Alphaproteobacteria fix nitrogen in a *Sphagnum*-dominated peat bog using molybdenum-dependent nitrogenase

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Running Title: Molybdenum-based nitrogen fixation in a *Sphagnum* peat bog

Abstract

Microbial N_2 fixation (diazotrophy) represents an important nitrogen source to oligotrophic peatland ecosystems, which are important sinks for atmospheric CO_2 and susceptible to changing climate. The objective of this study was to investigate the pathways and controls of diazotrophy, as well as the active microbial groups that mediate the process in *Sphagnum*-dominated peat bogs. In an ombrotrophic peat bog (S1) in the Marcell Experimental Forest (Minnesota, USA), low (μM) levels of inorganic nitrogen were observed, suggesting that diazotrophy could have a significant influence on ecosystem properties. Despite higher dissolved vanadium (V) (11 nM) than molybdenum (Mo) (3 nM) in surface peat, a combination of metagenomic, amplicon sequencing and activity measurements indicated that Mo-containing nitrogenases dominate over the V-containing form. Diazotrophy was only detected in surface peat exposed to light, with the highest rates observed in peat collected from hollows with the highest water content. Rates were suppressed by O_2, and unaffected by CH_4 and CO_2 amendments. Acetylene fully inhibited CH_4 consumption under oxic conditions, but only partially inhibited $^{15}N_2$ incorporation in degassed incubations, and had a minimal effect on oxic $^{15}N_2$ incorporation. Through a close coupling of process rate measurements with molecular analysis of the metabolically active microbial communities, our findings suggest that diazotrophy in surface layers of the S1 bog is mediated by *Alphaproteobacteria* (*Bradyrhizobiaceae* and *Beijerinckiaceae*) supported by photosynthate, rather than methane, for carbon and/or energy.

Importance

Previous studies indicate that diazotrophy provides an important nitrogen source and is linked to methanotrophy in *Sphagnum*-dominated peatlands. However, the environmental controls and enzymatic pathways of diazotrophy, as well as the metabolically active microbial populations that catalyze this process in peatlands, remain in question. Our findings indicate that oxygen levels and photosynthetic activity override low nutrient availability in limiting diazotrophy, and that primarily non-methanotrophic members of the *Alphaproteobacteria* (*Bradyrhizobiaceae* and *Beijerinckiaceae*) catalyze this process primarily at the bog surface.

50 **Introduction**

51 High-latitude peatlands store approximately one-third of global soil carbon and may pose a
52 climatic threat if rising global temperatures accelerate the release of this stored carbon in gaseous
53 forms as either carbon dioxide or methane (32, 59, 94). Mineral-poor (ombrotrophic) peatlands
54 receive most of their nutrient inputs from atmospheric deposition and contain *Sphagnum* moss as
55 their primary plant cover (11, 59). The peatmoss *Sphagnum* is a keystone genus in these
56 ecosystems with its biological function and abiotic environment responsible for much of the
57 primary production and recalcitrant dead organic matter (12, 92). *Sphagnum* mosses also host
58 complex microbiomes (8, 53, 69, 70), including N_2-fixers (diazotrophs) that are significant
59 nitrogen sources to peatland ecosystems (4).

60 Despite decades of research, there is still much debate on the identity of the dominant
61 diazotrophs in ombrotrophic peatlands. Early work implicated Cyanobacteria (1, 33, 34) or
62 heterotrophic bacteria (78) based primarily on microscopic studies, while more recent molecular
63 analyses argue for the importance of methanotrophic *Beijerinckiaceae* (18) as major diazotrophs
64 in *Sphagnum* peat bogs (7, 21, 40, 89). Possible contributions from other potential diazotrophs,
65 such as strictly anaerobic methanogenic *Euryarchaeaota*, remain unknown. However, it is quite
66 possible that diverse diazotrophs exist within defined niches of peatland environments (55).

67 Diazotrophy is catalyzed by the nitrogenase metalloenzyme, a complex of thee subunits
68 (H, D and K) that contains abundant iron as Fe-S clusters. This enzyme is extremely O_2 sensitive
69 (93), and must be protected from exposure to O_2 for diazotrophy to occur (24). The most
70 common form of nitrogenase, encoded by *nif* genes, contains molybdenum (Mo) as its cofactor.
71 When Mo is scarce, some species of Bacteria and Archaea express nitrogenases containing
72 vanadium (V; *vnf* genes) or iron (Fe; *anf* genes) in place of Mo, but these "alternative"

nitrogenases are less efficient than the Mo form (67, 90). Phylogenetic analyses of the most conserved nitrogenase gene, *nifH*, revealed four clusters: aerobic bacteria (cluster I); alternative nitrogenases (cluster II); anaerobic bacteria and archaea (cluster III); uncharacterized sequences (cluster IV), and paralogs related to chlorophyll biosynthesis (cluster V; 76). Consistent with higher concentrations of V than Mo in most rocks (91), microbes from diverse soils contain *vnf* (cluster II) genes (3, 6, 16, 41, 65). Given the oligotrophic conditions that predominate in peatlands, trace metals may limit diazotrophy. However, little is known about trace metal availability and the role of alternative nitrogenase pathways in ombrotrophic peatlands.

Similarly and importantly, methane monooxygenase (MMO) (the enzyme that catalyzes the first step of methane oxidation) occurs in particulate (copper (Cu)-containing pMMO) and soluble (Fe-containing sMMO) forms. While pMMO has more specific substrate requirements, pathways that employ sMMO can use a wider range of compounds (14). Both forms of MMO are inhibited by acetylene (C_2H_2) (13, 73). In organisms with both sets of genes, pMMO is expressed when Cu is abundant, whereas Cu limitation induces sMMO expression (80). The dominant peatland methanotrophs in the *Alphaproteobacteria* and *Gammaproteobacteria* tend to possess both MMOs (10, 23, 35, 47, 63), although *Methylocella* species containing solely sMMO have been isolated from peat bogs (19, 20). While most studies have primarily targeted the *pmoA* gene (23, 47), *mmoX* genes and transcripts have also been reported in peatlands (58, 63, 75), raising questions about the relative importance of each form for peatland methane oxidation.

The acetylene reduction assay (ARA) is commonly used as a proxy for diazotroph activity (37, 38). This assay is effective for capturing the potential activity of diazotrophic bacteria that are not inhibited by C_2H_2, such as *Cyanobacteria* and non-methanotrophic *Proteobacteria* (e.g. *Bradyrhizobiaceae*) (45). However, a number of functional guilds of

96 microorganisms (including methanotrophs, methanogens, sulfate reducers, and nitrifiers) are
97 inhibited by C_2H_2 (15, 44, 71, 72, 82, 84). If these or other C_2H_2-sensitive microbes (see
98 Fulweiler, et al. (25)) perform diazotrophy and/or provide substrates to other diazotrophs, ARA
99 may underestimate N_2 fixation activity in that system. Thus, recent studies have shifted to
100 tracking N_2 fixation by incorporation of the stable isotope tracer, $^{15}N_2$ (50, 55, 56, 89).

101 In this study of the S1 peat bog at the Marcell Experimental Forest in Minnesota, USA,
102 dissolved macro- (NH_4^+, NO_3^-, PO_4^{3-}) and micro- (Fe, Cu, V, Mo) nutrients were profiled along
103 with the community composition and abundance of diazotrophic microorganisms. We also
104 performed separate laboratory incubation experiments to measure potential rates of ARA and
105 $^{15}N_2$ incorporation to: (a) assess environmental controls (light, O_2, CH_4) on diazotrophy; (b)
106 quantify the effect of C_2H_2 on rates of diazotrophy and methanotrophy; and (c) search for
107 diagnostic markers for alternative nitrogenase activity such as a low conversion factor of ARA to
108 $^{15}N_2$-incorporation (3) and C_2H_2 reduction to ethane (22). Finally, we make recommendations on
109 universal *nifH* primers for amplicon sequencing and quantitative PCR based on our findings.

110

111 **Materials and Methods**

112 ***Site description and sample collection.*** Samples were collected from the S1 (black spruce-
113 *Sphagnum* spp.) peat bog at Marcell Experimental Forest (MEF; 47°30.476' N; 93°27.162' W),
114 the site of the DOE SPRUCE (Spruce and Peatland Responses Under Climatic and
115 Environmental Change) experiment in northern Minnesota, USA (36). The S1 bog is
116 ombrotrophic and acidic (average pH 3.5-4; 52, 79). Dissolved O_2 levels decrease to below
117 detection (~20 ppb) within the top 5 cm of the bog. Three locations were sampled along S1 bog
118 transect 3 (T3) at near, middle and far sites (see Lin, et al. (64) for further details). Surface (0-10

119 cm depth) peat was collected from hollows dominated by a mixture of *Sphagnum fallax* and *S.*
120 *angustifolium*, and hummocks dominated by *S. magellanicum*. Peat depth cores (0-200 cm) were
121 sampled from hollows where the water level reached the surface of the *Sphagnum* layer.

122 ***Macronutrients.*** Peat porewater was collected using piezometers from 0, 10, 25, 50, 75,
123 150 and 200 cm depth. Piezometers were recharged the same day as collection, and porewater
124 was pumped to the surface, filtered through sterile 0.2 µm polyethersulfone membrane filters,
125 and stored frozen until analysis. Nitrate (NO_3^-) and nitrite (NO_2^-) were analyzed using the
126 spectrophotometric assay as described by García-Robledo, et al. (28). Ammonium (NH_4^+)
127 concentrations were determined with the indophenol blue assay (85). Phosphate concentrations
128 were measured with the molybdate-antimony ascorbic acid colorimetric assay (68).

129 ***Micronutrients.*** Peat porewater was collected from two locations in the S1 bog from
130 cores at 0-30 cm, 30-50 cm, and 100-150 cm depths by filtration through 0.15 µm Rhizon soil
131 samplers (Rhizosphere Research Products). All plastics were HCl washed prior to sampling;
132 Rhizon soil samplers were cleaned by pumping 10 mL of 1 N HCl through them, followed by a
133 rinsing with ultrapure water until the pH returned to neutral (~100 mL/filter). After collection,
134 samples were acidified to 0.32 M HNO_3 (Fisher Optima) and analyzed using a Thermo
135 ELEMENT 2 HR-ICP-MS (National High Magnetic Field Laboratory, Florida State University).
136 Initial analyses resulted in frequent clogging of the nebulizer, likely due to abundant dissolved
137 organic carbon. Therefore, samples were diluted 1:10 to minimize interruptions from nebulizer
138 clogs. Concentrations were quantified with a 7-point external calibration using standards
139 prepared in 0.32 N HNO_3 from a multi-element standard mix (High Purity Standards). In order to
140 measure lower concentrations but avoiding sample dilution, subsequent samples were digested
141 before ICP-MS analysis. Briefly, 1 mL porewater samples were digested for 36 hours at 250°C

142 in a trace metal clean polypropylene exhausted laminar flow hood with 1 mL of 16 N NHO_3
143 (Ultrex II, JT Baker) and 100 μL of 30% H_2O_2 (Ultrex II, JT Baker) in acid-washed Teflon vials.
144 Samples were then evaporated to near dryness, resuspended in 0.32 N HNO_3, and analyzed by
145 ELEMENT2 ICP-MS along with parallel blank solutions.

146 ***Quantification and sequencing of gene and transcript amplicons***. Peat was frozen on
147 dry ice at the field site in July 2013, or in liquid N_2 after 7 days at 25°C in the light under
148 degassed (80% N_2 + 20% CO_2) headspace with 1% C_2H_2, with or without 1% CH_4 for June 2014
149 incubations (see ARA section for more details). DNA and RNA extractions were performed with
150 MoBio PowerSoil DNA and total RNA extraction kits, respectively, as described in Lin, et al.
151 (63). cDNA was prepared by reverse transcription as in Esson, et al. (23). Primer pairs were
152 polF/polR for *nifH* (25), A189f/Mb661r for *pmoA* (51), mmoX-206f/mmoX-886r for *mmoX* (43),
153 Mlas/mcrA-rev for *mcrA* (83), nifD820F/nifD1389R followed by nifD820F/nifD1331R for *nifD*
154 (3), and vnfD_anfD548F/vnfD_anfD1337R followed by vnfD_anfD548F/vnfD_anfD1291R for
155 *vnfD/anfD* (3). PCR conditions were based on (27), (23), (58), (83), (3) and (9) for *nifH*, *pmoA,*
156 *mmoX, mcrA, nifD/vnfD/anfD* and 16S rRNA, respectively.

157 Gene fragments for qPCR were amplified from genomic DNA and plasmid standards
158 were constructed according to Lin, et al. (61). To prepare cDNA standards, plasmid DNA with a
159 positive gene insert was linearized with NcoI restriction enzyme following the manufacturer's
160 protocol (Promega), and purified by MinElute PCR purification kit (Qiagen). RNA was
161 synthesized from the linearized plasmid DNA using the Riboprobe in vitro transcription system
162 according to the manufacturer's protocol (Promega). Then, cDNA standards and cDNA of
163 environmental RNA samples were synthesized using the GoScript Reverse Transcription system
164 following the manufacturer's protocol (Promega). Samples were run in duplicate against a

165 plasmid DNA standard curve on a StepOnePlus Real-Time PCR System (ABI) using Power
166 SYBR Green PCR master mix. Functional gene and transcript copy numbers were normalized to
167 dry weight of peat as well as 16S rRNA transcript copies for incubation samples. Amplicons
168 were sent to the University of Illinois at Chicago for DNA sequencing using a 454 platform. Raw
169 sequences were demultiplexed, trimmed, and quality filtered in CLCbio. The phylogeny of
170 *vnfD/anfD* sequences was inferred using the Maximum Likelihood method based on the Kimura
171 2-parameter model in MEGA5 (86).

172 ***Acetylene reduction and methane consumption rates.*** Samples of bulk peat (*Sphagnum*
173 spp. and surrounding soil) was collected from 0-10 and 10-30 cm depths in September 2013,
174 April 2014, June 2014, September 2014 and August 2015, and stored at 4°C until the start of
175 laboratory incubations. Samples from 0-10 cm depth were gently homogenized so as not to
176 rupture *Sphagnum* spp. tissues, while peat samples from 10-30 cm depth were fully
177 homogenized. For each sample, 5 g of bulk peat were placed in 70 mL glass serum bottles,
178 stoppered with black butyl stoppers (Geo-Microbial Technologies) and sealed with an aluminum
179 crimp seal. Headspaces were oxic (room air, 80% N_2 + 20% O_2) or degassed (100% N_2 or 80%
180 N_2 + 20% CO_2) with or without 1% C_2H_2 or 1% CH_4. Treatments were incubated for one week at
181 25°C in the light or dark. A gas chromatograph with a flame ionization detector (SRI
182 Instruments, Torrance, CA, USA) equipped with a HayeSep N column was used to quantify CH_4,
183 C_2H_2 and C_2H_4. Samples were measured for C_2H_4 production daily until C_2H_4 production was
184 linear (~7 days). Controls not amended with C_2H_2 did not produce ethylene (C_2H_4). Incubations
185 of hollow peat from June 2014 incubated in oxic headspace with and without 1% C_2H_2 were also
186 monitored for consumption of 1% CH_4. Statistical analysis was performed with JMP Pro (v.
187 12.1.0) using the Student's T-test.

188 ***$^{15}N_2$ incorporation rates.*** In September 2014, samples were quantified for N_2 fixation
189 rates by $^{15}N_2$ incorporation in parallel with ARA measurements. Incubations were set up as
190 described above and supplemented with 7 mL of 98% $^{15}N_2$ (Cambridge Isotope Laboratories,
191 Tewksbury, MA, USA). After 7 days, samples were dried at 80°C, homogenized into a fine
192 powder, and analyzed for N content and $\delta^{15}N$ by isotope-ratio mass spectrometry (IRMS) with a
193 MICRO cube elemental analyzer and IsoPrime100 IRMS (Elementar) at the University of
194 California, Berkeley, corrected relative to National Institute of Standards and Technology (NIST,
195 Gaithersburg, MD, USA) standards.

196 ***Metagenomic analyses.*** Metagenomes were generated in a previous study (62).
197 Diazotrophic and methanotrophic pathways were interrogated using the following bioinformatics
198 approaches. Briefly, Illumina reads were filtered by quality (Phred33 score threshold of Q25)
199 using Trim Galore (Babraham Bioinformatics) and a minimum sequence length cut off of 100
200 bp. The sequences were then queried using RAPSearch2 (95) against the NCBI-nr database of
201 non-redundant protein sequences as of November 2013. Sequences with a bit-score of 50 and
202 higher were retained to determine the total number of functional genes for normalization across
203 the different samples. The taxonomic composition of protein-coding sequences was determined
204 based on the taxonomic annotation of each gene according to the NCBI-nr taxonomy in
205 MEGAN5 (42) (min score: 50; max expected: 0.01; top percent: 10; min complexity: 0.3).

206 To classify sequences by nitrogenase cluster type, genes were analyzed using BLASTX
207 (e-value 0.1; bit-score 50) versus a custom *nifH* database that includes a phylogenetic tree to
208 distinguish the principal clusters (I, V, III) in the *nifH* phylogeny, as well as paralogous cluster
209 IV, *nifH*-like sequences (26). *nifH* genes from the four clusters were normalized to total protein-
210 coding genes from RAPSearch2 output sequences. The relative abundance of particulate vs.

211 soluble methane monooxygenase (*pmoA* vs. *mmoX*, respectively) was based on previous analyses
212 in Lin, et al. (63).

213 **Nucleotide sequence accession numbers.** Metagenomes were reported in a previous
214 study (62) and deposited in BioProject XXXXXXX. *pmoA* cDNA amplicons were reported in a
215 previous study (23) and deposited in BioProject PRJNA311735. *nifH*, *mcrA*, *nifD*, and
216 *vnfD/anfD* cDNA amplicons were deposited in BioProject XXXXXXX.

217

218 **Results**

219 ***Macro/micro-nutrients and functional genes.*** In S1 bog hollows, NH_4^+ remained at 2 μM from
220 the surface to 25 cm depth, and increased at greater depths (Fig. 1; Fig. S1a). Nitrate was <1 μM
221 in surface peat and decreased with depth (Fig. S1b). Phosphate was <0.1 μM from the surface to
222 25 cm depth, and then increased with depth (Fig. S1c). With the polF/polR primer pair, we
223 measured 1.2×10^7 copies g^{-1} *nifH* genes at 1 and 20 cm, and 0.2×10^7 copies g^{-1} at 30 and 75 cm;
224 *nifH* transcripts (12.2×10^7 copies g^{-1}) were only detected at 1 cm (a 10:1 transcript: gene ratio),
225 and not at deeper depths (Fig. 1; Fig. S2a). Sequencing of cDNA from surface peat amplified
226 with polF/polR (*nifH*) and nifD820F/nifD1331R (*nifD*) primers showed that the vast majority of
227 nitrogenase transcripts belonged to cluster I (*Alphaproteobacteria*), with *Bradyrhizobiaceae*
228 dominating *nifH* sequences and *Beijerinckiaceae* dominating *nifD* sequences (Fig. S2). *nifH*
229 transcripts from *Gammaproteobacteria*, *Cyanobacteria* (*Oscillatoriophycideae*) and *Nitrospira*
230 were also observed at lower abundance in cDNA amplicon libraries (data not shown). Like *nifH*,
231 *pmoA* and *mcrA* transcripts showed the highest abundance in surface peat (Fig. S2b,c). Surface
232 *pmoA* transcripts mapped to *Methylocystaceae* (75%) and *Methylococcaceae* (25%). Surface

233 *mcrA* transcripts mapped to *Methanosarcina* (58%), *Methanocella* (28%), and *Methanoregula*
234 (11%). Attempts to amplify *mmoX* from cDNA were unsuccessful (data not shown).

235 Vanadium (5-21 nM) was consistently more abundant than Mo (1-7 nM) at 0-30, 30-50
236 and 100-150 cm depth intervals (Fig. 2). In metagenomes, *nifH* genes were roughly equally
237 distributed between Mo-dependent clusters I and III, and cluster IV/V paralogs (Fig. 2; Table
238 S1). Sequences from Cluster II (alternative nitrogenases, *vnfH*/*anfH*) were scarce at all depths
239 (<5% overall); two *vnfD* genes from metagenomes showed phylogenetic similarity to those from
240 soil *Proteobacteria* (Fig. S3). Attempts to amplify *vnfD/anfD* from cDNA yielded few reads;
241 those recovered were most similar to *Alphaproteobacteria anfD* from *Rhodopseudomonas*
242 species (Fig. S4). Iron (7-35 μM) was three orders of magnitude higher than Cu (7-38 nM; Fig.
243 3). Trace nutrients Co (5-20 nM), Ni (10-80 nM), Zn (50-250 nM) and Mn (60-2220 nM) were
244 also quantified (data not shown). In metagenomes, genes for *pmoA* were dominant in surface
245 peat, whereas *mmoX* sequences became more abundant with depth (Fig. 3). In hollow surface
246 waters, other dissolved metals analyzed included Mn (~500 nM), Ni (~30 nM) and Co (~10 nM).

247 ***Rates of diazotrophy and methanotrophy.*** Potential rates of acetylene reduction were
248 measured for peat collected from S1 bog hollows and hummocks in April, June, August and
249 September 2013-2015 and incubated for 1 week at 25°C. Acetylene reduction to ethylene was
250 only detected in surface (0-10 cm) peat samples incubated in the light, and not in deep (10-30
251 cm) peat, nor in surface peat incubated in the dark; acetylene reduction to ethane was not
252 detected in any incubation (data not shown). *Sphagnum* in peat incubations exposed to light
253 became visibly greener over the course of the incubation (Fig. 4). In hollows, where surface peat
254 was dominantly covered by a mix of *S. fallax* and *S. angustifolium*, ARA rates were higher and
255 more variable in degassed in comparison to oxic incubations (0-163 vs. 2-23 μmol C_2H_4 g^{-1} h^{-1}),

256 and were unaffected by the presence or absence of 20% CO_2. *nifH* transcripts were also higher
257 and more variable in hollows than hummocks (Fig. S5). ARA in hollows incubated with
258 degassed headspace was positively correlated ($P < 0.0001$) with peat water content (93 to 96%).
259 In hummocks with lower water content (90-91%), surface peat was dominantly covered by *S.*
260 *magellanicum*, and oxic and degassed treatments had similarly low ARA rates (0-8 μmol C_2H_4 g^{-}
261 ^{1} h^{-1}). In both hollows and hummocks, ARA rates were not affected by addition of 1% CH_4.
262 $^{15}N_2$ incorporation showed similar overall trends as ARA (e.g. >90% higher rates in
263 degassed vs. oxic conditions, and no significant CH_4 effect; Fig. 5). Using the four sites
264 measured with both methods, a conversion factor of 3.9 for $^{15}N_2$-to-ARA was calculated (Fig.
265 S6). In degassed treatments, 1% C_2H_2 inhibited $^{15}N_2$ incorporation by 55% but had no effect on
266 oxic treatments. In oxic treatments, CH_4 consumption rates were 100 times higher than $^{15}N_2$
267 incorporation rates, and 1% C_2H_2 addition suppressed CH_4 oxidation rates by 95% (Fig. 5). *nifH*
268 transcripts in surface peat from hollows incubated under degassed headspace with 1% C_2H_2, with
269 or without 1% CH_4, ranged from 10^4 - 10^7 copies g^{-1} (July 2014; data not shown), which was 1-4
270 orders of magnitude lower than field samples from the previous summer (10^8 copies g^{-1}; Fig. 1).
271

272 **Discussion**

273 ***Peat bog nutrient concentrations and metalloenzymes.*** By definition, the only source of
274 nutrients to ombrotrophic peat bogs is the atmosphere, and thus these ecosystems are well known
275 to be oligotrophic (60). Scarce macronutrients in S1 peat pore water and low rates of atmospheric
276 deposition in Minnesota peat bogs (39) are consistent with oligotrophic conditions (60, 79, 87).
277 Micronutrient concentrations (~15 μM Fe, ~1 μM Zn and nM-level Ni, Cu, V, Mo and Co)
278 reported for acidic peatlands in Northern Europe (48) were similar to those we measured in the
279 S1 bog. Molybdenum sorption to peat is enhanced at low pH (5), and Mo concentrations (<10

280 nM) here were within the same range as in other freshwaters worldwide (30). In laboratory
281 cultures, <10 nM Mo can either limit growth (31) or induce expression of alternative nitrogenase
282 (2). Although S1 peat bog waters contained higher V (11 nM) than Mo (3 nM), the majority of
283 nitrogenase H-subunit genes retrieved from metagenomes belonged to Mo-containing clusters I
284 and III. A significant number of sequences was also obtained from uncharacterized Cluster IV,
285 recently shown to contain a functional nitrogenase (96) that likely binds a Mo-Fe cofactor (66).
286 Cluster I (*Alphaproteobacteria*; *Rhizobiales*) showed the highest relative abundance in transcript
287 libraries, with *Bradyrhizobiaceae* and *Beijerinckiaceae* dominating *nifD* and *nifH* amplicons,
288 respectively, as seen in prior studies (58, 89). Overall, evidence for alternative nitrogenases was
289 scant: (a) very few cluster II *vnfD/anfD* genes were found in metagenomes; (b) minimal
290 *vnfD/anfD* amplification was obtained from peat cDNA; (c) ethane was undetectable in ARA
291 incubations; and (d) the $^{15}N_2$-to-ARA conversion factor (3.9) – within the same range (3-4) as
292 other peat bogs (37, 57, 89) – matched Mo-nitrogenase in pure culture experiments (3), as
293 opposed to the lower values measured for alternative nitrogenases. Thus, in spite of lower
294 abundance of Mo than V in S1 bog porewaters, our collective evidence strongly suggests N_2
295 fixation by the Mo-containing form of the nitrogenase enzyme.

296 Like diazotrophy, methanotrophy was apparently also mediated by the enzyme requiring
297 the scarcer metal; dissolved Fe was consistently orders of magnitude higher than Cu in peat
298 porewater, yet *pmoA* sequences were more abundant than *mmoX* sequences in surface peat in
299 which the highest CH_4 consumption was observed (23, 63). Overall, these findings suggest that
300 trace metal availability is not the overriding environmental pressure driving selection for
301 diazotrophs and methanotrophs in *Sphagnum*-dominated peat bogs. Rather, water potential and
302 oxygen appear to be more important. Low metal and phosphate concentrations add further

303 evidence to previous suggestions that these nutrients may limit *Sphagnum* productivity (55)
304 and/or complex mechanisms may exist for nutrient scavenging at ultra-low concentrations.
305 Additionally, the inherent nature of the peat matrix, with characteristically high levels of
306 particulate and dissolved organic matter, likely also affects metal bioavailability in complex
307 ways not addressed in our study (88).

308 ***Environmental controls on diazotrophy.*** Methanotrophs have the potential to serve as a
309 biofilter to suppress methane emissions, along with providing a nitrogen source for the dominant
310 primary producers in peatland ecosystems. Previous field and laboratory studies point to a close
311 coupling of diazotrophy and methanotrophy in *Sphagnum*-dominated peatlands (49, 55, 74, 89).
312 This work has led others to suggest that methanotrophs provide the unaccounted nitrogen input
313 resulting from an imbalance in atmospheric nitrogen deposition and accumulation in *Sphagnum*
314 mosses (40). However, while substantial evidence indicates that methanotrophs are a source of
315 nitrogen to peat mosses, the ecological relevance of diazotrophic methanotrophs and their
316 importance to nitrogen flow still remains unclear because the two Alphaproteobacteria families
317 that appear to be the dominant diazotrophs in *Sphagnum* peat bogs grow on complex organics
318 (*Bradyrhizobiaceae* (29)) as well as simple alkanes and C1 compounds (*Beijerinckiaceae* (17)).

319 Our results from laboratory incubations of native peats revealed that diazotrophy was
320 stimulated by light, suppressed by O_2, and minimally affected by CH_4 and CO_2. Consistent with
321 previous reports (77), ARA and *nifH* transcription were only detected in surface samples
322 incubated in the light, although it is possible that our methods excluded C_2H_2-sensitive
323 diazotrophs at deeper depths (50). *nifH* transcripts were 1-4 orders of magnitude lower in
324 laboratory incubations than in the field, suggesting either that the diazotrophs in our incubations

were stressed (e.g. nutrient-limited), and/or that a different assemblage of diazotrophs grew in the incubations than in the field due to primer specificity (see next section).

Complete CH_4 consumption in air with 1% CH_4 showed that methanotrophs were active in our peat incubations. If these aerobic methanotrophs were the dominant diazotrophs, as previously proposed (55, 89), the presence of O_2 and CH_4 should have stimulated $^{15}N_2$ incorporation. Instead, our study and previous work (56) found the highest rates of diazotrophy at low O_2 and independent of CH_4 content. Acetylene fully inhibited CH_4 consumption under oxic conditions, but only partially inhibited $^{15}N_2$ incorporation under degassed conditions (as in Kox, et al. (54)) and had a minimal effect on oxic $^{15}N_2$ incorporation. Assuming that these incubations reflect field conditions, our data imply: (a) peatland diazotrophy is mediated by O_2-sensitive microorganisms that rely on photosynthesis for carbon and/or energy sources; and (b) at least part of the diazotrophic community is C_2H_2 sensitive. When we consider this in concert with the molecular information from the native peats, our data indicate that heterotrophic *Bradyrhizobiaceae* (C_2H_2 insensitive, O_2 sensitive (46)) and *Beijerinckiaceae* (C_2H_2 insensitive, O_2 insensitive (81)) were the primary diazotrophs in our incubations. Lesser contributions likely came from methanotrophic *Beijerinckiaceae* (C_2H_2 sensitive, O_2 insensitive), photosynthetic cyanobacteria from the *Oscillatoriophycideae* (C_2H_2 insensitive, O_2 sensitive (24)), and C_2H_2-sensitive, O_2-sensitive members of anaerobic cluster III. Future studies are warranted to interrogate contrasts in the activity and gene expression of diazotrophs when comparing laboratory microcosm experiments to the field, since microcosm experiments are most often used as a proxy for *in situ* activity.

Molecular markers for diazotrophy. We end with a word of caution with regard to the molecular detection of diazotrophs. The majority of studies in peatlands have employed PCR

348 amplification and sequencing of nitrogenase genes (nifH) for studying the dynamics of
349 diazotrophs in peatlands. A wide range of *nifH* primer sets exist, with varying universality (27).
350 Peat bog sequencing efforts have used polF/polR (this study; 58), F1/R6 (89),
351 FGPH19/polR+polF/AQER (56), and 19F/nifH3+nifH1(1)/nifH2(2) with nested PCR (7, 54).
352 We discourage use of polF/polR for peatland samples because *in silico* evaluation predicts that
353 this primer set will not amplify the majority of *Proteobacteria* and/or *Cyanobacteria* and Group
354 III *nifH* sequences (27). Of the *nifH* primer sets used previously, F1/R6, 19F/nifH3 and
355 nifH1(1)/nifH2 are predicted to have the highest coverage for soils (>80% predicted primer
356 binding for sequences from soil ecosystems). However, it is important to be aware that the F1/R6
357 primer set contains a number of mismatches with cluster III, including methanogens represented
358 in peatlands (Table S2). In order to maximize sequence coverage, we suggest the primer set
359 IGK(3)/DVV for future studies (27).

Acknowledgments

This research was funded by DOE support to J.E.K. under grant numbers DE-SC0007144 and DE-SC0012088. We thank Loren Dean Williams for inspiration with visuals, and Heather Dang (UC Berkeley) for IRMS analysis. Work at LLNL was conducted under the auspices of DOE Contract DE-AC52-07NA27344, with funding provided by LDRD 14-ER-038.

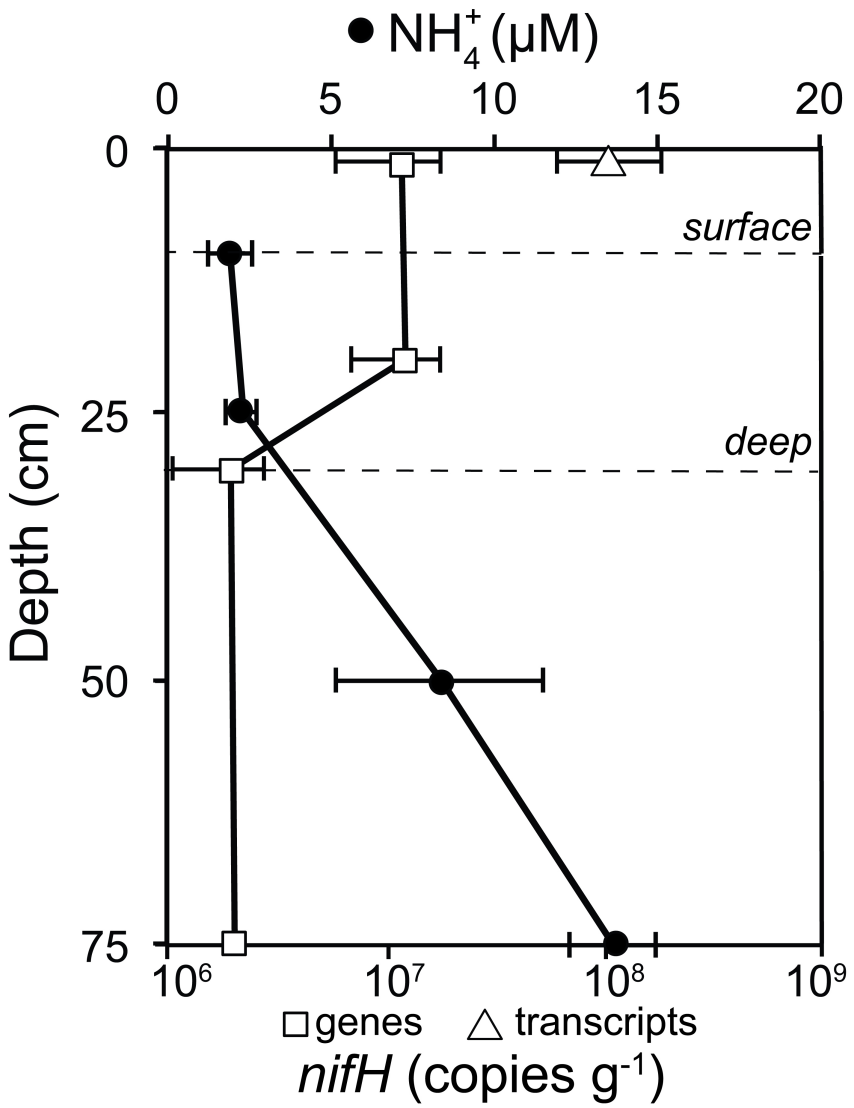
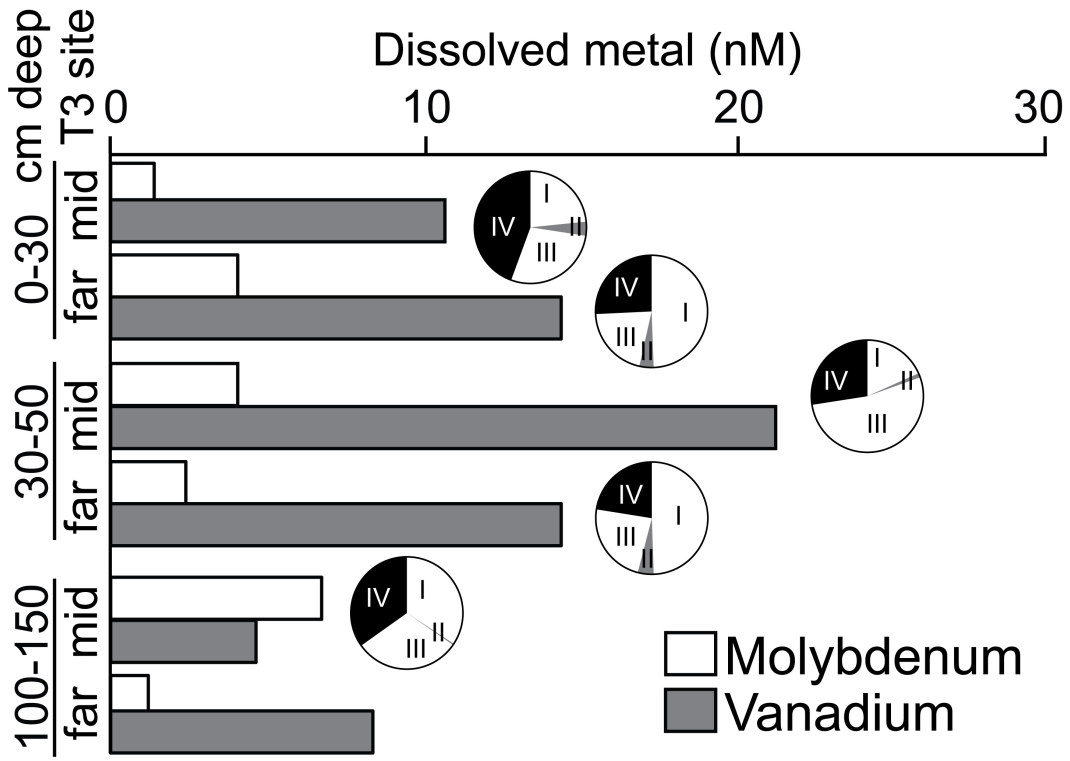


Figure 1. Depth profiles of NH_4^+ concentrations (black circles) and *nifH* gene copies (white squares) and transcripts (white triangle) in units of copies per gram of dry weight for the S1 bog T3 mid site. Concentrations are means of measurements from May, June and September 2014. *nifH* copy numbers are for July 2013. Error bars are standard errors. *nifH* transcripts were not detected at 20, 30 and 75 cm depths. Surface (0-10 cm) and deep (10-30 cm) peat depth intervals used for rate measurements are designated by dashed lines.



374

375 **Figure 2. Dissolved molybdenum (white) and vanadium (gray) concentrations in pore water**
376 **from three depths in S1 peat hollows (mid and far sites along T3 transect).** Pie charts show
377 the relative abundance of genes encoding the five nitrogenase H-subunit clusters from
378 metagenomes for each depth; clusters I and III encode Mo-Fe nitrogenases (*nifH)*; cluster II
379 encodes alternative (*vnfH, anfH*) nitrogenases; cluster IV encode nitrogenase paralogs. Deepest
380 metagenomes were from 75 cm; insufficient numbers of nitrogenase H-subunit sequences were
381 recovered from the far site for cluster analysis.

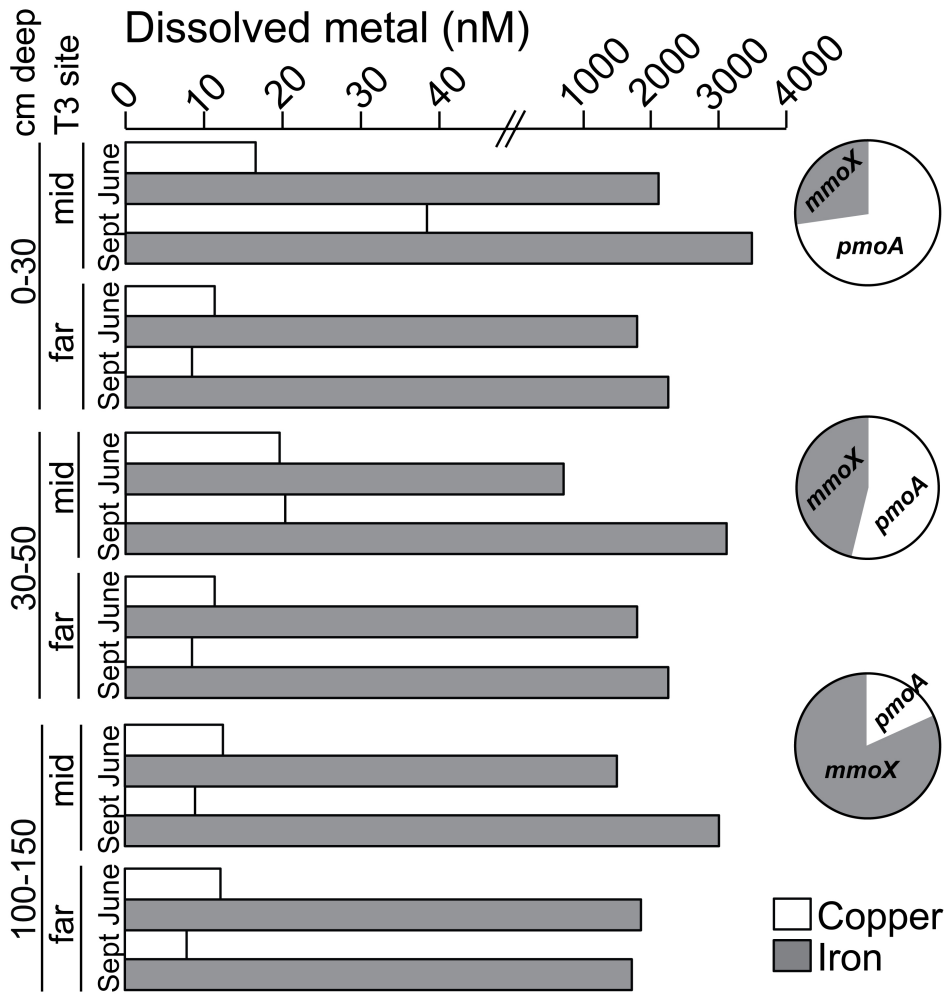


Figure 3. Dissolved copper (white) and iron (shaded) concentrations in pore water from three depths in S1 peat hollows (mid and far sites along T3 transect). Pie charts show the relative abundance of *pmoA* (Cu-containing particulate methane monooxygenase) and *mmoX* (Fe-containing soluble methane monooxygenase) sequences recovered from metagenomes from T3 mid sites (0-10, 25-50, and 75-100 cm).

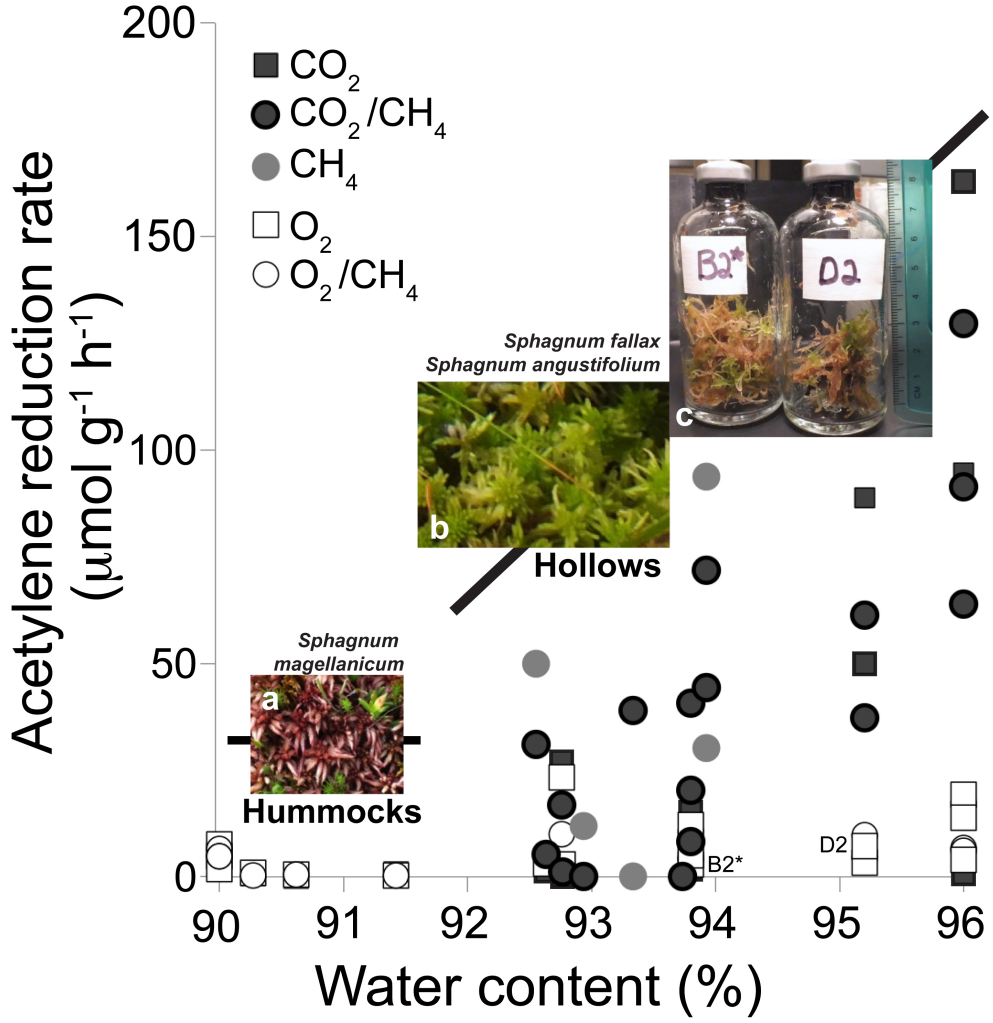


Figure 4. Acetylene reduction rates for hummocks (90-91% water content) and hollows (93-96% water content) at the S1 bog, T3 transect (0-10 cm depth incubated in the light at 25°C for 7 days). ARA units are µmol ethylene produced per gram of dry weight per hour. Photo insets show dominant *Sphagnum species* in (a) hummocks (*S. magellanicum*) and (b) hollows (*S. fallax* and *S. angustifolium*). Photo inset (c) shows *Sphagnum* greening after incubation of hollow samples in the light for 7 days at 25°C; bottle B2* (April 2014) received air headspace with 1% CH_4 and treatment D2 (Sept 2013) received air headspace without CH_4.

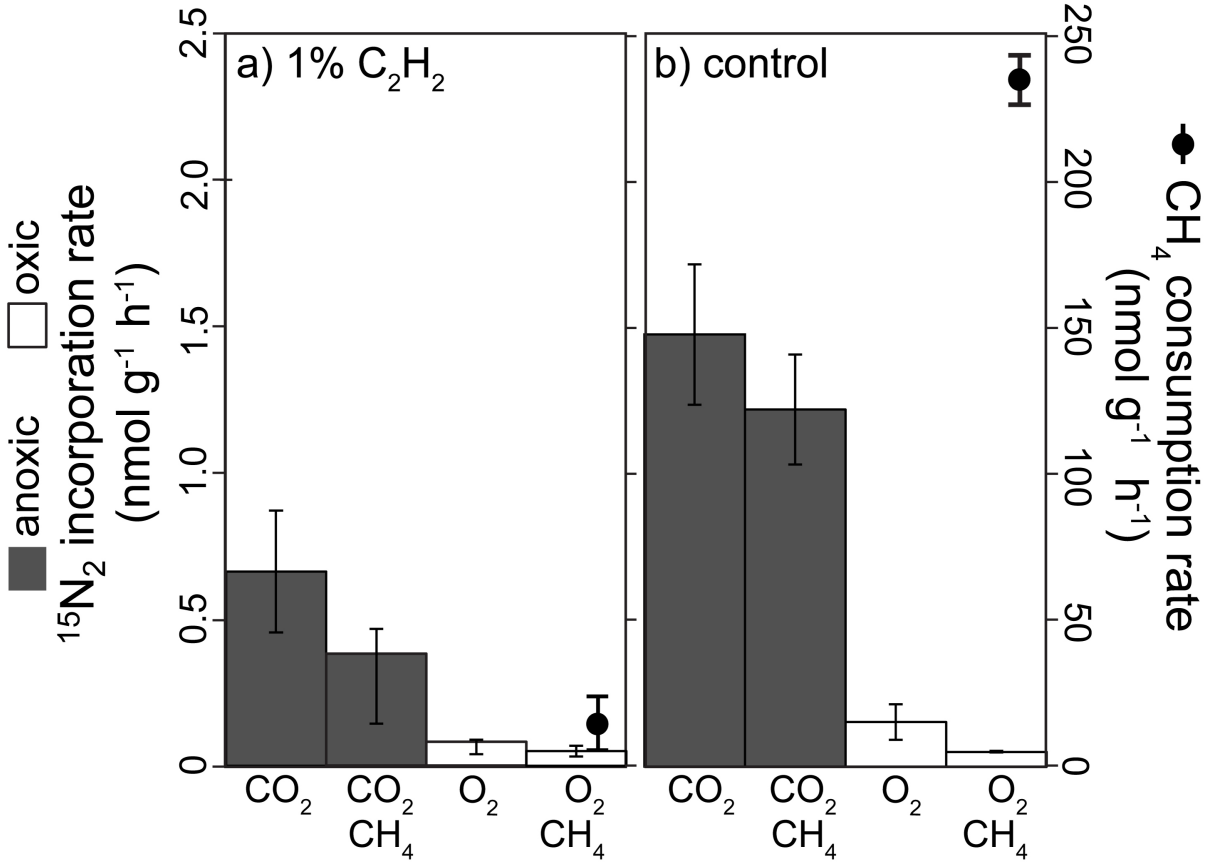


Figure 5. Effect of 1% C_2H_2 on $^{15}N_2$ incorporation and CH_4 consumption for S1 bog surface peat. Rates were measured for samples collected from the NW S1 bog transect in September 2014. $^{15}N_2$ incorporation treatment conditions were 80%N_2+20% CO_2±1% CH_4 (shaded bars) or 80%N_2+20% O_2±1% CH_4 (white bars), with (a) and without (b) 1% C_2H_2; units are nmol $^{15}N_2$ incorporated per gram of dry weight per hour. CH_4 consumption treatments were 80%N_2+20% CO_2+1% CH_4 (black circles) with (a) and without (b) 1% C_2H_2; units are nmol CH_4 consumed per gram of dry weight per hour. Error bars are standard errors.

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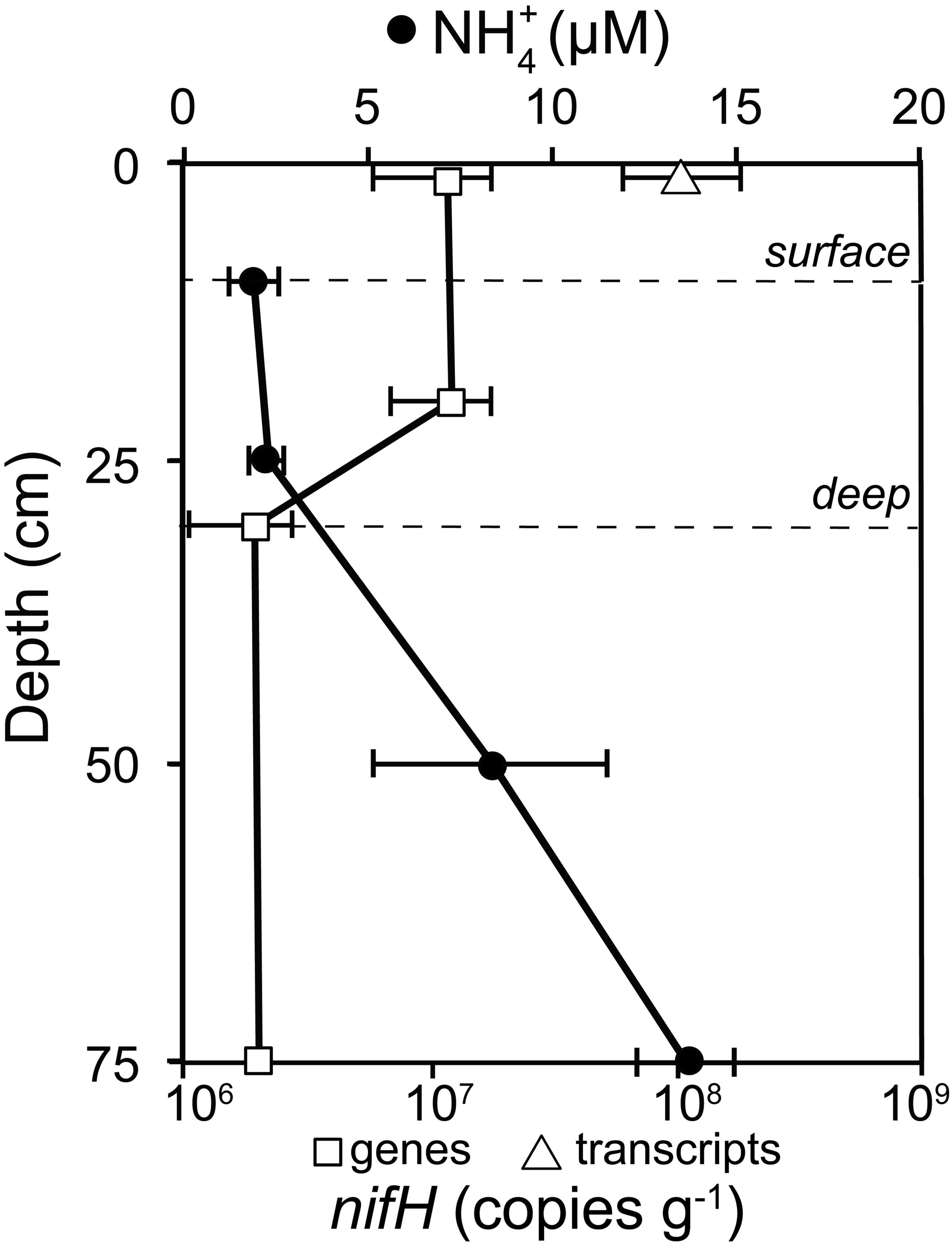
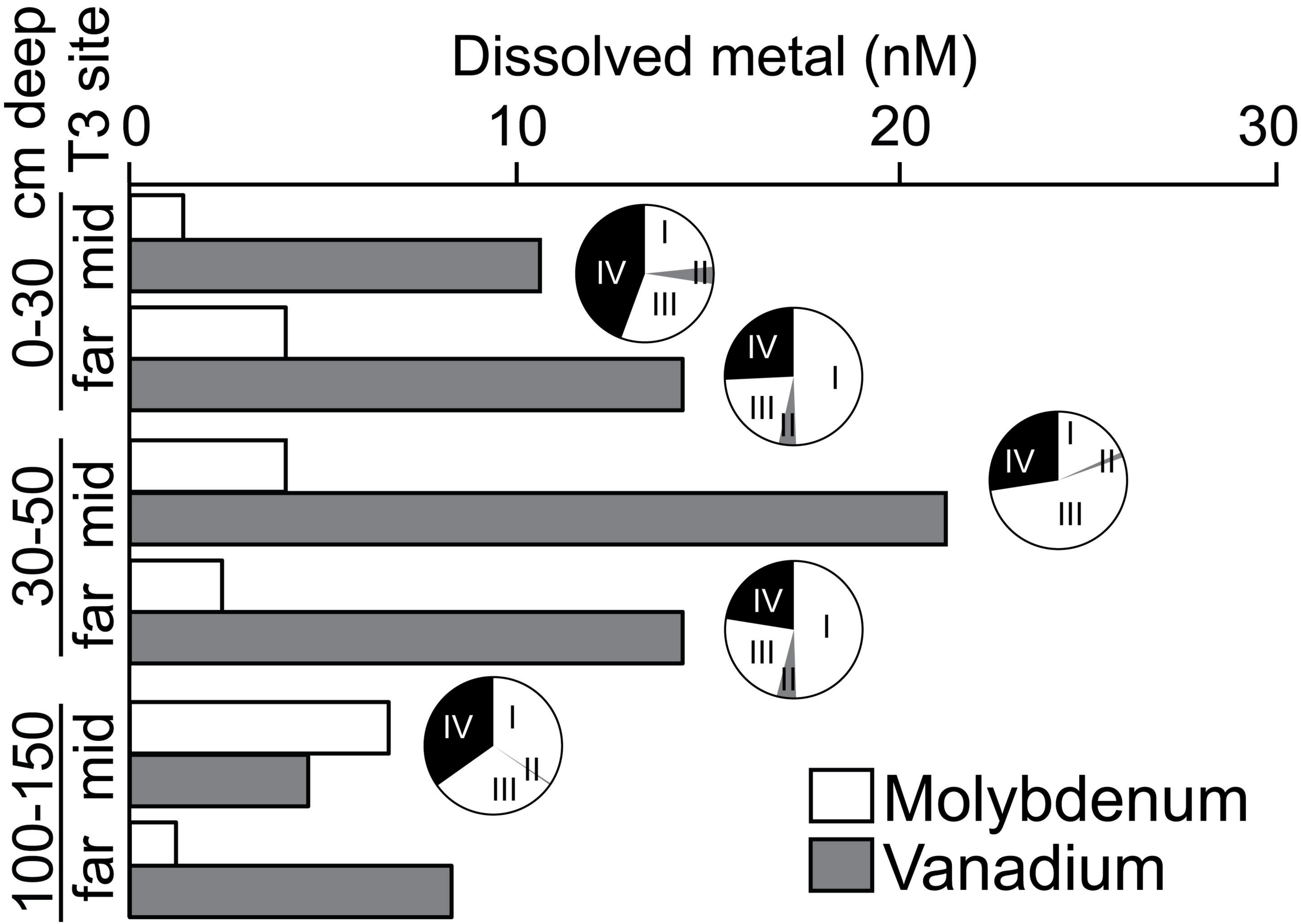
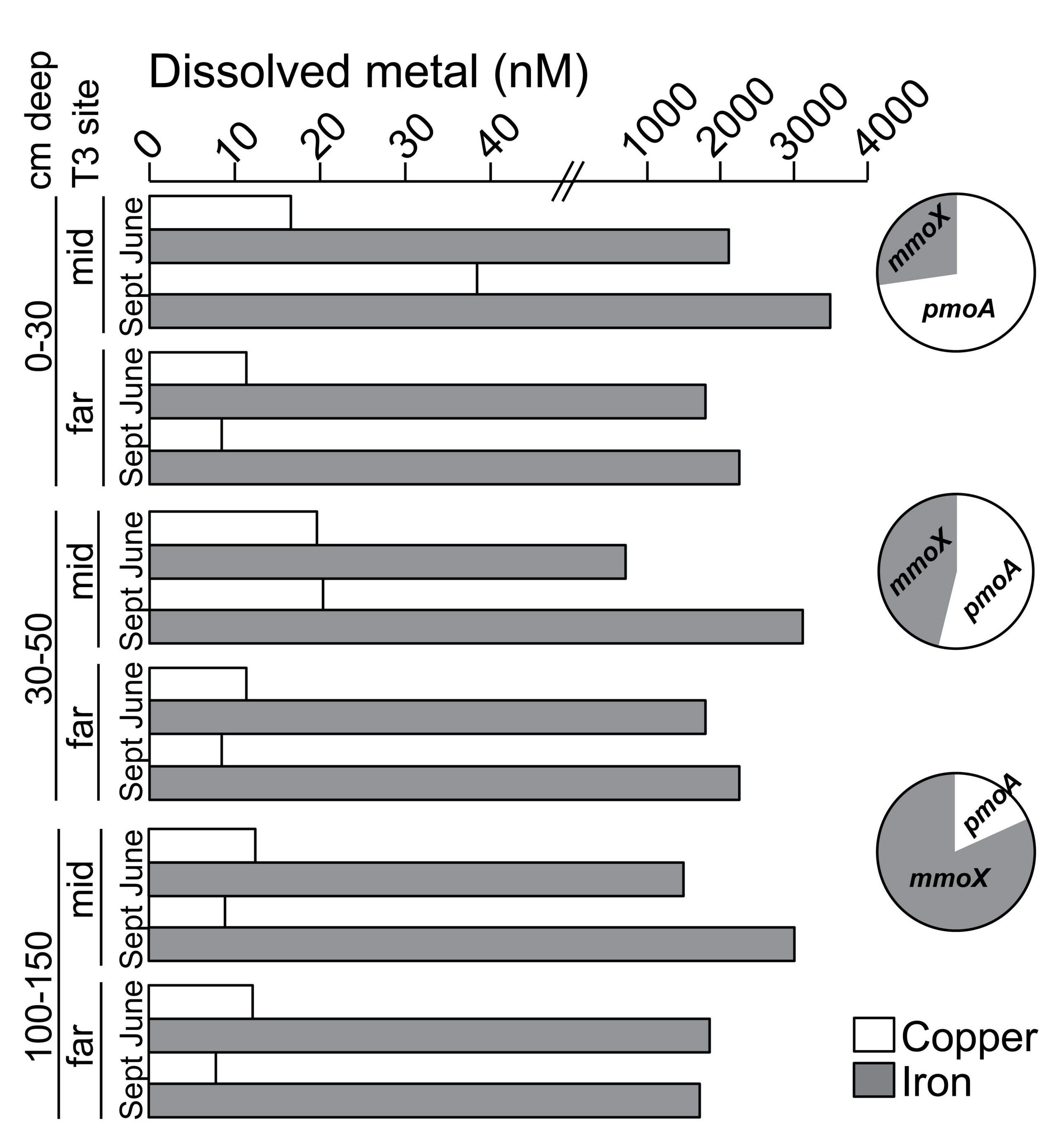
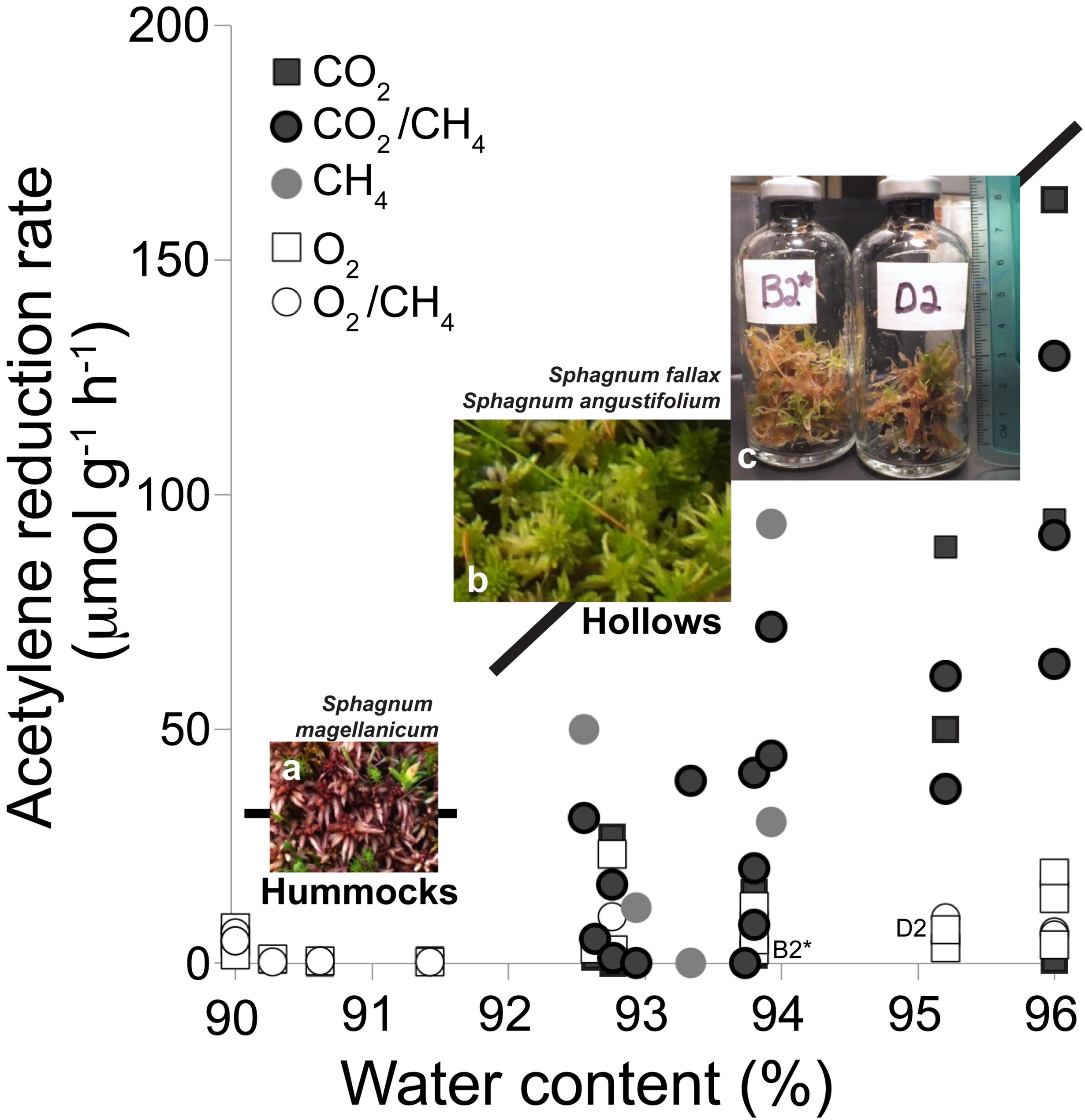
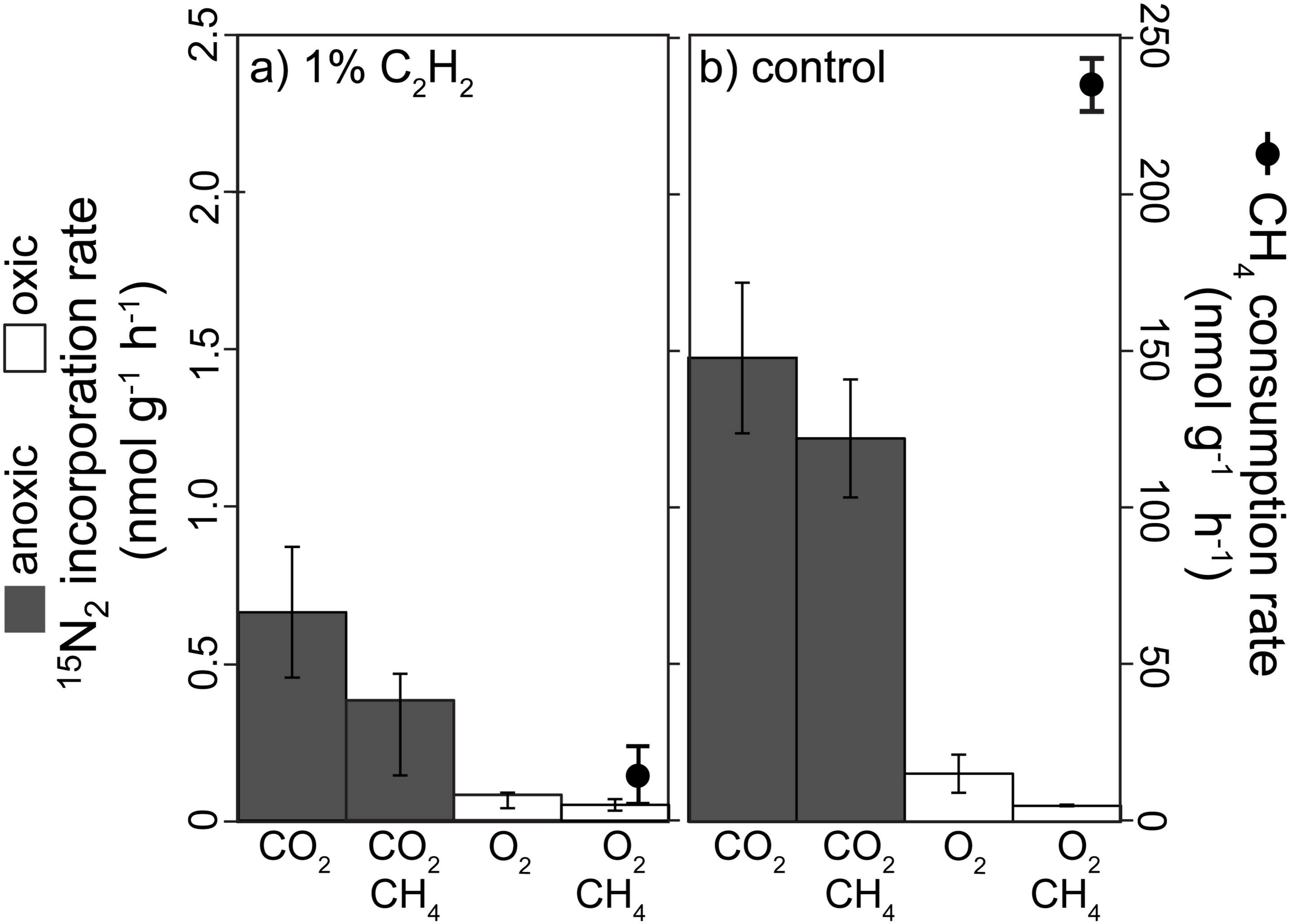

● NH_4^+ (μM)
0
5
10
15
20
0
25
50
75
Depth (cm)
surface
deep
10^6
10^7
10^8
10^9
□ genes
△ transcripts
nifH (copies g^{-1})


Dissolved metal (nM)
0
10
20
30
T3 site
cm deep
0-30
30-50
100-150
far
mid
far
mid
far
mid
far
I
II
III
IV
Molybdenum
Vanadium


Dissolved metal (nM)
0
10
20
30
40
1000
2000
3000
4000
cm deep
T3 site
0-30
mid
far
30-50
mid
far
100-150
mid
far
June
Sept
mmoX
pmoA
Copper
Iron


Acetylene reduction rate
(µmol g-1 h-1)
200
150
100
50
0
CO2
CO2/CH4
CH4
O2
O2/CH4
Sphagnum fallax
Sphagnum angustifolium
b
c
B2*
D2
Hollows
Sphagnum
magellanicum
a
Hummocks
D2
B2*
90
91
92
93
94
95
96
Water content (%)


anoxic
oxic
$^{15}N_2$ incorporation rate (nmol g^{-1} h^{-1})
CH_4 consumption rate (nmol g^{-1} h^{-1})
a) 1% C_2H_2
b) control
0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
200
250
CO_2
CO_2 CH_4
O_2
O_2 CH_4