

Biological nitrogen fixation in peatlands

Saiz, Ernesto; Sgouridis, Fotis; Drifjhout, Falko; Ullah, Sami

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7

8 BIOLOGICAL NITROGEN FIXATION IN PEATLANDS: COMPARISON BETWEEN
9 ACETYLENE REDUCTION ASSAY AND $^{15}\text{N}_2$ ASSIMILATION METHODS

10 Ernesto Saiz^a, Fotis Sgouridis^b, Falko P. Drijfhout^c, and Sami Ullah^{d*}

11 ^a School of Geography, Geology, and the Environment, Keele University, Staffordshire, ST5
12 5BG, UK

13 ^b School of Geographical Sciences, University of Bristol, BS8 1SS, UK

14 ^c Chemical Ecology Group, School of Physical and Chemical Sciences, Keele University,
15 Staffordshire, ST5 5BG, UK

16 ^d School of Geography, Earth, and Environmental Sciences, and Birmingham Institute of
17 Forest Research, University of Birmingham, B15 2TT, UK

18 *Corresponding author: Sami Ullah (s.ullah@bham.ac.uk).

19

21 Biological Nitrogen Fixation (BNF) is an essential microbial process supplying available
22 nitrogen (N) to *Sphagnum* mosses in ombrotrophic peatlands. Acetylene Reduction Assay
23 (ARA) and the $^{15}\text{N}_2$ assimilation are the main methods used for the measurement of BNF.
24 ARA is used as a proxy where the moles of ethylene (C_2H_4) produced from acetylene (C_2H_2)
25 during incubation of mosses and peat are used to estimate the moles of N being fixed using a
26 conversion factor (CF), thus relating the moles of C_2H_4 produced to the moles of N fixed. A
27 theoretical CF of 3:1 originally developed for agricultural soils using pure nitrogenase
28 enzymes is in use; in some cases a site specific CF is determined through parallel incubation
29 of mosses and peat with ARA and $^{15}\text{N}_2$ assimilation methods to enable the application of
30 ARA for subsequent BNF measurement at high resolution and low cost. However, in recent
31 literature, the reported site and/or species specific CF for peatlands varies by an order of
32 magnitude, thus raising the question if measured CFs are robust and consistent enough for the
33 estimation of BNF in peatlands. Thus, we measured BNF using the ARA and the direct $^{15}\text{N}_2$
34 assimilation methods in three different peatlands across the UK during the growing season
35 over two years. The incubations were carried out in parallel on the dominant *Sphagnum* spp.
36 (*S. cuspidatum*, *S. fallax*, *S. capillifolium*, and *S. papillosum*) and top bulk peat (0-15 cm).
37 Additional incubations were performed using the direct $^{15}\text{N}_2$ assimilation method with and
38 without C_2H_2 addition to evaluate if C_2H_2 was suppressing N assimilation through BNF all
39 together in peatlands. According to the results, the CF varied from 0.001 to 5.363, with a
40 median CF of 0.028 for both mosses and peat, which is far lower than the theoretical 3:1 CF.
41 The CF was also highly variable with differences up to 3 orders of magnitude across the
42 different *Sphagnum* species. The measured CF between years for the same species and across
43 the three peatland sites varied significantly suggesting an inconsistent performance of ARA
44 against the ^{15}N assimilation method. The generally low but highly varied CFs measured under

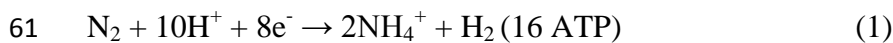
45 this study shows that C₂H₂ differentially interferes with the activity of diazotrophic microbes,
46 which results in an inconsistent CF at species, and site scales, and over time. In conclusion,
47 ARA is not suitable as a proxy method for estimating and/or modelling BNF in peatlands

48 Key words: Acetylene Reduction Assay, ARA, ¹⁵N₂ assimilation method, biological nitrogen
49 fixation, *Sphagnum*, diazotrophs, nitrogenase enzymes.

50

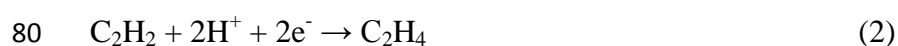
51 1. Introduction

52 Biological nitrogen fixation (BNF) is an essential microbial process for the provision of
53 available nitrogen (N) to plants in nutrient-poor ombrotrophic peatlands that otherwise rely
54 on atmospheric deposition of reactive N (Berg et al., 2013; Bragina et al., 2013).
55 Ombrotrophic peatlands are usually dominated by *Sphagnum* spp. (mosses), which are
56 adapted to acidic and nutrient-poor conditions (van den Elzen, et al., 2018). Moss-associated
57 cyanobacteria and free-living diazotrophic bacteria fix atmospheric N₂ into the bioavailable
58 NH₄⁺ form, thus enabling the plants to meet their N demands for capturing atmospheric
59 carbon (Postgate, 1982). Nitrogenase enzyme in the diazotrophs is responsible for reducing
60 N₂ into ammonium as below (Smith and Gallon, 1993):



62 There are two main methods for measuring BNF: direct ¹⁵N₂ assimilation (¹⁵N₂ method) and
63 acetylene reduction assay (ARA; Sprent, 1979; Bellenger et al., 2014). The ¹⁵N₂ method
64 allows for the direct quantification of BNF rates; however, the high cost of isotopic tracing is
65 prohibitive for a widespread repeated use. It involves the incubation of samples with ¹⁵N₂ gas,
66 for direct measurement of ¹⁵N incorporation into biomass by N fixers, followed by the
67 determination of ¹⁵N signature in the incubated samples through mass spectrometry (Zehr and
68 Montoya, 2007). Although it is a robust method, some practical problems have been reported
69 when using this method. For example incubation chamber leakage (Chalk et al., 2017),
70 oxygen depletion during long term incubation (over weeks) (Myrold et al., 1999), incomplete
71 and slow equilibration between the ¹⁵N₂ gas and the water sample (Großkopf et al., 2012),
72 and ¹⁵N₂ gas contamination (Dabundo et al., 2014) can result in under or overestimation of
73 BNF.

74 The ARA is the most common method for measuring BNF in different ecosystem types. It is
75 based on the nitrogenase enzyme preferential reduction of acetylene (C₂H₂) to ethylene
76 (C₂H₄) that involves just two electron transfer, instead of reducing N₂ which involves eight,
77 when C₂H₂ is present at relatively high concentrations (10% v/v; Koch and Evans, 1966;
78 Schöllhorn and Burris, 1967; Hardy et al., 1968), according to the following equation
79 (Bergersen, 1970; Staal et al., 2001):



81 Despite its simplicity ARA is an indirect method and a conversion factor (CF) is needed to
82 estimate BNF rate equivalents based on the number of moles of C₂H₄ produced. The
83 theoretical CF obtained from the formulas (1) and (2) relating the number of reducing
84 equivalents is 4:1 (moles of C₂H₄ produced per mole of nitrogen fixed) (Zehr and Montoya,
85 2007). Empirical measurements in *in vitro* experiments of nitrogen fixing bacteria
86 (*Azotobacter* and *Clostridium*) as well as *in situ* have found that the ratio of C₂H₄ produced to
87 N fixed was between 3 and 4.5 (Hardy et al., 1968). However, some authors consider that a
88 couple of electrons and protons are used to release one molecule of H₂ in equation 1, and
89 therefore, the theoretical CF should be 3:1, which is what has been traditionally used in the
90 soil literature (Postgate, 1982). Many authors have reported deviations from the theoretical
91 CF when measuring BNF in peatlands (Chapman and Hemond, 1982; Schwintzer, 1983), in
92 the laboratory with forest soil cores (Nohrstedt, 1983), or in different nitrogen-fixing systems
93 (Bergersen, 1970). As a result, these authors strongly recommended site specific calibration
94 of the ARA method using the ¹⁵N₂ method (Bergersen, 1970; Roskoski, 1981; Nohrstedt,
95 1983) for subsequent application of ARA at large scale over time.

96 Although there have been several studies relating ARA and the ¹⁵N₂ method in legumes and
97 laboratory cultures (Bergersen, 1970), in forests (Roskoski, 1981; Nohrstedt, 1983), and in

98 arctic habitats (Liengen, 1999), the effect of C₂H₂ on diazotrophic microbial activity in
99 peatlands and hence BNF has been overlooked. It is known that C₂H₂ interferes with different
100 microbial processes typical of peatlands such as nitrification, blocking it; denitrification,
101 inhibiting the respiratory reduction of N₂O to N₂ (Ryden, 1982); and methanotrophy,
102 inhibiting the oxidation of methane (Kip et al., 2010). These metabolic processes provide
103 energy to diazotrophs (Raghoebarsing et al., 2005) and substrate (for example, coupled
104 and/or direct respiratory N₂O reduction and N fixation; Desloover et al., 2014; Farias et al.,
105 2013), thus their inhibition or suppression in the presence of C₂H₂ might affect C₂H₄
106 production and hence estimation of BNF rates given that the presence of these microbes in
107 the incubated media (mosses and peat) may vary over time (Raghoebarsing et al., 2005). In
108 recent literature, it has been shown that methanotrophs play an important role in BNF
109 (Larmola et al., 2014; Vile et al., 2014), and that they are present in association of *Sphagnum*-
110 mosses other than cyanobacteria as a key N fixing microbe (Larmola et al., 2010) in
111 peatlands all over the world (Kip et al., 2010).

112 Many studies have applied site specific CFs in peatlands where *Sphagnum* spp. were present
113 such as 3.11 (Kox et al., 2016), 0.85 (Stewart et al., 2011) or 0.32 (Vile et al., 2014) for
114 mosses, and 1.1 (Knorr et al., 2015) for peat, albeit all of them reported high variability,
115 which raises the question if the site-specific CF is reliable and reproducible over time. In
116 other cases, ARA is applied for *Sphagnum* mosses using the theoretical 3:1 CF (Rousk et al.,
117 2018) or using a CF previously reported for the site (Rousk et al., 2015), whilst indicating
118 that BNF rates for the sites would be underestimated because of the inhibitory effects of C₂H₂
119 on methanotrophs. The only study that focused on the effects of ARA on BNF rates
120 estimation (Warren et al., 2017) was on peat soil, and therefore, no information exists on the
121 effect of C₂H₂ on diazotrophic microbes associated with *Sphagnum* mosses including
122 cyanobacteria and hence the usefulness of ARA as a proxy of BNF in peatlands.

123 Our main aim was to evaluate the usefulness of BNF method in peatlands. In this study, ARA
124 was calibrated against the $^{15}\text{N}_2$ assimilation method with the objectives to assess: (1) if the
125 conversion factor is consistent across *Sphagnum* species and peat at each site, across sites,
126 and across wider geographic temperate peatland regions, (2) if the CF is consistent over time,
127 and (3) if $^{15}\text{N}_2$ assimilation through BNF is completely suppressed in the presence of C_2H_2 .
128 Knowing the reported interference of C_2H_2 with microbial activities that have a direct and/or
129 indirect bearing on BNF activity such as methanotrophy, respiratory reduction of N_2O and
130 nitrification (Larmola et al., 2014; Desloover et al., 2014; Farias et al., 2013; Ho and
131 Bodelier, 2015; Sgouridis et al., 2016), we hypothesized that ARA as a proxy method will
132 underestimate BNF rates in peatlands.

133 2. Material and methods

134 2.1. Study sites and sampling

135 *Sphagnum* mosses and peat samples were collected from three ombrotrophic peatlands in the
136 UK: Migneint ($52^\circ 59' 20.8'' \text{ N} - 3^\circ 48' 09.8'' \text{ W}$) in Wales, Fenn's and Whixall ($52^\circ 55'$
137 $20.8'' \text{ N} - 2^\circ 45' 58.6'' \text{ W}$) in England, and Forsinard ($58^\circ 23' 42.2'' \text{ N} - 3^\circ 56' 47.0'' \text{ W}$) in
138 Scotland (Fig. 1). These sites had different characteristics regarding mean annual
139 temperature, rainfall, and atmospheric reactive nitrogen (Nr) deposition rates (Table 1) so that
140 the comparative performance of the two methods could then be representative at large
141 geographic scale.



142

143 *Figure 1. Location of the study sites in the United Kingdom.*

144 Moreover, the selected sites were exposed to variable atmospheric Nr deposition and the
145 rationale for the selection was also to evaluate the comparative performance of the two
146 techniques and to know if chronic Nr deposition might be affecting the CF given that a recent
147 paper reported suppression of BNF in feather mosses exposed to high Nr deposition
148 (Ackermann et al., 2012, Rousk and Michelsen, 2016). Two sampling campaigns were
149 undertaken at Migneint and Fenn's and Whixall sites during the growing season, in 2016 and
150 2017, respectively; and one campaign at Forsinard in 2017 for *in situ* method performance
151 incubations. Additionally, at Migneint site, samples were collected in spring 2016 for
152 laboratory-based CF determination.

153 The vegetation in these sites consisted of mosses and ericoid shrubs. For moss associated
154 BNF quantification, samples of four dominant *Sphagnum* species were collected at each site:
155 *Sphagnum cuspidatum* and *Sphagnum fallax* (most common in hollows); and *Sphagnum*
156 *papillosum* and *Sphagnum capillifolium* (in hummocks). During the 2016 campaigns, bulk

157 peat (0-15 cm) was also collected from hollows and hummocks, while in 2017 peat was
 158 collect only from hollows. *Sphagnum* and peat samples were collected from five random
 159 locations within each site to capture the wider inherent spatial variability of each site.

160 Table 1. Mean annual temperature, precipitation and reactive nitrogen (Nr) deposition
 161 in the study sites.

Site	Mean annual temperature (°C)	Mean annual precipitation (mm)	Atmospheric Nr deposition (kg N ha ⁻¹ yr ⁻¹)
Forsinard (Scotland)	6.9	1104	6
Migneint (Wales)	7.3	2236	17
Fenn's and Whixall (England)	9.5	747	26
Source: Met Office, Air Pollution Information System (APIS).			

162

163 2.2. ¹⁵N₂ assimilation method

164 For each species and peat four out of five replicates were incubated with ¹⁵N₂ (98 atom%
 165 Cambridge Isotope Laboratories Inc., USA), with the fifth being the control (incubated using
 166 ambient air). Each replicate consisted of 20 live moss shoots (~ upper 5 cm) of the selected
 167 moss species or 10 g of peat after passing it through a 2 mm sieve. Shoots and peat were
 168 placed in 50 ml serum vials which were capped with air tight rubber septa. Following the
 169 closure, 5 ml headspace air was drawn using gas tight syringe and replaced with 5 ml of the
 170 ¹⁵N₂ (98 atom%) gas (10% headspace concentration) and the bottles were then placed 'upside

171 down' (avoiding cap shade) in the same area from where the samples were collected and
172 incubated for 24 hours to avoid issues of oxygen depletion during long-term incubation
173 (Myrold et al., 1999). In case of peat, samples were placed under the moss carpet (dark
174 conditions). Parallel incubations were run for ARA (details below). Additionally, in order to
175 control some of the main factors affecting BNF (temperature, light), one set of moss and peat
176 samples from Migneint were incubated under laboratory conditions with the temperature set
177 at 20 °C (± 2) and the light/dark cycle of 12 hour, while maintaining light intensity through
178 artificial light (photosynthetically active radiation of $\sim 2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) to determine CF
179 under optimal laboratory conditions for comparison with field-based incubations.

180 After the 24 hour incubation, the vials were opened and aerated to flush out any remaining
181 $^{15}\text{N}_2$ gas and bring it to ambient conditions. Immediately after aeration, the samples were
182 transported in a cool box to the laboratory and then weighed and dried at 70 °C for 72 hours.
183 Dried samples were manually pulverised ($< 2 \text{ mm}$) and subsamples ($\sim 1 \text{ g}$) were sent to the
184 Life Sciences Mass Spectrometry Facility at the Centre for Ecology and Hydrology,
185 Lancaster for ^{15}N content analysis by Isotope Ratio Mass Spectrometry (IRMS) using a Carlo
186 Erba NA1500 (Italy) elemental analyser coupled to a Dennis Leigh Technologies (UK)
187 isotope ratio mass spectrometer. In-house working standards of natural abundance (flour and
188 soil) were analysed every twelfth sample giving an analytical precision of 0.36 ‰. They were
189 calibrated against the certified reference material International Atomic Energy Agency standard
190 AEA-N1 (nitrogen isotope in ammonium sulphate). All the control and enriched moss and
191 peat samples were analysed in duplicate (Jardine and Cunjak, 2005) and their variability was
192 within the limits of the analytical precision of the IRMS. We also analysed duplicates of three
193 non-enriched tissues samples on the IRMS and the resulting reproducibility of the analysis
194 including a cross-laboratory check (details in section 2.3 below) was within the analytical
195 precision of the IRMS. Subsequently, we only ran one control and four enriched incubations

196 during BNF measurements while ensuring that the duplicate runs of each sample on the
197 IRMS was within the analytical precision limits. This check was critical given that the
198 experiment relied on the incubation of one non-enriched sample in the field for calculating
199 BNF rates using the following formula (Liengen, 1999):

$$Y = \left(\frac{\text{atom}\% \text{ } ^{15}\text{N}_{\text{excess}}}{100} \right) \times \left(\frac{\text{totalN}_{\text{sample}} \times 10^9}{t \times 28} \right) \times \left(\frac{100}{\% \text{ } ^{15}\text{N}_{\text{headspace}}} \right)$$

200

201 where Y ($\text{nmol N g}^{-1} \text{ dw h}^{-1}$) is the amount of N fixed during the experiment, $\text{atom}\%$
202 $^{15}\text{N}_{\text{excess}}$ is the difference between $\text{atom}\% \text{ } ^{15}\text{N}_{\text{sample}}$ and $\text{atom}\% \text{ } ^{15}\text{N}_{\text{control}}$, total N is the
203 total amount of nitrogen in the sample ($\text{g N } 100 \text{ g}^{-1} \text{ dw}$), t is the incubation time, 28 is the
204 molecular weight of N_2 (g mol^{-1}), and $\% \text{ } ^{15}\text{N}$ is the percentage of ^{15}N out of the total amount
205 of N gas in each incubation bottle.

206

207 2.3. $^{15}\text{N}_2$ Gas quality control

208 Contamination of commercial $^{15}\text{N}_2$ gas with ^{15}N -labelled nitrate and ammonium can interfere
209 with the detection of BNF (Dabundo et al., 2014). We evaluated the potential contamination
210 of the $^{15}\text{N}_2$ gas as well as the possibility of abiotic uptake of $^{15}\text{N}_2$ gas by incubating six
211 samples of dried ($105 \text{ }^\circ\text{C}$) mosses for 24 hours, three with $^{15}\text{N}_2$ enriched gas as above and
212 three without. After the incubation, the samples were processed as above and were sent to
213 two different laboratories (CEH Lancaster and Bristol University) for ^{15}N analysis using
214 IRMS to ensure cross laboratory checks (Bahlmann et al., 2010). The results obtained
215 (average $\delta \text{ } ^{15}\text{N}$ in sample of enriched ones: -0.360 ; and of non-enriched ones: -0.387) showed
216 a difference of $-0.03 \delta \text{ } ^{15}\text{N}$ between the treatments. Therefore, we used this averaged

217 difference ($-0.03 \delta^{15}\text{N}$) as a threshold below which any difference between the control and
218 enriched samples incubated for direct BNF measurement was not considered.

219

220 *2.4. Acetylene reduction assay (ARA)*

221 Following the placement of 20 shoots of mosses or 10 g of field moist peat in serum bottles
222 ($n = 5$) and capping with septa, 10 % of the headspace was replaced with (10 % v/v) pure and
223 fresh C_2H_2 obtained by adding deionised water to calcium carbide. Immediately after doing
224 so, a 3 ml gas sample was obtained (T_0), and replaced by the same gas mixture to maintain
225 atmospheric pressure within the vials. Gas samples were subsequently collected following the
226 same procedure at 6 and 24 hours.

227 To check for possible contamination of the acetylene gas with ethylene and endogenous
228 production of ethylene by mosses and peat, we carried out quality control incubations.
229 Mosses and peat were incubated with and without the addition of C_2H_2 (each with three
230 replicates), whereas three bottles received C_2H_2 but no sample and three bottles were
231 incubated under ambient air without sample and C_2H_2 . The results showed no endogenous
232 C_2H_4 production or gas contamination, and negligible level of C_2H_4 in air was detected which
233 was later used for background correction while calculating ethylene production rates.

234 The gas samples were analysed for C_2H_4 concentration using a gas chromatograph (Varian
235 39000) equipped with a Restek-Alumina BOND/MAPD column (30 mm x 0.53 mm x10 μm)
236 and a flame ionization detector (FID) using He as a carrier gas. The temperatures of the
237 injector and detector were 200 °C, and for the column was 135 °C. The head pressure was 3.4
238 psi and the carrier flow 3.2 ml min^{-1} . The injection was manual. C_2H_4 production rates were
239 calculated by linear regression between time intervals T_0 - T_{24} using a standard calibration

240 curve for each of the daily batch samples. Using standards injection after 10 samples each,
241 the quality of the runs were checked and where needed, corrected for any drift in the signal.

242 2.5. ARA – $^{15}\text{N}_2$ direct assimilation conversion factor (CF ratio)

243 The ARA conversion factor was calculated by dividing moles of C_2H_4 produced (ARA
244 method) by the moles of N fixed ($^{15}\text{N}_2$ direct assimilation) for each parallel incubation for
245 different species and peat collected from the Migneint site and incubated under laboratory
246 conditions (Vile et al. 2014) in 2016. Following the CF determination under laboratory
247 conditions, CFs were then estimated for the field-based incubations for the Fenn's and
248 Whixall (2016-17), Migneint (2016-17), and Forsinard (2017) sites. CFs were calculated per
249 site as well as per species and peat type within each site.

250 2.6. BNF determination with $^{15}\text{N}_2$ assimilation method with and without C_2H_2 addition

251 In 2017, during the growing season, samples (*Sphagnum* mosses and peat) collected from
252 Fenn's and Whixall, Migneint, and Forsinard sites, were incubated for BNF measurement
253 using the $^{15}\text{N}_2$ assimilation method as described above, where 3 replicates were further
254 amended with C_2H_2 (10 % v/v) and 3 without to evaluate if the presence of C_2H_2 will
255 completely inhibit N_2 reduction to NH_4^+ by diazotrophs given that under high C_2H_2
256 concentration, diazotrophs have been shown to preferentially reduce C_2H_2 than reducing N_2
257 (Koch and Evans, 1966; Schöllhorn and Burris, 1967).

258 2.7. Statistical analysis

259 Data were tested for normality and homogeneity of variance and since they were found non-
260 normal and non-homogeneous, only non-parametric statistical tests were applied. To test
261 differences in paired samples, we used bootstrapped t-test. Differences in C_2H_4 production
262 (ARA) and BNF ($^{15}\text{N}_2$ method) and the effect of C_2H_2 on BNF rates were tested using the

263 Wilcoxon ranked sum test. To evaluate the effect of the site and the species we used the
264 Kruskal-Wallis test. All the analysis was performed on the IBM SPSS Statistics program,
265 version 24.

266

267 **3. Results**

268

269 *3.1. Conversion factors*

270

271 The CFs obtained showed great variability across species, sites and time ranging from 0.001
272 to 5.363 moles of C₂H₄ produced per mol of N fixed (Table 2). The mean (\pm standard
273 deviation) obtained for all the species and sites was 0.45 ± 2.373 , while the median (\pm median
274 absolute deviation) is 0.028 ± 0.022 (see supplementary Table S1) which is far lower than the
275 theoretical ratio 3:1. Across *Sphagnum* species within sites and between years, the CF varied
276 orders of magnitude (Table 2). CF values in peat also differed substantially between
277 laboratory-based, and *in situ* incubations in 2016 and 2017 at the Migneint site, while in case
278 of Fenn's and Whixall and Forsinard the data were <LOD except in once instance (Table 2).
279 Overall, the available data suggest high variability in CF values of peat between sites and
280 years (Table S1). In case of *S. cuspidatum* we observed extreme CF values during the
281 laboratory incubations in 2016 from the Migneint site, *in situ* incubation at the Migneint site
282 in 2016, and *in situ* incubations at the Forsinard site in 2017, which resulted in variations up
283 to three orders of magnitude. Exclusion of these extreme CF values still resulted in
284 significant differences where the median CFs by species and year and between lab-based and
285 *in situ* incubations exhibited variations of up to two orders of magnitude (see supplementary
286 Table S2).

287 Table 2. Conversion factors obtained as the ratio between C₂H₄ production measured by the ARA method divided by the N fixed using the ¹⁵N₂
 288 method. Data shown for species (rows) is the median (± median absolute deviation - MAD) of the three replicates (four for Migneint and Fenn's
 289 and Whixall 2016). Mean conversion factors (± standard deviation) for the *Sphagnum* species and peat are also shown. LOD: under the limit of
 290 detection. ND: no data.

Site	Lab incubations Migneint	Migneint		Fenn's & Whixall		Forsinard	Median of the three sites for the different species (±MAD)
Name / Year	2016	2016	2017	2016	2017	2017	2017
<i>S. cuspidatum</i>	0.457 (±0.071)	5.363 (±1.740)	0.056 (±0.014)	0.002 (±0.001)	0.002 (±0.001)	0.114 (±0.003)	0.056 (±0.054)
<i>S. capillifolium</i>	0.18 (±0.087) ⁴	0.025 (±0.011)	0.010 (±0.0002)	0.035 (±0.005)	0.002 (±0.001)	0.010 (±)	0.010 (±0.0002)
<i>S. fallax</i>	0.053 (±0.035)	0.047 (±0.020)	0.036 (±0.004)	0.033 (±)	0.005 (±0.002)	0.008 (±0.008)	0.008 (±0.003)
<i>S. papillosum</i>	0.015 (±0.015)	0.014 (±0.008)	0.043 (±0.003)	0.010 (±0.003)	0.031 (±0.020)	0.094 (±0.026)	0.043 (±0.012)
Median (±MAD)	0.095 (±0.084)	0.045 (±0.036)	0.039 (±0.010)	0.012 (±0.011)	0.005 (±0.003)	0.081 (±0.060)	0.026 (±0.018)

Mean (\pmSD)	0.205 (\pm 0.252)	1.989 (\pm 5.334)	0.091 (\pm 0.191)	0.018 (\pm 0.016)	0.010 (\pm 0.015)	0.203 (\pm 0.323)	0.089 (\pm 0.089)		
Peat hollows	0.001 (\pm 0.0001)	0.011 (\pm 0.007)	LOD	LOD	LOD	LOD			
Peat hummocks	0.005 (\pm 0.004)	0.027 (\pm 0.022)	ND	0.020 (\pm 0.013)	ND	ND			
Median (\pmMAD)	0.001 (\pm 0.0002)	0.018 (\pm 0.010)		0.020 (\pm 0.013)					
Mean (\pmSD)	0.004 (\pm 0.005)	0.019 (\pm 0.019)		0.020 (\pm 0.018)					

291

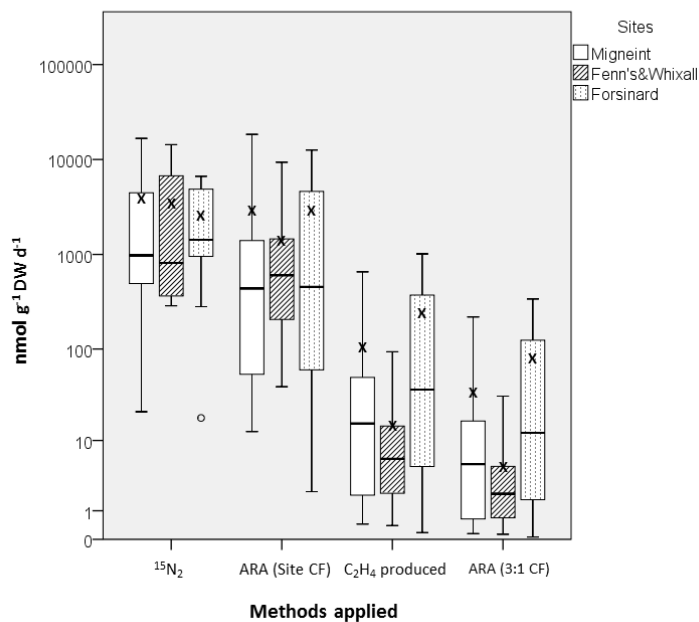
292 BNF rates measured by the $^{15}\text{N}_2$ method and the ARA method showed significant differences.
293 The median ARA rates of mosses and peat per sites in 2017 estimated by applying the
294 theoretical CF of 3:1 for the C_2H_4 produced during parallel incubations, were significantly
295 lower (Fig. 2; C_2H_4 produced also shown) than the direct BNF rates measured based on the
296 $^{15}\text{N}_2$ assimilation method. The difference between the median BNF rates based on ARA and
297 ^{15}N assimilation measurements was more prominent for the Fenn's and Whixall site (ARA
298 being four hundred times lower than $^{15}\text{N}_2$ method). Moreover, by applying the median field-
299 based CF obtained for each of the sites, the BNF rates obtained by the ARA method were
300 also relatively lower than the ones obtained using the $^{15}\text{N}_2$ assimilation method (Fig. 2),
301 which suggests an important underestimation of BNF by even applying the species, peat and
302 site specific CFs values that we measured.

303 BNF measured by the $^{15}\text{N}_2$ assimilation method was higher than the ARA (C_2H_4 produced, no
304 CF applied) for each *Sphagnum* species in each site, albeit the difference varied widely
305 among species (Fig.3). At Migneint the ^{15}N assimilation rate was 20 (*S. cuspidatum*) to 98 (*S.*
306 *capillifolium*) times larger than the C_2H_4 production; at Fenn's and Whixall it was 54 (*S.*
307 *papillosum*) to 271 (*S. cuspidatum*) times larger; and at Forsinard it was 7 (*S. capillifolium*) to
308 50 (*S. fallax*) times. Note that no detectable fixation was found in peat. This large range in the
309 ratio of ^{15}N fixed to C_2H_4 produced signified lack of consistency among species and sites.

310 3.2. BNF by $^{15}\text{N}_2$ method with and without C_2H_2

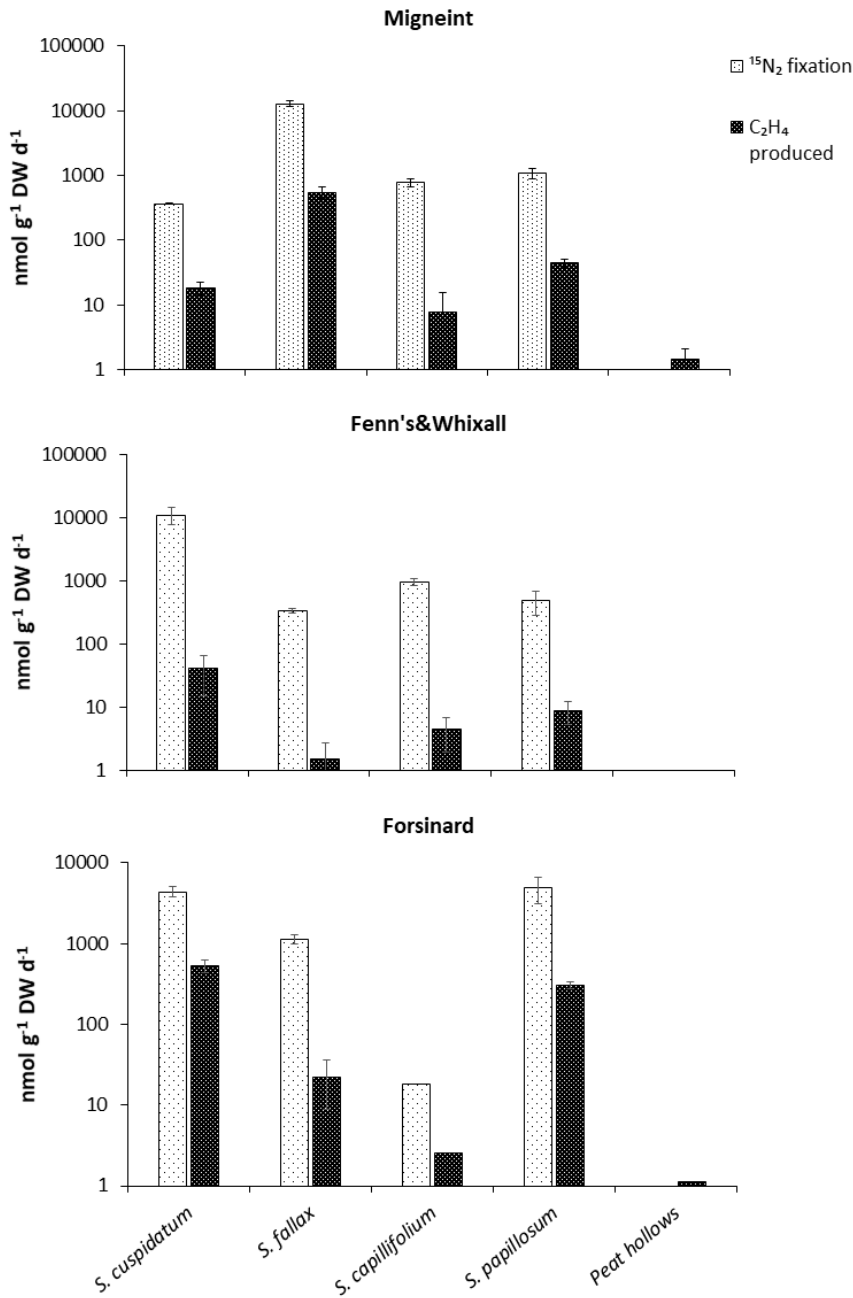
311 C_2H_2 failed to completely inhibit N_2 reduction to NH_4^+ using the ^{15}N uptake as a direct
312 evidence (Fig. 4). The partial suppression of BNF by the C_2H_2 was also inconsistent across
313 the three sites compared to BNF rates in the absence of C_2H_2 . The relative percentage of BNF
314 suppression calculated at each site (Suppression % = $[(\text{BNF with } \text{C}_2\text{H}_2 - \text{BNF without } \text{C}_2\text{H}_2)$

315 / BNF without C_2H_2] * 100) based on the mean was 74% at Migneint, 87% at Fenn's and
 316 Whixall, and 99% at Forsinard. However, based on the median, we found that in Migneint
 317 there was no suppression but a relative enhancement of 6%, whilst in Fenn's and Whixall, the
 318 relative percentage of BNF suppression was 64% and in Forsinard 99%, showing a marked
 319 variability. The differences in the percentage of BNF suppression among species within each
 320 site also varied substantially (ranging from none to complete suppression and even
 321 enhancements) showing an inconsistent suppression pattern (Fig. S1).



322

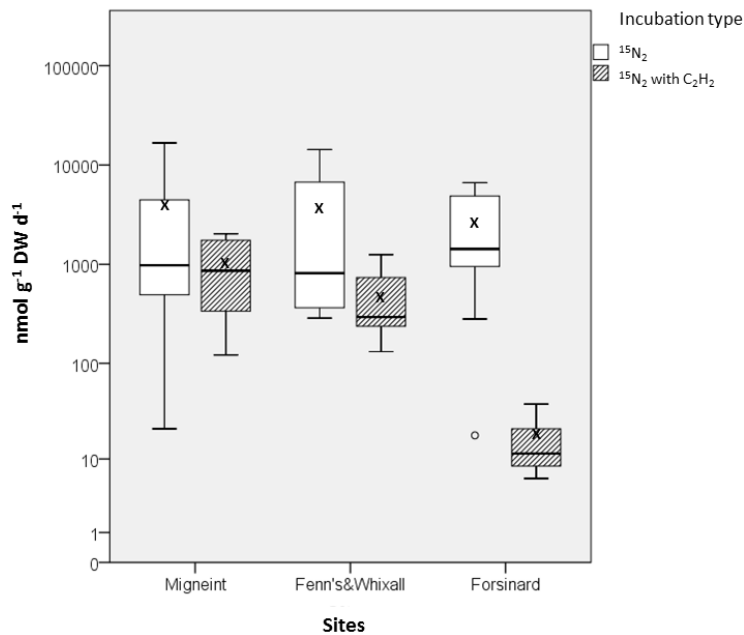
323 *Figure 2. Rates of C_2H_4 produced ($C_2H_4\ nmol\ g^{-1}\ DW\ d^{-1}$), BNF rates estimated ($nmol\ N\ g^{-1}\ DW\ d^{-1}$)*
 324 *using the theoretical 3:1 CF of ARA method, BNF rates estimated ($nmol\ N\ g^{-1}\ DW\ d^{-1}$)*
 325 *using site specific CFs (0.039 for Migneint; 0.005 for Fenn's & Whixall; and 0.081 for*
 326 *Forsinard), and direct BNF rates measured using $^{15}N_2$ assimilation method ($nmol\ N\ g^{-1}\ DW\ d^{-1}$)*
 327 *at each site in 2017. The box shows 25th, 50th (central line) and 75th percentile with*
 328 *whiskers showing the min and maximum values. The x sign show mean value and the dot an*
 329 *outlier (<math><1.5\ IQR</math>). Note the log-scale y-axis (n=15).*



Sphagnum species and peat

330

331 *Figure 3. Estimated C₂H₄ produced (C₂H₄ nmol g⁻¹ DW d⁻¹) using ARA method and direct*
 332 *BNF measurements (nmol N g⁻¹ DW d⁻¹) using ¹⁵N₂ assimilation method, for each of the*
 333 *Sphagnum species and peat within each site in 2017. Shown are the median values, ± median*
 334 *absolute deviation (n=3). No bars mean no N₂ fixation or no C₂H₂ reduction detected due to*
 335 *values being <LOD. Note the different log-scale y-axis.*



336

337 *Figure 4. Estimated BNF rates (nmol N g⁻¹ DW d⁻¹) in Sphagnum mosses and peat, by site,*
 338 *in 2017, using the ¹⁵N₂ assimilation method with and without C₂H₂ addition. The box shows*
 339 *25th, 50th (central line) and 75th percentile with whiskers showing the min and maximum*
 340 *values. The x sign show the mean value and the dot an outlier (<1.5 IQR). Note the log-*
 341 *scale y-axis (n=15).*

342 4. Discussion

343 Both for *Sphagnum* spp. and peat, the rates of C₂H₄ produced were lower than the direct
 344 assimilation of N determined through the ¹⁵N₂ assimilation method. This resulted in
 345 extremely low species-specific CFs (Table 2) signifying potentially serious underestimation
 346 of BNF rates by the ARA method, particularly in *Sphagnum* dominated peatlands. Hardy et
 347 al. (1968) reported CF ratios between 3 and 4.5 after empirical measurements of BNF activity
 348 in bacterial cultures and nitrogenase enzyme preparations as well as in free-living bacteria
 349 using parallel ARA and ¹⁵N₂ direct assimilation methods. Following this publication, the
 350 estimated theoretical CF of 3:1 has been applied in various ecosystems including peatlands
 351 (Basilier, 1979; Markham, 2009; Rousk et al., 2018). However, many studies have reported
 352 greater CFs than the theoretical one ranging from 3.11 to 4.5 for peat and *Sphagnum* spp.
 353 together (Basilier, 1980; Chapman and Hemond, 1982; Urban and Eisenreich, 1988; Kox et

354 al., 2016; Warren et al., 2017). One potential plausible explanation for the discrepancies over
355 the theoretical CF was the possibility of significant endogenous C₂H₄ production in
356 subsurface peat (Schwintzer, 1983; Kox et al., 2016). Conversely, lower CFs than the
357 theoretical CF have also been reported for *Sphagnum* spp. such as 2.48 (Sorensen et al.,
358 2006), 0.85 (Stewart et al., 2011), 0.32 (Vile et al., 2014), or *Sphagnum* peat 1.1 (Knorr et al.,
359 2015), as well as for bryophytes 0.25 (Menge and Hedin, 2009). Therefore the reported site
360 specific CF of peatlands show marked deviations from the theoretical CF and this is
361 consistent with the range of CF measured under this study, albeit we have gone further in
362 estimating CFs per species and in different peatlands across an Nr deposition gradient. High
363 rates of Nr deposition did not shut down BNF and we did not detect any effect on the
364 comparative performance of the methods and the resulting calculation of the CFs. The high
365 variability of these measured CFs suggests that the *Sphagnum* microbiome and its species-
366 specific distribution (Kostka et al., 2016), as well as the inhibitory effects of C₂H₂ on
367 microbial processes such as methanotrophy, nitrification and nitrous oxide reduction may be
368 at play, thus leading to highly inconsistent CFs across species, sites and time. We speculate
369 that such a differential effect could be responsible for the extreme CF values estimated in
370 case of *S. cuspidatum* (Lab incubations Migneint 2016, Migneint 2016) and hence as a *hot*
371 *spot* of biogeochemical processes (McClaine et al., 2003).

372 The variability in the measured CFs in this study is further substantiated by the fact that the
373 presence of C₂H₂ differentially affected the suppression of N₂ fixation, but did not completely
374 suppress it across the sites as demonstrated under pure in vitro incubations of nitrogenase
375 enzymes in the presence of C₂H₂ (Koch and Evans, 1966; Schöllhorn and Burris, 1967). This
376 differential suppression response under the ARA must have led to the variable CF ratios we
377 estimated in this study. It appears that the diversity of diazotrophic communities from
378 autotrophic cyanobacteria to chemolithotrophic methanotrophs associated with *Sphagnum*

379 mosses in peatlands may have been affected differentially by C_2H_2 with varied C_2H_4
380 production across species, sites and time. The CF is estimated to save resources for
381 widespread application of ARA in peatlands; however, the difference in CFs at species and
382 site level and over time is not consistent and thus we recommend the use of ^{15}N assimilation
383 method for measuring BNF in peatlands.

384 The very low rates of C_2H_4 production by the ARA in our study could be explained by the
385 presence of methanotrophs and the inhibitory effects of C_2H_2 on the methane monooxygenase
386 enzyme in these bacteria; thus depriving the methanotrophs of a significant bacterial energy
387 to fuel BNF (Flett et al., 1975; De Bont and Mulder, 1976). Widespread presence of
388 methanotrophs associated with *Sphagnum* species has been established (Basiliko et al., 2004;
389 Raghoebarsing et al., 2005; Kip et al., 2010; Larmola et al., 2010). Furthermore, Larmola et
390 al. (2014) and Vile et al. (2014) have demonstrated that methanotrophs can contribute up to
391 40% to total BNF in peatlands, therefore the use of the ARA method could underestimate the
392 BNF rates up to a similar percentage and even more, as shown by our study (~53% on
393 average), which is in agreement with a peat BNF study (~55%) in Minnesota, USA (Warren
394 et al., 2017). C_2H_2 also shuts down nitrification, and stops the reduction of N_2O to N_2 (Ryden,
395 1982). The inhibition of these processes, particularly of nitrification, leads to an increase in
396 plant available nitrogen (NH_4^+) that may in turn limit biological nitrogen fixation (Stewart et
397 al. 2013) particularly in ecosystems such as peatlands with a tightly coupled N cycle such as
398 peatlands. Moreover, inhibition of N_2O reduction which could potentially provide energy
399 and substrate for BNF, might also be a factor in affecting C_2H_4 production rates and hence
400 low and inconsistent CF in the end as observed in this study. For example, Farias et al. (2013)
401 and Desloover et al. (2014) reported respiratory reduction of N_2O to N_2 and its subsequent
402 fixation by diazotrophs in pure bacterial cultures and thus inhibition of N_2O reduction to N_2
403 by C_2H_2 in peatlands might be affecting N fixation rates.

404 High microbial diversity has been found in *Sphagnum* species between different habitats (e.g.
405 hummocks vs hollows) within peatlands (Opelt et al., 2007). Two main functional groups of
406 bacteria have been studied in *Sphagnum* mosses, nitrogen-fixers and methane-oxidizers
407 (some of which are able to fix nitrogen as well; Auman et al., 2001), and important
408 differences in the community diversity of these two types of bacteria between *Sphagnum*
409 species have been reported (Bragina et al., 2013). This microbial community diversity could
410 potentially explain the high variability of the CF between *Sphagnum* spp. observed in our
411 study, due to the differential presence of these kinds of bacteria, and the different level of
412 interference in their associated microbiological processes in the presence of C₂H₂. Further
413 microbiological studies are recommended to verify the net inhibitory impact against the
414 abundance and expression of N fixers, methanotrophs, nitrifiers and N₂O reducers.

415 4.1. Conclusions

416 The conversion factors measured under this study using the direct ¹⁵N assimilation and ARA
417 methods were inconsistent across species, site, and peat and over time. This lack of
418 reproducibility and deviation from the theoretical CF of 3:1 show that ARA as a proxy
419 method cannot fully reflect the BNF activity and hence fixation rates in peatlands. This lack
420 of consistency and partial, yet differential suppression of N₂ fixation in the presence of C₂H₂
421 led to lower CF values and hence underestimation of BNF. Direct interference and/or
422 inhibition of microbes, particularly methanotrophs seem to result in the differential
423 suppression of N₂ fixation. Therefore, caution is needed when estimating and modelling BNF
424 rates based on the ARA method in peatlands. We conclude that the ARA method is not
425 suitable for BNF measurement in *Sphagnum*-dominated peatlands.

426

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435 **References**

- 436 Ackermann, K., Zackrisson, O., Rousk, J., Jones, D.L., DeLuca, T.H. 2012. N₂ fixation in
437 feather mosses is a sensitive indicator of N deposition in boreal forests. *Ecosystems* 15,
438 986-998.
- 439 Auman, A., Speake, C., Lidstrom, M., 2001. nifH sequences and nitrogen fixation in type I
440 and type II methanotrophs. *Applied and Environmental Microbiology* 67, 4009-4016.
- 441 Bahlmann, E., Bernasconi, S.M., Bouillon, S., Houtekamer, M., Korntheuer, M., Langenberg,
442 F., Mayr, C., Metzke, M., Middelburg, J.J., Nagel, B., Struck, U., Voß, M., Emeis, K.C.,
443 2010. Performance evaluation of nitrogen isotope ratio determination in marine and
444 lacustrine sediments: an inter-laboratory comparison. *Organic Geochemistry* 41, 3–12.
- 445 Basilier, K., 1979. Moss-associated nitrogen fixation in some mire and coniferous forest
446 environments around Uppsala, Sweden. *Lindbergia* 5, 84-88.
- 447 Basilier, K. 1980. Fixation and Uptake of Nitrogen in *Sphagnum* Blue-Green-Algal
448 Associations. *Oikos* 34, 239-242.

449 Basiliko, N., Knowles, R., Moore, T., 2004. Roles of moss species and habitat in methane
450 consumption potential in a northern peatland. *Wetlands* 24, 178-185.

451 Bellenger, J.P., Xu, Y., Zhang, X., Morel, F.M.M., Kraepiel, A.M.L., 2014. Possible
452 contribution of alternative nitrogenases to nitrogen fixation by asymbiotic N₂-fixing
453 bacteria in soils. *Soil Biology & Biochemistry* 69, 413-420.

454 Berg, A., Danielsson, Å., Svensson, B., 2013. Transfer of fixed-N from N-fixing
455 cyanobacteria associated with the moss *Sphagnum riparium* results in enhanced growth
456 of the moss. *Plant and Soil* 362, 271-278.

457 Bergersen F. J., 1970. The quantitative relationship between nitrogen fixation and the
458 acetylene-reduction assay. *Australian Journal of Biological Sciences* 23, 1015-1025.

459 Bragina, A., Berg, C., Mueller, H., Moser, D., Berg, G., 2013. Insights into functional
460 bacterial diversity and its effects on Alpine bog ecosystem functioning. *Scientific*
461 *Reports* 3, 1955.

462 Chalk, P.M., He, J.Z., Peoples, M.B., Chen, D., 2017. ¹⁵N₂ as a tracer of biological N₂
463 fixation: A 75-year retrospective. *Soil Biology & Biogeochemistry* 106, 36-50.

464 Chapman, R.R., Hemond, H.F., 1982. Dinitrogen Fixation by Surface Peat and *Sphagnum* in
465 an Ombrotrophic Bog. *Canadian Journal of Botany-Revue Canadienne De Botanique* 60,
466 538-543.

467 Dabundo, R., Lehmann, M.F., Treibergs, L., Tobias, C.R., Altabet, M.A., Moisander, P.H.,
468 Granger, J., 2014. The Contamination of Commercial N-15(2) Gas Stocks with N-15-
469 Labeled Nitrate and Ammonium and Consequences for Nitrogen Fixation
470 Measurements. *PLoS ONE* 9, e110335.

471 De Bont, J.A., Mulder, E.G., 1976. Invalidity of the acetylene reduction assay in alkane-
472 utilizing, nitrogen-fixing bacteria. *Applied and Environmental Microbiology* 31, 640-
473 647.

474 Desloover, J., Roobroeck, D., Heylen, K., Puig, S., Boeckx, P., Verstraete, W., Boon, N.,
475 2014. Pathway of nitrous oxide consumption in isolated *Pseudomonas stutzeri* strains
476 under anoxic and oxic conditions. *Environmental Microbiology* 16, 3143-3152.

477 Farias, L., Faundez, J., Fernandez, C., Cornejo, M., Sanhueza, S., Carrasco, C. (2013).
478 Biological N₂O fixation in the eastern south Pacific Ocean and marine cyanobacterial
479 cultures. *PLoS ONE* 8, UNSP e63956.

480 Flett, R., Rudd, J., Hamilton, R., 1975. Acetylene-Reduction Assays for Nitrogen-Fixation in
481 Freshwaters - Note of Caution. *Applied Microbiology* 29, 580-583.

482 Grosskopf, T., Mohr, W., Baustian, T., Schunck, H., Gill, D., Kuypers, M.M.M., Lavik, G.,
483 Schmitz, R.A., Wallace, D.W.R., LaRoche, J., 2012. Doubling of marine dinitrogen-
484 fixation rates based on direct measurements. *Nature* 488, 361–4.

485 Hardy, R.W.F., Holsten, R.D., Jackson, E.K., Burns, R.C., 1968. The acetylene–ethylene
486 assay for N₂ fixation: laboratory and field evaluation. *Plant Physiology* 43, 1185–1207.

487 Ho, A., Bodelier, P.L.E., 2015. Diazotrophic methanotrophs in peatlands: the missing link?
488 *Plant and Soil* 389, 419-423.

489 Jardine, T., Cunjak, R., 2005. Analytical error in stable isotope ecology. *Oecologia* 144, 528–
490 533.

491 Kip, N., van Winden, J.F., Pan, Y., Bodrossy, L., Reichart, G., Smolders, A.J.P., Jetten,
492 M.S.M., Damste, J.S.S., Op den Camp, H.J.M., 2010. Global prevalence of methane
493 oxidation by symbiotic bacteria in peat-moss ecosystems. *Nature Geoscience* 3, 617-621.

494 Knorr, K., Horn, M.A., Borken, W., 2015. Significant nonsymbiotic nitrogen fixation in
495 Patagonian ombrotrophic bogs. *Global Change Biology* 21, 2357-2365.

496 Koch, B., Evans, H.J., 1966. Reduction of acetylene to ethylene by soybean root nodules.
497 *Plant Physiology* 41, 1748-49.

498 Kostka, J.E., Weston, D.J., Glass, J.B., Lilleskov, E.A., Shaw, A.J., Turetsky, M.R., 2016.
499 The *Sphagnum* microbiome: new insights from an ancient plant lineage. *New*
500 *Phytologist* 211, 57-64.

501 Kox, M.A.R., Luke, C., Fritz, C., van den Elzen, E., van Alen, T., Op den Camp, H.J.M.,
502 Lamers, L.P.M., Jetten, M.S.M., Ettwig, K.F., 2016. Effects of nitrogen fertilization on
503 diazotrophic activity of microorganisms associated with *Sphagnum magellanicum*. *Plant*
504 *and Soil* 406, 83-100.

505 Larmola, T., Leppanen, S.M., Tuittila, E., Aarva, M., Merila, P., Fritze, H., Tirola, M., 2014.
506 Methanotrophy induces nitrogen fixation during peatland development. *Proceedings of*
507 *the National Academy of Sciences of the United States of America* 111, 734-739.

508 Larmola, T., Tuittila, E., Tirola, M., Nykänen, H., Martikainen, P.J., Yrjälä, K., Tuomivirta,
509 T., Fritze, H., 2010. The role of *Sphagnum* mosses in the methane cycling of a boreal
510 mire. *Ecology* 91, 2356-2365.

511 Liengen, T., 1999. Conversion factor between acetylene reduction and nitrogen fixation in
512 free-living cyanobacteria from high arctic habitats. *Canadian Journal of Microbiology*
513 45, 223-229.

514 Markham, J.H., 2009. Variation in moss-associated nitrogen fixation in boreal stands.
515 *Oecologia* 161, 353–359.

516 McClain, M. E., Boyer, E. W., Dent, C. L., Gergel, S. E., Grimm, N. B., Groffman, P. M.,
517 Hart, S. C., Harvey, J. W., Johnston, C. A., Mayorga, E., McDowell, W. H., Pinay. G.,
518 2003. Biogeochemical hot spots and hot moments at the interface of terrestrial and
519 aquatic ecosystems. *Ecosystems* 6, 301-312.

520 Menge, D.N.L., Hedin, L.O., 2009. Nitrogen fixation in different biogeochemical niches
521 along a 120 000-year chronosequence in New Zealand. *Ecology* 90, 2190-2201.

522 Myrold, D.D., Ruess, W.R., Klug, M.J., 1999. Dinitrogen fixation. In: Robertson, G.P.,
523 Coleman, D.C., Bledsoe, C.S., Sollins, P. (Eds.), *Standard soil methods for long-term*
524 *ecological research*. Oxford University Press, Oxford, pp. 241-257.

525 Nohrstedt, H. 1983. Conversion factor between acetylene reduction and nitrogen-fixation in
526 soil: effect of water content and nitrogenase activity. *Soil Biology & Biochemistry* 15,
527 275-279.

528 Opelt, K., Berg, C., Schoenmann, S., Eberl, L., Berg, G., 2007. High specificity but
529 contrasting biodiversity of *Sphagnum*-associated bacterial and plant communities in bog
530 ecosystems independent of the geographical region. *ISME Journal* 1, 502-516.

531 Postgate, J.R., 1982. *The fundamentals of nitrogen fixation*, Cambridge University Press,
532 Cambridge.

533 Raghoebarsing, A., Smolders, A., Schmid, M., Rijpstra, W., Wolters-Arts, M., Derksen, J.,
534 Jetten, M., Schouten, S., Damste, J., Lamers, L., Roelofs, J., den Camp, H., Strous, M.,
535 2005. Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. *Nature*
536 436, 1153-1156.

537 Roskoski, J., 1981. Comparative C₂H₂ reduction and ¹⁵N₂ fixation in deciduous wood litter.
538 *Soil Biology & Biochemistry* 13, 83-85.

539 Rousk, K., Michelsen, A., 2016. The sensitivity of moss-associated nitrogen fixation towards
540 repeated nitrogen input. *PLoS ONE* 11, e0146655.

541 Rousk, K., Sorensen, P.L., Lett, S., Michelsen, A., 2015. Across-Habitat Comparison of
542 Diazotroph Activity in the Subarctic. *Microbial Ecology* 69, 778-787.

543 Rousk, K., Vestergard, M., Christensen, S., 2018. Are nitrous oxide emissions and nitrogen
544 fixation linked in temperate bogs? *Soil Biology & Biochemistry* 123, 74-79.

545 Ryden, J. 1982. Effects of Acetylene on Nitrification and Denitrification in 2 Soils during
546 Incubation with Ammonium-Nitrate. *Journal of Soil Science* 33, 263-270.

547 Schöllhorn, R., Burris, R.H., 1967. Acetylene as a competitive inhibitor of N₂ fixation.
548 *Proceedings of the National Academy of Sciences of the United States of America* 58,
549 213-16.

550 Schwintzer, C.R., 1983. Non-Symbiotic and Symbiotic Nitrogen-Fixation in a Weakly
551 Minerotrophic Peatland. *American Journal of Botany* 70, 1071-1078.

552 Sgouridis, F., Stott, A., Ullah, S., 2016. Application of the ¹⁵N gas-flux method for measuring
553 in situ N₂ and N₂O fluxes due to denitrification in natural and semi-natural terrestrial

554 ecosystems and comparison with the acetylene inhibition technique. *Biogeosciences* 13,
555 1821-1835.

556 Smith, R.J., Gallon, J.R., 1993. Nitrogen fixation. In Lea, P.J. & Leegood, R.C.(Eds), *Plant*
557 *biochemistry and molecular biology*, John Wiley & Sons, Chichester, pp. 129-153.

558 Sorensen, P.L., Jonasson S., Michelsen A., 2006. Nitrogen fixation, denitrification, and
559 ecosystem nitrogen pools in relation to vegetation development in the subarctic. *Arctic*
560 *Antarctic and Alpine Research* 38, 263–272.

561 Sprent, J.I., 1979. *The biology of nitrogen-fixing organisms*, McGraw-Hill, London.

562 Staal, M., Lintel-Hekkert, S., Harren, F., Stal, L., 2001. Nitrogenase activity in cyanobacteria
563 measured by the acetylene reduction assay: a comparison between batch incubation and
564 on-line monitoring. *Environmental Microbiology* 3, 343-351.

565 Stewart, K.J., Coxson, D., Grogan, P., 2011. Nitrogen Inputs by Associative Cyanobacteria
566 across a Low Arctic Tundra Landscape. *Arctic Antarctic and Alpine Research* 43, 267-
567 278.

568 Stewart, K.J., Brummell, M.E., Coxson, D.S., Siciliano, S.D., 2013. How is nitrogen fixation
569 in the high arctic linked to greenhouse gas emissions? *Plant and Soil* 362, 215–229.

570 Urban N., Eisenreich, S., 1988. Nitrogen cycling in a forested Minnesota bog. *Canadian*
571 *Journal of Botany* 66, 435– 449.

572 van den Elzen, E., van den Berg, L.J.L., van der Weijden, B., Fritz, C., Sheppard, L.J.,
573 Lamers, L.P.M., 2018. Effects of airborne ammonium and nitrate pollution strongly

574 differ in peat bogs, but symbiotic nitrogen fixation remains unaffected. *Science of the*
575 *Total Environment* 610, 732-740.

576 Vile, M., Kelman Wieder, R., Živkovic, T., Scott, K., Vitt, D., Hartsock, J., Iosue, C., Quinn,
577 J., Petix, M., Fillingim, H., Popma, J., Dynarski, K., Jackman, T., Albright, C., Wykoff,
578 D., 2014. N-fixation by methanotrophs sustains carbon and nitrogen accumulation in
579 pristine peatlands. *Biogeochemistry* 121, 317-328.

580 Warren, M.J., Lin, X., Gaby, J.C., Kretz, C.B., Kolton, M., Morton, P.L., Pett-Ridge, J.,
581 Weston, D.J., Schadt, C.W., Kostka, J.E., Glass, J.B., 2017. Molybdenum-Based
582 Diazotrophy in a *Sphagnum* Peatland in Northern Minnesota. *Applied and*
583 *Environmental Microbiology* 83, UNSP e01174.

584 Zehr, J.P., Montoya, J.P., 2007. Measuring N₂ fixation in the field. In Bothe, H., Ferguson,
585 S.J., Newton, W.E. (Eds), *Biology of the nitrogen cycle*. Amsterdam, Netherlands:
586 Elsevier, pp. 193-205.

587