Effect of freshwater mussels on the vertical distribution of anaerobic ammonia oxidizers and other nitrogen-transforming microorganisms in upper Mississippi river sediment

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Targeted qPCR and non-targeted amplicon sequencing of 16S rRNA genes within sediment layers identified the anaerobic ammonium oxidation (anammox) niche and characterized microbial community changes attributable to freshwater mussels. Anammox bacteria were normally distributed (Shapiro-Wilk normality test, W-statistic=0.954, p=0.773) between 1-15 cm depth and were increased by a factor of 2.2 (p<0.001) at 3 cm below the water-sediment interface when mussels were present. Amplicon sequencing of sediment at depths relevant to mussel burrowing (3 and 5 cm) showed that mussel presence reduced observed species richness (p=0.005), Chao1 diversity (p=0.005), and Shannon diversity (p<0.001), with more pronounced decreases at 5 cm depth. A non-metric, multidimensional scaling model showed that intersample microbial species diversity varied as a function of mussel presence, indicating that sediment below mussels harbored distinct microbial communities. Mussel presence corresponded with a 4-fold decrease in a majority of operational taxonomic units (OTUs) classified in the phyla Gemmatimonadetes, Actinobacteria, Acidobacteria, Plantomycetes, Chloroflexi, Firmicutes, Crenarcheota, and Verrucomicrobia. 38 OTUs in the phylum Nitrospirae were differentially abundant (p<0.001) with mussels, resulting in an overall increase from 25% to 35%. Nitrogen (N)-cycle OTUs significantly impacted by mussels belonged to anammmox genus *Candidatus* Brocadia, ammonium oxidizing bacteria family Nitrosomonadaceae, ammonium oxidizing archaea genus *Candidatus* Nitrososphaera, nitrite oxidizing bacteria in genus *Nitrospira*, and nitrate- and nitrite-dependent anaerobic methane oxidizing organisms in the archaeal family "ANME-2d" and bacterial phylum "NC10", respectively. Nitrosomonadaceae (0.9-fold (p<0.001)) increased with mussels, while NC10 (2.1-fold (p<0.001)), ANME-2d (1.8-fold (p<0.001)), and *Candidatus* Nitrososphaera (1.5-fold (p<0.001)) decreased with mussels. Co-occurrence of 2-fold increases in *Candidatus* Brocadia and *Nitrospira* in shallow sediments suggests that mussels may enhance microbial niches at the interface of

oxic-anoxic conditions, presumably through biodeposition and burrowing. Furthermore, it is likely that the niches of *Candidatus* Nitrososphaera and n-damo were suppressed by mussel biodeposition and sediment aeration, as these phylotypes require low ammonium concentrations and anoxic conditions, respectively. As far as we know, this is the first study to characterize freshwater mussel impacts on microbial diversity and the vertical distribution of N-cycle microorganisms in upper Mississippi river sediment. These findings advance our understanding of ecosystem services provided by mussels and their impact on aquatic biogeochemical N-cycling.

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2   **and other nitrogen-transforming microorganisms in upper Mississippi river sediment**

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12 **Abstract**

13 Targeted qPCR and non-targeted amplicon sequencing of 16S rRNA genes within sediment

14 layers identified the anaerobic ammonium oxidation (anammox) niche and characterized

15 microbial community changes attributable to freshwater mussels. Anammox bacteria were

16 normally distributed (Shapiro-Wilk normality test, W-statistic=0.954, p=0.773) between 1-15 cm

17 depth and were increased by a factor of 2.2 (p<0.001) at 3 cm below the water-sediment interface

18 when mussels were present. Amplicon sequencing of sediment at depths relevant to mussel

19 burrowing (3 and 5 cm) showed that mussel presence reduced observed species richness

20 (p=0.005), Chao1 diversity (p=0.005), and Shannon diversity (p<0.001), with more pronounced

21 decreases at 5 cm depth. A non-metric, multidimensional scaling model showed that intersample

22 microbial species diversity varied as a function of mussel presence, indicating that sediment

23 below mussels harbored distinct microbial communities. Mussel presence corresponded with a 4-

24 fold decrease in a majority of operational taxonomic units (OTUs) classified in the phyla

25 Gemmatimonadetes, Actinobacteria, Acidobacteria, Plantomycetes, Chloroflexi, Firmicutes,

26 Crenarcheota, and Verrucomicrobia. 38 OTUs in the phylum Nitrospirae were differentially

27 abundant (p<0.001) with mussels, resulting in an overall increase from 25% to 35%.

28   Nitrogen (N)-cycle OTUs significantly impacted by mussels belonged to anammmox

29 genus *Candidatus* Brocadia, ammonium oxidizing bacteria family Nitrosomonadaceae,

30 ammonium oxidizing archaea genus *Candidatus* Nitrososphaera, nitrite oxidizing bacteria in

31 genus *Nitrospira*, and nitrate- and nitrite-dependent anaerobic methane oxidizing organisms in

32 the archaeal family "ANME-2d" and bacterial phylum "NC10", respectively.

33 Nitrosomonadaceae (0.9-fold (p<0.001)) increased with mussels, while NC10 (2.1-fold

34 (p<0.001)), ANME-2d (1.8-fold (p<0.001)), and *Candidatus* Nitrososphaera (1.5-fold (p<0.001))

35 decreased with mussels. Co-occurrence of 2-fold increases in *Candidatus* Brocadia and

36 *Nitrospira* in shallow sediments suggests that mussels may enhance microbial niches at the

37 interface of oxic-anoxic conditions, presumably through biodeposition and burrowing.

38 Furthermore, it is likely that the niches of *Candidatus* Nitrososphaera and n-damo were

39 suppressed by mussel biodeposition and sediment aeration, as these phylotypes require low

40 ammonium concentrations and anoxic conditions, respectively. As far as we know, this is the

41 first study to characterize freshwater mussel impacts on microbial diversity and the vertical

42 distribution of N-cycle microorganisms in upper Mississippi river sediment. These findings

43 advance our understanding of ecosystem services provided by mussels and their impact on

44 aquatic biogeochemical N-cycling.

Introduction

46 Native freshwater mussels (Order Unionida) are ecosystem engineers that significantly alter

47 benthic habitats through biodeposition of feces and pseudofeces, rich in ammonium (NH_4^+) and

48 organic carbon (C), into sediment (Thorp 1998, Vaughn 2004 and 2008, Bril, 2014). The

49 estimated mussel filtration capacity in a 480 km, Upper Mississippi River (UMR) segment, as a

50 percentage of river discharge, is up to 1.4% at high flows, up to 4.4% at moderate flows and up

51 to 12.2% during low flows (Newton et al. 2011). The mussels in this river segment collectively

52 filter over 14 billion gallons of water, remove tons of biomass from the overlying water, and

53 deposit tons of reduced C and nitrogen (N) at the water-sediment interface each day (Newell

54 2004). The pocketbook mussel (*Lampsilis cardium*) and threeridge mussel (*Amblema plicata*)

55 comprise up to 38% and 56% of the mussel biomass in the UMR, respectively (Newton et al.

56 2011). A habitat near Buffalo, Iowa, in UMR Pool 16, had mean densities of 1.56 *L. cardium*-m^{-2}

57 and 7.18 *A. plicata*-m^{-2} that correlated with fine sediment diameters (d_{50}=0.300 ± 0.121 mm)

58 which were presumably influenced by mussel burrowing (Young 2006). Mussels live primarily

59 buried in sediment, with their posterior end often flush with the sediment surface (Haag 2012), or

60 slightly below the surface in soft sediments (Allen & Vaughn 2009; Allen 1923; Matteson 1955).

61 This positions adult freshwater mussels 6-10 cm into the sediment with tendencies toward more

62 shallow burrowing during the spring and summer (Schwalb & Pusch 2007). Extensive

63 observations in the UMR concluded that *A. plicata* were often found with portions of their shell

64 above the water-sediment interface, while *L. cardium* burrow a few cm into the sediment during

65 the summer (Newton et al. 2015). Additionally, *A. plicata* often burrowed up to 2.5 cm vertically

66 (Allen & Vaughn 2009) in response to stressors while *L. cardium* moved more horizontally when

67 stressed (Newton et al. 2015). Two common stressors, that happen to be created by the mussels

68   themselves, are low dissolved oxygen (DO) and elevated ammonia (NH_3) and NH_4^+ (Bril et al.

69   2017; Haag 2012). We hypothesize that this frequent vertical and horizontal movement by

70   mussels, many times as an indirect and/or direct response to their own waste production, has a

71   significant impact on porewater chemistry and microbiology in UMR sediments.

72   The evidence for freshwater mussel impacts on aquatic chemistry is compelling,

73   especially for nutrients. A dense mussel population can sequester 2 g C day^{-1} m^{-2}, 200 mg N day^{-1}

74   m^{-2}, and 50 mg phosphorus day^{-1} m^{-2} from river water into sediment (Strayer 2014). During the

75   summer months, biodeposition-derived N from mussels was roughly 67% NH_4^+, 28% amino

76   acids, and 5% urea (Bayne 1973). Mussel biodeposition accounted for up to 40% of total N

77   demand in freshwaters and up to 74% of N in the food web, but was sometimes dampened

78   (Atkinson et al. 2014) in high nutrient environments (Atkinson et al. 2014). Our previous work

79   showed mussel burrowing and biodeposition, just below the water-sediment interface, increased

80   porewater NH_4^+, nitrate (NO_3^-), nitrite (NO_2^-), and total organic C concentrations by 160%, 38%,

81   40%, and 26%, respectively (Bril 2016; Bril et al. 2014). But, the experimental design of our

82   previous work limited our ability to assess the effects of mussels on the broad microbial

83   community that was transforming N simultaneously and, quite likely, synergistically.

84   The UMR is an N-rich agro-ecosystem (Hill et al. 2011; Hill 2008; Houser & Richardson

85   2010; Ikenberry et al. 2014; Schilling et al. 2015) shown to foster high microbial N

86   transformations (Mason et al. 2016; Millar et al. 2015) potentially making the effects of mussels

87   on a variety of N-transforming bacteria and archaea more pronounced than in any other

88   freshwater environment. The first step in transforming biologically active N is nitrification by

89   aerobic ammonium oxidizing bacteria (AOB) (Figure 1, yellow arrows), such as the genera

90   *Nitrosomonas* and *Nitrosospira* in the Nitrosomonadaceae family (Aakra et al. 2001; Burrell et

91   al. 2001; Hayatsu et al. 2008; Prosser et al. 2014), and aerobic ammonium oxidizing archaea

92   (AOA) in multiple candidate genera. AOB and AOA are metabolically diverse (Leininger et al.

93   2006) and serve a functionally important role of catalyzing the rate limiting step of nitrification

94   (Martens-Habbena et al. 2009) in various freshwater niches. For example, *Candidatus*

95   Nitrososphaera (phylum Thaumarchaetoa), a group of thermophilic AOA (Hatzenpichler et al.

96   2008), and AOB species in genera *Nitrosospira* and *Nitrosococcus* (Koper et al. 2004) can use

97   urea as an alternative source of NH_4^+ (Spang et al. 2012). AOA often outnumber AOB (Prosser

98   & Nicol 2008) due to their ability to grow at NH_4^+ concentrations below 10 nM (Martens-

99   Habbena et al. 2009), compared to 10 μM for some AOB species (Bollmann et al. 2002). In the

100 second step of nitrification, nitrite oxidizing bacteria (NOB), such as *Nitrospira* (phylum

101 Nitrospirae), and *Nitrobacter*, *Nitrococcus*, and *Nitrospina* (phylum Proteobacteria) (Prosser et

102 al. 2014), aerobically oxidize NO_2^- to NO_3^- (Figure 1, yellow arrows).

103   *Nitrospira* are the most abundant and diverse group of NOB and dominate numerous

104 habitats, ranging from freshwater sediment to engineered wastewater treatment plants (Daims et

105 al. 2015; Koch et al. 2015; Lucker et al. 2010). Furthermore, NOB species *Nitrospira*

106 *moscoviensis* and *Nitrospira lenta* can derive NH_4^+ from urea hydrolysis, provide NH_4^+ to AOB,

107 and subsequently may oxidize NO_2^- from AOB in a process deemed "reciprocal feeding of

108 nitrifiers" (Daims et al. 2015; Koch et al. 2015). *Candidatus* Nitrospira inopinata, can also use

109 urea as an alternative NH_4^+ source (Daims et al. 2015) and has all the genes necessary for

110 complete ammonia oxidation (comammox) to NO_3^- (van Kessel et al. 2015) (Figure 1, yellow

111 curved arrow). Denitrifiers complete the conventional N-cycle by sequentially reducing NO_3^- to

112 nitric oxide (NO), nitrous oxide (N_2O), and nitrogen gas in anoxic and high C environments

113 (Figure 1, blue arrows) (Hayatsu et al. 2008).

114   A specialized group of bacteria in the phylum Planctomycetes, anaerobic ammonium

115 oxidizing (anammox) bacteria, oxidize NH_4^+, utilize NO_2^- as a terminal electron acceptor, and

116 produce N_2 gas (Kartal et al. 2011; Kuenen 2008) (Figure 1, gray arrows). Anammox bacteria

117 thrive at the interface of oxic-anoxic conditions due to dependence on NO_2^- production by AOB

118 or AOA (Thamdrup 2012). Anammox and NOB compete for NO_2^- in low substrate

119 environments, and this is especially true for *Nitrospira* NOB, which share a homologous form of

120 the key enzyme catalyzing NO_2^- oxidation with anammox (Lucker et al. 2010). In another

121 example, *N. moscoviensis* can adapt to a range of oxygen concentrations by coupling formate

122 oxidation and NO_3^- reduction (Koch et al. 2015). Recently, an N-cycling enrichment culture

123 revealed comammox bacteria co-occurring with anammox bacteria in the genus *Candidatus*

124 Brocadia, presumably enhanced by the ability of commamox organisms to oxidize NH_4^+ in low

125 oxygen conditions (<3.1μM) (van Kessel et al. 2015). *Nitrospira* species, *N. moscoviensis* and

126 "*Ca*. Nitrospira inopinata" in particular, are examples of NOB which harbor a unique ability to

127 assist or compete with anammox for N-substrate in a variety of niches (Lucker et al. 2010).

128   Shallow sediments also pose a competitive niche for anammox bacteria because of high

129 NH_4^+ fluxes into oxic sediment and NO_2^- limitations from denitrification (Thamdrup 2012;

130 Trimmer & Engstrom 2011). Another addition to the suite of known N-transformations includes

131 prokaryotic coupling of anaerobic oxidation of methane with denitrification (Raghoebarsing et

132 al. 2006). In nitrite- and nitrate-dependent anaerobic methane (CH_4) oxidation (n-damo)

133 (Raghoebarsing et al. 2006; Thamdrup 2012; Welte et al. 2016), NO_3^- reduction to NO_2^- and

134 NO_2^- reduction to N_2 are coupled with CH_4 oxidation to CO_2 (Figure 1, gray line and curved

135 arrows). Nitrate-damo biochemical processes have been linked to family "ANME-2d"(Ding et al.

136 2016; Haroon et al. 2013), while nitrite-damo was discovered for "*Candidatus* Methylomirabilis

137   oxyfera"(Ettwig et al. 2010) in phylum "NC10" (Ettwig et al. 2009; Luesken et al. 2011; Padilla

138   et al. 2016), and both are widespread in anoxic freshwater sediments (Deutzmann & Schink

139   2011; Ding et al. 2016; Ettwig et al. 2009; Hu et al. 2009).

140   Mollusks have been shown to influence the diversity of microbial communities and

141   abundance of N-transforming microorganisms. For example, metagenomic profiling revealed a

142   marine California mussel (*Mytilus californianus*) shell provided a niche for N- and C-

143   transforming microorganism populations (Pfister et al. 2010), and a restored oyster reef enhanced

144   nitrification and denitrification rates greater than 10-fold (Kellogg et al. 2013). Furthermore, an

145   experimental microcosm study reported enhanced prokaryotic metabolic activity and diversity

146   following a biodeposition rate of 10 g m^{-2} d^{-1} of mussel feces and pseudofeces (Pollet et al.

147   2015). Additionally, clusters of the zebra mussel (*Dreissena polymorpha*) in a lake increased

148   heterotrophic bacteria density, activity, and diversity (Lohner et al. 2007). Since the impact of

149   native freshwater mussels on prokaryotic diversity and abundance in the UMR is largely

150   unknown, this study utilized targeted and non-targeted sequencing of the 16S rRNA gene to

151   determine how N-transforming microorganisms and microbial community structure differs in

152   sediments with mussels compared to sediments without mussels.

153   **Materials and Methods**

154   UMR sediments were collected within a dense, well-characterized mussel assemblage in the

155   Buffalo Habitat of UMR Pool 16 (Young 2006) (41.452804, -90.763299), and from a slightly up-

156   river location with no mussels (41.451540, -90.753275) using a 3-inch diameter, hammer-driven,

157   acrylic tube (Batch 1 samples) or a 2-inch diameter, post-driver sediment sampler with a

158   polypropylene liner (Multi-Stage Sediment Sampler, Art's Manufacturing and Supply, Inc.,

159   American Falls, ID, USA; Batch 2 samples). Batch 1 sediment was used to identify the vertical

160   distribution of anammox bacteria below freshwater mussels. For Batch 1, the acrylic tube for

161   each core (n=3 with-mussels) was penetrated at 1, 3, 5, 7, 11, and 15 cm sediment depths with a

162   3/8th-inch diameter, ethanol flame-sterilized drill bit to enable sediment collection. In

163   comparison, Batch 2 sediment was used to characterize anammox abundance, microbial

164   diversity, and community structure in shallow sediments below mussels. For Batch 2, the

165   polypropylene liner for each sediment core (n=5 with-mussels, n=5 no-mussels) was penetrated

166   at depths of 3 cm and 5 cm. Sediment was sampled for DNA isolation (in quadruplicate) for a

167   combined sample size of n=20 for 3 cm depth with-mussels, n=20 for 5 cm depth with-mussels,

168   n=20 for 3 cm depth without mussels, and n=20 for 5 cm depth without mussels. Genomic DNA

169   was isolated from 0.25 g of each sediment sample (PowerSoil® DNA Isolation Kit, MoBio

170   Laboratories, Inc., Carlsbad, CA, USA) and stored at -20ºC. Batch 2 Genomic DNA was used

171   for anammox-targeted qPCR (n=20 for each treatment) and 16S rRNA gene amplicon

172   sequencing (n=10 for each treatment).

173   ***Anammox 16S rRNA gene quantification***

174   Microbial culture from a sidestream deammonification process (Hampton Roads Sanitation

175   District, Virginia Beach, VA) served as a source of anammox genetic material for qPCR standard

176   curve construction. PCR products (primers A483f (5'-GTCRGGAGTTADGAAATG-3') and

177   A684r (5'-ACCAGAAGTTCCACTCTC-3') (Sonthiphand & Neufeld 2013)) of the anammox

178   16S rRNA gene was purified with Qiaquick PCR purification Kit (Qiagen Inc., Valencia, CA,

179   USA), and cloned into the pCR 2.1-TOPO® vector using the TOPO® TA cloning Kit

180   (Invitrogen Corp., Carlsbad, CA, USA). Clones were Sanger sequenced at the University of Iowa

181   Institute of Human Genetics with M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-

182   CAGGAAACAGCTATGAC-3') primers to ensure anammox 16S rRNA PCR products were

183   inserted into the vector. Nucleotide sequences were aligned using the Standard Nucleotide Basic

184   Local Alignment Search Tool (Altschul et al. 1997) (GenBank Accession: KU047953) and

185   classified as *Candidatus* Brocadiales (of the Planctomycetes phylum) with a 95% confidence

186   threshold using RDP Naïve Bayesian rRNA Classifier Version 2.10 (Wang et al. 2007). Plasmid

187   DNA concentration was quantified with Qubit® Fluorometer 1.0 (Thermo Fisher Scientific,

188   Inc.), serially diluted, and used to construct qPCR calibration curves.

189   The anammox 16S rRNA gene from batches 1 and 2 was quantified (Wang et al. 2015)

190   with qPCR using QuantStudio^{TM} 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Inc.,

191   Waltham, MA, USA) with primers A483f and A684r (Sonthiphand & Neufeld 2013) and

192   analyzed with QuantStudio^{TM} Real-Time PCR Software (Thermo Fisher Scientific, Inc.). The

193   threshold cycle (C_t) curves were satisfactory (slope=-3.374, Y-int=36.702, R^2=0.998, and

194   amplification efficiency=97.99%), and PCR product dissociation curves revealed singe peaks

195   centered at a melting temperature of 83°C. The statistical significance of 16S rRNA gene copies

196   was determined via a one-way, repeated measures analysis of variance (ANOVA) (SigmaPlot

197   13.0, Systat Software, Inc., Chicago, IL, USA) between the 4 treatment groups (n=20) following

198   a passed normality test (p=0.826, Shapiro-Wilk) and an equal variance test (p=0.073, Brown-

199   Forsythe). Pairwise multiple comparison procedures were completed via the Holm-Sidak method

200   with a significance level of 0.050 and a power of 0.990.

201   ***Non-targeted amplicon sequencing of the 16S rRNA gene***

202   Batch 2 genomic DNA (20 μL, 1-50 ng/μL) was analyzed by the Argonne National Laboratory,

203   Environmental Sample Preparation and Sequencing Facility (ESPSF) utilizing the Earth

204   Microbiome Project protocol (http://www.earthmicrobiome.org/emp-standard-protocols/16s/).

205   All samples were analyzed together in one batch. The v4 region of prokaryotic 16S rRNA gene

206   (515F-806R) was amplified using the following conditions: 3 minutes at 94°C, 35 cycles of 94°C

207   for 45 seconds, 50°C for 60 seconds, and 72 °C for 90 seconds, followed by 10 minutes at 72 °C

208   (Caporaso et al. 2012). The PCR mixture consisted of 13.0 μL PCR grade water, 10.0 μL 5

209   PRIME HotMasterMix (Quanta Biosciences, Beverly, MA), 1.0 μL genomic DNA, and 0.5 μL

210   forward and reverse primers (10 μM). 16S rRNA gene amplicon libraries were sequenced by

211   ESPSF using Illumina MiSeq paired end reads (2x151 bp) (Caporaso et al. 2012) and uploaded

212   to MG-RAST (ID's: 4705672.3-4705709.3) and NCBI (BioProject ID PRJNA374585).

213   Determining the operational taxonomic units ("OTUs") in each sample from the raw 16S

214   rRNA gene amplicon reads was accomplished using the default Quantitative Insights into

215   Microbial Ecology (QIIME) open-reference pipeline (Navas-Molina et al. 2013). Briefly, the

216   QIIME open-reference pipeline takes paired-end reads as input, which are then joined,

217   demultiplexed, filtered, and clustered into OTUs with uclust (Edgar 2010). Representative

218   sequences from each cluster were aligned (Caporaso et al. 2010) to GreenGenes 13.5 reference

219   database (DeSantis et al. 2006) with a 97% similarity threshold. RDP classifier (Wang et al.

220   2007) was used for taxonomy assignment, PyNAST (Caporaso et al. 2010) was used for multiple

221   sequence alignment. Phylogenetic trees were constructed using FastTree2.1.3 with default

222   settings (Price et al. 2010). The OTU table from QIIME open reference picking

223   ('otu_table_mc2_w_tax_no_pynast_failures_json.biom' in the standard QIIME workflow) was

224   imported into R using the phyloseq package (McMurdie & Holmes 2013) for downstream

225   analysis, along with the corresponding phylogenetic tree ('rep_set.tre') and a metadata mapping

226   file. These datasets were merged to create a single 'physeq' object representing the experiment.

227   Alpha-diversity was calculated on the unfiltered OTU abundance data using the Observed

228   species, Chao1 (Chao & Chiu 2001), and Shannon (Li et al. 2011) metrics. Beta-diversity was

229   calculated using a matrix of bray-curtis (Bray & Curtis 1957) intersample distances and

230   ordination plots calculated with non-metric multidimensional scaling (NMDS). Differential

231   abundance analysis was carried out using the DESeq2 (Love et al. 2014) R package with default

232   settings (test type was "Wald," fit type was "parametric"). Translating physeq objects into a

233   compatible DESeq2 object was performed with the "phyloseq_to_deseq2" function. The

234   complete data analysis R script can be downloaded from the public github repository:

235   https://github.com/mchimenti/black_chimenti_just_phyloseq/blob/master/phyloseq.r.

236   Analysis at the OTU level provided a fine scale resolution for significant differences in

237   microbial ecology between mussel and no mussel treatments. To put these results into a

238   biological context, the genus-level OTU file was used to compare relative abundances for N-

239   cycle phylotypes. These groups include AOA genus *Candidatus* Nitrososphaera, nitrate-damo

240   family "ANME-2d", NOB genus *Nitrospira*, anammox genus *Candidatus* Brocadia, AOB family

241   Nitrosomonadaceae, and nitrite-damo phylum "NC10". Relative abundance counts for each N-

242   cycle group was tested for statistical significance between treatments, using metadata groups "3

243   cm with-mussels" (n=10), "5 cm with-mussels" (n=10), "3 cm no-mussels" (n=10), and "5 cm

244   no-mussels" (n=10). 1-way ANOVA's of each N-cycle group was performed using the Kruskal-

245   Wallis test (p<0.05) with Dunn's multiple correction test (Padj<0.05) (GraphPad Prism 7.0, La

246   Jolla, CA). Similarly, multiple comparisons were made between all N-cycle phylotype groups

247   and their respective treatments (n=10); significant differences between relative abundances were

248   tested using the Kruskal-Wallis test (P<0.0001) and Dunn's multiple comparison test

249   (Padj<0.05).

250   **Results**

251   ***Anammox-targeted 16S rRNA gene quantification***

252 The targeted 16S rRNA gene data from Batch 1 (n=3, with-mussels) indicated an anammox

253 bacterial gene copy maximum (~3×10^5 copies g^{-1} sediment) between 3 cm and 7 cm sediment

254 depth in the presence of mussels (Figure 2A). The Batch 1 data was normally distributed

255 between 1 cm and 15 cm (Shapiro-Wilk normality test, W-statistic=0.954, p=0.773). Only one

256 sediment core went beyond 7 cm leaving anammox bacterial gene copy data at 11 cm and 15 cm

257 without replicates. The Batch 2 data (n=20 for 3 cm with-mussels, n=20 for 5 cm with-mussels,

258 n=20 for 3 cm no-mussels, n=20 for 5 cm no-mussels) showed that anammox bacteria

259 experienced a 2.2-fold increase (p<0.001) at 3 cm with-mussels compared to the no-mussels

260 control (Figure 2B). The anammox gene copies measured at 5 cm were statistically

261 indistinguishable between the with-mussels and no-mussels treatments.

262 ***Non-targeted sequencing of the 16S rRNA gene***

263 Summing across all samples, a total of 2,103,661 amplicon sequences were analyzed and about

264 76,000 unique OTUs were reported by QIIME. Of the unique OTUs, 18,777 had 10 or more

265 reads and 3,916 OTUs had counts exceeding 100 reads. Mussel bed samples had read counts of

266 45,290 (±15,271) at 3 cm sediment depth and 52,451 (±7,044) at 5 cm sediment depth, while no-

267 mussel samples had 48,920 (±7,517) read counts at 3 cm depth and 63,706 (±25,379) at 5 cm

268 sediment depth (read depths depicted in Figure S1). The top phyla in mussel bed sediments were

269 Proteobacteria (40.7%), Nitrospirae (35.2%), Chloroflexi (5.9 %), Euryarchaeota (5.0%),

270 Chlorobi (4.2%), and Bacteroidetes (2.3%). Proteobacteria decreased by about 6% with mussels

271 while Nitrospirae increased by 10% with mussels. The most abundant taxonomic families in the

272 Nitrospirae phylum were *Thermodesulfovibrionaceae* (55%), "FW" (33%), and Nitrospiraceae

273 (13%), and were 5% less, 3% and 2 % greater than in no-mussel samples, respectively. With

274 mussels, Proteobacteria taxonomic classes consisted of the following proportions: 68%

275   Deltaproteobacteria (8% less than without-mussels), 16% Gammaproteobacteria, and 15%

276   Betaproteobacteria. A majority of these Deltaproteobacteria OTUs were from "BPC076",

277   Desulfarculales, and Syntrophobacterales taxanomic orders, while orders Burkholderiales and

278   Xanthomonodales made up a majority of Betaproteobacteria and Gammaproteobacteria taxons.

279   Species richness was analyzed using three common measures: Observed species, Chao1

280   and Shannon indices (n=20 with-mussels and n=20 without mussels). Together, the three

281   measures indicated a decrease in microbial community richness and evenness in the presence of

282   mussels as compared to sediments without mussels (Figure 3A). The observed decrease in alpha-

283   diversity reached significance for each of the three measures tested (p=0.0054 or lower). A

284   similar result was obtained when calculating alpha-diversity measures in samples exclusively

285   from 3 cm (n=10) or exclusively from 5 cm (n=10) depths in the presence and absence of

286   mussels. However, the decrease in richness was more pronounced at 5 cm than at 3 cm depth

287   (Figures S2 and S3).

288   To compare intersample diversity in species abundances and community composition

289   ("beta diversity"), we employed NMDS scaling to accurately visualize, in 2D space, the higher-

290   order community structure between with-mussels and no-mussels samples (Figure 3B). The

291   NMDS model produced an excellent representation of the bray-curtis distances for all samples

292   (convergence in 20 iterations, stress ~ 0.06; shepard plot shown in Figure S4). The beta diversity

293   clearly differentiated as a function of mussel presence, but not sediment depth (Figure 3B).

294   Taken together, these data show that mussel presence had a pronounced influence on the

295   microbial community evenness, richness, and composition within the sediment.

296   Differential abundances in OTUs did not reach significance for metadata values of

297   sediment depth or comparisons between sediment cores. On the other hand, there were numerous

298 differences in OTU abundances when comparing sediment with mussels and without mussels.

299 We performed a differential abundance estimation with the DESeq2 R package using mussel

300 presence status (n=20 with-mussels, n=20 no-mussels) as our covariate. 734 OTUs (or 0.94% of

301 the 77,288 OTUs tested) reached significance with a false discovery rate of 0.01. The vast

302 majority of OTUs belonging to the phyla Gemmatimonadetes, Actinobacteria, Acidobacteria,

303 Plantomycetes, Chloroflexi, Firmicutes, Crenarcheota, and Verrucomicrobia decreased by at

304 least 4-fold in the presence of mussels. In contrast, Proteobacteria showed a marked decrease in

305 order Alphaproteobacteria, while showing mixed increasing and decreasing OTUs among Beta-,

306 Delta-, and Gammaproteobacteria. Phylum Nitrospirae also had 38 OTUs which were

307 differentially abundant with p-adj<0.001. OTUs assigned to the GreenGenes taxonomic family

308 of "0319-6A21" were the most abundant among those OTUs increasing without mussels, while

309 families *Thermodesulfovibrionaceae* and "FW" were most abundant among those OTUs

310 increasing with mussels.

311   Many of the Nitrospirae taxons that increased without mussels did so from a smaller

312 average abundance (17 average counts for Nitrospira and up to 126 average counts for

313 *Thermodesulfovibrionaceae*) relative to those that were increased with mussels (209 average

314 counts for *Nitrospira* and up to 581 average counts for *Thermodesulfovibrionaceae*). This

315 explains the 10% increase in Nitrospirae abundance when summing across all samples with

316 mussels. Figure 4 shows the Log2FC categorized by phyla for OTUs with p-adj<0.0001 (to

317 enhance visual clarity). Significant differences within the Nitrospirae phylum were represented

318 by increases of genus "HB118" in family *Thermodesulfovibrionaceae* (2.0Log2FC from a mean

319 count of 52, p<0.001) and unclassified *Nitrospira* species (0.8Log2FC from an average count of

320 209, p<0.001) with mussels. No-mussel treatments showed increases in genus "LCP-6" from

321   family *Thermodesulfovibrionaceae* (3.6Log2FC from an average count of 126, p<0.001) and

322   unclassified *Nitrospira* species (2.1Log2FC from an average count of 17, p<0.001).

323   Despite seemingly even representation of phylum Thaumarchaeota between treatments,

324   unclassified species from *Candidatus* Nitrososphaera were enhanced from an average abundance

325   of 126 (1.73Log2FC, p<0.001) without mussels, and AOA species, *Candidatus* Nitrososphaera

326   gargensis increased from an average count of 16 (2.85Log2FC, p<0.001) without mussels. One

327   OTU classified in the anammox genus, *Candidatus* Brocadia, increased from an average count of

328   17 (3.72Log2FC, p<0.001) without mussels, while another OTU classified as an unknown

329   *Candidatus* Brocadia species increased from a mean count of 16 (1.2Log2FC, p=0.001) with-

330   mussels. Furthermore, OTUs belonging to the AOB family Nitrosomonadaceae increased from

331   an average abundance of 6 (1.9Log2FC, p<0.001) with mussels. Without mussels, taxonomic

332   groups capable of nitrite-damo, phylum "NC10", increased from average abundances up to 130

333   (4.4 Log2FC, p<0.001), and nitrate-damo family "ANME-2d" increased from average

334   abundances up to 59 (3.4 Log2FC, p<0.001). A summary of Log2FC values for OTUs relevant

335   to N-transformations are listed in Table S1.

336   N-cycle phylotypes were examined for statistically significant relative abundances between

337   treatments of mussel presence and sediment depth (Table 1). *Candidatus* Nitrososphaera

338   experienced a 2.6-fold decrease (p=0.047) with mussels at 5 cm sediment depth. ANME-2d was

339   3 times greater (p=0.049) at 5 cm sediment depth without mussels, compared to 3 cm sediment

340   depth without mussels. Within the mussel bed, *Nitrospira* were 1.7 times greater (p=0.0497) at 3

341   cm depth, and experienced a 1.9-fold increase (p=0.025) with mussels at 3 cm sediment depth

342   versus control. *Candidatus* Brocadia was 3 times greater (p=0.013) at 5 cm depth without

343   mussels versus 3 cm without mussels, and the 3 cm sediment showed a 2-fold increase (p=0.002)

344 with mussels versus control. Nitrosomonadaceae was 2.7 times greater (p=0.015) at 3 cm with

345 mussels versus 5 cm depth with mussels.

346   Relative abundances of N-cycle phylotypes were compared within each treatment (Figure 5,

347 Boxes A, B, D, E) and between treatments (Figure 5, Boxes C, F, G-I). Within 3 cm sediment

348 samples with mussels (Figure 5, Box A), *Nitrospira* was statistically greater in abundance than

349 *Candidatus* Brocadia, and ANME-2d was less abundant than *Nitrospira*. Sediment without

350 mussels at 3 cm depth (Figure 5, Box B) contained statistically greater abundances of

351 *Candidatus* Nitrososphaera than *Candidatus* Brocadia, and greater *Nitrospira* abundances

352 compared to *Candidatus* Brocadia, Nitrosomonadaceae, and ANME-2d.

353   Relative abundance comparisons between mussel and no-mussel treatments at 3 cm depth

354 (Figure 5, Box C) showed that *Candidatus* Nitrososphaera was reduced in the mussel treatment,

355 while *Nitrospira* and *Candidatus* Brocadia were enhanced with mussels. Within mussel sediment

356 samples at 5 cm depth, *Nitrospira* was more abundant than *Candidatus* Brocadia, ANME-2d, and

357 Nitrosomonadaceae. (Figure 5, Box D). On the other hand, *Candidatus* Nitrososphaera and

358 *Nitrospira* were both more abundant than Nitrosomonadaceae without mussels at 5 cm sediment

359 depth (Figure 5, Box E). Comparing microbial communities at 5 cm depth between mussel and

360 no-mussel treatments (Figure 5, Box F) revealed that *Candidatus* Nitrososphaera was less

361 abundant with mussels versus the no-mussel population. *Nitrospira* and Nitrosomonadaceae

362 phylotypes were more prominent with mussels in shallow sediment depths (Figure 5, Box G).

363   Overall, *Nitrospira* made up larger proportions of microbial communities with and without

364 mussels compared to many N-cycle organisms, especially *Candidatus* Brocadia and

365 Nitrosomonadaceae (Figure 5, Box C, F, and G-I). Without mussels at 3 cm sediment depth,

366 *Candidatus* Brocadia made up a smaller proportion of the N-cycling microbial community,

367   especially when compared to *Candidatus* Nitrososphaera, ANME-2d, NC10, and *Nitrospira* in

368   deeper sediments (Figure 5, Box H).

369   **Discussion**

370   Numerous studies have found Proteobacteria to be the most abundant phylum in freshwater

371   sediments (Bucci et al. 2014; Dai et al. 2016; Wakelin et al. 2008; Zeng et al. 2008; Zhang et al.

372   2015), sediments with mollusks (Fernandez et al. 2014; Lee et al. 2015), and also mollusk

373   microbiomes (Frischer et al. 2000; Neta et al. 2015; Ngangbam et al. 2015; Trabal et al. 2012).

374   Although our results showed Proteobacteria were the most abundant phylum, we observed a

375   decrease in Proteobacteria by 6% and an increase in Nitrospirae by 10% in the presence of

376   mussels. Families *Thermodesulfovibrionaceae* and "FW" accounted for many of the Nitrospirae

377   OTUs that increased with mussels and helps explain decreases in species richness for mussel bed

378   sediment.

379   Sediments contain the most phylogenetically diverse microbial communities (Lozupone

380   & Knight 2007) and structure and diversity of soil microbial communities is often determined by

381   soil biogeochemistry (Fierer & Jackson 2006), further supporting the impact mussels have on

382   biogeochemical cycling. In support of our hypothesis, our data indicated that mussel presence in

383   the UMR had a pronounced influence on the microbial community evenness, richness, and

384   composition within the sediment. The observed changes in sediment microbial community

385   structure and diversity showed mussels created a niche for specific microorganisms and may be

386   attributable to the diverse chemical composition of mussel biodeposits, mixing of sediment from

387   mussel burrowing, or the microbes living on mussels. Our findings of distinct microbial

388   communities in mussel bed sediment are corroborated by a study of the California mussel

389   (Pfister et al. 2014) where taxonomic richness increased and taxa evenness increased following

390   the removal of mussels from a rocky shore habitat.

391   In contrast to our results of decreased microbial diversity with freshwater mussels,

392   research has shown invasive zebra mussels (*Dreissena polymorpha*) increased bacterial

393   community diversity and richness (Lee et al. 2015), and metabolic diversity and activity in

394   freshwater sediments (Lohner et al. 2007). Increased microbial diversity and activity has been

395   attributed to the variety of C and N components in feces and pseudofeces, and also selects for the

396   dominant microbial species (Lohner et al. 2007; Pollet et al. 2015). An experiment combining

397   estuarine bivalve species (*N. virens, M. arenaria, and M. balthica*) implicated mussel-induced

398   changes in O_2, NH_4^+ and NO_3^- fluxes for the alteration of microbial community composition

399   (Michaud et al. 2009). On the other hand, investigation of microbiota in Thick-shelled River

400   Mussel (*Unio crassus*) beds did not find any difference in microorganism diversity, abundance,

401   and composition (Richter et al. 2016). This may be explained by the drastic differences in the

402   study site, with high mussel densities (23-433 mussels/m^2) and control plots containing low

403   microbial diversities with mean species richness of 48 OTUs/sample with high evenness (Richter

404   et al. 2016). The contrasting findings of microbial community diversity and composition indicate

405   that mussel density and/or mollusk species may produce different responses by microorganism

406   communities.

407   Additionally, alterations in sediment microbial community structure may arise from

408   exposure to the mussel shell, tissues, or fecal microbiome. Mussel tissue and fecal material has

409   been shown to contain less diverse microbiomes than the surrounding water and sediment for the

410   zebra mussel (Frischer et al. 2000), tropical oyster (*Crassostrea rhizophorae*) (Neta et al. 2015),

411   and marine mussel, *Mytilus californianus* (Frischer et al. 2000; Pfister et al. 2014). Some studies

412 have attributed immediate increased sediment microbial activity to the mussel intestinal

413 microbiome (Grenz et al. 1990). Furthermore, mollusk biodeposition rates and biodeposit

414 chemical compositions are highly dependent on mollusk species (Hegaret et al. 2007; Tenore &

415 Dunstan 1973), and food availability (Bril et al. 2017; Cranford et al. 2007; Vaughn &

416 Hakenkamp 2001), so it makes sense that our results differ from studies with dissimilar mollusk

417 species, densities, and study location.

418   Changes in mussel bed sediment microbial communities was also likely enhanced by

419 mussel burrowing, because diffusion of substrates across the water-sediment interface is a

420 relatively slow process (Kristensen et al. 2012) and is increased by mollusk burrowing (Vaughn

421 & Hakenkamp 2001), which ultimately affects microbial communities. For example, the burrow

422 of shrimp species *Upogebia deltaura* and *Callianassa subterranean* contained distinct bacterial

423 communities and a 3-fold increase in taxon richness (Laverock et al. 2010), and the estuarine

424 bivalve, *C. fluminea*, stimulated microbial diversity via bioturbation (Novais et al. 2016). It is

425 likely that UMR mussel bed sediments also experience the benefits from bioturbation, such as

426 sediment mixing (McCall et al. 1979) and aeration (Vaughn & Hakenkamp 2001). Furthermore,

427 bioturbation has been linked to increased NH_4^+ concentrations which alters the N-transforming

428 microbial community (Chen & Gu 2017), with greatest effects on bacteria growth found at 4 to 6

429 cm depth below the water-sediment interface (McCall et al. 1986).

430 ***N-cycle microbial community***

431 Our research revealed an increase in anammox bacteria abundance 3 cm below the water-

432 sediment interface when mussels were present, shown for the anammox community using

433 anammox-targeted qPCR (2.2-fold increase) and for *Candidatus* Brocadia using non-targeted

434 16S rRNA gene amplicon sequencing (2-fold increase). The significance of agreement between

435   these techniques is finding that increases in the genus *Candidatus* Brocadia are representative for

436   the anammox phylotype as a whole. *Candidatus* Brocadia may also make up a majority of the

437   anammox community in UMR sediment, as amplicon sequencing did not detect anammox

438   bacteria belonging to other genera. We are confident in these conclusions, as *Candidatus*

439   Brocadia is often the dominant anammox genus in freshwater sediments (Humbert et al. 2009;

440   Shen et al. 2016; Sonthiphand et al. 2014). One study showed that feeding of NH_4^+, NO_2^-, NO_3^-,

441   and acetate led to an 80% enrichment of *Candidatus* 'Brocadia fulgida', signifying that B.

442   *fulgida* could outcompete anammox species in genera *Candidatus* Anammoxoglobus and

443   *Candidatus* Kuenenia, species *Candidatus* 'Brocadia anammoxidans', and even denitrifiers when

444   acetate is present (Kartal et al. 2008). This indicates that *Candidatus* Brocadia has a distinct

445   ecological niche and can utilize intermediates from anaerobic degradation of organic C to reduce

446   NO_3^- (Kartal et al. 2008). Therefore, it is possible that a portion of our observed increases in

447   *Candidatus* Brocadia with mussels was attributable to C biodeposition in the UMR.

448   Our research also revealed a vertical distribution of anammox bacteria with higher

449   abundances near the sediment surface, which reflects the vertical distribution found in an

450   agricultural field (Shen et al. 2017), oxygen minimum zone (Galán et al. 2009), flooded paddy

451   fields (Shen et al. 2017; Zhu et al. 2011), and an urban wetland (Shen et al. 2015). A vertical

452   anammox distribution has been shown to coincide with NH_4^+ presence and NO_2^- production

453   (Oshiki et al. 2016; Shen et al. 2014; Shen et al. 2015; Sun et al. 2014) and anammox "hotspots"

454   occur in zones of low, but not entirely absent, O_2 availability (Zhu et al. 2013). Anammox

455   abundance in freshwater sediment can range between $7x10^4$ -8 $x10^6$ gene copies g^{-1} sediment

456   (Shen et al. 2016), or between 10^6-10^7 gene copies g^{-1} sediment in peak NO_2^- microniches at the

457   oxic-anoxic interface (Nie et al. 2015; Shen et al. 2015; Zheng et al. 2016). Studies have shown

458   anammox bacteria increase 1.5 to 2-fold within their niche (Nie et al. 2015; Zheng et al. 2016),

459   similar to our findings of a 2.2-fold increase in anammox bacteria 3 cm below the water-

460   sediment interfacewith mussels.

461   Co-occurrence of aerobic NH_4^+ oxidation and anammox niches are likely due to linked

462   NO_2^- oxidation and reduction, respectively (Shen et al. 2014). Interestingly, we found that

463   mussels also enhanced taxa from the AOB family Nitrosomonadaceae and the OTUs made up a

464   greater proportion of mussel bed sediment populations near the water-sediment interface. To this

465   point, the pacific oyster (*C. gigas*) was found to increase porewater NH_4^+ and elevate the

466   concentration of NH_4^+ oxidizing microorganisms (Green et al. 2012). Furthermore, our previous

467   research (Bril et al. 2014; Bril et al. 2017) showed elevated NH_4^+ and NO_2^- in porewater of a

468   similar depth below mussels. It makes sense that these groups of N-transforming bacteria co-

469   occur where their substrate microniches overlap, and is likely enhanced by mussels periodically

470   aerating the sediment (Chen & Gu 2017). Intermittent aeration has shown to enrich microbial

471   cultures in AOB and anammox bacteria in engineered partial nitritation-anammox processes

472   (Shannon et al. 2015; Yang et al. 2015), and similar to our findings, enriches the anammox genus

473   *Candidatus* Brocadia (Shannon et al. 2015).

474   On the other hand, we saw a decrease in *Candidatus* Nitrososphaera (AOA) with mussels

475   at 3 cm (1.7-fold) and 5 cm (2.6-fold) sediment depths. It makes sense that mussels suppress

476   abundance of AOA since these organisms typically dominate sediment niches with low NH_4^+

477   concentrations (Hatzenpichler 2012; Martens-Habbena et al. 2009). Furthermore, a group of

478   OTUs suppressed by mussels were classified at the species level as *Candidatus* 'Nitrososphaera

479   gargensis', which are partially inhibited by NH_4^+ concentrations (3.08 mM) much lower than

480   AOB (Hatzenpichler 2012; Hatzenpichler et al. 2008; Nakagawa & Takahashi 2015; Pester et al.

481   2011). Furthermore, nitrifier niche partitioning studies using agricultural soil showed that AOB

482   increased in abundance and activity following the addition of urine-derived N, while AOA

483   remained unchanged (Di et al. 2009; Hatzenpichler 2012; Jia & Conrad 2009). Therefore, it is

484   possible that mussel biodeposits and an increased flux of agriculturally-fed water into sediment

485   by mussel burrowing enhanced porewater NH_4^+ composition such that Nitrosomonadaceae out

486   competed *Candidatus* Nitrososphaera. Our results agree with Chen et al.(2017), who found

487   bioturbated sediment corresponded with a greater diversity of AOB and lower diversity of AOA

488   microbial communities (Chen & Gu 2017). On the other hand, our results of decreased

489   abundance of *Candidatus* Nitrososphaera co-occurring with an increase in *Nitrospira* is in

490   contrast to previous findings that these organisms may exhibit similar niche partitioning (Pester

491   et al. 2011). For example, some species in *Candidatus* Nitrososphaera can adjust their

492   metabolism for low oxygen availability (Zhalnina et al. 2014) and *Nitrospira* species are adapted

493   to low oxygen concentrations (Maixner et al. 2006; Nowka et al. 2015; Zhalnina et al. 2014) and

494   microoxic environments (Schramm et al. 2000). Alternatively, our detected increased abundance

495   of *Nitrospira* may include species with a variety of environmental niches.

496   Some *Nitrospira* species have shown to occupy a niche at oxic-anoxic interfaces, in

497   opposition to NOB with higher O_2 tolerances such as those in genus *Nitrobacter* (Schramm et al.

498   2000). This supports our mussel-attributed increases in relative *Nitrospira* abundances (1.9-fold)

499   at 3 cm sediment depths. Although we saw two different *Nitrospira* OTUs suppressed and

500   enhanced by mussels, the mussel-enhanced OTUs had a larger mean abundance by about 12%.

501   Different NOB OTUs enhanced with and without mussels further suggests that mussel bed

502   sediments harbor specific NOB strains sensitive to microoxic niches. Despite OTU variability,

503 we can conclude that mussels enhance the *Nitrospira* phylotype, especially near the water-

504 sediment interface where *Nitrospira* were 1.7-times greater than deeper mussel bed depths.

505   On the other hand, we did not expect to see an increase in both NOB and anammox

506 phylotypes due to competition of NO_2^- as a substrate. The co-occurrence of *Nitrospira* and

507 anammox bacteria may be explained by the metabolic versatility of *Nitrospira* species, especially

508 if mussel-derived urea provided an additional source of NH_3 and NO_2^- via reciprocal feeding

509 between ammonia oxidizers and *Nitrospira*. Furthermore, these phylotypes have been shown to

510 coexist in an oxygen minimum zone, where anammox bacteria obtained a majority of NO_2^- from

511 NO_3^- reducers (Lam et al. 2009). The similar effect size of mussels on *Nitrospira* (1.9-fold) and

512 *Candidatus* Brocadia (2-fold) at 3 cm depth suggests that mussels may exert similar influences

513 on the niches of these phylotypes. It is possible that these anammox and *Nitrospira* phylotypes

514 were functionally linked in shallow mussel bed sediment, which has been shown for microoxic

515 niches (van Kessel et al. 2015). Furthermore, it is possible that the *Nitrospira* co-occurring with

516 *Candidatus* Brocadia were *Nitrospira* species with the genetic potential for comammox, as a

517 fluorescence in-situ hybridization study confirmed the extensive aggregation of the 2 phylotypes

518 in hypoxic conditions (<3.1 µM O_2) (van Kessel et al. 2015). Despite *Nitrospira* comammox

519 being identified in numerous aquatic environments (Chao et al. 2016; Daims et al. 2015; Pinto et

520 al. 2016), we cannot conclusively identify comammox without sequencing the ammonia

521 monooxygenase gene (Pinto et al. 2016; van Kessel et al. 2015).

522   In contrast to studies which found significant N-reduction on both a marine mussel

523 (*Mytilus californianus*) (Pfister et al. 2010) and a freshwater mussel (*Limnoperna fortunei*)

524 (Zhang et al. 2014), our results showed that mussels suppressed n-damo OTUs in phylum

525 "NC10" (2.1-fold) and family "ANME-2d" (1.8-fold). One study determined NO_3^--damo was

526 responsible for NO_3^- reduction and anammox for NO_2^- reductions in a bioreactor supplied with

527 NH_4^+, NO_2^-, NO_3^-, CH_4, and anoxic conditions, thus concluding anammox outcompeted NO_2^--

528 damo (Hu et al. 2015). These findings make sense, because anammox bacteria have a higher

529 affinity for NO_2^- (Luesken et al. 2011), anammox outperform n-damo in bioturbated sediments

530 with higher NH_4^+ and lower NO_2^- and NO_3^- (Chen & Gu 2017), and anammox and n-damo

531 communities have a competitive relationship in burrowed mangrove sediment (Chen & Gu

532 2017). Furthermore, NC10 bacteria in a peatland were most prevalent at depths with porewater

533 CH_4 concentrations near 300 μM, where NO_3^- consumption exceeds production, and in

534 completely anoxic conditions (Zhu et al. 2012). According to the literature, it makes sense that

535 we found UMR mussels enhanced *Candidatus* Brocadia and suppressed NO_2^- reducing-NC10.

536 Perhaps n-damo organisms did not have a favorable niche in mussel bed sediment because

537 biodeposition products created an excess of NH_4^+ in sediment porewater (Winkler et al. 2015), or

538 burrowing activity increased oxygen concentrations and made methane oxidation unfavorable

539 (van Bodegom et al. 2001). Our finding that no-mussel sediment contained 3 times more ANME-

540 2D in deeper, and presumably anoxic sediment, further suggests that mussels broaden the oxic-

541 anoxic interface niche (Chen & Gu 2017; Luesken et al. 2011). However, we cannot extrapolate

542 these findings to all denitrifying organisms, since denitrifying species are sporadically distributed

543 among various taxonomic lineages, and are difficult to identify solely with16S rRNA amplicon

544 sequencing (Ishii et al. 2011).

545   Although we observed greater relative abundances of *Nitrospira* than *Candidatus*

546 Brocadia in a majority of treatments, both phylotypes increased by a factor of 2 with mussels at 3

547 cm depth. No-mussel samples contained a significantly smaller proportion of *Candidatus*

548 Brocadia in shallow sediments compared to almost all N-transformers found in the deeper

549   control sediments. Our phylotype-level analyses revealed similarities with the OTU-level

550   differential abundance comparisons. For example, phylotype comparisons showed ANME-2d

551   was less abundant than *Nitrospira* in 3 cm sediments with mussels, and *Candidatus*

552   Nitrososphaera was more abundant than *Candidatus* Brocadia in 3 cm sediment samples without

553   mussels. These results relate to DESeq2 OTU comparisons which found *Candidatus* Brocadia

554   and Nitrospira enhanced with mussels while ANME-2d and *Candidatus* Nitrososphaera were

555   suppressed with mussels.

556   Extending our focus beyond N-cycling organisms, we demonstrated that mussels

557   promoted a large effect size for OTUs classified as *Thermodesulfovibrionaceae* (Nitrospirales

558   order). In contrast to *Nitrospira*, the Nitrospirales genus *Thermodesulfovibrio* contains multiple

559   sulfate reducing species (Kirchman 2012; Sekiguchi et al. 2008) and can outcompete other

560   anaerobic organisms when sulfate is present (He et al. 2015). These findings are corroborated by

561   discoveries of significantly greater C and sulfate concentrations from mussel biodeposits and

562   63% greater sulfate reduction in sediments with mussels (McKindsey et al. 2011). Biodeposition

563   products often lead to increasingly anoxic sediment and greater activity of anoxic

564   microorganisms (Kellogg et al. 2013; McKindsey et al. 2011), presumably due to consumption

565   of excretion products by oxygen-consuming microorganisms (Pollet et al. 2015). Interestingly,

566   Fdz-Polanco et al. (2001) observed simultaneous N and sulfate removal in an anaerobic

567   fluidized-bed reactor and proposed simultaneous anammox and sulfate reduction (Fdz-Polanco et

568   al. 2001). Coupled biological sulfate reduction and anammox reactions are metabolically feasible

569   (Schrum et al. 2009; Strous et al. 2002) and have been of interest in the recent history (Ali et al.

570   2013; Cai et al. 2010; Rikmann et al. 2016; Rios-Del Toro & Cervantes 2016), therefore

571   warranting further research. Therefore, we showed that *Thermodesulfovibrionaceae* are

572 significantly increased in the presence of mussels which may affect sulfate reduction (Mahmoudi

573 et al. 2015) in tandem with anammox reactions in UMR sediments.

574   As a whole, mussels do have an impact on microbial niches and lower the overall

575 community diversity. Mussel-influenced changes in microbiological diversity may have larger

576 ecosystem implications, such as macrobiota richness and diversity (Arribas et al. 2014;

577 Borthagaray & Carranza 2007). Native freshwater mussels are capable of increasing macrobiota

578 diversity as a result of being keystone species (Hartmann et al. 2016) and ecosystem engineers

579 (Chowdhury et al. 2016; Lopes-Lima et al. 2014). Mussel biogeochemical hotspots can lead to a

580 bottom-up trophic cascade by enhancing N substrates normally limiting primary productivity,

581 ultimately leading to increased richness (Atkinson et al. 2013) and biodiversity (Allen et al.

582 2012) of higher trophic levels.

583 **Conclusion**

584 As far as we know, this is the first study to characterize freshwater mussel effects on microbial

585 community diversity, composition, and the vertical distribution of N-cycle microorganisms in the

586 UMR. qPCR of the anammox-specific 16S rRNA gene revealed an increase in anammox bacteria

587 abundance 3 cm below the water-sediment interface when mussels were present, and confirmed

588 anammox bacteria were normally distributed with depth. Non-targeted 16S rRNA gene amplicon

589 sequencing revealed mussel presence suppressed AOA (*Candidatus* Nitrososphaera) and that the

590 families *Thermodesulfovibrionaceae* and "FW" (Nitrospirales order) were overrepresented

591 among the enhanced OTUs with-mussels. Mussel bed sediment contained microbial

592 communities with 10% greater Nitrospirae and 6% fewer OTUs belonging to the phylum

593 Proteobacteria, which ultimately had a pronounced influence on microbial community evenness,

594 richness, and composition. This was indicated by lower observed species richness, Chao1

595   diversity, Shannon diversity, and clustering of mussel samples in an NMDS analysis. We have

596   shown that native freshwater mussels affect niche differentiation of N-cycle microorganisms, as

597   evidenced by increased abundances of AOB family Nitrosomonadaceae, anammox genus

598   *Candidatus* Brocadia, and NOB genus *Nitrospira*, while exhibiting a decrease in AOA genus

599   *Candidatus* Nitrososphaera, and n-damo organisms in the phylum NC10 and family ANME-2d.

600   Co-occurring 2-fold increases in *Candidatus* Brocadia and *Nitrospira* in shallow sediment

601   suggests that mussels may enhance microbial niches at the interface of oxic-anoxic conditions,

602   presumably through biodeposition and burrowing. Ultimately, this study demonstrates the large

603   impact mussels have on biogeochemical N-cycling and ecosystem services in freshwater

604   agroecosystems.

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1058   Table 1: Percent relative abundance of N-cycle organisms for mussel and depth treatments.

Taxonomic Classification	N-Cycle Classification	Mean Percent Relative Abundance			
		3 cm with-mussels	3 cm no-mussels	5 cm with-mussels	5 cm no-mussels
Candidatus Nitrososphaera	AOA	0.26	0.44	0.22	0.58
ANME-2D	nitrate-damo	0.12	0.21	0.11	0.63
NC10	nitrite-damo	0.0039	0.02	0.0035	0.08
Nitrospira	NOB/comammox	1.92	1.00	1.11	0.85
Candidatus Brocadia	anammox	0.10	0.05	0.07	0.15
Nitrosomonadaceae	AOB	0.27	0.13	0.10	0.08

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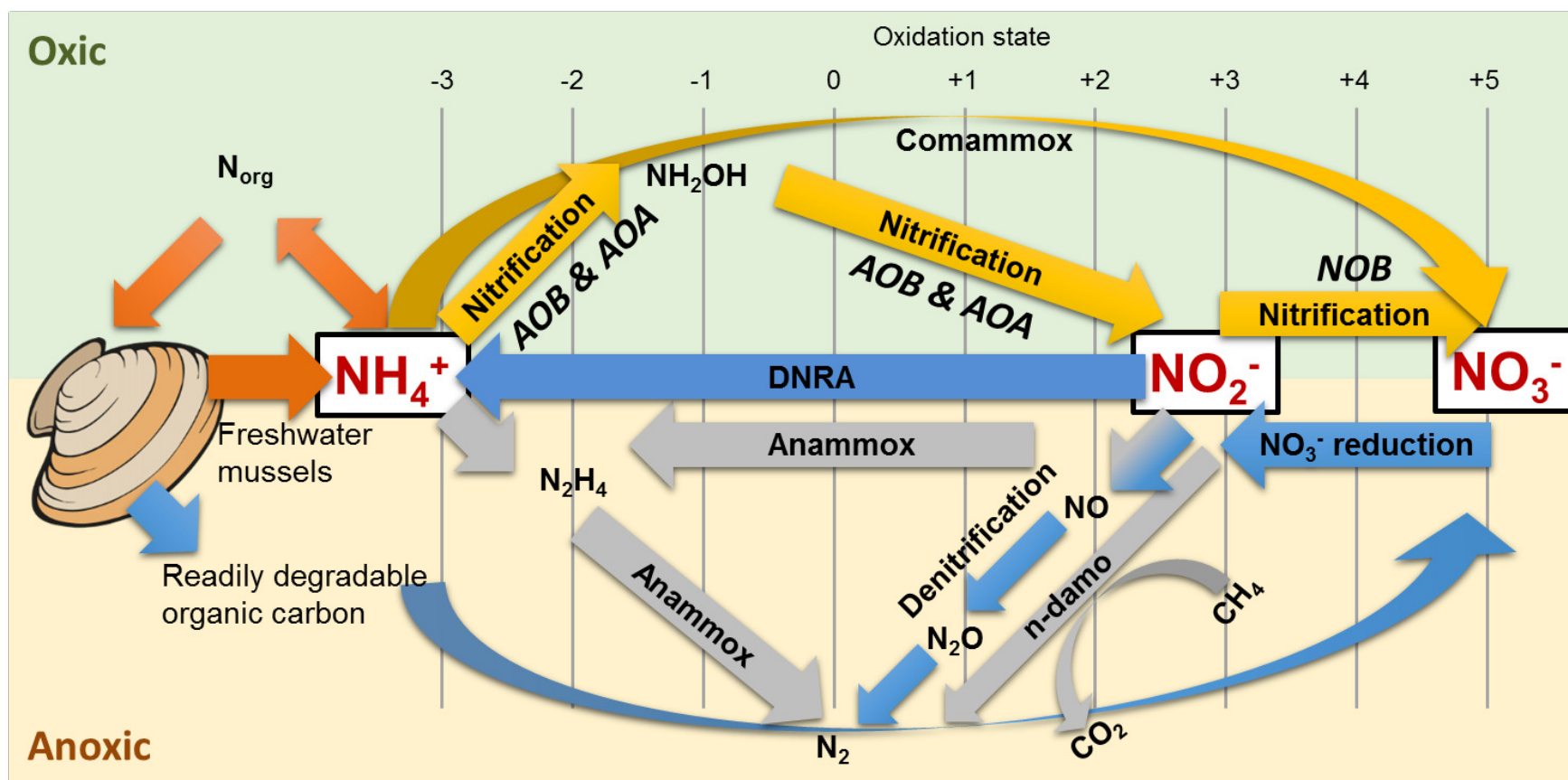
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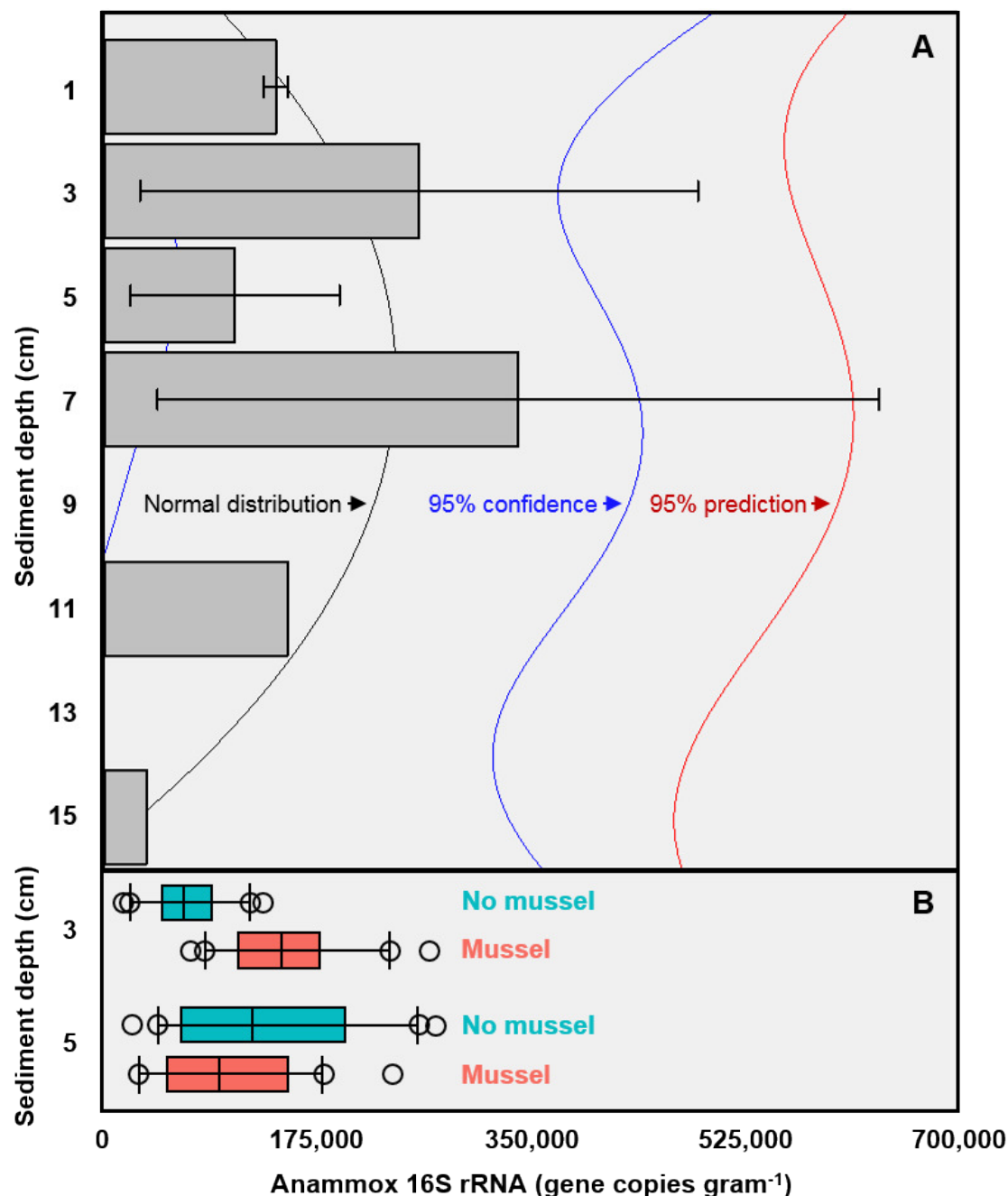
1062   Figure 1: Freshwater mussels deposit feces and pseudofeces containing nitrogen and carbon at the water-sediment interface (i.e. oxic-
1063   anoxic transition). NH_4^+ resulting from mussel biodeposits may be oxidized via nitrification and comammox in oxic conditions
1064   (yellow arrows), and/or by anammox and n-damo near the oxic-anoxic interface (gray arrows). Oxidized nitrogen species (NO_2^- and
1065 NO_3^-) may be reduced by dissimilatory nitrate reduction to ammonium (DNRA) and denitrification pathways (blue).

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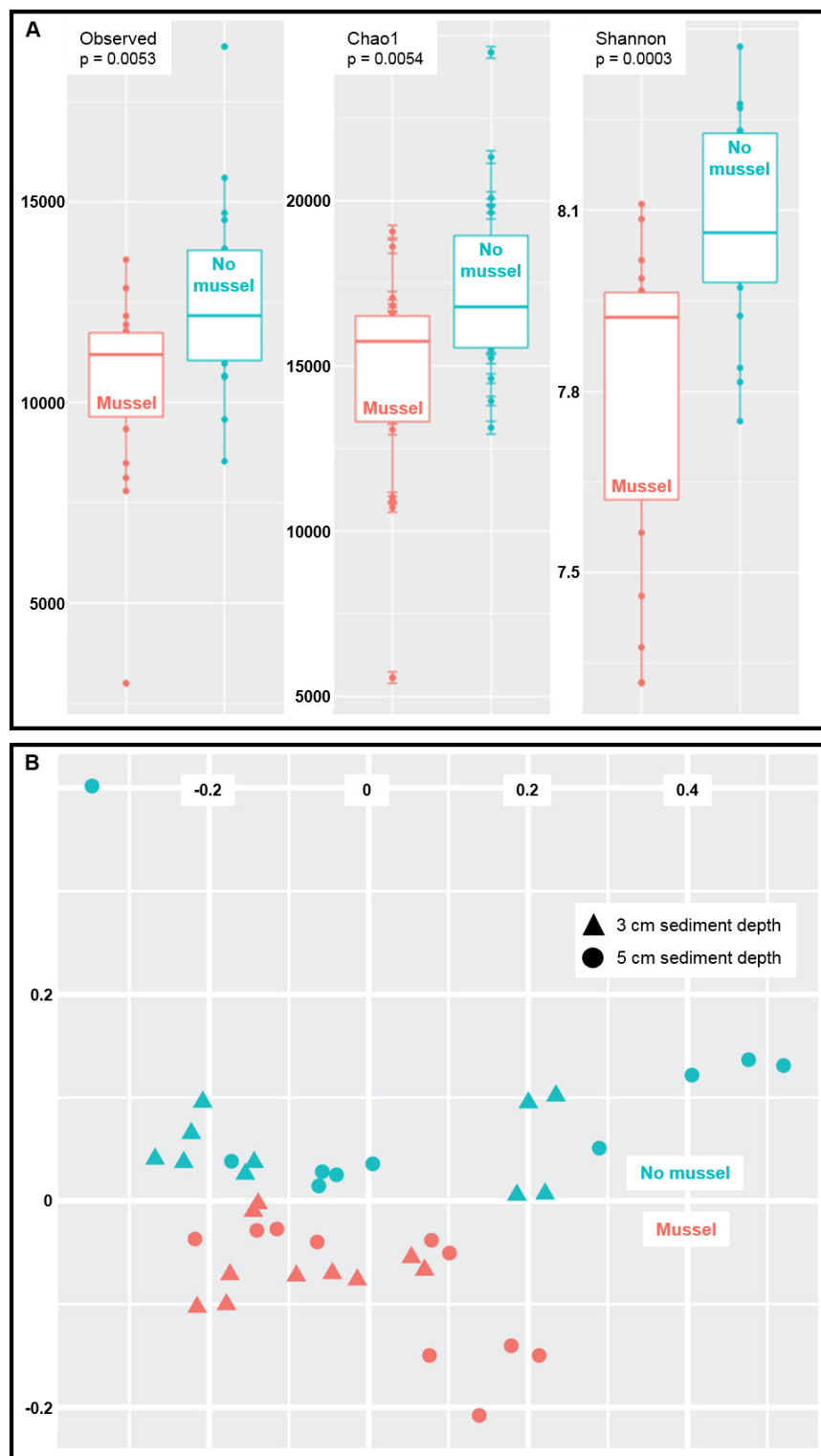
1067 Figure 2: A) The mean anammox 16S rRNA gene copies (per gram of sediment) in the presence
1068 of mussels were normally distributed (Shapiro-Wilk normality test, W-statistic=0.954, p=0.773)
1069 with depth (Batch 1 data). Error bars represent 1 standard deviation from the mean. B) Mussels
1070 (**salmon-colored data**) significantly increased the anammox 16S rRNA gene copies at 3 cm
1071 depth (p<0.001; Batch 2 data). The anammox gene copies were statistically indistinguishable
1072 with mussels as compared to the no mussels (**turquoise-colored data**) sediments at 5 cm (Batch
1073 2 data). The outer most open circles in Figure 2B represent data outliers, box boundaries
1074 represent the 25th and 75th percentile, the line within the box is the median, and error bars
1075 indicate 10th and 90th percentiles.

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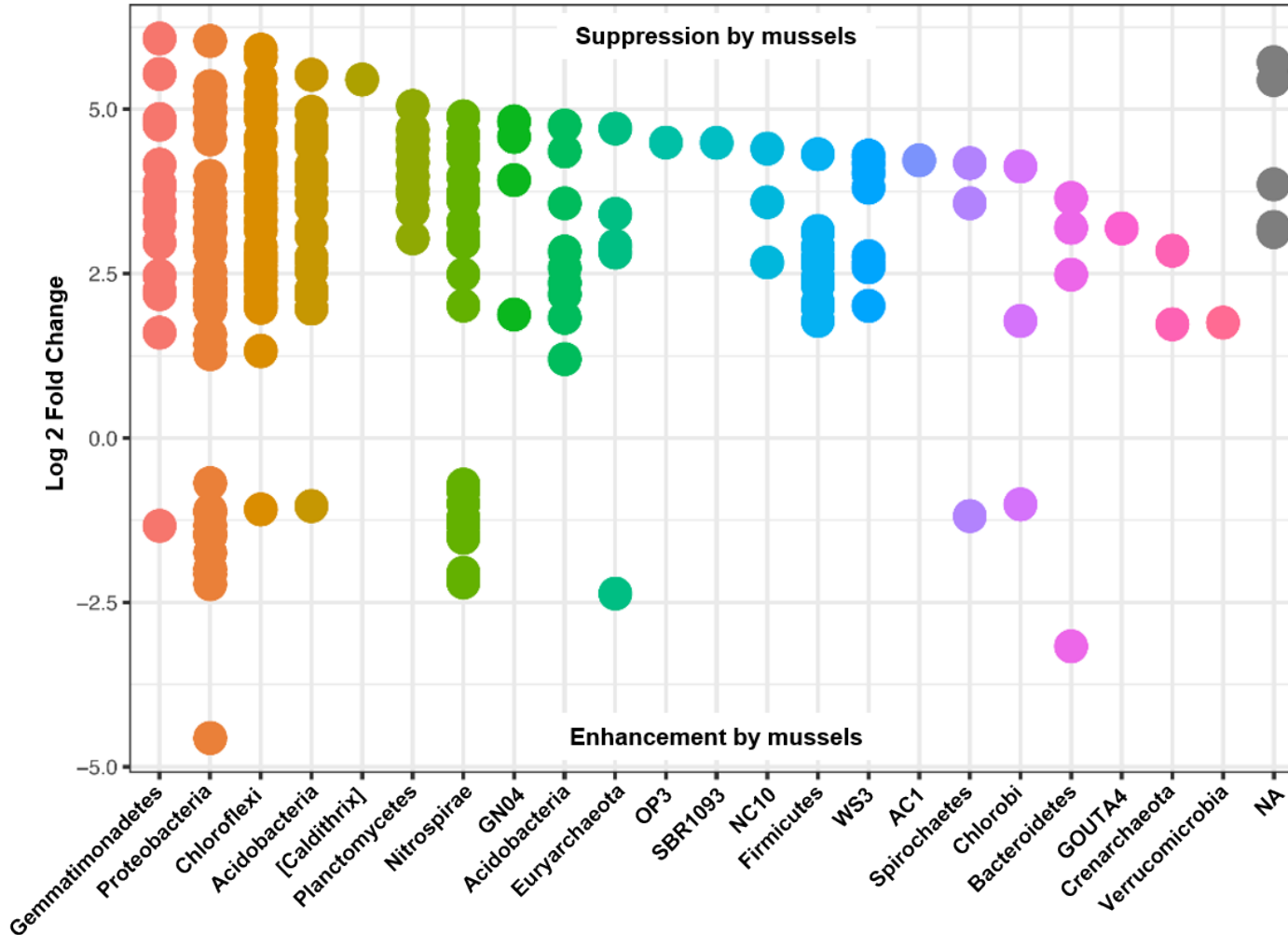
Figure 3: A) Sediments with mussels have lower observed species richness (p=0.005), Chao1 diversity (p=0.005), and Shannon (p=0.0003) diversity than no-mussel sediments. B) NMDS analysis using bray-curtis distances revealed sample clustering as a function of mussel presence, but not sediment depth.

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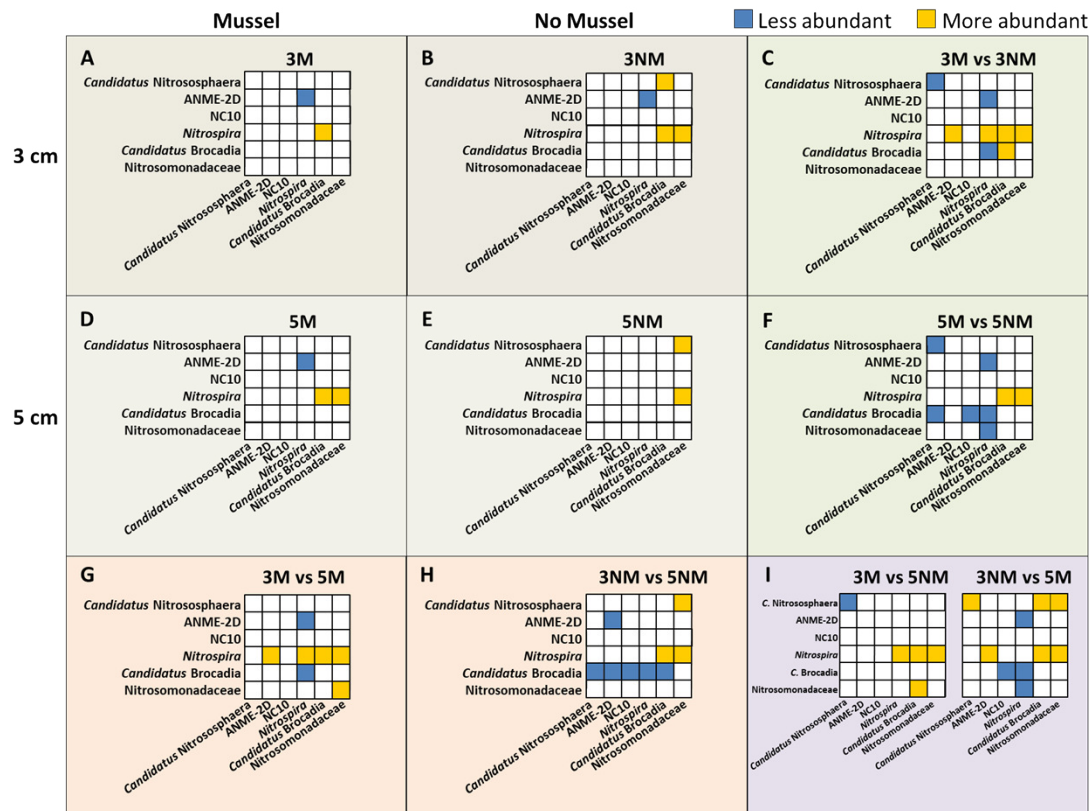
1082 Figure 4: Results from a DESeq2 differential abundance analysis expressed as Log2FC comparison of with-mussels and no-mussels
1083 samples. Negative Log2FC represent phyla enhanced in the mussel bed and each point represents an individual OTU. To enhance
1084 clarity, only those OTUs with p-adj < 0.0001 are shown.

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1086 Figure 5: Statistically significant differences in N-cycle phylotype relative abundances
1087 (Padj<0.05). All boxes show y-axes compared to the "baseline" x-axes, with no boxes
1088 representing comparisons not meeting significance. Boxes A-B, D-E: Comparisons within
1089 treatment conditions of mussel presence and depth. Box C: Differentially abundant phylotypes
1090 between 3 cm mussel and 3 cm no mussel treatments. Box F: Relative abundance comparisons
1091 between 5 cm mussel and 5 cm no mussel treatments. Box G: Differential N-cycle phylotype
1092 abundances between 3 cm mussel and 5 cm mussel samples. Box H: Comparisons between 3 cm
1093 no mussel and 5 cm no mussel treatments. Box I: Abundance comparisons of 3 cm Mussel
1094 versus 5 cm no mussel, and 3 no mussel versus 5 mussel samples.