soil organic matter extracts as electron shuttle, ranging from 0.82-3.05 mmol Fe(II) d⁻¹. All of these results are shown in **section 3.3, page 9** of the manuscript.

Regarding the NMR data, I agree that the spectra should be shown in the main paper and a table added to the SI.

According to the editor’s suggestion, we added all of the NMR spectra to the main manuscript as Figure 2 and a table showing the relative intensity distributions of specific chemical shifts is shown in the supporting information (Table S2). We also removed the row that showed the aromaticity of samples calculated from the NRM results from Table 1 and combined the aromaticity and the NMR spectra together in Figure 2.

The introduction is missing a lead to the microbial reduction experiment (the abstract too).

To introduce the microbial Fe(III) reduction experiment in the abstract, we added one sentence as “*we also performed a microbial Fe(III) reduction experiment with all of the different extracts and we found that higher EEC...*” (line 32, page 1).

For the introduction, we revised the manuscript from “*We analyzed the electron accepting and the electron exchange capacity (the sum of EAC and EDC) of all extracted water-extractable OM, FA and HA fractions and performed a microbial Fe(III) mineral reduction experiment with all of the different extracts*” to “*We analyzed the electron accepting capacity and the electron exchange capacity (the sum of EAC and EDC) of all extracted water-extractable OM, FA and HA fractions. To further compare their electron shuttling capacity, we performed a microbial Fe(III) mineral reduction experiment with all of the different extracts*” (line 85, page 3).

The logic flow leading to the microbial Fe(III) reduction in the introduction is, first, we introduced soil organic matter (SOM) and how scientists usually extract humic substance (HS) and use HS as a proxy for SOM (first paragraph). We then explain that participation in redox-active reactions is a very important property of SOM, for example, SOM as electron shuttle to stimulate microbial Fe(III) reduction. However, scientists have been using HS in electron shuttling studies to represent SOM, and since we do not know the impact of the extraction procedure on the redox activities of HS, we could not determine to which extent the electron shuttling capacity of HS is just coming from the extraction-induced changes on the functional groups thus the redox activity (paragraph 2). In paragraph 3 we refer to a previous study that clearly demonstrated higher reducing capacity of the chemically-extracted humic acid compared to water-extracted organic matter. However, in this study the humic acids and the water-extracted organic matter were extracted from different environments. Therefore, in the last paragraph, we explain that we would like to perform an experiment in which we extract SOM with either the traditional chemical method or with water at neutral pH, compare its redox activity by the electron exchange capacity analysis and further test its ability to act as electron shuttle in a microbial Fe(III) reduction experiment.

Include methods for extract chemical analysis.

Thank you for the suggestion. We assume the editor means the methods for the MP-AES analysis of the metal content of our extracts. The results of this analysis are shown in Table S5. Following the reviewer's suggestion, we added section 2.5 in the materials and methods part as "Microwave Plasma-Atomic Emission Spectrometer (MP-AES) analysis" (**Line 136-142, page 5**).

Suggest replacing "setups" with "experiments" or "treatments" throughout.

Thank you for the suggestion. We replaced all "setups" by "experiments" in the manuscript and the supporting information.

Line 12: serving as an electron shuttle, add "an".

Revised, thank you.

Line 18: "led to" instead of "lead to".

As suggested by reviewer 1, this sentence was removed from the abstract. But we carefully went through the manuscript and revised all "lead" to "led" where past tense is needed.

Line 55: Implication, need for research.

We added one sentence here as "*Therefore, studies that compare the stimulating effects of SOM extracted with either the traditional chemical extraction method or with water at neutral pH conditions on microbial Fe(III) reduction are needed*" (**line 65, page 3**).

Line 82: "world reference base for soil", is this correct? It's not capitalized?

Thank you. We revised it to "WORLD REFERENCE BASE FOR SOIL" (**line 94, page 4**).

Line 88: "5000 rpm", can you provide g-force?

Thank you for the suggestion. The rotor we used for centrifugation has a radius of 120 mm, therefore the converted g-force should be 3528×g (added to **line 100, page 4**).

Line 92: Change “doubly-deionized water” to “ultrapure water”.

Thank you, changed (line 103, page 4).

Line 97: “Filtration”, capitalized “F”.

Revised, thank you (line 111, page 4).

Line 136: samples were p though?

Revised to “*samples were filtered through*” (line 146, page 5).

Line 166: “14600 rpm”, please give g-force.

Thank you. Revised to 28649 ×g (line 179, page 6).

Line 168: Describe AQDS treatment in the section 2.7.

We added “*AQDS, i.e. 2,6-anthraquinone disulphonate, a quinone model compound commonly used in electron shuttling studies that can significantly increase the rates of microbial Fe(III) reduction, was used as 100 $\mu\text{mol L}^{-1}$ in our experiments as a reference for significant stimulation of Fe(III) reduction by our extracted OM via electron shuttling*” (line 174, page 6).

Line 197: “more than 90% of the Fe was removed by filtration”, how was this tested?

We first analyzed the concentration of Fe(II) in the water-extractable OM (anoxic) solution right upon finishing the water extraction, and it was 3 mM. When preparing these extracts for the chemical analyses, electron exchange capacity (EEC) analysis, and the microbial Fe(III) reduction experiments, solutions of the extracts were passed through 0.22 μm syringe filters. After this filtration step, we measured again the Fe(II) and Fe(total) concentration in all samples. Taking water-extractable OM under anoxic conditions as an example, the total Fe concentration after filtration was 123.1 $\mu\text{mol L}^{-1}$. This was also the highest Fe concentration we detected among all of our extracts after filtration (Table S3). We calculated that this represents $123.1 \mu\text{mol L}^{-1} / 3 \text{ mmol L}^{-1} = 4\%$ of the initial Fe

concentration. This means that the remaining Fe concentration in the water-extractable OM under anoxic condition is up to 4% of the Fe(II) that was detected right after the water extraction, therefore, more than 90% of Fe was removed during the filtration. We used total Fe concentrations instead of Fe(II) concentrations here because all of the extracts, before being prepared into solutions for any analysis or microbial Fe(III) reduction experiment, were freeze-dried under oxic conditions. Therefore, a large portion of Fe(II) was oxidized to Fe(III), and later during the preparation of extracts solution, the pH was adjusted to 7, therefore Fe(III) was not dissolved and got removed by the syringe filter.

In order to show how we got to the conclusion that more than 90% of Fe was removed by filtration, we revised the sentence (line 197, page 7) from “*Although more than 90% of the Fe was removed by filtration (0.22 μm , ...) and around 30% of the remaining Fe(II) was oxidized to Fe(III)...in the water-extractable OM, FA and HA fractions (Tab. S3).*” to “*Although, as shown in Tab. S3, more than 90% of the Fe was removed by filtration (0.22 μm , ...) and around 30% of the remaining Fe(II) ... in the water-extractable OM and FA/HA isolated from it*” (**line 208, page 7**).

Line 215: “The EEC of chemically-extracted FA under anoxic conditions was even 33 $\mu\text{mol e- mmol C}^{-1}$ higher than FA chemically-extracted under oxic conditions”. Was this observed in everything with anoxic preparation?

Yes, higher EEC values were observed in all extracts extracted under anoxic conditions. As also shown in section 3.2 in the manuscript, **line 225-234, page 8**, “*The EEC of water-extractable OM obtained under oxic conditions was 32 $\mu\text{mol e- mmol C}^{-1}$ (with ca. 4 $\mu\text{mol e- mmol C}^{-1}$ from Fe), whereas when extracted anoxically, it increased to 44 $\mu\text{mol e- mmol C}^{-1}$ (with 14.8 $\mu\text{mol e- mmol C}^{-1}$ from Fe). Higher EEC values under anoxic compared to oxic extraction conditions were also observed for all extracted FA: for FA isolated oxically from the water-extractable OM, the EEC was 13 $\mu\text{mol e- mmol C}^{-1}$ (2.3 $\mu\text{mol e- mmol C}^{-1}$ from Fe), while it increased to 24 $\mu\text{mol e- mmol C}^{-1}$ (2.7 $\mu\text{mol e- mmol C}^{-1}$ from Fe) when FA was isolated anoxically from the water-extractable OM. The EEC of FA isolated from soil under anoxic conditions was 33 $\mu\text{mol e- mmol C}^{-1}$ higher than FA isolated from soil under oxic conditions. Similar to FA, for the HA isolated from water-extractable OM, the EEC values*

increased from 15 $\mu\text{mol e- mmol C}^{-1}$ (1.9 $\mu\text{mol e- mmol C}^{-1}$ from Fe) under oxic conditions to 83 $\mu\text{mol e- mmol C}^{-1}$ (7.3 $\mu\text{mol e- mmol C}^{-1}$ from Fe) under anoxic conditions. For HA isolated from soil, EEC values increased from 40 $\mu\text{mol e- mmol C}^{-1}$ under oxic conditions to 127 $\mu\text{mol e- mmol C}^{-1}$ under anoxic conditions”.

Line 228: “strongly”.

Thank you, revised (**line 242, page 8**).

Line 282: “The presence of Fe(II) at the end of extraction in all anoxic extracts suggested that the higher amount of extracted OM is probably related to microbial Fe(III) mineral reduction and the release of mineral-bound OM during mineral dissolution”. This is only speculation since you don’t show data of Fe-reduction during the extraction.

First of all, we agree to the editor that this is a speculation. The reason that we do not have a figure or table to show the Fe reduction results during the anoxic water extraction is because we only had the Fe(II) concentration before and after the anoxic extraction, therefore only two data points. This data is shown.

We speculate that microbial Fe(III) reduction happened during the anoxic water extraction because we measured 3 mM Fe(II) after the anoxic water extraction compared to non-detectable Fe(II) concentrations at the beginning of the anoxic water extraction. Secondly, the soil we used for organic matter extraction was not sterilized, therefore, based on many previous studies, microbial Fe(III) reduction could happen under anoxic and neutral pH conditions. Thirdly, we also observed much more organic carbon extracted by water under anoxic compared to oxic conditions. We assume, according to previous studies (Burdige et al., 1992; Burdige et al., 1999; Chin et al., 1998; Dadi et al., 2017; Gu et al., 1994; Riedel et al., 2013; Shimizu et al., 2013), this is due to the occurrence of microbial Fe(III) reduction under anoxic condition and the release of organic carbon that was co-precipitated with or adsorbed to the Fe(III) minerals.

Line 325-331: Aren’t ESI-FTICR-MS the same as FTICR-MS?

ESI, electrospray ionization, is a low-fragmentation ionization technique that preferentially ionizes polar functional groups prior to mass spectrometric analysis. Coupling it to FT-ICR-MS is especially useful for the characterization of organic matter of polar or slightly polar constituents of environmental mixture. Therefore, they are not exactly the same.

Line 342: there are instead of there is.

The discussion part from **line 326-343** was deleted according to the second reviewer's suggestions.

Line 352: add "produced" after mineral phase.

Thank you. Added (**line 348, page 12**).

Table 1: "***The percentage of carbon extracted from soil of the samples are significantly different (n=2, two-sided t-test, P<0.05", the other half bracket missing.

Thank you. Added.

Figure 1: "Forest soil samples (Schönbuch forest, Baden-Wuerttemberg, Germany) dried under 30 °C and grinned to pass 2 mm sieve". (1) add 'were' before dried, (2) "grounded" instead of "grinned".

Thank you. The sentence is changed to "*Schematic diagram of soil organic matter (SOM) extraction process. Forest soil samples (Schönbuch forest, Baden-Wuerttemberg, Germany) were dried under 30 °C and grounded to pass 2 mm sieve*".

Figure 1: "after centrifugation, the supernatant was acidified by HCl to pH<2, HA then precipitated to be separated from FA". Revise to "HA was then precipitated out to be separated from FA".

Thank you. Revised.

Figure 1: Replace "doubly-deionized water" by "ultrapure water".

Thank you. Revised.

Figure 1: Last sentence in the caption, “This extraction was conducted under both oxic and anoxic conditions”, not clear, only the water extraction? Also consider defining abbreviations in the legend.

We revised the last sentence in the caption to “*All of the extractions were conducted under both oxic and anoxic conditions*”.

We also added definitions of all the extracts, as “*the supernatant was acidified by HCl to pH<2, HA was then precipitated to be separated from FA, we define these two extracts as HA (isolated from soil) and FA (isolated from soil)*”, “*100 g soil was incubated with 400 mL ultrapure water (<18.2 MΩ.cm; Milli-Q, Millipore) at pH 7. pH was monitored during the extraction and it remained stable (range between 7.02-7.33). The slurry was centrifuged after 24 h; we define the supernatant as water-extractable OM*” and “*the pH of the solution was adjusted to <2 by 6 M HCl to precipitate HA and to separate FA, and these two fractions are HA (isolated from water-extractable OM) and FA (isolated from water-extractable OM)*”.

Figure 2: Are these colors color-blind safe? Red-green is a known issue.

We agree with the editor that red and green are not good colors to present our data. Therefore, we changed FA in Figure2, Figure 3, Figure 4, Figure S2 and Figure S3 into orange and HA in Figure2, Figure 3, Figure 4, Figure S2 and Figure S3 into purple.

Figure 3: “The insert in panels a-f show the data points for the first 6 h of incubation”, revise “6h” to “2 days”.

Revised, thank you.

Figure 3: Consider using the same scale for y-axis (not for inserts).

Revised to same scale for all panels, thank you.

Reviewer 1 :

Bai et al. compare redox properties of a water extract with humic and fulvic acid extracts of a soil.

Additionally, fulvic acids and humic acids were extracted of the water extract. All extractions were performed under air as well as under N₂. The final conclusion is that (oxic or anoxic) alkaline extracts are not suitable to substitute natural organic matter in redox-experiments. Although the concept of humic and fulvic extractions was often challenged, the method is still popular in current papers and textbooks, sometimes even described as the method of choice. The topic is therefore both important and in time. Also, I appreciate such a clear statement from groups which frequently used humic acids in the past. The paper is well written and nicely illustrated by figures and tables.

One general comment: In the manuscript the abbreviation “SOM” is used to address the water extractable fraction (tables, figures, text). This seems misleading to me: SOM comprises all non-living soil organic matter, while a water extract is done to gain something similar to the dissolved organic matter or the fraction which can be dissolved or mobilized (colloidal fraction) during rain events or rising groundwater. I think, a better name for the water extract would be “water extract (WE)” or “water extractable organic matter (WEOM)” instead of SOM. WE or WEOM also shows that one is aware of the difference between a water extract and the real DOM of a soilsolution.

We would like to thank the reviewer for taking the time to go through our manuscript and his/her useful comments, which helped improving our manuscript. We went through the manuscript thoroughly and made sure to carefully incorporate all suggestions and comments.

Following the reviewer’s suggestion, we changed the name of this extraction fraction from “water-extracted soil organic matter (SOM)” to “water-extractable organic matter (OM)” in the text, tables and figures in both the manuscript and the supporting information. As suggested by the reviewer, the term “soil organic matter” is usually used to indicate all of the non-living organic matter in soil, which is obviously not possible to be completely extracted by the water extraction method we applied. Instead, our results as presented in Table 1 showed that only about 0.41 % (under oxic conditions) and 2.74 % (under anoxic conditions) of the total organic carbon in the soil was extracted by the water extraction at pH 7. Therefore, in line with the comment provided by the reviewer, we believe that this “water-extracted OM” fraction is the fraction that can be easily mobilized by rain or rising ground water, which is only a small portion of the total soil organic matter (SOM).

To my knowledge, humic substances are meant to extract all HA and FA of a sample, while it is accepted that a large fraction may remain behind. I therefore wonder if it is meaningful to compare extracted C amounts from alkaline extraction with water extraction. With respect to the different extraction of functional groups or artefacts during exposure to pH12, the comparison between WEOM and FA/HA of WEOM are certainly more robust. (For example, does the sum of EAC (FA of WEOM, oxic) and EAC (HA of WEOM oxic) equals EAC(WEOM) or do we see artefacts? The sum of FA of WEOM+HA of WEOM (0.035 g C) is very close to the total WEOM (0.036 g C)).

We agree that the two extraction methods (water vs. alkali/acid) target different fractions of the soil. Actually, in the manuscript, we pointed out that chemical extraction at pH 12 leads to the extraction of different fractions of soil organic material compared to water extraction. In **line 322, page 11**, we wrote “However, we found that even under anoxic conditions, the chemical extraction extracted up to 100 times more carbon than the water extraction at neutral pH (Tab. 1), consistent with previous studies (Aiken, 1985). This higher extraction efficiency at high pH could be due to the deprotonation of carboxyl and phenol functional groups leading to both higher aqueous solubility and electrostatic repulsion of OM from negatively charged soil minerals (Kleber et al., 2015) or due to the hydrolysis of plant material and the formation of smaller oligo- and monomers (Sparks, 2003).” Nevertheless, we agree with the reviewer that comparing the chemical characteristics and redox properties of the FA and HA chemically isolated from the water-extractable OM (the reviewer calls it „FA/HA of WEOM“) to the water-extractable OM itself is more straightforward and informative than comparing the chemically extracted FA and HA from soil to water-extractable OM. Therefore, we will revise our manuscript and when presenting the data regarding the amount of carbon extracted, the SUVA₂₅₄ values, the NMR, emission-excitation matrix (EEM) and electron exchange capacity (EEC) results, we will compare and discuss the results between FA and HA isolated from the water-extractable OM to the water-extractable OM. We would like to make revisions in the manuscript as follows:

Line 190, page 7, change from

“Chemical extraction using NaOH and HCl under oxic conditions yielded ca. 100 times more carbon

for FA (1.451 g; 17.0% of the total carbon present in the soil) and HA (1.450 g; 17.0%) compared to water under oxic conditions at neutral pH. Under anoxic conditions, the chemical extraction also lead to a somewhat higher percentage of extracted carbon for FA (20.7%) and HA (22.0%) than under oxic conditions”,

to

“Chemical extraction directly from the soil using NaOH and HCl under oxic conditions yielded 1.451 g C in the extracted FA (17.0% of the total carbon present in the soil) and 1.450 g C in HA (17.0% of the total carbon present in the soil). Conducting the same chemical extraction from soil under anoxic condition lead to higher percentage of extracted carbon for FA (20.7%) and HA (22.0%) than under oxic conditions”.

Line 197, page 7, change from

“All HA extracts showed much higher $SUVA_{254}$ values than SOM and FA extracts, with the highest value of $0.265 \text{ mg}^{-1} \text{ C cm}^{-1}$ (HA chemically-extracted under anoxic conditions), followed by HA chemically isolated from the water- extracted SOM under oxic conditions ($0.207 \text{ mg}^{-1} \text{ C cm}^{-1}$). For HA chemically isolated from the water-extracted SOM under anoxic conditions and HA chemically extracted from soil under oxic conditions, the $SUVA_{254}$ values are 0.068 and $0.083 \text{ mg}^{-1} \text{ C cm}^{-1}$, respectively.”

to

“HA extracts isolated from the water-extractable OM under oxic condition showed a $SUVA_{254}$ value of $0.068 \text{ mg}^{-1} \text{ C cm}^{-1}$, higher than the $0.018 \text{ mg}^{-1} \text{ C cm}^{-1}$ of the water-extractable OM itself obtained under oxic conditions. A higher $SUVA_{254}$ value for the HA isolated from the water-extractable OM ($0.207 \text{ mg}^{-1} \text{ C cm}^{-1}$) than for the water-extractable OM itself ($0.027 \text{ mg}^{-1} \text{ C cm}^{-1}$) was also found under anoxic extraction conditions”.

The reviewer also suggested to compare the electron accepting capacity (EAC) of the FA and HA isolated from the water-extractable OM to that of the water-extractable OM to clarify whether the chemical extraction method modifies the chemical composition and the redox properties of the

extracts. In the manuscript, we therefore compare the “total number of electrons that can be transferred” (the electron exchange capacity) of FA and HA isolated from the water-extractable OM to the values of the water-extractable OM, these results are presented in Figure 2. In **line 235-242, page 8**, we write *“The total number of electrons that can be exchanged (that means transferred from Fe(III)-reducing bacteria to the OM, or from the OM to Fe(III) minerals) by water-extractable OM before and after the chemical separation of FA and HA from this water-extractable OM was also calculated (the recovery of EEC) under both oxic and anoxic conditions (Fig. 2). For the extracts obtained under anoxic conditions, the sum of total exchangeable electrons values of the FA and HA isolated from water-extractable OM (786 mmol e-) was almost identical to that of water-extractable OM itself (836 mmol e-). In contrast, under oxic conditions, the sum of the EEC values of the FA and HA separated from the water-extractable OM was 324 mmol e-, ca. 5-times higher than the EEC value of the water-extractable OM (64 mmol e-). This confirms that the traditional chemical extraction procedure conducted under oxic conditions strongly enhances the redox capacity of the samples”*. We compared the “total amount of electrons that can be transferred” (the recovery of electron exchange capacity (EEC)) instead of electron accepting capacity (EAC) as suggested by the reviewer because while the EAC represents mainly the content of quinone functional groups in the extracts, the electron donating capacity (EDC) can also provide information for the abundance of functional groups such as phenols in the extracts (Aeschbacher et al., 2012). Therefore, to understand the effects of the chemical extraction method on the content and abundance of functional groups of the soil extracts, we think that the total EEC should be used instead of EAC only.

It was also pointed out by the reviewer that under oxic extraction conditions, the sum of the carbon content of FA and HA from water-extractable OM ($0.021+0.014=0.035$ g C) is very close to the C of the total water-extractable OM (0.036 g C). Under anoxic extraction conditions, the carbon content of FA and HA isolated from water-extractable OM was 0.146 and 0,079 g, respectively. And the sum of them (0.225 g) is also very close to the carbon content of water-extractable OM under anoxic conditions (0.234 g). We think this result makes sense because under both oxic and anoxic conditions, the FA and HA are a sub-fraction of the water-extractable OM, therefore the carbon content of FA and HA should each be lower than the carbon content of the water-extractable OM (but

the sum of HA+FA should represent the OM). The fact that the sum of the carbon content of FA and HA is very close to the carbon content of the water-extractable OM indicates that the chemical treatment of the OM water extract with HA and HCl is very effective and complete without any significant C loss.

Other comments:

First sentence: I propose to delete “including humic substances”. The main point can be made without and if alkaline extraction causes that many artefacts, it may even be wrong.

As suggested, we would like to delete “including humic substances” and revise the sentence to “*Soil organic matter (SOM) is redox-active, can be microbially reduced, and transfers electrons in an abiotic reaction to Fe(III) minerals thus serving as electron shuttle.*”

Line 15: name soil, sampled depth/horizon and pH of the soil.

The sentence of **line 15** will be revised from “*we prepared HS and SOM extracts from a forest soil applying either a combination of 0.1 M NaOH and 6 M HCl, or water (pH 7)*”

to

“*we prepared HA, FA and water-extractable organic matter (OM) extracts, applying either a combination of 0.1 M NaOH and 6 M HCl or ultrapure water (pH 7), from soil samples collected from the subsoil (0-15 cm, A horizon, pH 6.5-6.8) in Schönbuch forest, Baden-Wuerttemberg, Germany.*”

(line 15, page 1).

Line 18/19: “...100 times more...” see general comment above.

As suggested by the reviewer, we would like to revise the sentence from “*We found that soil extraction with NaOH lead to ca. 100 times more extracted C and the extracted HS had 2-3 times higher electron exchange capacities (EEC) than SOM extracted by water.*”

to

“*We found that FA and HA chemically extracted from the soil can make up to 34-40% of the soil*

organic carbon pool while the water-extractable OM only represents 0.41-2.74% of the total soil organic carbon. The higher extraction efficiency of the chemical extraction is probably due to the deprotonation of carboxyl and phenol functional under high pH (line 21, page 1).

Line 25: . . .changes in functional groups . . . is there space to name observed or assumed changes in the abstract?

To include the reviewer's suggestion, we would like to revise the text from "*NaOH/HCl treatment of the water-extracted SOM lead to 2 times higher EEC values in the HA isolated from the SOM compared to the water-extracted SOM itself, suggesting the chemical treatment with NaOH and HCl caused changes of redox-active functional groups of the extracted organic compounds.*"

to

"under anoxic extraction condition, the HA chemically isolated from the water-extractable OM had 2 times higher EEC values compare to the water-extractable OM itself, suggesting the potential formation of redox-active aromatic functional groups during the extraction with NaOH under anoxic conditions by condensation reactions between amino acids, aldehydes, hydroxyl-and catechol-containing molecules." (line 29, page 1).

Line 27: Delete "at neutral pH". Rainwater has a pH of 5, soil solutions are rather variable in pH. The choice of pH should therefore be adopted to the soil and the specific research question. Ionic strength and ionic composition will also play a role. Was pH7 a good choice for the Cambisol?

We agree with the reviewer that rainwater has a pH of about 5.6 (Liljestrand, 1985), and the soil solution pH of Cambisol soils ranges between 5.8-6.2 (WRB). However, we chose pH 7 for the extraction because, first of all, in a paper published in 2014 by our group, the pH of the soil from exactly the same sampling site in the Schönbuch forest was measured 24 h after the addition of 0.01 M CaCl₂, and was reported as 7.0±0.0 (Porsch et al., 2014). In our experiment, the pH of the native soil slurry in Milli-Q water ranged from 6.5-6.8. Therefore, we think that pH 7 as the extraction pH is relevant to the native soil pH conditions. Additionally, we also believe that the extraction pH has significant impact on the results from the electrochemical analysis and the microbial experiments

(Aeschbacher et al., 2012). The standard pH for the electrochemical analysis is 7 (Aeschbacher et al., 2012; Aeschbacher et al., 2010) and for the microbial Fe(III) reduction experiments, also pH 7 is used because pH 7 is the optimal growth pH of *Shewanella oneidensis* MR-1 (Babauta et al., 2011). If we conduct water extraction of OM under the native pH of soil at around 6.5-6.8, and later adjust the pH of the water-extractable OM to 7 for the analyses, due to the possible alteration of the functional groups of the OM by changing of pH and by the use of NaOH for the pH adjustment (Dadi et al., 2017; Guigue et al., 2014; Kappler and Brune, 1999; Schnitzer and Skinner, 1968), it is difficult to judge whether and to which extent the electrochemical capacities and the extent and rate of microbial Fe(III) reduction are a result of the artifacts caused by the pH-adjusting step. We would therefore like to add one sentence to explain this in section 2.1 (**line 104, page 4**) as follows: “*The pH of the water extraction was adjusted to 7 to avoid any possible artefacts resulting from further pH adjustments prior to analyses and experiments that require the sample pH to be 7 such as the electrochemical analysis or the microbial Fe(III) reduction*”.

However, we are aware that adjusting the pH using 1 M NaOH from pH 6.5-6.8 to pH 7 will increase the ionic strength of the solution used for the extraction. To adjust 400 mL of soil slurry solution to pH 7, we used maximally 0.5 mL 1 M NaOH. Therefore, the final concentration of NaOH in the soil slurry is:

$$1 \text{ mol L}^{-1} \times 0.5 \times 10^{-3} \text{ L} = 0.5 \times 10^{-3} \text{ mol} \div 400 \times 10^{-3} \text{ L} = 0.00175 \text{ mol L}^{-1}$$

The ionic strength of 0.00175 mol L⁻¹ NaOH is:

$$I = \frac{1}{2} \{0.00175 \times (+1)^2 + 0.00175 \times (-1)^2\} = 0.00175 \text{ mol L}^{-1}$$

The measured ionic strength of the soil solution of Schönbuch forest is 0.005±0.003 mol L⁻¹ (Rothe et al., 2002), and therefore an increase by 0.00175 mol L⁻¹ from the NaOH used to adjust the pH is smaller than the ionic strength stemming of the soil. Furthermore, although it is known that the amount of OM extracted decreases with increasing ionic strength, significant changes in the amount of OM that was extracted was only seen when the increase of the ionic strength was at least one order of magnitude (Evans and Sorensen, 1985). Therefore, we think in our system, increasing the ionic strength by 0.00175 mol L⁻¹ on top of the 0.005 mol L⁻¹ stemming from the native soil ionic strength should not have a significant impact on the amount of carbon that can be extracted from the soil.

Line 81: Was the mineral topsoil horizon sampled or the mineral topsoil plus litter layer? Do the upper 15 cm include material of the B horizon? Is anything known about the Fe oxide content of the soil? If yes, does the amount of Fe oxides fit to the observed Fe concentration in the extracts?

We only sampled the topsoil horizon without any of the litter layer. The soil sample 0-15 cm was classified as A horizon according to the German soil classification (Classification, 1997; Weigold et al., 2016) therefore we believe it does not include any material from the B horizon. Consequently, the sentence in **line 92, page 3**, “*Top soil (0-15 cm) was collected from the Schönbuch forest, Baden-Wuerttemberg, Germany.*” will be revised to “*Top soil (0-15 cm) without leaf litter from A horizon was collected form Schönbuch forest, Baden-Wuerttemberg, Germany.*”

The 0.5 M-HCl extractable total Fe (poorly-crystallized Fe) mass fraction of the soil is 0.2 wt%. Therefore, in the 100 g soil we used for the extraction, the poorly-crystallized Fe is:

$$100 \text{ g} \times 0.2\% = 0.2 \text{ g}$$

The molecular weight of Fe is 56 g/mol, therefore, the molar amount of the Fe in 100 g soil is $0.2 \text{ g} \div 56 \text{ g/mol} = 0.00357 \text{ mol} = 3.57 \text{ mmol}$

The extraction solution was 400 mL, therefore the concentration of Fe in the water-extractable OM solution is:

$$3.57 \text{ mmol} \div 400 \text{ mL} = 0.0089 \text{ mol L}^{-1} = 8.9 \text{ mmol L}^{-1}$$

The total Fe concentration after 1 h of extraction by 1 M HCl was 3 mmol L^{-1} , measured right after the water extraction under anoxic conditions. Under oxic extraction conditions, the total Fe concentration decreased to 0.8 mmol L^{-1} . The reason for the higher Fe concentration in the extracts under anoxic conditions is the microbial reduction of Fe(III) minerals, as also discussed in the manuscript. As calculated above, both Fe concentrations measured under anoxic and oxic extraction conditions were in the range of the amount of poorly-crystalline Fe present in the soil sample (8.9 mmol L^{-1}).

The total Fe concentrations in the water-extractable OM samples extracted under both oxic and anoxic conditions were analyzed again after filtration of the samples through $0.22 \mu\text{m}$ syringe filters before electrochemical analyses and before addition to the microbial Fe(III) reduction (see

values in Table S3). Because the filtration step removed a large amount of Fe from the extracted OM sample, after filtration the total Fe concentration in the water-extractable OM was 32.18 $\mu\text{mol L}^{-1}$ and 123.1 $\mu\text{mol L}^{-1}$, respectively. These concentrations were also used for the calculation of the Fe contribution to the electron exchange capacity of the extracted OM samples because the OM samples were also filtered through 0.22 μm syringe filters before electrochemical analysis.

Line 63: please give ferrihydrite concentration in mM Fh-Fe or in weight %. As the composition of ferrihydrite is still under debate and as a mineral structure can be given in different ways, mM Fh is not unambiguous.

We would like to thank the reviewer for this suggestion (he/she probably meant line 163). The composition/formula of ferrihydrite is indeed still under debate so giving the concentration as 15 mM ferrihydrite is somehow ambiguous. Therefore, to be more accurate, we would like to replace all “15 mM ferrihydrite” in the manuscript by “15 mM Fe(III)”. The reason is that after we synthesized the ferrihydrite, we analyzed the Fe(III) concentration in the ferrihydrite by ferrozine assay (Stookey, 1970) and for the microbial Fe(III) reduction experiment, the amount of ferrihydrite added was calculated based on the measured Fe(III) concentration of ferrihydrite. Therefore the targeting concentration (15 mM) in the microbial Fe(III) reduction setups represent Fe as Fe(III).

Line 194: delete the “e”

Thank you. Deleted. (line 205, page 7).

Line 211: please correct, the table in Fig.2 give 44 $\mu\text{mol e}^- \text{mmolC}^{-1}$, instead of 45.

Corrected. Thank you. (Line 226, page 8).

Line 304-307: sentence?

We split the long sentence, “Consistent with previous studies, our SUVA_{254} (Tab. 1), EEM (Fig. S1), $^{13}\text{C-NMR}$ (Tab. S2) and EEC (Fig. 2) results showed lower aromaticity and EEC of chemically-extracted FA and HA under oxic conditions compared to chemically-extracted FA and HA

extracted under anoxic conditions, indicating the degradation of aromatic structures and functional groups in the OM to smaller molecules in the presence of O₂ under high pH conditions” into two sentences: “Consistent with previous studies, our SUVA₂₅₄ (Tab. 1), ¹³C-NMR (Tab. S2) and EEC (Fig. 2) results showed that, FA and HA isolated from soil under oxic conditions had lower aromaticity and EEC compare to the FA and HA isolated from soil under anoxic condition. This indicates that degradation of aromatic structures and functional groups in the OM to smaller molecules occurs in the presence of O₂ under higher pH conditions” (line 317, page 11).

Line 400: “adsorption” instead of “absorption”

Revised. Thank you (Line 398, page 13).

Table 1 is unclear. What was analyzed, DOC (after 0.45 filtration) or TOC? Why are concentrations and masses of C needed? Can the descriptive first and second rows of the table be optimized? “Water extracted” above columns 4,5,8, and 9 is misleading, as the treatment is a chemical extraction of a water extract. The latter also applies for the figures. Replace SOM by WE or WEOM or the like.

First, we agree with the reviewer that naming the FA and HA isolated from the water-extracted OM as “*water-extracted HA/FA*” is misleading. Therefore, we would like to name the FA (and the HA) isolated from the water-extractable OM as “*FA (or HA) isolated from water extract*”, whereas the FA (or HA) extracted chemically from soil directly as “*FA (or HA) isolated from soil*”. The terminology for all extracts will be revised in the method section 2.1 (pages 3-4) and throughout the manuscript, the supporting information and in all tables and figures.

Second, regarding the question whether dissolved organic carbon (DOC) or total organic carbon (TOC) of the extracts was analyzed, we analyzed DOC directly from the extracted solution (after passing through 0.45 mm syringe filter) for the water-extractable OM (oxic or anoxic), and for the FA isolated from soil (oxic or anoxic). For the FA isolated from the water-extractable OM (oxic, anoxic) and of all the precipitated HA samples, the TOC was analyzed from the freeze-dried samples. This is explained in the first sentence below Table 1. However, to make it clearer, we will revise this sentence to “*TOC of all HA extracts and of the FA isolated from the water-extracted OM was directly*

quantified from the freeze-dried powders. The carbon content of all other liquid samples was determined as DOC, measured directly from the solutions after passing through 0.45 mm syringe filter”.

In the second row, we show the “*total organic carbon of the extracts*”, that means the total mass of carbon that was extracted. This value was calculated by multiplying the measured OC concentration of the extracted solutions with the volume of the extracted solution. In order to make it clearer, and as suggested by the reviewer “to optimize the first two rows”, we revised the second sentence below Table 1 to “*total organic carbon content in the extract was directly quantified from the freeze-dried samples of FA isolated from the water-extractable OM (oxic, anoxic) and of the HA extracts*”. For the other extract solutions, the total organic carbon was calculated by $DOC (mg C L^{-1}) \times volume of the extracted solution (L)$.”

The revised Table 1 is shown below, all revisions are shown in blue.

	Water-extractable OM		FA				^a HA			
	Water-extracted, oxic	Water-extracted, anoxic*	^a Isolated from water-extractable OM, oxic	^a Isolated from water-extractable OM, anoxic*	Isolated from soil, oxic	Isolated from soil, anoxic*	Isolated from water-extractable OM, oxic	Isolated from water-extractable OM, anoxic*	Isolated from soil, oxic	Isolated from soil, anoxic*
DOC concentration in extract (mg C L ⁻¹)	0.149 ± 0.036	0.890 ± 0.041	–	–	5.800 ± 0.025	6.320 ± 0.071	–	–	–	–
^b Total organic carbon in extract (g)	0.036 ± 0.012	0.234 ± 0.015	0.021 ± 0.002	0.146 ± 0.013	1.451 ± 0.008	1.770 ± 0.028	0.014 ± 0.003	0.079 ± 0.000	1.450 ± 0.002	1.881 ± 0.029
^c Percentage of carbon extracted from soil (%)	0.41 ± 0.14	2.74 ± 0.18	0.24 ± 0.02	1.69 ± 0.15	17.0 ± 0.09	20.7 ± 0.32	0.15 ± 0.03	0.90 ± 0.00	17.0 ± 0.02	22.0 ± 0.34
^d SUVA ₂₅₄ (mg ⁻¹ C cm ⁻¹)	0.018	0.027	0.017	0.029	0.023	0.042	0.068	0.207	0.083	0.265

^aTOC of all HA extracts and of the FA isolated from the water-extractable OM was directly quantified from the freeze-dried powders. The carbon content of all other liquid samples was determined as DOC, measured directly from the solutions after passing through 0.45 µm syringe filter

·Total organic carbon content in the extract was directly quantified from the freeze-dried samples of FA isolated from the water-extractable OM (oxic, anoxic) and of the HA extracts". For the other extract solutions, the total organic carbon was calculated by $\text{DOC (mg C L}^{-1}) \times \text{volume of the extracted solution (L)}$.

·Percentage of carbon extracted from soil = $\text{Total carbon extracted (mg g}^{-1}) / \text{soil carbon content (8.54 mg g}^{-1})$

·Specific UV absorbance 254 nm (SUVA_{254}) = $\text{UV}_{254} \times \text{DOC (mg C L}^{-1})$, b is the optical path length in centimeter (1 cm in this experiment)

**The percentage of carbon extracted from soil of the samples are significantly different (n=2, two-sided t-test, $P < 0.05$)

Figure 3 is very confusing due to the complex legend.

The revised Figure 3 according to the reviewer's suggestion is shown below. We added a legend for each single figure right on top or beneath that figure.

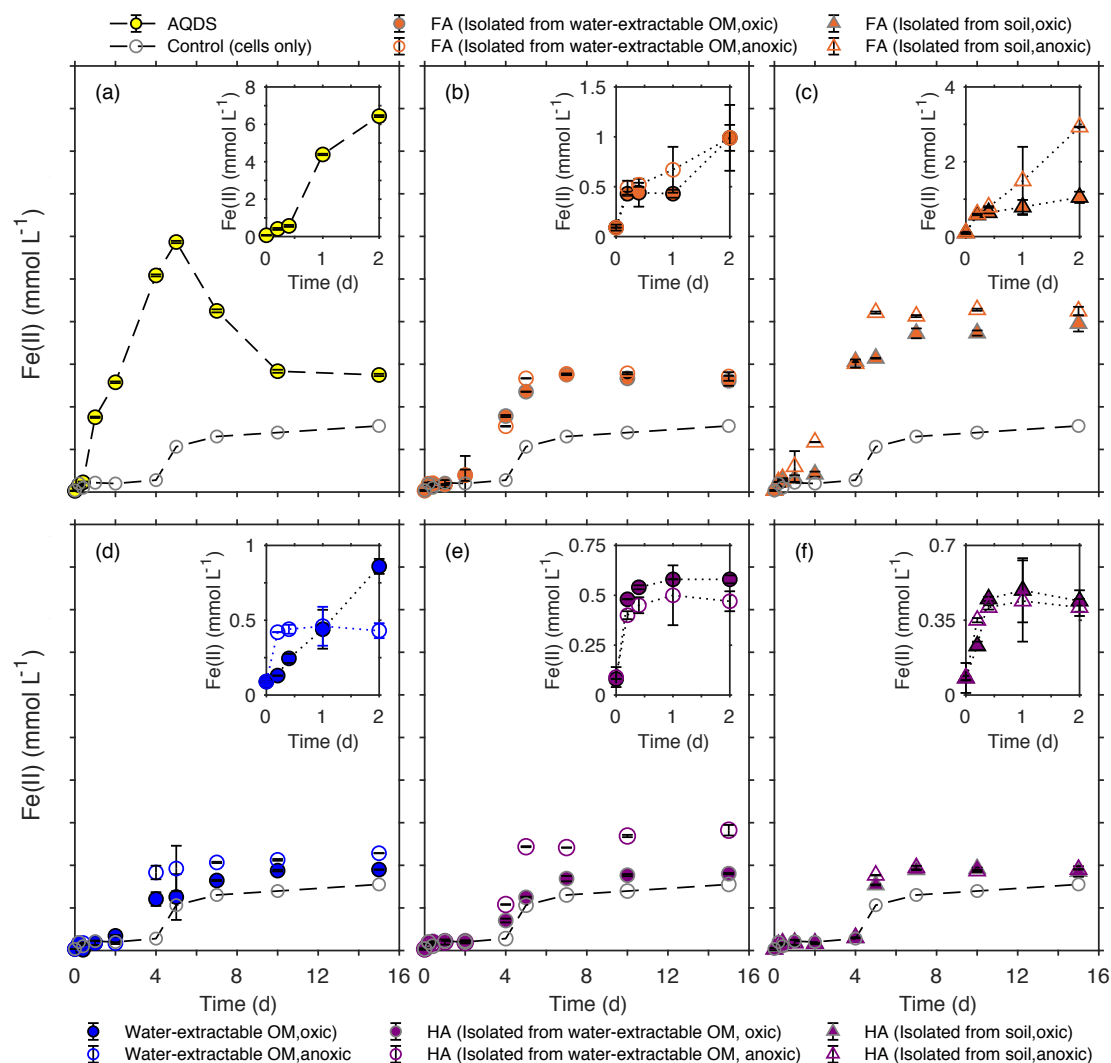


Figure 1, although nice, could be sacrificed to show NMR or EEMS data. It's a pity, that these ended up in the SI. Can NMR spectra be given at least in the SI?

We would like to thank the reviewer for this suggestion. However, if possible (if the reviewer and editor agree) we would prefer to keep Figure 1 due to the following reasons: we believe that this figure clearly shows the extraction process, so even readers who will not read the methods section in

detail will get an idea about what all of the extracts are and how were they extracted. We decided to put the EEM spectra in the SI because there have been debates about the suitability of EEMs, specifically whether EEM analysis reflects the redox state and thus the abundance of redox-active functional groups and aromaticity of humic substances (HS) and natural organic matter (NOM). For example, Maurer et al. (Maurer et al., 2010) compared the EEM spectra of native, electrochemically reduced and re-oxidized humic acid (HA) and they did not find any difference in the emission peak position and density. Identical EEM spectra between native and electrochemically reduced lake organic matter were also shown in the study of Fimmen et al. (Fimmen et al., 2007). Therefore, instead of putting too much emphasis on the EEMs, we use $SUVA_{254}$ (results shown in Figure 1), specific UV absorbance at 254 nm, to characterize the aromaticity of our extracts. $SUVA_{254}$ is a well-recognized method and it has been shown to be a very useful proxy for OM aromatic content (Hansen et al., 2016; Weishaar et al., 2003) and molecular weight (Chowdhury, 2013).

Regarding NMR: as suggested by the reviewer, we would like to show all the NMR spectra in the manuscript (Figure 2) and show the calculated aromaticity of the extracts from the NMR spectra (as suggested by the study of Abelmann et al. (Abelmann et al., 2005)) by adding an extra table in Figure 2.

Title: While the manuscript is concise and clear, the title is less straight. How about having the main message in the title?

As suggested by the reviewer, we would like to revise the title to “High pH and anoxic conditions during soil organic matter extraction increase its electron exchange capacity and its ability to stimulate microbial Fe(III) mineral reduction by electron shuttling”.

Reviewer 2:

This paper compares how different extraction methods influence the composition of soil organic matter (SOM) derived from the process. They compared SOM extracted by neutral pH water and mediated by alkaline extraction followed by acid precipitation (the standard approach used to delineate soil humic and fulvic acids) under oxic and anoxic conditions. The authors determined carbon recovered, specific UV absorbance @ 254 nm (SUVA), and most importantly the exchangeable electron capacity (EEC) as well as electron accepting and donating capacities (EAC and EDC). The manuscript is well-organized, and easy to read (even though there are a couple of typos that spell checker did not catch). The most important contribution, however, is the electrochemical analyses that were conducted, which makes this paper really unique. There are a few major issues that I have, and specific comments are below.

We would like to thank the reviewer for going through our manuscript and providing useful suggestions to help us to improve the manuscript. We also appreciate the overall positive response and in particular his/her appreciation of the electrochemical analyses of the SOM extracts. We will go through the manuscript carefully and incorporate all suggestions and comments.

1. The SUVA data seems fine for the water extracted SOM and fall well within the range of values reported by others (e.g., Weishaar et al., 2003). However, the FA and HA alkaline extraction conducted anoxically were off the charts and many factors higher than the highest value reported by Weishaar et al., 2003. These numbers appear unrealistic and could be due to the presence of iron (both (II) and (III)) in the extracts that reached 3 mM. Given that Weishaar et al., reported iron interference (they use Fe(III) as an example, but noted that Fe(II) can also interfere) at levels of only a few mg/L (or 10's of μM) this could be a positive interference to their SUVA data.

We agree with the reviewer that the SUVA_{254} values measured in our study, especially for the HA isolated from the water-extracted OM ($0.207 \text{ L mg}^{-1} \text{ C cm}^{-1}$), are almost one order of magnitude higher than the reported SUVA_{254} values for HA chemically extracted from Coal Creek

soil in Weishaar et al., 2003 ($0.039 \text{ mg}^{-1} \text{ C cm}^{-1}$) (Weishaar et al., 2003), and also higher than the typical SUVA_{254} values of HA analyzed in many other studies (Beckett et al., 1987; Chen et al., 2003; Fox et al., 2017). However, the SUVA_{254} of all our FA extracts range from 0.017 to $0.042 \text{ L mg}^{-1} \text{ C cm}^{-1}$, and these values are in line with previous studies (Beckett et al., 1987; Chen et al., 2003; Fox et al., 2017).

One reason for the higher SUVA_{254} values for the HA isolated from water-extracted SOM under anoxic conditions in our study compared to others, in addition to the differences in the soils from which the HA were extracted, as suggested by the reviewer, could be the presence of Fe(II) and Fe(III). As shown by Weishaar et al. (2003), the presence of 4 mg L^{-1} Fe(III) showed an absorbance value about 0.65 cm^{-1} at 254 nm wavelength, and the absorbance increased with increasing Fe(III) concentrations. Based on this study, we can hypothesize that also in our case the presence of Fe(III) influenced the measured SUVA_{254} value of the HA isolated from the water-extracted SOM under anoxic conditions. The Fe(III) concentration in the HA isolated from water-extracted SOM under anoxic condition was $33 \text{ } \mu\text{mol L}^{-1}$. Please note that we did not use the concentration of 3 mmol L^{-1} as suggested by the reviewer, because 3 mmol L^{-1} was the Fe(II) concentration determined right after the anoxic water extraction of SOM. However, all of the samples were passed through 0.45 mm syringe filters under oxic conditions before the SUVA analyses. Therefore, a large amount of the Fe(II) was oxidized to Fe(III) and removed as particulate Fe(III) by the filtration, as explained in the manuscript **line 208-211, page 7**. The remaining Fe(II) and Fe(III) in the samples were analyzed and shown in Table S3 and the Fe(III) concentration for HA isolated from the water-extracted SOM under anoxic condition was $33 \text{ } \mu\text{mol L}^{-1}$.

$$33 \text{ } \mu\text{mol L}^{-1} \times 56 \text{ g mol}^{-1} = 1.848 \text{ mg L}^{-1}$$

According to Weishaar et al. (2003), 1.848 mg L^{-1} Fe(III) has an absorbance value of 0.15 cm^{-1} at 254 nm wavelength. With the additional $28 \text{ } \mu\text{mol L}^{-1}$ Fe(II), the contribution of iron to the

measured SUVA₂₅₄ value should be even higher. Therefore, we would like to add one sentence in the results section 3.1, **line 212, page 7**, as “*A previous study showed that 4 mg L⁻¹ Fe(III) yielded an absorbance value of 0.65 cm⁻¹ at 254 nm wavelength (Weishaar et al., 2003). Therefore, we believe that the high SUVA₂₅₄ value of HA isolated from the water-extractable OM (please note, this is the term used to replace ‘water-extracted SOM’ as suggested by reviewer 1) compared to SUVA₂₅₄ values of HA shown in previous studies could be caused by the presence of Fe(II) and Fe(III) in the sample due to the microbial Fe(III) reduction that occurred under the anoxic extraction conditions.*”

2. There was only passing mention of the NMR and fluorescence data. Why wasn't this data more prominently discussed in the paper (as opposed to a glancing mention in the SI)? For example, how does the EEM data “confirm higher contents of aromatic carbon” (the explanation in the SI caption was inadequate)? Further, the relatively smaller differences in NMR determined aromaticity between anoxically extracted vs oxic extraction SOM is not reflected in the much larger (order of magnitude) spread observed for SUVA (see above). Further, the EEMs from Figure S2 look really odd and I suspect that this caused by the really high DOC levels used by the authors (100 mg/L!). At those levels inner-filter-effects will become dominant as the solution will be optically dense to the point where inner-filter corrections will likely no longer work. Typically, fluorescence EEMs are collected at much lower (nearly two orders of magnitude) DOC concentrations to minimize inner-filter-effects (see papers by Stedmon et al., in L and O). Thus, because the data is likely improperly collected I would simply eliminate it from the discussion.

We would like to thank the reviewer for the comments and suggestions. First, we would like to clarify that the concentration of all samples used for the EEM analysis was not 100 mg C L⁻¹. In the manuscript, **line 113, page 4**, it says “*Freeze-dried SOM/FA/HA powders were dissolved in Milli-Q water (pH 7) at a concentration of 100 mg C L⁻¹ and the solutions were agitated for 12 h at 300 rpm at room temperature, samples were then filtered through 0.45 mm syringe filter*

(mixed cellulose ester (MCE, Millipore, Germany). For fluorescence analyses, samples were prepared by stepwise dilution of extract solution with Milli-Q water (pH 7) until absorbance values of 0.300 at 254 nm wavelength were reached". Therefore, after the stepwise dilution, the final concentration of samples used for the EEM analyses was much lower than 100 mg C L⁻¹ and inner-filter effects can be neglected.

However, as the reviewer pointed out, we did not draw any conclusions directly from the EEM data. As also explained in the reply letter to reviewer 1, we did this because there are many debates about whether, to any extent, the EEM spectra can reflect the redox state and the aromaticity of the OM samples (Fimmen et al., 2007; Maurer et al., 2010). Since only very briefly mentioned the results of the EEM spectra and since leaving the EEMs out completely will not impact the conclusion of the manuscript at all, as suggested by the reviewer, we would like to completely remove the EEM results from our manuscript.

Regarding the NMR, as we commented already in the reply letter to reviewer 1, we would like to show in the revised manuscript the NMR spectra of all samples as Figure 2.

Finally, regarding the reviewer question "why the very distinct difference between different OM extracts as shown by the SUVA results could not be seen in the calculated aromaticity from NMR": in previous studies that applied NMR to characterize the aromaticity of OM, the differences between different OM samples are usually in the range of 10%. For example, in the study of Lorenz et al. (2006), seven different OM samples extracted from different sampling sites were analyzed, and the aromaticity of these samples ranged from 21-32%. Inbar and co-authors compared the aromaticity of native SOM and the same SOM after 147 days of composting, and the aromaticity only changed from 35% to 37% (Inbar et al., 1990). In our study, all of the OM extracts, although extracted in different ways, were from the same soil. Therefore, we believe that, for example, the 4% difference in the aromaticity between chemically-extracted HA under oxic and chemically-extracted HA under anoxic condition does indicate a potential difference in

the aromaticity of these two samples. Moreover, although the differences of the aromaticity among different OM samples calculated from NMR are not as significant as the differences of $SUVA_{254}$ values among different samples, the aromaticity of SOM/HA/FA extracted under anoxic conditions was higher than the aromaticity of SOM/HA/FA extracted under oxic conditions. Furthermore, under anoxic conditions, FA and HA isolated from the water-extracted SOM (water-extractable OM) both have higher aromaticity than the water-extractable OM itself. Therefore, our NMR results are perfectly in line with the $SUVA_{254}$ results and the electron exchange capacity analysis of the OM samples and can be used to support and strength our argument that the chemical extraction and the presence of oxygen impacts the aromaticity thus the redox activity of the SOM extracts.

3. I think the discussion regarding the comparison between Suwannee River reverse osmosis dissolved organic matter (DOM) to the fulvic acid fraction isolated by XAD-8 chromatography (as opposed to acid precipitation) does not add any value to the paper because you are basically comparing apples and oranges (i.e., SOM vs. aquatic DOM). The methods are totally different from alkaline and neutral extraction and there are no mineral phases involved. The authors can delete the entire discussion and it will not affect the conclusions or the quality of this paper.

Following the reviewer's suggestion, we would like to remove this part of the discussion:
line 326-343, page 11.

4. While the authors point to several studies demonstrating correlations between DOC and Fe(II) formed from the dissolution of iron oxides in batch incubation studies, evidence for this relationship has also been reported in benthic pore waters. See papers by Burdige (et al.), Chin (et al.), plus many others. I think showing that this phenomenon occurs in real aquatic systems strengthens the arguments put forth by the authors for this paper.

As suggested by the reviewer, to strengthen our argument of the correlation between DOC and Fe(III) mineral dissolution, we would like to add one sentence as follows (at **line 297, page 10**):

“In-situ monitoring of the DOC flux in pore water of marine sediment or freshwater wetland also suggested an increase in DOC with increasing amount of microbial iron(III) mineral reduction (Burdige et al., 1992; Burdige et al., 1999; Chin et al., 1998)”.

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Tuebingen, December 20th, 2019

Dear Denise,

Enclosed is our revised manuscript (bg-2019-308) entitled “High pH and anoxic conditions during soil organic matter extraction increases its electron exchange capacity and ability to stimulate microbial Fe(III) reduction by electron shuttling” for publication in *Biogeosciences*.

We appreciate the interest you and the reviewers have taken in our manuscript and the constructive suggestions they have given. We have incorporated all comments and advice in the revised manuscript. Specifically, we changed the title of the manuscript to make sure it reflects the main message of our study. As suggested by reviewer 2, the emission-excitation spectra (EEM) were removed from the manuscript. Instead, as suggested by you, we have added the NMR spectra in the manuscript.

We have also included a point-by-point response to the reviewers and the revised supporting information. Both manuscript and supporting information are available also as marked-up versions to show all the changes we made in the files.

Thank you very much for your consideration of our revised manuscript.

Yours sincerely,

Prof. Dr. Andreas Kappler

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High pH and anoxic conditions during soil organic matter extraction increases its electron exchange capacity and ability to stimulate microbial Fe(III) reduction by electron shuttling

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Abstract. Soil organic matter (SOM) is redox-active, can be microbially reduced, and transfers electrons in an abiotic reaction to Fe(III) minerals thus serving as an electron shuttle. The standard procedure to isolate organic matter (OM) from soil involves the use of alkaline and acidic solutions and the separation of humic acids (HA) and fulvic acids (FA). This process potentially leads to unwanted changes in SOM chemical and redox properties. To determine the effects of extraction conditions on the redox and electron shuttling properties of SOM extracts, we prepared HA, FA and water-extractable organic matter (OM) extracts applying either a combination of 0.1 M NaOH and 6 M HCl or ultrapure water (pH 7), from soil samples collected from the subsoil (0-15 cm, A horizon, pH 6.5-6.8) in Schönbuch forest, Baden-Wuerttemberg, Germany. Both chemical extractions (NaOH/HCl) and water extractions were done in separate experiments under either oxic or anoxic conditions. Furthermore, we applied the NaOH/HCl treatment to a subsample of the water-extractable OM to separate HA and FA from the water-extractable OM. When comparing the amount of carbon extracted from soil by different extraction methods, we found that FA and HA chemically extracted from the soil can make up to 34-40% of the soil organic carbon pool while the water-extractable OM only represents 0.41-2.74% of the total soil organic carbon. The higher extraction efficiency of the chemical extraction is probably due to the deprotonation of carboxyl and phenol functional groups under high pH. Anoxic extraction condition also led to more extracted carbon. For water-extractable OM, 7 times more C was extracted under anoxic condition compared to oxic condition. This difference was probably due to the occurrence of microbial reduction and dissolution of Fe(III) minerals in the soil during the anoxic water extraction thus the concomitant release of Fe(III) mineral-bound organic matter. To compare the redox activity of different SOM extracts, the electron exchange capacity (EEC) of all extracted HA, FA and water-extractable OM was analyzed and our results showed that, under anoxic extraction condition, the HA chemically isolated from the water-extractable OM had 2 times higher EEC values compare to the water-extractable OM itself, suggesting the potential formation of redox-active aromatic functional groups during the extraction with NaOH under anoxic conditions by condensation reactions between amino acids, aldehydes, hydroxyl- and catechol-containing molecules. We also performed a microbial Fe(III) reduction experiment with all extracts

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Gelöscht: a forest soil applying either a combination of 0.1 M NaOH and 6 M HCl, or water (pH 7)

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55 | and found that higher EEC of extracts in turn resulted in a higher stimulation of microbial Fe(III) mineral reduction by
electron shuttling, i.e. faster initial Fe(III) reduction rates, and in most cases also in higher reduction extents. Our findings
60 | suggest that OM extracted with water at neutral pH should be used to better reflect environmental SOM redox processes in
lab experiments and that potential artefacts of the chemical extraction method and anoxic extraction condition need to be
considered when evaluating and comparing abiotic and microbial SOM redox processes.

1 Introduction

65 | Soil organic matter (SOM) contains more organic carbon than the sum of the atmosphere and living plants (Fischlin, 2007)
and can influence greenhouse gas emission, plant growth and water quality (Lal, 2004; Marin-Spiotta et al., 2014). Studying
SOM is challenging because it needs to be separated from other soil components before doing laboratory experiments
(Lehmann and Kleber, 2015). One of the most commonly used methods is a chemical extraction of humic substances (HS) at
pH >12 (Achard, 1786). Although the concept of HS as large-molecular-weight molecules formed by degradation and
70 | repolymerization of biomolecules has been challenged by seeing SOM as a continuum of progressively decomposing organic
compounds (Lehmann and Kleber, 2015), HS extraction is still applied by many laboratories and the extracted HS are still
widely used as a proxy for SOM. Briefly, HS are extracted by adjusting the pH to >12 using NaOH, followed by
acidification of the alkaline extract to pH <2 to separate humic acids (HA) from fulvic acids (FA) (Achard, 1786). Ion
exchange resins, dialysis, and even hydrofluoric acid (HF) treatment are used to further purify the extracts (IHSS, 2017).
Concerns regarding the effectiveness of this harsh chemical extraction method were already raised in 1888 (van Bemmelen,
1888) and last until today (Lehmann and Kleber, 2015; Kleber and Lehmann, 2019).

75 | It has been shown that alkaline extraction influences the chemical composition and the content of redox-active quinoid
moieties of the extracted SOM (Piccolo, 1988; Engebretson and Von Wandruszka, 1999). Participation in redox reactions is
a key property of SOM and relevant for many biogeochemical processes in the environment (Murphy et al., 2014). For
example, under anoxic conditions, SOM can accept electrons from microorganisms, transfer electrons to other electron
acceptors such as Fe(III) minerals, and be reoxidized to accept electrons again from microorganisms (Lovley et al., 1996;
Kappler et al., 2004; Bauer and Kappler, 2009; Wolf et al., 2009). This electron shuttling process, which is facilitated by
80 | SOM, can significantly increase microbial reduction rates of poorly soluble Fe(III) minerals (Lovley et al., 1996; Jiang and
Kappler, 2008), enable microbial reduction of otherwise inaccessible Fe(III) minerals (Lovley et al., 1998), and stimulate
indirect reduction of minerals that are spatially separated from the bacteria (Lies et al., 2005). Highly purified FA and HA
are used in most electron shuttling studies to represent SOM (Lovley et al., 1998; Lovley and Blunt-Harris, 1999; Lies et al.,
2005; Bauer and Kappler, 2009; Wolf et al., 2009; Klupfel et al., 2014). However, currently it is not known if and to which
85 | extent the SOM electron shuttling capacity is based on protocol-induced changes caused by the harsh chemical isolation

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Gelöscht: We found that soil extraction with NaOH lead to ca. 100 times more extracted C and the extracted HS had 2-3 times higher electron exchange capacities (EEC) than SOM extracted by water. For water-extracted SOM, anoxic extraction conditions lead to about 7 times more extracted C and 1.5 times higher EEC than under oxix extraction conditions. This difference was probably due to the occurrence of microbial reduction and dissolution of Fe(III) minerals in the soil during the water extraction at neutral pH and the concomitant release of Fe(III) mineral-bound organic matter. NaOH/HCl treatment of the water-extracted SOM lead to 2 times higher EEC values in the HA isolated from the SOM compared to the water-extracted SOM itself, suggesting the chemical treatment with NaOH and HCl caused changes of redox-active functional groups of the extracted organic compounds.

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procedure. [Therefore, studies that compare the stimulating effects of SOM extracted with either the traditional chemical extraction method or with water at neutral pH conditions on the microbial Fe\(III\) reduction are needed.](#)

Piepenbrock and co-authors [extracted SOM](#) from a forest soil at circumneutral pH using water (Piepenbrock et al., 2014).

10 Compared to chemically extracted Pahokee Peat humic acids (PPHA), the extracted SOM had a significantly lower reducing capacity ($\mu\text{eq/g C}$), which was calculated from the concentration of reduced Fe(II) after the abiotic reaction of PPHA/SOM with Fe(III)-citrate. This potentially indicates different types and proportions of functional groups in these samples. However, due to the different origin of the extracted soils, it remains unclear whether and to which extent the differences in reducing capacities of the SOM extract and PPHA was caused by the chemical extraction methods. Furthermore, in this
15 study the water extraction was conducted only under oxic conditions. Although it is known that the presence of O_2 causes oxidation of certain organic compounds under alkaline conditions and therefore chemical extraction with NaOH should be conducted under anoxic conditions (Bauer and Kappler, 2009; Maurer et al., 2010), it remains unclear whether and how the presence of O_2 influences the abundance of different (redox-active) functional groups and therefore the redox activity of the water-extracted organic compounds under neutral pH.

20 To determine the effect of these chemicals on the SOM redox properties, we extracted OM from a forest soil using several methods (Fig. 1). The first was the traditional chemical extraction method (1 M NaOH followed by 6 M HCl) yielding HA and FA under either oxic or anoxic conditions. The second was [OM extraction by ultrapure water at neutral pH \(water-extractable OM\)](#) under either oxic or anoxic conditions. Additionally, we treated the [water-extractable OM](#) with NaOH and HCl to further separate HA and FA from the [water-extractable OM](#) (also under either oxic or anoxic conditions). We
25 analyzed the electron accepting capacity (EAC, i.e. the number of electrons that can be accepted), the electron donating capacity (EDC, i.e. the number of electrons that can be donated by the [OM](#)) and the electron exchange capacity (the sum of EAC and EDC) of all extracted [water-extractable OM, FA and HA](#) fractions. [To further compare their electron shuttling capacity, we](#) performed a microbial Fe(III) mineral reduction experiment with all of the different extracts. The goals of this study were, first, to identify the effects of alkali and oxygen on the EEC values of the [water-extractable OM, FA and HA](#)
30 samples, and second, to compare the rates and extents of microbial ferrihydrite reduction in the presence of the different extracts.

2 Materials and methods

2.1 Soil organic matter (SOM) extraction

35 Top soil (0-15 cm) [without leaf litter from A horizon](#) was collected from the Schönbuch forest, Baden-Wuerttemberg, Germany. The forest is dominated by beech with populations of oak, spruce and bald cypress and the soil is qualified as vertic cambisol ([WORLD REFERENCE BASE FOR SOIL, WRB](#)) (WRB). Soil was dried (30°C), ground to pass through a

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Gelöscht: extracted SOM

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Gelöscht: world reference base for soil

2 mm sieve by automatic soil grinder (ball mill, FRITSCH, Germany) and stored in the dark at 4°C. Chemical extraction of FA/HA was modified from the IHSS protocol (IHSS, 2017) as follows. In the IHSS protocol, soil samples are incubated with 0.1 M HCl with a ratio of 10 mL liquid per g dry soil and after 1 h of extraction and XAD-8 resin separation, a fraction called "FA_extract_1" is collected. In our experiment, we did not extract this fraction to avoid using XAD-8 separation. As shown in Fig. 1, 100 g soil was incubated with 400 mL of 0.1 M NaOH (pH 12) for 4 h (overhead shaker, 90 rpm, 25°C). The slurry was centrifuged (3528 ×g, 30 min) and the supernatant was acidified (pH 2) by 6 M HCl to separate FA (dissolved in the supernatant) and HA (precipitated). Within the present study we define these extracts as FA (isolated from soil) and HA (isolated from soil). Water-extractable OM was prepared following Piepenbrock et al. (Piepenbrock et al., 2014) (Fig. 1). 100 g of soil were incubated with 400 mL of ultrapure water (resistivity=18.2 MΩ.cm, 25°C; Milli-Q, Millipore) at pH 7 (pH was monitored during the extraction and it ranged between 7.02-7.33). The pH of the water extraction was adjusted to 7 to avoid any possible artefacts resulting from further pH adjustment prior to analyses and experiments that require the sample pH to be 7 such as the electrochemical analysis or the microbial Fe(III) reduction. The slurry was centrifuged after 24 h; we collected and defined the supernatant as water-extractable OM. A 200 mL aliquot of the supernatant containing the water-extractable OM was amended with 0.1 M NaOH until pH 12. After 4 h of incubation, the pH of the solution was adjusted to <2 by 6 M HCl to precipitate HA and to separate FA, this FA and HA are defined as FA (isolated from water-extractable OM) and HA (isolated from water-extractable OM). Anoxic chemical extractions (NaOH/HCl) and water extractions were performed in a N₂-filled anoxic glovebox; Filtration (0.45 μm, polyethersulfone membrane (PES), Millipore, Germany) was used instead of centrifugation to remove remaining soil after the extraction. All collected extracts were freeze-dried under oxic conditions and stored (4°C, dark) until use.

2.2 Total organic carbon (TOC) quantification

TOC of water-extractable OM and FA isolated from soil was quantified directly from their extracted solutions after dilution, whereas the precipitated HA and FA isolated from the water-extractable OM were first freeze-dried and then re-dissolved in Milli-Q water (pH 7, stirring at 300 rpm overnight). All solutions were analyzed for TOC (TOC analyzer, model 2100S, Analytik Jena, Germany).

2.3 Nuclear magnetic resonance (NMR) measurements

NMR analysis was conducted from freeze-dried water-extractable OM, HA and FA samples. Solid-state ¹³C NMR spectra were obtained with a Bruker Avance III HD 400 MHz Wideboard operating at a frequency of 100.63 MHz using zirconium rotors of 4 mm OD with KEL-F-caps. The cross polarization magic angle spinning (CPMAS) technique was applied during magic-angle spinning of the rotor at 14 kHz. A ramped ¹H-pulse was used during a contact time of in order to circumvent spin modulation of Hartmann-Hahn conditions. A contact time of 1 ms and a 90° ¹H-pulse width of 2.2 μs were used for all

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Yuge Bai 16.12.2019 11:52	Gelöscht: SOM water extracts
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Yuge Bai 16.12.2019 11:47	Gelöscht: doubly-deionized
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Yuge Bai 16.12.2019 11:51	Gelöscht: water-extracted
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Yuge Bai 17.12.2019 12:02	Gelöscht: extracts
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spectra. The ¹³C-chemical shifts were calibrated to tetramethylsilane (0 ppm) and were calibrated with glycine (176.04 ppm). The aromaticity of samples were calculated following a previous study (Abelmann et al., 2005).

2.4 Specific UV absorbance at 254 (SUVA₂₅₄) analysis

SUVA₂₅₄ analysis was conducted from [water-extractable OM, HA and FA](#) solutions dissolved in Milli-Q water at concentrations of 10 mg C L⁻¹. All solutions were filtered with 0.45 mm syringe filter (polyethersulfone membrane (PES), Millipore, Germany) and the dissolved organic carbon (DOC) concentration of all samples was analyzed prior to the SUVA₂₅₄ analysis (DOC analyzer, model 2100S, Analytik Jena, Germany). The SUVA₂₅₄ values of all samples were measured in a 1 cm rectangular quartz cuvette with a fluorescence spectrophotometer (Fluoromax-4, Jobin-Yvon-SPEX instruments, New Jersey, USA). The final SUVA₂₅₄ values of all extracts were calculated with equation (1):

$$\text{SUVA}_{254} = \text{UV}_{254} / \text{DOC} \quad (1)$$

where UV₂₅₄ is the absorbance at 254 nm and 1 cm optical path length.

2.5 Microwave Plasma-Atomic Emission Spectrometer (MP-AES) analysis

[Metal contents were analyzed by Microwave Plasma-Atomic Emission Spectrometer \(MP-AES\) \(4100, Agilent Inc., Santa Clara, CA, USA\) in the extracted water-extractable OM, HA and FA samples. Prior to the MP-AES analysis, 0.5 g sample was digested with 10 mL 2% HNO₃ in microwave oven at 190°C \(800 w\) for 10 min, after cooling down to room temperature, centrifuged for 10 min at 14000 rpm and the supernatant was used for the analysis. The data for FA \(isolated from soil, oxic\) and HA \(isolated from water-extractable OM, oxic\) are missing due to the lack of enough samples. The unit of all metal concentrations is mg kg⁻¹, blank means the concentration of the corresponding metal is too low to be detected.](#)

2.6 Electrochemical analysis

Electrochemical analysis followed the method described by Aeschbacher et al (Aeschbacher et al., 2010). Freeze-dried extracts (powders) were dissolved in 100 mM of phosphate buffer (pH 7) at a concentration of 100 mg C L⁻¹. After overnight agitation at 300 rpm at room temperature, samples were [filtered](#) through 0.22 μm syringe filters (mixed cellulose ester (MCE), Millipore, Germany). All preparations and measurements were conducted in an anoxic glovebox. The number of electrons transferred to and from all extracts were quantified by integration of reductive and oxidative current responses after baseline correction in mediated electrochemical reduction (MER; at E_h = -0.49 V) and mediated electrochemical oxidation (MEO; E_h = +0.61 V) with 1'-ethylene-2,2'-bipyridyldiylum di-bromide (DQ) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as electron transfer mediators, respectively. To obtain the EAC and EDC values, the integrated current response was normalized to the measured DOC of all extracts prior to the EEC analysis (DOC_{SOM/FA/HA} [mg C/L], DOC analyzer, model 2100S, Analytik Jena, Germany) as shown in Eq. 2 and 3:

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45 $EAC = I_{red} dt / (F \times DOC_{SOM/FA/HA})$ (2)

$EDC = \int I_{ox} dt / F \times DOC_{SOM/FA/HA}$ (3)

where I_{red} and I_{ox} ([A]) are baseline-corrected reductive and oxidative currents in MER and MOR, respectively (F=Faraday constant).

2.7 Microbial Fe(III) reduction experiment and calculation of microbial Fe(III) reduction rates

50 Solutions of organic matter extracts for the microbial Fe(III) reduction experiment were prepared by dissolving freeze-dried powders in 50 mM phosphate buffer (pH 7.0-7.2) at 500 mg C L⁻¹, agitation overnight (300 rpm, room temperature), filtration and sterilization (0.22 µm syringe filters, mixed cellulose ester (MCE), Millipore, Germany), as described before (Jiang and Kappler, 2008). Although the chosen phosphate concentration is higher than typically observed in nature and can potentially lead to the formation of Fe(II) phosphate minerals (e.g., vivianite) during our microbial Fe(III) reduction experiment, this phosphate buffer was chosen to enable comparison of our study to previous studies (Jiang and Kappler, 2008; Bauer and Kappler, 2009; Klupfel et al., 2014; Piepenbrock et al., 2014). All solutions were deoxygenated 3 times (each time 3 min vacuum and 3 min N₂-flushing) and stored in dark bottles to avoid photochemical reactions. Ferrihydrite was prepared as described before (Amstaetter et al., 2012) and stored no more than 2 months (4°C) before use.

60 *Shewanella oneidensis* MR-1 cells from a frozen stock were streaked on oxyc lysogeny broth (LB) agar plates (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl and 15 g/L agar). Colonies were transferred to liquid LB medium and incubated at 30°C for 14 h, harvested by centrifugation (10 min, 8602 ×g) and then washed three times with anoxic SBM medium (0.225 g/L K₂HPO₄, 0.225 g/L KH₂PO₄, 0.46 g/L NaCl, 0.225 g/L (NH₄)₂SO₄, 2.18 g/L Na-lactate, 0.117 g/L MgSO₄•7H₂O, 2.38 g/L HEPES, pH 7.2-7.5). For the Fe(III) reduction experiments, washed cells were added at a final concentration of 10⁷ cells mL⁻¹ to solutions of water-extractable OM, FA, HA (50 mg C L⁻¹) and ferrihydrite (15 mM Fe(III)) in SBM medium (phosphate in the SBM medium was 5 mmol L⁻¹). AQDS, i.e. 2,6-anthraquinone disulphonate, a quinone model compound commonly used in electron shuttling studies that can significantly increase the rates of microbial Fe(III) reduction, was used as 100 µmol L⁻¹ in our experiments as a reference for a significant stimulation of Fe(III) reduction by our extracted OM via electron shuttling. The headspace was flushed with N₂ and the bottles were incubated in the dark (30°C). At each sampling point, an 100 µl aliquot was taken from each bottle, acidified and incubated with 900 µl of 1 M HCl for 1 h to facilitate mineral dissolution, centrifuged (28649 ×g, 5 min), and the total Fe(II) concentration was quantified with the spectrophotometric ferrozine assay in a microtiterplate (Stookey, 1970; Hegler et al., 2008). The fastest reduction rates of the microbial Fe(III) reduction experiments were calculated as shown and explained in the supporting information (SI) (Fig. S3).

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3 Results

3.1 Quantity of soil organic carbon extracted by different methods and characterization of extracted OM, HA and FA

Comparison of different extraction methods revealed that the amount of soil-extracted carbon varied depending on the presence/absence of O₂ during the extraction and on the type of extraction liquid (Tab. 1). Extraction with H₂O at neutral pH under oxic conditions followed by NaOH and HCl treatment to separate HA and FA in the water-extractable OM yielded 0.036, 0.021 and 0.014 g C in the water-extractable OM, FA and HA fractions, respectively, corresponding to 0.41, 0.24 and 0.15% of the total carbon present in the soil. In contrast, anoxic water extraction significantly increased the fraction of extracted carbon to 0.234, 0.146, and 0.079 g C in the water-extractable OM, FA and HA fractions, respectively, corresponding to 2.74, 1.69 and 0.90% of the total soil C. Chemical extraction directly from soil using NaOH and HCl under oxic conditions yielded 1.451 g in the extracted FA (17.0% of the total carbon present in the soil) and 1.450 g C in HA (17.0% of the total carbon present in the soil). Conducting the same chemical extraction from soil under anoxic conditions lead to a higher percentage of extracted carbon for FA (20.7%) and HA (22%) than under oxic conditions.

Specific UV absorbance at 254 nm (SUVA₂₅₄) indicates the aromaticity of the extracted OM, FA and HA (Tab. 1). Water-extractable OM showed a SUVA₂₅₄ value of 0.018 mg⁻¹ C cm⁻¹ under oxic conditions and the value increased to 0.027 mg⁻¹ C cm⁻¹ when extracted under anoxic conditions. Similarly, for both FA isolated from water-extractable OM and FA isolated from soil, the SUVA₂₅₄ values were higher under anoxic conditions than under oxic conditions. HA extracts isolated from the water-extractable OM under oxic conditions showed a SUVA₂₅₄ value of 0.068 mg⁻¹ C cm⁻¹, higher than the 0.018 mg⁻¹ C cm⁻¹ of the water-extractable OM itself obtained under oxic conditions. A higher SUVA₂₅₄ value for the HA isolated from the water-extractable OM (0.207 mg⁻¹ C cm⁻¹) than for the water-extractable OM itself (0.027 mg⁻¹ C cm⁻¹) was also found under anoxic extraction conditions. A 2-way ANOVA statistical analysis revealed that both oxic versus anoxic conditions and the extraction method (neutral pH water versus chemical extraction) resulted in different SUVA₂₅₄ values at a significance level of P<0.05 (Tab. S1). In general, anoxic conditions and the chemical extraction method led to higher SUVA₂₅₄ values of the extracts, suggesting that these extracts had higher degree of aromaticity (Korshin et al., 1997). ¹³C-NMR analysis of extracted OM, FA and HA (Fig. 2) confirmed higher contents of aromatic carbon in samples subject to chemical extraction or anoxic conditions.

Furthermore, after 24 h water extraction of OM, we found a maximum Fe(II) concentration of 3 mmol L⁻¹ in the water-extractable OM solution. Although, as shown in Tab. S3, more than 90% of the Fe was removed by filtration (0.22 μm, mixed cellulose ester (MCE), Millipore, Germany) and around 30% of the remaining Fe(II) was oxidized to Fe(III) during the oxic freeze drying process, there was still 15-123 μmol L⁻¹ total Fe present in the water-extractable OM and FA/HA isolated from it. This Fe is potentially redox-active and can contribute to the redox properties of the extracted OM. For example, A previous study showed that 4 mg L⁻¹ of Fe(III) yielded an absorbance value of 0.65 cm⁻¹ at 254 nm wavelength (Weishaar et al., 2003). Therefore, we believe that the high SUVA₂₅₄ value of HA isolated from the water-extractable OM

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Gelöscht: S

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Gelöscht: SOM ...ater-extractable OM ext... [2]

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Gelöscht: S...M, FA and HA (Tab. 1). SO... [3]

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Gelöscht: All HA extracts showed much higher SUVA₂₅₄ values than SOM and FA extracts, with the highest value of 0.265 mg⁻¹ C cm⁻¹ (HA chemically-extracted under anoxic conditions), followed by HA chemically isolated from the water-extracted SOM under oxic conditions (0.207 mg⁻¹ C cm⁻¹). For HA chemically isolated from the water-extracted SOM under anoxic conditions and HA chemically extracted from soil under oxic conditions, the SUVA₂₅₄ values are 0.068 and 0.083 mg⁻¹ C cm⁻¹, respectively

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Gelöscht: a... to higher SUVA₂₅₄ values of... [5]

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Gelöscht: S...M, we found a maximum Fe... [6]

75 compared to $SUVA_{254}$ values of HA shown in previous studies could be caused by the presence of Fe(II) and Fe(III) in the sample due to the microbial Fe(III) reduction that occurred under the anoxic extraction conditions. However, since this Fe is an integral part of the OM in the environment and we were interested in determining the role of environmentally relevant OM extracts in electron shuttling, we decided not to further purify the extracts (also because this probably would have changed the properties of the present redox-active organic matter).

3.2 Redox properties of extracted water-extractable OM, FA and HA

80 We used mediated electrochemical reduction and oxidation to quantify the EAC, EDC and thus the EEC of all water-extractable OM, FA, and HA extracts (Fig. 3). Based on the Fe content we calculated the contribution of the Fe to the redox properties of all extracts (Tab. S3). A 2-way ANOVA statistical analysis revealed that both the extraction condition (anoxic versus oxic) and the extraction method (neutral pH water versus NaOH) resulted in significantly different EEC values ($P < 0.05$; Tab. S4). The EEC of water-extractable OM obtained under oxic conditions was $32 \mu\text{mol e}^- \text{mmol C}^{-1}$ (with ca. $4 \mu\text{mol e}^- \text{mmol C}^{-1}$ from Fe), whereas when extracted anoxically, it increased to $44 \mu\text{mol e}^- \text{mmol C}^{-1}$ (with $14.8 \mu\text{mol e}^- \text{mmol C}^{-1}$ from Fe). Higher EEC values under anoxic compared to oxic extraction conditions were also observed for all extracted FA: for FA isolated oxically from the water-extractable OM, the EEC was $13 \mu\text{mol e}^- \text{mmol C}^{-1}$ ($2.3 \mu\text{mol e}^- \text{mmol C}^{-1}$ from Fe), while it increased to $24 \mu\text{mol e}^- \text{mmol C}^{-1}$ ($2.7 \mu\text{mol e}^- \text{mmol C}^{-1}$ from Fe) when FA was isolated anoxically from the water-extractable OM. The EEC of FA isolated from soil under anoxic conditions was $33 \mu\text{mol e}^- \text{mmol C}^{-1}$ higher than FA isolated from soil under oxic conditions. Similar to FA, for the HA isolated from water-extractable OM, the EEC values increased from $15 \mu\text{mol e}^- \text{mmol C}^{-1}$ ($1.9 \mu\text{mol e}^- \text{mmol C}^{-1}$ from Fe) under oxic conditions to $83 \mu\text{mol e}^- \text{mmol C}^{-1}$ ($7.3 \mu\text{mol e}^- \text{mmol C}^{-1}$ from Fe) under anoxic conditions. For HA isolated from soil, EEC values increased from $40 \mu\text{mol e}^- \text{mmol C}^{-1}$ under oxic conditions to $127 \mu\text{mol e}^- \text{mmol C}^{-1}$ under anoxic conditions.

95 The total number of electrons that can be exchanged (that means transferred from Fe(III)-reducing bacteria to the OM, or from the OM to Fe(III) minerals) by water-extractable OM before and after the chemical separation of FA and HA from this water-extractable OM was also calculated (the recovery of EEC) under both oxic and anoxic conditions (Fig. 2). For the extracts obtained under anoxic conditions, the sum of total exchangeable electrons values of the FA and HA isolated from water-extractable OM ($786 \mu\text{mol e}^-$) was almost identical to that of water-extractable OM itself ($836 \mu\text{mol e}^-$). In contrast, under oxic conditions, the sum of the EEC values of the FA and HA separated from the water-extractable OM was $324 \mu\text{mol e}^-$, ca. 5-times higher than the EEC value of the water-extractable OM ($64 \mu\text{mol e}^-$). This confirms that the traditional chemical extraction procedure conducted under oxic conditions strongly enhances the redox capacity of the samples.

00 In addition to the EEC that represents the total amount of electrons that can be stored by the extracted organic compounds, the individual EAC and EDC values can be used to characterize the redox state of the water-extractable OM, HA and FA.

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Gelöscht: SOM

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Gelöscht: SOM...ater-extractable OM, FA... [7]

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Gelöscht: ted...S...M before and after the... [8]

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Gelöscht: S

The EDC and EAC quantify how many electrons are already stored in the molecules (EDC) and how many electrons can still be taken up by functional groups that can be reduced (EAC) (Fig. 3). Surprisingly, the EAC values were larger for all FA and HA extracts obtained under anoxic extraction conditions than under oxic conditions (Fig. 3). The higher EAC under anoxic conditions suggests the presence of more functional groups that can be reduced in FA and HA extracted in the absence of oxygen, meaning that the additional amount of organic compounds that was extracted under anoxic conditions compared to oxic conditions contain more oxidized functional groups.

Yuge Bai 18.12.2019 15:06

Gelöscht: 2...). Surprisingly, the EAC val... [9]

3.3 Effects of different organic matter extracts on rate and extent of microbial ferrihydrite reduction and mineral transformation during reduction

To determine the effects of water-extractable OM, FA and HA extracts on microbial Fe(III) reduction, the Fe(III) mineral-ferrihydrite was incubated with the Fe(III)-reducing bacterium *Shewanella oneidensis* MR-1 in experiments amended with our extracts and total Fe(II) concentration was monitored over time (Fig. 4). The highest initial microbial Fe(III) reduction rates were determined as shown in the supporting information (Fig. S3). The presence of AQDS stimulated ferrihydrite reduction to Fe(II) with a maximum reduction rate of 3.12 ± 0.38 mmol Fe(II) d⁻¹ compared to experiments without electron shuttle with a rate of 0.79 ± 0.31 mmol Fe(II) d⁻¹ (Fig. 4(a), Fig. S3). The observed decrease of total Fe(II) after 5 days of incubation (from 14.67 mM to 6.87 mM) in the AQDS-amended setup was caused by Fe(II) loss due to sorption of Fe(II) or precipitation of Fe(II) (e.g. as Fe(II)-phosphate mineral due to the presence of phosphate buffer) at the wall of the glass bottles (Fig. S1). After the addition of oxically and anoxically water-extractable OM (Fig. 4(d)), Fe(III) was reduced at maximum rates of 1.53 ± 0.20 mmol Fe(II) d⁻¹ and 2.07 ± 0.43 mmol Fe(II) d⁻¹, respectively, suggesting higher reduction rates than without any electron shuttle (0.79 ± 0.31 mmol Fe(II) d⁻¹).

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Gelöscht: SOM...ater-extractable OM, F... [10]

Yuge Bai 16.12.2019 12:18

[1] nach oben verschoben: AQDS, i.e. 2,6-anthraquinone disulphonate, a quinone model compound commonly used in electron shuttling studies that can significantly increase the extent of microbial Fe(III) reduction, was used as a reference for a significant stimulation of Fe(III) reduction by our extracted OM via electron shuttling.

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Gelöscht: S4...3). The presence of AQDS... [11]

When comparing Fe(III) reduction in the presence of the different HA extracts (Fig. 4(e), 3(f)), we found that amendment with HA isolated from soil under anoxic conditions showed the fastest reduction rate (1.83 ± 0.03 mmol Fe(II) d⁻¹) followed by HA isolated from water-extractable OM anoxically (1.70 ± 0.25 mmol Fe(II) d⁻¹) and HA isolated from soil oxically (1.55 ± 0.08 mmol Fe(II) d⁻¹). The reduction rate of the experiment amended with HA isolated oxically from the water-extractable OM was 0.82 ± 0.27 showing slight stimulation effect compare to the setup without electron shuttle (0.79 ± 0.31 mmol Fe(II) d⁻¹). Addition of FA increased Fe(III) reduction rates significantly in all cases (Fig. 4(b), 3(c)). In the presence of FA isolated oxically and anoxically from water-extractable OM, the fastest rates were 2.03 ± 0.54 and 2.22 ± 0.36 mmol Fe(II) d⁻¹, respectively. After addition of FA isolated from soil under oxic and anoxic conditions, the maximum reduction rates were even faster with 2.31 ± 0.15 and 3.05 ± 0.07 mmol Fe(II) d⁻¹. Control samples with only OM and ferrihydrite (without bacteria) did not show any ferrihydrite reduction (Fig. S2).

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Gelöscht: 3...(e), 3(f)), we found that am... [12]

In addition to differences in reduction rates depending on the identity of the added organic extract, we also found differences in reduction extents. In most cases, the reduction extent was higher in the presence of OM compared to OM-free experiments

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Gelöscht: setups

15 (3.87 mM of Fe(II) after 15 days). Specifically, [experiments](#) amended with FA showed higher microbial Fe(III) reduction extents than with HA. After 25 days of incubation, [experiments](#) with FA extracted chemically from soil under anoxic conditions reduced 10.87 mmol L⁻¹ Fe(III) to Fe(II), while the maximum Fe(III) reduction extent in the presence of added HA (chemically isolated from anoxically water-extracted SOM) was about 7.08 mmol L⁻¹ Fe(II) (Fig. [4\(c\)](#) and (e)).

20 Since the used OM extracts contained some Fe(II) and Fe(III), we evaluated the contribution of these ions to the observed Fe(III) reduction (Tab. S3). First, the Fe(II) present in the water-extractable OM, FA and HA ranged from 7.2 (FA isolated from water-extractable OM, oxic) to 79.2 μmol L⁻¹ (water-extractable OM, anoxic) (Tab. S3) and made up between 1-17.6% of the measured Fe(II) concentration after 30 minutes of incubation. With the increase of Fe(II) concentration over time, the percentage of Fe(II) present in the extracts to the measured Fe(II) concentration decreased to less than 0.1% and is therefore negligible. Second, the influence of Fe(III) initially present in the water-extracted SOM, FA and HA (Tab. S3) can be neglected as well, because the Fe(III) concentration of the extracted organic matter fractions ranged from 8.7-43.9 μmol L⁻¹

25 (Tab. S3), but the ferrihydrite concentration used in the [experiments](#) was 15 mmol L⁻¹.

4 Discussion

4.1 Effects of the presence of oxygen on the amount and properties of SOM extracts

30 The presence and absence of oxygen impacted the amount of water-extractable OM. Under anoxic conditions, water at neutral pH extracted about 6.7 times more organic carbon than under oxic conditions (Tab. 1). The presence of Fe(II) at the end of extraction in all anoxic extracts suggested that the higher amount of extracted OM is probably related to microbial Fe(III) mineral reduction and the release of mineral-bound OM during mineral dissolution. A correlation between the dissolved organic carbon (DOC) concentration and the amount of Fe(II) in pore water was reported before for sediment samples that were incubated in the dark under anoxic conditions for 14 days (Dadi et al., 2017). [In-situ monitoring of the DOC flux in pore water of marine sediment or freshwater wetland also suggested an increase in DOC with increasing microbial iron\(III\) mineral reduction \(Burdige et al., 1992; Burdige et al., 1999; Chin et al., 1998\)](#). Other studies also suggested an increase in DOC under anoxic conditions due to the microbial iron(III) mineral reduction and dissolution and the concomitant release of organic carbon (OC) that was co-precipitated with and adsorbed to the iron(III) minerals (Gu et al., 1994; Riedel et al., 2013; Shimizu et al., 2013).

40 In addition to differences in the amount of extracted OM, the presence or absence of oxygen also influenced the aromaticity of the extracted SOM, as shown by the SUVA₂₅₄ values (Tab. 1) and ¹³C-NMR data (Fig. 2). Water-extractable OM extracted under anoxic conditions showed a higher aromaticity, suggesting that the additional organic matter mobilized by reductive dissolution of iron minerals possesses a higher degree of aromaticity. This is in line with findings described by other studies (Gu et al., 1994; Lv et al., 2016; Avneri-Katz et al., 2017; Coward et al., 2019). Kothawala and co-authors

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Gelöscht: setups

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Gelöscht: setups

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Gelöscht: 3

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Gelöscht: extracted

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Gelöscht: S

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Gelöscht: water-extracted

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Gelöscht: SOM, water-extracted

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Gelöscht: 2

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Gelöscht: setups

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Gelöscht: S

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Gelöscht: , the EEMs, (Fig. S1)

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Gelöscht: to some extent also by the

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Gelöscht: Tab. S2

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Gelöscht: The SOM

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Gelöscht: anoxically

60 (Kothawala et al., 2012) incubated oxically-extracted soil solution with soils with different mineral composition. SUVA and fluorescence index analysis of the remaining non-sorbed organic matter showed that regardless of the soil type, the aromatic functional groups were preferentially adsorbed to the soil minerals.

4.2 Effect of extraction pH on the amount and properties of extracted organic matter

65 The practice of extracting and isolating HA and FA using NaOH and HCl under anoxic conditions has been the established standard protocol (IHSS, 2017). As early as in 1972, Swift and Posner (Swift and Posner, 1972) showed that by incubating a peat HA with 1 M NaOH under oxic conditions for 30 days, more than half of the HA was degraded to low-molecular-weight molecules and amino acid N was lost from the HA. Later studies also reported the hydrolysis of esters in NOM to carboxylic acid groups when exposing NOM to NaOH under oxic conditions (Ritchie and Perdue, 2008). Consistent with previous studies, our SUVA₂₅₄ (Tab. 1), ¹³C-NMR (Fig. 2) and EEC (Fig. 3) results showed that FA and HA isolated from soil under oxic conditions had lower aromaticity and EEC compared to the FA and HA isolated from soil under anoxic conditions. This indicates that degradation of aromatic structures and functional groups in the OM to smaller molecules occurs in the presence of O₂ under higher pH conditions.

70 However, we found that even under anoxic conditions, the chemical extraction extracted up to 100 times more carbon than the water extraction at neutral pH (Tab. 1), consistent with previous studies (Aiken, 1985). This higher extraction efficiency at high pH could be due to the deprotonation of carboxyl and phenol functional groups leading to both higher aqueous solubility and electrostatic repulsion of OM from negatively charged soil minerals (Kleber et al., 2015) or due to the hydrolysis of plant material and the formation of smaller oligo- and monomers (Sparks, 2003). Not only the amount of C extracted but also the properties of the extracted FA and HA are affected by the chemical extraction under anoxic conditions.

75 Our results indicate that the HA isolated from soil have higher aromaticity than water-extractable OM under anoxic extraction conditions. On the one hand, the higher aromaticity in HA isolated from soil can probably be explained by the extra amount of C extracted from soil by the chemical extraction method. On the other hand, this cannot be the only explanation, since the HA isolated from the water-extractable OM also had higher aromaticity than the water-extractable OM itself. This suggests the formation of aromatic functional groups during the extraction with NaOH under anoxic conditions

80 by condensation reactions between amino acids, aldehydes, hydroxyl- and catechol-containing molecules. Such condensation reactions could result in larger molecules with a higher degree of aromaticity (Gieseking, 1975; Golchin et al., 1994; Kappler and Brune, 1999; Kappler and Haderlein, 2003). A recent study comparing OM extraction from a freshwater sediment using water (acidified to pH 2 with 1 M HCl), with an extraction using 0.1 M sodium pyrophosphate (pH 10) and 0.5 M NaOH (pH 12) also revealed a higher aromaticity in the alkali-extracted OM (Fox et al., 2017). Using Fourier-transform infrared spectroscopy (FTIR) and electrospray ionization Fourier-transform ion cyclotron resonance spectrometry (ESI-FTICR-MS), these authors showed that OM extracted by sodium pyrophosphate and NaOH had more condensed aromatic compounds.

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Gelöscht: Consistent with previous studies, our SUVA₂₅₄ (Tab. 1), EEM (Fig. S1), ¹³C-NMR (Tab. S2) and EEC (Fig. 2) results showed lower aromaticity and EEC of chemically-extracted FA and HA under oxic conditions compared to chemically-extracted FA and HA extracted under anoxic conditions, indicating the degradation of aromatic structures and functional groups in the OM to smaller molecules in the presence of O₂ under high pH conditions

Yuge Bai 16.12.2019 13:38

Gelöscht: chemically-extracted

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Gelöscht: extracted

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Gelöscht: S

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Gelöscht: chemically-extracted

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Gelöscht: chemically separated

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Gelöscht: extracted

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Gelöscht: S

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Gelöscht: (water-extracted HA)

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Gelöscht: extracted

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Gelöscht: S

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Gelöscht: However, whether the chemically-extracted HA has higher aromaticity than water-extracted SOM due to the usage of NaOH has been questioned as well (Golchin et al., 1994; Olk et al., 2002). Recently, Olk and co-authors collected water samples from Suwannee River (Georgia, USA), extracted HA following the IHSS protocol and natural organic matter (NOM) by using the reverse osmosis (RO) method that includes no usage of chemicals. By using electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), they found that the molecular formulas of the HA and SOM extracts had a similarity of 66%. Therefore, they believed that very minor chemical modifications of NOM happened during the chemical isolation procedure (Olk et al., 2019). In contrast, in another stu... [13]

4.3 Electron exchange capacity (EEC) of soil extracts determines their ability to stimulate microbial Fe(III) reduction

Our data showed that the rates of microbial ferrihydrite reduction differed in the presence of different OM extracts. The observed differences in Fe(III) reduction rates can either be a result of the differences in OM redox activity (e.g. number and redox potentials of redox-active functional groups) and the resulting function of the OM as electron shuttle or due to different secondary mineral phases that can form during ferrihydrite reduction. However, a previous microbial Fe(III) mineral reduction study of ferrihydrite (5 mM Fe(III)) in the presence of 50 mg C L⁻¹ OM, 0.8 mM phosphate buffer and 2×10⁵ cells mL⁻¹ *Shewanella oneidensis* MR-1 showed no goethite or magnetite (based on ⁵⁷Fe-Moessbauer and XRD analysis) but vivianite as the major mineral phase produced (Amstaetter et al., 2012). The transformation of ferrihydrite to vivianite instead of goethite or magnetite in presence of phosphate buffer was also reported in other studies using similar concentration of OM, buffer, cells and ferrihydrite (Chen et al., 2003a; Piepenbrock et al., 2011; Shimizu et al., 2013). The formation of more crystalline secondary mineral phases such as goethite was only observed during ferrihydrite reduction in the absence of phosphate (Hansel et al., 2003; Borch et al., 2007). Abiotic experiments showed that phosphate inhibits the transformation of ferrihydrite to magnetite or goethite by blocking of surface sites of ferrihydrite, therefore prevents the sorption of the produced Fe(II) on the Fe(III) mineral, thus lowering the number of surface sites where conversion of ferrihydrite to magnetite or goethite can take place (Galvez et al., 1999). Therefore, the transformation of ferrihydrite to magnetite or goethite is not expected to happen in our experiments and the following discussion will focus on the influence of the redox activity of the extracted OM on the rate and extent of the microbial Fe(III) reduction.

As measures for the redox activity of the different extracted OM fractions, we determined their potential for accepting electrons (EAC) and for accepting and donating electrons (EEC). Correlating the EAC values of our different OM extracts and the maximum microbial ferrihydrite reduction rates showed that the higher the EAC values of the extracted OM, FA and HA, the faster the microbial Fe(III) reduction rates are (Fig. 5). As shown before (Aeschbacher et al., 2012), (hydro)quinone functional groups contribute mainly to the measured EAC values in OM and these quinone moieties are thought to be the major functional group responsible for electron transfer between Fe(III)-reducing bacteria and Fe(III) minerals during electron shuttling. Scott et al. (1998) reported a direct correlation between OM oxidation capacity and the stable free-radical content in the OM, stemming from semiquinone radicals (Lovley et al., 1996; Scott et al., 1998). However, we also found a correlation between EEC values and the maximum microbial ferrihydrite reduction rates in the presence of water-extractable OM, HA and FA. Since higher EEC values reflects higher contents of aromatic/polycondensed aromatic compounds in the OM (Aeschbacher et al., 2012), our results also indicate that, apart from quinones, also other aromatic functional groups were involved in the microbial Fe(III) reduction with OM as electron shuttles and these functional groups also influence the electron transfer efficiency between the Fe(III)-reducing bacteria, the OM and the Fe(III) minerals. Support for the

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Gelöscht: 5 mM

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Gelöscht: SOM

participation of non-quinone groups in such OM electron transfer studies also comes from previous analyses of redox properties and stable free-radical concentrations in OM (Struyk and Sposito, 2001; Chen et al., 2003a).

10 Faster Fe(III) mineral reduction rates in the presence of more aromatic functional groups (including quinones) was demonstrated previously in experiments with increasing concentrations of AQDS or HA (Jiang and Kappler, 2008; Wolf et al., 2009; Glasser et al., 2017). It was suggested that the microbial turnover of substrate (lactate as electron source) is limited by the availability of the electron acceptor, i.e. either by the Fe(III) in the absence of shuttles or by the OM when OM serves as electron shuttling compound (Jiang and Kappler, 2008; Poggenburg et al., 2018). Thus, with the same concentration of OM electron shuttle, the OM with more redox-active functional groups can accept more electrons per time from the microorganisms, therefore resulting in higher Fe(III) reduction rates. Additionally, when more quinone or other redox-active
15 functional groups are present per shuttle molecule, the distance between redox-active functional groups is smaller, therefore, electron transfer within the shuttle molecule and between the shuttle molecules can occur faster, thus further increasing the electron transfer rate from the microbial cells to the shuttle molecules and further to the Fe(III) minerals (Boyd et al., 2015; Glasser et al., 2017). The different types and proportions of functional groups in the different OM extracts may also influence their adsorption onto the ferrihydrite surface, and therefore also impact the rates of microbial ferrihydrite reduction amended with different OM. However, due to the high concentration of lactate and HEPES buffer in our experiment, we could not quantify the amount of adsorbed OM vs. dissolved OM. It has to be noted, however, that in our extracted OM (Fig. 2) different amounts of redox-active Fe ions were present and that the redox-active OM-bound Fe can potentially also influence the rates of Fe(III) mineral reduction. The OM-bound Fe(III) can also be reduced by the Fe(III)-reducing bacteria or by reduced organic functional groups in the OM to Fe(II), which can then transfer electrons further to the ferrihydrite. The
20 OM-bound Fe is subsequently reoxidized to Fe(III), and therefore contributes to electron shuttling between Fe(III)-reducing bacteria and ferrihydrite.

In addition to the differences in the reduction rates, we also observed that the extent of ferrihydrite reduction was influenced by the presence of different OM. Specifically, amendments with HA lead to lower extents of Fe(III) reduction than FA amendments. This difference could be caused by the higher content of aromatic functional groups in HA than in FA and the resulting differences in sorption properties. OM with higher aromaticity and larger molecular weight was shown to have a
30 higher adsorption affinity to ferrihydrite (Lv et al., 2016; Coward et al., 2019). Since our HA extracted under all conditions were more aromatic than the FA, the HA were probably preferentially adsorbed to ferrihydrite. On the one hand, the sorbed HA can block surface sites on the minerals and restrict the accessibility for bacteria. On the other hand, HA adsorption changes the net surface charge of ferrihydrite from positive to (partially) negative and thus leads to repulsion of negatively charged cells (Aeschbacher et al., 2012).
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Gelöscht: adsorption

5 Conclusions

In summary, our results clearly show that the extraction method determines the concentration of redox-active (aromatic) functional groups and the EEC of the soil extracts and the EEC is a key factor for the electron shuttling capacity of soil extracts in microbial Fe(III) mineral reduction. Therefore, it has to be carefully decided which SOM extraction method to apply and which SOM fraction to use in biogeochemical experiments to obtain soil extracts that can represent natural SOM. Representative SOM is necessary to obtain meaningful results that will prevent overestimating the reactivity of SOM in redox processes in the environment. Based on our experimental results we suggest that firstly, the NaOH extraction method should be avoided in general because it alters the chemical and redox properties of SOM. Additionally, soil pH values typically range from 3.5-8.5 (Sparks, 2003), therefore the organic matter that is soluble only at pH>12 will not be dissolved under in-situ soil conditions and might react differently in biogeochemical processes compared to solid-phase soil OM (Roden et al., 2010; Kappler et al., 2014). Secondly, when extracting SOM with water at neutral pH, the redox milieu (oxic or anoxic) during extraction needs to be carefully controlled. When targeting oxic environmental systems with the goal of obtaining relevant OM matter that participates in biogeochemical processes under such redox conditions, short extraction times (<24 h), small batches, aeration, and thorough stirring is recommended for the OM extraction. Thus, anoxic conditions during the OM extraction should be avoided that would lead to reductive dissolution of iron minerals with concomitant mobilization of OM that would not be available under oxic conditions (in the absence of microbial Fe(III) reduction). However, in case the target environmental systems undergo redox fluctuations or even permanent reducing conditions, yielding anoxic conditions with microbial Fe(III) mineral reduction during the OM extraction is appropriate.

Data availability. Raw data of all results presented in this study are available [DOI: 10.1594/PANGAEA.904416](https://doi.org/10.1594/PANGAEA.904416).

Supplement. Supporting information includes the statistical analysis of the specific UV absorbance at 254 nm (SUVA₂₅₄) data, the ¹³C-NMR analysis, the Fe(II) and Fe(III) content and their contribution to the electron exchange capacity (EEC) of the OM extracts, the statistical analysis of the EEC data, the fluorescence excitation-emission (EEM), the picture showing the sorption and precipitation of Fe(II) on the wall of glass bottles of the microbial Fe(III) reduction experiments containing AQDS, the results of the abiotic reduction of Fe(III) minerals with the addition of OM extracts, and the calculation of the rates of microbial Fe(III) reduction amended with different OM extracts. The supporting information is available online at XXX.

Author contribution. YB, AK, SH designed the experiment, YB conducted the experiments and analyzed the data of OM extraction, fluorescence spectra and microbial Fe(III) reduction together with AK. ES performed and analyzed data of the electron chemical analysis and helped with the fluorescence spectra. HK conducted the NMR analysis and processed the data. YB and AK prepared the paper, with great help from SH.

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<https://issues.pangaea.de/browse/PDI-21233>.

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Table 1. Total dissolved organic carbon (DOC) concentration of the water-extractable OM and FA isolated from soil, total organic carbon in all extracted OM, HA and FA fractions, as well as the calculated percentage of carbon extracted from the soil. DOC of water-extractable OM and FA isolated from soil fractions was quantified directly from their extracted solutions, whereas the FA isolated from water-extractable OM and all of the HA extracts were first freeze-dried and then re-dissolved in Milli-Q water at pH 7 for analysis. The difference between total carbon (mg) of water-extractable SOM and the sum of total carbon (mg) of FA and HA isolated from the water-extractable OM (under oxic and anoxic conditions) is due to the loss of material during the isolation of FA and HA. Values are means±standard deviation (SD) of triplicates. An unpaired two-sided T-test was done to analyze the statistical significance of each pair of samples that was treated with the same extraction method but under different conditions (i.e., FA isolated from water-extractable OM, oxic, was compared to FA isolated from water-extractable OM, anoxic)

	Water-extractable OM		FA				^a HA			
	Water-extracted, oxic	Water-extracted, anoxic**	^a Isolated from water-extractable OM, oxic	^a Isolated from water-extractable OM, anoxic**	Isolated from soil, oxic	Isolated from soil, anoxic**	Isolated from water-extractable OM, oxic	Isolated from water-extractable OM, anoxic**	Isolated from soil, oxic	Isolated from soil, anoxic**
DOC concentration in extract (mg C L ⁻¹)	0.149±0.036	0.890±0.041	-	-	5.800±0.025	6.320±0.071	-	-	-	-
^b Total organic carbon in extract (g)	0.036±0.012	0.234±0.015	0.021±0.002	0.146±0.013	1.451±0.008	1.770±0.028	0.014±0.003	0.079±0.000	1.450±0.002	1.881±0.029
^c Percentage of carbon extracted from soil (%)	0.41±0.14	2.74±0.18	0.24±0.02	1.69±0.15	17.0±0.09	20.7±0.32	0.15±0.03	0.90±0.00	17.0±0.02	22.0±0.34
^d SUVA ₂₅₄ (mg ⁻¹ C cm ⁻¹)	0.018	0.027	0.017	0.029	0.023	0.042	0.068	0.207	0.083	0.265

^aTOC of all HA extracts and of the FA isolated from the water-extractable OM was directly quantified from the freeze-dried powders. The carbon content of all other liquid samples was determined as DOC, measured directly from the solutions after passing through 0.45 µm syringe filter

^bTotal organic carbon content in the extract was directly quantified from the freeze-dried samples of FA isolated from the water-extractable OM (oxic, anoxic) and of the HA extracts".

For the other extract solutions, the total organic carbon was calculated by DOC (mg C L⁻¹) × volume of the extracted solution (L).

^cPercentage of carbon extracted from soil = Total carbon extracted (mg g⁻¹)/soil carbon content (8.54 mg g⁻¹)

^dSpecific UV absorbance 254 nm (SUVA₂₅₄) = UV₂₅₄ × DOC (mg C L⁻¹). b is the optical path length in centimeter (1 cm in this experiment)

**The percentage of carbon extracted from soil of the samples are significantly different (n=2, two-sided t-test, P < 0.05)

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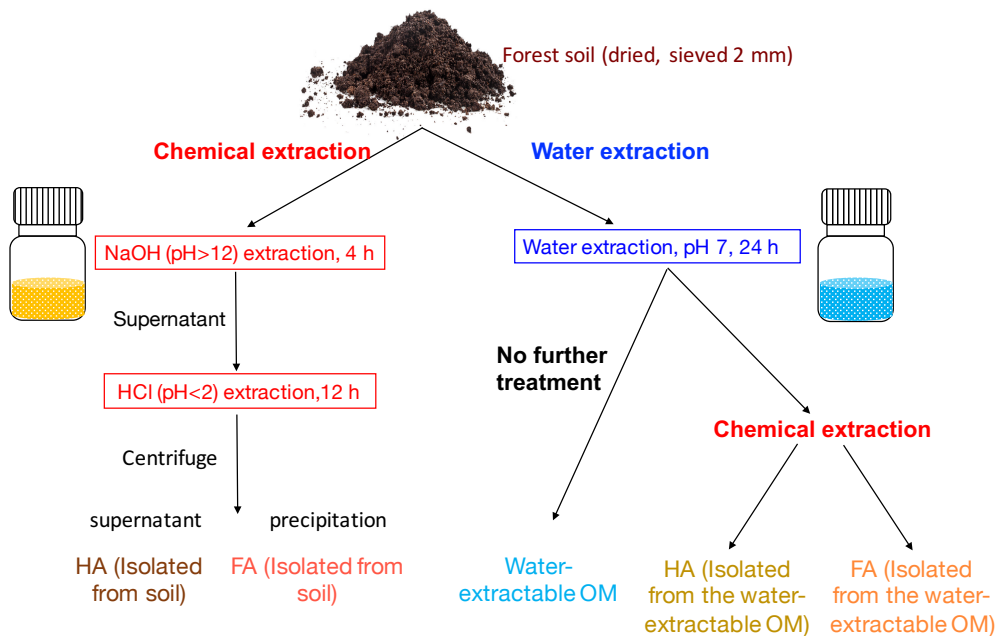
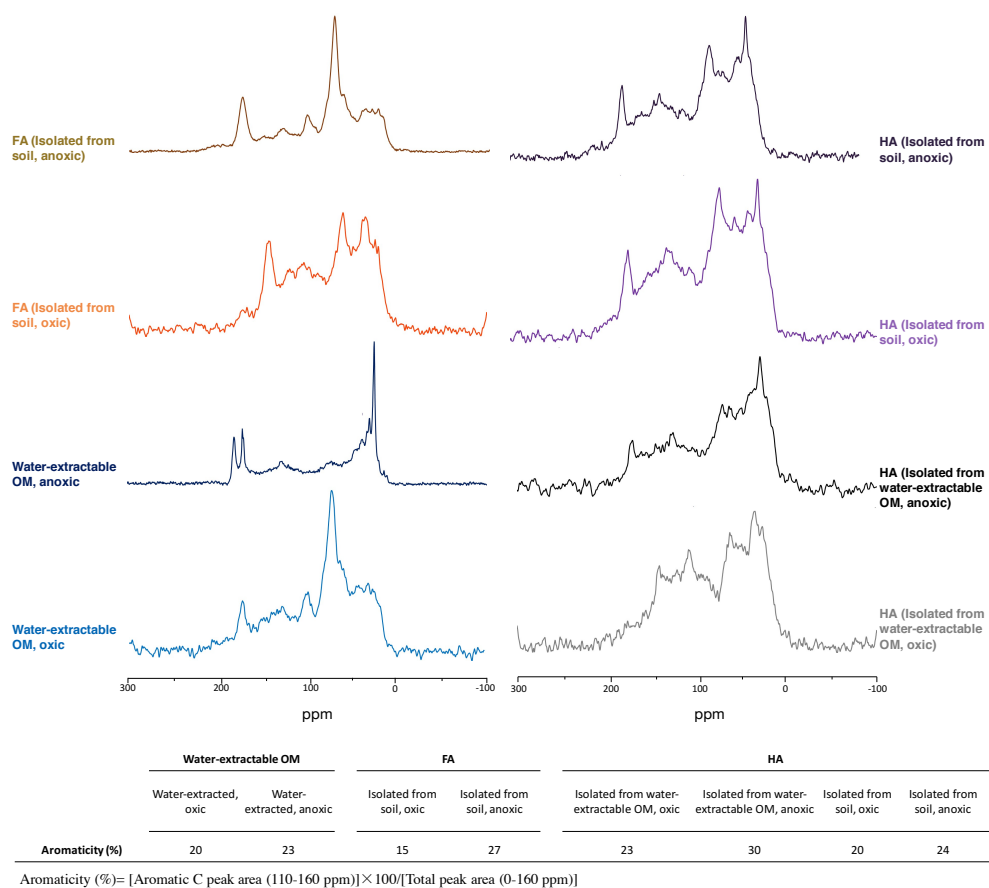


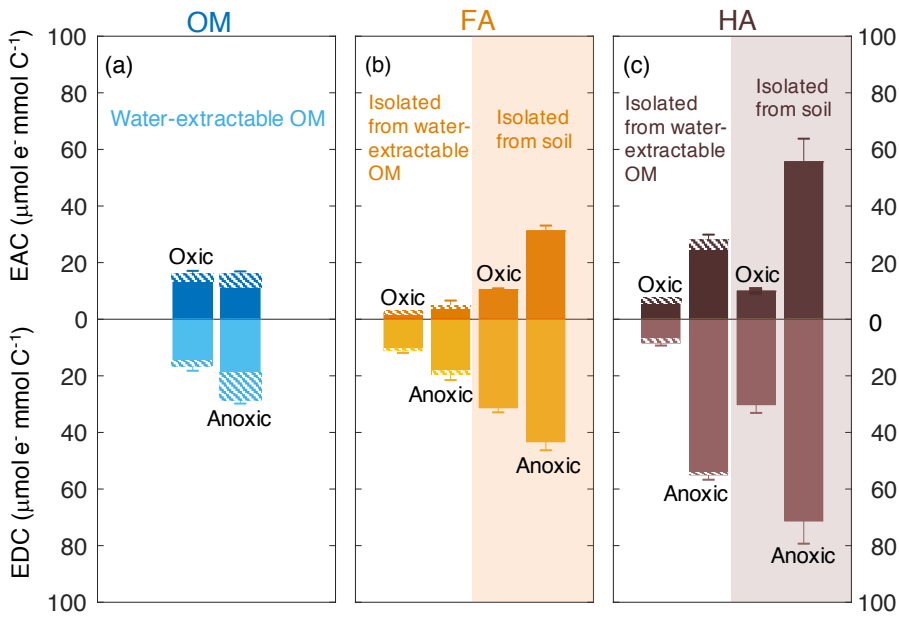
Figure 1. Schematic diagram of soil organic matter (SOM) extraction process. Forest soil samples (Schönbuch forest, Baden-Wuerttemberg, Germany) were dried under 30°C and ground to pass 2 mm sieve. To isolate FA and HA from soil, 100 g soil was incubated with 400 mL 0.1 M NaOH (pH 12) for 4 h, after centrifugation, the supernatant was acidified by HCl to pH<2, HA was then precipitated out to be separated from FA, we define these two extracts as HA (isolated from soil) and FA (isolated from soil). For water extraction, 100 g soil was incubated with 400 mL ultrapure water (<18.2 MΩ.cm; Milli-Q, Millipore) at pH 7. pH was monitored during the extraction and it remained stable (range between 7.02-7.33). The slurry was centrifuged after 24 h; we define the supernatant as water-extractable OM. A 200 mL aliquot of the supernatant containing the water-extractable OM was amended with 1 M NaOH until pH 12. After 4 h of incubation, the pH of the solution was adjusted to <2 by 6 M HCl to precipitate HA and to separate FA, and these two fractions are HA (isolated from water-extractable OM) and HA (isolated from water-extractable OM). All of the extractions were conducted under both oxic and anoxic conditions.

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Gelöscht: doubly-deionized
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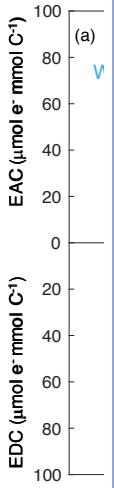
Aromaticity (%) = [Aromatic C peak area (110-160 ppm)] × 100 / [Total peak area (0-160 ppm)]

Figure 2. ^{13}C -NMR spectra of water-extractable OM, FA and HA. All spectra were collected from freeze-dried extracts and the aromaticity of samples was calculated as the percentage of aromatic C peak area to the total peak area. The relative intensity distributions of specific chemical shifts are shown in the supporting information, Tab. S2. Spectra for FA isolated from water-extractable OM cannot be presented due to the lack of an adequate amount of sample for analysis.



	EEC (mmol e ⁻ mmol C ⁻¹)	*Carbon content (mmol)	Total number of electrons that can be exchanged (mmol e ⁻)
FA (Isolated from water-extractable OM, oxic)	13	3	39
HA (Isolated from water-extractable OM, oxic)	15	19	285
SUM			324
Water-extractable OM, oxic	32	2	64
FA (Isolated from water-extractable OM, anoxic)	24	12	288
HA (Isolated from water-extractable OM, anoxic)	83	6	498
SUM			786
Water-extractable OM, anoxic	44	19	836

*carbon content was calculated from the total organic carbon content in extracts in Tab. 1



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Figure 3. Electron exchange capacity (EEC), the sum of EAC (electron accepting capacity) and EDC (electron donating capacity) of water-extractable OM(a), FA (b) and HA (c) extracted from Schönbuch forest soil under oxic or anoxic conditions. Areas with white background represent FA and HA isolated from water-extractable OM, whereas orange and purple shaded areas represent FA and HA isolated from soil, respectively. Hashed areas represent the EAC or EDC contribution stemming from redox-active Fe(III) and Fe(II) ions in the samples. The integrated current response was normalized to the DOC of all extracts. Despite the contribution of Fe to the redox activity, we decided to normalize the EAC/EDC values to C content first because in most cases the Fe contribution was small and second because normalizing the EAC/EDC values to total weight of material would be misleading since the fraction of inorganic, non-redox active constituents (e.g. Mg^{2+} , Ca^{2+} ions from salts present in the soil) varies significantly between the different extracts (Tab. S5). Error bars indicate the standard deviations of at least 4 replicates. 2-way ANOVA statistical analysis was conducted and the result suggested the measured EEC values of the extracts were significantly different from each other ($P < 0.005$, Tab. S4). The table underneath the figure shows the recovery of total number of electrons that can be exchanged (that means transferred from Fe(III)-reducing bacteria to the OM, or from the OM to Fe(III) minerals) by water-extractable OM before and after the chemical isolation of HA and FA.

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Gelöscht: (without further treatment in (a) and after NaOH and HCl treatment in (b) and (c), respectively)
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Gelöscht: green
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Gelöscht: chemically-extracted
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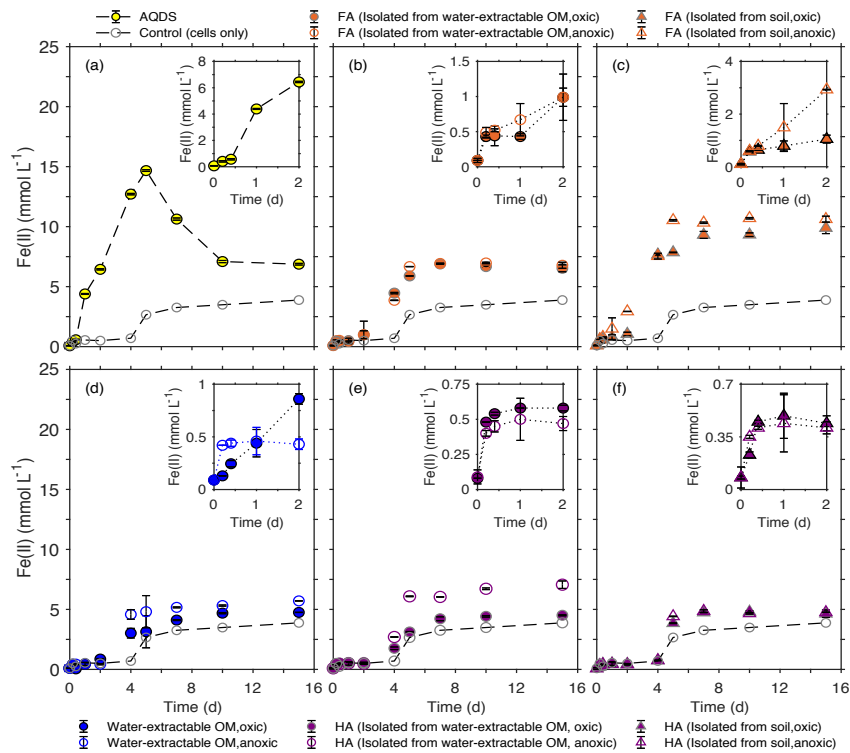
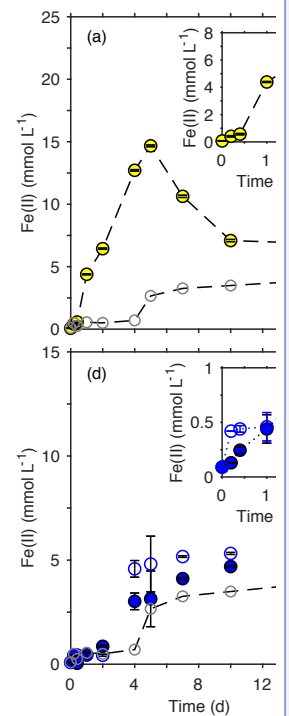


Figure 4. Microbial reduction of ferrihydrite (15 mmol L^{-1}) by *S. oneidensis* MR-1 ($10^7 \text{ cells mL}^{-1}$) in the presence of 15 mmol L^{-1} lactate as electron donor and 50 mg C L^{-1} FA (b, c), water-extractable OM (d), and HA (e, f) compared to $100 \text{ } \mu\text{mol L}^{-1}$ AQDS (a) presented as formation of total Fe(II) over time. The inserts in panels a-f show the data points for the first 2 days of incubation. All experiments were incubated in air-tight 100 mL glass serum bottles flushed with N_2 at 30°C in the dark. Control samples were incubated at the same condition in the absence of electron shuttles (ferrihydrite, lactate, and cells only). Error bars represent standard deviations of triplicate bottles.

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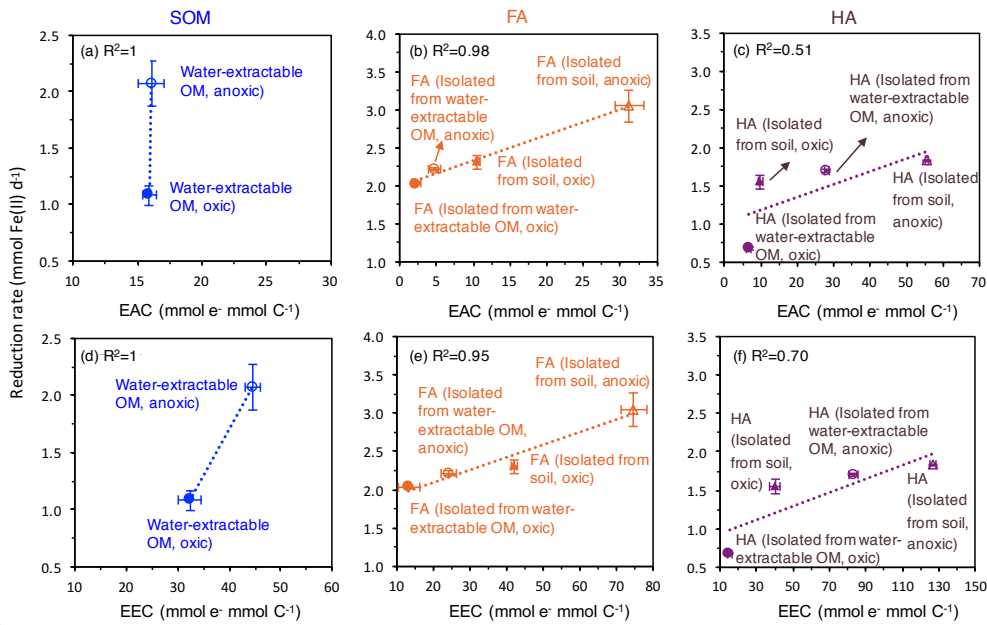


Figure 5. Correlation of the electron exchange capacity (EEC) (bottom) and electron accepting capacity (EAC) (top) with the fastest microbial ferrihydrite reduction rates (*Shewanella oneidensis* MR-1) in the presence of oxidically and anoxically prepared SOM (a), FA (b) and HA (c). EEC and EAC values are re-plotted from Fig. 2. Please note that the EEC and EAC values were determined by the electrochemical method described in the methods section and represent the contribution of both redox-active organic functional groups such as quinones and the redox-active Fe ions in the SOM, FA and HA extracts. Horizontal error bars represent standard deviations of the measured EEC values; vertical error bars are standard deviations of the reduction rates calculated as shown in Fig. S4.

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2.4 Fluorescence spectra and 2-D excitation-emission-matrices (EEMs)

The preparation of samples was modified from a previous study (Chen et al., 2003b). Freeze-dried SOM/FA/HA powders were dissolved in Milli-Q water (pH 7) at a concentration of 100 mg C L⁻¹ and the solutions were agitated for 12 h at 300 rpm at room temperature, samples were then filtered through 0.45 µm syringe filters (mixed cellulose ester (MCE), Millipore, Germany). For fluorescence analysis, samples were prepared by stepwise dilution of extract solutions with Milli-Q water (pH 7) until absorbance values of 0.300 at 254 nm wavelength were reached. 1.8 mL of sample were placed in a Suprasil UV quartz rectangular fluorescence cuvette; Milli-Q water was used as blank. All fluorescence spectra were recorded on a fluorescence spectrophotometer equipped with both excitation and emission monochromators (Fluoromax-4, Jobin-Yvon-SPEX instruments, New Jersey, USA). A 450 W Xenon arc lamp was used as the excitation source. A series of emission scans were collected over a range of excitation wavelengths to generate the fluorescence maps as 2-D excitation-emission-matrices (EEM). A wavelength step size of 10 nm was used for the collection of EEM spectra. The excitation and emission wavelength ranges were from 200-600 and 300-700 nm, respectively.

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Seite 11: [13] Gelöscht	Yuge Bai	16.12.19 13:40
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However, whether the chemically-extracted HA has higher aromaticity than water-extracted SOM due to the usage of NaOH has been questioned as well (Golchin et al., 1994; Olk et al., 2002). Recently, Olk and co-authors collected water samples from Suwannee River (Georgia, USA), extracted HA following the IHSS protocol and natural organic matter (NOM) by using the reverse osmosis (RO) method that includes no usage of chemicals. By using electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), they found that the molecular formulas of the HA and SOM extracts had a similarity of 66%. Therefore, they believed that very minor chemical modifications of NOM happened during the chemical isolation procedure (Olk et al., 2019). In contrast, in another study (Li et al., 2016), NOM was also extracted from Suwannee river using a styrene-divinylbenzene copolymer (PPL)-based solid phase extraction (SPE) (PPL-SPE_NOM) and the same RO method as used in Olk et al. (2019) (RO-NOM). ¹H-NMR analysis was conducted to compare the chemical structure of these two NOM extracts to the original Suwannee river samples, and it was found that the RO-NOM extracts had a remarkably higher abundance of aromatics and carboxyl-rich alicyclic molecules and less aliphatic groups comparing to the Suwannee river samples or the PPL-SPE-NOM extracts. These findings rise the question whether the RO extraction is selective to aromatic and alicyclic carbons. Finally, it is well known that aquatic NOM (e.g. from Suwannee river) differs a lot from soil NOM regarding the average molecular size and chemical structure (i.e., the aromaticity) (Chen et al., 2003a). Therefore, even if the HA chemically extracted from the Suwannee river had similar molecular properties than the Suwannee-river NOM itself, this still does not mean that in soil environments there is no significant differences in aromaticity between the differently extracted OM fractions.

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·TOC of all HA extracts and water-extracted FA were directly measured from the freeze-dried powders

·Total carbon (g) = TOC (g C L⁻¹) × volume of the extracted solution (L)

·Percentage of carbon extracted from soil = Total carbon extracted (mg g⁻¹)/soil carbon content (8.54 mg g⁻¹)

**The percentage of carbon extracted from soil of the samples are significantly different (n=2, two-sided t-test, P < 0.05)

·Specific UV absorbance 254 nm (SUVA₂₅₄) = UV₂₅₄ × DOC (mg C L⁻¹), b is the optical path length in centimeter (1 cm in this experiment)

High pH and anoxic conditions during soil organic matter extraction increases its electron exchange capacity and ability to stimulate microbial Fe(III) reduction by electron shuttling

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SUPPORTING INFORMATION

Number of tables in supporting information: 5

| Number of figures in supporting information: [3](#)

| Total number of pages of supporting information: [9](#) (including cover page)

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Gelöscht: Effects of extraction conditions on the redox properties of soil organic matter (SOM) and its ability to stimulate microbial iron(III) mineral reduction by electron shuttling

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Gelöscht: Effects of extraction conditions on the redox properties of soil organic matter (SOM) and its ability to stimulate microbial iron(III) mineral reduction by electron shuttling -

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Table S1. Two-way ANOVA statistical analysis of the measured specific UV absorbance values at 254 nm (SUVA₂₅₄) for all NOM extracts. The two-way ANOVA analysis was conducted using the SPSS software to test whether the oxalic extraction conditions, the extraction method, or both of them have significant impact on the SUVA₂₅₄ values of the extracts. P<0.05 indicates the rejection of the null hypotheses, meaning that the corresponding condition has significant influence on the SUVA₂₅₄.

^aDependent Variable: SUVA₂₅₄

Conditions	Sum of Squares	^b Degrees of freedom	^c Mean Square	^d F	P
Oxalic extraction conditions (oxalic vs. anoxic)	0.002	1	0.002	11.181 F (1.20) = 4.35	<0.05
Extraction method (water vs. chemical)	0.125	4	0.031	166.808 F (4.20) = 2.87	<0.05
Oxalic extraction conditions and extraction method	0.077	4	0.019	103.209 F (4.20) = 2.87	<0.05
Error (within group variances)	0.004	20	0.000 (0.00018)		

[†]Three null hypotheses of the two-way ANOVA analysis:

Hypothesis₁: the oxalic conditions do not have significant impact on the measured SUVA₂₅₄ values of the extracts

Hypothesis₂: the extraction method does not have significant impact on the measured SUVA₂₅₄ values of the extracts

Hypothesis₃: the oxalic conditions and the extraction method together do not have any significant impact on the measured SUVA₂₅₄ values of the extracts

[‡]Degree of Freedom was calculated based on the number of variables under each condition. For example, under oxalic extraction conditions, there are two variables, i.e., oxalic conditions or anoxic conditions, so the degree of freedom is 2-1=1. For the extraction method, there are five variables, i.e., water-extractable OM, FA isolated from water-extractable OM, FA isolated from soil, HA isolated from water-extractable OM, HA isolated from soil, therefore the degree of freedom is 5-1=4. The degree of freedom of both conditions (the third row: oxalic conditions and extraction method) was calculated by multiplying the degree of freedom of oxalic extraction conditions and the degree of freedom of extraction method, therefore 1 × 4 = 4.

[§]Mean Square = Sum of Square / Degree of Freedom

[¶]F ratio = variance of the group means (Mean Square) / mean of the within group variances (Mean Square Error). The calculated F ratio should be compared to the critical F ratio based on the degree of freedom (as shown in the brackets), and in all cases above, the F ratio was higher than the critical F ratio, indicating the null hypotheses should be rejected.

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Gelösch: S

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Gelösch: water-extracted

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Gelösch: chemically-extracted

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Gelösch: water-extracted

Yuge Bai 16.12.2019 18:47

Gelösch: chemically extracted

Table S2. ¹³C-NMR analysis of [OM](#), FA and HA. All extracts were isolated from a forest soil (Schönbuch forest, Baden-Wuerttemberg, Germany) under oxic or anoxic conditions. ¹³C-NMR was conducted from freeze-dried extracts. The relative intensities of the signals were obtained by integration of the specific chemical shift ranges by an integration routine with MESTRE NOVA. Data for [FA isolated from water-extractable OM](#) cannot be presented due to the lack of an adequate amount of sample for analysis.

Percentage distribution of ¹³C within indicated ppm regions (%)

Sample/ppm range	0-45	45-60	60-90	90-110	110-140	140-160	160-185	185-225	Aromaticity (%)
	Alkyl C	N-Alkyl C	O-Alkyl C	O-Alkyl & C/H-Aryl C	C/H-Aryl C	O-Aryl C	Carbonyl C	Aldehyde & Ketone	
Water-extractable OM, oxic	20	10	30	10	12	6	7	4	20
Water-extractable OM, anoxic	35	10	14	6	13	6	15	1	23
FA (isolated from soil, oxic)	21	9	32	9	9	4	11	3	15
FA (isolated from soil, anoxic)	22	10	19	8	14	8	12	6	27
HA (isolated from water-extractable OM, oxic)	31	11	19	7	13	7	7	2	23
HA (isolated from water-extractable OM, anoxic)	23	9	17	8	15	9	10	8	30
HA (isolated from soil, oxic)	24	11	22	8	15	8	9	3	20
HA (isolated from soil, anoxic)	27	11	21	8	14	7	8	3	24

Aromaticity (%) = [Aromatic C peak area (110-160 ppm)] × 100 / [Total peak area (0-160 ppm)]

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Gelöscht: SOM

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Gelöscht: water-extracted

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Gelöscht: a

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Gelöscht: extracted

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Gelöscht: extracted

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Gelöscht: Chem.-extracted

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Gelöscht: Chem.-extracted

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Gelöscht: Water-extracted

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Gelöscht: Water-extracted

Yuge Bai 16.12.2019 18:50

Gelöscht: anoxic

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Gelöscht: Chem.-extracted

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Gelöscht: Chem.-extracted

Table S3. Total phosphate-leachable Fe, Fe(II) and Fe(III) in the isolated OM, FA and HA samples. All samples were prepared by dissolving the freeze-dried OM, FA, and HA extracts (powder) in 50 mM of phosphate buffer (pH 7) at a concentration of 100 mg C L⁻¹. After overnight agitation at 300 rpm at room temperature, samples were filtered through 0.22 µm syringe filter (mixed cellulose ester (MCE), Millipore, Germany). Iron concentrations were quantified in the phosphate-buffer extracts with the spectrophotometric ferrozine assay in a microtiterplate assay. Errors represent the standard deviation calculated from 3 parallels. Missing data is due to concentration below detection.

	Fe total (µmol L ⁻¹)	Fe(II) (µmol L ⁻¹)	Fe(III) (µmol L ⁻¹)	Contribution of Fe(II) to electron accepting capacity (EDC) (µmol e mmol C ⁻¹)/%	Contribution of Fe(III) to electron accepting capacity (EAC) (µmol e mmol C ⁻¹)/%	Total contribution of Fe to electron exchange capacity (EEC) (µmol e mmol C ⁻¹)/%
Water-extractable OM, oxic	32.8±1.8	17.4±1.6	15.4±1.3	2.1±0.19/11.7-14.0	1.9±0.16/10.9-12.9	4.0±0.21/11.8-13.0
Water-extractable OM, anoxic	123.1±0.8	79.2±0.8	43.9±0.9	9.5±0.10/33.4-33.7	5.3±0.11/32.3-33.7	14.8±0.10/33.0-33.4
FA (isolated from water-extractable OM, oxic)	18.8±1.3	7.2±0.4	11.6±0.7	0.9±0.05/7.7-8.6	1.4±0.08/59.6-66.8	2.3±0.16/16.1-18.5
FA (isolated from water-extractable OM, anoxic)	22.6±1.0	10.0±1.0	12.6±1.6	1.2±0.12/5.5-6.8	1.5±0.19/27.5-35.4	2.7±0.12/10.6-11.6
FA (isolated from soil, oxic)	-	-	-	-	-	-
FA (isolated from soil, anoxic)	-	-	-	-	-	-
HA (isolated from water-extractable OM, oxic)	15.9±2.1	7.2±0.3	8.7±0.5	0.9±0.04/1.6-1.7	1.0±0.06/14.1-15.9	1.9±0.25/2.0-2.6
HA (isolated from water-extractable OM, anoxic)	60.7±2.6	27.8±0.4	32.9±0.6	3.3±0.05/38.5-39.7	3.9±0.07/13.6-14.1	7.3±0.31/46.3-50.4
HA (isolated from soil, oxic)	-	-	-	-	-	-
HA (isolated from soil, anoxic)	-	-	-	-	-	-

Calculation of contribution of Fe(II) to the EDC:

Take water-extractable OM, oxic as an example, Fe(II) concentration = 17.4 µmol L⁻¹
 The volume of OM solution used for EDC analysis is 200 µL
 Mole quantity of Fe(II) = 17.4 µmol L⁻¹ * 200 µL = 0.00348 µmol Fe(II)
 1 mol Fe(II) can donate 1 mol e, so 0.00348 µmol Fe(II) can donate 0.00348 µmol e
 Mole quantity of C = 100 mg C L⁻¹ * 10⁻³ * 200 µL / 12 g mol⁻¹ = 0.00167 mmol C
 Therefore, the contribution of Fe(II) to the EDC = 0.00348 µmol e / 0.00167 mmol C = 2.1 µmol e mmol C⁻¹

Calculation of contribution of Fe(III) to the EAC:

Take water-extractable OM, oxic as an example, Fe(III) concentration = 15.4 µmol L⁻¹
 The volume of OM solution used for EAC analysis is 200 µL
 Mole quantity of Fe = 15.4 µmol L⁻¹ * 200 µL = 0.00308 µmol Fe
 1 mol Fe(III) can accept 1 mol e, so 0.00308 µmol Fe(III) can accept 0.00308 µmol e
 Mole quantity of C = 100 mg C L⁻¹ * 10⁻³ * 200 µL / 12 g mol⁻¹ = 0.00167 mmol C
 Therefore, the contribution of Fe(III) to the EAC = 0.00308 µmol e / 0.00167 mmol C = 1.9 µmol e mmol C⁻¹

Calculation of contribution of Fe to the EEC:

Take water-extractable OM, oxic as an example, Fe total concentration = 32.8 µmol L⁻¹
 The volume of OM solution used for EEC analysis is 200 µL
 Mole quantity of Fe total = 32.8 µmol L⁻¹ * 200 µL = 0.00656 µmol Fe(II)
 1 mol Fe(II) can exchange (either donate or accept) 1 mol e, so 0.00656 µmol Fe(II) can donate 0.00656 µmol e
 Mole quantity of C = 100 mg C L⁻¹ * 10⁻³ * 200 µL / 12 g mol⁻¹ = 0.00167 mmol C
 Therefore, the contribution of Fe(II) to the EDC = 0.00656 µmol e / 0.00167 mmol C = 4.0 µmol e mmol C⁻¹

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Gelöscht: S

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Gelöscht: SOM (water-extracted, oxic)

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Gelöscht: SOM (water-extracted, anoxic)

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Gelöscht: water-extracted,

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Gelöscht: (water-extracted,

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Gelöscht: chem.-extracted

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Gelöscht: chem.-extracted

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Gelöscht: HA (water-extracted, oxic)

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Gelöscht: HA (water-extracted, anoxic)

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Gelöscht: HA (chem.-extracted, oxic)

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Gelöscht: HA (chem.-extracted, anoxic)

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Gelöscht: water-extracted S

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Gelöscht: S

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Gelöscht: water-extracted SOM

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Gelöscht: water-extracted SOM

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Gelöscht: S

Table S4. Summary table for the two-way ANOVA of the measured electron exchange capacity (EEC) of all extracts. The two-way ANOVA analysis was conducted using the SPSS software to test whether the oxalic extraction conditions or the extraction method, or both of them have significant impact on the EEC values of the extracts. $P < 0.05$ indicates the rejection of the null hypotheses, meaning that the corresponding condition has significant influence on the EEC.

Dependent Variable: Electron exchange capacity (EEC)

Condition	Sum of Squares	Degrees of freedom	Mean Square	F	P
Oxic extraction conditions (oxic vs. anoxic)	18073	1	18073.6	657.912 F (1,20) = 4.35	<0.05
Extraction method (water vs. chemical)	21448	4	5362.1	195 F (4,20) = 2.87	<0.05
Oxic extraction conditions and extraction method	9722	4	2430.8	.191 88.477 F (4,20) = 2.87	<0.05
Error (within group variances)	150	20	7.5		

Three null hypotheses of the two-way ANOVA analysis:

Hypothesis₁: the oxalic extraction conditions do not have significant impact on the measured EEC values of the extracts

Hypothesis₂: the extraction method does not have significant impact on the measured EEC values of the extracts

Hypothesis₃: the oxalic extraction conditions and the extraction method together do not have any significant impact on the measured EEC values of the extracts

Degree of Freedom was calculated based on the number of variables under each condition. For example, under oxalic extraction conditions, there are two variables, i.e., oxalic conditions and anoxic conditions, so the degree of freedom is $2-1=1$. For the extraction method, there are five variables, i.e., [water-extractable OM](#), [FA isolated from water-extractable OM](#), [FA isolated from soil](#), [HA isolated from water-extractable OM](#), [HA isolated from soil](#), therefore the degree of freedom is $5-1=4$. The degree of freedom of both conditions (the third row: oxalic extraction conditions and extraction method) was calculated by multiplying the degree of freedom of oxalic extraction conditions and the degree of freedom of extraction method, therefore $1 \times 4 = 4$.

Mean Square = Sum of Square / Degree of Freedom

F ratio = variance of the group means (Mean Square) / mean of the within group variances (Mean Square Error). The calculated F ratio should be compared to the critical F ratio based on the degree of freedom (as shown in the brackets), and in all cases above, the F ratio was higher than the critical F ratio, indicating the null hypotheses should be rejected.

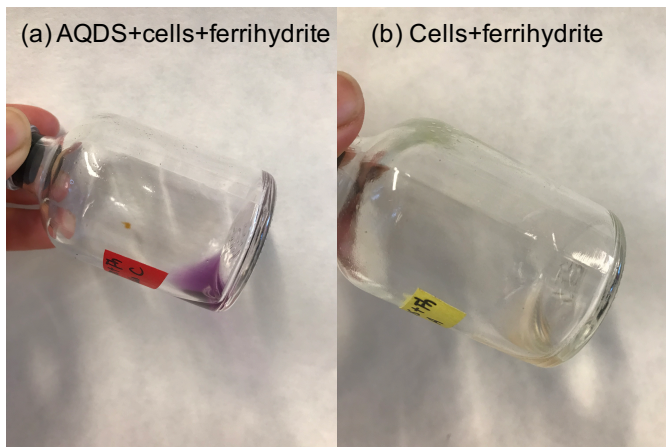
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Gelöscht: water-extracted SOM, water-extracted FA, chemically-extracted FA, water-extracted HA, chemically extracted HA,

Table S5. Metal content analyzed by Microwave Plasma-Atomic Emission Spectrometer (MP-AES) (4100, Agilent Inc., Santa Clara, CA, USA) in the extracted [water-extractable OM](#), HA and FA samples. Prior to the MP-AES analysis, 0.5 g sample was digested with 10 mL 2% HNO₃ in microwave oven at 190 °C (800 w) for 10 min, after cooling down to room temperature, centrifuged for 10 min at 14000 rpm and the supernatant was used for the analysis. The data for FA ([isolated from soil, oxic](#)) and HA ([isolated from water-extractable OM, oxic](#)) are missing due to the lack of enough samples. The unit of all metal concentrations is mg kg⁻¹, blank means the concentration of the corresponding metal is too low to be detected.

	B	Zn	Ca	Mg	Cu	Ag	Ba	Mn	Pb	Al	Ga
Water-extractable OM, oxic	1.65	0.04	10.09	3.03	0.10	0.02	0.07	3.82	0.10	0.99	0.33
Water-extractable OM, anoxic	0.11	0.13	-	8.97	0.11	0.01	0.68	10.64	0.14	12.64	0.01
FA (isolated from water-extractable OM, oxic)	0.34	0.01	14.02	0.20	0.08	0.01	0.03	0.45	0.07	0.26	0.03
FA (isolated from water-extractable OM, anoxic)	0.06	0.01	0.84	-	0.05	0.01	0.01	0.31	0.03	0.82	0.02
HA (isolated from soil, anoxic)	0.09	0.11	-	11.13	0.19	0.02	1.05	3.40	0.12	14.87	0.01
HA (isolated from water-extractable OM, anoxic)	0.03	0.03	6.41	2.78	0.01	0.01	0.26	2.07	0.10	19.64	0.02
HA (isolated from soil, oxic)	0.08	0.04	0.03	7.91	3.92	0.09	0.02	0.32	1.6	0.15	64.12
HA (isolated from soil, anoxic)	0.02	0.02	0.01	6.46	2.94	0.05	0.03	0.09	0.15	0.09	7.14

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Gelösch: chemically-extracted
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Gelösch: water-extracted
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Gelösch: SOM (water-extracted, oxic)
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Gelösch: SOM (water-extracted, anoxic)
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Gelösch: extracted
- Yuge Bai 16.12.2019 18:56
Gelösch: water-extracted
- Yuge Bai 16.12.2019 18:56
Gelösch: chemically-extracted
- Yuge Bai 16.12.2019 18:56
Gelösch: water-extracted
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Gelösch: chemically-extracted



| **Figure S1.** Addition of ferrozine to incubation bottles at the end of the experiment shows sorption or precipitation of Fe(II) at the glass walls of experiments containing AQDS, *Shewanella oneidensis* MR-1 and ferrhydrite (a) but not when containing *Shewanella oneidensis* MR-1 and ferrhydrite without AQDS (b). Before adding the ferrozine solution, original solutions in the bottles were discarded completely in the anoxic glovebox.

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Gelöscht: S2

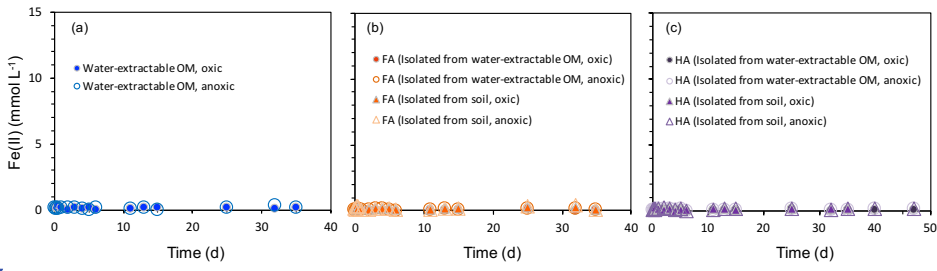


Figure S2. Abiotic reduction of ferrihydrite (15 mmol L^{-1}) in the presence of 50 mg C L^{-1} water-extractable OM (a), FA (b) and HA (c) and 15 mmol L^{-1} lactate, presented as total Fe(II) formed over time. All setups were incubated in air-tight 100 mL glass serum bottles flushed with N_2 at 30°C in the dark.

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Gelöscht: S3

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Gelöscht: SOM

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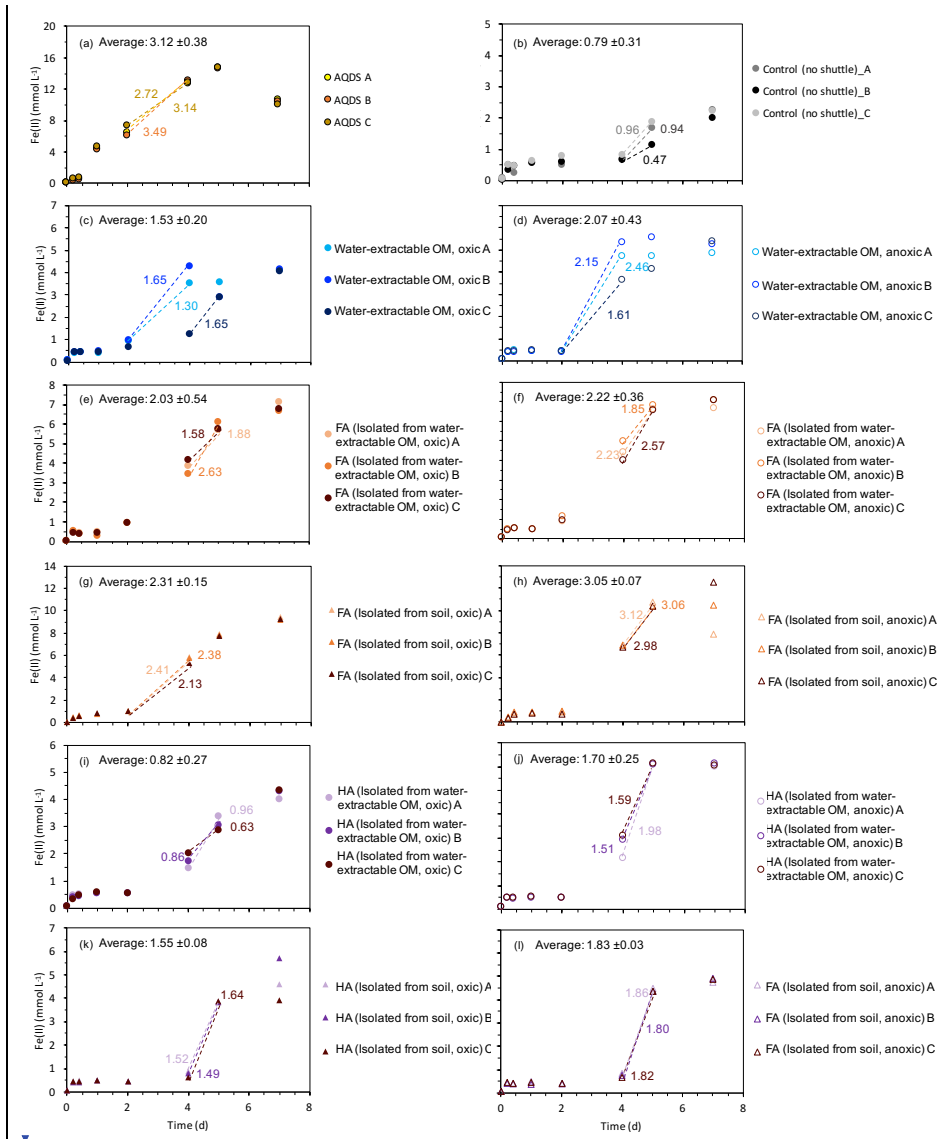
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Gelöscht: B

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Gelöscht: C



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Gelöscht: S4

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