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Sgouridis, Fotis; Ullah, Sami

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Relative Magnitude and Controls of *in situ* N_2 and N_2O Fluxes due to Denitrification in Natural and Seminatural Terrestrial Ecosystems Using ^{15}N Tracers

***Fotis Sgouridis*[1†] *and Sami Ullah*[1*]**

[1]School of Physical and Geographical Sciences, Keele University, Staffordshire, UK.

[*]Corresponding author: Sami Ullah, School of Physical and Geographical Sciences, Keele University, Staffordshire, ST5 5BG. Tel. +44(0) 1782 733987. Email: s.ullah@keele.ac.uk

[†]Present Address: School of Geographical Sciences, University of Bristol, BS8 1SS, Bristol, UK. Email: f.sgouridis@bristol.ac.uk

Key words

N cycling, ^{15}N tracer, nitrous oxide, *in situ* measurements, denitrification, N_2 and N_2O emission, N_2O:N_2 ratio, UK catchments, Natural and semi-natural terrestrial ecosystems.

Abstract

Denitrification is the most uncertain component of the nitrogen (N) cycle, hampering our ability to assess its contribution to reactive N (Nr) removal. This uncertainty emanates from the difficulty in measuring *in situ* soil N_2 production and from the high spatio-temporal variability of the process itself. *In situ* denitrification was measured monthly between April 2013 and October 2014 in natural (organic and forest) and semi-natural ecosystems (semi-improved and improved grasslands) in two UK catchments. Using the ^{15}N-Gas Flux method with low additions of $^{15}NO_3^-$ tracer, a minimum detectable flux rate of 4 μg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for N_2 and N_2O, respectively was achieved. Denitrification rates were lower in organic and forest (8 and 10 kg N ha^{-1} y^{-1}, respectively) than in semi-improved and improved grassland soils (13 and 25 kg N ha^{-1} y^{-1}, respectively). The ratio of N_2O/ N_2 + N_2O was low and ranged from <1% to 7% across the sites. Variation in denitrification was driven by differences in soil respiration, nitrate, C:N ratio, bulk density, moisture and pH across the sties. Overall, the contribution of denitrification to Nr removal in natural ecosystems was ~50% of the annual atmospheric Nr deposition, making these ecosystems vulnerable to chronic N saturation.

Introduction

Human activities have more than doubled the inputs of reactive nitrogen (Nr: all nitrogen species apart from the inert N_2) mainly through fertiliser use and fossil fuel combustion in terrestrial ecosystems[1]. Compared to the multiple pathways of Nr creation, microbial denitrification, the sequential reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitrous oxide (N_2O) and dinitrogen (N_2) gases, [2] is the only major process responsible for the permanent removal of Nr.[3] While the input of Nr (particularly due to human sources) into terrestrial ecosystems is relatively well constrained, it is the process of denitrification which is the most uncertain component of the N cycle, thus hampering our ability to fully account for the pools and fluxes of N from regional to global scales.[3,4]

The uncertainty associated with quantifying the magnitude of denitrification in terrestrial ecosystems emanate mainly from two factors. One is the difficulty in accurately measuring soil N_2 production rates against the high background atmospheric N_2 concentration and another is the notoriously high spatio-temporal variability of denitrification.[3] Available methods for measuring the gaseous end products (N_2 and N_2O) of denitrification are limited and can be categorised into the direct flux and ^{15}N isotope tracer techniques.[5] The acetylene inhibition technique (AIT) is considered a direct flux technique; however, N_2O and N_2 flux quantification needs two separate incubations (with and without C_2H_2) and the robustness of estimates suffers from issues associated with the effectiveness of C_2H_2 to completely block the reduction of N_2O to N_2 in case of intact soil core incubations.[6] These limitations preclude the use of the AIT for more reliable measurements of *in situ* denitrification rates.[7] The gas-flow soil core method[8,9] is a direct flux technique that allows the simultaneous quantification of N_2 and N_2O flux from soil cores where the soil pore N_2 atmosphere is replaced by a

mixture of He/O_2. Although this method is very sensitive and can detect small changes in soil N_2 production, in most cases it does not constitute an *in situ* technique due to soil disturbance during core collection and the subsequent laboratory incubation. Moreover, the AIT and the gas-flow soil core techniques cannot discriminate between sources of N_2O, potentially leading to overestimation of denitrification product ratios ($N_2O/ N_2 + N_2O$).[10,11]

The ^{15}N Gas-Flux method[12] can simultaneously measure N_2 and N_2O production due to denitrification under *in situ* conditions with minimal disturbance to soils. A ^{15}N-labelled tracer is added to soil enclosed by a gas-tight chamber and the chamber headspace is progressively enriched with ^{15}N-N_2 and ^{15}N-N_2O gases produced by denitrification.[13] An important criticism of this method has been the artificial stimulation of denitrification by the added tracer, which has limited its use in highly fertilised agro-ecosystems.[13,14] However, recent advances in analytical instrumentation[15,16] and flux calculations[17] have shown that the limit of detection for N_2 flux determination can be lowered so that only low levels of added tracer (<10% of the soil NO_3^- pool) are needed, permitting the application of this method in natural terrestrial ecosystems under laboratory[16,18] and field conditions.[5] Therefore, these methodological advancements offer the opportunity for expanding the application of this method across a range of natural and semi-natural terrestrial ecosystems for the measurement of *in situ* denitrification rates with adequate spatio-temporal replication, which was not previously possible.

Natural and semi-natural terrestrial ecosystems in the UK (i.e. peatlands, heathlands, deciduous and coniferous forests), where there is no fertiliser use and the impact from grazing and commercial forestry is minimal,[19] along with improved and unimproved

grasslands constitute approximately 49 % and 85 % of rural land use cover in England and Wales, respectively.[20] These land use types have been poorly investigated for their role in Nr loss through denitrification. Moreover, current estimates of atmospheric Nr deposition rates (~ 15 to 25 kg N ha^{-1} y^{-1})[21,22] suggest an increased threat of chronic Nr enrichment of natural terrestrial ecosystems in the UK, with important consequences for shifts in vegetation composition and the export of excess Nr to water resources.[23,24] Global models, largely dependent on broad scale mass-balance approaches, estimate average basin denitrification rates for UK soils between 11 and 200 kg N ha^{-1} y^{-1} [25,26]. Given this large range in modelled denitrification rates and its high spatio-temporal variability from plot to the catchment scale,[3] further studies are needed to quantify *in situ* denitrification and its controls in order to validate and constrain catchment and/or regional scale denitrification models. [24,27]

The high spatio-temporal variability of soil denitrification is commonly attributed to the heterogeneity of the proximal regulators of the process, such as nitrate and organic carbon availability, concentration of soil oxygen but also distal factors such as soil pH and texture, topography and landscape position.[3,28] Land management practices (e.g. fertilisation, liming, grazing) affect both the proximal and distal regulators of denitrification[29,30] with consequences for the relative proportion of the denitrification end products.[29] Traditional grassland management has been associated with increased denitrification activity due to the additional supply of Nr through fertilisation[31] and C through manure application.[32] Forest soils, particularly those developed under poorly-drained conditions, sustain a relatively high denitrification activity due to anoxic conditions.[11,33] Peat soils under natural conditions are generally nutrient limited, which limits their denitrification potential,[34-36] while their response to increased atmospheric Nr deposition is unclear. Therefore, quantitative understanding of the links between denitrification and its associated controls are necessary for improving

predictive models that can produce robust extrapolations of field denitrification rates to catchment or regional scales.[3]

The objectives of the present study were to: (1) apply the ^{15}N Gas-Flux method for measuring *in situ* N_2 and N_2O fluxes due to denitrification across a range of natural and semi-natural ecosystems, (2) assess the environmental controlling factors of denitrification activity, and (3) estimate annual denitrification rates for validating a national scale model for the UK to be developed as part of a UK Natural Environment Research Council consortium project on macronutrient cycling.

Materials and methods

Study sites

Two river catchments in the UK, the Conwy (N. Wales, 52°59'82" N, 3°46'06" W) and the Ribble - Wyre (NW England, 53°59'99" N, 2°41'79" W) were selected for this study, where more than 90 % of land cover consists of natural and semi-natural rural land use types.[20] In the Conwy catchment, four study sites (C-PB = peat bog; C-UG = unimproved grassland; C-IG = improved grassland; C-MW = mixed woodland) were chosen (Figure 1a, Supporting Information). The C-PB and C-UG are under light grazing regime, less than one sheep per hectare, while the C-IG, characterised by seasonally waterlogged cambric stagnogley soils, is intensively grazed perennially by both sheep and cattle, while fertiliser (range: 100 – 200 kg N ha^{-1}) and manure are applied twice per year during spring and summer months. The C-MW (mature mixed forest currently unmanaged) is characterised by typical brown podzolic soils

146 that are shallow and well drained, while bare rock is locally visible and steep slopes are
147 common.

148

149 In the Ribble-Wyre catchment, four study sites (R-IG = improved grassland; R-UG =
150 unimproved grassland; R-HL = heathland; and R-DW = deciduous woodland) were selected
151 (Figure 1b, Supporting Information). The dominant soils in the area have been described as
152 stagnopodzols to stagnohumic gleys. The R-IG has land management practices analogous to
153 the ones described for the improved grassland in the Conwy catchment. The R-UG was
154 fertilised with N in the last decade once and has not been fertilised since, while it is being
155 mowed twice per year and is perennially grazed. The R-HL is managed as a grouse moor and
156 grazed by sheep at low densities. The R-DW is an old growth forest developed on poorly
157 drained soils and has never been fertilised. Further details on dominant plant species and soil
158 texture of the study sites can be found in Sgouridis & Ullah.[36]

159

160 Sampling strategy

161 *In situ* denitrification and N_2O emissions were measured monthly between April 2013 and
162 October 2014 with the exception of November 2013 and January 2014. Flux measurements
163 were made using static chambers according to the ^{15}N Gas-Flux method[12] as it was adapted
164 for application in natural and semi-natural land use types.[37] Five plots were randomly
165 established in each site within each catchment. In each plot a round PVC collar (basal area
166 0.05 m^2; chamber volume 4 L) was inserted into the soil at c. 10 cm depth 2 - 4 weeks before
167 the measurement. The PVC collars were fitted with a circular groove of 25 mm depth to fit in
168 a cylindrical acrylic cover (chamber) providing a gas-tight seal when filled with water.[38] For

each monthly measurement, labelled $K^{15}NO_3^-$ (98 at. % ^{15}N, Sigma-Aldrich) was applied in each plot at a mean rate between 0.03 (±SE 0.005) and 0.50 (±SE 0.073) kg N ha^{-1}. For the natural land use types, the average tracer application rate reflected current daily estimates of atmospheric Nr deposition in the UK (0.05 kg N ha^{-1} d^{-1}),[22] whilst for the grassland soils the tracer application mimicked a daily fertiliser application rate of 0.5 kg N ha^{-1} d^{-1}. The tracer solution (volume 50 - 250 mL, adjusted within 5 % of the ambient soil volumetric water content) was applied in the soil volume enclosed by the collar via multiple injections of equal volume through an equally-spaced grid using custom-made 10 cm long lumber needles attached to a syringe.[39]

Following the tracer application, the collars were covered with the chamber and wrapped with reflective foil for minimising temperature increase within the chamber headspace during incubation.[38] Two sets of gas samples (20 mL each) were collected with a gas tight syringe (SGE Analytical science) through a septum in the chamber at T = 1h, T = 2h and T ≈ 20h after the tracer injection, while a T = 0h sample was collected immediately after tracer injection above the plot surface before fitting the chamber. The gas samples were transferred into pre-evacuated (<100 Pa) 12 mL borosilicate glass vials (Exetainer vials) and were analysed within 8 weeks of collection.[40]

During the gas flux measurements, soil temperature and volumetric water content at 10 cm depth were recorded next to each chamber using a soil thermometer and a soil moisture probe (Hydrosense II, CS659, Campbell Scientific), respectively. Five composite soil samples (0-10 cm) were collected with a hand auger from each study site at the end of the incubation within 50 cm of each plot. The collars were moved to new random plots within each study site every

three months to minimise any priming effects from repeated tracer application in the same plots. The volume and concentration of the labelled $K^{15}NO_3^-$ tracer solution was determined from measurements of soil nitrate and moisture content, as well as bulk density adjacent to each plot made at the previous measurement campaign.[18]

Soil properties

The soil samples were analysed for dry bulk density, water filled pore space (WFPS), pH, soil moisture and organic matter contents according to established methods. The gravimetric soil moisture content was reported as per wet basis for comparison purposes between very moist organic and mesic mineral soils.[41] The experimental procedures for the analysis of soil nitrate, ammonium, dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) are described in Sgouridis & Ullah.[36] Soils sampled in July 2013 and in May 2014 were also analysed for total carbon (TC) and total nitrogen (TN) using an elemental analyser (Flash EA 1112, Thermo-Finnigan). CO_2 production rate, representing soil respiration,[42] was measured in the second set of gas samples collected from the static chambers on a GC-FID (7890A GC Agilent Technologies Ltd., Cheshire, UK) and flux rates were determined by linear regression between 0 and 20 hours and expressed as mg C m^{-2} h^{-1}, while the precision determined from repeated analyses of 200 ppm CO_2 standards (n = 8) was <1%.

N_2 and N_2O fluxes

The ^{15}N content of the N_2 in each 12 mL vial was determined by automated isotope ratio mass spectrometry (IRMS) using an Isoprime IRMS (Isoprime Ltd, UK). Gas samples (4 μL) were injected into the N_2 preparation unit manually using a gas tight syringe and the mass to

charge ratios for *m/z* 28, *m/z* 29 and *m/z* 30 ($^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ respectively) as well as the ratios R29 ($^{29}N_2/^{28}N_2$) and R30 ($^{30}N_2/^{28}N_2$) were measured in both enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). The minimum detectable change (MDC) in R29 and R30 was defined with repeated manual analyses of air reference standards (n=10) and was calculated using the following equation[43]:

$$MDC = \mu_{pair\ diff} + (2\sigma_{pair\ diff}) \quad (1)$$

where μ is the mean difference of all possible unique pairs of air reference standards (n=45) and σ is the standard deviation between sample pairs. The MDC for R29 was 7.7 x 10^{-7} and for R30 was 6.1 x 10^{-7} and these values were used to determine if each time step sample was significantly different from ambient reference samples (T=0 hours) and if not they were excluded from the flux calculations.

For calculating the total N_2 flux from a uniformly labelled soil nitrate pool the 'non-equilibrium' equations were applied as originally described by Mulvaney[44] and subsequently revised by Stevens & Laughlin[17] for lowering the detection limit for N_2[37]. The contribution of anammox and co-denitrification to N_2 fluxes was found negligible after comparison with the equations proposed by Spott & Stange [45] (data not shown). Therefore, the N_2 flux was calculated using linear regression between the maximum evolved N_2 and the incubation time per plot surface area and was expressed in μg N m^{-2} h^{-1} representing the total N_2 flux from the mixture of the ^{15}N-labelled tracer and the soil NO_3^- at natural abundance.[13]

The ^{15}N content of the N_2O in the same 12 mL vials was determined using an Isoprime IRMS (GV instruments Ltd) interfaced with a TraceGas™ Preconcentrator. The mass to charge

ratios for *m/z* 44, *m/z* 45 and *m/z* 46 ($^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ respectively) as well as the ratios R45 ($^{45}N_2O$ /$^{44}N_2O$) and R46 ($^{46}N_2O$ /$^{44}N_2O$) were measured in both enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). The application of the 'non-equilibrium' equations to N_2O is analogous to N_2 after correcting for the naturally occurring oxygen isotopes.[46] Therefore, the ratios R45 and R46 were converted to ratios of R29 and R30, respectively by applying the corrections as described by Bergsma et al.[46]. The MDC was defined, for the converted R29 and R30, with repeated analyses of 0.5 ppm N_2O standards (n=15) as 3.4×10^{-5} and 2.9×10^{-5}, respectively. The limit of detection for N_2 and N_2O fluxes depends on the precision of R29 and R30 determination, the enrichment of the soil nitrate pool, the dimensions of the static chamber and the incubation time.[17] For our chamber design, an incubation time of 20 hours, a soil nitrate pool enrichment of 60 ^{15}N at%, calculated using the estimated MDC values; the limit of detection was calculated at 4 μg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for the N_2 and N_2O fluxes, respectively. Approximately 69 % of the N_2 and 89 % of the N_2O fluxes were above the respective MDC values and were subsequently used in process rate calculations. Annual fluxes were estimated by interpolating monthly measurements for each year and calculating the average between the two monitoring years.[38]

Statistical analysis

All statistical analyses were performed using SPSS® 21.0 for Windows (see Supporting Information for further details).

Results

Soil properties

Factor Analysis (FA) of the soil physico-chemical variables across the 8 study sites (n = 680), identified two principal components (PC), which together explained 88.3 % of the total

variance within the data set (Figure 1). The soil moisture and organic matter contents correlated significantly ($p < 0.01$) with the negative axis of PC1, whilst the bulk density correlated with the positive axis of PC1, explaining 73.8 % of the observed variance in the overall data. Soil nitrate and pH correlated with both PC1 and PC2, of which the latter explained an additional 14.5 % of the variance in the dataset.

Based on the FA results, the 8 study sites were grouped into 5 distinct land use types groups. The land use type groups are represented by cluster centroids (average score on each PC1 and PC2 with standard errors) in Figure 1. The sites C-PB, C-UG and R-HL formed a distinct group hereafter called organic soils (OS) characterised by high soil organic matter and moisture contents and low bulk density, soil nitrate and pH (Table 1). Opposite from the OS, the sites C-IG and R-IG clustered together, forming the improved grassland (IG) land use type. IG is closely associated with the mixed woodland (MW) in the Conwy catchment, with both land use types characterised by higher bulk densities and higher nitrate content; however, the MW had significantly lower soil moisture and pH compared to the IG (Table 1). The site R-UG formed a separate land use type, named as semi-improved grassland (SIG), with intermediate soil properties between OS and IG. Finally, the deciduous woodland (DW) was positioned away from all the other land use types mainly due to the significantly higher soil pH, whilst it displayed similar soil moisture and nitrate content to the SIG.

Denitrification, N_2O emission and $N_2O/ (N_2 + N_2O)$ product ratio

In situ denitrification, as represented by the N_2 flux, in the two catchments ranged between 3 and 3000 μg N m^{-2} $h^{-1}$and was significantly influenced by land use type (ANOVA; $F = 13.8$, $df = 4$, $p < 0.001$) with the improved grasslands showing on average 3.5 times higher denitrification rates than the organic soils (Fig. 2a). Seasonal variation in denitrification was

286 evident in most land use types (see Supporting Information). The N_2O emission due to
287 denitrification (range: $2x10^{-4}$ - 117.5 μg N m^{-2} h^{-1}) significantly differed between land use
288 types (ANOVA; F = 226.1, df = 4, $p < 0.001$) with the improved grasslands emitting on
289 average 100 times more N_2O than the organic soils and four times more than the semi-
290 improved grassland (Fig. 2b). Monthly interpolated annual total denitrification rates
291 (including both N_2 and N_2O fluxes) averaged between the two monitoring years were highest
292 in IG (25 kg N ha^{-1} y^{-1}) followed by the SIG, MW, OS and DW with 13, 13, 8 and 7 kg N ha^{-1}
293 y^{-1}, respectively. Measuring N_2 and N_2O fluxes due to denitrification separately, allowed us
294 to estimate the denitrification product ratio of $N_2O/ (N_2 + N_2O)$ and compare it between land
295 use types. On average 7 % of the denitrification product in improved grasslands was nitrous
296 oxide and this percentage dropped between 2 and 3 % in DW and SIG, while it was < 1% in
297 OS and MW (ANOVA; F = 103.3, df = 4, $p < 0.001$) (Fig 2c).

298

299 Controlling factors

300 Significant positive correlations (Table 2) were found between *in situ* denitrification,
301 averaged per plot (n = 40), and soil respiration, bulk density, nitrate and pH, whilst the
302 relationship with WFPS and the C:N ratio was negative. This was due to the fact that
303 denitrification maxima were measured between WFPS 60-70 % in the grassland sites, whilst
304 higher WFPS in the OS land use types was associated with lower denitrification rates (Fig.
305 3a). Highest denitrification rates were measured between pH 5 and 6 (Fig. 3b), whereas a
306 linear gradient of soil respiration, bulk density, nitrate content and C:N ratio (Fig. 3) among
307 land use types was driving the relationship with denitrification. The combination of soil
308 respiration, nitrate content and pH explained 61 % of the variance in *in situ* denitrification
309 rates, when averaged per plot (n = 40), according to multiple linear regression analysis (r^2 =

0.61, p <0.001), whilst significant error due to heteroscedasticity negated a similar analysis for the N_2O emission and the ratio of $N_2O/ (N_2 + N_2O)$. Multiple linear regression (MLR) of monthly rates per land use type of log-transformed denitrification, N_2O emission and the $N_2O/ (N_2 + N_2O)$ ratio with selected physico-chemical variables revealed more varied and complex process controls within each land use (data presented in Supporting Information).

Discussion

^{15}N Gas-Flux method

We have adapted the ^{15}N Gas-Flux method[12] for quantifying N_2 and N_2O fluxes due to denitrification in natural and semi-natural terrestrial ecosystems by using low tracer application rates and extending the incubation time up to ~20 hours where needed, which is described in detail in a separate publication[37]. The minimum detectable flux rates were 4 μg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for the N_2 and N_2O fluxes respectively, which were significantly better than the minimum rates (175 μg N_2-N m^{-2} h^{-1} and 0.21 μg N_2O-N m^{-2} h^{-1}) reported by Kulkarni et al.,[5] using a similar field ^{15}N tracer approach, and comparable to the rates measured by a high precision ^{15}N analysis system in a laboratory incubation[16] and the gas-flow soil core method (8 μg N_2-N m^{-2} h^{-1} and < 1 μg N_2O-N m^{-2} h^{-1}) by Wang et al.[47] Thus, our adapted ^{15}N Gas-Flux method constitutes a significant improvement in quantifying *in situ* N_2 emissions from natural and semi-natural terrestrial ecosystems. This advancement can facilitate the generation of data necessary to improve and validate predictive models of denitrification in response to climate and land use changes.[3,4]

N_2 and N_2O fluxes due to denitrification

In situ denitrification and N_2O emission rates varied significantly between land use types with higher rates measured in managed and/or high nitrate content land uses (IG, SIG, MW)

and lower rates measured in unmanaged and/or low nitrate content land use types (OS, DW). Within the UK context, our monthly interpolated annual denitrification rates fall within the lower range of rates reported for improved (14 - 287 kg N ha^{-1} y^{-1}) and unimproved (1 - 140 kg N ha^{-1} y^{-1}) grasslands in the UK,[48-52] where denitrification was measured by the C_2H_2 block technique, which has limited utility for *in situ* measurements.[4,7] With respect to organic and forest soils, most UK based studies have focused on bulk N_2O emissions rather than denitrification using either static chambers for measurements [53-55] or modelling approaches;[56] and the reported rates for forests (0.005 - 2 kg N_2O-N ha^{-1} y^{-1}) and peatlands (0.02 - 0.24 kg N_2O-N ha^{-1} y^{-1}) are higher than the N_2O emission rates measured in this study (0.006 – 0.07 kg N_2O-N ha^{-1} y^{-1}) due to the fact that these studies estimated bulk N_2O flux without discrimination of its microbial sources . Our mean annual denitrification rate of the two forest types is in line with the rates reported for Central European forests under similar atmospheric Nr deposition using the He/O_2 headspace method.[9] Global models estimate average soil denitrification rates for UK between 11 - 200 kg N ha^{-1} y^{-1}. [25,26] Comparing our results with these broad scale global estimates, it becomes evident that due to the significant differences in denitrification rates between land use types, modelled denitrification at regional scales may be overestimated if land use type differences at catchment scale are not taken into account.

A significant advantage of the ^{15}N gas flux method is the ability to quantify the relative amounts of N_2 and N_2O fluxes due to denitrification,[46] thus allowing the estimation of the true denitrification product ratio $N_2O/ (N_2 + N_2O)$, which is crucial in evaluating the role of denitrification as a Nr sink.[3] The denitrification product ratio ranged between <1 % to 7 % across land use types and was comparable to the N_2O yields reported from ^{15}N tracer studies in forest[5,18] and grassland soils,[14,46] while it was significantly lower compared to estimates obtained using the C_2H_2 block technique [10] and the gas-flow soil core methods,[11] which

cannot discriminate between N_2O sources, thus overestimating the denitrification product ratio. Our results indicate that denitrification is an effective Nr sink (i.e. low N_2O yield) in natural/ unmanaged land use types (OS, MW, DW), whilst increased N_2O yields should be expected as land management intensifies in terms of nitrate enrichment (SIG & IG).[10,57]

Controlling factors

Significant relationships emerged between denitrification and its proximal regulators (Table 2) such as nitrate content, soil respiration, C:N ratio, WFPS and also with distal factors such as soil pH and bulk density that affect denitrification activity,[3,29] when rates were averaged across the sampling period and kept separate by sampling plots (n = 40). This suggests that spatial variability between land use types was higher than temporal and thus more meaningful evaluations of the controlling factors of denitrification can be made at broader temporal scales across land use types. At this broad scale, the combination of nitrate, soil respiration and pH explained 61 % of the variability in denitrification highlighting the importance of these variables as key controls at the landscape scale for modelling purposes.

Denitrification was partly controlled by the nitrate gradient observed across land uses with higher nitrate in managed land use types (IG, SIG) and lower in natural land uses such as the OS and DW. The high soil nitrate in MW was most likely a result of high nitrification activity, as a consequence of more aerobic soils (mean WFPS 39 %). In a preliminary study, nitrification potential of the MW was > 4 times higher than the OS and twice as much as the grassland soils, which seem to have supported high denitrification activity.[36] The OS land use included an ombrotrophic peat-bog, an acid grassland and a heathland, which are generally

381 considered having low denitrification activity due to their inherent low nitrate availability.[34]
382 A 10-fold increase of denitrification potential in nitrate-amended compared to the un-
383 amended peat soils verified that nitrate availability was indeed limiting denitrification in
384 OS.[36] The DW displayed lower denitrification than the MW, possibly due to its relatively
385 lower nitrate content. Land management practices applied in grasslands such as fertilisation
386 and grazing seem to have supported higher denitrifier activity. Fertilisation has ceased at the
387 SIG land use since the previous decade; however, the IG land uses are currently fertilised at
388 an annual rate between 100 - 200 kg N ha^{-1}, a fertilisation intensity, which was shown to
389 increase denitrification activity and N_2O emissions from grasslands.[58] Both the SIG and the
390 IG land use types are intensively grazed throughout the year and grazing has been related to
391 increased denitrification activity, because of the additional inputs of N and C through the
392 deposition of urine and faeces.[31,59,60]

393

394 In addition to soil nitrate, a gradient of soil respiration across all the land use types, correlated
395 with denitrification and its end products. The emission of CO_2 (representing soil respiration)
396 measured simultaneously with denitrification has been used as a surrogate for organic carbon
397 availability in soils.[61,62] In a parallel study in the same sites, a negative correlation was found
398 between soil microbial respiration and the aromaticity and SUVA indices of extracted DOC,
399 where OS exhibited the highest and forest and grassland soils exhibited the lowest
400 aromaticity and SUVA (Ullah & Sgouridis, unpublished data). This suggests that low
401 availability of mineralisable carbon may have further limited denitrifier activity in OS.[63]
402 Land management activities including grazing and manure application have been shown to
403 increase the lability of organic C[64] leading to enhanced denitrification activity and N_2O
404 emissions from grasslands.[32,65] Mowing, which is applied to the SIG land use twice per year,
405 may have also supported enhanced denitrifier activity through the release of DOC and

406 mineral N in mown grasslands.[59,66] The gradient of nitrate and soil respiration in explaining
407 denitrification trends across the sites was further substantiated by the significant correlation
408 of soil C:N ratio with denitrification. The C:N ratio integrates the natural variability of
409 potentially available nitrate for denitrification in OS, MW and DW land uses and of land
410 management through fertilisation in grassland soils. Similar negative relationships between
411 C:N ratios and N cycling processes have been reported in the literature.[38,67-69] This
412 observation points to the potential utility of substrate-based variables of C:N ratio, nitrate and
413 C availability as broad and large scale predictors of denitrification.

414

415 Apart from the substrate-based controllers of denitrification, soil moisture as expressed by
416 WFPS, also correlated negatively with denitrification and N_2O emissions. This negative
417 relationship was mainly driven by denitrification and N_2O emission maxima measured
418 between 60 and 70 % WFPS, particularly in grassland soils, whereas at higher WFPS,
419 represented by OS, denitrification activity was low due to nitrate limitations. Significant
420 denitrification at WFPS around 40 % was measured in the MW forest, which was NO_3^- rich.[36]
421 Morse et al. [11] have measured appreciable denitrification rates in forest soils even at 20 %
422 pore air O_2 concentration that have been attributed to the presence of anaerobic microsites
423 present in 'aerated' soils acting as denitrification hotspots.[70] Our results show that the
424 maxima of denitrification and N_2O emissions vary across the soil moisture gradient of
425 different land use types, depending on nitrate availability among other factors, suggesting
426 complex controls of the process by substrate and edaphic variables.[71]

427

428 In addition to the proximal regulators of denitrification (e.g. organic C, N and soil moisture),
429 distal factors (e.g. soil pH and bulk density) significantly influenced denitrification and N_2O

emissions across land use types. Land management such as liming in grasslands, aimed at raising the soil pH for higher biomass productivity, also increases denitrification activity[72] up to the optimum pH, which in our land use types was around 6. The gradient of increasing soil bulk density across land use types as an index of land management intensity correlated positively with denitrification and N_2O emissions. Herbivore trampling in grazed grassland soils has been related to enhanced denitrification rates due to an increase in bulk density, which subsequently creates anoxic microsites in soils.[73]

Implications for natural terrestrial ecosystems

Comparing the measured *in situ* denitrification and contemporary atmospheric Nr deposition rates in the UK (~15 to 25 kg N ha^{-1} y^{-1}),[21,22] it is evident that denitrification accounts for ~32 to 53 % and 40 to 66 % of the Nr deposition rates in organic and forest soils, respectively. The current rate of C accumulation in the OS ranges from 560 to 720 kg C ha^{-1} y^{-1} [74], which equates to N accumulation rates of 20 to 25 kg N ha^{-1} y^{-1} using a C:N ratio of 28 (Table 1). These rates are higher than the global northern peatland C and N accumulation rates of 220 kg C ha^{-1} y^{-1} and 3.4 kg N ha^{-1} y^{-1}, respectively.[75] The high N accumulation rates in OS seem to be indicative of chronic N saturation given that denitrification cannot match the increased Nr input through atmospheric deposition and biological nitrogen fixation in OS (~10 to 25 kg N ha^{-1} y^{-1})[76,77] posing threats to changes in ecology and water quality through export of Nr to water resources.[23,78] Similar to OS, the total input of Nr of 22 to 51 kg N ha^{-1} y^{-1} to forests through biological nitrogen fixation (7-26 kg N ha^{-1} y^{-1})[79] and atmospheric Nr deposition, is in excess of the measured removal capacity of denitrification.

453 This study presents the most comprehensive measurements to date of *in situ* denitrification
454 and N_2O emissions from natural and semi-natural land use types replicated in both space and
455 time in two UK catchments, using an adapted ^{15}N Gas-Flux method. Denitrification activity
456 was controlled by a combination of proximal and distal factors as influenced by their natural
457 variability and land management, which highlights the need for consideration of land
458 management when modelling and/or predicting the response of denitrification to land use and
459 climate change. Denitrification rates were lower in organic and forest than in semi-improved
460 and improved grassland soils and its overall contribution to N_2O emissions was significantly
461 lower than previously reported in literature. However, denitrification accounted for ~ 50% of
462 the contemporary annual atmospheric Nr deposition in natural ecosystems suggesting
463 increased threat of chronic N saturation in natural ecosystems alike the fertilised grasslands.

464

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473

474 **Table 1**: Soil physico-chemical properties in the five land use types in the Conwy and Ribble-Wyre River catchments.

	Organic soils (n = 255)	Mixed woodland (n = 85)	Deciduous woodland (n = 85)	Semi-improved grassland (n = 85)	Improved grassland (n = 170)
Bulk Density ($g\ cm^{-3}$)	0.09 (0.006)^{a}	0.56 (0.015)^{b}	0.38 (0.010)^{c}	0.29 (0.009)^{d}	0.59 (0.008)^{b}
WFPS (%)	69 (0.5)^{a}	39 (1.0)^{b}	65 (0.4)^{c}	67 (1.0)^{ac}	60 (0.8)^{d}
Moisture content (% on w/w)	86 (0.5)^{a}	38 (0.5)^{b}	60 (0.6)^{c}	64 (1.0)^{d}	42 (0.5)^{e}
pH	4.2 (0.03)^{a}	5.2 (0.08)^{b}	7.0 (0.04)^{c}	5.8 (0.03)^{d}	6.0 (0.01)^{e}
Soil Temperature (^{o}C)	10 (0.2)^{a}	10 (0.3)^{ab}	11 (0.3)^{b}	12 (0.4)^{bc}	12 (0.3)^{c}
Clay (%)*	7 (0.4)^{a}	37 (2.1)^{c}	24 (3.1)^{b}	31 (0.6)^{d}	26 (1.5)^{b}
Organic matter (%)	90 (0.9)^{a}	12 (0.3)^{b}	25 (0.5)^{c}	51 (1.4)^{d}	18 (0.4)^{e}
DOC ($g\ m^{-2}$)	6.0 (0.27)^{a}	4.3 (0.46)^{b}	4.5 (0.30)^{b}	10.5 (0.67)^{c}	4.3 (0.23)^{b}
Soil respiration ($mg\ C\ m^{-2}\ h^{-1}$)	43.8 (1.99)^{a}	36.6 (2.00)^{a}	53.3 (3.45)^{b}	75.9 (4.87)^{c}	160.3 (5.96)^{d}

C:N*	28 (0.8)[a]	11 (0.6)[b]	12 (0.5)[b]	18 (0.5)[c]	13 (0.6)[b]
NO_3^--N ($g\ m^{-2}$)	0.02 (0.002)[a]	0.43 (0.046)[b]	0.14 (0.010)[c]	0.17 (0.013)[c]	0.38 (0.020)[b]
NH_4^+-N ($g\ m^{-2}$)	0.11 (0.012)[a]	0.32 (0.030)[b]	0.05 (0.007)[c]	0.19 (0.075)[a]	0.42 (0.053)[b]
TDN ($g\ m^{-2}$)	0.37 (0.018)[a]	0.74 (0.052)[b]	0.41 (0.030)[c]	0.80 (0.059)[b]	0.80 (0.034)[b]

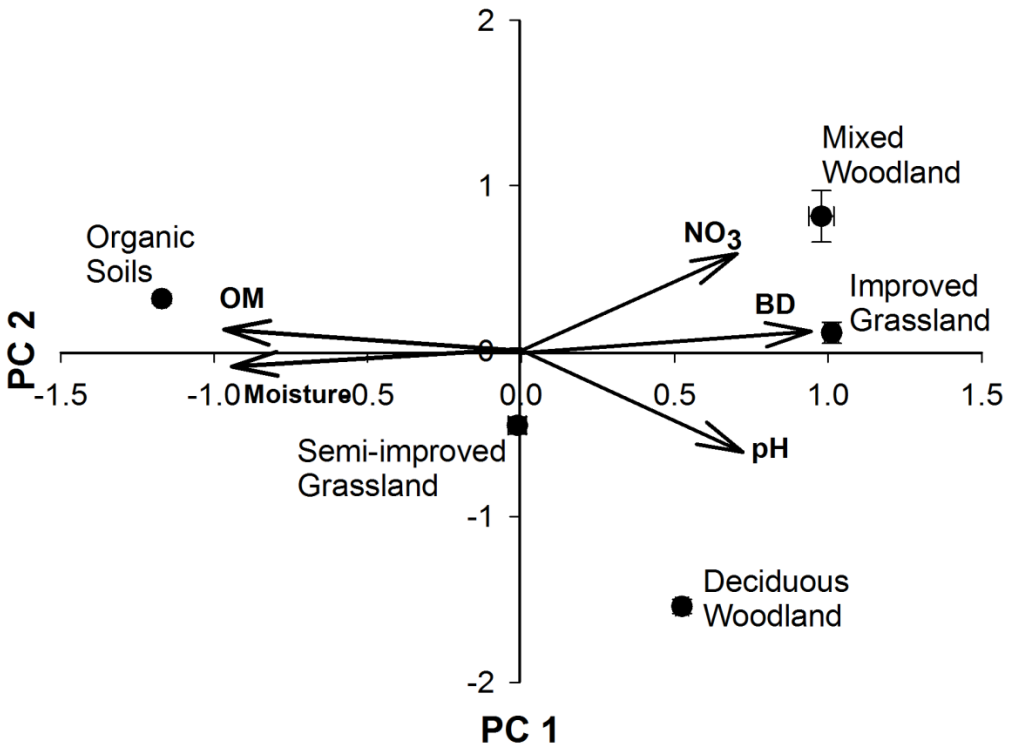
*n = 30 for organic soils, n=10 for mixed and deciduous woodland and semi-improved grassland and n=20 for improved grassland. Data are mean ± standard error (SE) in parenthesis. Same lower case letters indicate no significant differences ($p > 0.05$) between land use types according to 3-way ANOVA (One-way ANOVA for C:N ratio and clay) and the Hochberg's GT2 *post hoc* test (see Table 1 in supporting information for further details).

479 **Table 2**: Spearman's rank correlation coefficients between soil physico-chemical properties
480 and mean *in situ* denitrification, N_2O emission due to denitrification and the denitrification
481 product ratio $N_2O/ (N_2 + N_2O)$.

	Denitrification	N_2O	$N_2O/ (N_2 + N_2O)$
NO_3^--N	0.53	0.74	0.65
Soil respiration	0.65	0.73	0.72
pH	0.47	0.70	0.66
WFPS	-0.44	-0.52	-0.42
Bulk Density	0.63	0.76	0.65
C:N	-0.42	-0.61	-0.54

482 $p < 0.01$ probability level, n = 40.

483



484

485 **Figure 1**: Correlation bi-plot from the factor analysis scores on soil physico-chemical
486 variables. Soil properties represented by arrows and land use type groups represented by
487 cluster centroids. PC1; Horizontal principal component, PC2; Vertical principal component,
488 BD; Bulk density, OM; Organic matter content.

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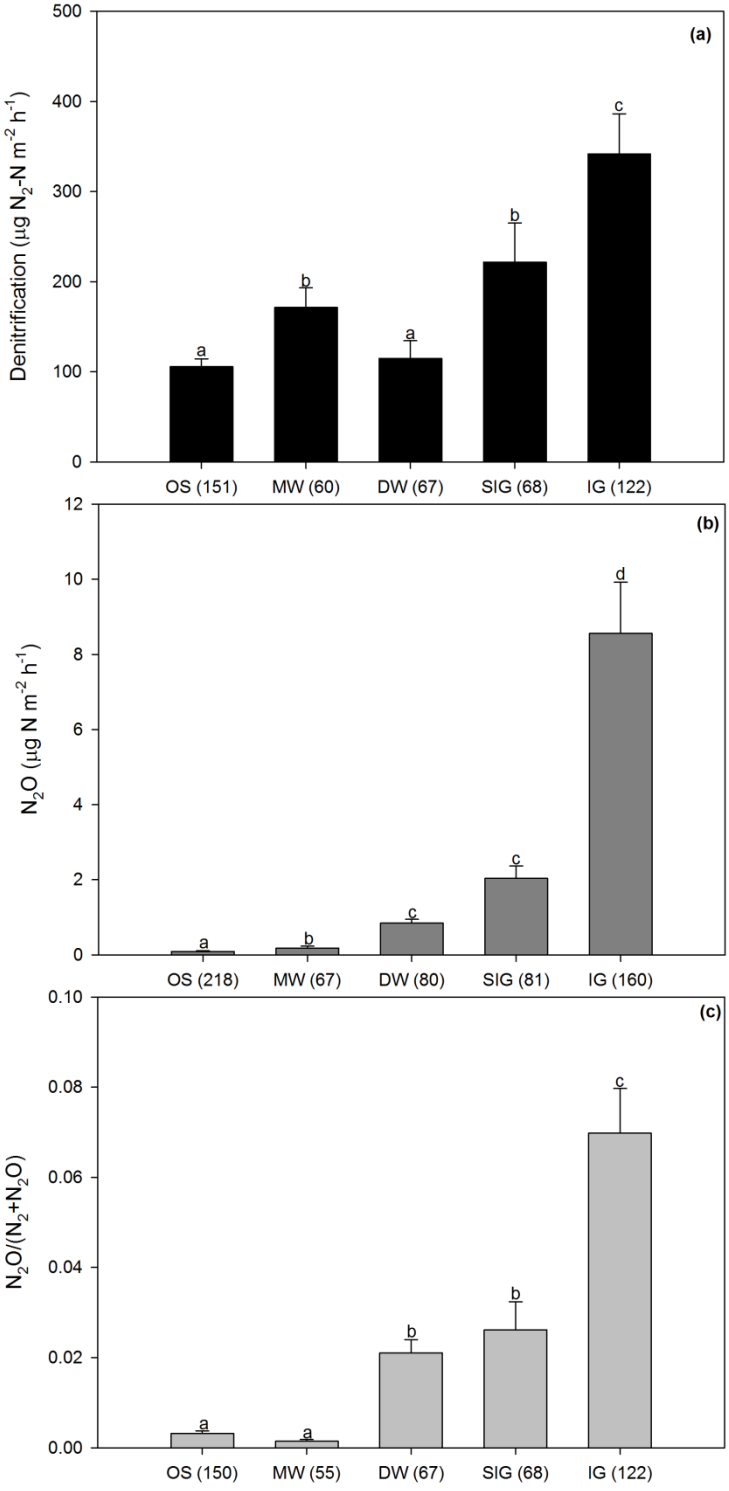
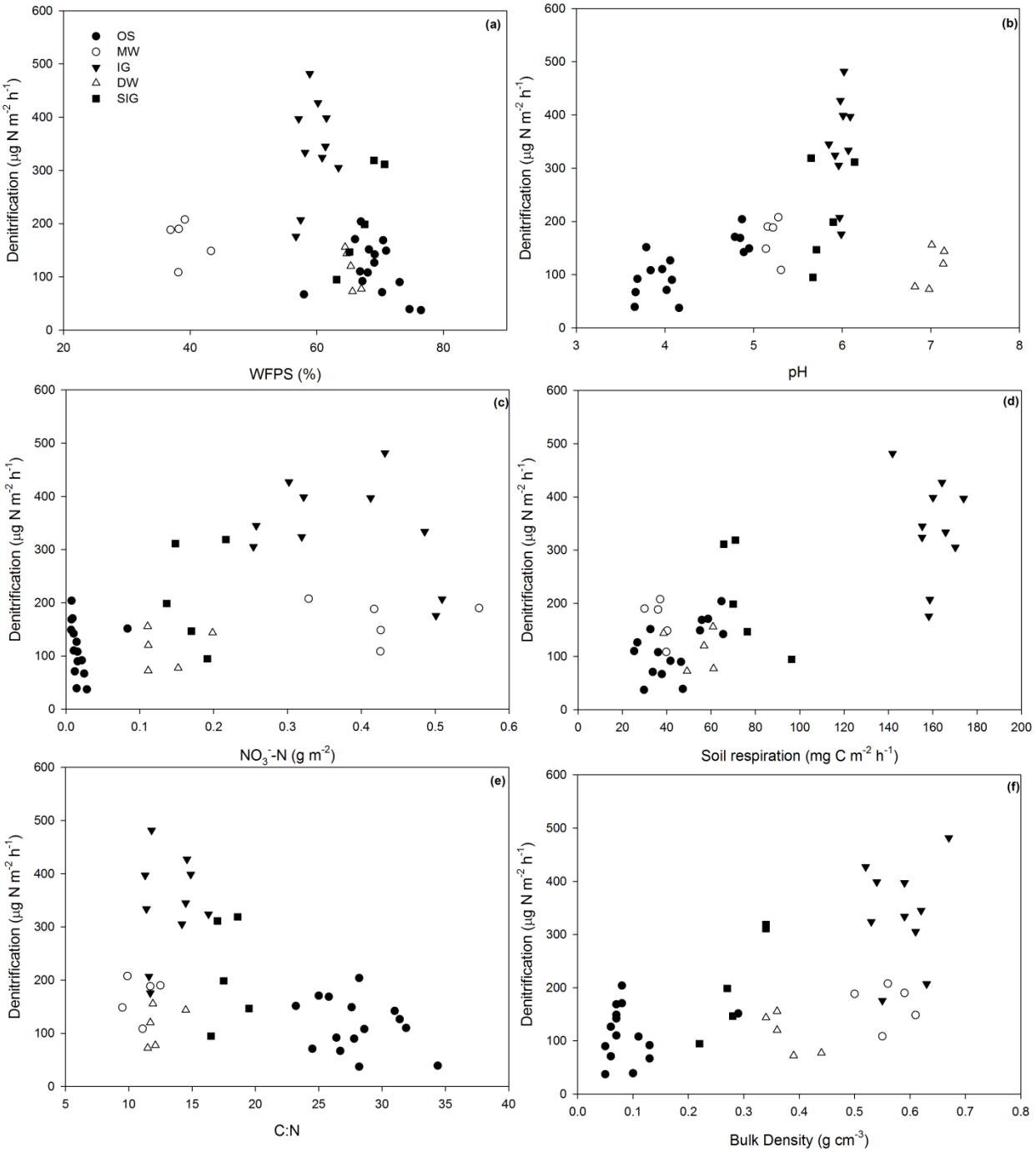


Figure 2: Mean rates of: (a) *in situ* denitrification, (b) N_2O emission due to denitrification and (c) the denitrification product ratio N_2O/ (N_2 + N_2O) in the five land use types in the Conwy and Ribble-Wyre River catchments. Same lower case letters indicate no significant differences (p > 0.05) between land use types according to One-way ANOVA and the Games-Howell *post hoc* test. The sample size (n) is given in parenthesis for each land use type on the x-axis.



503

504 **Figure 3**: Relationships between mean denitrification per plot (n=40) and: (a) Water filled
505 pore space, (b) pH, (c) soil nitrate content, (d) soil respiration, (e) soil C:N ratio and (f) bulk
506 density. OS = Organic Soils; MW = Mixed Woodland; DW = Deciduous Woodland; SIG =
507 Semi-Improved Grassland; IG = Improved Grassland.

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