Title

Trace metal availability affects greenhouse gas emissions and microbial functional group abundance in freshwater wetland sediments

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Abstract

We investigated the effects of trace metal additions on microbial nitrogen and carbon cycling using freshwater wetland sediment microcosms amended with µM concentrations of copper (Cu), molybdenum (Mo), iron (Fe), and all combinations. In addition to monitoring inorganic nitrogen transformations (NO_3^-, NO_2^-, N_2O, NH_4^+) and carbon mineralization (CO_2, CH_4), we tracked changes in functional gene abundance associated with denitrification (*nirS*, *nirK*, *nosZ*), DNRA (*nrfA*), and methanogenesis (*mcrA*). Greater availability of Cu led to more complete denitrification (i.e., less N_2O accumulation) and a higher abundance of the *nirK* and *nosZ* genes, which encode for Cu-dependent reductases. We found sparse evidence of DNRA activity and no consistent effect on CO_2 production. Contrary, net CH_4 production was stimulated by the trace metal amendments and the Mo additions, in particular, led to increased *mcrA* gene abundance. Taken together, these findings demonstrate that trace metal effects on microbial physiology, which have heretofore only been studied in pure culture, can impact community-level function. We observed direct and indirect effects on both nitrogen and carbon biogeochemistry that culminated in increased production of greenhouse gasses, and the shifts in functional group abundance that we documented suggest these responses may have been mediated through changes in microbial community composition. Overall, this work supports a more holistic consideration of metal effects on environmental microbial communities that recognizes the key role that metal limitation plays in microbial physiology.

Keywords: trace metals, denitrification, methane, carbon mineralization, greenhouse gases, wetlands, *nirK, nirS, nosZ, nrfA, mcrA*

Introduction

Wetland microbes are important for removing many anthropogenic pollutants from surface waters, effectively preventing the contaminants from entering downstream coastal and marine ecosystems. Nitrogen (N) pollutant release (> 60 Tg N y^{-1} globally) to the environment is of particular concern because it is 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). In freshwater wetland sediments, the microbial processes of nitrification, denitrification or coupled-denitrification, methanogenesis, and methanotrophy involve N_2O and CH_4 as metabolic byproducts, in addition to CO_2 (Soares et al., 2016). Nitrous oxide is an especially potent greenhouse gas, with a global warming potential (GWP) 298 and 25 times greater per 100 years than CO_2 and CH_4, respectively, and with important implications in ozone depletion (Ravishankara et al., 2009;IPCC, 2013). Some microbes can reduce N_2O to inert N_2 as part of denitrification, though there is only one enzyme known to facilitate this reaction: the nitrous oxide reductase (NOS)(Thomson et al., 2012). It is estimated that ~17 Tg N y^{-1} is returned to the atmosphere via denitrification in wetlands and terrestrial ecosystems globally, releasing ~48 Tg N y^{-1} to coastal and marine ecosystems (Schlesinger, 2009). Wetlands are equally important for their role in carbon (C) cycling. Despite their relatively small coverage (~10 % of global land area), these ecosystems store large amounts of C and emit considerable amounts of CH_4 (144 Tg CH_4 y^{-1})(IPCC, 2014). These net CH_4 emissions represent a balance between the microbial processes of methanogenesis and methanotrophy.

The microbial production and consumption of N_2O and CH_4 is catalyzed by oxy-reductases that utilize metal co-factors (Mo, Cu, Fe) (Glass and Orphan, 2012). For denitrification, the pathway includes the reduction of NO_3^- to NO_2^-, catalyzed by a periplasmic or membrane bound molybdenum (Mo) nitrate reductase (NAR) (Schwarz et al., 2009), and the further reduction of NO_2^- to NO by either a non-metal cytochrome cd_1 (Einsle et al., 1999) or a Cu co-factor (Adman et al., 1995) nitrite reductase (NIR), expressed by *nirS* and *nirK,* respectively. NO is further reduced to N_2O by nitric oxide reductase (NOR), which contains a heme-Fe co-factor (Shiro, 2012). Finally, N_2O is reduced by a Cu-containing NOS (Rosenzweig, 2000). Similar metal complexes are key in methane cycling. For example, Fe-, Ni-, and Zn- dependent ferrodoxins and dehydrogenases catalyze the early steps of methanogenesis, and all three classes of methanogens utilize a common methyl-coenzyme M reductase (MCR; *mcrA*) to catalyze the terminal steps (Glass and Orphan, 2012). Anaerobic methanotrophs such as members of the NC10 phylum (e.g., *M. oxyfera*) are able to couple CH_4 oxidation to denitrification (NO_2^--dependent methane oxidation (N-DAMO)) utilizing a Cu-dependent particulate methane monoxygenase (pMMO) and a Fe-rich cd_1 nitrite reductase (Deutzmann et al., 2014;Cheng et al., 2019). Through these sorts of interconnected pathways and processes, the abundance

and bioavailability of trace metals in the environment can impact C and N biogeochemistry and help regulate associated greenhouse gas emissions.

Although extensive research has considered the relative contribution of key environmental factors (e.g., pH, $NO_{3/2}^-$, O_2, and C/N) in regulating anaerobic microbial processes and specifically denitrification (Wallenstein et al., 2006), our understanding of the impact of metal availability is more limited. Metals in the environment have been traditionally seen as unwanted pollutants, and trace metal accumulation has been linked to toxicity and inhibition of ecosystem processes (Samanidou and Papadoyannis, 1992). For example, concentrations exceeding $mg{\cdot}L^{-1}$ range for Cu, Mo, Fe, Zn and Pb severely inhibited denitrification in soils, sediments, surface water bodies, and waste waters (Labbé et al., 2003;Magalhães et al., 2007;Liu et al., 2016). However, given the dependence of many microbial enzymes on metal co-factors, it is also possible for the opposite problem to occur – wherein function is limited due to an inadequate supply of trace metals. This scenario has received limited study in the environment, but is well documented in case studies with model organisms. In fact, *in vitro* studies have shown that lack of Cu, Mo, or Fe severely inhibits denitrification or methane cycling due to the formation of non-functional enzymes typically lacking the respective metal co-factor. For example, in the soil bacteria *Paracoccus denitrificans* and *Pseudomonas stutzeri*, Cu is required to express to a functional NOS dimer and reduce N_2O to N_2 (Granger and Ward, 2003;Felgate et al., 2012;Black et al., 2016). In methanotrophs oxidizing CH_4 to methanol (CH_3OH), the switch between a Cu-dependent pMMO or Fe-dependent soluble MMO is regulated by the environmental availability of each metal (Murrell et al., 2000;Bollinger Jr, 2010).

Despite the great progress in understanding metal ecotoxicity and metalloenzyme biochemistry, our knowledge of how trace metal availability affects microbial activity in the environment is generally limited to selected processes (e.g., NH_4^+/NO_3^- and CO_2 assimilation) in aquatic ecosystems (Twining et al., 2007;Glass et al., 2012;Moore et al., 2013;Romero et al., 2013;Schoffman et al., 2016). A broader understanding of how metal availability regulates microbial processes, especially in soils and sediments, is necessary if we are to fully appreciate environmental controls on ecosystem C and N cycling. In this study, we investigated the effects of trace metal additions on the microbial biogeochemistry of freshwater wetland sediments focusing primarily on NO_3^-/NO_2^- reduction and greenhouse gas kinetics, and used quantitative (qPCR) to assess changes in the abundance of key functional groups associated with these processes. We hypothesized that greater bioavailability of Mo, Fe, and Cu in the sediments would increase denitrification and that greater Cu availability would reduce N_2O emissions. We also predicted that C mineralization rates would be impacted by metal additions, specifically higher CO_2 emissions driven by greater denitrification rates. Furthermore, we expected net CH_4 emissions to be lower due to a combination of decreased CH_4 production, since denitrifiers

will outcompete methanogens for C substrates once metal limitations are removed, and increased CH_4 consumption, because of stimulation of methanotrophs through Fe and Cu additions.

Materials and Methods

Soil samples

Five soil samples (~350 g each, 5 – 15 cm from surface and ~2 m apart) were collected using a PVC pipe (tube dia. 10 cm and length ~15 cm) from a tidal-fluvial point bar deposit in the Pamunkey River (N 37.557451, W -76.972521) during October 2016. Samples were kept in a cooler on ice during transport to the lab and stored overnight (4°C) until experimental setup (below) and characterization using standard methods (SSSA, 1996). Sediments had 25 (± 4 S.E.) % organic matter (OM), 79 (± 6) % gravimetric moisture content, 0.18 (± 0.05) g.cm^{-3} bulk density, C/N ratio of 11.0 (± 1.5), and pH 6.1 (± 0.3). Porewater, collected from aliquots of soil via centrifugation, contained 0.10 (± 0.03) mM NO_3^-, 0.07 (± 0.02) mM NO_2^-, 0.16 (± 0.04) mM SO_4^{2-}, 0.4 (± 0.1) mM Cl^-, 0.3 (± 0.1) mM NH_4^+, 0.11 (± 0.01) mM K^+, 0.26 (± 0.10) mM Mg^{2+}, and 0.29 (± 0.10) mM Ca^{2+}.

Experimental set-up

After large stones and roots were removed, the five soil samples were combined in approximately equal parts to form a composite sample for subsequent experiments. An aliquot (1000 g) of this composite soil was homogenized briefly with an additional 1500 ml of filtered porewater (Cellulose, 10-µm pore-size, Millipore, Burlington, Massachusetts, USA) using a commercial blender (30 s at max speed). Soil slurry aliquots (50 ml) were then transferred to sterile 125-ml glass bottles (Wheaton, Millville, New Jersey, USA). Bottles were crimped with rubber septa and incubated for 3 days in the dark (25°C) to allow residual O_2 to be consumed by basal respiration. Then, each bottle received a 250-µl aliquot of 2 M KNO_3 for a final calculated NO_3^- concentration of 10 mM. According to the treatment plan (**Table 1**), replicate (*n*=4) bottles also received aliquots (200 µl) from aqueous stock solutions of ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}$; 1 mM), iron sulfate ($FeSO_4$; 18.5 mM) and copper sulfate ($CuSO_4$; 6.5 mM), yielding final concentrations of 28, 74 and 26 µM of Mo, Fe, and Cu respectively. Bottles were vortexed briefly (30 s at 6000 rpm), flushed with N_2 (60 min), and incubated in the dark (25°C). Gas (5 ml) and soil slurry (1.5 ml) samples were taken anaerobically using a sterile syringe after 0, 6, 12, 24, 48, 72, and 96 h. Following the 96 h sampling, bottles were opened in an O_2-free chamber and 1 g of wet soil was removed and immediately frozen (-20°C) for future molecular analyses.

Analytical techniques

142 Gas samples were stored in 3-ml Exetainer vials (Labco, UK) that were previously flushed (N_2; 5 min) and
143 vacuumed. Concentrations of N_2O, CO_2, and CH_4 were determined with gas chromatography (GC-14A
144 equipped with FID, TCD and ECD; Shimadzu, Columbia, Maryland, USA) as in Morrissey and Franklin (2015).
145 For each gas, total production was determined as the sum of the gas accumulated in the headspace and the
146 gas dissolved in the slurry; the latter was estimated using the relationships described by Heincke and
147 Kaupenjohann (1999). Liquid samples were obtained from each slurry sample by centrifugation (10,000 x *g*
148 for 5 min) and the supernatant was diluted 10-fold, filtered (0.22-µm pore size), and stored frozen (-20°C)
149 until ion analysis. The concentrations of NO_3^-, NO_2^-, SO_4^{2-} and NH_4^+ were determined by ion-chromatography
150 (ICS – 5000+, Dionex, Sunnydale, California, USA) according to the manufacturer's instructions using columns
151 Dionex IonPac AS17 (2 × 250 mm) and IonPac CS12 (A – 5 µm, 3 × 150 mm) and a Dionex conductivity sensor.
152

153 ***Molecular techniques***

154 DNA was extracted from frozen soil samples (~1 g wet weight, equivalent to ~0.3 g dry weight) using the
155 DNEasy Kit from Qiagen (Germantown, Maryland, USA) following the manufacturer's instructions. The
156 retrieved DNA was quantified using a Nanodrop spectrometer (Thermo, Wilmington, Delaware, USA) and
157 visualized on a 1.2 % agarose gel for integrity. The DNA yields were, on average, 42 (± 2) ng·µl^{-1}. DNA
158 samples were diluted to 3 ng·µl^{-1} prior to use in quantitative PCR (qPCR) to determine the abundance of
159 microbial groups typically found in wetland sediments: total eubacteria (*eub-518*), nitrite reducers
160 (denitrification: *nirK* and *nirS*; DNRA: *nrfA*), nitrous oxide reducers (*nosZ*), and methanogens (*mcrA*).
161 SensiFAST™ SYBR® No-ROX Kit Polymerase 2X mix (Bioline, UK) was used according to the manufacturer's
162 instructions. Primers were purchased from IDT (Integrative DNA Technologies, Skokie, Illinois, USA) and the
163 DNA for the standard curves was extracted from isolates obtained from ATCC (American Type Culture
164 Collection, Manassas, Virginia, USA). Data were collected using a BioRad CFX-384 Real Time System (BioRad,
165 Hercules, California, USA) and analyzed using CFX Manager software (Ver 3.1).
166

167 ***Estimation of the combined GHG emissions (CO_2 equivalents)***

168 To compare overall GHG emissions across treatments, we estimated the combined "CO_2-equivalents" for
169 CO_2, N_2O, and CH_4 based on the 100 y GWP factors (IPCC, 2013). The combined CO_2-equivalents as g C per
170 bottle were calculated as follows:

$$CO_{2-eqv} = V * \{(12 * [CO_2]) + (298 * 14 * [N_2O]) + (25 * 12 * [CH_4])\}$$

171 where V is the volume, 298 and 25 is the global warming potential for 100 y per weight basis in CO_2-
172 equivalents for N_2O and CH_4, respectively; 12 the molecular weight of C per mole CO_2 and CH_4; 14 the
173 molecular weight of N per mole N_2O; [CO_2] and [CH_4] are the M concentrations for CO_2 and CH_4, respectively;
174 and, lastly, [N_2O] the M concentration of N_2O per mole N.

175

176 ***Statistical analyses***

177 Kinetic and molecular datasets were tested for normality (RStudio [*base*]; Shapiro.test). Non-parametric
178 *Kruskal-Wallis* with *Bonferroni* correction statistical tests were applied to assess differences in the mean of
179 ranks between the treatments (RStudio [*agricolae*]). Basic statistics (mean and standard error) were
180 summarized with RStudio [*dyplr*] (Boston, Massachusetts, USA) and plotted with SigmaPlot 14 (Systat
181 Software Inc. San Jose, California, USA). For all statistical tests, $p \leq 0.05$ was considered significant.

182

183 **Results**

184 ***The effect of trace metal additions on NO_3^-, NO_2^-, and NH_4^+ kinetics***

185 At the end of the incubation, all treatments had visible gas bubbles on the surface and within the slurry,
186 indicating microbial activity and gas production. We observed yellow and black patches within the slurry
187 possibly due to Fe and residual Mn reduction, indicating anaerobic reducing conditions. Nitrate reduction
188 commenced at the same time for all the treatments (12-24 h, **Figure 1**), and ~12 mM NO_3^- was consumed by
189 the end of the incubation. At that time, 0.3 mM NO_2^- remained across all treatments while NH_4^+ ranged from
190 0.20 to 0.75 mM (**Figure 1**). The treatments receiving additional Mo+Fe ($p \leq 0.01$) and Mo+Fe+Cu ($p \leq 0.01$) had
191 significantly higher NH_4^+ than the control, and an increasing trend in NH_4^+ concentration for all treatments
192 was observed towards the end of the incubation.

193

194 ***The effect of trace metal additions on N_2O, CH_4 and CO_2 cumulative gas kinetics***

195 Matching the NO_3^- consumption in the slurry, N_2O emissions increased rapidly between 24 to 48 h (**Figure**
196 **2.a)**. Notably, the control, Mo, Fe, and Mo+Fe treatments accumulated significantly more N_2O than the
197 remaining treatments (Cu, Fe+Cu, Mo+Cu and Mo+Fe+Cu) that all contained Cu. Total N_2O concentrations
198 reached a plateau between 48 to 96 h, most likely due to exhaustion of the available NO_3^-. CH_4 accumulated
199 quickly in the microcosms (6 to 12 h) and continued to accumulate up to ~6 mM. By the end of the
200 incubation, all metal additions caused an increase in CH_4 production relative to the control, though the
201 differences were only significant between the control and the Cu, Mo+Cu and Mo+Fe+Cu treatments (**Figure**
202 **2.b**).

203 Cumulative CO_2 production increased most rapidly between 12 to 24 h, and continued to increase up to ~20
204 mM CO_2 at the end of the incubation. There were no significant differences, except between the control and
205 the Cu, Mo+Cu, Mo+Fe+Cu treatments (Figure 2.**c**).

206

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

208 The average bacterial abundance was 6.7 x 10^8 *eub* copies·g^{-1} sediment dry weight with the Fe, Fe+Cu and
209 Mo+Cu treatments having significantly (p=0.005) more *eub* gene copies than the control (*eubacteria;* **Figure**
210 **3**). Trace metal addition affected the abundance of *nirK* (p<0.001), *nirS* (p<0.001), and *nosZ* (p=0.001)
211 denitrifying microbial groups. Reducers of NO_2^- utilizing Cu-NIR (*nirK*) were more abundant in all treatments
212 when compared to the control, though the increase due to Fe addition was not significant. Microbial groups
213 having a cytochrome cd_1-NIR (*nirS*) were relatively more abundant in the Mo and Fe+Cu treatments. The
214 *nosZ* microbial group responsible for the reduction of N_2O to N_2 was more abundant in the treatments that
215 received Cu. We observed no effect of trace metals on DNRA NO_2^- reducers (*nrfA*; **Figure 3**) but found that
216 methanogens were more abundant in the treatments containing additional Mo (Mo, Mo+Cu, Mo+Fe and
217 Mo+Fe+Cu; *mcrA*; **Figure 3**) when compared to the control.

218

219 **Discussion**

220 We observed that trace metal (Mo, Fe, Cu, and their combinations) addition to wetland sediments regulated
221 denitrification kinetics (**Figure 1**) and greenhouse gas emissions (**Figure 2**), and altered the abundance of
222 microbial functional groups typically associated with N removal and C cycling (**Figure 3**). Previous studies
223 have examined the effects of trace metal availability on these processes, often reporting negative effects
224 (Giller et al., 1998;Magalhães et al., 2007;Magalhães et al., 2011;Deng et al., 2018;Keller and Wade, 2018).
225 The range of metal concentrations previously tested far exceeds the levels applied in the current study. The
226 key interpretation of this experiment is that trace metal availability, specifically Cu, enhances the reduction
227 of N_2O to N_2, stimulates the production of CH_4, and has no effect on CO_2 emissions from wetland sediments.
228 When N_2O, CH_4, and CO_2 were combined and expressed as CO_2-equivalents, all treatments receiving a Cu
229 addition (Cu, Mo+Cu, Fe+Cu and Mo+Fe+Cu) had significantly lower CO_2-equivalent emissions (**Figure 4**),
230 indicating the major role of Cu in anaerobic respiration (denitrification and methanogenesis) as a regulator
231 of nutrient cycling and greenhouse gas emissions.

232

233 ***Denitrification***

234 Denitrification is an anaerobic respiratory process that utilizes NO_3^- as a terminal electron acceptor with
235 sequential reduction: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. In our experiments, none of the trace metal additions
236 had an effect on NO_3^- removal (**Figure 1**), which is somewhat surprising since all bacterial nitrate reductases
237 contain a Mo cofactor at their active sites (Moreno-Vivian et al., 1999). This may indicate that Mo was not

limiting to the denitrifiers in this set of soils. Following NO_2^- reduction, the production and consumption of NO is an important denitrification intermediate. Although we didn't measure NO in our experiments, we assume that it was quickly utilized or reduced to N_2O due to its potent cytotoxicity at µM levels (Chaudhari et al., 2017;Hartop et al., 2017). In contrast, we saw a dramatic effect of Cu addition. Regardless of what other metals were included, Cu amendments significantly enhanced the reduction of N_2O to N_2 (**Figure 2**) and led to an increased abundance of nitrite (*nirK*) and nitrous oxide (*nosZ*) reductace genes (**Figure 3**), which both code for Cu-containing enzymes. The especially strong effect of Cu is because the last step of denitrification is catalyzed by a Cu-containing reductase (NOS) and, thus far, no other alternative pathway has been suggested (Thomson et al., 2012). In laboratory experiments, Cu has similarly been found to be a strong driver of denitrifying metabolism. For example, Cu-deficient cells can have more transcripts of *nosZ* and Cu-scavenging genes to compensate for the loss of N_2O reduction due to depleted Cu availability (Felgate et al., 2012). Also, strains deficient in Cu-transporters and chaperones may be unable to reduce N_2O to N_2, even when *nosZ* is expressed and forming a non-functional NOR (Sullivan et al., 2013).

In the treatments accumulating notable amounts of N_2O (≥ 1 mM), N_2O cytotoxicity may occur and it can be relieved by the addition of vitamin B12, Co, or methionine (Sullivan et al., 2013). We do not exclude other partial denitrification processes or other anaerobic processes simultaneously co-occurring with denitrification as subjected by the residual N_2O in the Cu-containing treatments (≤2.3 mM N_2O). The continuous accumulation of CO_2 in all treatments firstly, as proxy for microbial activity, suggests against any indirect acute cytotoxicity from NO due to NO_2^- reduction or N_2O accumulation. Secondly, cumulative CO_2 emissions indicate that the available electron donor (organic C) was in excess of the added NO_3^-. The magnitute of CO_2 accumulation was predictable; treatments with less residual N_2O, and thus a more complete denitrifation pathway, had accumulated more CO_2 by the end of the incubation.

DNRA

Another possible fate of the added NO_3^- is reduction to NH_4^+ via dissimilatory nitrite reduction to ammonium (DNRA). Over the course of the study, we saw small accumulations of NH_4^+ (<1 mM; **Figure 1**), which suggests this process could be happening to some degree, and observed variable effects depending on the type of metal added. The accumulation of NH_4^+ was greatest in treatments that contained both Mo and Fe (i.e., Mo+Fe and Mo+Fe+Cu in **Figure 1**), which may indicate a synergistic response of the enzymes that reduce NO_3^- (all nitrate reductases are Mo dependent) and the DNRA-specific nitrite reductase (*nrfA)*, which includes a multi-heme complex. The addition of either Mo or Fe alone did not result in NH_4^+ accumulation. The addition of Cu (added alone, or in combination with Mo or Fe) only led to a modest increase in NH_4^+. Overall, the accumulated NH_4^+ (<1 mM) was relatively less to the amount of NO_3^- removed (>10 mM) and the

271 abundance of the DNRA marker gene *nrfA* was equally low (**Figure 3**), so it is unclear whether DNRA was
272 occurring to an appreciable extent in our microcosms. Alternatively, one could postulate that anaerobic
273 organic matter mineralization contributed to the accumulation of NH_4^+ and that observed differences due to
274 metal addition were indirect responses. However, if this were the case, one would expect to see a
275 correlation between final NH_4^+ concentration and total CO_2 production, a finding which is not evident. DNRA
276 and N mineralization commonly co-occur in wetland sediments (White and Reddy, 2009), and additional
277 analyses would be necessary to disentangle the two.

278

279 ***Effect on carbon mineralization***

280 Wetland soils are large C sinks because anaerobic conditions constrain organic matter mineralization and
281 oxidation. The lack of electron acceptors, such as: O_2, NO_3^-, and SO_4^{2-} in freshwater wetlands, may lead to
282 fermentative and reductive conditions were organic C will be reduced to CH_4 (Kim et al., 2015;Cheng et al.,
283 2019). In this study, the pattern of CO_2 emissions primarily reflected the magnitude of denitrification, as
284 discussed earlier. Also, the addition of a more bio-energetically favourable substrate (NO_3^-) did not inhibit
285 methanogenesis, nor did the addition of Fe and Cu increase CH_4 consumption as hypothesized, considering
286 Fe- and Cu-methane monoxygenase pathways; rather, CH_4 accumulated throughout the incubations. The
287 addition of 10 mM NO_3^-, 5 mM NO_2^-, and 1 mM N_2O in anoxic rice field slurries inhibited methanogenesis for
288 30, 25, and 20 d, respectively, and after that methanogenesis resumed (Klüber and Conrad, 1998). Perhaps,
289 the addition of 10 mM NO_3^- was not sufficient to fully inhibit methanogenesis and/or the ample electron
290 donors from organic matter alleviated any inhibitory effects.

291 In terms of trace metal amendment, Mo addition enhanced the abundance of *mcrA* genes (Mo, Mo+Cu,
292 Mo+Fe and Mo+Fe+Cu; **Figure 3**). This was expected because a Mo co-factor is typically required for the
293 formylmethanofuran dehydrogenase (*fwd*) that catalyzes the reduction of CO_2 to formyl-methanofuran in
294 the first step of methanogenesis by reduction of CO_2 with electrons from H_2 (Glass and Orphan, 2012). The
295 subsequent step in methanogenesis is catalyzed by MCR, which is a Ni-containing enzyme. Since we did not
296 add Ni to any in any of our treatments, we assumed that MCR would not change (or would decrease due to
297 compentation with denitrification). Mo addition and the increasing partial pressure of CO_2 in the bottles
298 during the incubation could have triggered a shift towards a Mo-based methanogenesis pathway. In general,
299 methanogens tend to grow more slowly than denitrifers. It could be that the *mcrA* response was not as
300 strong due to insufficient time to increase in abundance (Enzmann et al., 2018). Another interesting aspect
301 of Cu effects on CH_4 emissions is the competition between methanotrophs and denitrifiers for the "Cu
302 monopoly" (Chang et al., 2018), which should be further investigated in environmental samples. Thus, the

303 higher abundance of methanogens (*mcrA*) and noticable accumulation of CH_4 in all metal treatments than
304 the control indicates an important role of trace metal availability in CH_4 cycling in environmental samples.

305

306 ***Potential environmental implications***

307 Previous studies have examined the effects of trace metal abundance or addition on C- and N- cycling in
308 sediments, peat, and agricultural soils. Typically, those studies report a negative effect; however, the range
309 of trace metal concentrations in those studies far exceed the levels of the current study. For instance, Keller
310 and Wade (2018) amended peat slurries with ~200 µM trace element solution and saw significant decrease
311 in CH_4 emission, most likely due to Cu-induced toxicity. Similarly, Magalhães et al. (2007) observed 85%
312 inhibition of denitrification accompanied with accumulation of substantial N_2O and NO_2^- at 79 µg $Cu \cdot g^{-1}$
313 sediment (equiv. 1200 µM Cu) in estuaries. In a follow-up study, lower denitrification rates due to Cu (~900
314 µM) were accompanied by a decline in the abundance and *β*-diversity associated with the *nirK*, *nirS*, and
315 *nosZ* microbial groups (Magalhães et al., 2011). A more recent study that tested the effects of Cu pollution
316 (addition of 100 $\mu g \cdot L^{-1}$, ~1.6 µM) on urban freshwater wetland sediments found significantly reduced CH_4
317 emissions, but no change in N_2O emissions (Doroski et al. (2019). This divergent observation could be due to
318 low NO_3^- concentration (18 µM) in relation to available C or the presence of substantial amounts of soil Cu
319 (9.4 $\mu g \cdot g^{-1}$ sediment) at the start of the experiment in both control and Cu treatment.

320 The abundance of bioavailable metals in the environment, typically in their ion forms, is often observed at
321 several magnitute orders less compared to the routinely reported total metal content. Such trivial amounts
322 could be unavailable to microorganisms due to physiochemical interactions and plant uptake (Giller et al.,
323 1998). At neutral and alkaline pH levels, metals tend to be effectively immobilized as inorganic compounds
324 (metal-oxides, -hydroxides and -carbonates). Additionally, organic complexes such humic ligands are known
325 to bind metal cations lessening their availablity to microbes and plants. For example, the particularly low
326 concentrations of dissolved Cu, Fe, and Mo (~5, 1 and 0.2 $\mu g \cdot L^{-1}$, respectively) and the copious dissolved
327 organic C (~80 $mg \cdot L^{-1}$) in Siberian peatlands (Raudina et al., 2017) could trigger significant emissions of CH_4
328 and N_2O with climate warming (Basiliko and Yavitt, 2001;Voigt et al., 2017). On the other hand, in temperate
329 salt marshes with reducing conditions, ample SO_4^{2-} may support abundant communities of sulfate-reducing
330 bacteria that outcompete methanogens and thus suppress CH_4 emissions, but the H_2S produced due to SO_4^{2-}
331 reduction may limit the availability of trace metals through the production of insoluble metal-sulfide
332 complexes with potential implications in N cycling and N_2O emissions (Gauci et al., 2004;Butterbach-Bahl et
333 al., 2013). In this study, we added 26 µM and 74 µM of SO_4^{2-} as $CuSO_4$ and $FeSO_4$, respectively, that could
334 potentially have been reduced to H_2S; however, the initial and final porewater SO_4^{2-} concentrations remained

335 at comparable levels (average change over time across treatments was only 2.8%, **Table 3**) and no sulfide
336 odor was detected when microcosms were opened.

337 Other well-known environmental factors controlling denitrification are O_2, NH_4^+, NO_3^-/NO_2^-, and C (Thomson
338 et al., 2012). Synthesizing the findings from other chemostat (Baumann et al., 1996;Felgate et al.,
339 2012;Giannopoulos et al., 2017;Hartop et al., 2017;Conthe et al., 2018) and incubation (Koike and Hattori,
340 1975;Okereke, 1993;Morley et al., 2008) studies, it appears that the relative importance of the
341 aforementioned factors in control of denitrification is O_2> NH_4^+, $NO_{3/2}^-$> C/N or C quality, with trace metal
342 bioavailability exerting an additional effect on each factor. The present results suggest that the effect Cu
343 exerts on N_2 and N_2O emissions from the environment may be more extensive than previously thought.
344 These findings are important not only for understanding fundamental controls on N_2O production, but also
345 for making predictions about potential future emissions given increasing nutrient and metal loads associated
346 with urban pollution. Interestingly, the effect of trace metal availability on denitrification enzymology is also
347 of considerable interest in the context of agriculture and food production, and it has even been suggested
348 that metal additions could be a possible strategy to mitigate high N_2O emissions associated with crop
349 production (Richardson et al., 2009).

350

351 ***Conclusions and future challenges***

352 Our results demonstrate that the importance of trace metal availability in microbial physiology can affect
353 community-level function, including rates of anaerobic respiration, and manifest as differences in ecosystem
354 processes. We found that even short-term (96 hr) exposure to increased concentrations of trace metals led
355 to changes in community structure (functional group abundance) and greenhouse gas emissions (N_2O, CH_4,
356 and CO_2). In particular, our results suggest that trace metal bioavailability may, directly or indirectly, regulate
357 or co-limit denitrification kinetics and favor more active microbial communities that are able to quickly
358 acquire the bioavailable metals and incorporate them in functional oxy-reductases. Disentangling the
359 potential controls of various metals on denitrification, and co-varying effects on C cycling, requires more
360 experiments across a range of metal concentrations and biochemical processes in a variety of different soil
361 types and ecosystems. Future studies should investigate microbial diversity and meta-transcriptomic profiles
362 under comparative treatments to understand and evaluate the ecological importance of trace metal
363 limitation. Such 'omic studies should be accompanied by high-throughput metabolite or process rate analysis
364 either in the lab or in the field.

365

366 **Acknowledgements**

367 We thank Dr. Ioannis Ipsilantis for his comments on an earlier version of this manuscript. We also thank
368 Colin McKenny for assistance in field and lab, Gabriella Balasa for assistance with qPCR, Olivia De Meo for
369 setting up the GC and HPLC procedures, and Dr. Scott Neubauer for equipment access. This work was
370 supported by a grant from the National Science Foundation to Franklin, Brown, and Neubauer (DEB
371 1355059). This paper is contribution [# to be inserted at time of publication] from the VCU Rice Rivers
372 Center.

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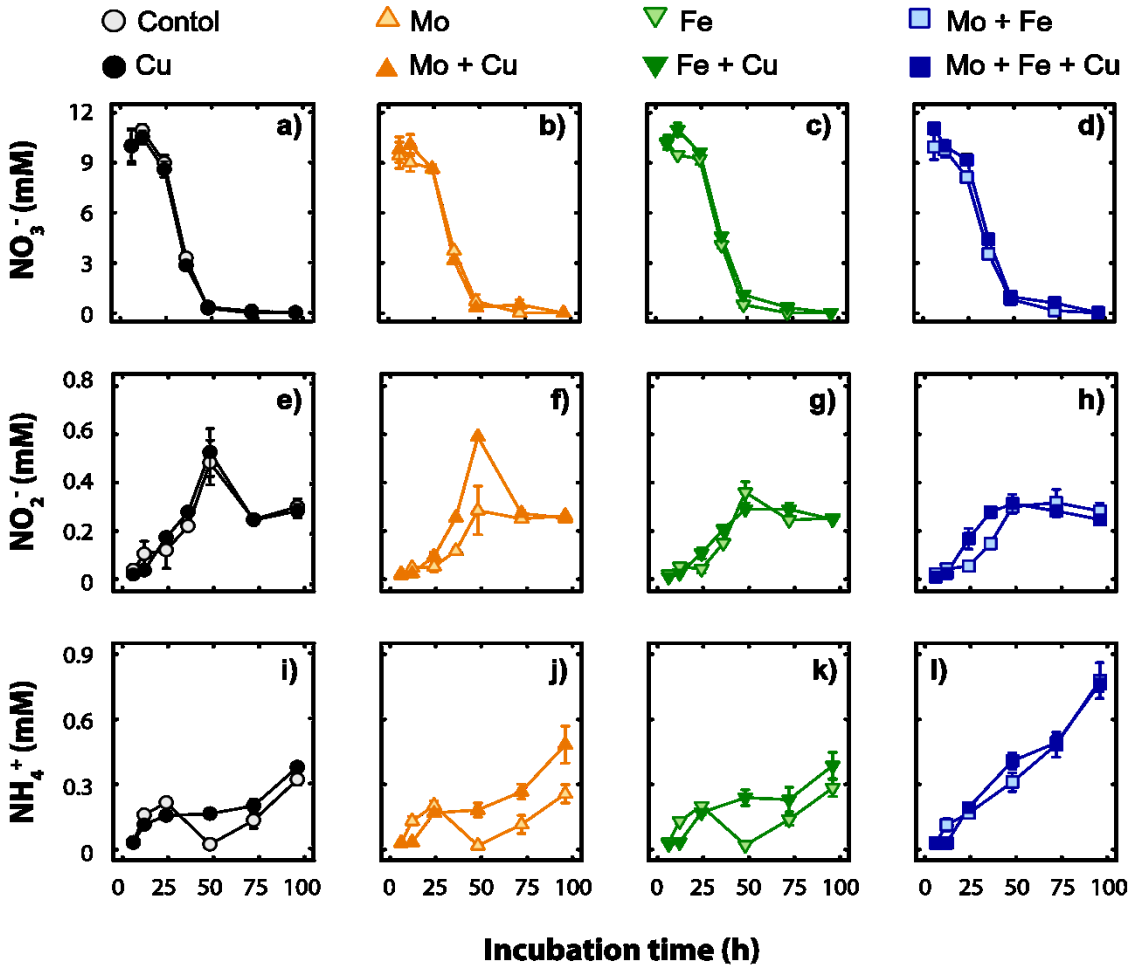

376

377 **Figure 1** Average NO_3^- (a, b, c, d), NO_2^- (e, f, g, h), and NH_4^+ (i, j, k, l) concentrations (mM, n=4, mean ± SE) per
378 treatment throughout the incubations.

379

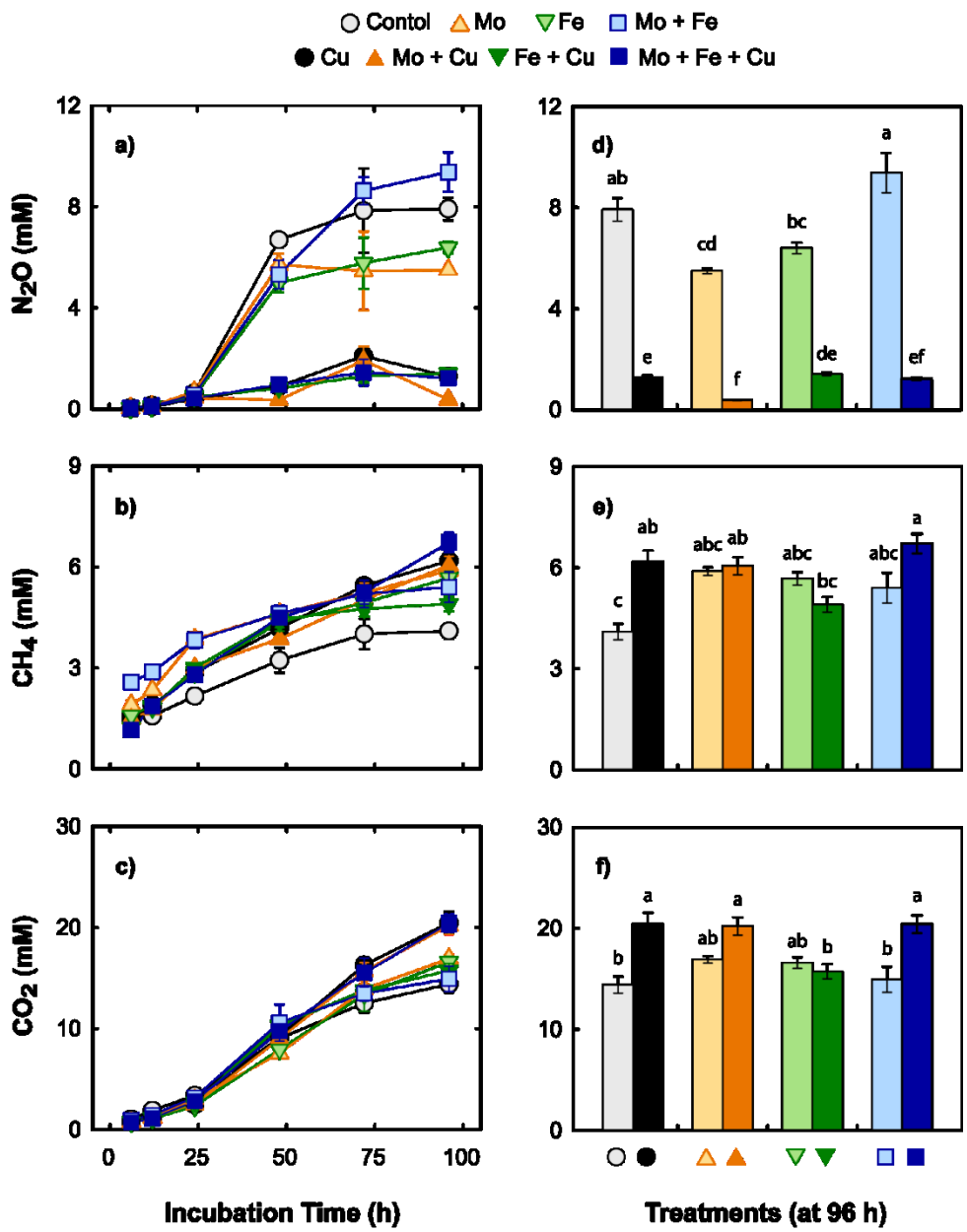

Figure 2 Cumulative gas concentrations of N_2O (a, d), CH_4 (b, e) and CO_2 (c, f), through the incubation and at 96 h, respectively (*n*=4; mean ± SE). Within each bar graph (d, e, f), treatments with the same letter are not significantly different as determined via Kruskal-Wallis and Bonferroni *post hoc* testing.

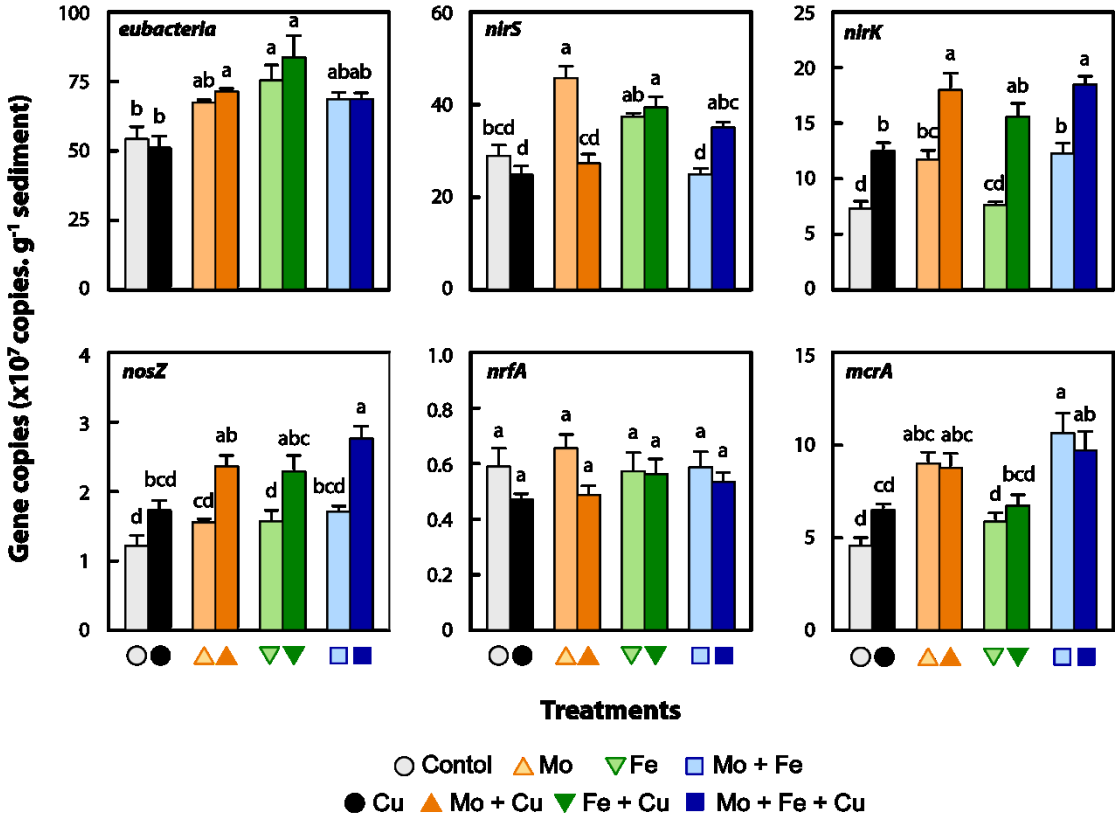

Figure 3 Average gene copies per g sediment (dry weight) at the end of the incubation (96 h; *n*=4; mean ± SE). Within each graph, treatments with the same letter are not significantly different as determined via Kruskal-Wallis and Bonferroni *post hoc* testing.

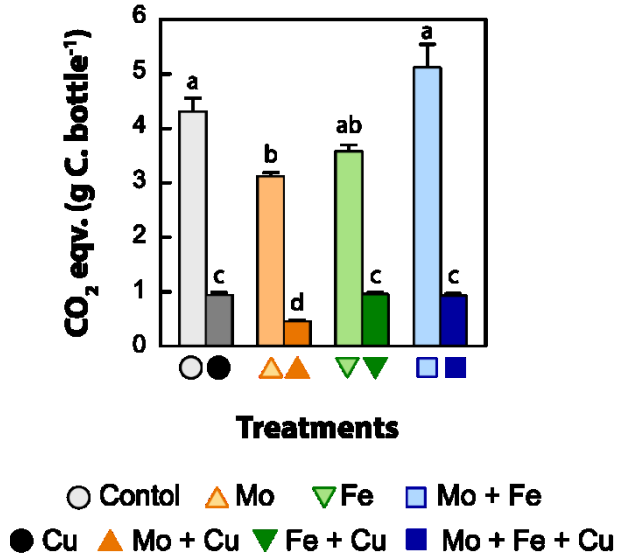

Figure 4 Average cumulative CO_2-eqv. emissions (100 y^{-1}) per treatment at the end of the incubation (96 h; *n*=4; mean ± SE). Treatments with the same letter are not significantly different.

394

395 **Table 1** Experimental treatments (*n*= 4).

Treatment	**Nitrate** (KNO_3; mM)	**Molybdenum** ($(NH_4)_6Mo_7O_{24}$; µM)	**Iron** ($FeSO_4$; µM)	**Copper** ($CuSO_4$; µM)
Control	10	--	--	--
Cu	10	--	--	26
Fe	10	--	74	--
Fe+Cu	10	--	74	26
Mo	10	28	--	--
Mo+Cu	10	28	--	26
Mo+Fe	10	28	74	--
Mo+Fe+Cu	10	28	74	26

396

397

Table 2 Primers and reaction conditions for qPCR assays

Target	Primer	PCR Mix (15 µl)	Standard	Reaction Conditions	Reference
Eubacteria (*16S rRNA*)	*eub*338 *eub*518	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*)	*nirK*q-F *nirK*1040	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*)	*nosZ*1F *nosZ*2F	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*)	*nrfA*6F *nrfA*6R	10 ng DNA template 0.3 µM each primer	*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 *mlas* 0.7 µM *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)

Table 3 Initial (0 h) and final (96 h) SO_4^{2-} concentrations in the various treatments (*n*=4, mean ± SE).

Treatment	Initial [SO_4^{2-}] µM		Final [SO_4^{2-}] 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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