Author's response to comments from reviewers

We would like to deeply appreciate David Emerson and Katrin Wendt-Potthoff for reviewing our manuscript and for their helpful comments on it. In below we respond to each comment.

Comments from David Emerson (Referee #1)

General comments

This paper presents a somewhat surprising finding, the occurrence of neutrophilic iron-oxidizing bacteria associated with algal filaments in a stream with very high iron concentrations. This is surprising because we normally think of these bacteria as being adapted for microaerophilic conditions, and unable to compete with the abiotic oxidation of iron under the fully saturated O2 conditions that occur in association with photosynthetic algae. The authors do a convincing job of showing the presence of these bacteria. It is possible to argue these findings are circumstantial, because they do not show the bacteria actually live by iron-oxidation; however this group has pretty much only been found in high iron environments with all cultured representatives able to grow on iron, and, as yet, there are no definitive functional assays for iron oxidation so they have done a nice job based on current knowledge.

The authors could do a better job of placing this work in context and emphasizing some of the more important and surprising aspects of the work, i.e. that it goes against current dogma of these bacteria being strict microaerophiles, and I would argue some of these concepts are more important than ones that have been addressed in introduction and discussion.

Thank you for pointing out the important and surprising aspects of our work which are against current dogma of *Gallionella* being strict microaerophiles. Thus, we emphasized this aspect more in the revised version of our manuscript.

They do not mention if characteristic structures of FeOB were observed, these are a typical diagnostic for FeOB; however not all FeOB produce them, so it is interesting if they were not observed.

We did not observe stalks of *Gallionella* on the algae as we described in the discussion, and also other characteristic structures of FeOB were not confirmed. The following sentence was added.

***'Neither stalks of Gallionella nor other characteristic extracellular structures of FeOB were found on the algae.'* (Page 13, Line 302)**

There was no observation of diurnal cycles, is it possible that at night the O2 levels go to a much lower level allowing an opportunity for FeOB to grow under low O2?

Yes, we agree. We added this statement to the text as follows:

***'However, it is possible that at night the oxygen levels go to a much lower level allowing an opportunity for FeOB to grow under low oxygen.'* (Page 18, Line 432)**

Specific comments

Abstract, could be better written, and it is not proven that high iron kills these algae, at best evidence is circumstantial; therefore would not conclude this.

We agree that there is no conclusive evidence of fatal effect of Fe encrustation on algae. In addition, we emphasized our findings of neutrophilic FeOB under presumable O_2-saturation around the algae. Thus, the abstract was changed.

RC: p4 (7708), l15 Better not to suggest specific O2 saturations, since these are not well quantified.

Specific values of O_2 saturations were removed (Page 5, Line 79).

RC: p4 (7708) l23, This is speculation and has not been proven, best to not mention.

Sentence removed (Page 5).

RC: p4-5 (7708-7709), Don't follow the significance of mentioning EPS here, presumably algal production of EPS will dwarf production by bacteria, besides much of this discussion deals with acidic systems, yet the significance of the work is really related to more circumneutral systems.

We removed these sentences to have a stronger focus on circumneutral systems (Page 5).

RC: P5 (7709), Introduction would be better to mention other work done related to iron-oxidation and algae, specifically discuss Chocolate pots in Yellowstone and work of Kapplers group on spring in Switzerland with cyanobacteria and iron oxidizers. CP work suggests there should be co-existence of photosynthetic algae and FeOB.

Thank you for pointing out these important references. We included them as follows:

'Few studies have addressed the relationship between Fe(II)-oxidation and algae. A previous study reported that oxygen production by cyanobacteria appeared to control Fe(II)-oxidation in iron-rich microbial mats at Chocolate Pots in Yellowstone despite

co-existence of anoxygenic photosynthetic FeOB (Trouwborst et al., 2007), but there was no evidence of biogenic Fe(II)-oxidation by chemolithotrophic neutrophilic FeOB. Another study examining a bicarbonate Fe(II)-rich spring in the Swiss Alps showed the co-existence but physical separation of cyanobacteria and Gallionella (Hegler et al., 2012). Since the presence and activity of neutrophilic FeOB close to oxygen-generating photosynthetic organisms has not been documented, we applied...' **(Page 6, Line 117).**

RC: p7 (7711) l18 Should give the specific glycoconjugate this lectin binds to.

The *Aleuria aurantia* Lectin (AAL) was used in this study binds preferentially to fucose linked (α-1, 6) to *N*-acetylglucosamine or to fucose linked (α-1, 3) to *N*-acetyllactosamine related structures. AAL also reversibly binds fucose attached to nucleic acid. This information was added (Page 8, Line 180).

RC: p11 (7715) l3 The increase in O2 was presumably a result of algal photosynthesis, while the increase in pH likely resulted from a combination of CO2 outgassing, and draw down of CO2 via algal growth.

The increase in O_2 and pH was also observed without the presence of algae (Fabisch et al., 2015; Johnson et al., 2014). Sentences were rephrased as follows:
'The increase in oxygen could be caused by both turbulent mixing with air and photosynthetic activities of the algae and increase of pH likely resulted from a combination of CO_2 outgassing from the initial anoxic outflow water and draw down of CO_2 via algal growth.' **(Page 12, Line 261).**

RC: p16 (7720) l24 I would not say the stalks are composed of polysaccharides should cite work of Chan and Japanese workers who have explicitly looked a stalk composition, not Hanert.

Very recent studies clearly indicated that the stalks were mainly composed of polysaccharides and long-chain saturated aliphatic compounds (Fabisch et al., 2015; Picard et al., 2015), which was also suggested in previous studies (Chan et al., 2011; Suzuki et al., 2011). We removed the reference of Hanert and rephrased our statement as follows:
'Gallionella species form long stalks that are mainly composed of polysaccharides and long-chain saturated aliphatic compounds during Fe(II)-oxidation with the purpose of deposition of Fe-oxides apart from the cells (Chan et al., 2011; Suzuki et al., 2011; Fabisch et al., 2015; Picard et al., 2015).' **(Page 19, Line 456)**

RC: p11 (7715) l7 Need to explain what expected temps would be without exothermic input.

Sentence was removed (Page 12, Line 265).

RC: p11 (7715) l15 Why? You state below you could reach it in August.

In July 2013 we could not reach to site O because it was fenced due to the construction work. This information was added (Page 7, Line 146).

RC: p15 (7719) l12 This doesn't make a whole lot of sense, FeOB normally need very little O2, the algae to supersaturating O2.

We agree with your comment. However, this was the argument by Chapman 1941. To avoid confusion, we deleted part of the sentence (Page 17, Line 394).

RC: p15 (7719) l20 Could the iron coatings being acting as buffers that help prevent the plant from taking up metals, this as been suggested as a type of protection for root plaque

We added the following sentences:

'Thus, the iron coatings could also act as buffers that help prevent the plant from taking up these heavy metals, similar to what has been suggested as a type of protection for root plaques (Tripathi et al., 2014) and references therein).' **(Page 17, Line 402)**

RC: p16 (7720) l5 What about relative abundances of heavy metals, Gall may be more resistant, Sideroxydans less so. See recent Frontiers paper on this.

Thank you for your advice. In the reference (Emerson et al., 2013) it was reported that *G. capsiferriformans* ES-2 should be more resistant to heavy metals than *S. lithotrophicus* ES-1 based upon their genome information. As we showed in Figure S2 the stream water was polluted with heavy metals through the entire flow path, and concentrations of Fe, Zn and Cd (data not shown) in the water were higher at site O than the other sites. In addition, higher amounts of Mn and other metals (Co, Cr, Cu, and Pb, data not shown) were precipitated in the sediment at site O than those of other sites. We added following sentences:

'Based on genome information, G. ferruginea ES-2 should be more resistant to heavy metals than S. lithotrophicus ES-1 (Emerson et al., 2013) and thus should dominate the outflow site which showed the highest metal loads in the water. Unfortunately, we could not link the dominance of these species with the heavy metals precipitated on the algae due to shortage of the present sample amount for ICP-MS.' **(Page 18, Line 419)**

RC: p17 (7721) l25, Still need a better discussion of how FeOB deal with the high oxygen. Especially in terms of Fenton chemistry. Also should cite work of de Vet who showed that Gallionella in water treatment systems was growing at surprisingly high O2 concentrations.

We added following sentences in the discussions:

'In an Fe(II)-rich and oxygenated environment, bacteria potentially face the problem of highly reactive oxygen species due to reaction of hydrogen peroxide with Fe(II) (Imlay, 2008). Both G. capsiferriformans ES-2 and S. lithotrophicus ES-1 were reported to encode enzymes that presumably act as catalase or peroxidase to prevent production of reactive oxygen species (Emerson et al., 2013).' **(Page 19, Line 441)**

'In water treatment system and dewatering wells in open cast mines, Gallionella have been also reported to grow at surprisingly high O_2 concentrations at the low water temperature of 13°C (or even higher) which slows down abiotic Fe(II) oxidation (de Vet et al., 2011; J. Wang et al., 2014).' **(Page 18, Line 434)**

RC: p18 (7722) l5 Some confusion here, are the green algae a separate genus or species from the yellow-green algae?

Green and brown algae are the same species. *Tribonema* belongs to the yellow-green algae. To avoid confusion we deleted 'yellow-green' from the manuscript (Page 2, Line 10; Page 13, Line 285; Page 21, Line 497).

RC: p18 (7722) l13 I would say detrimental not fatal, since for most of the season the algae appeared to grow.

Changed as requested (Page 2, Line 17; Page 20, Line 470; Page 21, Line 507).

Comments from Katrin Wendt-Potthoff (Referee #2)

General comments

The manuscript reports on the occurrence of algae and prokaryotes with a focus on FeOB in a slightly acidic polluted stream in Germany. This is a very interesting study site for two reasons: (1) there are still fewer reports on iron cycling in mildly acidic than extremely acidic sites and (2) the study design provides gradients both in geochemistry (from site O to C) and time (July to September). The authors should more clearly mention these important aspects in the introduction and discussion.

We included the following sentence:

'In this study, T. viride colonized metal-rich (Fe, Mn, Ni, Zn and U) and less acidic (pH 5.9 to 6.5) mine-water outflow which showed variations in geochemistry over time and along the flow paths from site O to C.' **(Page 16, Line 375)**

The authors apply a comprehensive set of modern methods to samples directly obtained from the field, thereby producing an impressive and novel dataset which is very well presented. The findings are discussed in the context of a comprehensive selection of international literature. Regarding the text, the authors should again carefully check the use of present tense/past tense and singular/plural. In several places, small words like “a” or “in” are missing.

We asked a native speaker to correct our English.

Specific comments:

P 7706 lines 24-25: misleading statement, as production of the EPS by algae could not be proved.

This is correct. We weakened our statement as follows:

'Lower photosynthetic activities of the brown algae could have led to reduced EPS production which is known to affect predator colonization.' **(Page 2, Line 23)**

P 7707 lines 10-12: better separate these statements and references and explain in a more specific way – apparently in this study the EPS production was higher in “fresh” (green) than senescent (brown) cells?

We deleted this statement to avoid confusion (Page 4, Line 50).

P 7708, lines 14-15: the strict chemolithotrophy of Gallionella in general is still under dispute, please check Spring & Kämpfer 2005 (Bergey’s Manual). As this might be considered important in the present study (organic-rich exudates of algae?), the authors should clearly explain their point of view on this.

We respectfully disagree with this comment, because recent findings by Emerson showed that both *G. capsiferriformans* ES-2 and *S. lithotrophicus* ES-1 have pathways for CO_2-fixation and do not grow heterotrophically (Emerson et al., 2013), and any genes for organic carbon degradation have not been reported in genomes of *Gallionellaceae*. We added this statement to the discussion (Page 19, Line 461).

P 7710 line 10: PVDF should be explained

Explained (Page 7, Line 149).

P 7710 line 13: how long were samples stored?

Samples were analyzed at the same day and stored at 4˚C during transportation. This information was added (Page 8, Line 170).

P 7715 line 14: apparently the site O could not be reached at all. Has this something to do with the flood occurring in Germany in June 2013? Did the water level change?

See also comment above. We could not reach the site O in July 2013 because it was fenced due to the construction. It has nothing to do with the flood in July 2013.

P 7715 lines 21-22: Tribonema sp. is a genus, not a species

P 7715 lines 26 and 28: autofluorescence should be used throughout

Corrected (Page 13, Line 285; Page 13, Line 289, 290).

P 7716 line 8: minerals with irregular shape or with rough appearance?

The shape, size and location of the minerals were irregular on the green algae, in contrast to those on the brown algae. Text was modified as follows:

***'In contrast, the surface of the green algae was encrusted with minerals in irregular shape, size and location (Fig. 3C, D, 4A, B).'* (Page 13, Line 298)**

P 7716 line 15: figure 4 suggests that there was more EPS with green algae, is this true? Sentence should be revised.

Unfortunately we could not quantify the amount of EPS on green vs. brown algae. We modified out statements as follows:

***'In addition, algal or bacterial EPS-like glycoconjugates were likely associated with the minerals (Fig. 4C), whereas the amount of EPS could not be quantified or compared between the green and brown algae.'* (Page 14, Line 313)**

P 7719 lines 1-3: it could be discussed what cause and effect is. Why not: decreasing light reduces photosynthetic activity and EPS production (if produced by algae), thereby facilitating Fe precipitation, which further impedes photosynthesis (positive feedback)?

Unfortunately, we did not measure the photosynthetic activity and O_2 production of

the algae. Thus, we cannot prove a positive feedback effect.

P 7719 line 28: hard to say without photosynthetic rates or microscale data. It should be discussed that Gallionella has previously found to grow and be active under fully aerated conditions (de Vet et al. 2011, Water Research).

We included this reference (Page 19, Line 439). See also our comments to Referee #1.

P 7720 lines 1-3: which geochemistry parameters make the difference?

The water pH or heavy metal concentrations can be responsible. See also our comments to Referee #1.

P 7720 line 17: the mechanisms presented in Schädler et al. 2009 (Geomicrobiology Journal) could also be discussed here.

We included this reference (Page 19, Line 451).

P 7721 line 18 and following: in August and September, the brown algae apparently harbored more beta-proteobacteria (Figure 7). Is there any evidence that Fe(III)-reducing communities developed with time?

No, there is no conclusive evidence for the presence of Fe(III)-reducing bacteria. Beta-proteobacteria colonizing the brown algae in August and September were composed of *Gallionellaceae* (95.3~96.4% RNA, 90.7~94.8% DNA), *Comamonadaceae* (0.7~2.8% RNA, 0.7~3.3% DNA), *Neisseriaceae* (0.8~2.1% RNA, 1.0~4.7% DNA) and *Rhodocyclaceae* (0.6~1.3% RNA, 1.3~3.9% DNA). The family *Comamonadaceae* may contain Fe(III)-reducing *Albidiferax* spp., but their presence could not be confirmed.