
Article:
Langarica-Fuentes, A., Manrubia, M., Giles, M.E. et al. (2 more authors) (2018) Effect of model root exudate on denitrifier community dynamics and activity at different water-filled pore space levels in a fertilised soil. Soil Biology & Biochemistry, 120. pp. 70-79. ISSN 0038-0717

https://doi.org/10.1016/j.soilbio.2018.01.034

Highlights:

- Controlled microcosms used to assess exudate C x WFPS effects on denitrification
- The response of different denitrifier communities (*nirK*, *nirS*, *nosZ*) to C is distinct
- *nirS* and *nosZ-I* community size and structure strongly affected by carbon
- WFPS is a key factor modulating denitrifier community changes via C addition
- Higher WFPS and C concentrations resulted in greater denitrification rates

Title: Effect of model root exudate on denitrifier community dynamics and activity at different water-filled pore space levels in a fertilised soil

Authors and affiliations: Adrian Langarica-Fuentes[1], Marta Manrubia[2,3], Madeline E. Giles[1], Susan Mitchell[1], Tim J. Daniell[1,4]*

[1]The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK; [2]Department of Soil Quality, Wageningen University, PO Box 47, 6700 AA, Wageningen, the Netherlands; [3]Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), PO Box 50, 6700 AB Wageningen, the Netherlands; [4]Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, UK

*Corresponding author information:
E-mail address: t.j.daniell@sheffield.ac.uk
Full postal address: Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, UK
Telephone number: +44 (0)114 2220137

Article Type: Regular paper

Keywords: Denitrification, root exudate addition, microbial community dynamics, nitrous oxide emissions, *nirK*, *nirS*, *nosZ*

Conflict of interest: The authors declare no conflict of interest.

Abstract

Although a “rhizosphere effect” on denitrification rates has been established, a clear understanding of the effects of exudate addition on denitrifier community dynamics remains elusive. A microcosm experiment was designed to explore the interaction between exudate addition and soil moisture on community dynamics and denitrification rates. Artificial root exudate at 5 different carbon concentrations was added daily to soil microcosms at contrasting target WFPS (50, 70 and 90%). After a 7-day period, total denitrification and N_2O emission rates were measured and community dynamics assessed using molecular methods. The response of denitrifier genes to exudate addition was different, with *nirS* and *nos*Z-I showing a stronger effect than *nirK* and *nos*Z-II. Distinct community structures were observed for *nirS* and *nosZ*-I at 90% target WFPS when compared to 50% and 70%. *Nir*S denitrifier population size showed a ca. 5-fold increase in gene copy number at 90% WFPS when exudate was added at the highest C input. Significant total denitrification and N_2O emission rates were observed only at 90% WFPS, which increased with C input. Our study improves the understanding of the complex interaction between microbial communities, the abiotic environment and process rates which can inform management practices aimed at increasing complete denitrification and controlling greenhouse gas production from agriculture.

1. Introduction

Denitrification is a respiratory microbial process in which nitrate (NO_3^-) or nitrite (NO_2^-) are reduced to nitrogen gases (NO, N_2O and N_2) under oxygen limiting conditions (Tiedje, 1988; Philippot et al., 2007). It allows the maintenance of respiration through the use of nitrogen oxides as alternative electron acceptors (Zumft, 1997). This process is of great importance as, in addition to leading to significant N losses in agricultural systems (Rheinbaben, 1990; Shcherbak et al., 2014), it has negative environmental effects due to nitrous oxide (N_2O) production (Mosier et al., 1998; Bouwman et al., 2013). N_2O is an important greenhouse gas with a 100-year warming potential ≈ 300 greater than that of carbon dioxide (Forster et al., 2007) and has also been shown to contribute to ozone layer depletion (Cicerone, 1987). Atmospheric N_2O levels have increased by 19% since pre-industrial times with current estimates suggesting that agricultural soils contribute as much as 50-60% of N_2O global emissions (IPCC, 2007; Galloway et al., 2008; Bouwman et al., 2013).

The denitrification process occurs through a series of enzymatic steps, each performed by a specific reductase encoded by *narG*/*napA*, *nirK*/*nirS*, *norB* and *nosZ* genes (Zumft, 1997). Key process enzymes are nitrite reductase (encoded by *nirK* or *nirS*) catalysing the reduction of NO_2- to NO and N_2O reductase (encoded by *nosZ*) converting N_2O into inert N_2 (Zumft, 1997; Shapleigh, 2013). Graf et al. (2014) confirmed that denitrification is a modular pathway, with some bacteria possessing the full complement and others lacking genes thus performing a subset of the pathway being exclusively N_2O producers (K and S-denitrifier types), N_2O consumers (Z-types) or capable of both processes (KZ and SZ-types) (Jones et al., 2013; Graf et al., 2014). In general, denitrification is promoted under anaerobic conditions, high levels of NO_3^- and the presence of readily available carbon sources (Philippot et al., 2007) but overall denitrification rates, and the proportions of N_2 and N_2O produced, in soil are affected by the interaction of many environmental factors, such as pH, water holding capacity, pore structure, carbon and NO_3^- availability and O_2 levels (Morley

and Baggs, 2010; Attard et al., 2011; Butterbach-Bahl et al., 2013; Hu et al., 2015). Water-filled pore space (WFPS) has been widely used in denitrification studies as it integrates information about the water content and total porosity of a soil system and has been shown to be closely related to microbial activity and denitrification-derived N_2O emissions, which increase at levels above 60-70% WFPS (Clayton et al., 1997; Bateman and Baggs, 2005).

Plants alter denitrification rates in soil via the growth and activity of the root system (Woldendorp, 1962; Philippot et al., 2013) with increased denitrification in rhizospheric soil compared to bulk soil (Klemedtsson et al., 1987; Bakken, 1988; Mahmood et al., 1997). Rhizodeposition has been suggested as the main factor behind this "rhizosphere effect" (Philippot et al., 2007). It is estimated to account for approximately 11% of the net carbon fixed by photosynthesis or 27% of the allocated C to the roots (Jones et al., 2009) with exuded carbon stimulating the denitrifier community by providing a source of electron donor. Additionally, root respiration and exudation decreases O_2 availability, favouring denitrification (Bateman and Baggs, 2005; Hu et al., 2015). However, the high spatial and temporal variability of the denitrification process and the many factors affecting plant-microbe interactions in the rhizospheric soil (Groffman et al., 2009; Giles et al., 2012), have limited the understanding of the dynamics of this and other soil processes. To date, a handful of studies have attempted to elucidate the effect of carbon addition on the denitrifier community (e.g. Murray et al. (2004); Henry et al. (2008); Morley et al. (2014); Giles et al. (2017)). However, no conclusive links between C input quantity or quality, community dynamics and denitrification fluxes were demonstrated. Here, we carried out a controlled 7-day soil microcosm incubation with contrasting target WFPS levels (50, 70, 90%) in which the effect of daily additions of artificial root exudate (ARE) at variable carbon concentration (0 to 3 mg C day^{-1}, equivalent to a range between 0x and 2x the normal exudation rate of *L. perenne* (Paterson and Sim, 1999) on the denitrifier community size and structure, total denitrification ($N_2O + N_2$) and N_2O emission rates in soils was studied in an effort to reveal linkage

between the amount of C potentially exuded by plants, denitrifier community dynamics and activity.

2. Materials and Methods

2.1. Field soil

A brown forest soil of the Carpow series (Laing, 1976) with a sandy loam texture and a pH (measured in 0.01 M $CaCl_2$) of 5.95 was collected on April 2013 from an arable field at The James Hutton Institute (56°27'20"N, 3°04'35"W). Soil characteristics were 60% sand, 25% silt, 11% clay, 3.68% C and 0.12 % N. Soil dissolved organic carbon (DOC) was 14.38 ± 2.65 mg kg^{-1} dry soil. Soil was sieved to 4mm mesh size and stored at 4°C until microcosm preparation the following week. Moisture content was determined by drying at 105°C.

2.2. Experimental setup

A fully factorial soil microcosm experiment consisting of three target WFPS levels (50, 70 and 90%) and five root exudate C inputs (0, 0.375, 0.75, 1.5 and 3 mg C day^{-1}) was established. C input levels selected were equivalent to 0x (water-only control), 0.25x, 0.5x, 1x and 2x the normal exudation rate of the model grass species *Lolium perenne*, as previously estimated by Paterson and Sim (1999). Prior to microcosm set-up, potassium nitrate (KNO_3) was added to the soil as a solution and mixed thoroughly to obtain a rate of 100 mg N kg^{-1} dry soil and water content adjusted to 50% WFPS. The amount of N added is representative of the recommended fertiliser N addition rates under current UK agricultural practices (Defra, 2010) and is similar to that used in other developed countries (Potter et al., 2010). Additionally, it also falls within the range of N rates previously shown to stimulate denitrification and lead to the production of N_2O and N_2 (Giles et al., 2012; Morley et al., 2014). To establish the microcosms, 120 g dry soil equivalent was added to 150 identical plastic pots (6.6 cm height x 5 cm diameter) with 10 replicates and packed to a bulk density of 1.2 g cm^{-3}. Subsequently, water was added as required to establish the 70 and 90%

143 WFPS treatments. A completely randomized block design was established in a controlled
144 temperature room (darkness, 21°C) and pre-incubated for seven days prior to ARE addition.
145 Additional microcosms were established for each moisture level and sampled upon the start
146 of the experiment (day 0) to allow the evaluation of changes in community dynamics and soil
147 parameters between the start and the end of the experiment in water-only (0 C) controls.
148
149 **2.3. Artificial root exudate addition**
150 A single artificial root exudate mix containing five sugars (glucose, sucrose, fructose, ribose
151 and arabinose), five amino acids (glycine, valine, glutamine, serine and alanine) and five
152 organic acids (malic, citric, malonic, oxalic and fumaric acid) at equimolar C concentrations
153 was used for the experiment (Paterson et al., 2007). This ARE mix was previously designed
154 to represent a model mixture of the major chemical classes of carbon compounds (sugars,
155 amino acids and organic acids) found in plant root exudates (Uren, 2001; Paterson et al.,
156 2007; Dennis et al., 2010). Although amino acids also provided an additional N input to the
157 system, amino-N accounted for less than 25% of the total N added as KNO_3 in the highest C
158 input treatment. The KNO_3 added is known to be sufficient to support unrestricted
159 denitrification over the time frame of the experiment (Giles et al., 2012), and the amino-N
160 supplied was not expected to affect or alter this process.
161 Distinct ARE solutions at the different final C input levels previously mentioned (0 - 3 mg C
162 ml^{-1}) were prepared daily. After degassing, 1 ml of the corresponding ARE solution for each
163 treatment was applied to the centre of the soil microcosms using a syringe at a depth of 2
164 cm depth (simulating root exudation and generating a model “rhizospheric” zone in the
165 centre of the microcosm, Supplementary Fig S1). Microcosms were then immediately
166 adjusted to the target WFPS by further addition of water to the surface based on the
167 overnight weight loss from each microcosm. This rapid water adjustment ensured WFPS
168 changes around the injection zone due to ARE addition were kept to a minimum. ARE
169 solution application and moisture adjustment were repeated daily for seven days, thus
170 allowing sufficient time for the bacterial and denitrifier community to be affected by their

171 respective C inputs. After this period, gas sampling and soil sampling were carried out to
172 evaluate the effect of the 7-day ARE application regime on gas emissions and community
173 dynamics.
174
175 **2.4. Gas sampling and analysis**
176 After ARE addition and WFPS adjustment on day 7, microcosms were placed in 500 ml
177 Kilner jars and sealed. In half the microcosms for each treatment, 10% v/v of the headspace
178 air was replaced with acetylene (C_2H_2) to measure total denitrification rates ($N_2O + N_2$) by
179 inhibiting N_2O reductase activity. In the remaining half of the microcosms, headspace air was
180 not modified to allow the measurement of actual N_2O emission rates. All microcosms were
181 then incubated at 21 °C for a 4-hour period after which a 5.9 ml gas sample was taken using
182 a syringe and placed in a sealed Exetainer® vacuum vial (Labco Limited, Lampeter, UK).
183 Preliminary tests were run on the same soil and with extremes of treatments to determine
184 the headspace incubation time for the entire experiment taking samples every 30 minutes for
185 8 hours. N_2O was found to accumulate linearly over 8 hours in this system and a 4-hour
186 incubation reliably provided measurable N_2O concentrations. Although ideally several time
187 points should be taken our approach was used to allow the high number of samples required
188 in this experiment (data not shown). Samples were analysed for N_2O concentrations using a
189 TRACE™ Gas Chromatograph (GC) fitted with an electron capture detector (Thermo
190 Scientific, Hemel Hempstead, UK) maintained at 300 °C. GC response was calibrated using
191 certified standard N_2O gas mixtures with N_2O concentrations of 0.0001, 0.0002, 0.001,
192 0.0025 and 0.1 % v/v (Scientific & Technical Gases Ltd, Staffordshire, UK).
193
194 **2.5. Soil sampling**
195 After gas sampling, each soil microcosm was divided into two separate model compartments
196 ("bulk" and "rhizospheric" soil) for subsequent analyses. A core of 2.5 cm diameter and 4 cm
197 depth around the ARE application point was taken to represent the model "rhizospheric" soil,

198 while the remaining soil represented the model “bulk” soil (Supplementary Fig S1). Each soil
199 compartment was homogenised within each microcosm, and 1.5 g subsamples taken and
200 stored at -80 ℃ for DNA extraction. The remaining soil was stored at 4 ℃ prior to chemical
201 analyses. Preliminary experiments using methylene blue dye as a tracer suggested that ARE
202 added during the experiment predominantly remained in the “rhizospheric” zone.
203
204 **2.6. Soil chemical analyses**
205 Soil samples were extracted with 1M of KCl solution and analysed for inorganic N (NO_3-N
206 and NH_4-N) and dissolved organic carbon (DOC) using a Konelab Aqua 20 Chemistry
207 Analyzer (Thermo Scientific, Hemel Hempstead, UK) and a 1010 OI Total Organic Carbon
208 Analyser (OI Analytical, TX, USA), respectively. Separately, pH was measured in 0.01M
209 $CaCl_2$ using a MP220 pH meter (Mettler-Toledo GmbH, Switzerland) and moisture content
210 was assessed by drying at 105℃ for 24 hours (Hendershot et al., 2008).
211
212 **2.7. DNA extraction and community analyses**
213 Total DNA was extracted from “rhizospheric” and “bulk” soil samples in a 96-well format
214 using a phenol/chloroform extraction method as described in Deng et al. (2010) with addition
215 of an internal reference DNA target. Terminal Restriction Fragment Length Polymorphism (T-
216 RFLP) and relative real time PCR were used to assess changes in community structure and
217 size for the total bacterial community (16S rRNA) and the denitrifier community (*nirS*, *nirK*,
218 *nosZ*-I *and nosZ*-II denitrification genes). Due to the large number of samples processed (5
219 targeted genes x 300 samples), T-RFLP was selected for this study due to its cost-effective,
220 high sample throughput nature and its ability to produce highly comparable community
221 structure results to next generation sequencing technologies (Camarinha-Silva et al., 2012;
222 Pilloni et al., 2012).
223 For T-RFLP, gene fragments were amplified using the labelled primers and cycling
224 conditions described in Supplementary Table S1. A nested PCR approach was required for
225 *nirK* and *nirS*, while 16S, *nosZ*-I and *nosZ*-II were amplified in a single step. Each 15 µl PCR

reaction mix contained the following reagents: 1X PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM of each dNTP, 0.4 µg/µl of BSA, 1 U of Platinum® Taq DNA Polymerase (Invitrogen) and 1 µl of a 1:10 dilution of extracted total genomic DNA or first round product. T-RFLP was conducted by subjecting the final round PCR product to restriction enzyme digestion using *Alu*I for 16S, *Hae*III for *nirK*, *Ava*I for *nirS*, *Bsa*JI for *nosZ*-I and *Hpy*188III for *nosZ*-II selected through preliminary analyses of a restricted sample set. Restriction separation and post run analysis in GeneMapper® v 3.7 (Applied Biosystems, CA, USA) were as described in Cooper et al. (2011).

For relative real time PCR, 16S rRNA, *nirK, nirS*, *nosZ*-I and *nosZ*-II gene copy numbers were quantified using the added reference target as described in Daniell et al. (2012). The primer pairs and reaction conditions used are shown in Supplementary Table S1. Primer pairs selected have been consistently used to date in the literature for qPCR amplification of the different denitrifier groups (Jones et al., 2013; Domeignoz-Horta et al., 2015; Zhao et al., 2017). All amplifications were performed using the SYBR green I master mix (Roche, Burgess Hill, UK) under recommended conditions, 0.5 µM of each primer and 0.3 µg/µl of BSA on a Lightcycler® 480 Real time PCR system (Roche) with associated relative quantification software. Standard curves were generated using linearised plasmids containing gene fragments of interest from model organisms (*E. coli* for 16S, *Paracoccus denitrificans* for *nirS, Sinorhizobium meliloti* for *nirK* and *nosZ*-I, and an environmental clone for *nosZ*-II). The environmental clone sequence used as reference to generate the standard curve for *nosZ*-II has been deposited to the GenBank (NCBI) sequence database under accession number MG495619.

2.8 Data analyses

All statistical analyses were carried out using Genstat 16th edition (VSN International, Hemel Hempstead, UK), except the multivariate analysis of T-RFLP data, for which the vegan package (version 2.4.0) (Oksanen et al., 2016) in R was used (R Core Team, 2016).

ANOVA was used to determine the significance of WFPS, C Input and Compartment effects on gas emissions, soil chemical data, relative real time PCR gene copy number data and principal coordinate scores from the T-RFLP analysis. Total denitrification rates ($N_2O + N_2$), N_2O emission rates and NH_4-N data were log transformed prior to ANOVA in order to achieve an approximate Gaussian distribution. The 'Block/Pot' block structure was used in ANOVA when the Compartment factor was present, to account for the nested structure of the experimental design. For the T-RFLP data analysis, relative abundance matrices were log transformed using the 'decostand' function in vegan and then subjected to principal coordinate analysis (PCoA) using the alternative Gower dissimilarity matrix (Anderson et al., 2006) as implemented in the 'vegdist' function.

3. Results

3.1. Soil physico-chemical analyses

DOC, NO_3-N, NH_4-N, pH and soil water content data for the microcosms at the end of the experiment are summarised in the below subsections and in Fig 1, Supplementary Fig S2 and Supplementary Tables S2 and S3. Additional analyses assessing these parameters for water-only controls and day 0 samples, showed no changes in most parameters except DOC and NO_3-N, which are discussed below (see Supplementary Information for a detailed description for details). Additionally, acetylene addition was shown to have no significant effect on any measured parameter.

3.1.1. Dissolved organic carbon (DOC)

At the end of the experiment, equal or lower DOC values to those found at day 0 were observed for all treatments, despite the high C amounts supplied in the ARE (Fig 1A). A compartment effect was observed, with "rhizospheric" soil having lower DOC values than "bulk" soil ($p<0.001$) at the end of the experiment (Supplementary Table S2). Additionally,

280 WFPS was also significant ($p<0.001$) with lower DOC values for the 50% treatment than
281 those observed for 70% and 90%.
282
283 **3.1.2. Nitrate (NO_3-N)**
284 NO_3-N levels in the water-only controls were the same as those observed in day 0 for the 90
285 and 70% WFPS, but showed a significant decrease ($p<0.01$) at 50% WFPS (84.21 ± 3.0 mg
286 kg^{-1}). When C was supplemented in the ARE, NO_3-N showed a highly significant decline
287 ($p<0.001$) at 90% WFPS as C input increased while the other treatments showed no overall
288 variation (Fig 1B and Supplementary Table S2). This effect was strongest in the
289 "rhizospheric" soil (from ≈100 to 60 mg N kg^{-1} dry soil) but "bulk" soil also showed a
290 significant decrease in NO_3-N levels as C amount increased.
291
292 **3.1.3. Ammonium (NH_4-N)**
293 At the end of the experiment, the "rhizospheric" soil in all treatments showed higher NH_4-N
294 levels than "bulk" soil as C and amino acid input increased ($p<0.001$) (Supplementary Table
295 S2). Although net N mineralisation was observed, final ammonium levels in the
296 "rhizospheric" soils were much lower than nitrate levels for all treatments (Supplementary Fig
297 2A), with the highest values observed at 50% (32 mg N kg^{-1} dry soil) and 90% WFPS (21 N
298 kg^{-1} dry soil) for 3 mg C day^{-1} input, while all other treatments had values below 10 mg N kg^{-1}
299 dry soil (Supplementary Table S3).
300
301 **3.1.4. pH and water content**
302 A small but significant pH rise ($p<0.001$) in the "rhizospheric" soil at 90% WFPS was
303 observed (6.02 to 6.32) as C input increased (Supplementary Fig 2B and Table S2). For all
304 other treatments, pH remained unchanged from the water-only controls. WFPS of the
305 microcosms averaged 46, 65 and 81 % at the end of the experiment, slightly lower than the
306 respective targets of 50, 70 and 90% WFPS indicating a small but substantial drying of the
307 microcosms during gas and soil sampling. Minor but significant differences (<2% WFPS)

were found among some treatments between “bulk” and “rhizospheric” soil (Supplementary Fig 2C).

3.2. Total denitrification (N_2O and N_2) and N_2O emissions rates

Total denitrification rates assessed using the acetylene inhibition technique showed denitrification activity was very low (<1.5 ng N_2O-N g dry soil^{-1} h^{-1}) in the water-only controls and all the 50 and 70% WFPS treatments (Fig 2A). Significant denitrification rates were only observed at the 90% WFPS treatment and increased with C input ($p<0.001$) (ranging from 17.9 to 118.2 ng N_2O-N g dry soil^{-1} h^{-1}) (Fig 2A and Table S2).

Comparable to the denitrification rates result, the water-only controls and 50 and 70% WFPS treatments showed no significant N_2O emission rates (Fig 2B). Significant although highly variable N_2O emission rates were only observed at 90% WFPS after C addition ($p<0.05$, Table S2), ranging from 6.5 to 27.6 ng N_2O-N g dry soil^{-1} h^{-1} and increasing with C concentration. N_2O emission rates at 90% WFPS were 3-4 times lower than total denitrification rates and more variable (Fig 2B). As actual WFPS at the time of soil sampling differed from the target WFPS, linear regression of actual WFPS against both total denitrification rates and N_2O emission rates was performed. The test was performed with all target WFPS and C treatments were included in this analysis and with each WFPS treatment separately. There was no significant relationship between WFPS and either total denitrification rates or rates of N_2O emission.

3.3. Soil microbial community structure and size

The results of the denitrifier and general microbial community size and structure analyses, performed using relative real time PCR and T-RFLP, respectively, are summarised in Fig 3, Tables 1 and 2 and Supplementary Tables S4 and S5. Similarly to the physico-chemical parameters measured, microbial community data in the water-only controls showed no significant differences to the values from day 0 (Supplementary Information).

3.3.1. Total bacterial community (16S rRNA)

The two-way interactions Compartment × C input ($p<0.001$) and Compartment × WFPS ($p<0.01$) (Table 1) were highly significant for the 16S rRNA gene copy number at the end of the experiment and were driven by a marked increase in "rhizospheric" soil gene copy count with C input (Fig 3A). This did not occur in "bulk" soils. At the highest C input (3 mg C day^{-1}), "rhizospheric" soil gene copy numbers were significantly different between WFPS levels, with 50% WFPS showing a higher copy number (mean 3.90 x 10^9 copies g^{-1} dry weight soil), than 70% (3.0 x 10^9 copies g^{-1} dry weight soil) and 90% WFPS (2.7 x 10^9 copies g^{-1} dry weight soil). PCoA analysis of the 16S rRNA T-RFLP data revealed significant differences in bacterial community structure (Fig 3B, Table 2). Both PC1 (16% of variation) and PC2 (12% of variation) showed a significant three-way interaction ($p<0.001$) driven by "rhizospheric" soils responding to C input differently between WFPS levels while "bulk" soils did not vary from water-only controls. In PC2, significant separation between the 90, 70 and 50% WFPS rhizospheric soils can be observed ($p<0.001$). In PC1, increasing separation from the water-only controls can be observed in the rhizospheric soils as C input increases ($p<0.001$).

3.3.2. Nitrite-reducing community (*nirS* and *nirK*)

The two groups of nitrite reducers (*nirS* and *nirK*) responded differently to C addition. The effect of C addition and WFPS on the *nirS* community was much more pronounced than in *nirK* (Fig 3C-3F). All the interactions and their main factors had strong effects on the *nirS* copy number ($p<0.001$) (Table 1). These responses were driven by "rhizospheric" soil copy number being differently affected at the three WFPS levels as C input increased (Fig 3E). The strongest effect was observed at 90% WFPS, with 3.2, 5.5 and 6-fold increase in *nirS* copy numbers with higher C input (0.75, 1.5 and 3 mg C day^{-1}, respectively) compared to the control. In these treatments, the *nirS/nirK* ratio was shifted with *nirS* numbers surpassing those of *nirK (*1.5, 1.7 and 2.4-fold), contrary to that observed in the controls. PCoA analysis of the *nirS* T-RFLP showed that PC1 (14% of variation) and PC3 (7% of variation) had strong effects in community structure in relation to three main factors and all their two-way

364 interactions (Fig 3F and Table 2). In PC1, “rhizospheric” samples at 50 and 70% WFPS
365 showed significant separation from “bulk” and water-only samples at C inputs above 0.75 mg
366 C day^{-1} ($p<0.05$), but 90% did not. In contrast, in PC3, it is the 90% “rhizospheric” samples at
367 C inputs above 0.375 mg C day^{-1} that show significant separation from the rest of the
368 samples ($p<0.05$).
369 In contrast, the *nirK* community showed moderate to minor changes in size and structure
370 (Fig 3C and 3D). the interaction Compartment × WFPS ($p<0.05$) was shown to have a
371 significant effect on *nirK* copy numbers (Table 1). Upon examination, this can be attributed to
372 differences in copy number at the different WFPS in the “bulk” soil samples despite high
373 variation (Fig 3C). PCoA analysis of *nirK* T-RFLP data showed the most significant effects of
374 C and WFPS were found in PC1 and PC3 (13 and 7% of the variation, respectively) although
375 they were mostly driven by the distinct and unsystematic response of different WFPS and
376 compartment treatments to C input. In both PCs, the most relevant response observed is an
377 overall separation between “bulk” and “rhizospheric” samples ($p<0.001$), however this effect
378 is not uniform to all treatments.
379
380 **3.3.3. N_2O-reducing community (*nosZ*-I and *nosZ*-II)**
381 The effect of C addition on the community dynamics of the two *nosZ* gene clades was also
382 contrasting, with *nosZ*-I responding strongly to C addition while *nosZ*-II remained mostly
383 unaffected (Fig 3G-3J). All interactions and main factors had significant effects on *nosZ*-I
384 gene copy number ($p<0.001$) (Table 1). Responses were driven by rhizospheric soil being
385 differently affected by increases in C input across WFPS (Fig 3G). The strongest response
386 to C input was at 50% WFPS, followed by 70% and 90%. At 3 mg C day^{-1}, the three WFPS
387 levels showed significantly different copy numbers ($p<0.001$), with 50% WFPS at 6.20×10^7
388 copies g^{-1} dry weight soil, followed by 70% (4.76×10^7 copies g^{-1} dry weight soil) and 90%
389 (2.48×10^7 copies g^{-1} dry weight soil). These values represented a 9.5, 4.9 and 4.4-fold
390 increase from the water-only controls, respectively. *NosZ*-II community size showed minor
391 changes in response to ARE (Fig 3I). WFPS was the only factor affecting gene copy number

392 ($p<0.05$) (Table 1), with 70% WFPS having significantly higher values (2.18 x 10^7 copies g^{-1}
393 dry weight soil) than 50 or 90% WFPS (1.91 x 10^7 copies g^{-1} dry weight soil and 1.84 x 10^7
394 copies g^{-1} dry weight soil, respectively). In the *nosZ-I* T-RFLP data, PC1 (18% of variation)
395 showed community structure effects connected mainly to WFPS ($p<0.001$) and the three-
396 way interaction ($p<0.01$), while in PC3 (6.32% of variation) the Compartment effect was
397 strongest ($p<0.001$) (Table 2). In PC1, clear separation between 90% WFPS and the other
398 two WFPS treatments can be observed (Fig 3H). Meanwhile, in PC3, "bulk" and
399 "rhizospheric" samples show clear separation ($p<0.001$) while 50% WFPS samples also
400 show lower values in this component than 90% and 70% ($p<0.001$). *NosZ*-II T-RFLP data
401 showed very minor or no significant effects (Table 2). In both PC1 (18% of variation) and
402 PC2 (14%), there was no significant separation pattern between water-only controls and
403 most "bulk" and "rhizospheric" samples at the different WFPS levels (Fig 3J).
404
405 **4. Discussion**
406
407 Several studies have demonstrated a "rhizosphere effect" on denitrification rates, with
408 Woldendorp (1962) being the first comprehensive study to show that the living root system
409 increased the amount of N lost via denitrification in grassland soils. Subsequently, other
410 work has confirmed the generality of this effect in different plants groups (Bakken, 1988) and
411 quantified the increase in denitrification activity both in microcosm experiments
412 (Klemedtsson et al., 1987; Philippot et al., 2006) and at the field scale (Mahmood et al.,
413 1997). While several explanations have been suggested for this effect, such as changes in
414 oxygen pressure caused by the roots via respiration and changes in water content, carbon
415 and nitrogen availability via rhizodeposition (Philippot et al., 2013), the individual effects of
416 these factors have been difficult to separate. To date, a clear understanding of the effects of
417 carbon addition from root exudates on denitrifier community dynamics and their subsequent
418 influence on N_2O production remains elusive. To our knowledge, two studies have attempted
419 to use artificial exudate addition to isolate effects of carbon release in soil. Mounier et al.

(2004) studied the effects of adding maize mucilage on soil denitrification rates and *narG* *nosZ*-I diversity using RFLP and clone libraries, while Henry et al. (2008) investigated the effect of four different ARE solutions on denitrification activity, N_2O emissions and *narG*, *napA*, *nirK*, *nirS* and *nosZ*-I community dynamics. Both studies observed a strong increase in denitrification rates but minor changes of the denitrifier community dynamics, prompting the suggestion by the authors that carbon was not a strong driver of soil denitrifier structure and abundance (Philippot et al., 2007). Here, a short-term microcosm experiment evaluated changes in soil community dynamics with daily ARE addition. In contrast to previous attempts, different target WFPS levels were included as well as ARE with different total C additions allowing a variable set of conditions with a higher opportunity to detect differences. The rationale behind the selected target WFPS levels was to yield highly aerobic (50% WFPS), low oxygen (70%) and largely anaerobic (90%) systems providing a range of conditions from the unfavourable to optimal for denitrification activity. By using an ARE model system, plant effects (e.g. oxygen reduction via respiration, root architecture, nutrient uptake, variation in exudate amount and quality) were removed allowing evaluation of exudate carbon effects in isolation.

The soil used in the experiment had a moderate to low total C content (approx. 3.6%) which lies within the normal C range found in Scottish arable soils (Lilly et al., 2012). C supplied during the experiment would therefore greatly stimulate soil microbial activity. Soil DOC levels measured before and after the 7-day incubation showed the readily available carbon added in the ARE was fully utilised (Fig 1A), as previously seen in other studies in which carbon compounds added to soil are quickly consumed within hours of being added (Gilmour and Gilmour, 1985; Giles et al., 2017). The significantly lower values in the "rhizospheric" soil when compared with "bulk" soil would also suggest a possible positive priming effect on C utilisation (Cheng, 2009; Wang et al., 2016), although this effect was minor.

Microbial community analyses provided conclusive evidence that C addition drives changes in total bacteria and denitrifier community size and structure. A highly dynamic, differential

response to C input was observed among the four functional targets studied, with *nirS* and *nosZ-I* shown to be much more responsive than *nirK* and *nosZ-II* to C addition. Moreover, contrasting community changes were observed depending on the abiotic conditions (WFPS). Strongest effects were observed at microcosms watered to 50% and 90% target WFPS with high C input suggesting that previous attempts, using microcosms at 70% WFPS, may have been too variable in oxygen status to affect denitrifier community dynamics significantly especially without the tight localisation of exudates undertaken here.

While the "rhizospheric" *nirK* community size showed a moderate response to ARE addition (Fig 3C), the "rhizospheric" *nirS* community was strongly stimulated as C input increased, particularly under anaerobic conditions (90% target WFPS) (Fig 3E) which was distinct from the greater response of the total bacterial community (16S) at lower target WFPS levels. Analysis of community structure showed that changes were again more pronounced in *nirS* than *nirK*, showing a distinct community shift between aerobic or partially aerobic (50 and 70% WFPS) and predominantly anaerobic conditions (90%). Niche differentiation between *nirK* and *nirS* denitrifiers has been suggested with differential response to environmental factors such as pH, N and P availability, Cu soil content and moisture (Priemé et al., 2002; Enwall et al., 2010; Hamonts et al., 2013; Xie et al., 2014; Ma et al., 2016). This study confirms that *nirS* denitrifiers are sensitive to C addition under anaerobic conditions when N supply is abundant with copy numbers enriched at 90% WFPS relative to both total bacteria and *nosZ* genes, demonstrating selection of denitrifier populations that can produce but not consume N_2O (S-type denitrifiers). Similar results where the *nirS* community responded strongly to exudation under anoxic conditions has been observed in the rhizosphere of wheat (Hamonts et al., 2013), and maize (Florio et al., 2017). The underlying reasons for the strong effect of C on the *nirS* community need to be studied further but could be that this subgroup contains several copiotrophic bacterial taxa which thrive at anaerobic conditions (Hamilton et al., 2015). A differentiated response to ARE addition was also observed between the two clades of *nosZ*, with *nosZ-I* community dynamics being strongly affected by C input, while the *nosZ-II* community was virtually unresponsive. *NosZ-I* community size

476 responded in a similar manner to the general bacterial community (with the largest size
477 increase occurring at lower WFPS) although with a stronger response in *nosZ-I* with a
478 consequent enriching effect. *NosZ-I* community structure changes were similar to those of
479 16S and *nirS*, shifting into separate directions between aerobic or partially aerobic (50 and
480 70% target WFPS) and mostly anaerobic (90%), indicating selection within the community in
481 response to C and abiotic conditions. The recent characterisation of *nosZ-II* limits the
482 available knowledge of this clade but Jones et al. (2014) and Domeignoz-Horta et al. (2015)
483 have suggested niche partitioning between the clades, with the *nosZ-I* community being
484 more sensitive to changes in soil textural factors (e.g. SOM, C/N ratio) than *nosZ-II*, and
485 *nosZ-II* being more responsive to changes in pH and agricultural practices than *nosZ-I*. This
486 study provides further evidence of partitioning driven by response to C addition.
487 For the measurement of total denitrification rates ($N_2O + N_2$), the acetylene block method
488 was used (Tiedje et al., 1989). Although this method is known to have some limitations, such
489 as the potential for C_2H_2 to be used as a carbon source for denitrification (Butterbach-Bahl et
490 al., 2013), the absence of denitrification activity in the water-only controls indicates that over
491 the short incubation time of the experiment there was no acetylene effect. N_2O emissions
492 and total denitrification rates ($N_2O + N_2$) at 50 and 70% target WFPS showed no significant
493 denitrification activity or N_2O production from other sources (e.g. nitrification). Soil chemical
494 analyses (Fig 1 and Supplementary Fig S2) suggested that the absence of denitrification
495 activity and N_2O production was not driven by shifts towards unfavourable pH or nitrate
496 limitation, as nitrate after the 7-day experiment was abundant and relatively stable at all C
497 addition levels for these target WFPS. No denitrification activity was expected at 50% target
498 WFPS, where aerobic respiration would be favoured due to high oxygen availability (Zumft,
499 1997; Hu et al., 2015). However, at 70% target WFPS we expected to observe denitrification
500 activity as previous studies have reported this WFPS level as conducive to denitrification and
501 N_2O production (Bateman and Baggs, 2005; Ullah et al., 2005). The absence of
502 denitrification at 70% target WFPS was surprising but could be linked to the water loss
503 observed by the water content at sampling, as strong reductions in activity have been

observed when WFPS drops from 70 to 60% WFPS (Dobbie and Smith, 2001; Bateman and Baggs, 2005). Denitrifier community size and structure changes observed at lower WFPS levels demonstrate this facultative functional group is highly dynamic even when the community is respiring aerobically. Observed changes in size and structure were a result of growth from aerobic respiration with *nosZ-I* type denitrifiers being more effective than other types when competing for C with other aerobic bacteria and increasing their ratio when compared to 16S after ARE addition (Supplementary Table S5). Further research is required to determine whether denitrifier community changes occurring at aerobic conditions have an impact on N_2O emissions and denitrification activity as a system transiently shifts to an anaerobic state due to, for example, rain events or flooding.

Significant total denitrification rates ($N_2O + N_2$) were only observed in the 90% target WFPS treatment (Fig 2A) where activity was expected to be high as the system was mostly anaerobic. High rates were driven by C addition, relieving limitation under anaerobic conditions as previously suggested (Philippot et al., 2007; Philippot et al., 2013). Denitrification activity was also reflected in the soil chemical analyses of the rhizospheric soil, which showed a small pH increase (likely caused by conversion of OH^- ions produced in denitrification to bicarbonate/carbonate ions (Drtil et al., 1995)) and a strong decrease in NO_3-N levels as C input increased (Fig 1B). Actual N_2O emission rates measured at 90% target WFPS, demonstrate that most of the N_2O produced by denitrification was being converted to N_2 although the large variation suggests that this conversion is not uniform potentially due to spatial variability in structure driving variation in nitrous oxide reductase activity (Morley and Baggs, 2010; Giles et al., 2012)

Comparison of the community dynamics information with total denitrification and N_2O emission rates at 90% target WFPS suggests a connection between the strong "rhizospheric" *nirS* community dynamics shift and the flux observed with C input. Although *nosZ-I* and *nirK* communities also showed important community dynamics shifts, the *nirS* response was more pronounced resulting in an enrichment of denitrifiers possessing *nirS* but neither *nosZ* clade (1.38 ratio total nosZ:nirS at 90%WFPS with no carbon added vs. 0.46

with highest carbon addition) (Supplementary Table S5). It is possible that his enrichment in near *nirS* may be indicative of an increase in S-type denitrifiers, if so it is unclear if this is transient and decreases to its initial level once C is no longer supplied or if these changes remain in the long term.

Having established a clear response between C addition, changes in community dynamics and flux, further work will need to understand the generality of the response across soil types and how it is moderated by other factors such as the C composition of root exudates, C/N balance and land use history. Additionally, the interaction of this effect with other root/plant-related changes in the rhizosphere (formation of biofilm and mycorrhizal networks, secondary metabolite signalling, water uptake and root respiration) could affect the results observed and experiments including plant systems should be performed to determine the real significance of C release in a "true" rhizospheric zone. However despite these limitations these findings of our study could have important implications, especially under conditions with poor N_2O sink capacity where N_2O production may be higher than consumption.

4.1. Conclusions

For denitrification the response and sensitivity of the different gene complement communities to C addition is distinct at different target WFPS. *NirS* containing denitrifiers in particular appear to be strongly stimulated by C input when denitrification conditions are optimal. We suggest that the differences in soil microbial communities between our treatments are not solely structural but that they can have an impact on soil processes, as shown by the differences in denitrification rates and N_2O emissions observed. Increased understanding at this fundamental level helps reveal the interaction between microbial communities, the abiotic environment and process rates which can inform management practices aimed at increasing complete denitrification aiding control of greenhouse gas production from agriculture.

5. Acknowledgements

This work was funded by the Scottish Government through the Rural and Environment Science and Analytical Services division (RESAS). We thank Katharine Preedy (Biomathematics and Statistics Scotland) for statistical advice.

6. References

Anderson, M.J., Ellingsen, K.E., McArdle, B.H., 2006. Multivariate dispersion as a measure of beta diversity. Ecology Letters 9, 683-693.

Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., Philippot, L., Schmid, B., Le Roux, X., 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology 17, 1975-1989.

Bakken, L.R., 1988. Denitrification under different cultivated plants - Effects of soil moisture tension, nitrate concentration, and photosynthetic activity. Biology and Fertility of Soils 6, 271-278.

Bateman, E.J., Baggs, E.M., 2005. Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. Biology and Fertility of Soils 41, 379-388.

Bouwman, A.F., Beusen, A.H.W., Griffioen, J., Van Groenigen, J.W., Hefting, M.M., Oenema, O., Van Puijenbroek, P.J.T.M., Seitzinger, S., Slomp, C.P., Stehfest, E., 2013. Global trends and uncertainties in terrestrial denitrification and N_2O emissions. Philosophical Transactions of the Royal Society of London B: Biological Sciences 368, 1621.

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical Transactions of the Royal Society of London B: Biological Sciences 368, 1621.

Camarinha-Silva, A., Wos-Oxley, M.L., Jáuregui, R., Becker, K., Pieper, D.H., 2012. Validating T-RFLP as a sensitive and high-throughput approach to assess bacterial diversity patterns in human anterior nares. FEMS Microbiology Ecology 79, 98-108.

Cheng, W., 2009. Rhizosphere priming effect: Its functional relationships with microbial turnover, evapotranspiration, and C–N budgets. Soil Biology & Biochemistry 41, 1795-1801.

Cicerone, R.J., 1987. Changes in stratospheric ozone. Science 237, 35-42.

Clayton, H., McTaggart, P.I., Parker, J., Swan, L., Smith, A.K., 1997. Nitrous oxide emissions from fertilised grassland: A 2-year study of the effects of N fertiliser form and environmental conditions. Biology and Fertility of Soils 25, 252-260.

Cooper, J.M., Burton, D., Daniell, T.J., Griffiths, B.S., Zebarth, B.J., 2011. Carbon mineralization kinetics and soil biological characteristics as influenced by manure addition in soil incubated at a range of temperatures. European Journal of Soil Biology 47, 392-399.

598 Daniell, T.J., Davidson, J., Alexander, C.J., Caul, S., Roberts, D.M., 2012. Improved real-
599 time PCR estimation of gene copy number in soil extracts using an artificial reference.
600 Journal of Microbiological Methods 91, 38-44.

601 Defra, 2010. Fertiliser manual RB209. London, UK: Department for Environment, Food and
602 Rural Affairs.

603 Deng, H., Zhang, B., Yin, R., Wang, H.-l., Mitchell, S., Griffiths, B., Daniell, T., 2010. Long-
604 term effect of re-vegetation on the microbial community of a severely eroded soil in sub-
605 tropical China. Plant and Soil 328, 447-458.

606 Dennis, P.G., Miller, A.J., Hirsch, P.R., 2010. Are root exudates more important than other
607 sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS
608 Microbiology Ecology 72, 313-327.

609 Dobbie, K.E., Smith, K.A., 2001. The effects of temperature, water-filled pore space and land
610 use on N_2O emissions from an imperfectly drained gleysol. European Journal of Soil Science
611 52, 667-673.

612 Domeignoz-Horta, L., Spor, A., Bru, D., Breuil, M.-C., Bizouard, F., Leonard, J., Philippot, L.,
613 2015. The diversity of the N_2O reducers matters for the N_2O:N_2 denitrification end-product
614 ratio across an annual and a perennial cropping system. Frontiers in Microbiology 6, 971.

615 Drtil, M., Németh, P., Kucman, K., Bodík, I., Kašperek, V., 1995. Acidobasic balances in the
616 course of heterotrophic denitrification. Water Research 29, 1353-1360.

617 Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M., Hallin, S., 2010. Soil resources
618 influence spatial patterns of denitrifying communities at scales compatible with land
619 management. Applied and Environmental Microbiology 76, 2243-2250.

620 Florio, A., Pommier, T., Gervaix, J., Bérard, A., Le Roux, X., 2017. Soil C and N statuses
621 determine the effect of maize inoculation by plant growth-promoting rhizobacteria on
622 nitrifying and denitrifying communities. Scientific Reports 7, 8411.

623 Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W., Haywood, J.,
624 Lean, J., Lowe, D.C., Myhre, G., 2007. Changes in atmospheric constituents and in radiative
625 forcing, In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Tignor, M.,
626 Miller, H. (Eds.), Climate Change 2007. The Physical Science Basis. Contribution of Working
627 Group I o the Fourth Assessment Report of the Intergovernmental Panel on Climate
628 Change. Cambridge University Press.

629 Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z.C., Freney, J.R.,
630 Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle:
631 Recent trends, questions, and potential solutions. Science 320, 889-892.

632 Giles, M.E., Daniell, T.J., Baggs, E.M., 2017. Compound driven differences in N_2 and N_2O
633 emission from soil; the role of substrate use efficiency and the microbial community. Soil
634 Biology & Biochemistry 106, 90-98.

635 Giles, M.E., Morley, N.J., Baggs, E.M., Daniell, T.J., 2012. Soil nitrate reducing processes –
636 drivers, mechanisms for spatial variation and significance for nitrous oxide production.
637 Frontiers in Microbiology 3, 407.

638 Gilmour, C.M., Gilmour, J.T., 1985. Assimilation of carbon by the soil biomass. Plant and
639 Soil 86, 101-112.

640 Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic comparisons highlight modularity of
641 the denitrification pathway and underpin the importance of community structure for N_2O
642 emissions. PLoS ONE 9, e114118.

643 Groffman, P.M., Butterbach-Bahl, K., Fulweiler, R.W., Gold, A.J., Morse, J.L., Stander, E.K.,
644 Tague, C., Tonitto, C., Vidon, P., 2009. Challenges to incorporating spatially and temporally
645 explicit phenomena (hotspots and hot moments) in denitrification models. Biogeochemistry
646 93, 49-77.

647 Hamilton, S.K., Doll, J.E., Robertson, G., 2015. The ecology of agricultural landscapes:
648 Long-term research on the path to sustainability. Oxford University Press, USA.

649 Hamonts, K., Clough, T.J., Stewart, A., Clinton, P.W., Richardson, A.E., Wakelin, S.A.,
650 O'Callaghan, M., Condron, L.M., 2013. Effect of nitrogen and waterlogging on denitrifier
651 gene abundance, community structure and activity in the rhizosphere of wheat. FEMS
652 Microbiology Ecology 83, 568-584.

653 Hendershot, W., Lalande, H., Duquette, M., 2008. Soil reaction and exchangeable acidity, In:
654 Carter, M., Gregorich, E. (Eds.), Soil Sampling and Methods of Analysis, Second Edition.
655 CRC Press, London, pp. 173-196.

656 Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Cheneby, D., Bizouard, F.,
657 Germon, J.C., Philippot, L., 2008. Disentangling the rhizosphere effect on nitrate reducers
658 and denitrifiers: insight into the role of root exudates. Environmental Microbiology 10, 3082-
659 3092.

660 Hu, H.-W., Chen, D., He, J.-Z., 2015. Microbial regulation of terrestrial nitrous oxide
661 formation: understanding the biological pathways for prediction of emission rates. FEMS
662 Microbiology Reviews 39, 729-749.

663 IPCC, 2007. Climate Change 2007: The Physical Science Basis. Contribution of Working
664 Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate
665 Change, In: Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor
666 and H.L. Miller (Ed.), Cambridge, UK and New York, NY, USA.

667 Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., Hallin, S., 2013. The unaccounted yet
668 abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISME J
669 7, 417-426.

670 Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.-C., Bru, D., Lemanceau, P., Griffiths, B.,
671 Hallin, S., Philippot, L., 2014. Recently identified microbial guild mediates soil N_2O sink
672 capacity. Nature Climate Change 4, 801-805.

673 Jones, D.L., Nguyen, C., Finlay, R.D., 2009. Carbon flow in the rhizosphere: carbon trading
674 at the soil–root interface. Plant and Soil 321, 5-33.

675 Klemedtsson, L., Svensson, B.H., Rosswall, T., 1987. Dinitrogen and nitrous oxide produced
676 by denitrification and nitrification in soil with and without barley plants. Plant and Soil 99,
677 303-319.

678 Laing, D., 1976. The soils of the country round Perth, Arbroath and Dundee. Sheets 48 and
679 49. HMSO, Edinburgh.

680 Lilly, A., Baggaley, N., Donnelly, D., 2012. Map of soil organic carbon in top soils of
681 Scotland. Map prepared for EU project GS-SOIL -Assessment and strategic development of
682 INSPIRE compliant Geodata-Services for European Soil Data. ECP-2008-GEO-318004.

683 Ma, W., Jiang, S., Assemien, F., Qin, M., Ma, B., Xie, Z., Liu, Y., Feng, H., Du, G., Ma, X., Le
684 Roux, X., 2016. Response of microbial functional groups involved in soil N cycle to N, P and
685 NP fertilization in Tibetan alpine meadows. Soil Biology and Biochemistry 101, 195-206.

686 Mahmood, T., Ali, R., Malik, K.A., Shamsi, S.R.A., 1997. Denitrification with and without
687 maize plants (*Zea mays L.*) under irrigated field conditions. Biology and Fertility of Soils 24,
688 323-328.

689 Morley, N., Baggs, E.M., 2010. Carbon and oxygen controls on N_2O and N_2 production
690 during nitrate reduction. Soil Biology & Biochemistry 42, 1864-1871.

691 Morley, N.J., Richardson, D.J., Baggs, E.M., 2014. Substrate induced denitrification over or
692 under estimates shifts in soil N_2/N_2O Ratios. PLoS ONE 9, e108144.

693 Mosier, A., Kroeze, C., Nevison, C., Oenema, O., Seitzinger, S., van Cleemput, O., 1998.
694 Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen
695 cycle - OECD/IPCC/IEA phase II development of IPCC guidelines for national greenhouse
696 gas inventory methodology. Nutrient Cycling in Agroecosystems 52, 225-248.

697 Mounier, E., Hallet, S., Cheneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C.,
698 Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J.C., Philippot, L., 2004. Influence of
699 maize mucilage on the diversity and activity of the denitrifying community. Environmental
700 Microbiology 6, 301-312.

701 Murray, P.J., Hatch, D.J., Dixon, E.R., Stevens, R.J., Laughlin, R.J., Jarvis, S.C., 2004.
702 Denitrification potential in a grassland subsoil: effect of carbon substrates. Soil Biology &
703 Biochemistry 36, 545-547.

704 Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin,
705 P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H.,
706 2016. vegan: Community Ecology Package, version 2.4-0, https://CRAN.R-
707 project.org/package=vegan.

708 Paterson, E., Gebbing, T., Abel, C., Sim, A., Telfer, G., 2007. Rhizodeposition shapes
709 rhizosphere microbial community structure in organic soil. New Phytologist 173, 600-610.

710 Paterson, E., Sim, A., 1999. Rhizodeposition and C-partitioning of *Lolium perenne* in axenic
711 culture affected by nitrogen supply and defoliation. Plant and Soil 216, 155-164.

712 Philippot, L., Hallin, S., Schloter, M., 2007. Ecology of denitrifying prokaryotes in agricultural
713 soil. Advances in Agronomy 96, 249-305.

714 Philippot, L., Kuffner, M., Chèneby, D., Depret, G., Laguerre, G., Martin-Laurent, F., 2006.
715 Genetic structure and activity of the nitrate-reducers community in the rhizosphere of
716 different cultivars of maize. Plant and Soil 287, 177-186.

717 Philippot, L., Raaijmakers, J.M., Lemanceau, P., van der Putten, W.H., 2013. Going back to
718 the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology 11, 789-
719 799.

720 Pilloni, G., Granitsiotis, M.S., Engel, M., Lueders, T., 2012. Testing the limits of 454 pyrotag
721 sequencing: reproducibility, quantitative assessment and comparison to T-RFLP
722 fingerprinting of aquifer microbes. PLoS ONE 7, e40467.

723 Potter, P., Ramankutty, N., Bennett, E.M., Donner, S.D., 2010. Characterizing the spatial
724 patterns of global fertilizer application and manure production. Earth Interactions 14, 1-22.

725 Priemé, A., Braker, G., Tiedje, J.M., 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene
726 fragments in forested upland and wetland soils. Applied and Environmental Microbiology 68,
727 1893-1900.

728 R Core Team, 2016. R: A Language and Environment for Statistical Computing. R
729 Foundation for Statistical Computing: Vienna, Austria, https://http://www.R-project.org/.

730 Rheinbaben, W.V., 1990. Nitrogen losses from agricultural soils through denitrification - a
731 critical evaluation. Zeitschrift für Pflanzenernährung und Bodenkunde 153, 157-166.

732 Shapleigh, J.P., 2013. Denitrifying prokaryotes, In: Rosenberg, E., DeLong, E.F., Lory, S.,
733 Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes: Prokaryotic Physiology and
734 Biochemistry. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 405-425.

735 Shcherbak, I., Millar, N., Robertson, G.P., 2014. Global metaanalysis of the nonlinear
736 response of soil nitrous oxide (N_2O) emissions to fertilizer nitrogen. Proceedings of the
737 National Academy of Sciences 111, 9199-9204.

738 Tiedje, J.M., 1988. Ecology of denitrification and dissimilatory nitrate reduction to
739 ammonium, In: Zehnder, A.J.B. (Ed.), Environmental Microbiology of Anaerobes. John Wiley
740 and Sons, N.Y.

741 Tiedje, J.M., Simkins, S., Groffman, P.M., 1989. Perspectives on measurement of
742 denitrification in the field including recommended protocols for acetylene based methods.
743 Plant and Soil 115, 261-284.

744 Ullah, S., Breitenbeck, G.A., Faulkner, S.P., 2005. Denitrification and N_2O emission from
745 forested and cultivated alluvial clay soil. Biogeochemistry 73, 499-513.

746 Uren, N., 2001. Types, amounts, and possible functions of compounds released into the
747 rhizosphere by soil-grown plants, In: R. Pinton, Z.V.a.P.N. (Ed.), The Rhizosphere:
748 Biochemistry and Organic Substances at the Soil-Plant Interface. Marcel Dekker Inc., New
749 York, pp. 19-40.

750 Wang, X., Tang, C., Severi, J., Butterly, C.R., Baldock, J.A., 2016. Rhizosphere priming
751 effect on soil organic carbon decomposition under plant species differing in soil acidification
752 and root exudation. New Phytologist 211, 864-873.

753 Woldendorp, J.W., 1962. The quantitative influence of the rhizosphere on denitrification.
754 Plant and Soil 17, 267-270.

755 Xie, Z., Le Roux, X., Wang, C., Gu, Z., An, M., Nan, H., Chen, B., Li, F., Liu, Y., Du, G.,
756 Feng, H., Ma, X., 2014. Identifying response groups of soil nitrifiers and denitrifiers to
757 grazing and associated soil environmental drivers in Tibetan alpine meadows. Soil Biology
758 and Biochemistry 77, 89-99.

759 Zhao, M., Jones, C.M., Meijer, J., Lundquist, P.-O., Fransson, P., Carlsson, G., Hallin, S.,
760 2017. Intercropping affects genetic potential for inorganic nitrogen cycling by root-associated

761 microorganisms in Medicago sativa and Dactylis glomerata. Applied Soil Ecology 119, 260-
762 266.

763 Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology and
764 Molecular Biology Reviews 61, 533-616.
765
766

767

768

769

770

771

772

773

774

775

776

777

778

779

780

781

782

783

784

785

786

787

788

789

790

7. Tables

Table 1. P-values for the main effects of the different treatments (Compartment, C Input, and WFPS) and their interactions for relative real time PCR gene copy number estimation (16S, *nirK*, *nirS*, *nosZ-I* and *nosZ-II*) as determined by three-way ANOVA. Significance level of p-values is indicated as follows: *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$, n.s. = $p>0.05$ (not significant).

	DF	16S	*nirK*	*nirS*	*nosZ*-I	*nosZ*-II
Compartment	1	***	***	***	***	n.s.
C Input	4	***	*	***	***	n.s.
WFPS	2	***	*	***	***	*
Compartment × C Input	4	***	n.s.	***	***	n.s.
Compartment × WFPS	2	**	*	***	***	n.s.
C input × WFPS	8	n.s.	n.s.	***	***	n.s.
Compartment × C input × WFPS	8	n.s.	n.s.	***	***	n.s.

Table 2. P-values for the main effects of the different treatments (Compartment, C Input, and WFPS) and their interactions for T-RFLP principal component scores (PC 1 through PC 5) as determined by three-way ANOVA. Significance level of p-values is indicated as follows: *** = p<0.001, ** = p<0.01, * = p<0.05, n.s. = p>0.05 (not significant). Percent of total variation explained by each principal component is indicated.

		Principal Component				
	DF	**1**	**2**	**3**	**4**	**5**
16S						
Compartment	1	***	***	n.s.	n.s.	n.s.
C Input	4	***	**	n.s.	n.s.	n.s.
WFPS	2	**	***	***	**	***
Compartment × C Input	4	***	***	n.s.	n.s.	n.s.
Compartment × WFPS	2	***	***	***	***	***
C input × WFPS	8	n.s.	***	n.s.	*	**
Compartment × C input × WFPS	8	**	***	n.s.	n.s.	n.s.
% Variation explained by PC		16.25	12.75	8.18	7.33	6.87
nirK						
Compartment	1	***	***	***	n.s.	n.s.
C Input	4	*	n.s.	**	n.s.	**
WFPS	2	n.s.	n.s.	***	n.s.	***
Compartment × C Input	4	***	n.s.	**	***	n.s.
Compartment × WFPS	2	*	n.s.	***	**	n.s.
C input × WFPS	8	n.s.	n.s.	n.s.	n.s.	n.s.
Compartment × C input × WFPS	8	***	*	n.s.	**	n.s.
% Variation explained by PC		13.05	9.56	7.84	6.63	5.02
nirS						
Compartment	1	***	n.s.	*	n.s.	n.s.
C Input	4	***	n.s.	n.s.	n.s.	n.s.
WFPS	2	***	***	***	n.s.	n.s.
Compartment × C Input	4	***	n.s.	***	n.s.	n.s.
Compartment × WFPS	2	**	n.s.	***	n.s.	n.s.
C input × WFPS	8	***	n.s.	*	n.s.	*
Compartment × C input × WFPS	8	n.s.	n.s.	n.s.	n.s.	n.s.
% Variation explained by PC		14.14	10.21	7.30	5.93	4.91
nosZ-I						
Compartment	1	n.s.	n.s.	***	***	n.s.
C Input	4	**	***	n.s.	n.s.	**
WFPS	2	***	n.s.	***	n.s.	n.s.
Compartment × C Input	4	n.s.	n.s.	n.s.	n.s.	**
Compartment × WFPS	2	**	n.s.	n.s.	*	*
C input × WFPS	8	*	***	n.s.	**	n.s.
Compartment × C input × WFPS	8	**	*	n.s.	*	n.s.
% Variation explained by PC		18.52	10.86	6.32	5.93	5.47

nosZ-II						
Compartment	1	n.s.	n.s.	n.s.	n.s.	n.s.
C Input	4	n.s.	n.s.	n.s.	*	n.s.
WFPS	2	n.s.	n.s.	n.s.	n.s.	n.s.
Compartment × C Input	4	n.s.	n.s.	n.s.	n.s.	n.s.
Compartment × WFPS	2	n.s.	n.s.	n.s.	n.s.	*
C input × WFPS	8	*	n.s.	n.s.	n.s.	n.s.
Compartment × C input × WFPS	8	n.s.	*	**	n.s.	n.s.
% Variation explained by PC		18.02	13.95	9.82	7.83	6.55

8. Figure captions

Figure 1. Interaction plots showing dissolved organic carbon (A) and NO_3-N (B) data for the "bulk" and model "rhizospheric" soil compartments after daily addition of ARE (0 to 3 mg C day^{-1}) for 7 consecutive days at 50, 70 and 90% WFPS. Filled symbols (■) represent "rhizospheric" soil and empty symbols "bulk" soil (□). The different WFPS levels are given by the symbol shape (50%: ■, 70%: ● and 90%: ▲). Error bars indicate the 5% Fisher's protected LSDs calculated using ANOVA.

Figure 2. Interaction plots showing total denitrification rates (A) and N_2O emission rates (B) for the different soil microcosm treatments after daily addition of ARE (0 to 3 mg C day-1) for 7 consecutive days at 50 (■), 70 (●) and 90% (▲) WFPS. Measurements were taken for the whole microcosm before they were destructively separated into "rhizospheric" and "bulk" soil. Error bars indicate the 5% LSDs calculated using ANOVA.

Figure 3. Gene copy number and PCoA analysis of the 16S rRNA (A,B), *nirK* (C,D), *nirS* (E,F), *nosZ*-I (G,H) and *nosZ*-II (I,J) genes in the "bulk" and "rhizospheric" soil compartments after daily addition of ARE (0 to 3 mg C day^{-1}) for 7 consecutive days at 50, 70 and 90% WFPS. For the gene copy number data, filled symbols (■) represent rhizospheric soil and empty symbols bulk soil (□). The different WFPS levels are given by shape (50%: ■, 70%: ● and 90%: ▲). In the PCoA analysis plots, the same symbol convention was used with the addition of different colours to indicate the C input added (mg C day^{-1} 0: ■, 0.375: ■, 0.75: ■, 1.5: ■ and 3.0: ■). Error bars indicate the 5% Fisher's protected LSDs calculated using ANOVA. Percentage variation accounted for by each PC is indicated in parentheses in PCoA plots.

9. Supplementary Information

The following Supporting Information is available for this article:

Figure S1. Schematic representation of the soil microcosm setup used in the experiment.

Figure S2. Interaction plots showing NH_4-N (A), pH (B), and WFPS (C) data for the "bulk" and "rhizospheric" soil compartments after daily addition of ARE (0 to 3 mg C day^{-1}) for 7 consecutive days at 50, 70 and 90% WFPS.

Table S1. Primer pairs and reaction conditions used for T-RFLP and relative real time PCR analysis.

Table S2. P-values for the main effects of the different factors (Compartment, C Input, and WFPS) and their interactions for the different physicochemical parameters measured as determined by three-way ANOVA.

Table S3. Soil physicochemical data (NH_4-N, NO_3-N, moisture, pH and DOC) for the "bulk" and "rhizospheric" compartments after daily addition of ARE (0 to 3 mg C day^{-1}) for 7 days at 50, 70 and 90% WFPS.

Table S4. Gene copy number of the 16S rRNA, *nirK*, *nirS*, *nosZ*-I and *nosZ*-II genes in the "bulk" and "rhizospheric" soil compartments.

Table S5. Gene ratios of the different denitrification genes studied when compared to 16S rRNA in the "bulk" and "rhizospheric" soil compartments.

Figure

Click here to download high resolution image

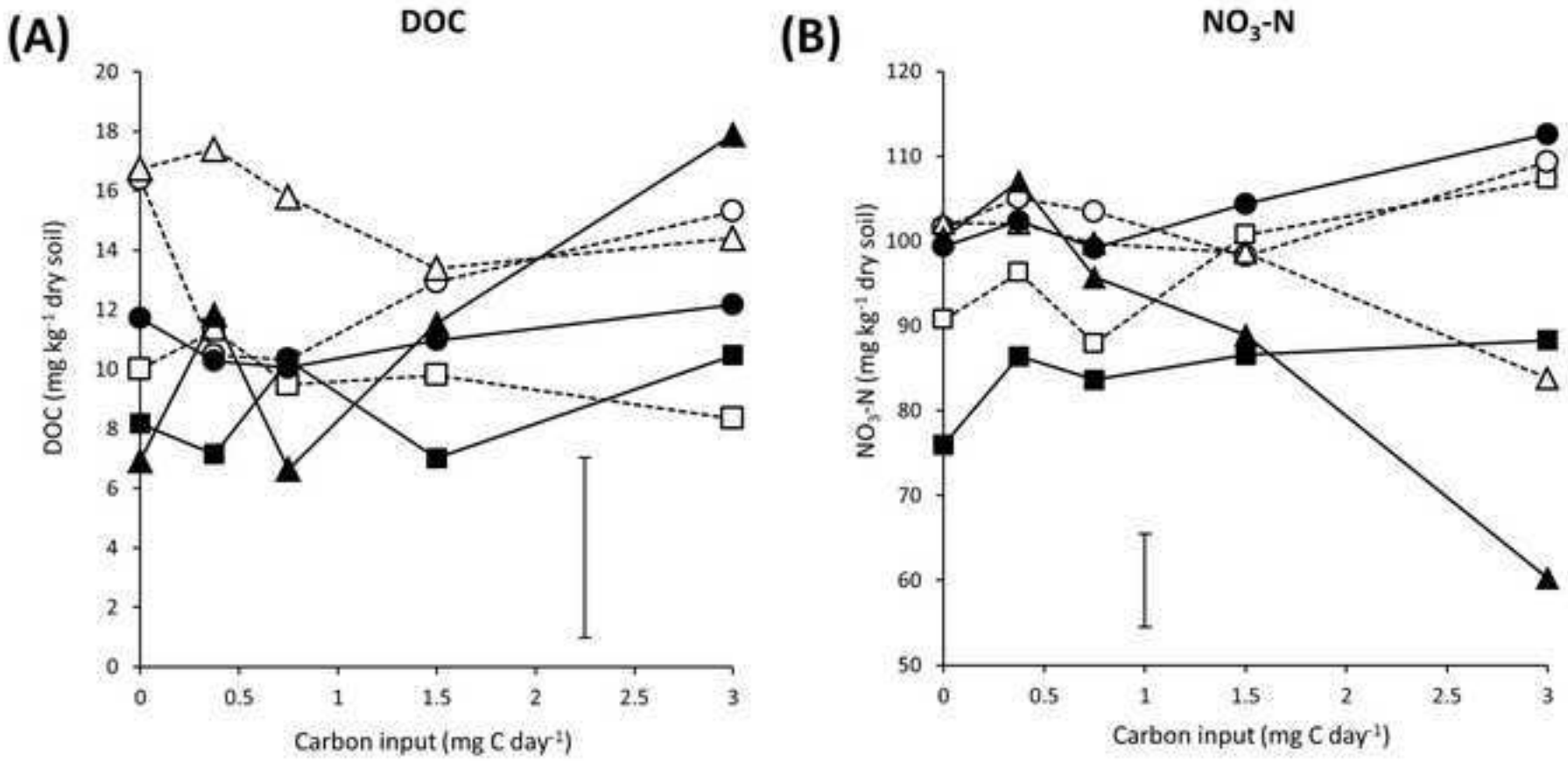


Figure 2

Click here to download high resolution image

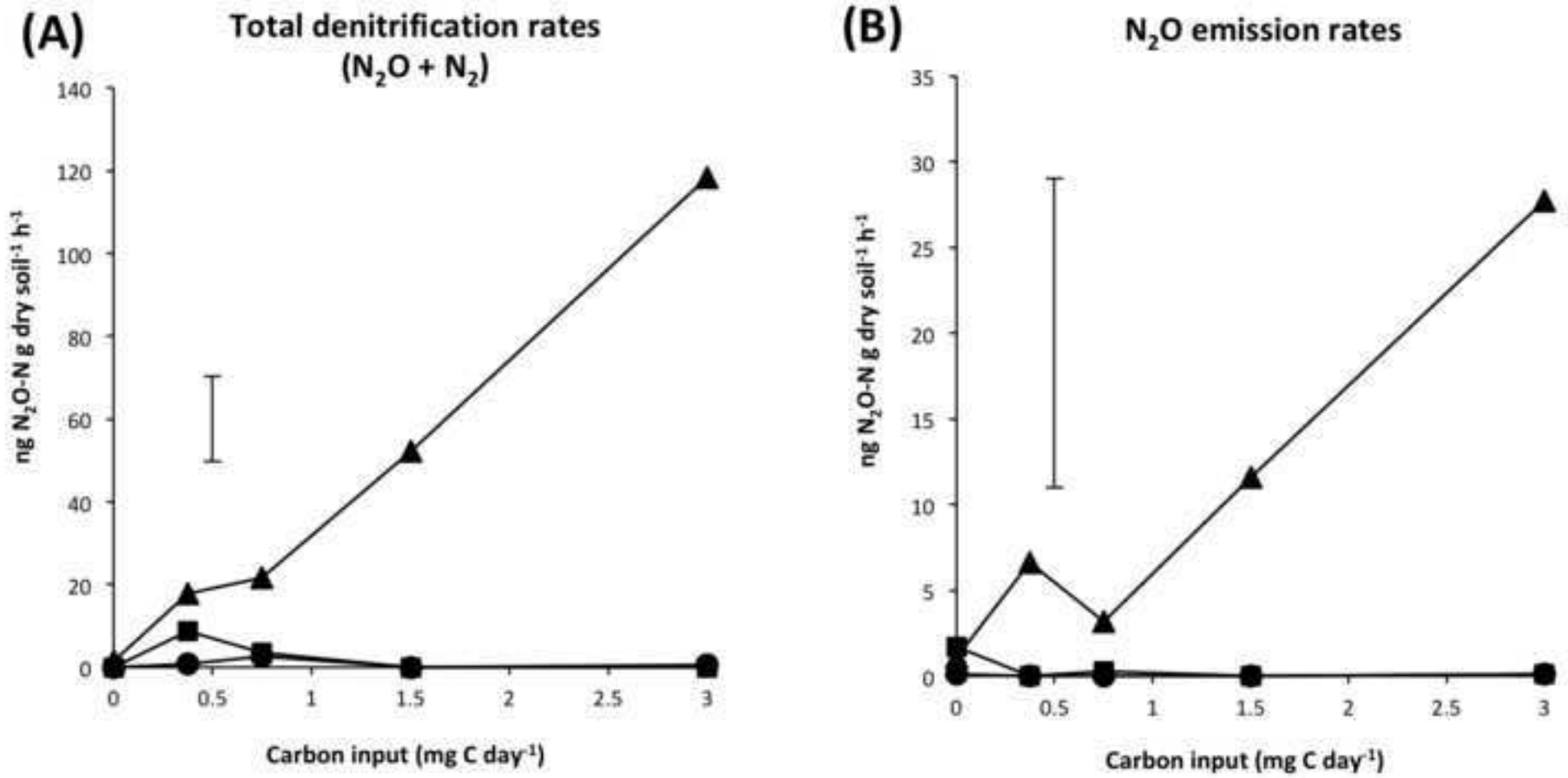
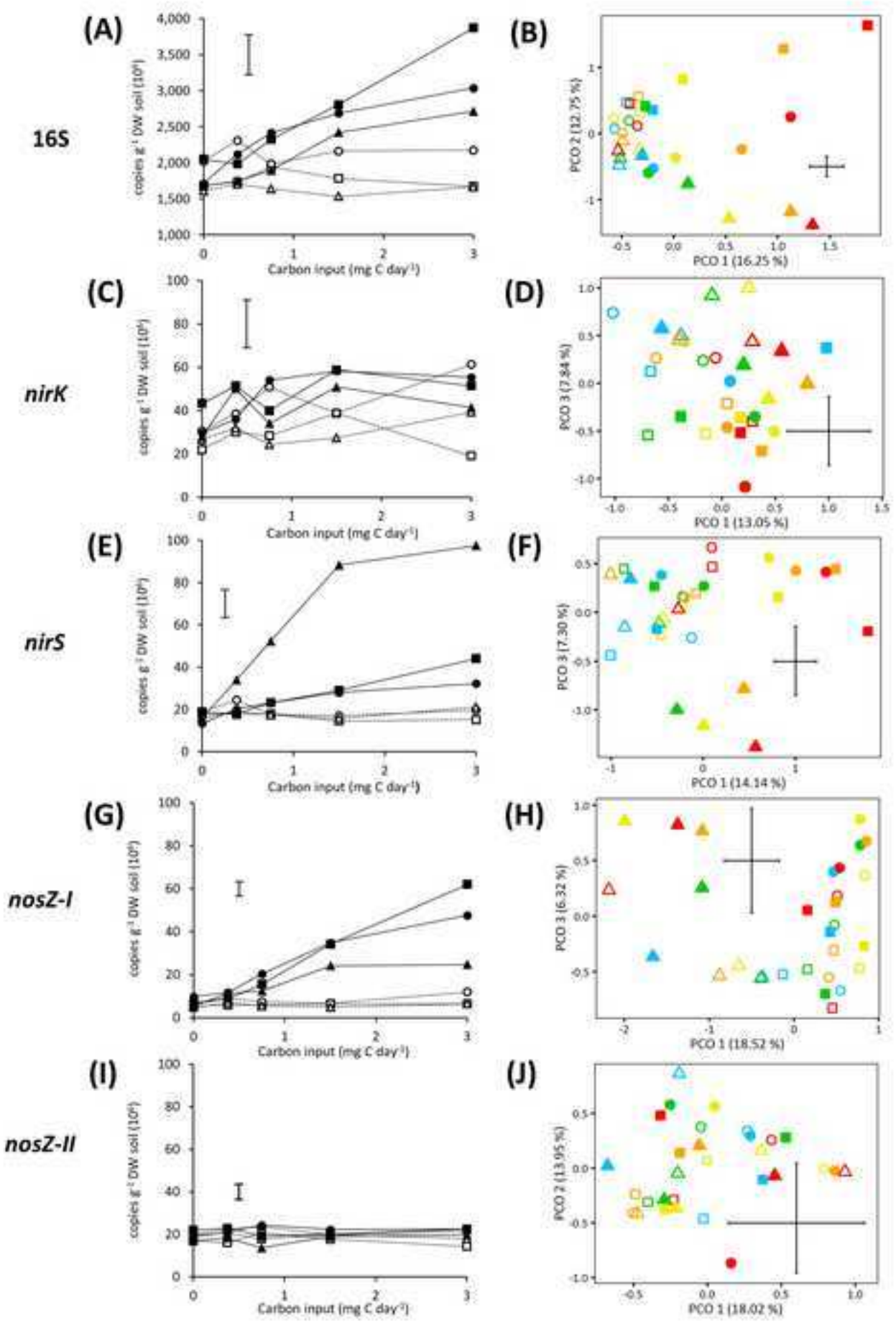


Figure 3

Click here to download high resolution image



Supplementary Material for online publication only

Click here to download Supplementary Material for online publication only: ARE paper_SBB_Supp_InformationSubmitted.docx