Title

Response relationships between CO_2, CH_4 and N_2O emissions and microbial functional groups in wetland sediments after trace metal addition.

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Abstract (176 words)

Biochemical nitrogen removal relies heavily on wetland microbes, prior to entering downstream ecosystems. Microbes utilize oxy-reductases that contain metal-cofactors to reduce NO3 and NO2 to N2. These transformations are linked also to other C cycling processes and may emit N2O, CH4 and CO2 as well. In this incubation study we tested the effects of supplementing wetland sediments with trace-metals (Cu, Mo, Fe) at µM levels on nitrogen kinetics, gaseous emissions (N2O, CO2 and CH4) and genes abundances for the overall microbial community, related to NO2 reduction (denitrification and DNRA), N2O reduction and methanogenesis. Trace metal availability and specifically Cu, enhances the reduction of N2O to N2, increases the production of CH4 and has no effect on CO2 emissions from wetland sediments. When sediments were supplied with Cu, higher gene abundances were observed for Cu-reductases *nirK* and *nosZ*. Mo addition increased CH4 emissions from sediments and higher *mcrA* gene abundances were observed. Emerging from this investigation, trace-metals bio-availability may directly or indirectly regulate denitrification in the environment; an effect observed so far only in pure microbial cultures.

Keywords: trace metals, denitrification, greenhouse gases, sediments, microbial groups

Introduction

The removal of many upstream anthropogenic pollutants (industrial, urban and agricultural) prior to entering downstream coastal and marine ecosystems relies on wetland microbes. Out of those pollutants, nitrogen (N) pollutants (exceeding 60 Tg N y^{-1}) are of major concern because they are linked to water quality degradation, eutrophication and increased greenhouse emissions (carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O)) (Anderson et al., 2010;Tian et al., 2015). Wetland microbes reduce the N load through various biochemical pathways i.e. assimilation, reduction and oxidation, but often this ecosystem service comes with the release of significant amounts of CO_2, N_2O and CH_4.

Microbial processes of nitrification, denitrification or nitrifier-denitrification, methanogenesis and methanotrophy are responsible for the production of N_2O and CH_4 in wetland sentiments (Soares et al., 2016). In particular N_2O is a very potent greenhouse gas, with a global warming potential ~ 300 and ~ 60 greater than CO_2 and CH_4, respectively, with important implications in ozone depletion. N_2O can be further reduced to inert N_2 by microbes that possess the only known enzyme to reduce N_2O; the nitrous oxide reductase (NOS)(Thomson et al., 2012). It is estimated that ~ 17 Tg N p.a. is returned to the atmosphere via denitrification in wetlands and terrestrial ecosystems globally, releasing ~ 48 Tg N p.a. to coastal and marine ecosystems (Schlesinger, 2009). Besides N cycling, wetlands are important ecosystems in C cycling as well. Despite their relative small coverage (~ 10 % globally), wetlands store large amounts of C and emit considerable amounts of CH_4 (144 Tg CH_4 p.a.)(IPPC, 2014). CH_4 emissions are the trade-off balance between the microbial processes of methanogenesis and methanotrophy.

The production and consumption of N_2O and CH_4 is catalyzed by oxy-reductases that utilize a metal co-factor (Mo, Cu, Fe and S) (Glass and Orphan, 2012). The reduction of NO_3^- is catalyzed by a periplasmic or membrane bound molybdenum (Mo) nitrate reductase (NAR) (Schwarz et al., 2009), NO_2^- is reduced to NO by either a non-metal cytochrome cd_1 (Einsle et al., 1999) or Cu-cofactor (Adman et al., 1995) nitrite reductase (NIR; expressed by *nirS* and *nirK* respectively). NO is reduced to N_2O by nitric oxide reductase (NOR) and contains heme-Fe co-factor; two types of NOR are distinguished, cNOR which is found in denitrifying bacteria and receives electrons from cytochrome *c* and qNOR which is typically found in pathogenic non-denitrifying bacteria as well as in denitrifiers and receives electrons directly from quinol (Shiro, 2012). Finally N_2O is reduced by the Cu-containing NOS (Rosenzweig, 2000). NO_2^-, besides being a denitrification intermediate, it is also a substrate for dissimilatory nitrite reduction to ammonium (DRNA) and this permutation has been linked to C/N (Kraft et al., 2014;Yoon et al., 2015;van den Berg et al., 2017). Likewise, denitrification may be linked or co-occur with other C cycling processes.

65 Interestingly, members of the NC10 phylum (e.g. *M. oxyfera*) are able to couple methane oxidation to
66 denitrification (NO_2^--dependent methane oxidation (N-DAMO)) utilizing a Cu-dependent particulate
67 methane monoxygenase (pMMO) and a Fe-rich cd_1 nitrite reductase (Deutzmann et al., 2014). While
68 extensive research has tested the relative contribution of key environmental factors (pH, NO_3^-/NO_2^-, O_2,
69 C/N) in regulating anaerobic microbial processes and specifically denitrification (Thomson et al., 2012),
70 we argue that the abundance and bio-availability of trace metals in the environment could considerably
71 control greenhouse gas emissions in the C and N biochemical cycle.
72
73 Metals in the environment have been traditionally seen as unwanted pollutants. This is because trace
74 metal accumulation is linked to toxicity and inhibition of ecosystem processes (Samanidou and
75 Papadoyannis, 1992). Contrariwise, low levels of trace metals may limit crop production, and impede
76 animal and human nutrition (Teklić et al., 2013). Concentrations exceeding $mg.L^{-1}$ range for Cu, Mo, Fe,
77 Zn and Pb severely inhibited denitrification in soils, sediments, water-bodies and waste waters (Labbé et
78 al., 2003;Magalhães et al., 2007;Liu et al., 2016). The recent advances in our understanding of
79 denitrification using model organisms demonstrated the complete reduction of NO_3^- to N_2 requires trace
80 metals at µM to pM levels. In fact, *in vitro* studies have shown that lack of Cu, Mo or Fe severely inhibits
81 denitrification or methane cycling, due to the formation of non-functional enzymes typically lacking the
82 respective metal co-factor. In soil bacteria *P. denitrificans* and *Pseudomonas stutzeri* Cu is required to
83 express to a functional Cu-containing NOS dimer (Granger and Ward, 2003;Felgate et al., 2012;Black et
84 al., 2016). In methanotrophs oxidizing CH_4 to methanol (CH_3OH) by MMO, the switch over between a Cu-
85 or Fe-dependent MMO is regulated by the environmental availability of Cu and Fe respectively (Murrell
86 et al., 2000;Bollinger Jr, 2010). All three classes of methanogens utilize a common cobalt dependent
87 Methyl-coenzyme M reductase (MCR; *mcrA*), whereas in the earlier steps of methanogenesis are
88 catalyzed by Fe-, Ni- and Zn- dependent ferrodoxins and dehydrogenases (Glass and Orphan, 2012).
89 Based on the above observations, few studies have optimized the trace metal concertation for
90 denitrification in a culture medium (Hahnke et al., 2014) and suggested the optimal levels of trace
91 metals in biological wastewater treatment plants (He et al., 2015;Wintsche et al., 2018).
92
93 Despite the great progress in metal ecotoxicity and metalloenzyme biochemistry, our understanding of
94 trace-metal availability in ecosystem C and N biochemical cycles is generally limited to aquatic
95 ecosystems primarily focusing on productivity, NH_4^+/NO_3^- and CO_2 assimilation rates (Twining et al.,
96 2007;Glass et al., 2012;Moore et al., 2013;Romero et al., 2013;Schoffman et al., 2016). Here, we
97 investigated the effects of trace metal (µM) addition on the microbial biochemistry of wetland

98 sediments focusing primarily on NO_2- reduction and greenhouse gas kinetics. We applied molecular
99 techniques (qPCR) to quantify changes in the microbial ecology of wetland sentiment in an effort to
100 better understand the intrinsic link between microbial ecology and process rates. We hypothesized that
101 **(*i*) the bio-availability of metals (Mo, Fe and Cu) at µM levels will increase denitrification rates and**
102 **specifically Cu availability will reduce N_2O emissions. Also, considering C respiration, (*iia*) CO_2**
103 **emissions will be driven by denitrification and (*iib*) no effect on CH_4 emissions is expected.**
104
105 **Materials and Methods**
106 **Soil samples and experimental set-up**
107 A combined (5 sites; 5-10 cm from surface) soil sample and soil water were collected from the tidal-
108 fluvial point bar deposit of the Pamunkey River (N 37.557451, W -76.972521) using a PVC pipe (Ø 10
109 cm). Soil sediments had 25 ±4 %DW organic matter (OM), 79 ±6 % moisture, 0.18 ±0.05 $g.cm^{-3}$ bulk
110 density, C/N ratio of 11 ±1.5 and pH 6.1 ±0.3. Soil water, at the collection time, contained 0.1 ±0.03 mM
111 NO_3^-, 0.1 ±0.02 mM NO_2^-, 0.16 ±0.04 mM SO_4^-, 0.4 ±0.1 mM Cl^-, and 0.3 ±0.1 mM NH_4^+, 0.11 ±0.01 mM
112 K^+, 0.26 ±0.1 mM Mg^{2+} and 0.29 ±0.1 mM Ca^{2+}. Samples were kept cool (ice-cooler) during transport and
113 stored at 4 °C overnight prior to processing. Any large stones and roots were removed, then 1000 g wet
114 soil was homogenized with an additional 1500 ml of filtered soil-water (Cellulose, 10 µm, Millipore,
115 USA). Soil slurry aliquots of 50 ml were transferred to 125 ml bottles (Wheaton, USA). Bottles were
116 crimped with rubber septa and were kept for 3 days in dark (25 °C) to let any residual O_2 to be
117 consumed by basal respiration. Then, all treatments received 250 µl aliquots of 2 M KNO_3 reaching a
118 final concentration of 10 mM. A 400 µl volume of ammonium molybdate ($(NH_4)Mo_7O_{24}$; 1 mM), iron
119 sulfate ($FeSO_4$; 18.5 mM) and copper sulfate ($CuSO_4$; 6.5 mM) stock solution were injected to the
120 bottles, adding 4, 74 and 26 µM respectively, according to the treatment plan (Table 1). Bottles were
121 vortexed briefly (30 s at 6000 rpm), flushed with N_2 (130 mins) and incubated at 25 °C in dark. Through-
122 out the incubation several gas and liquid samples were taken anaerobically (as indicated in the graphs).
123 At 96 hours, bottles were un-crimped in an O_2-free chamber, then 1 g of wet soil was taken
124 anaerobically and was frozen instantly for further molecular analyses.
125
126 **Analytical techniques**
127 Gas samples of 5 ml were stored in 3 ml exetainer vials (Labco, UK), that were previously flushed (N_2; 5
128 mins) and vacuumed. Gas samples were analyzed for N_2O, CO_2, CH_4 and O_2 concentration with gas
129 chromoatography (GC – 2014 equipped with FID, TCD and ECD; Shimadzu, USA) as in Morrissey and
130 Franklin (2015). For each gas, total production was determined as the sum of the gas accumulated in the

131 headspace and the gas dissolved in the slurry; the latter was estimated using the relationships described
132 by Heincke and Kaupenjohann (1999). From each bottle, 1.5 ml slurry samples were collected,
133 centrifuged (10000 x *g* for 5 mins) and the supernatant was diluted 10x, filtered (0.22 µm) and stored (-4
134 °C) prior to ion analysis. The concentration of NO_3^-, NO_2^-, SO_4^{2-} and NH_4^+ was determined by ion-
135 chromatography (ICS – 5000+, Dionex, USA) according to the manufacturer's instructions using columns
136 Dionex IonPac AS17 (2 × 250 mm) and IonPac CS12 (A – 5 µm, 3 × 150 mm) and a Dionex conductivity
137 sensor. Soil water content and OM were determined gravimetrically, soil pH was determined with a
138 glass electrode pH probe (Laquact; Horiba, USA) in soil-water extract (after centrifugation 4000 *x g* for
139 20 mins).
140

141 **Molecular techniques**

142 DNA was extracted from frozen soil samples (0.3 g) using the DNEasy Kit from Qiagen (USA) following
143 the manufacturer's instructions. The DNA yields were, on average, 42 ±2 $ng.\mu l^{-1}$. The retrieved DNA was
144 quantified using a Nanodrop spectrometer (Thermo, USA) and visualized on a 1.2 % agarose gel for
145 integrity. DNA samples were diluted to 3 $ng.\mu l^{-1}$ and were further tested (see qPCR protocols on Table 2)
146 for the abundance of microbial groups typically found in wetlands: total eubacteria (*eub-518*), nitrite
147 reducers (denitrification: *nirK*, *nirS* and DNRA: *nrfA*), nitrous oxide reducers (*nosZ*) and methanogens
148 (*mcrA*). SensiFAST™ SYBR® No-ROX Kit (Bioline, UK) polymerase x2 mix was used according to the
149 manufacturer's instructions and real time data were collected using a Bio-rad CFX-384 (Bio-rad, USA)
150 real time PCR engine.
151

152 **Statistical analyses**

153 Kinetic and molecular datasets were tested for normality (RStudio [*base*]; Shapiro.test). Non-parametric
154 statistical tests were applied to assess any differences in the mean of ranks between the treatments
155 (RStudio [*agricolae*]; *Kruskal-Wallis* with *Bonferroni* correction). Basic statistics (mean and standard
156 error) were summarized with RStudio [*dyplr*] and plotted with RStudio [*ggplot2*]. For all statistical tests
157 $p<0.05$ was considered significant.

158

159 **Results**

160 **The effects of trace metal addition on NO_3^-, NO_2^- and NH_4^+ kinetics**

161 At the end of the incubation, all treatments had visible gas bubbles on the surface and within the slurry,
162 indicating microbial activity and gas production. We observed yellow and black patches within the slurry
163 possibly due to Fe and Mn reduction indicating anaerobic redusive conditions. Denitrfication
164 commenced with out any lag between the control and trace metal additions. Approximatelly, 12 mM
165 NO_3^- were consumed and 0.3 mM NO_2^- remained, while NH_4+ ranged from 200 to 750 µM at the end of
166 the incubation (**Figure 1**). At 48 h we observed a reduction in the remaining NO_2^- and a simultaneous
167 increase in the NH_4^+ concentration for all treatments. The remaining NO_3^- and NO_2^- at the end of the
168 incubation did not differ significantly when compared to the control. The treatments receiving additonal
169 Mo + Fe ($p \leq 0.01$) and Mo + Fe + Cu ($p \leq 0.01$) had significantly higher NH_4^+ than the control, and an
170 increasing trend in NH_4+ concentration for all treatments was observed towards the end of the
171 incubation, perhaps due to OM mineralization.

172

173 **The effects of trace metal addition on CO_2, CH_4 and N_2O cumulative gas kinetics**

174 Cumulative CO_2 production increased rapidly between 12 to 24 h, and continued to increase up to ~ 24
175 mM C-CO_2 at the end of the incubation. There were no significant differences between the control and
176 each metal treatment, except the Cu and the Mo + Fe treatment (Figure 2.a). A faster rate of CH_4
177 accumulation in the microcosms was observed between 6 to 12 h of incubation, earlier than CO_2. CH_4
178 continued to accumulate during the incubation and reached levels ranging from ~ 4 to 6.5 mM C-CH_4. At
179 the end of the incubation, we observed that the Cu treatment had significantly accumulated more CH_4
180 than the control treatment (Figure 2.b). Cumulative N_2O emissions rapidly increased between 24 to 48 h,
181 matching the increased NO_3^- consumption. Interestingly, the control, Mo, Fe and Mo + Fe treatment
182 accumulated significantly more N_2O than the remaining treatments that contained Cu (Cu, Fe + Cu, Mo +
183 Cu and Mo + Fe + Cu). These differences remained until the end of the incubation (Figure 2.c). Contrary
184 to CO_2 and CH_4 emissions, N_2O emissions reached a plateau between 48 to 96 h, indicating that N_2O
185 emissions were limited by the exhaustion of the available NO_3^- and not by C.

186

187 **The effects of trace metal addition on targeted microbial groups**

188 Average total DNA was ~ 52 $ng.\mu l^{-1}.g^{-1}$ sediment with the Mo + Fe + Cu treatment having significantly
189 (p=0.03) greater DNA yield (62 ±2 SE) that the control (44 ±5 SE). The average bacterial abundance was
190 6.75E+8 *eub* copies g^{-1} sediment with the Fe, Fe + Cu and Mo + Cu treatments having significantly
191 (p=0.005) more *eub* gene copies than the control (**Figure 3.a**). The microbial group of methanogens

192 (*mcrA*) was relatively more abundant in the treatments containing additional Mo (Mo, Mo + Cu, Mo + Fe
193 and Mo + Fe + Cu; **Figure 3.c**) when compared to the control. Regarding the microbial groups involved in
194 N cycling we observed no effect of trace metals on DNRA NO_2^- reducers (*nrfA*; **Figure 3.b**). Interestingly,
195 trace metal addition affected the abundance of *nirK* (p<0.001; **Figure 3.d**), *nirS* (p<0.001; **Figure 3.e**) and
196 *nosZ* (p=0.001; **Figure 3.f**) denitrifying microbial groups. NO_2^- reducers utilizing Cu-NIR (*nirK*) were more
197 abundant in all treatments when compared to the control, except when Fe was added. Microbial groups
198 having a cytochrome cd_1-NIR (*nirS*) were relatively more abundant in the Mo and Fe + Cu treatment.
199 Lastly, the *nosZ* microbial group responsible for the reduction of N_2O to N_2 was more abundant in the
200 treatments Fe+Cu, Mo+Cu and Mo+Fe+Cu than the control. Although *nosZ* abundance was relatively
201 higher in all trace metal additions, the effects of single Cu addition on *nosZ* did not differ significantly.

202

203 **Discussion**

204 We observed that trace metal additions (Mo, Fe, Cu and their combinations) in wetland sediments
205 regulated denitrification kinetics and greenhouse gas emissions, and altered the abundance of targeted
206 microbial groups that are typically found in wetlands. The key interpretation of the incubation
207 experiments is that trace metal availability and specifically Cu, enhances the reduction of N_2O to N_2, the
208 production of CH_4 and has no effect on CO_2 emissions from wetland sentiments. The increase in the
209 denitrifiers' associated physiological activity to reduce N_2O when supplied with additional Cu
210 corresponded to the relative higher abundance of Cu-containing genes - nitrous oxide (*nosZ*) and
211 nititrite (*nirK*) reductase, but not to the abundance of nitrite reduces *nirS* and *nrfA,* suggesting a direct
212 relationship between Cu availiabilty and denitrification process in soils.

213 The absence of substantial amounts of NO_2 and N_2O in the bottles for the Cu-containing treatments (Cu,
214 Fe + Cu, Mo + Cu and Mo + Fe + Cu) and an increase in the respective gene abundance indicates that a
215 balanced denitrification process NO_3^- to N_2. Microbes with a complete denitrification strategy harvest
216 the energy from the last reduction step (N_2O to N_2) in addition to the rest of the denitrification steps,
217 which may be particularly advantageous when there are limited electron acceptors available in the
218 environment. We do not exclude other partial denitrification processes or other anaerobic processes
219 simultaneously co-occurring with denitrification as subjected by the residual N_2O in the treatments Cu-
220 containing treatments (≤2.3 mM N-N_2O). The increasing NH_4+ concentration (<1 mM) towards the end
221 of the incubation could indicate DNRA and /or N mineralization processes commonly co-occurring in
222 wetland sediments (White and Reddy, 2009). However, the lack of significant differences in DNRA gene
223 abundance indicates towards anaerobic N mineralization. Although we didn't measure NO in the

224 treatments, we assume that it was quickly utilized due to its potent cytotoxicity at µM levels (Chaudhari
225 et al., 2017). In the treatments accumulating notable amounts of N_2O (≥ 1mM) N_2O cytotoxicity may
226 occur and it can be releaved by the addition of vitamin B12, Co or methionine (Sullivan et al., 2013). The
227 continuous accumulation of CO_2 in all treatments, as proxy for microbial activity, firstly, excludes any
228 indirect acute cytotoxicity from NO due to NO_2^- reduction or N_2O accumulation and secondly idicates
229 that the availible electron donor (organic C) was in excess of the added NO_3^-. The magnitute of CO_2
230 accumulation was predictable; treatments with complete dentrification, thus having less residual N_2O
231 had accumulated more CO_2 by the end of the incubation. We assumed that the lack of additional Ni
232 would inhibit MCR, key enzyme in methanogenesis (see *Possible mechanisms and other regulators*).
233 Contrary to our hypothesis *iib,* trace metals had a significant effect on CH_4 emissions.

234 ***Other studies and trace metal levels***

235 Several studies have looked into the effects of trace metal abundance or addition on C and N cycling in
236 sediments and peat soils. Typically those studies report a negative effect of trace metals on
237 biogeochemical processes, however the range of trace metal concentration far exceed the levels of the
238 current study. For instance, Keller and Wade amended peat slurries with ~ 100 µM trace element
239 solution and saw significant decrease in CH_4 emission, probably due to Cu induced toxicity. Similarly,
240 Magalhães et al. (2007) observed an 85% inhibition of denitrification accompanied with accumulation of
241 substantial N_2O and NO_2^- amounts at 79 Cu $\mu g.g^{-1}$ sediment (equiv. 1.2 mM Cu) in estuaries. In a follow-
242 up study, lower denitrification rates due to Cu (~ 0.9 mM) were accompanied with a decline in *nirK*, *nirS*
243 and *nosZ* microbial group abundance and *β*-diversity (Magalhães et al., 2011). A more recent study that
244 tested the effects of Cu pollution (addition of 100 µg.L-1 ~ 1.6 µM) on urban wetland sediments, found
245 that Cu addition significantly reduced CH_4 emissions, but had no effect on N_2O emissions when
246 compared to the control (freshwater sediments; Doroski et al. (2019)). This divergent observation could
247 be due to low NO_3^- concentration (18 µM) in relation to available C or the presence of substantial
248 amounts of soil Cu (9.4 $\mu g.g^{-1}$ sediment) at the start of the experiment in both control and Cu treatment.

249

250 ***Possible mechanisms***

251 Denitrification is an anaerboic respiratory process that utilizes NO_3^- as a terminal electron acceptor
252 (Equation (2)) accompanied by energy transfer (Thomson et al., 2012). Thus, for a complete
253 denitrification reaction, two molecules of NO_3^- will be converted to one molecule of N_2 with the
254 transferring of 10 electrons to the electron recipient (NO_3^-, NO_2^-, NO and N_2O). Therefore, microbial

255 groups unable to utlize the complete denitrification pathway will harvest less energy, and/or will have
256 to compensate the energy loss by adjusting their metabolic rates for example by enhancing the rate of
257 NO_3^- reduction. Such bioenergetic adjustment was observed in chemostat cultures of *P. denitrificans;*
258 the *nosZ* deficient strain was unable to complete the last step of denitrification and had lower biomass
259 and higher NO_3^- reduction rates (Giannopoulos et al., 2017). At RNA level, Cu-decifient cells had more
260 transcripts of *nosZ* and Cu-scavenging genes to compensate for the loss of N_2O reduction due to
261 depleted Cu availiability (Felgate et al., 2012). And finally, strains deficient in Cu – transporters and
262 chaperones were unable to reduce N_2O to N_2, although nosZ was expressed, forming a non-functional
263 NOR (Sullivan et al., 2013). Therefore we postulate that, at community level, microbes utilizing the
264 complete dentrification pathway will have a physiological advantage than those unbale to do so. This
265 was supported by the increased abundances of NO_2^- reducers (*nirK*) and partly of the N_2O reducers
266 (nosZ) and of the overal microbial abundance (eub); corroborating the fact that denitrifcation genes
267 were up-regulated in single denitrifiers (*P.stutezi* at 0.05 Cu $mg.L^{-1}$; Black et al. (2016)).

268 Considering net CH_4 emissions, Mo clearly enhanced CH_4 accumulation (Mo) and abundance of *mcrA*
269 genes in the Mo containing treatments. A Mo co-factor is typically required for the formylmethanofuran
270 dehydrogenase (*fwd*) that catalyzes the reduction of CO_2 to formyl-methanofuran in the first step of
271 methanogenesis by reduction of CO_2 with electrons from H_2 (Glass and Orphan, 2012). Mo availioability
272 and the increasing partial pressure of CO_2 in the bottles during the incubation could have triggered a
273 shift towards a Mo-based methanogenesis pathway. Another interesting aspect in CH_4 emissions and Cu,
274 is the competition between methanotrophs and denitrifiers for the "Cu monopoly" (Chang et al., 2018),
275 which should be further investigated in environmental samples. Eventhough the purpose of this study
276 wasn't to quantify the relative contribution between methanotrophs (MMO) and methanogens (MCR) in
277 the net CH_4 emissions, the higher abundance of methanogens (*mcrA*) and noticable accumulation of CH_4
278 in all metal treatments indicates the important role of trace metal availiability in CH_4 cycling in
279 environmental samples.

280

281 ***Trace metal limitation in the environment and other regulators***

282 The abundance of bio-availiable metals in the environment, typically in the ion form, is often observed
283 at several magnitute orders less compared to the routinely reported total metal content. Such trivial
284 amounts could be unavailiable to microorganisms due to physiochemical interactions and plant uptake
285 (Giller et al., 1998). At neutral and alkaline pH levels, metals tend to be effectively immobilized as
286 inorganic compounds (metal – oxides, –hydroxides and – carbonates). Additionally, organic complexes

287 such humic ligands may bind metal cations making them unavailiable to microbes and plants. For
288 example, the particularly low concentrations of dissolved Cu, Fe and Mo (~ 5, 1 and 0.2 $\mu g.L^{-1}$,
289 respectively) and the copious dissolved organic C (~ 80 $mg.L^{-1}$) in siberian peatlands (Raudina et al.,
290 2017), could trigger significant emissions of CH_4 and N_2O as our climate is warming due to metal
291 limitations (Basiliko and Yavitt, 2001;Voigt et al., 2017). On the other hand, in temperate salt marshes
292 with reducing conditions, SO_4^- may suppress CH_4 emissions, but the abundant H_2S may reduce the
293 availiability of trace metals with important implications in N cycling and N_2O emissions (Gauci et al.,
294 2004;Butterbach-Bahl et al., 2013). In this study we added 26 µM and 74 µM of SO_4^- as $CuSO_4$ and $FeSO_4$
295 respectively that could potentially be reduced to H_2S, however the initial and final SO_4^- concentration
296 remained at comperable levels (Table 4).

297 Other well known environmental factors controlling denitrification is O_2, NH_4^+, NO_3^-/NO_2^- and C
298 (Thomson et al., 2012). Synthesizing the findings from other chemostat (Baumann et al., 1996;Felgate et
299 al., 2012;Giannopoulos et al., 2017;Hartop et al., 2017;Conthe et al., 2018) and incubation (Koike and
300 Hattori, 1975;Okereke, 1993;Morley et al., 2008) studies, it appears that the relative importance of the
301 aforementioned factors as control of denitrification is O_2> NH_4, NO_3/NO_2> C/N or C quality, with trace
302 metal bio-availability exerting an additional effect on each factor. In this study we did not indent to
303 quantify the relative contribution of each environmental factor on denitrification. Instead we
304 demonstrated the importance of trace metal availability on ecosystem processes contributing to the
305 release of N_2O, CH_4 and CO_2.

306 ***Future challenges***

307 Trace metal limitition in denitrification biochemistry and in general the C and N cycle may also be
308 important in agricultural ecosystems besides wetlands. The continous fertilization with macronutrients
309 (NPK) and intentisive cropping has resulted in low concentrations of microelements (trace metals) in
310 soils and edible crops (hidden hunger; (Alloway, 2008);Teklić et al. (2013)). The additon of essential
311 micronutrients particularly Cu, Mo and Fe to agricultural lands has been previously suggested as a
312 possible strategy to mitigate N_2O emissions (Richardson et al., 2009), but few assessments have been
313 reported. Felgate et al. (2012) and Black et al. (2016) recomended a Cu concentration of 13 µM and 50
314 µM (equiv. 150–200 $\mu g.g^{-1}$ Cu in soils) based on studies with *P. denitrificans* and *Ps. Stutezi*
315 respectively. Building upon prior knowledge, we demonstrate that effects of metal addition (2.5 Cu, 26.7
316 Fe and 7.8 Mo $\mu g.g^{-1}$ sediment) on greenhouse gas emissions in environmental samples (sediments) are
317 due to subsequent changes in the microbial community structure. We are currently missing quantitative
318 data on optimal levels of soluble trace metals for biochemical processes in C and N cycling in a variety of

319 different soil types. Agricultural and flooded soils are particularly interesting because they tend to have
320 higher N_2O yields than marine and aquatic ecosystems (Beaulieu et al., 2011). Future studies should
321 investigate the effects of trace metal bio-fertilization in soils under commercial crops to assess any
322 beneficial effects in greenhouse gas mitigation and crop quality, as in Montoya et al. (2018). The type of
323 trace metal fertilizer is particularly interesting to investigate because chelators based fertilizers may
324 scavenge the metal co-factors and dissable key enzymes of the N cycle (urease, ammonia
325 monooxygenase and nitrite reductase) prior to N_2O reduction (Moffett et al., 2012;Montoya et al.,
326 2018).

327 Assessing the abundance of microbial groups using qPCR showed significant differences between the
328 control and the treatments. In this short term batch experiment, differences in microbial group
329 abundance could also illustrate changes in in the diversity of the microbial groups, for example between
330 the abundance of copiotrophic and / or fast growing (*r* strategists) microbes upon NO_3^- and trace metal
331 addtion, relative to slow growing (*k* strategists) microbes (Fierer et al., 2012). Further information on
332 microbial diversity and meta-transcriptomic profiles under comperative treatments is needed to
333 understand and evaluate the ecological importance of trace metal limitation. Such 'omic studies should
334 be accompanied by high-throughput metabolite or process rate analysis either in the lab or in the field.

335

336 ***Conlcusion***

337 Emerging from this investigation, our results suggest that trace metal bio-availability may, directly or
338 indirectly, regulate or co-limit denitrification kinetics and induce a shift in the microbial community in
339 environmental samples, favoring a more active microbial community that is able to quickly acquire the
340 bio-available metals and incorporate them in functional oxy-reductases. This observation has important
341 implications in wetland C and N cycling. Considering also agro-ecosystems that are becoming limited in
342 trace metals trace metal amendments should be evaluated and considered as an alternative way to
343 mitigate N_2O emissions and improve the nutritional quality of crops.

344

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347

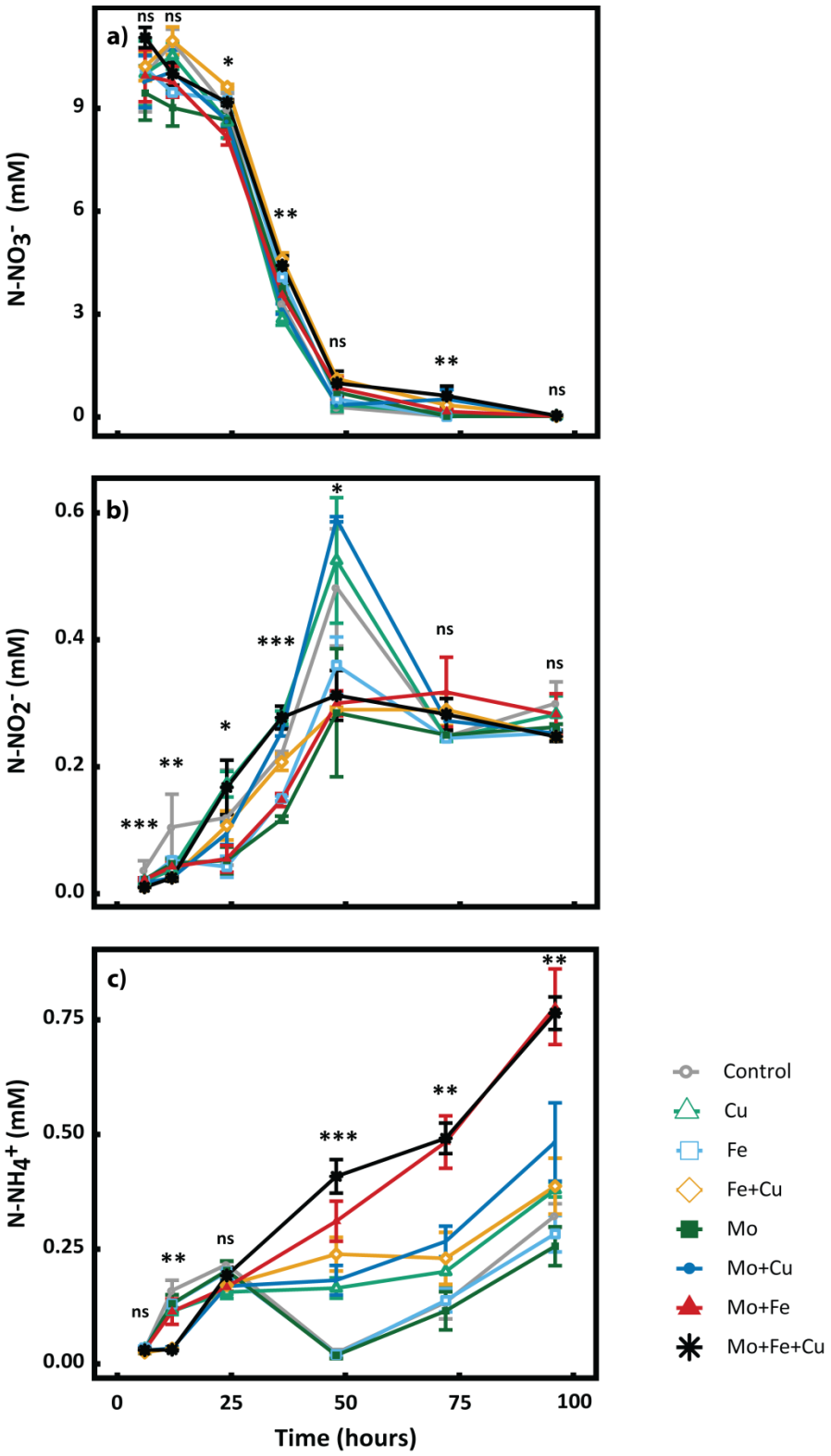


Figure 1 Average a) NO_3^-, b) NO_2^- and c) NH_4^+ concentration (mM; n=4, ±SE) per treatment throughout the incubation (96 h). Levels of significance: *≤0.5, ** ≤0.01, *** ≤0.001 and *ns* denotes non-significant. For Bonferroni test results, see SI.

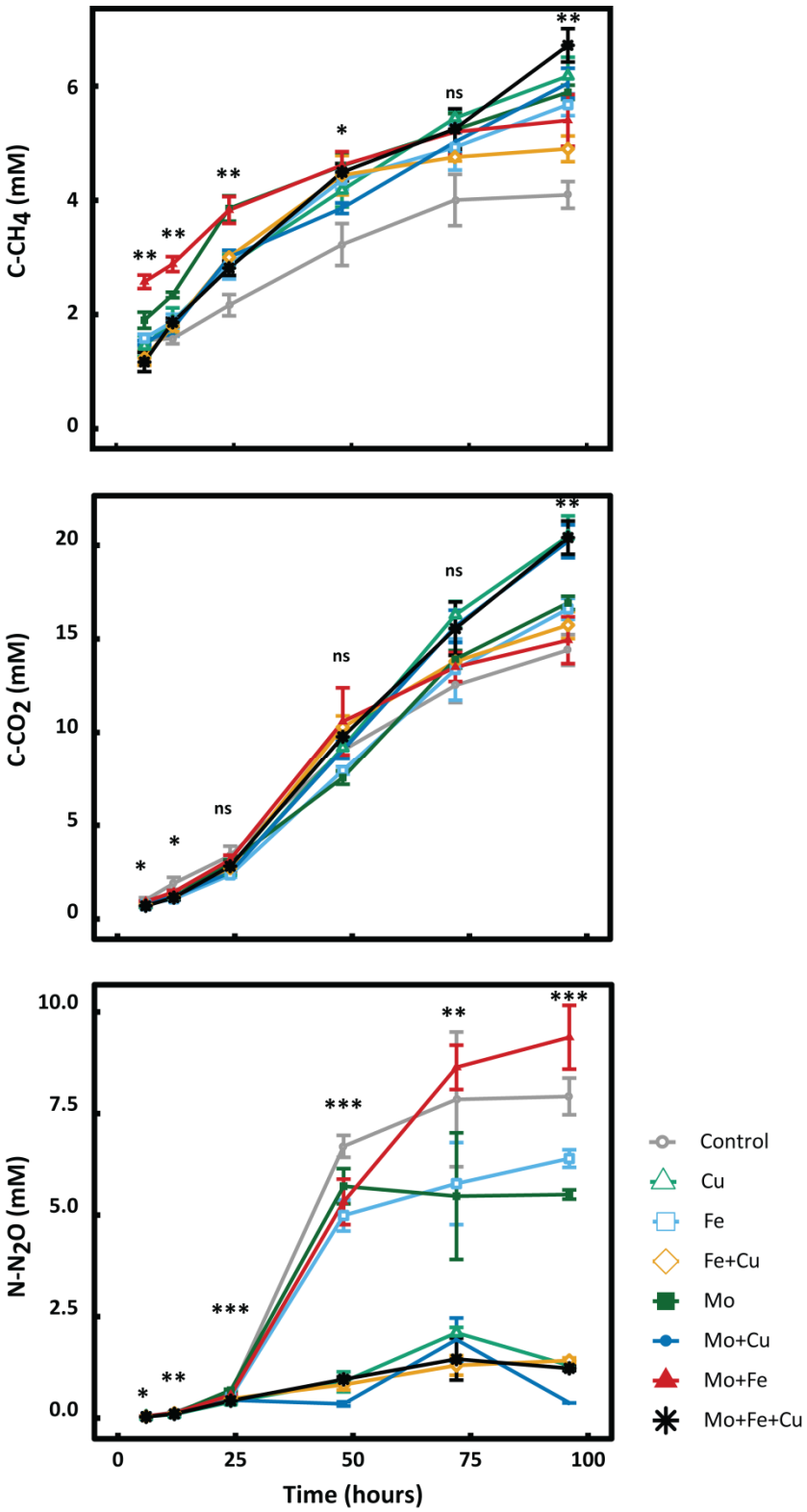
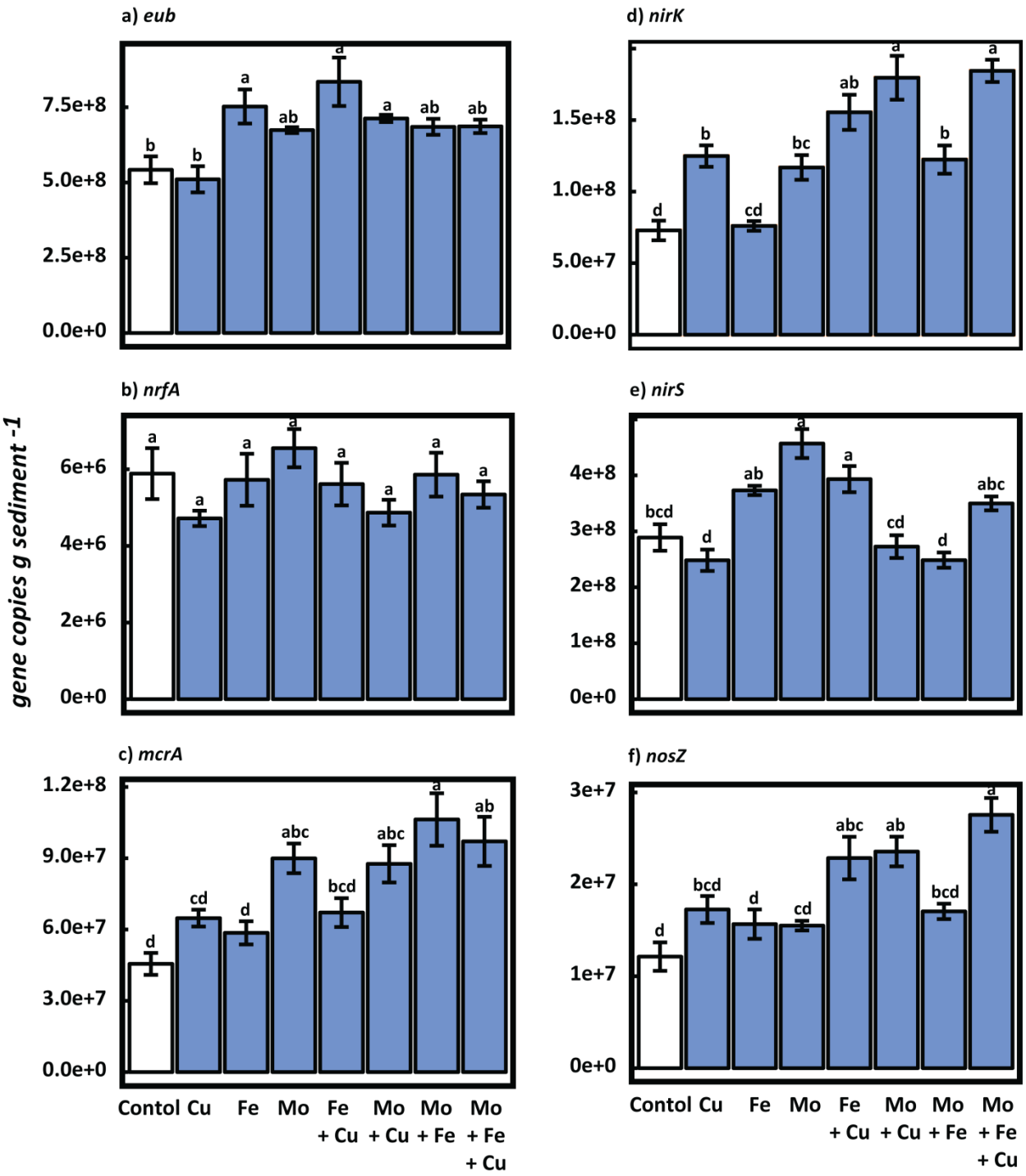


Figure 2 Cumulative gas concentration of CH_4, CO_2 and N_2O (n=4; ±SE). Levels of significance: *≤0.5, ** ≤0.01, *** ≤0.001 and ns denotes non-significant. For Bonferroni test results, see SI.



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357 **Figure 3** Average gene copies per g sediment per treatment at the end of the incubation (96 h; *n*=4; ±SE).
358 Treatments with the same letter are not significantly different.

359

360 Table 1 Experimental treatments.

Treatment	**Replicates**	**Nitrate** (KNO_3; mM)	**Molybdenum** ($(NH_4)Mo_7O_{24}$; µM)	**Iron** ($FeSO_4$; µM)	**Copper** ($CuSO_4$; µM)
Control	4	10	--	--	--
Cu	4	10	--	--	26
Fe	4	10	--	74	--
Mo	4	10	4	--	--
Fe + Cu	4	10	--	74	26
Mo + Cu	4	10	4	--	26
Mo + Fe	4	10	4	74	--
Mo + Fe + Cu	4	10	4	74	26

361

362 Table 2 Primers and reaction conditions for qPCR assays

Target	Primer	PCR Mix (15 µl)	Standard	Reaction Conditions	Reference
Eubacteria (*16S rRNA*)	Eub338 Eub518	1.2 ng template 0.1 µM each primer	*Desulfovibrio desulfuricans* ATCC 27774	95°C for 4 min, then 40 cycles of 30 s at 95°C, 30 s at 55.5°C, and 60 s at 72°C	(Fierer et al., 2005)
Denitrifiers (*nirS*)	cd3aF R3cd	10 ng DNA template 0.1 µM each primer	*Paracoccus dentrificans* ATCC 17741	95°C for 4 min, then 50 cycles of 30 s at 95°C, 30 s at 56°C and 60 s at 72°C	(Throback et al., 2004)
Denitrifiers (*nirK*)	nirKq-F nirK1040	1.5 ng DNA template 0.35 µM each primer	*Pseudomonas nitroreducens* ATCC 13867	15 min at 95°C, 9 touchdown cycles of 95°C for 15 s, 68°C for 60 s, and 81.5°C for 30 s (-1° per cycle for annealing); then 28 cycles of 95°C for 15 s, 60 °C for 60 s, and 81.5 °C for 30 s	(Smith et al., 2007)
Denitrifiers (*nosZ*)	nosZ1F nosZ2F	10 ng DNA template 1 µM each primer	*Pseudomonas fluorescens* C7R12	15 min at 95°C, 6 cycles of 95°C for 15 s, 67°C for 30 s with a touchdown of –1°C by cycle, 72°C for 30 s, and 80°C for 15 s (acquisition data step); 40 cycles of 95°C for 15 s and 62°C for 15 s, 72°C for 30 s, and 80°C for 15 s; and 1 cycle at 95°C for 15 s and 60°C for 15 s, to 95°C for 15 s	(Henry et al., 2006)
DNRA (*nrfA*)	nrfA6F nrfA6R	10 ng DNA template 0.3 µM conc. primers	*Escherichia coli* ATCC 11775	50°C for 2 min, 95°C for 8.5 min, and 50 cycles of 20 s at 94°C, 40 s at 54.5°C, and 10 s at 72°C	(Takeuchi, 2006)
Methanogens (*mcrA*)	mlas mcrA-rev	2 ng DNA template 0.6 µM conc. mlas 0.7 µM conc. mcrA-rev	*Methanococcus voltae* ATCC Strain A3	95°C for 5 min, then 50 cycles of 20 s at 95°C, 20 s at 59°C, and 45 s at 72°C	(Steinberg and Regan, 2009)

363 **Table 3** Average DNA yield per treatment and post-hoc analysis results of the probability of treatment
364 differences (n=4, ±SE).

Treatment	Total DNA ($ng.\mu l^{-1}$)		Means of the ranks*	
Control	44.3	±5	8.8	b
Cu	44.0	±2	6.0	b
Fe	50.6	±1	15.6	ab
Mo	57.0	±3	22.0	ab
Mo + Cu	52.8	±3	17.4	ab
Mo + Fe	53.3	±5	16.9	ab
Fe + Cu	53.3	±4	17.4	ab
Mo + Fe + Cu	62.3	±2	28.0	a

365 **Treatments with the same letter are not significantly different.*

366

367

368 **Table 4** Initial (0 h) and final (96 h) SO_4^- concentration in the treatments (n=4, ±SE).

Treatment	Initial [SO_4^-] µM		Final [SO_4^-] µM	
Control	4.9	±0.8	8.5	±3.5
Cu	23.4	±2.9	20.25	±2.0
Fe	68.9	±2.3	56.95	±8.4
Fe + Cu	99.2	±6.3	78.0	±33.3
Mo	15.0	±3.8	17.5	±2.7
Mo + Cu	21.4	±0.9	25.4	±2.7
Mo + Fe	68.1	±3.9	63.5	±5.0
Mo + Fe + Cu	133.0	±18.0	138.3	±21.0

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