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Compound driven differences in N_2 and N_2O emission from soil; the role of substrate use efficiency and the microbial community

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Abstract

Organic C is an important control on the process of denitrification, a process that can result in the production and reduction of the potent greenhouse gas nitrous oxide (N_2O). This study identified the influence of different low molecular weight C (LMW-C) compounds on the production of nitrous oxide (N_2O) and dinitrogen (N_2) and the associated role of the size and structure of the microbial community. We examined this following application of glucose, glutamine or citric acid (250 mg C kg^{-1} dry soil) and ^{15}N-KNO_3 (100 mg N kg^{-1} dry soil) to a sandy loam soil and measured the production of N_2 and N_2O by denitrifiers using ^{15}N labeling techniques, changes in the bacterial community as measured by T-RFLP on *16SrDNA* fragments and changes in the gene copy number of *16SrDNA*, *nirK*, *nirS* and *nosZ* over 144 hours. Addition of glucose, citric acid and glutamine all increased emissions of ^{15}N-N_2 above that found in the control ($P < 0.05$) while the addition of glucose and glutamine resulted in higher emissions of $^{14+15}N$-N_2O ($P < 0.001$) than the addition of citric acid, resulting in a lower ^{15}N-N_2O to ^{15}N-N_2 ratio in the citric acid treatment. The *16SrDNA* gene copy number increased after addition of citric acid and glutamine, whilst *16SrDNA* showed significant shifts in community composition in all C treatments although over different time periods. The gene copy number of *nosZ* only significantly increased at 120 hours in the glutamine treatment ($P < 0.05$) and *nirS* at 120 hours in the citric acid and glutamine treatments ($P < 0.05$). This suggests that where C is added as a single input, differences in N_2 and N_2O emissions between LMW-C compounds were not caused by selection for denitrifiers but likely driven by differences in substrate use efficiency and subsequent differences in C partitioning between growth and respiration. The differing influence of the three selected C compounds on denitrification indicates the potential for lowering net N_2O emissions through regulation of C compound availability.

Key words; Carbon, denitrification, nitrous oxide, *nosZ*, *nirK*, *nirS*

1 Introduction

Soils are an important source of N_2O, a greenhouse gas with a global warming potential around 300 times greater than that of CO_2 over a 100 year period (Forster et al., 2007). The global atmospheric concentration of N_2O has increased from pre-industrial levels of 270 ppb to 319 ppb as of 2005 (Forster et al., 2007), with emissions from agricultural soils increasing from 2.4 Tg $N\text{-}N_2O\ yr^{-1}$ in 1990 to 6.6 Tg $N\text{-}N_2O\ yr^{-1}$ in 2000 and representing one of the most important sources of N_2O (Bouwman et al., 2013). The microbial process of denitrification, the stepwise reduction of NO_3^- to N_2, is thought to be responsible for ~ 60% of global N_2O emissions from agricultural soils (Smith et al., 2007). It is believed that emissions from soils can be lowered under conditions favorable to the final step in denitrification, the reduction of N_2O to N_2 (Richardson et al., 2009). Denitrification is performed by facultative anaerobic microorganisms as a means of maintaining respiration under O_2 limited conditions where NO_3, NO_2^-, NO and N_2O are used as alternative electron receptors (Zumft, 1997). Carbon remains as the electron donor for all of the possible reduction steps so the presence of organic C is an important control of both the production and reduction of N_2O, with high concentrations of labile C found to promote the reduction of N_2O to N_2 when NO_3^- is limiting (Weier et al., 1993). As denitrification is predominately a biological process the relative abundance and structure of the denitrifying community plays a crucial role in facilitating the production and reduction of N_2O. However, whilst there is a growing body of evidence of the role of C quantity in regulating N_2O production in soil (Morley et al., 2014), we know comparatively little about the effects of the form of C substrate, or on the interaction between C substrate and the denitrifying bacterial community.

Dissolved organic C (DOC) in soil is comprised of a wide variety of C compounds and within the soil matrix the quantity and composition of this DOC can vary over small distances (zu Schweinsberg-Mickan et al., 2010; Kuzyakov and Blagodatskaya

2015). In the rhizosphere plant roots provide a wide range of low molecular weight C (LMW-C) compounds, through rhizodeposition (Nguyen, 2003), to soil surrounding a root. This, together with root respiration driving down oxygen availability, results in higher denitrification rates and subsequently higher N_2O production in the rhizosphere compared to the bulk soil (Højberg et al., 1996; Mahmood et al., 1997). Controls on denitrification in the rhizosphere are likely to be driven not only by increased C availability but also the form this C takes. The range of rhizodeposited compounds is broad and comprises sugars, organic acids and amino acids (Nguyen, 2003), which are highly labile and can have half-lives as short as minutes (Paterson et al., 2008).

Studies have demonstrated differences in the rates and potential rates of denitrification as well as in the reduction of N_2O to N_2 between different LMW-C compound amendments to soil (Morley et al., 2014; Murray et al., 2004; Dendoovan et al., 1996). Morley et al. (2014) found the addition of organic acids to soil resulted in higher N_2O-to-N_2 ratios than sugars or amino acids and suggested that there was variation in the efficiency of nitrate metabolism by nitrate reductase with different LMW-C compounds. However efficiency of C compound use is likely to be one of a number of complex interacting controls on the effects of substrate dependence on denitrification and may act through alteration of microbial community dynamics either in terms of structure or abundance. Environmental variables are known to be important controls on process rates but the composition of the microbial community has also been shown to be an important predictor (Graham et al. 2016), so to lower net N_2O emissions from soils by promoting reduction to N_2 (Richardson et al., 2009), it is important to understand the drivers shaping the denitrifier community and how this relates to denitrification N_2O-to-N_2 product ratios.

106 The community may play a key role in determining the effects of different C
107 compounds by controlling the efficiency with which soil substrates can be used and
108 subsequently the fate of soil C. Studies on aerobic respiration have shown
109 differences in the partitioning of C compounds between growth and respiration
110 (Fischer et al., 2010). Greater substrate use efficiency promotes growth in
111 microorganisms (Manzoni et al., 2012; Sinsabaugh et al., 2013), which can lower the
112 C available for respiration. A similar mechanism is likely to affect denitrification with
113 compounds that promote growth lowering the C and N available for respiration via
114 denitrification and thus lowering the rates of denitrification and the reduction of NO_3^-
115 to N_2O and N_2. However, C form may provide a selective pressure for the microbial
116 community, selecting communities able to most efficiently utilise the C compound
117 added; consequently the addition of different forms of C is known to result in the
118 formation of C compound specific communities (Fierer et al., 2007; Eilers et al.,
119 2010). It remains unclear whether, under conditions that induce denitrification,
120 increased substrate use efficiency and lower C availability could alter rates of
121 denitrification and N_2O-to-N_2 ratios and if this is driven by the presence of
122 communities more able to rapidly utilize the form of available C.
123
124 Different LMW-C additions have been found to lead to varying abundances of *narG*,
125 encoding for nitrate reductase and *nosZ* encoding for N_2O reductase (Henry et al.,
126 2008). This initial finding suggests that LMW-C compounds have the ability to select
127 for organisms able to carry out specific denitrification steps. Whilst relationships have
128 been found between denitrifier abundance and both potential denitrification rates and
129 N_2O fluxes (Petersen et al. 2012; Lammel et al. 2015), the high functional
130 redundancy involved in denitrification (Wallenstein et al., 2006), the facultative nature
131 of the process and competition for LMW-C within the soil community as a whole may
132 limit the relationship between denitrifiers and net N_2O emission. This necessitates
133 consideration of the impact of different LMW-C compounds on denitrifier community

dynamics as well as N_2O production and reduction in order to better understand the complex interaction between LMW-C, the microbial community and resulting net N_2O emission.

The objectives of our study were 1) to determine if the addition of 3 different commonly rhizodeposited LMW-C compounds result in differences in N_2 and N_2O emissions from soil incubated under controlled conditions, 2) to identify relationships between N_2, N_2O and CO_2 emissions and *16SrDNA* gene copy number, as an indirect measure of substrate use efficiency and C partitioning, 3) to determine if changes in the bacterial community as measured by T-RFLP on *16SrDNA* fragments and abundance of denitrifiers are more important regulators of N_2O emissions than the substrate use efficiency and C partitioning of the initial soil community. We hypothesised that a) addition of C would increase N_2O emissions compared with the un-amended control, and there would be differences in the quantity of N_2 and N_2O produced between each of the C treatments; b) the structure of the microbial community would change following addition of C, but that this change would be slower than the production of N_2O so the substrate use efficiency of the initial soil community and C partitioning between growth and respiration would be the main determinates of N_2O and N_2 emissions.

2. Materials and methods

2.1 Experimental set-up

The soil used was an agricultural Dystric Cambisol soil (sandy loam) from Insch Aberdeenshire, North East Scotland (57°33′ N; 2°63′ W) and was selected as it represented a typical Scottish agricultural soil which had been well characterised and used in other studies (Morley et al., 2014). The soil had a starting pH of 6.7 and was comprised of 57.7% sand, 30.8% silt and 11.5% clay (Morley et al., 2014). Soil was sieved to 2 mm and 100 g dry weight soil was weighed into 156 cm^3 plastic pots (6

cm high, 5.75 cm diameter), and packed to a bulk density of 0.64 g cm^{-3}. The soil water-filled pore space (WFPS) was brought to 90 % and maintained by watering to weight daily, to ensure the soil maintained anaerobic conditions.

Treatments consisted of an addition of 100 mg N kg^{-1} dry weight soil, added as ^{15}N-labeled KNO_3 (19 atom % ^{15}N excess) and 250 mg C kg^{-1} dry weight soil as glucose, glutamine or citric acid (69.4, 83.3 and 69.4 *mM*, respectively), representing one of each of the most commonly rhizodeposited groups of C compounds; sugars, amino acids and organic acids (Nguyen, 2003). A control was established with the same N addition but no C added. Treatments and control were replicated five times for each time point (0, 12, 24, 36, 48, 72, 96, 120 and 144 hours after amendment). At time 0 N and C compounds were added by mixing 5 ml of a combined C and N solution described above with the soil whilst maintaining water contents at 90% WFPS. Samples for $^{14+15}$N-N_2O and C-CO_2 analysis were taken every 12 hours from time 0, and pots for molecular analyses, ^{15}N-N_2, ^{15}N-N_2O, N-NO_3^-, DOC and pH analyses were destructively sampled every 24 hours.

2.2 Gas sampling and analysis

Gas samples were taken by placing pots into 500 cm^3 gas-tight Kilner jars, allowing gas to accumulate in the closed head space for an hour. Linearity of gas accumulation over this time period had previously been determined. At each designated gas sampling time point one 12 ml gas sample was taken from the headspace of the Kilner jar (5 replicates per treatment), using a gas-tight syringe, and stored in an evacuated 12 ml gas vial (Labco) prior to $^{14+15}$N-N_2O and C-CO_2 analyses. Every 24 hours an additional 120 ml gas sample was taken from each jar and stored in a He-flushed, pre-evacuated 120 ml bottle (Supelco) for ^{15}N-N_2 and ^{15}N-N_2O analysis.

190 $^{14+15}N$-N_2O and C-CO_2 concentrations were determined on an Agilent 6890 gas
191 chromatograph, fitted with a flame ionization detector, an electron capture detector
192 and a methaniser. The column was a Haysep Q, with N_2 carrier gas and an oven
193 temperature of 100 ºC. CO_2 was converted to CH_4 in the methaniser followed by
194 detection on the flame ionization detector. The 120 ml gas samples were analysed
195 for ^{15}N enrichment of N_2 and N_2O using a Sercon Ltd isotope ratio mass spectrometer
196 following cryofocusing in an ANCA TGII gas preparation module. The concentration
197 of ^{15}N-N_2O was taken as indicative of N_2O production from nitrate reduction, primarily
198 denitrification (Baggs et al., 2003), while $^{14+15}N$-N_2O concentrations were
199 representative of both nitrate reducing and ammonia oxidizing processes.
200
201 *2.3 Soil mineral N, DOC and pH determination*
202 Every 24 hours individual pots that had been sampled for ^{15}N-N_2, ^{15}N-N_2O, $^{14+15}N$-
203 N_2O and C-CO_2 were homogenized by mixing and subsequently destructively
204 sampled (5 replicates per treatment). NO_2^- and NO_3^- were extracted from 20 g of soil
205 using 1 *M* KCl and measured on a Fiastar 500 Flow Injection Analyser (Foss
206 Analytical Ltd, Warrington UK). The ^{15}N enrichment of NO_3^- in these extracts was
207 determined by analysis on the isotope ratio mass spectrometer following gas
208 diffusion (Brooks et al., 1989). From the same pot of soil, a separate 20 g sample of
209 soil was taken for determination of DOC. DOC was extracted in 0.5 *M* K_2SO_4 and
210 measured on a LABTOC analyser (Pollution and process monitoring Ltd, Nottingham
211 UK). The pH was determined from soil suspended in 0.01 *M* $CaCl_2$ (F.8L, Horiba Ltd,
212 Kyoto Japan).
213
214 *2.4 Relative real time PCR and 16SrDNA TRFLP*
215 At 12, 36, 60, 96 and 120 hours 5 replicate pots for each treatment were sampled for
216 $^{14+15}N$-N_2O and CO_2 before being destructively sampled as described above. 0.5 g of
217 homogenized soil from each of these pots were separately snap frozen in liquid N_2

and stored at - 80 ℃ prior to analysis. Nucleic acid was extracted from the 0.5 g soil samples using a phenol chloroform extraction (Deng et al., 2010) with 1 x 10^8 copies of a mutated DNA spike added to the lysis buffer to act as an internal standard for relative real time-PCR (Daniell et al., 2012).

The gene copy number of the mutated spike standard, *16SrDNA* gene, *nirK*, *nirS* and *nosZ* was quantified using relative real time-PCR (Light cycler 480, Roche, Basel, Switzerland) using primers and conditions listed in Table 1. For all four primers 2 µl of template was added to 6 µl of sterile milli Q water, 10 pmol of each of the forward and the reverse primers and 10 µl of SYBR green I master mix (Roche).

T-RFLP was performed on the *16SrDNA* gene. The fluorescently labelled 16F27 – FAM AGAGTTTGATCCTGGCTCAG and 1392R – HEX ACGGGCGRTGTGT ACA primers were used (Blackwood et al., 2003) as were the following conditions 94 °C for 4.5 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 68 °C for 90 s and a final step of 68 °C for 10 min. T-RFLP digests were performed as described in Deng et al. (2010).

2.5 Statistical analyses

Data were analysed using the R statistical language in the R base environment (version 3.0.1). As pots were destructively sampled at each time point, data were assumed to be independent. Differences in means between grouping variables were tested using ANOVA, the presence of linear relationships between variables were tested using correlations and linear regression depending on whether a causative relationship was assumed. All data were checked for normality and homogeneity of variance. T-RFLP data were processed using GeneMapper (Applied Biosystems, Paisley, UK) after which peaks that contributed less than 1 % of the fluorescence in each sample were removed. The resulting data were Hellinger transformed and used in a principal component analysis (PCA) ANOVA was used to test for differences in

246 mean PC scores between grouping variables (Deng et al., 2009). Gas samples were
247 initially collected as rate measurements this was converted to total concentrations of
248 $^{15}N\text{-}N_2$, $^{14+15}N\text{-}N_2O$, $^{15}N\text{-}N_2O$ and $C\text{-}CO_2$ by plotting graphs of rate of gas production
249 against time in Microsoft Excel and calculating the area under the graphs.
250
251 3 Results
252 *3.1 Emissions of $^{14+15}N\text{-}N_2O$, $^{15}N\text{-}N_2O$, $^{15}N\text{-}N_2$ and $C\text{-}CO_2$*
253 At 144 hours the cumulative concentration of $^{14+15}N\text{-}N_2O$ was significantly higher than
254 the control in all 3 C treatments, while both glucose and glutamine treatments had
255 significantly higher concentrations of $^{14+15}N\text{-}N_2O$ than citric acid treatments ($P <$
256 0.001). $^{15}N\text{-}N_2O$ produced in the glucose treatments was significantly higher ($P <$
257 0.05) than those of the control, with the citric acid and glutamine treatments
258 intermediate but not significantly different from the control (Fig. 1a). Concentrations
259 of $^{15}N\text{-}N_2$ at 144 hours were significantly higher than the control in all three C
260 treatments ($P < 0.05$) (Fig. 1b), while ratios of $^{15}N\text{-}N_2O$-to-$^{15}N\text{-}N_2$ were significantly
261 higher than the control in the glucose and glutamine treatments ($P < 0.05$) (Fig. 1c).
262 The emissions of $^{15}N\text{-}N_2O$ were lower than that of $^{15}N\text{-}N_2$ in all treatments, making
263 $^{15}N\text{-}N_2$ the predominant product of denitrification in this experiment. Concentrations of
264 $C\text{-}CO_2$ at 144 hours were significantly greater ($P < 0.001$) than the control in all three
265 C treatments but did not differ between the C treatments (Fig. 1d). In all three C
266 treatments the concentrations of $^{14+15}N\text{-}N_2O$, $^{15}N\text{-}N_2O$, $^{15}N\text{-}N_2$ increased over time
267 (glucose; $^{14+15}N\text{-}N_2O$ $P < 0.001$, $^{15}N\text{-}N_2O$ $P < 0.005$, $^{15}N\text{-}N_2$ $P < 0.01$, citric acid;
268 $^{14+15}N\text{-}N_2O$ $P < 0.001$, $^{15}N\text{-}N_2O$ $P < 0.01$, $^{15}N\text{-}N_2$ $P < 0.005$, glutamine; $^{14+15}N\text{-}N_2O$ $P <$
269 0.001, $^{15}N\text{-}N_2O$ $P < 0.005$, $^{15}N\text{-}N_2$ $P < 0.001$). This was not the case in control
270 treatments where only $^{14+15}N\text{-}N_2O$ showed a significant increase in concentrations
271 over time ($P < 0.001$) (data not shown).
272

There were positive relationships between the concentrations of $^{14+15}N\text{-}N_2O$ and C-CO_2 in all C treatments (Table 2), with the greatest increase in $^{14+15}N\text{-}N_2O$ per unit increase in C-CO_2 in the glucose treatment and lowest in citric acid treatments. Similarly there were significant linear relationships between $^{15}N\text{-}N_2$ and C-CO_2 in both glucose and citric acid treatments (Table 2).

3.2 Soil DOC concentrations, N-NO_3^- concentrations and soil pH

Soil DOC concentrations decreased over time in all three C treatments. The most rapid decrease occurred between 0 and 24 hours in the C treatments (Fig. 2). At 144 hours there was no significant difference in DOC concentrations between any of the C treatments or the control suggesting that all added C had been utilized by the end of the experiment. DOC concentrations remained raised for longest in the citric acid treatments. Regression analysis showed that DOC concentrations were significantly related to $^{14+15}N\text{-}N_2O$ concentrations in the glutamine treatment and to $^{15}N\text{-}N_2$ in all the C treatments (Table 2). Neither the soil NO_3^- concentrations nor the DOC-to-NO_3^- significantly correlated with concentrations of C-CO_2, $^{14+15}N\text{-}N_2O$, $^{15}N\text{-}N_2O$ or $^{15}N\text{-}N_2$.

At 24 hours soil pH varied between treatments ($P < 0.001$), with both glucose and citric acid treatment showing a significantly lower pH than the control (glucose 5.64 ± 0.05, citric acid 5.35 ± 0.15). By 144 hours there was no difference in pH between any of the treatments. Regression analysis showed that pH did not significantly correlate with concentrations of $^{14+15}N\text{-}N_2O$, $^{15}N\text{-}N_2O$, $^{15}N\text{-}N_2$ and C-CO_2.

3.3 16SrDNA, *nirK*, *nirS and nosZ* gene copy number *and 16SrDNA* T-RFLP

The *16SrDNA* gene copy number increased linearly over time in the citric acid ($P < 0.001$, $R^2 = 55$ %) and glutamine ($P < 0.05$, $R^2 = 42$ %) treatments in contrast to the gene copy numbers of *nirK*, *nirS* and *nosZ* which did not increase linearly over time in any of the C treatments or in the control (Fig. 3). However there were significant

changes in these gene copy numbers over the course of the experiment. In both glucose and citric acid treatments there was a significant dip in the gene copy numbers of *nirK*. This occurred at 96 hours in the glucose treatment when copy numbers were lower than at 12 and 120 hours ($P < 0.05$) and at 60 hours in the citric acid treatment when copy numbers were lower than at 12, 36 or 120 hours ($P < 0.05$). In both cases the population of *nirK* containing organisms recovered by 120 hours. In addition, the ratio of *16SrDNA*-to-*nirK* increased with time in both citric acid ($P < 0.05$, $R^2 = 31$ %) and glutamine treatments ($P < 0.05$, $R^2 = 36$ %).

More dramatic changes in gene copy number were observed with *nirS* where glutamine and citric acid drove higher counts of this gene at 120 hours (citric acid $P < 0.05$, glutamine $P < 0.05$). *nosZ* gene copy numbers also showed a significant increase in the glutamine treatment where they increased over time, with the exception of 96 hours, and were higher at 120 hours than at 12 or 36 hours ($P < 0.05$).

PCA of the *16SrDNA* T-RF's and an ANOVA on the resulting PC scores for components 1 and 2 indicated that differences in T-RF profiles were driven by both compounds and time and the interaction between them (Fig. 4) (PC 1 $P < 0.005$, PC 2 $P < 0.001$). There was no shift in PC scores in the control treatment. In contrast the citric acid treatment changed along PC 1 from 36 to 60 hours and when compared to the control showed the greatest difference in T-RF profiles at 60 hours. By 96 hours community composition appeared to be reverting back to the composition found in the control. Glucose and glutamine treatments initially showed a shift in T-RF profiles along PC 2, although this shift occurred faster in the glutamine treatment. T-RF profiles were most different from the control at 12 hours in the glutamine treatment and 36 hours in the glucose treatment. By 96 hours both treatments were showing T-

328 RF profiles closer to that of the control suggesting community composition changes
329 were transient.
330
331 4. Discussion
332 The quantity of N_2O and N_2 produced and the ^{15}N-N_2O-to-^{15}N-N_2 ratio varied between
333 the C treatments applied in this study, indicating the importance of considering the
334 form of C as a control on denitrification. In many studies available soil C is
335 considered as a single compound and glucose is often used as the sole
336 representative of LMW-C (Dandie et al., 2007; Miller et al., 2008), despite plant roots
337 being able to produce a complex mix of LMW-C compounds that, within soil, can vary
338 over small spatial and temporal scales (Giles et al., 2012). While there is growing
339 recognition of compound dependent effects on denitrification, that have been found
340 to vary from responses to glucose (Murray et al., 2004; Henry et al., 2008; Morley et
341 al., 2014), little consideration has been given to the cause of these effects. Here we
342 found relationships between DOC, CO_2, N_2O and N_2 which varied between LMW-C
343 compounds suggesting that the efficiency with which the soil community is able to
344 use a C compound and the control that exerts on partitioning C between assimilatory
345 and dissimilatory processes may be one of a number of interacting factors that
346 determine LMW-C compound driven differences in N_2 and N_2O emissions. The
347 addition of LMW-C did select for *nirS* and *nosZ* containing denitrifiers but only in two
348 of three C treatments and the response of N_2O and N_2 production to a single C input
349 event was not commensurate in time with the shift in denitrification genes. The
350 control exerted by the microbial community is likely to be greater over larger time
351 scales and where more continuous C addition occurs such as in the rhizosphere.
352 Here, where C was in a single addition, the efficiency of C use and its fate was a
353 strong control on the response of denitrification to different C compounds.
354
355 4.1 N_2 and N_2O emission

356 $^{14+15}N$-N_2O production from the glucose and glutamine treatments fell within the
357 range of N_2O concentration found in similar studies, with comparable conditions
358 where glucose was used as a C source (Murray et al., 2004; Miller et al., 2008;
359 Henderson et al., 2010). In this study N_2 was the predominant product of
360 denitrification and in all three C treatments there was between 10-100 times greater
361 emission of N_2 than N_2O. Consequently there were also differences in the ^{15}N-N_2O-
362 to-^{15}N-N_2 ratios between C treatments, indicating compound dependent differences in
363 the reduction of N_2O to N_2. Citric acid, an organic acid, was the most efficient
364 compound at reducing N_2O. Differences in N_2 and N_2O emissions between LMW-C
365 compounds occurred in spite of equal amounts of C being present in the glucose,
366 citric acid and glutamine treatments. The reasons for compound dependent
367 differences in N_2 and N_2O emissions are likely to be complex and driven by N
368 limitation, the way C is used for competing processes and the interaction of C with
369 both the wider soil matrix and the microbial community present in it. Morley et al.
370 (2014) suggested that controls exerted by the form of C were caused by compound
371 dependent differences in the efficiency of the nitrate reductase. If NO_3^- becomes
372 limiting to denitrification it is advantageous for denitrifiers to undertake N_2O reduction
373 to N_2, the least energetically favorable of the denitrification reduction steps (Blackmer
374 and Bremner, 1978). The availability of NO_3^- will not only be limited by the efficiency
375 of uptake but also by whether NO_3^- is used in assimilatory or dissimilatory processes.
376 Where NO_3^- is used for processes such as growth it may result in increased C-to-N
377 ratios which promote the reduction of N_2O.
378
379 Soil communities are known to exhibit different C substrate use efficiencies, relating
380 to their ability to use a given substrate for growth as well as cell maintenance. A
381 community with a greater substrate use efficiency will show greater growth per unit
382 consumption of C than a community where low efficiency means that C is
383 predominately used for maintenance (Manzoni et al., 2012). In aerobic respiration

384 there is also evidence that the form of LMW-C can affect the fate of C, with Fischer et
385 al. (2010) finding that C was partitioned between growth and respiration differently
386 between diverse LMW-C substrates. Here in both glutamine and citric acid
387 treatments, C appeared to be being used for growth with *16SrDNA* gene copy
388 numbers in these treatments increasing over the course of the experiment. However
389 the NO_3^- concentrations at the end of the experiment differed between these two
390 treatments with 70 μg $N\text{-}NO_3^-$ per g dry weight soil less in the citric acid treatment
391 than in the glutamine treatment. As growth requires N, NO_3^- limitation in the citric acid
392 treatment may have resulted in the lower $^{15}N\text{-}N_2O$-to-$^{15}N\text{-}N_2$ ratio, while growth in the
393 glutamine treatment did not become N-limited most likely because of the presence of
394 the amine group in glutamine, resulting in higher $^{15}N\text{-}N_2O$-to-$^{15}N\text{-}N_2$ ratios despite
395 growth in the bacterial community. The efficiency of a community is believed to be
396 affected by the composition of soil dissolved organic matter (DOM) (Manzoni et al.,
397 2012; Bölscher et al., 2016) with the C-to-N ratio of DOM thought to be responsible
398 for substrate based differences in use efficiencies (Sinsabaugh et al., 2013).
399 Differences in substrate use efficiency in this study are indicated by the variation in
400 the amount of DOC required to support N_2 production in the LMW-C treatments; for
401 every 0.1 mg decrease in DOC concentration there was a 46.7μg increase in $^{15}N\text{-}N_2$
402 emission in the citric acid treatment, while this was 175.7μg $^{15}N\text{-}N_2$ in the glutamine
403 and 177.3 μg $^{15}N\text{-}N_2$ in the glucose treatments. As $^{15}N\text{-}N_2$ emissions at 144 hours
404 were similar between all three LMW-C treatments this strongly indicates that C was
405 not solely being used for denitrification. The presence of higher CO_2 emissions and
406 highest production of $^{15}N\text{-}N_2$ per μg $C\text{-}CO_2$ in the citric acid treatment highlights the
407 importance of considering the fate of C when identifying compound driven differences
408 in denitrification. The role of substrate use efficiency has not been expressly looked
409 at for denitrification, but the results from this study would indicate that there are

differences in the efficiency with which different LMW-C compounds are used when denitrification is the predominant respiratory pathway.

Soil is a complex environment and biological, chemical and physical controls will all act in conjunction with each other to determine process rates. Consequently substrate use efficiency will be one of a number of controls on denitrification that will include both the microbial community and the interaction of C compounds with the soil matrix. The interaction of these controls could be seen in the speed at which DOC was depleted; of the three C treatments citric acid was utilised the most slowly. The slow depletion of citric acid is likely linked to the adsorption of citrate ions to soil particles and from studies on aerobic respiration it is known that sorption of organic acids to soil particles can lower their availability for microbial consumption (Van Hees et al., 2003; Osburger et al., 2011).

4.2 The microbial community as a driver of N_2O production

The measured bacterial community response differed between the four treatments. In the glucose treatment where there was a lower increase in the bacterial population, there was the slowest shift in the community structure of all the C treatments, the co-occurrence of these two responses may suggest that there was a link between the substrate use efficiency and consequently the partitioning of C between growth and respiration which was important in determining how fast community structure changed in response to a C input.

It is likely the shifts in bacterial community as measured by *16SrDNA* represent the formation of communities able to reproduce quickly by rapidly utilising the added C and N (Eilers et al., 2010; Blagodatskaya et al., 2014; Cederlund et al., 2014).It is probable that the effect of changing community structure had only a limited effect on the production of N_2 and N_2O in this study due to time limitation and the addition of C

438 in one pulse. Denitrifiers in the glucose treatment were able to rapidly produce N_2O
439 despite showing the slowest community shift.
440
441 With the exception of *nosZ* gene copy numbers in the glutamine treatment, over the
442 first 96 hours of the experiment there was no indication for an increase in denitrifier
443 population size, as measured by the gene abundance of the denitrification genes
444 *nirK*, *nirS* or *nosZ*. It is also possible that *nirK* containing denitrifiers were
445 outcompeted by other members of the heterotrophic community as ratios of
446 *16SrDNA*-to-*nirK* decreased over time. While initially it appears that glucose, citric
447 acid and glutamine failed to provide sufficient selective pressure for denitrifiers, by
448 120 hours there was an increase in the abundance of *nirS* in the citric acid treatment
449 and *nirS* and *nosZ* in the glutamine treatment. The response of denitrifiers to a single
450 C input occurred 96 hours after the addition of C when soil DOC concentrations had
451 returned to their pre-experiment levels and at a point in time when the majority of
452 N_2O and N_2 had already been produced. This slow response may have been due to
453 the presence of residual O_2 in soil micropores that is likely to have been consumed
454 by 120 hours, but there is also known to be the potential for a disconnect in time
455 between soil environmental conditions and the structure of the microbial community
456 present. This is most markedly seen in the form of soil legacy effects (Marschner et
457 al., 2015; Banerjee et al., 2016), where historical soil conditions can shape current
458 microbial communities. This temporal disconnect may account for failure to create a
459 selective pressure for denitrifiers on the same time scale to $^{15}N\text{-}N_2$ and N_2O
460 emissions. However, it is important to note that the speed or magnitude of change in
461 *nosZ* may have altered if *nosZ* clade II containing denitrifiers had also been included
462 in this study (Domeignoz-Horta et al., 2015). Several studies have found no link
463 between denitrification measures and the abundance of denitrification genes (Dandie
464 et al., 2007; Miller et al., 2008; Henderson et al., 2010).
465

466 The short time period of this experiment may in part explain the failure to link
467 changes in community dynamics to denitrification although shifts in community size
468 were observed late in the time course; this may be indicative of a mismatch between
469 the time of most rapid nutrient utilisation and the point where community dynamics
470 were changed. This is not always the case and Henry et al., (2008) found that the
471 copy numbers of *narG* and nosZ varied between treatments amended with artificial
472 rhizodeposits formed of different C compounds added over several days. While it is
473 likely that the form of C can exert a control over denitrification by affecting the rate of
474 community change and selection for denitrifiers, the effects of this may not be seen
475 where C inputs are an isolated event and used rapidly because of the slower
476 response from denitrifiers. In such cases the substrate use efficiency of the initial soil
477 bacterial community and the partitioning of C will play a stronger role in determining
478 compound dependent differences in N_2 and N_2O emissions.
479
480 The differences in the emission of N_2 and N_2O following addition of LMW-C
481 compounds highlights the importance of considering the composition of DOC when
482 predicting its effects on denitrification. This is of special importance in the biologically
483 active rhizosphere where plant roots contribute a wide range of rapidly utilised LMW-
484 C compounds to soil (Nguyen 2003). Understanding which C compounds promote
485 the reduction of N_2O and the mechanisms by which they do this may help in the
486 selection of crop cultivars with rhizodeposit biochemical compositions that promote
487 the reduction of N_2O. However an understanding of how LMW-C compounds
488 influence the microbial community over longer time scales is needed.
489
490 Our results show that compound dependent differences in N_2 and N_2O emissions are
491 potentially linked to the differences in the substrate use efficiency of initial microbial
492 community between C compounds. Compounds that promote growth resulted in
493 lower ^{15}N-N_2O-to-^{15}N-N_2 ratios, most likely as a result of N limitation. However the

494 controls exerted by different C compounds are also influenced by their interaction
495 with the soil matrix. LMW-C compounds that stimulated growth resulted in faster
496 community change, but selection for *nirS* and *nosZ* containing denitrifiers occurred
497 after LMW-C additions had been utilised and therefore were unlikely to affect N_2 and
498 N_2O emissions. It is likely that the mechanisms by which the form of LMW-C
499 influences denitrification will be dependent on the type of C input and in this study
500 where C addition was as a single event, the substrate use efficiency of the initial
501 microbial community was a more important control on the N_2O-to-N_2 ratio, ^{15}N-N_2 and
502 N_2O emissions than compound driven differences in the selection for denitrifiers.
503
504
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511
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721 Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology
722 and Molecular Biology Reviews 61, 533-616.

723 Table 1 – Gene amplified, sequence and source of primers used in RT-PCR

724

725 Table 2 – Results of regression analysis (*P* value, R^2 and slope) for $^{14+15}N\text{-}N_2O$ and

726 $^{15}N\text{-}N_2$ emissions against $C\text{-}CO_2$ emissions and DOC concentrations in soil treated

727 with 250 mg C kg^{-1} dry weight soil in the form of either glucose, citric acid and

728 glutamine treatments and the control soil.

729

730

731 Figure 1 - a) Total $^{14+15}N$-N_2O and ^{15}N-N_2O emissions produced by 144 hours, b)
732 total ^{15}N-N_2 emissions produced by 144 hours, c) ^{15}N-N_2O-to-^{15}N-N_2 at 144 hours
733 and d) total C-CO_2 emissions produced by 144 hours in soil treated with 250 mg C
734 kg^{-1} dry weight soil in the form of either glucose, citric acid and glutamine and the
735 control soil. Data shown are mean ± one SE. Columns with different letters denote
736 significant differences ($P < 0.05$) between treatments.
737

738 Figure 2 - Dissolved organic C (DOC) concentrations over 144 hours in soil treated
739 with 250 mg C kg^{-1} dry weight soil of either glucose, citric acid or glutamine and in
740 control soil treatments. Data shown are mean ± one SE.
741

742 Figure 3 - Gene copy numbers of a) *nirK* b) *nirS* c) *nosZ* and d) *16SrDNA* in soil
743 treated with 250 mg C kg^{-1} dry weight soil of either glucose, citric acid or glutamine
744 and in control soil treatments. Data shown are mean ± one SE.
745

746 Figure 4 - Principal component scores for control, glucose, citric acid and glutamine
747 treatments at 12, 36, 60 and 96 hours after carbon addition. Means for each
748 treatment and time combination are displayed and the overall least significant
749 difference for each dimension represented by a cross. PCA was performed on
750 *16SrDNA* T-RFLP fragments. Shade denotes the sampling time, while the shape
751 denotes the treatment.
752

753

754 Table 1.

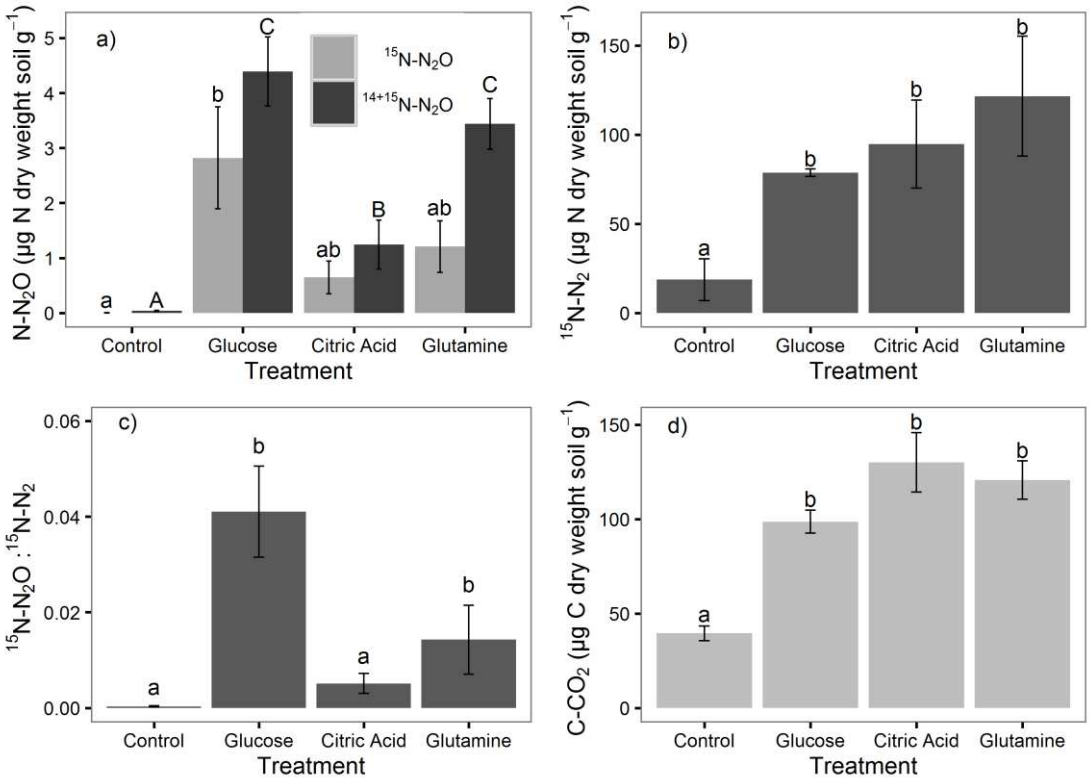
Target	primer	Sequence and conditions	Source
DNA spike	Mut342F Mut534R	CCTACG GGA GGC AGC AG ATT ACC GCG GCT GGA CC 95 ℃ 15min, 40 cycles of 95 ℃ for 10 sec, 54 ℃ for 10 sec, 72 ℃ for 20 sec, acquisition at 81℃ for 5 sec	Daniell et al., 2012
16SrDNA	342F 634R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG 95 ℃ 15min, 40 cycles of 95 ℃ for 10 sec, 54 ℃ for 10 sec, 72 ℃ for 20 sec, acquisition at 81℃ for 5 sec	Muyzer et al., 1993
nirK	876 1040	ATYGGCGGVCAYGGC GA GCCTCGATCAGRTTRTGGTT 95 ℃ 15 min, 6 cycles of 95 ℃ for 10 sec, 63 ℃ for 10 sec, 72 ℃ for 10 sec, 40 cycles of 60 ℃ for 10 sec, 72 ℃ for 20 sec, acquisition at 86 ℃ for 5 sec	Hallin et al., 2009
nirS	cd3aF R3cd	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA 95 ℃ 10 min, 40 cycles of 95 ℃ for 30 sec, 57 ℃ for 20 sec, 72 ℃ for 20 sec, acquisition at 72 ℃ for 5 sec	Michotey et al., 2000; Throback et al., 2004
nosZ	nosZ2F nosZ2R	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA 95 ℃ 10 min, 40 cycles of 95 ℃ for 30 sec, 62 ℃ for 15 sec, 72 ℃ for 30 sec, acquisition at 82 ℃ for 5 sec	Henry et al., 2006

755

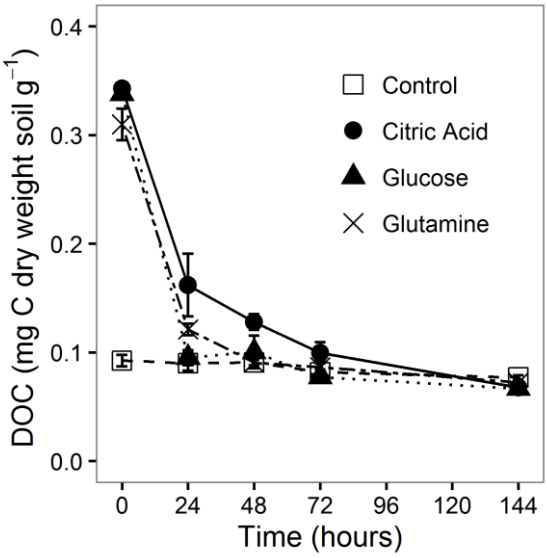
Table 2.

		$^{14+15}N\text{-}N_2O$			$^{15}N\text{-}N_2$		
		P value	R^2	Slope	*P* value	R^2	Slope
$C\text{-}CO_2$	Control	< 0.05	0.24	0.008		ns	
	Glucose	< 0.001	0.78	0.049	< 0.001	0.78	0.74
	Citric acid	< 0.001	0.53	0.010	< 0.001	0.84	0.85
	Glutamine	< 0.001	0.89	0.030		ns	
DOC	Control		ns			ns	
	Glucose		ns		< 0.05	0.39	-1773
	Citric acid		ns		< 0.001	0.73	-467
	Glutamine	< 0.001	0.49	-42.87	< 0.001	0.32	-1757

Figure 1



761 Figure 2



762
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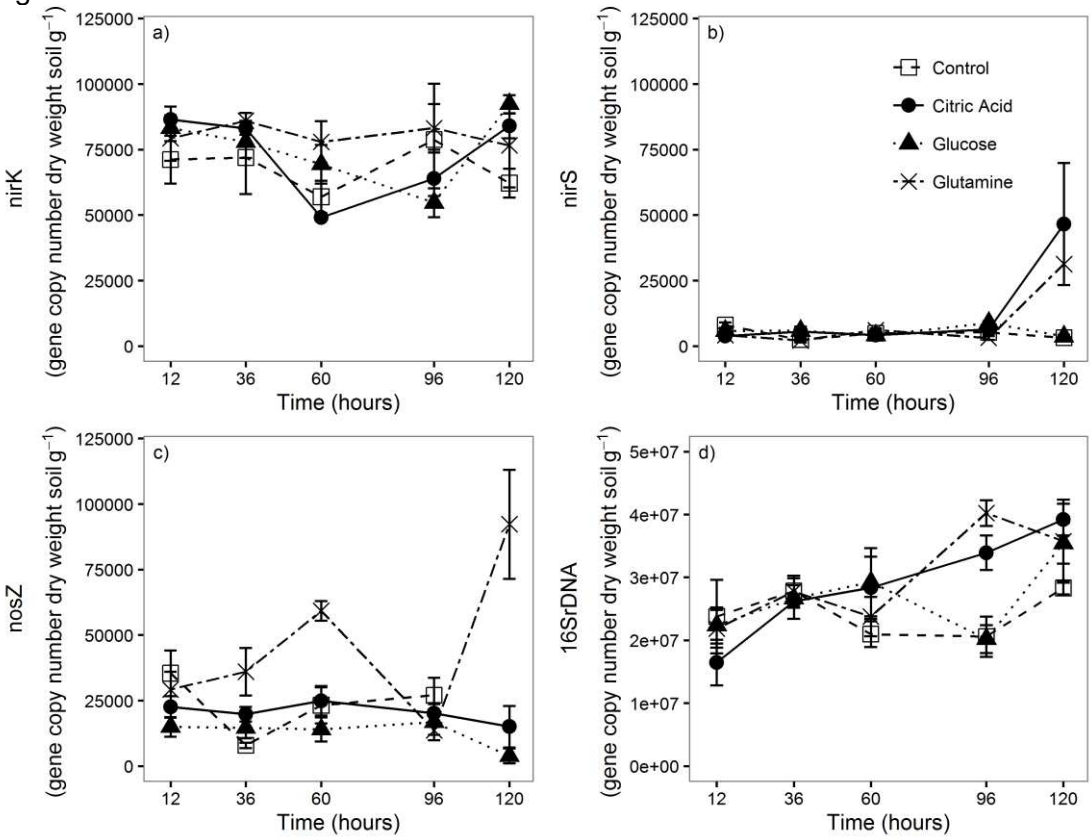
764 Figure 3



765
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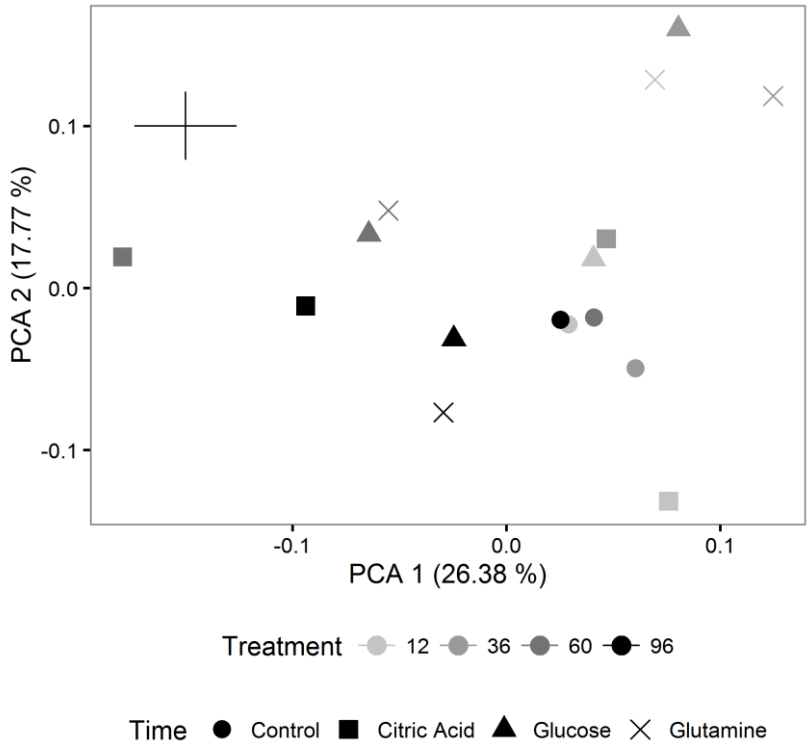
767 Figure 4



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