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BIOLOGICAL NITROGEN FIXATION IN PEATLANDS: COMPARISON BETWEEN ACETYLENE REDUCTION ASSAY AND $^{15}N_2$ ASSIMILATION METHODS

Ernesto Saiz[a], Fotis Sgouridis[b], Falko P. Drijfhout[c], and Sami Ullah[d*]

[a] School of Geography, Geology, and the Environment, Keele University, Staffordshire, ST5 5BG, UK

[b] School of Geographical Sciences, University of Bristol, BS8 1SS, UK

[c] Chemical Ecology Group, School of Physical and Chemical Sciences, Keele University, Staffordshire, ST5 5BG, UK

[d] School of Geography, Earth, and Environmental Sciences, and Birmingham Institute of Forest Research, University of Birmingham, B15 2TT, UK

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

Abstract

Biological Nitrogen Fixation (BNF) is an essential microbial process supplying available nitrogen (N) to *Sphagnum* mosses in ombrotrophic peatlands. Acetylene Reduction Assay (ARA) and the $^{15}N_2$ assimilation are the main methods used for the measurement of BNF. ARA is used as a proxy where the moles of ethylene (C_2H_4) produced from acetylene (C_2H_2) during incubation of mosses and peat are used to estimate the moles of N being fixed using a conversion factor (CF), thus relating the moles of C_2H_4 produced to the moles of N fixed. A theoretical CF of 3:1 originally developed for agricultural soils using pure nitrogenase enzymes is in use; in some cases a site specific CF is determined through parallel incubation of mosses and peat with ARA and $^{15}N_2$ assimilation methods to enable the application of ARA for subsequent BNF measurement at high resolution and low cost. However, in recent literature, the reported site and/or species specific CF for peatlands varies by an order of magnitude, thus raising the question if measured CFs are robust and consistent enough for the estimation of BNF in peatlands. Thus, we measured BNF using the ARA and the direct $^{15}N_2$ assimilation methods in three different peatlands across the UK during the growing season over two years. The incubations were carried out in parallel on the dominant *Sphagnum* spp. *(S. cuspidatum, S. fallax, S. capillifolium,* and *S. papillosum*) and top bulk peat (0-15 cm). Additional incubations were performed using the direct $^{15}N_2$ assimilation method with and without C_2H_2 addition to evaluate if C_2H_2 was supressing N assimilation through BNF all together in peatlands. According to the results, the CF varied from 0.001 to 5.363, with a median CF of 0.028 for both mosses and peat, which is far lower than the theoretical 3:1 CF. The CF was also highly variable with differences up to 3 orders of magnitude across the different *Sphagnum* species. The measured CF between years for the same species and across the three peatland sites varied significantly suggesting an inconsistent performance of ARA against the ^{15}N assimilation method. The generally low but highly varied CFs measured under

this study shows that C_2H_2 differentially interferes with the activity of diazotrophic microbes, which results in an inconsistent CF at species, and site scales, and over time. In conclusion, ARA is not suitable as a proxy method for estimating and/or modelling BNF in peatlands

Key words: Acetylene Reduction Assay, ARA, $^{15}N_2$ assimilation method, biological nitrogen fixation, *Sphagnum*, diazotrophs, nitrogenase enzymes.

1. Introduction

Biological nitrogen fixation (BNF) is an essential microbial process for the provision of available nitrogen (N) to plants in nutrient-poor ombrotrophic peatlands that otherwise rely on atmospheric deposition of reactive N (Berg et al., 2013; Bragina et al., 2013). Ombrotrophic peatlands are usually dominated by *Sphagnum* spp. (mosses), which are adapted to acidic and nutrient-poor conditions (van den Elzen, et al., 2018). Moss-associated cyanobacteria and free-living diazotrophic bacteria fix atmospheric N_2 into the bioavailable NH_4^+ form, thus enabling the plants to meet their N demands for capturing atmospheric carbon (Postgate, 1982). Nitrogenase enzyme in the diazotrophs is responsible for reducing N_2 into ammonium as below (Smith and Gallon, 1993):

$$N_2 + 10H^+ + 8e^- \rightarrow 2NH_4^+ + H_2\,(16\ ATP) \qquad (1)$$

There are two main methods for measuring BNF: direct $^{15}N_2$ assimilation ($^{15}N_2$ method) and acetylene reduction assay (ARA; Sprent, 1979; Bellenger et al., 2014). The $^{15}N_2$ method allows for the direct quantification of BNF rates; however, the high cost of isotopic tracing is prohibitive for a widespread repeated use. It involves the incubation of samples with $^{15}N_2$ gas, for direct measurement of ^{15}N incorporation into biomass by N fixers, followed by the determination of ^{15}N signature in the incubated samples through mass spectrometry (Zehr and Montoya, 2007). Although it is a robust method, some practical problems have been reported when using this method. For example incubation chamber leakage (Chalk et al., 2017), oxygen depletion during long term incubation (over weeks) (Myrold et al., 1999), incomplete and slow equilibration between the $^{15}N_2$ gas and the water sample (Großkopf et al., 2012), and $^{15}N_2$ gas contamination (Dabundo et al., 2014) can result in under or overestimation of BNF.

The ARA is the most common method for measuring BNF in different ecosystem types. It is based on the nitrogenase enzyme preferential reduction of acetylene (C_2H_2) to ethylene (C_2H_4) that involves just two electron transfer, instead of reducing N_2 which involves eight, when C_2H_2 is present at relatively high concentrations (10% v/v; Koch and Evans, 1966; Schöllhorn and Burris, 1967; Hardy et al., 1968), according to the following equation (Bergersen, 1970; Staal et al., 2001):

$$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4 \qquad (2)$$

Despite its simplicity ARA is an indirect method and a conversion factor (CF) is needed to estimate BNF rate equivalents based on the number of moles of C_2H_4 produced. The theoretical CF obtained from the formulas (1) and (2) relating the number of reducing equivalents is 4:1 (moles of C_2H_4 produced per mole of nitrogen fixed) (Zehr and Montoya, 2007). Empirical measurements in *in vitro* experiments of nitrogen fixing bacteria (*Azotobacter* and *Clostridium*) as well as *in situ* have found that the ratio of C_2H_4 produced to N fixed was between 3 and 4.5 (Hardy et al., 1968). However, some authors consider that a couple of electrons and protons are used to release one molecule of H_2 in equation 1, and therefore, the theoretical CF should be 3:1, which is what has been traditionally used in the soil literature (Postgate, 1982). Many authors have reported deviations from the theoretical CF when measuring BNF in peatlands (Chapman and Hemond, 1982; Schwintzer, 1983), in the laboratory with forest soil cores (Nohrstedt, 1983), or in different nitrogen-fixing systems (Bergersen, 1970). As a result, these authors strongly recommended site specific calibration of the ARA method using the $^{15}N_2$ method (Bergersen, 1970; Roskoski, 1981; Nohrstedt, 1983) for subsequent application of ARA at large scale over time.

Although there have been several studies relating ARA and the $^{15}N_2$ method in legumes and laboratory cultures (Bergersen, 1970), in forests (Roskoski, 1981; Nohrstedt, 1983), and in

arctic habitats (Liengen, 1999), the effect of C_2H_2 on diazotrophic microbial activity in peatlands and hence BNF has been overlooked. It is known that C_2H_2 interferes with different microbial processes typical of peatlands such as nitrification, blocking it; denitrification, inhibiting the respiratory reduction of N_2O to N_2 (Ryden, 1982); and methanotrophy, inhibiting the oxidation of methane (Kip et al., 2010). These metabolic processes provide energy to diazotrophs (Raghoebarsing et al., 2005) and substrate (for example, coupled and/or direct respiratory N_2O reduction and N fixation; Desloover et al., 2014; Farias et al., 2013), thus their inhibition or suppression in the presence of C_2H_2 might affect C_2H_4 production and hence estimation of BNF rates given that the presence of these microbes in the incubated media (mosses and peat) may vary over time (Raghoebarsing et al., 2005). In recent literature, it has been shown that methanotrophs play an important role in BNF (Larmola et al., 2014; Vile et al., 2014), and that they are present in association of *Sphagnum*-mosses other than cyanobacteria as a key N fixing microbe (Larmola et al., 2010) in peatlands all over the world (Kip et al., 2010).

Many studies have applied site specific CFs in peatlands where *Sphagnum* spp. were present such as 3.11 (Kox et al., 2016), 0.85 (Stewart et al., 2011) or 0.32 (Vile et al., 2014) for mosses, and 1.1 (Knorr et al., 2015) for peat, albeit all of them reported high variability, which raises the question if the site-specific CF is reliable and reproducible over time. In other cases, ARA is applied for *Sphagnum* mosses using the theoretical 3:1 CF (Rousk et al., 2018) or using a CF previously reported for the site (Rousk et al., 2015), whilst indicating that BNF rates for the sites would be underestimated because of the inhibitory effects of C_2H_2 on methanotrophs. The only study that focused on the effects of ARA on BNF rates estimation (Warren et al., 2017) was on peat soil, and therefore, no information exists on the effect of C_2H_2 on diazotrophic microbes associated with *Sphagnum* mosses including cyanobacteria and hence the usefulness of ARA as a proxy of BNF in peatlands.

Our main aim was to evaluate the usefulness of BNF method in peatlands. In this study, ARA was calibrated against the $^{15}N_2$ assimilation method with the objectives to assess: (1) if the conversion factor is consistent across *Sphagnum* species and peat at each site, across sites, and across wider geographic temperate peatland regions, (2) if the CF is consistent over time, and (3) if $^{15}N_2$ assimilation through BNF is completely suppressed in the presence of C_2H_2. Knowing the reported interference of C_2H_2 with microbial activities that have a direct and/or indirect bearing on BNF activity such as methanotrophy, respiratory reduction of N_2O and nitrification (Larmola et al., 2014; Desloover et al., 2014; Farias et al., 2013; Ho and Bodelier, 2015; Sgouridis et al., 2016), we hypothesized that ARA as a proxy method will underestimate BNF rates in peatlands.

2. Material and methods

2.1. Study sites and sampling

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

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

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

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

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

Table 1. Mean annual temperature, precipitation and reactive nitrogen (Nr) deposition 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).			

2.2. $^{15}N_2$ assimilation method

For each species and peat four out of five replicates were incubated with $^{15}N_2$ (98 atom% Cambridge Isotope Laboratories Inc., USA), with the fifth being the control (incubated using ambient air). Each replicate consisted of 20 live moss shoots (~ upper 5 cm) of the selected moss species or 10 g of peat after passing it through a 2 mm sieve. Shoots and peat were placed in 50 ml serum vials which were capped with air tight rubber septa. Following the closure, 5 ml headspace air was drawn using gas tight syringe and replaced with 5 ml of the $^{15}N_2$ (98 atom%) gas (10% headspace concentration) and the bottles were then placed 'upside

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

After the 24 hour incubation, the vials were opened and aerated to flush out any remaining $^{15}N_2$ gas and bring it to ambient conditions. Immediately after aeration, the samples were transported in a cool box to the laboratory and then weighed and dried at 70 ºC for 72 hours. Dried samples were manually pulverised (<2 mm) and subsamples (~ 1 g) were sent to the Life Sciences Mass Spectrometry Facility at the Centre for Ecology and Hydrology, Lancaster for ^{15}N content analysis by Isotope Ratio Mass Spectrometry (IRMS) using a Carlo Erba NA1500 (Italy) elemental analyser coupled to a Dennis Leigh Technologies (UK) isotope ratio mass spectrometer. In-house working standards of natural abundance (flour and soil) were analysed every twelfth sample giving an analytical precision of 0.36 ‰. They were calibrated against the certified reference material Internal Atomic Energy Agency standard AEA-N1 (nitrogen isotope in ammonium sulphate). All the control and enriched moss and peat samples were analysed in duplicate (Jardine and Cunjak, 2005) and their variability was within the limits of the analytical precision of the IRMS. We also analysed duplicates of three non-enriched tissues samples on the IRMS and the resulting reproducibility of the analysis including a cross-laboratory check (details in section 2.3 below) was within the analytical precision of the IRMS. Subsequently, we only ran one control and four enriched incubations

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

$$Y = \left(\frac{atom\%\ ^{15}Nexcess}{100}\right) x \left(\frac{totalNsample\ x\ 10^9}{t\ x\ 28}\right) x \left(\frac{100}{\%^{15}N\ headspace}\right)$$

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

2.3. $^{15}N_2$ Gas quality control

Contamination of commercial $^{15}N_2$ gas with ^{15}N-labelled nitrate and ammonium can interfere with the detection of BNF (Dabundo et al., 2014). We evaluated the potential contamination of the $^{15}N_2$ gas as well as the possibility of abiotic uptake of $^{15}N_2$ gas by incubating six samples of dried (105 ^oC) mosses for 24 hours, three with $^{15}N_2$ enriched gas as above and three without. After the incubation, the samples were processed as above and were sent to two different laboratories (CEH Lancaster and Bristol University) for ^{15}N analysis using IRMS to ensure cross laboratory checks (Bahlmann et al., 2010). The results obtained (average $\delta\ ^{15}N$ in sample of enriched ones: -0.360; and of non-enriched ones: -0.387) showed a difference of -0.03 $\delta\ ^{15}N$ between the treatments. Therefore, we used this averaged

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

2.4. Acetylene reduction assay (ARA)

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

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

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

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

2.5. ARA – $^{15}N_2$ direct assimilation conversion factor (CF ratio)

The ARA conversion factor was calculated by dividing moles of C_2H_4 produced (ARA method) by the moles of N fixed ($^{15}N_2$ direct assimilation) for each parallel incubation for different species and peat collected from the Migneint site and incubated under laboratory conditions (Vile at al. 2014) in 2016. Following the CF determination under laboratory conditions, CFs were then estimated for the field-based incubations for the Fenn's and Whixall (2016-17), Migneint (2016-17), and Forsinard (2017) sites. CFs were calculated per site as well as per species and peat type within each site.

2.6. BNF determination with $^{15}N_2$ assimilation method with and without C_2H_2 addition

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

2.7. Statistical analysis

Data were tested for normality and homogeneity of variance and since they were found non-normal and non-homogeneous, only non-parametric statistical tests were applied. To test differences in paired samples, we used bootstrapped t-test. Differences in C_2H_4 production (ARA) and BNF ($^{15}N_2$ method) and the effect of C_2H_2 on BNF rates were tested using the

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

3. Results

3.1. Conversion factors

The CFs obtained showed great variability across species, sites and time ranging from 0.001 to 5.363 moles of C_2H_4 produced per mol of N fixed (Table 2). The mean (± standard deviation) obtained for all the species and sites was 0.45 ± 2.373, while the median (± median absolute deviation) is 0.028 ± 0.022 (see supplementary Table S1) which is far lower than the theoretical ratio 3:1. Across *Sphagnum* species within sites and between years, the CF varied orders of magnitude (Table 2). CF values in peat also differed substantially between laboratory-based, and in situ incubations in 2016 and 2017 at the Migneint site, while in case of Fenn's and Whixall and Forsinard the data were <LOD except in once instance (Table 2). Overall, the available data suggest high variability in CF values of peat between sites and years (Table S1). In case of *S. cuspidatum* we observed extreme CF values during the laboratory incubations in 2016 from the Migneint site, *in situ* incubation at the Migneint site in 2016, and *in situ* incubations at the Forsinard site in 2017, which resulted in variations up to three orders of magnitude. Exclusion of these extreme CF values still resulted in significant differences where the median CFs by species and year and between lab-based and *in situ* incubations exhibited variations of up to two orders of magnitude (see supplementary Table S2).

Table 2. Conversion factors obtained as the ratio between C_2H_4 production measured by the ARA method divided by the N fixed using the $^{15}N_2$ method. Data shown for species (rows) is the median (± median absolute deviation - MAD) of the three replicates (four for Migneint and Fenn's and Whixall 2016). Mean conversion factors (± standard deviation) for the *Sphagnum* species and peat are also shown. LOD: under the limit of 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**
S. cuspidatum	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)
S. capillifolium	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)
S. fallax	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)
S. papillosum	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 (±SD)	0.205 (±0.252)	1.989 (±5.334)	0.091 (±0.191)	0.018 (±0.016)	0.010 (±0.015)	0.203 (±0.323)	0.089 (±0.089)
Peat hollows	0.001 (±0.0001)	0.011 (±0.007)	LOD	LOD	LOD	LOD	
Peat hummocks	0.005 (±0.004)	0.027 (±0.022)	ND	0.020 (±0.013)	ND	ND	
Median (±MAD)	0.001 (±0.0002)	0.018 (±0.010)		0.020 (±0.013)			
Mean (±SD)	0.004 (±0.005)	0.019 (±0.019)		0.020 (±0.018)			

BNF rates measured by the $^{15}N_2$ method and the ARA method showed significant differences. The median ARA rates of mosses and peat per sites in 2017 estimated by applying the theoretical CF of 3:1 for the C_2H_4 produced during parallel incubations, were significantly lower (Fig. 2; C_2H_4 produced also shown) than the direct BNF rates measured based on the $^{15}N_2$ assimilation method. The difference between the median BNF rates based on ARA and ^{15}N assimilation measurements was more prominent for the Fenn's and Whixall site (ARA being four hundred times lower than $^{15}N_2$ method). Moreover, by applying the median field-based CF obtained for each of the sites, the BNF rates obtained by the ARA method were also relatively lower than the ones obtained using the $^{15}N_2$ assimilation method (Fig. 2), which suggests an important underestimation of BNF by even applying the species, peat and site specific CFs values that we measured.

BNF measured by the $^{15}N_2$ assimilation method was higher than the ARA (C_2H_4 produced, no CF applied) for each *Sphagnum* species in each site, albeit the difference varied widely among species (Fig.3). At Migneint the ^{15}N assimilation rate was 20 (*S. cuspidatum*) to 98 (*S. capillifolium*) times larger than the C_2H_4 production; at Fenn's and Whixall it was 54 (*S. papillosum*) to 271 (*S. cuspidatum*) times larger; and at Forsinard it was 7 (*S. capillifolium*) to 50 (*S. fallax*) times. Note that no detectable fixation was found in peat. This large range in the ratio of ^{15}N fixed to C_2H_4 produced signified lack of consistency among species and sites.

3.2. BNF by $^{15}N_2$ method with and without C_2H_2

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

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

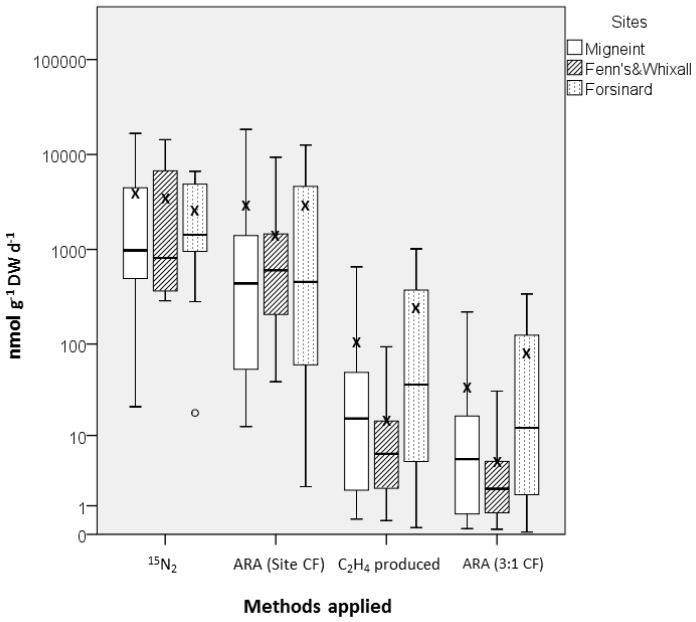
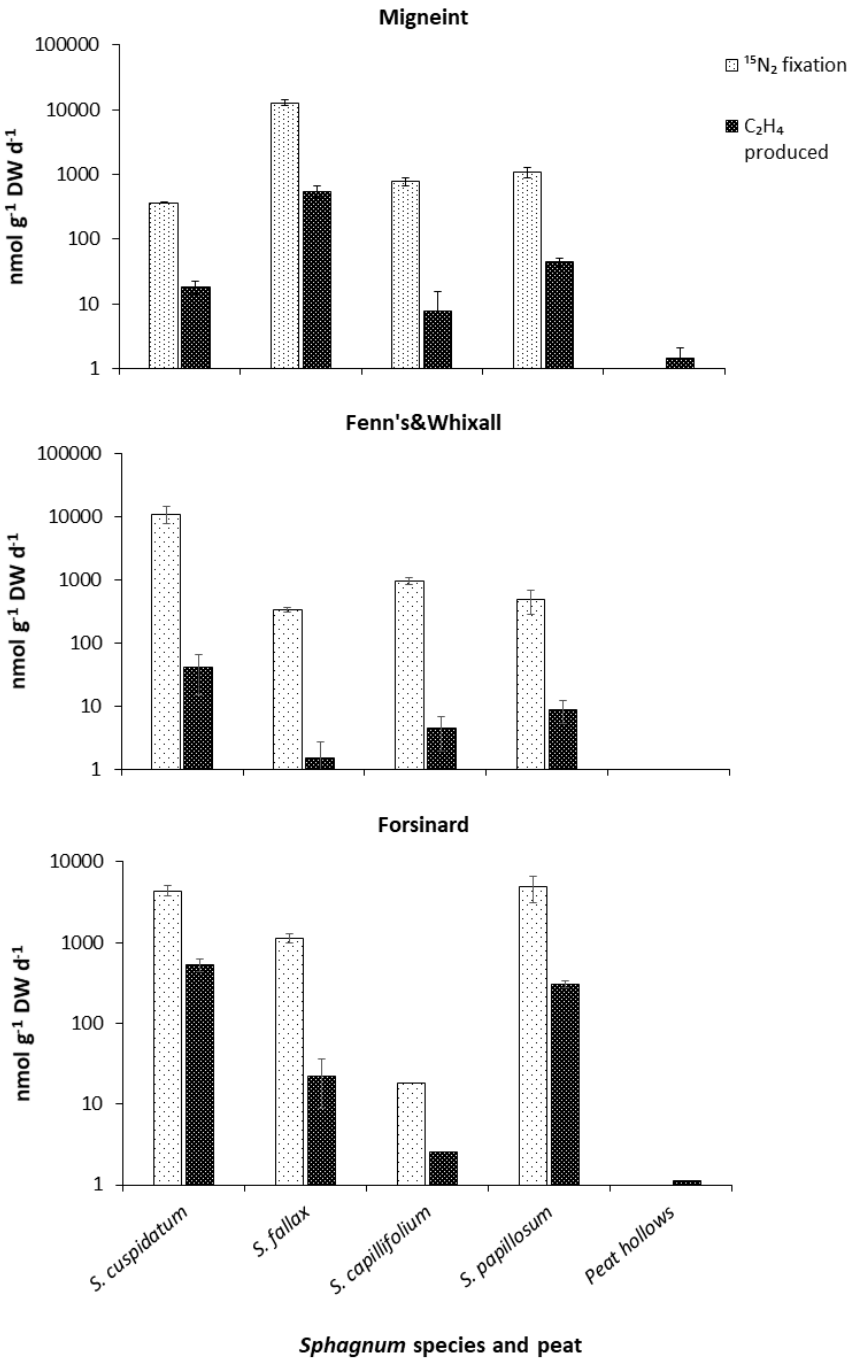


Figure 2. Rates of C_2H_4 produced (C_2H_4 nmol g^{-1} DW d^{-1}), BNF rates estimated (nmol N g^{-1} DW d^{-1}) using the theoretical 3:1 CF of ARA method, BNF rates estimated (nmol N g^{-1} DW d^{-1}) using site specific CFs (0.039 for Migneint; 0.005 for Fenn's & Whixall; and 0.081 for Forsinard),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



330

331 *Figure 3. Estimated C_2H_4 produced (C_2H_4 nmol g^{-1} DW d^{-1}) using ARA method and direct*
332 *BNF measurements (nmol N g^{-1} DW d^{-1}) using $^{15}N_2$ 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 N_2 fixation or no C_2H_2 reduction detected due to*
335 *values being <LOD. Note the different log-scale y-axis.*

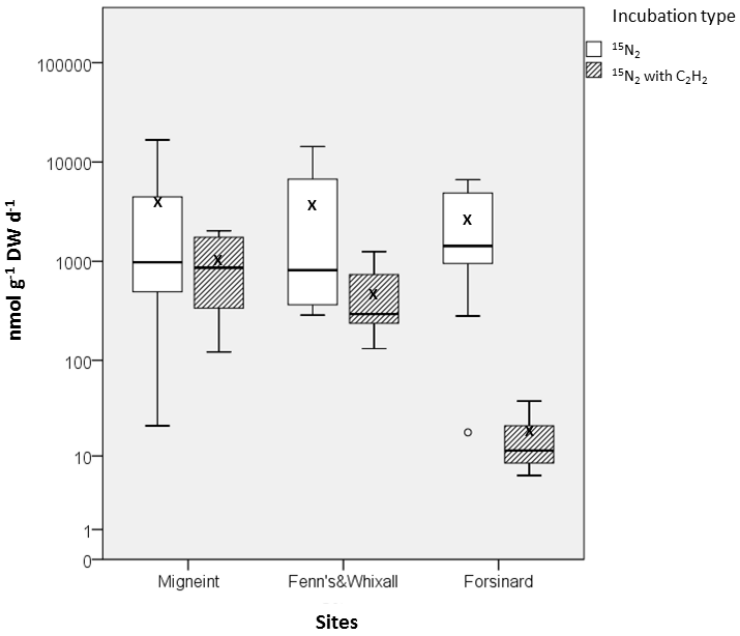


Figure 4. Estimated BNF rates (nmol N g^{-1} DW d^{-1}) in Sphagnum mosses and peat, by site, in 2017, using the $^{15}N_2$ assimilation method with and without C_2H_2 addition. The box shows 25th, 50th (central line) and 75th percentile with whiskers showing the min and maximum values. The x sign show the mean value and the dot an outlier (<1.5 IQR). Note the log-scale y-axis (n=15).

4. Discussion

Both for *Sphagnum* spp. and peat, the rates of C_2H_4 produced were lower than the direct assimilation of N determined through the $^{15}N_2$ assimilation method. This resulted in extremely low species-specific CFs (Table 2) signifying potentially serious underestimation of BNF rates by the ARA method, particularly in *Sphagnum* dominated peatlands. Hardy et al. (1968) reported CF ratios between 3 and 4.5 after empirical measurements of BNF activity in bacterial cultures and nitrogenase enzyme preparations as well as in free-living bacteria using parallel ARA and $^{15}N_2$ direct assimilation methods. Following this publication, the estimated theoretical CF of 3:1 has been applied in various ecosystems including peatlands (Basilier, 1979; Markham, 2009; Rousk et al., 2018). However, many studies have reported greater CFs than the theoretical one ranging from 3.11 to 4.5 for peat and *Sphagnum* spp. together (Basilier, 1980: Chapman and Hemond, 1982; Urban and Eisenreich, 1988; Kox et

al., 2016; Warren et al., 2017). One potential plausible explanation for the discrepancies over the theoretical CF was the possibility of significant endogenous C_2H_4 production in subsurface peat (Schwintzer, 1983; Kox et al., 2016). Conversely, lower CFs than the theoretical CF have also been reported for *Sphagnum* spp. such as 2.48 (Sorensen et al., 2006), 0.85 (Stewart et al., 2011), 0.32 (Vile et al., 2014), or *Sphagnum* peat 1.1 (Knorr et al., 2015), as well as for bryophytes 0.25 (Menge and Hedin, 2009). Therefore the reported site specific CF of peatlands show marked deviations from the theoretical CF and this is consistent with the range of CF measured under this study, albeit we have gone further in estimating CFs per species and in different peatlands across an Nr deposition gradient. High rates of Nr deposition did not shut down BNF and we did not detect any effect on the comparative performance of the methods and the resulting calculation of the CFs. The high variability of these measured CFs suggests that the *Sphagnum* microbiome and its species-specific distribution (Kostka et al., 2016), as well as the inhibitory effects of C_2H_2 on microbial processes such as methanotrophy, nitrification and nitrous oxide reduction may be at play, thus leading to highly inconsistent CFs across species, sites and time. We speculate that such a differential effect could be responsible for the extreme CF values estimated in case of *S. cuspidatum* (Lab incubations Migneint 2016, Migneint 2016) and hence as a *hot spot* of biogeochemical processes (McClaine et al., 2003).

The variability in the measured CFs in this study is further substantiated by the fact that the presence of C_2H_2 differentially affected the suppression of N_2 fixation, but did not completely suppress it across the sites as demonstrated under pure in vitro incubations of nitrogenase enzymes in the presence of C_2H_2 (Koch and Evans, 1966; Schöllhorn and Burris, 1967). This differential suppression response under the ARA must have led to the variable CF ratios we estimated in this study. It appears that the diversity of diazotrophic communities from 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 pealands 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.

High microbial diversity has been found in *Sphagnum* species between different habitats (e.g. hummocks vs hollows) within peatlands (Opelt et al., 2007). Two main functional groups of bacteria have been studied in *Sphagnum* mosses, nitrogen-fixers and methane-oxidizers (some of which are able to fix nitrogen as well; Auman et al., 2001), and important differences in the community diversity of these two types of bacteria between *Sphagnum* species have been reported (Bragina et al., 2013). This microbial community diversity could potentially explain the high variability of the CF between *Sphagnum* spp. observed in our study, due to the differential presence of these kinds of bacteria, and the different level of interference in their associated microbiological processes in the presence of C_2H_2. Further microbiological studies are recommended to verify the net inhibitory impact against the abundance and expression of N fixers, methanotrophs, nitrifiers and N_2O reducers.

4.1. Conclusions

The conversion factors measured under this study using the direct ^{15}N assimilation and ARA methods were inconsistent across species, site, and peat and over time. This lack of reproducibility and deviation from the theoretical CF of 3:1 show that ARA as a proxy method cannot fully reflect the BNF activity and hence fixation rates in peatlands. This lack of consistency and partial, yet differential suppression of N_2 fixation in the presence of C_2H_2 led to lower CF values and hence underestimation of BNF. Direct interference and/or inhibition of microbes, particularly methanotrophs seem to result in the differential suppression of N_2 fixation. Therefore, caution is needed when estimating and modelling BNF rates based on the ARA method in peatlands. We conclude that the ARA method is not suitable for BNF measurement in *Sphagnum*-dominated peatlands.

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