

1 **Note: This paper is published in the Environmental Science and**
2 **Technology Journal on October 28, 2015 and thus should be**
3 **referenced as**

4 **Sgouridis F, and S Ullah. 2015. Relative Magnitude and Controls of *in situ* N₂ and N₂O**
5 **Fluxes due to Denitrification in Natural and Seminatural Terrestrial Ecosystems Using**
6 **¹⁵N Tracers. Environmental Science and Technology, DOI: 10.1021/acs.est.5b03513.**

7

8 **This is the accepted version of the manuscript.**

9

10 **Relative Magnitude and Controls of *in situ* N₂ and N₂O**
11 **Fluxes due to Denitrification in Natural and Seminatural**
12 **Terrestrial Ecosystems Using ¹⁵N Tracers**

13

14

15 *Fotis Sgouridis*^{1†} and *Sami Ullah*^{1*}

16

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

18

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

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

23

24 **Key words**

25 N cycling, ¹⁵N tracer, nitrous oxide, *in situ* measurements, denitrification, N₂ and N₂O
26 emission, N₂O:N₂ ratio, UK catchments, Natural and semi-natural terrestrial ecosystems.

27

28

29 **Abstract**

30 Denitrification is the most uncertain component of the nitrogen (N) cycle, hampering our
31 ability to assess its contribution to reactive N (Nr) removal. This uncertainty emanates from
32 the difficulty in measuring *in situ* soil N₂ production and from the high spatio-temporal
33 variability of the process itself. *In situ* denitrification was measured monthly between April
34 2013 and October 2014 in natural (organic and forest) and semi-natural ecosystems (semi-
35 improved and improved grasslands) in two UK catchments. Using the ¹⁵N-Gas Flux method
36 with low additions of ¹⁵NO₃⁻ tracer, a minimum detectable flux rate of 4 μg N m⁻² h⁻¹ and 0.2
37 ng N m⁻² h⁻¹ for N₂ and N₂O, respectively was achieved. Denitrification rates were lower in
38 organic and forest (8 and 10 kg N ha⁻¹ y⁻¹, respectively) than in semi-improved and improved
39 grassland soils (13 and 25 kg N ha⁻¹ y⁻¹, respectively). The ratio of N₂O/ N₂ + N₂O was low
40 and ranged from <1% to 7% across the sites. Variation in denitrification was driven by
41 differences in soil respiration, nitrate, C:N ratio, bulk density, moisture and pH across the
42 sites. Overall, the contribution of denitrification to Nr removal in natural ecosystems was
43 ~50% of the annual atmospheric Nr deposition, making these ecosystems vulnerable to
44 chronic N saturation.

45

46

47

48

49

50

51 **Introduction**

52 Human activities have more than doubled the inputs of reactive nitrogen (Nr: all nitrogen
53 species apart from the inert N₂) mainly through fertiliser use and fossil fuel combustion in
54 terrestrial ecosystems¹. Compared to the multiple pathways of Nr creation, microbial
55 denitrification, the sequential reduction of nitrate (NO₃⁻) to nitrite (NO₂⁻), nitrous oxide (N₂O)
56 and dinitrogen (N₂) gases,² is the only major process responsible for the permanent removal
57 of Nr.³ While the input of Nr (particularly due to human sources) into terrestrial ecosystems
58 is relatively well constrained, it is the process of denitrification which is the most uncertain
59 component of the N cycle, thus hampering our ability to fully account for the pools and
60 fluxes of N from regional to global scales.^{3,4}

61

62 The uncertainty associated with quantifying the magnitude of denitrification in terrestrial
63 ecosystems emanate mainly from two factors. One is the difficulty in accurately measuring
64 soil N₂ production rates against the high background atmospheric N₂ concentration and
65 another is the notoriously high spatio-temporal variability of denitrification.³ Available
66 methods for measuring the gaseous end products (N₂ and N₂O) of denitrification are limited
67 and can be categorised into the direct flux and ¹⁵N isotope tracer techniques.⁵ The acetylene
68 inhibition technique (AIT) is considered a direct flux technique; however, N₂O and N₂ flux
69 quantification needs two separate incubations (with and without C₂H₂) and the robustness of
70 estimates suffers from issues associated with the effectiveness of C₂H₂ to completely block
71 the reduction of N₂O to N₂ in case of intact soil core incubations.⁶ These limitations preclude
72 the use of the AIT for more reliable measurements of *in situ* denitrification rates.⁷ The gas-
73 flow soil core method^{8,9} is a direct flux technique that allows the simultaneous quantification
74 of N₂ and N₂O flux from soil cores where the soil pore N₂ atmosphere is replaced by a

75 mixture of He/O₂. Although this method is very sensitive and can detect small changes in soil
76 N₂ production, in most cases it does not constitute an *in situ* technique due to soil disturbance
77 during core collection and the subsequent laboratory incubation. Moreover, the AIT and the
78 gas-flow soil core techniques cannot discriminate between sources of N₂O, potentially
79 leading to overestimation of denitrification product ratios (N₂O/ N₂ + N₂O).^{10,11}

80

81 The ¹⁵N Gas-Flux method¹² can simultaneously measure N₂ and N₂O production due to
82 denitrification under *in situ* conditions with minimal disturbance to soils. A ¹⁵N-labelled
83 tracer is added to soil enclosed by a gas-tight chamber and the chamber headspace is
84 progressively enriched with ¹⁵N-N₂ and ¹⁵N-N₂O gases produced by denitrification.¹³ An
85 important criticism of this method has been the artificial stimulation of denitrification by the
86 added tracer, which has limited its use in highly fertilised agro-ecosystems.^{13,14} However,
87 recent advances in analytical instrumentation^{15,16} and flux calculations¹⁷ have shown that the
88 limit of detection for N₂ flux determination can be lowered so that only low levels of added
89 tracer (<10% of the soil NO₃⁻ pool) are needed, permitting the application of this method in
90 natural terrestrial ecosystems under laboratory^{16,18} and field conditions.⁵ Therefore, these
91 methodological advancements offer the opportunity for expanding the application of this
92 method across a range of natural and semi-natural terrestrial ecosystems for the measurement
93 of *in situ* denitrification rates with adequate spatio-temporal replication, which was not
94 previously possible.

95

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

99 grasslands constitute approximately 49 % and 85 % of rural land use cover in England and
100 Wales, respectively.²⁰ These land use types have been poorly investigated for their role in Nr
101 loss through denitrification. Moreover, current estimates of atmospheric Nr deposition rates
102 (~ 15 to 25 kg N ha⁻¹ y⁻¹)^{21,22} suggest an increased threat of chronic Nr enrichment of natural
103 terrestrial ecosystems in the UK, with important consequences for shifts in vegetation
104 composition and the export of excess Nr to water resources.^{23,24} Global models, largely
105 dependent on broad scale mass-balance approaches, estimate average basin denitrification
106 rates for UK soils between 11 and 200 kg N ha⁻¹ y⁻¹ ^{25,26}. Given this large range in modelled
107 denitrification rates and its high spatio-temporal variability from plot to the catchment scale,³
108 further studies are needed to quantify *in situ* denitrification and its controls in order to
109 validate and constrain catchment and/or regional scale denitrification models. ^{24,27}

110

111 The high spatio-temporal variability of soil denitrification is commonly attributed to the
112 heterogeneity of the proximal regulators of the process, such as nitrate and organic carbon
113 availability, concentration of soil oxygen but also distal factors such as soil pH and texture,
114 topography and landscape position.^{3,28} Land management practices (e.g. fertilisation, liming,
115 grazing) affect both the proximal and distal regulators of denitrification^{29,30} with
116 consequences for the relative proportion of the denitrification end products.²⁹ Traditional
117 grassland management has been associated with increased denitrification activity due to the
118 additional supply of Nr through fertilisation³¹ and C through manure application.³² Forest
119 soils, particularly those developed under poorly-drained conditions, sustain a relatively high
120 denitrification activity due to anoxic conditions.^{11,33} Peat soils under natural conditions are
121 generally nutrient limited, which limits their denitrification potential,³⁴⁻³⁶ while their response
122 to increased atmospheric Nr deposition is unclear. Therefore, quantitative understanding of
123 the links between denitrification and its associated controls are necessary for improving

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

126

127 The objectives of the present study were to: (1) apply the ¹⁵N Gas-Flux method for measuring
128 *in situ* N₂ and N₂O fluxes due to denitrification across a range of natural and semi-natural
129 ecosystems, (2) assess the environmental controlling factors of denitrification activity, and
130 (3) estimate annual denitrification rates for validating a national scale model for the UK to be
131 developed as part of a UK Natural Environment Research Council consortium project on
132 macronutrient cycling.

133

134 **Materials and methods**

135 Study sites

136 Two river catchments in the UK, the Conwy (N. Wales, 52°59'82" N, 3°46'06" W) and the
137 Ribble - Wyre (NW England, 53°59'99" N, 2°41'79" W) were selected for this study, where
138 more than 90 % of land cover consists of natural and semi-natural rural land use types.²⁰ In
139 the Conwy catchment, four study sites (C-PB = peat bog; C-UG = unimproved grassland; C-
140 IG = improved grassland; C-MW = mixed woodland) were chosen (Figure 1a, Supporting
141 Information). The C-PB and C-UG are under light grazing regime, less than one sheep per
142 hectare, while the C-IG, characterised by seasonally waterlogged cambic stagnogley soils, is
143 intensively grazed perennially by both sheep and cattle, while fertiliser (range: 100 – 200 kg
144 N ha⁻¹) and manure are applied twice per year during spring and summer months. The C-MW
145 (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 N₂O 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 ¹⁵N Gas-Flux method¹² 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 m²; 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

169 each monthly measurement, labelled $\text{K}^{15}\text{NO}_3^-$ (98 at. % ^{15}N , Sigma-Aldrich) was applied in
170 each plot at a mean rate between $0.03 (\pm\text{SE } 0.005)$ and $0.50 (\pm\text{SE } 0.073)$ kg N ha^{-1} . For the
171 natural land use types, the average tracer application rate reflected current daily estimates of
172 atmospheric N_r deposition in the UK ($0.05 \text{ kg N ha}^{-1} \text{ d}^{-1}$),²² whilst for the grassland soils the
173 tracer application mimicked a daily fertiliser application rate of $0.5 \text{ kg N ha}^{-1} \text{ d}^{-1}$. The tracer
174 solution (volume 50 - 250 mL, adjusted within 5 % of the ambient soil volumetric water
175 content) was applied in the soil volume enclosed by the collar via multiple injections of equal
176 volume through an equally-spaced grid using custom-made 10 cm long lumber needles
177 attached to a syringe.³⁹

178

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

187

188 During the gas flux measurements, soil temperature and volumetric water content at 10 cm
189 depth were recorded next to each chamber using a soil thermometer and a soil moisture probe
190 (Hydrosense II, CS659, Campbell Scientific), respectively. Five composite soil samples (0-10
191 cm) were collected with a hand auger from each study site at the end of the incubation within
192 50 cm of each plot. The collars were moved to new random plots within each study site every

193 three months to minimise any priming effects from repeated tracer application in the same
194 plots. The volume and concentration of the labelled $\text{K}^{15}\text{NO}_3^-$ tracer solution was determined
195 from measurements of soil nitrate and moisture content, as well as bulk density adjacent to
196 each plot made at the previous measurement campaign.¹⁸

197

198 Soil properties

199 The soil samples were analysed for dry bulk density, water filled pore space (WFPS), pH, soil
200 moisture and organic matter contents according to established methods. The gravimetric soil
201 moisture content was reported as per wet basis for comparison purposes between very moist
202 organic and mesic mineral soils.⁴¹ The experimental procedures for the analysis of soil
203 nitrate, ammonium, dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) are
204 described in Sgouridis & Ullah.³⁶ Soils sampled in July 2013 and in May 2014 were also
205 analysed for total carbon (TC) and total nitrogen (TN) using an elemental analyser (Flash EA
206 1112, Thermo-Finnigan). CO_2 production rate, representing soil respiration,⁴² was measured
207 in the second set of gas samples collected from the static chambers on a GC-FID (7890A GC
208 Agilent Technologies Ltd., Cheshire, UK) and flux rates were determined by linear
209 regression between 0 and 20 hours and expressed as $\text{mg C m}^{-2} \text{ h}^{-1}$, while the precision
210 determined from repeated analyses of 200 ppm CO_2 standards ($n = 8$) was $<1\%$.

211

212 N_2 and N_2O fluxes

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

216 charge ratios for m/z 28, m/z 29 and m/z 30 ($^{28}\text{N}_2$, $^{29}\text{N}_2$, and $^{30}\text{N}_2$ respectively) as well as the
217 ratios R29 ($^{29}\text{N}_2/^{28}\text{N}_2$) and R30 ($^{30}\text{N}_2/^{28}\text{N}_2$) were measured in both enriched (T=1, 2 and 20
218 hours) and reference samples (T=0 hours). The minimum detectable change (MDC) in R29
219 and R30 was defined with repeated manual analyses of air reference standards (n=10) and
220 was calculated using the following equation⁴³:

$$221 \quad MDC = \mu_{pair\ diff} + (2\sigma_{pair\ diff}) \quad (1)$$

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

227

228 For calculating the total N_2 flux from a uniformly labelled soil nitrate pool the ‘non-
229 equilibrium’ equations were applied as originally described by Mulvaney⁴⁴ and subsequently
230 revised by Stevens & Laughlin¹⁷ for lowering the detection limit for N_2 ³⁷. The contribution of
231 anammox and co-denitrification to N_2 fluxes was found negligible after comparison with the
232 equations proposed by Spott & Stange⁴⁵ (data not shown). Therefore, the N_2 flux was
233 calculated using linear regression between the maximum evolved N_2 and the incubation time
234 per plot surface area and was expressed in $\mu\text{g N m}^{-2} \text{h}^{-1}$ representing the total N_2 flux from the
235 mixture of the ^{15}N -labelled tracer and the soil NO_3^- at natural abundance.¹³

236

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

239 ratios for m/z 44, m/z 45 and m/z 46 ($^{44}\text{N}_2\text{O}$, $^{45}\text{N}_2\text{O}$, and $^{46}\text{N}_2\text{O}$ respectively) as well as the
240 ratios R45 ($^{45}\text{N}_2\text{O} / ^{44}\text{N}_2\text{O}$) and R46 ($^{46}\text{N}_2\text{O} / ^{44}\text{N}_2\text{O}$) were measured in both enriched (T=1, 2
241 and 20 hours) and reference samples (T=0 hours). The application of the ‘non-equilibrium’
242 equations to N_2O is analogous to N_2 after correcting for the naturally occurring oxygen
243 isotopes.⁴⁶ Therefore, the ratios R45 and R46 were converted to ratios of R29 and R30,
244 respectively by applying the corrections as described by Bergsma et al.⁴⁶. The MDC was
245 defined, for the converted R29 and R30, with repeated analyses of 0.5 ppm N_2O standards
246 (n=15) as 3.4×10^{-5} and 2.9×10^{-5} , respectively. The limit of detection for N_2 and N_2O fluxes
247 depends on the precision of R29 and R30 determination, the enrichment of the soil nitrate
248 pool, the dimensions of the static chamber and the incubation time.¹⁷ For our chamber design,
249 an incubation time of 20 hours, a soil nitrate pool enrichment of 60 ^{15}N at%, calculated using
250 the estimated MDC values; the limit of detection was calculated at $4 \mu\text{g N m}^{-2} \text{h}^{-1}$ and 0.2 ng
251 $\text{N m}^{-2} \text{h}^{-1}$ for the N_2 and N_2O fluxes, respectively. Approximately 69 % of the N_2 and 89 % of
252 the N_2O fluxes were above the respective MDC values and were subsequently used in process
253 rate calculations. Annual fluxes were estimated by interpolating monthly measurements for
254 each year and calculating the average between the two monitoring years.³⁸

255 Statistical analysis

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

258 Results

259 Soil properties

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

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

267

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

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

282 *In situ* denitrification, as represented by the N_2 flux, in the two catchments ranged between 3
283 and $3000 \mu\text{g N m}^{-2} \text{h}^{-1}$ and was significantly influenced by land use type (ANOVA; $F = 13.8$,
284 $df = 4$, $p < 0.001$) with the improved grasslands showing on average 3.5 times higher
285 denitrification rates than the organic soils (Fig. 2a). Seasonal variation in denitrification was

286 evident in most land use types (see Supporting Information). The N₂O emission due to
287 denitrification (range: 2×10^{-4} - $117.5 \mu\text{g N m}^{-2} \text{ h}^{-1}$) 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 N₂O 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 N₂ and N₂O fluxes) averaged between the two monitoring years were highest
292 in IG ($25 \text{ kg N ha}^{-1} \text{ y}^{-1}$) followed by the SIG, MW, OS and DW with 13, 13, 8 and 7 kg N ha^{-1}
293 y^{-1} , respectively. Measuring N₂ and N₂O fluxes due to denitrification separately, allowed us
294 to estimate the denitrification product ratio of N₂O/ (N₂ + N₂O) 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 ($r^2 =$

310 0.61, $p < 0.001$), whilst significant error due to heteroscedasticity negated a similar analysis
311 for the N_2O emission and the ratio of $\text{N}_2\text{O}/(\text{N}_2 + \text{N}_2\text{O})$. Multiple linear regression (MLR) of
312 monthly rates per land use type of log-transformed denitrification, N_2O emission and the
313 $\text{N}_2\text{O}/(\text{N}_2 + \text{N}_2\text{O})$ ratio with selected physico-chemical variables revealed more varied and
314 complex process controls within each land use (data presented in Supporting Information).

315 **Discussion**

316 ^{15}N Gas-Flux method

317 We have adapted the ^{15}N Gas-Flux method¹² for quantifying N_2 and N_2O fluxes due to
318 denitrification in natural and semi-natural terrestrial ecosystems by using low tracer
319 application rates and extending the incubation time up to ~20 hours where needed, which is
320 described in detail in a separate publication³⁷. The minimum detectable flux rates were $4 \mu\text{g}$
321 $\text{N m}^{-2} \text{h}^{-1}$ and $0.2 \text{ ng N m}^{-2} \text{h}^{-1}$ for the N_2 and N_2O fluxes respectively, which were
322 significantly better than the minimum rates ($175 \mu\text{g N}_2\text{-N m}^{-2} \text{h}^{-1}$ and $0.21 \mu\text{g N}_2\text{O-N m}^{-2} \text{h}^{-1}$)
323 reported by Kulkarni et al.,⁵ using a similar field ^{15}N tracer approach, and comparable to the
324 rates measured by a high precision ^{15}N analysis system in a laboratory incubation¹⁶ and the
325 gas-flow soil core method ($8 \mu\text{g N}_2\text{-N m}^{-2} \text{h}^{-1}$ and $< 1 \mu\text{g N}_2\text{O-N m}^{-2} \text{h}^{-1}$) by Wang et al.⁴⁷
326 Thus, our adapted ^{15}N Gas-Flux method constitutes a significant improvement in quantifying
327 *in situ* N_2 emissions from natural and semi-natural terrestrial ecosystems. This advancement
328 can facilitate the generation of data necessary to improve and validate predictive models of
329 denitrification in response to climate and land use changes.^{3,4}

330 N_2 and N_2O fluxes due to denitrification

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

333 and lower rates measured in unmanaged and/or low nitrate content land use types (OS, DW).
334 Within the UK context, our monthly interpolated annual denitrification rates fall within the
335 lower range of rates reported for improved ($14 - 287 \text{ kg N ha}^{-1} \text{ y}^{-1}$) and unimproved ($1 - 140$
336 $\text{kg N ha}^{-1} \text{ y}^{-1}$) grasslands in the UK,⁴⁸⁻⁵² where denitrification was measured by the C_2H_2
337 block technique, which has limited utility for *in situ* measurements.^{4,7} With respect to organic
338 and forest soils, most UK based studies have focused on bulk N_2O emissions rather than
339 denitrification using either static chambers for measurements⁵³⁻⁵⁵ or modelling approaches,⁵⁶
340 and the reported rates for forests ($0.005 - 2 \text{ kg N}_2\text{O-N ha}^{-1} \text{ y}^{-1}$) and peatlands ($0.02 - 0.24 \text{ kg}$
341 $\text{N}_2\text{O-N ha}^{-1} \text{ y}^{-1}$) are higher than the N_2O emission rates measured in this study ($0.006 - 0.07$
342 $\text{kg N}_2\text{O-N ha}^{-1} \text{ y}^{-1}$) due to the fact that these studies estimated bulk N_2O flux without
343 discrimination of its microbial sources. Our mean annual denitrification rate of the two forest
344 types is in line with the rates reported for Central European forests under similar atmospheric
345 Nr deposition using the He/O_2 headspace method.⁹ Global models estimate average soil
346 denitrification rates for UK between $11 - 200 \text{ kg N ha}^{-1} \text{ y}^{-1}$.^{25,26} Comparing our results with
347 these broad scale global estimates, it becomes evident that due to the significant differences
348 in denitrification rates between land use types, modelled denitrification at regional scales may
349 be overestimated if land use type differences at catchment scale are not taken into account.

350

351 A significant advantage of the ^{15}N gas flux method is the ability to quantify the relative
352 amounts of N_2 and N_2O fluxes due to denitrification,⁴⁶ thus allowing the estimation of the
353 true denitrification product ratio $\text{N}_2\text{O}/(\text{N}_2 + \text{N}_2\text{O})$, which is crucial in evaluating the role of
354 denitrification as a Nr sink.³ The denitrification product ratio ranged between $<1\%$ to 7%
355 across land use types and was comparable to the N_2O yields reported from ^{15}N tracer studies
356 in forest^{5,18} and grassland soils,^{14,46} while it was significantly lower compared to estimates
357 obtained using the C_2H_2 block technique¹⁰ and the gas-flow soil core methods,¹¹ which

358 cannot discriminate between N₂O sources, thus overestimating the denitrification product
359 ratio. Our results indicate that denitrification is an effective Nr sink (i.e. low N₂O yield) in
360 natural/ unmanaged land use types (OS, MW, DW), whilst increased N₂O yields should be
361 expected as land management intensifies in terms of nitrate enrichment (SIG & IG).^{10,57}

362

363 Controlling factors

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

373

374 Denitrification was partly controlled by the nitrate gradient observed across land uses with
375 higher nitrate in managed land use types (IG, SIG) and lower in natural land uses such as the
376 OS and DW. The high soil nitrate in MW was most likely a result of high nitrification
377 activity, as a consequence of more aerobic soils (mean WFPS 39 %). In a preliminary study,
378 nitrification potential of the MW was > 4 times higher than the OS and twice as much as the
379 grassland soils, which seem to have supported high denitrification activity.³⁶ The OS land use
380 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 N₂O 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 CO₂ (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 C⁶⁴ leading to enhanced denitrification activity and N₂O
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 N₂O emissions. This negative
417 relationship was mainly driven by denitrification and N₂O 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 NO₃⁻ rich.³⁶
421 Morse et al.¹¹ have measured appreciable denitrification rates in forest soils even at 20 %
422 pore air O₂ 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 N₂O 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 N₂O

430 emissions across land use types. Land management such as liming in grasslands, aimed at
431 raising the soil pH for higher biomass productivity, also increases denitrification activity⁷² up
432 to the optimum pH, which in our land use types was around 6. The gradient of increasing soil
433 bulk density across land use types as an index of land management intensity correlated
434 positively with denitrification and N₂O emissions. Herbivore trampling in grazed grassland
435 soils has been related to enhanced denitrification rates due to an increase in bulk density,
436 which subsequently creates anoxic microsites in soils.⁷³

437

438 Implications for natural terrestrial ecosystems

439 Comparing the measured *in situ* denitrification and contemporary atmospheric Nr deposition
440 rates in the UK (~15 to 25 kg N ha⁻¹ y⁻¹),^{21,22} it is evident that denitrification accounts for ~32
441 to 53 % and 40 to 66 % of the Nr deposition rates in organic and forest soils, respectively.
442 The current rate of C accumulation in the OS ranges from 560 to 720 kg C ha⁻¹ y⁻¹ ⁷⁴, which
443 equates to N accumulation rates of 20 to 25 kg N ha⁻¹ y⁻¹ using a C:N ratio of 28 (Table 1).
444 These rates are higher than the global northern peatland C and N accumulation rates of 220
445 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
446 to be indicative of chronic N saturation given that denitrification cannot match the increased
447 Nr input through atmospheric deposition and biological nitrogen fixation in OS (~10 to 25 kg
448 N ha⁻¹ y⁻¹)^{76,77} posing threats to changes in ecology and water quality through export of Nr to
449 water resources.^{23,78} Similar to OS, the total input of Nr of 22 to 51 kg N ha⁻¹ y⁻¹ to forests
450 through biological nitrogen fixation (7-26 kg N ha⁻¹ y⁻¹)⁷⁹ and atmospheric Nr deposition, is
451 in excess of the measured removal capacity of denitrification.

452

453 This study presents the most comprehensive measurements to date of *in situ* denitrification
454 and N₂O emissions from natural and semi-natural land use types replicated in both space and
455 time in two UK catchments, using an adapted ¹⁵N 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 N₂O 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

465 **Acknowledgements**

466 The authors are grateful to Mr Edward Ritchie at Conwy and Mr Richard Rhodes at Ribble-
467 Wyre River catchments for their collaboration and permission to access their land, as well as
468 the National Trust in Conwy, the Abbeystead Estate in the Trough of Bowland and the
469 Forestry Commission in Gisburn Forest for their guidance and advice. We are also thankful
470 to Dr Andy Stott for his extended analytical assistance at the NERC's Stable Isotope Facility.
471 This research is funded by the UK Natural Environment Research Council grant
472 (NE/J011541/1) awarded to Keele University.

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 (°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

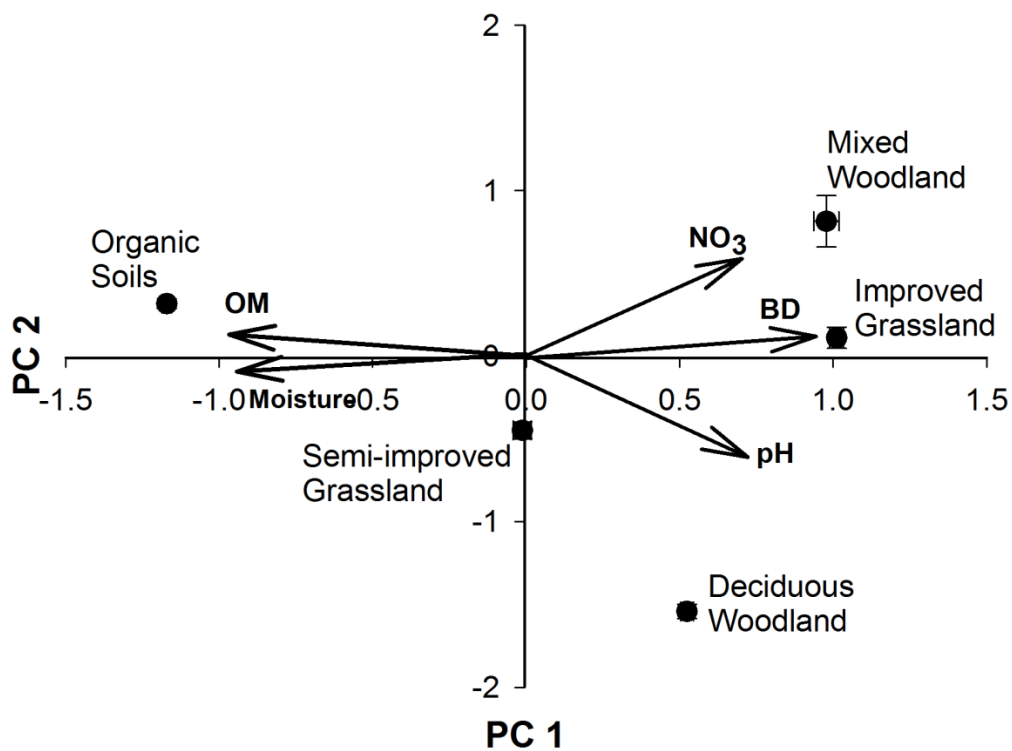
475 *n = 30 for organic soils, n=10 for mixed and deciduous woodland and semi-improved grassland and n=20 for improved grassland. Data are
476 mean ± standard error (SE) in parenthesis. Same lower case letters indicate no significant differences ($p > 0.05$) between land use types
477 according to 3-way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

479 **Table 2:** Spearman's rank correlation coefficients between soil physico-chemical properties
 480 and mean *in situ* denitrification, N₂O emission due to denitrification and the denitrification
 481 product ratio N₂O/ (N₂ + N₂O).

	Denitrification	N ₂ O	N ₂ O/ (N ₂ + N ₂ O)
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.

489

490

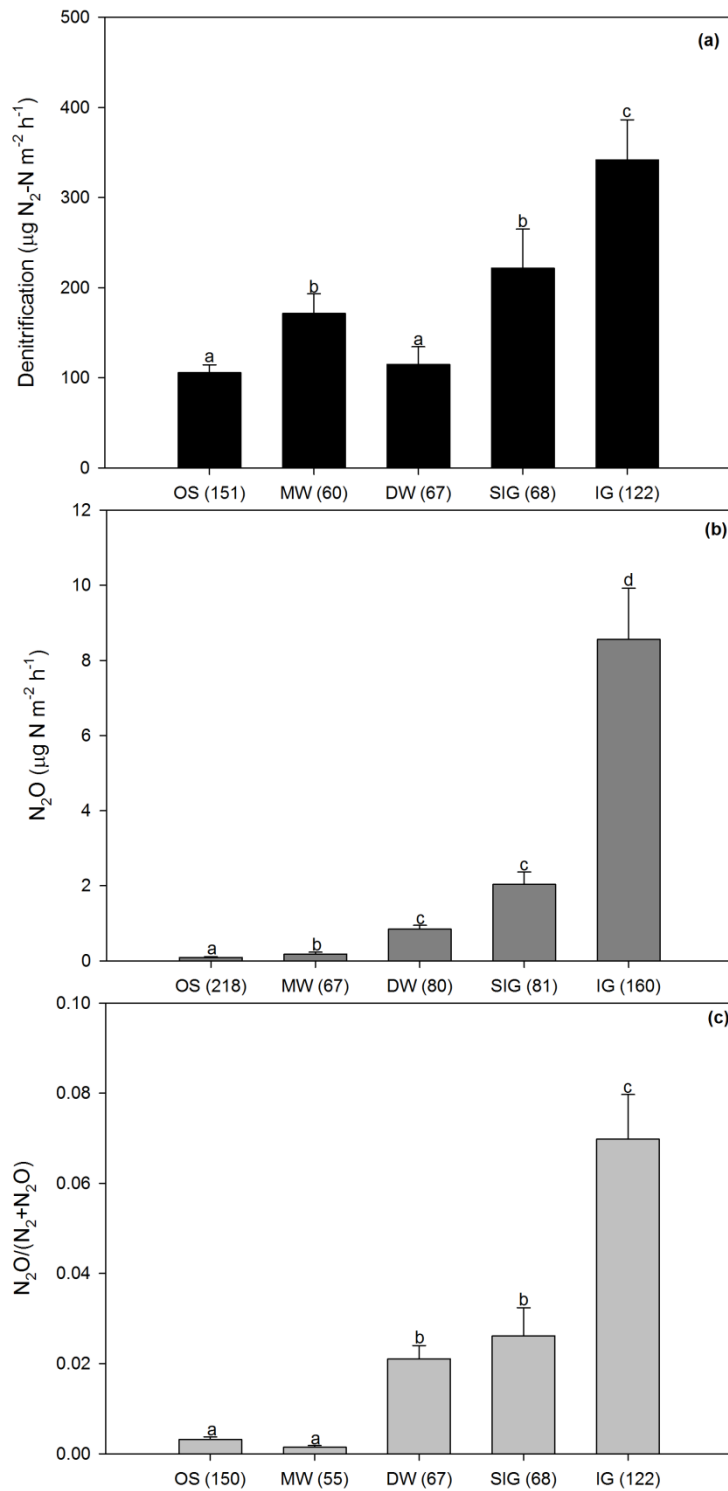
491

492

493

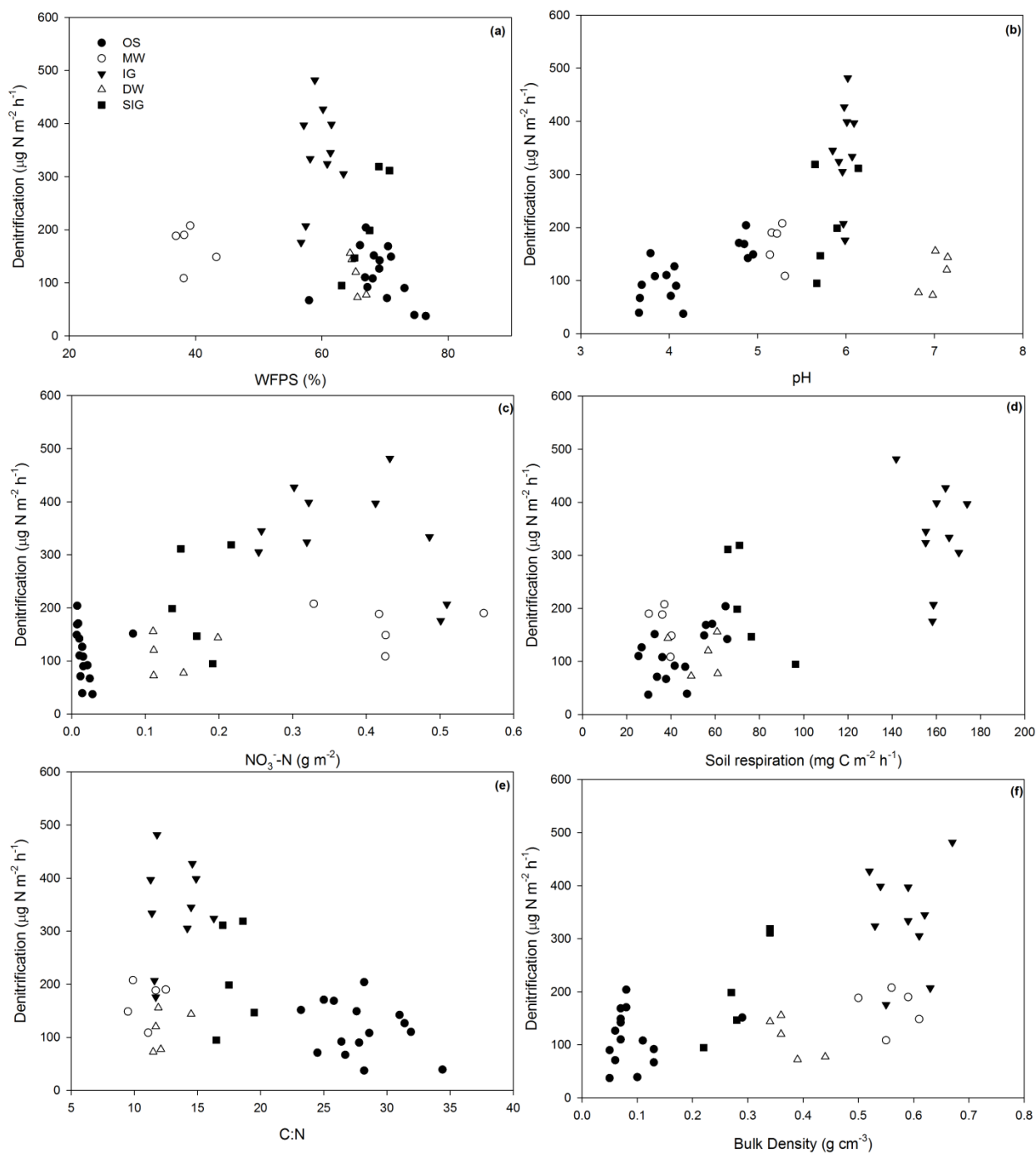
494

495



496

497 **Figure 2:** Mean rates of: (a) *in situ* denitrification, (b) N_2O emission due to denitrification
 498 and (c) the denitrification product ratio $\text{N}_2\text{O}/(\text{N}_2 + \text{N}_2\text{O})$ in the five land use types in the
 499 Conwy and Ribble-Wyre River catchments. Same lower case letters indicate no significant
 500 differences ($p > 0.05$) between land use types according to One-way ANOVA and the
 501 Games-Howell *post hoc* test. The sample size (n) is given in parenthesis for each land use
 502 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.

508

509

510

- 512 1. Fowler, D.; Pyle, J. A.; Raven, J. A.; Sutton, M. A. The global nitrogen cycle in the
513 twenty-first century: introduction. *Philosophical Transactions of the Royal Society B:
514 Biological Sciences* **2013**, *368*, 20130165.
- 515 2. Knowles, R. Denitrification. *Microbiol. Rev.* **1982**, *46*, 43-70.
- 516 3. Kulkarni, M. V.; Groffman, P. M.; Yavitt, J. B. Solving the global nitrogen problem: it's a
517 gas! *Frontiers in Ecology and the Environment* **2008**, *6*, 199-206.
- 518 4. Groffman, P. M.; Altabet, M. A.; Bohlke, J. K.; Butterbach-Bahl, K.; David, M. B.;
519 Firestone, M. K.; Giblin, A. E.; Kana, T. M.; Nielsen, L. P.; Voytek, M. A. Methods for
520 measuring denitrification: Diverse approaches to a difficult problem. *Ecol. Appl.* **2006**,
521 *16*, 2091-2122.
- 522 5. Kulkarni, M. V.; Burgin, A. J.; Groffman, P. M.; Yavitt, J. B. Direct flux and N-15 tracer
523 methods for measuring denitrification in forest soils. *Biogeochemistry* **2014**, *117*, 359-
524 373.
- 525 6. Yu, K.; Seo, D.; DeLaune, R. D. Incomplete Acetylene Inhibition of Nitrous Oxide
526 Reduction in Potential Denitrification Assay as Revealed by using ¹⁵N-Nitrate Tracer.
527 *Commun. Soil Sci. Plant Anal.* **2010**, *41*, 2201-2210.
- 528 7. Felber, R.; Conen, F.; Flechard, C. R.; Neftel, A. Theoretical and practical limitations of
529 the acetylene inhibition technique to determine total denitrification losses.
530 *Biogeosciences* **2012**, *9*, 4125-4138.
- 531 8. Scholefield, D.; Hawkins, J.; Jackson, S. Development of a helium atmosphere soil
532 incubation technique for direct measurement of nitrous oxide and dinitrogen fluxes
533 during denitrification. *Soil Biology & Biochemistry* **1997**, *29*, 1345-1352.
- 534 9. Butterbach-Bahl, K.; Willibald, G.; Papen, H. Soil core method for direct simultaneous
535 determination of N₂ and N₂O emissions from forest soils. *Plant Soil* **2002**, *240*, 105-
536 116.
- 537 10. Butterbach-Bahl, K.; Baggs, E. M.; Dannenmann, M.; Kiese, R.; Zechmeister-
538 Boltenstern, S. Nitrous oxide emissions from soils: how well do we understand the
539 processes and their controls? *Philosophical Transactions of the Royal Society B-
540 Biological Sciences* **2013**, *368*.
- 541 11. Morse, J. L.; Duran, J.; Beall, F.; Enanga, E. M.; Creed, I. F.; Fernandez, I.; Groffman, P.
542 M. Soil denitrification fluxes from three northeastern North American forests across a
543 range of nitrogen deposition. *Oecologia* **2015**, *177*, 17-27.
- 544 12. Mosier, A. R.; Klemetsson, L. Measuring denitrification in the field. In *Methods of Soil
545 Analysis, Part 2, Microbiological and Biochemical Properties*; Weaver, R. W., Angle, J.

- 546 S. and Bottomley, P. S., Eds.; Soil Science Society of America, Inc.: Wisconsin, USA,
547 1994; pp 1047.
- 548 13. Stevens, R.; Laughlin, R. Measurement of nitrous oxide and di-nitrogen emissions from
549 agricultural soils. *Nutr. Cycling Agroecosyst.* **1998**, *52*, 131-139.
- 550 14. Baily, A.; Watson, C. J.; Laughlin, R.; Matthews, D.; McGeough, K.; Jordan, P. Use of
551 the ^{15}N gas flux method to measure the source and level of N_2O and N_2 emissions
552 from grazed grassland. *Nutr. Cycling Agroecosyst.* **2012**, *94*, 287-298.
- 553 15. Lewicka-Szczebak, D.; Well, R.; Giesemann, A.; Rohe, L.; Wolf, U. An enhanced
554 technique for automated determination of N-^{15} signatures of N_2 , ($\text{N}_2+\text{N}_2\text{O}$) and N_2O
555 in gas samples. *Rapid Communications in Mass Spectrometry* **2013**, *27*, 1548-1558.
- 556 16. Yang, W. H.; McDowell, A. C.; Brooks, P. D.; Silver, W. L. New high precision
557 approach for measuring N-^{15} - N_2 gas fluxes from terrestrial ecosystems. *Soil Biology &*
558 *Biochemistry* **2014**, *69*, 234-241.
- 559 17. Stevens, R. J.; Laughlin, R. J. Lowering the detection limit for dinitrogen using the
560 enrichment of nitrous oxide. *Soil Biol. Biochem.* **2001**, *33*, 1287-1289.
- 561 18. Morse, J. L.; Bernhardt, E. S. Using N-^{15} tracers to estimate N_2O and N_2 emissions
562 from nitrification and denitrification in coastal plain wetlands under contrasting land-
563 uses. *Soil Biology & Biochemistry* **2013**, *57*, 635-643.
- 564 19. Mills, R. T. E.; Tipping, E.; Bryant, C. L.; Emmett, B. A. Long-term organic carbon
565 turnover rates in natural and semi-natural topsoils. *Biogeochemistry* **2013**, 1-16.
- 566 20. Morton, D.; Rowland, C.; Wood, C.; Meek, L.; Marston, C.; Smith, G.; Wadsworth, R.;
567 Simpson, I. C. Final Report for LCM2007 - the new UK Land Cover Map. **2011**, *11/07*.
- 568 21. Dore, A. J.; Kryza, M.; Hall, J. R.; Hallsworth, S.; Keller, V. J. D.; Vieno, M.; Sutton, M.
569 A. The influence of model grid resolution on estimation of national scale nitrogen
570 deposition and exceedance of critical loads. *Biogeosciences* **2012**, *9*, 1597-1609.
- 571 22. Payne, R. J. The exposure of British peatlands to nitrogen deposition, 1900-2030. *Mires*
572 *and Peat* **2014**, *14*, 04.
- 573 23. Limpens, J.; Berendse, F.; Klees, H. N deposition affects N availability in interstitial
574 water, growth of Sphagnum and invasion of vascular plants in bog vegetation. *New*
575 *Phytol.* **2003**, *157*, 339-347.
- 576 24. Galloway, J. N.; Townsend, A. R.; Erisman, J. W.; Bekunda, M.; Cai, Z.; Freney, J. R.;
577 Martinelli, L. A.; Seitzinger, S.; Sutton, M. A. Transformation of the Nitrogen Cycle:
578 Recent trends, questions and potential solutions. *Science* **2008**, *320*, 889-892.

- 579 25. Seitzinger, S.; Harrison, J. A.; Bohlke, J. K.; Bouwman, A. F.; Lowrance, R.; Peterson,
580 B.; Tobias, C.; Van Drecht, G. Denitrification across landscapes and waterscapes: A
581 synthesis. *Ecol. Appl.* **2006**, *16*, 2064-2090.
- 582 26. Bouwman, A. F.; Beusen, A. H. W.; Griffioen, J.; Van Groenigen, J. W.; Hefting, M. M.;
583 Oenema, O.; Van Puijenbroek, P. J. T. M.; Seitzinger, S.; Slomp, C. P.; Stehfest, E.
584 Global trends and uncertainties in terrestrial denitrification and N₂O emissions.
585 *Philosophical Transactions of the Royal Society B-Biological Sciences* **2013**, *368*.
- 586 27. Boyer, E. W.; Alexander, R. B.; Parton, W. J.; Li, C.; Butterbach-Bahl, K.; Donner, S. D.;
587 Skaggs, R. W.; Del Grosso, S. J. Modeling denitrification in terrestrial and aquatic
588 ecosystems at regional scales. *Ecol. Appl.* **2006**, *16*, 2123-2142.
- 589 28. Groffman, P. M.; Butterbach-Bahl, K.; Fulweiler, R. W.; Gold, A. J.; Morse, J. L.;
590 Stander, E. K.; Tague, C.; Tonitto, C.; Vidon, P. Challenges to incorporating spatially
591 and temporally explicit phenomena (hotspots and hot moments) in denitrification
592 models. *Biogeochemistry* **2009**, *93*, 49-77.
- 593 29. Sagar, S.; Jha, N.; Deslippe, J.; Bolan, N. S.; Luo, J.; Giltrap, D. L.; Kim, D. -.; Zaman,
594 M.; Tillman, R. W. Denitrification and N₂O:N₂ production in temperate grasslands:
595 Processes, measurements, modelling and mitigating negative impacts. *Sci. Total*
596 *Environ.* **2013**, *465*, 173-195.
- 597 30. Philippot, L.; Cuhel, J.; Saby, N. P. A.; Cheneby, D.; Chronakova, A.; Bru, D.; Arrouays,
598 D.; Martin-Laurent, F.; Simek, M. Mapping field-scale spatial patterns of size and
599 activity of the denitrifier community. *Environ. Microbiol.* **2009**, *11*, 1518-1526.
- 600 31. van Beek, C. L.; Pleijter, M.; Jacobs, C. M. J.; Velthof, G. L.; van Groenigen, J. W.;
601 Kuikman, P. J. Emissions of N₂O from fertilized and grazed grassland on organic soil in
602 relation to groundwater level. *Nutr. Cycling Agroecosyst.* **2010**, *86*, 331-340.
- 603 32. Graham, C. J.; van Es, H. M.; Melkonian, J. J. Nitrous oxide emissions are greater in silt
604 loam soils with a legacy of manure application than without. *Biol. Fertility Soils* **2013**,
605 *49*, 1123-1129.
- 606 33. Ullah, S.; Frasier, R.; King, L.; Picotte-Anderson, N.; Moore, T. R. Potential fluxes of
607 N₂O and CH₄ from soils of three forest types in Eastern Canada. *Soil Biol. Biochem.*
608 **2008**, *40*, 986-994.
- 609 34. Hayden, M.; Ross, D. Denitrification as a nitrogen removal mechanism in a Vermont
610 peatland. *J. Environ. Qual.* **2005**, *34*, 2052-2061.
- 611 35. Francez, A.; Pinay, G.; Josselin, N.; Williams, B. L. Denitrification triggered by nitrogen
612 addition in *Sphagnum magellanicum* peat. *Biogeochemistry* **2011**, *106*, 435-441.
- 613 36. Sgouridis, F.; Ullah, S. Denitrification potential of organic, forest and grassland soils in
614 the Ribble-Wyre and Conwy River catchments, UK. *Environ. Sci. -Process Impacts*
615 **2014**, *16*, 1551-1562.

- 616 37. Sgouridis, F.; Stott, A.; Ullah, S. Application of the ^{15}N -Gas Flux method for measuring
617 in situ N_2 and N_2O fluxes due to denitrification in natural and semi-natural terrestrial
618 ecosystems and comparison with the acetylene inhibition technique. *Biogeosciences*
619 **2015**, *12*, 12653-12653–12689.
- 620 38. Ullah, S.; Moore, T. R. Biogeochemical controls on methane, nitrous oxide, and carbon
621 dioxide fluxes from deciduous forest soils in eastern Canada. *J. Geophys. Res. -*
622 *Biogeosci.* **2011**, *116*, G03010.
- 623 39. Ruetting, T.; Huygens, D.; Staelens, J.; Mueller, C.; Boeckx, P. Advances in N-15-tracing
624 experiments: new labelling and data analysis approaches. *Biochem. Soc. Trans.* **2011**, *39*,
625 279-283.
- 626 40. Laughlin, R. J.; Stevens, R. J. Changes in composition of nitrogen-15-labeled gases
627 during storage in septum-capped vials. *Soil Sci. Soc. Am. J.* **2003**, *67*, 540-543.
- 628 41. Gardner, W. H. Water Content. In *Methods of Soil Analysis Part 1: Physical and*
629 *Minerological Properties, Including Statistics of Measurement and Sampling*; Black, C.
630 A., Evans, D. D., White, J. L., Ensminger, L. E. and Clark, F. E., Eds.; American Society
631 of Agronomy: Madison, Wisconsin, USA, 1965; pp 82-125.
- 632 42. Strom, L.; Falk, J.; Skov, K.; Jackowicz-Korczynski, M.; Mastepanov, M.; Christensen,
633 T.; Lund, M.; Schmidt, N. Controls of spatial and temporal variability in CH_4 flux in a
634 high arctic fen over three years. *Biogeochemistry* **2015**.
- 635 43. Matson, A.; Pennock, D.; Bedard-Haughn, A. Methane and nitrous oxide emissions from
636 mature forest stands in the boreal forest, Saskatchewan, Canada. *For. Ecol. Manage.*
637 **2009**, *258*, 1073-1083.
- 638 44. Mulvaney, R. L. Determination of ^{15}N -Labeled Dinitrogen and Nitrous Oxide With
639 Triple-collector Mass Spectrometers. *Soil Sci. Soc. Am. J.* **1984**, *48*, 690-692.
- 640 45. Spott, O.; Stange, C. F. A new mathematical approach for calculating the contribution of
641 anammox, denitrification and atmosphere to an N_2 mixture based on a ^{15}N tracer
642 technique. *Rapid Communications in Mass Spectrometry* **2007**, *21*, 2398-2406.
- 643 46. Bergsma, T.; Ostrom, N.; Emmons, M.; Robertson, G. Measuring simultaneous fluxes
644 from soil of N_2O and N_2 in the field using the $(^{15}\text{N})\text{N}$ -Gas "nonequilibrium"
645 technique. *Environ. Sci. Technol.* **2001**, *35*, 4307-4312.
- 646 47. Wang, R.; Willibald, G.; Feng, Q.; Zheng, X.; Liao, T.; Brueggemann, N.; Butterbach-
647 Bahl, K. Measurement of N_2 , N_2O , NO , and CO_2 Emissions from Soil with the Gas-
648 Row-Soil-Core Technique. *Environ. Sci. Technol.* **2011**, *45*, 6066-6072.
- 649 48. Ryden, J. C. Denitrification loss from a grassland soil in the field receiving different rates
650 of nitrogen as ammonium nitrate. *J. Soil Sci.* **1983**, *34*, 355-365.

- 651 49. Ellis, S.; Yamulki, S.; Dixon, E.; Harrison, R.; Jarvis, S. Denitrification and N₂O
652 emissions from a UK pasture soil following the early spring application of cattle slurry
653 and mineral fertiliser. *Plant Soil* **1998**, *202*, 15-25.
- 654 50. Burt, T. P.; Matchett, L. S.; Goulding, K. W. T.; Webster, C. P.; Haycock, N. E.
655 Denitrification in riparian buffer zones: the role of floodplain hydrology. *Hydrol.*
656 *Process.* **1999**, *13*, 1451-1463.
- 657 51. Abbasi, M.; Adams, W. Estimation of simultaneous nitrification and denitrification in
658 grassland soil associated with urea-N using N-15 and nitrification inhibitor. *Biol.*
659 *Fertility Soils* **2000**, *31*, 38-44.
- 660 52. Jordan, C.; Smith, R. Methods to predict the agricultural contribution to catchment nitrate
661 loads: designation of nitrate vulnerable zones in Northern Ireland. *J. Hydrol.* **2005**, *304*,
662 316-329.
- 663 53. Emmett, B.; Cosby, B.; Ferrier, R.; Jenkins, A.; Tietema, A.; Wright, R. Modelling the
664 ecosystem effects of nitrogen deposition: Simulation of nitrogen saturation in a Sitka
665 spruce forest, Aber, Wales, UK. *Biogeochemistry* **1997**, *38*, 129-148.
- 666 54. Curtis, C. J.; Emmett, B. A.; Reynolds, B.; Shilland, J. How important is N₂O
667 production in removing atmospherically deposited nitrogen from UK moorland
668 catchments? *Soil Biology & Biochemistry* **2006**, *38*.
- 669 55. Dinsmore, K. J.; Skiba, U. M.; Billett, M. F.; Rees, R. M.; Drewer, J. Spatial and
670 temporal variability in CH₄ and N₂O fluxes from a Scottish ombrotrophic peatland:
671 Implications for modelling and up-scaling. *Soil Biology and Biochemistry* **2009**, *41*,
672 1315-1323.
- 673 56. Wade, A.; Durand, P.; Beaujouan, V.; Wessel, W.; Raat, K.; Whitehead, P.; Butterfield,
674 D.; Rankinen, K.; Lepisto, A. A nitrogen model for European catchments: INCA, new
675 model structure and equations. *Hydrol. Earth Syst. Sci.* **2002**, *6*, 559-582.
- 676 57. Bedard-Haughn, A.; Matson, A. L.; Pennock, D. J. Land use effects on gross nitrogen
677 mineralization, nitrification, and N₂O emissions in ephemeral wetlands. *Soil Biology &*
678 *Biochemistry* **2006**, *38*, 3398-3406.
- 679 58. Cardenas, L. M.; Thorman, R.; Ashlee, N.; Butler, M.; Chadwick, D.; Chambers, B.;
680 Cuttle, S.; Donovan, N.; Kingston, H.; Lane, S.; Dhanoa, M. S.; Scholefield, D.
681 Quantifying annual N₂O emission fluxes from grazed grassland under a range of
682 inorganic fertiliser nitrogen inputs. *Agric. , Ecosyst. Environ.* **2010**, *136*, 218-226.
- 683 59. Rafique, R.; Anex, R.; Hennessy, D.; Kiely, G. What are the impacts of grazing and
684 cutting events on the N₂O dynamics in humid temperate grassland? *Geoderma* **2012**,
685 *181-182*, 36-44.

- 686 60. Cowan, N. J.; Norman, P.; Famulari, D.; Levy, P. E.; Reay, D. S.; Skiba, U. M. Spatial
687 variability and hotspots of soil N₂O fluxes from intensively grazed grassland.
688 *Biogeosciences* **2015**, *12*, 1585-1596.
- 689 61. Beauchamp, E. G.; Trevors, J. T.; Paul, J. W. Carbon sources for bacterial denitrification.
690 *Adv. Soil Sci.* **1989**, *10*, 113-142.
- 691 62. Mathieu, O.; Leveque, J.; Henault, C.; Milloux, M. J.; Bizouard, F.; Andreux, F.
692 Emissions and spatial variability of N₂O, N₂ and nitrous oxide mole fraction at the field
693 scale, revealed with ¹⁵N isotopic techniques. *Soil Biology and Biochemistry* **2006**, *38*,
694 941-951.
- 695 63. Amha, Y.; Bohne, H. Denitrification from the horticultural peats: effects of pH, nitrogen,
696 carbon, and moisture contents. *Biol. Fertility Soils* **2011**, *47*, 293-302.
- 697 64. Wu, H.; Wiesmeier, M.; Yu, Q.; Steffens, M.; Han, X.; Koegel-Knabner, I. Labile organic
698 C and N mineralization of soil aggregate size classes in semiarid grasslands as affected
699 by grazing management. *Biol. Fertility Soils* **2012**, *48*, 305-313.
- 700 65. Sgouridis, F.; Heppell, C. M.; Wharton, G.; Lansdown, K.; Trimmer, M. Denitrification
701 and dissimilatory nitrate reduction to ammonium (DNRA) in a temperate re-connected
702 floodplain. *Water Res.* **2011**, *45*, 4909-4922.
- 703 66. Robson, T. M.; Lavorel, S.; Clement, J. C.; Le Roux, X. Neglect of mowing and
704 manuring leads to slower nitrogen cycling in subalpine grasslands. *Soil Biology and*
705 *Biochemistry* **2007**, *39*, 930-941.
- 706 67. Ollinger, S.; Smith, M.; Martin, M.; Hallett, R.; Goodale, C.; Aber, J. Regional variation
707 in foliar chemistry and N cycling among forests of diverse history and composition.
708 *Ecology* **2002**, *83*, 339-355.
- 709 68. Klemmedtsson, L.; Von Arnold, K.; Weslien, P.; Gundersen, P. Soil CN ratio as a scalar
710 parameter to predict nitrous oxide emissions. *Global Change Biol.* **2005**, *11*, 1142-1147.
- 711 69. Ullah, S.; Moore, T. R. Soil drainage and vegetation controls of nitrogen transformation
712 rates in forest soils, southern Quebec. *Journal of Geophysical Research-Biogeosciences*
713 **2009**, *114*, G01014.
- 714 70. Sexstone, A.; Revsbech, N.; Parkin, T.; Tiedje, J. Direct Measurement of Oxygen Profiles
715 and Denitrification Rates in Soil Aggregates. *Soil Sci. Soc. Am. J.* **1985**, *49*, 645-651.
- 716 71. Kulkarni, M. V.; Groffman, P. M.; Yavitt, J. B.; Goodale, C. L. Complex controls of
717 denitrification at ecosystem, landscape and regional scales in northern hardwood forests.
718 *Ecol. Model.* **2015**, *298*, 39-52.
- 719 72. Cuhel, J.; Simek, M.; Laughlin, R. J.; Bru, D.; Cheneby, D.; Watson, C. J.; Philippot, L.
720 Insights into the Effect of Soil pH on N₂O and N₂ Emissions and Denitrifier
721 Community Size and Activity. *Appl. Environ. Microbiol.* **2010**, *76*, 1870-1878.

- 722 73. Schrama, M.; Heijning, P.; Bakker, J. P.; van Wijnen, H. J.; Berg, M. P.; Olf, H.
723 Herbivore trampling as an alternative pathway for explaining differences in nitrogen
724 mineralization in moist grasslands. *Oecologia* **2013**, *172*, 231-243.
- 725 74. Billett, M. F.; Charman, D. J.; Clark, J. M.; Evans, C. D.; Evans, M. G.; Ostle, N. J.;
726 Worrall, F.; Burden, A.; Dinsmore, K. J.; Jones, T.; McNamara, N. P.; Parry, L.;
727 Rowson, J. G.; Rose, R. Carbon balance of UK peatlands: current state of knowledge
728 and future research challenges. *Climate Research* **2010**, *45*, 13-29.
- 729 75. Loisel, J.; Yu, Z.; Beilman, D. W.; Camill, P.; Alm, J.; Amesbury, M. J.; Anderson, D.;
730 Andersson, S.; Bochicchio, C.; Barber, K.; Belyea, L. R.; Bunbury, J.; Chambers, F. M.;
731 Charman, D. J.; De Vleeschouwer, F.; Fialkiewicz-Koziel, B.; Finkelstein, S. A.; Galka,
732 M.; Garneau, M.; Hammarlund, D.; Hinchcliffe, W.; Holmquist, J.; Hughes, P.; Jones,
733 M. C.; Klein, E. S.; Kokfelt, U.; Korhola, A.; Kuhry, P.; Lamarre, A.; Lamentowicz, M.;
734 Large, D.; Lavoie, M.; MacDonald, G.; Magnan, G.; Makila, M.; Mallon, G.;
735 Mathijssen, P.; Mauquoy, D.; McCarroll, J.; Moore, T. R.; Nichols, J.; O'Reilly, B.;
736 Oksanen, P.; Packalen, M.; Peteet, D.; Richard, P. J. H.; Robinson, S.; Ronkainen, T.;
737 Rundgren, M.; Sannel, A. B. K.; Tarnocai, C.; Thom, T.; Tuittila, E.; Turetsky, M.;
738 Valiranta, M.; van der Linden, M.; van Geel, B.; van Bellen, S.; Vitt, D.; Zhao, Y.;
739 Zhou, W. A database and synthesis of northern peatland soil properties and Holocene
740 carbon and nitrogen accumulation. *Holocene* **2014**, *24*, 1028-1042.
- 741 76. Whitehead, D. C. *Grassland nitrogen*; CAB International: Wallingford; UK, 1995; .
- 742 77. Vile, M. A.; Wieder, R. K.; Zivkovic, T.; Scott, K. D.; Vitt, D. H.; Hartsock, J. A.; Iosue,
743 C. L.; Quinn, J. C.; Petix, M.; Fillingim, H. M.; Popma, J. M. A.; Dynarski, K. A.;
744 Jackman, T. R.; Albright, C. M.; Wykoff, D. D. N₂-fixation by methanotrophs sustains
745 carbon and nitrogen accumulation in pristine peatlands. *Biogeochemistry* **2014**, *121*,
746 317-328.
- 747 78. Curtis, C. J.; Heaton, T. H. E.; Simpson, G. L.; Evans, C. D.; Shilland, J.; Turner, S.
748 Dominance of biologically produced nitrate in upland waters of Great Britain indicated
749 by stable isotopes. *Biogeochemistry* **2012**, *111*, 535-554.
- 750 79. Cleveland, C.; Townsend, A.; Schimel, D.; Fisher, H.; Howarth, R.; Hedin, L.; Perakis,
751 S.; Latty, E.; Von Fischer, J.; Elseroad, A.; Wasson, M. Global patterns of terrestrial
752 biological nitrogen (N₂) fixation in natural ecosystems. *Global Biogeochem. Cycles*
753 **1999**, *13*, 623-645.
- 754