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Variable response of nirK and nirS containing denitrifier communities to long term pH manipulation and cultivation

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3 1 **Variable response of *nirK* and *nirS* containing denitrifier communities to long term pH**
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5 2 **manipulation and cultivation**
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8
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41 19 Running title: pH and cultivation effects on soil denitrifier community
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45 20 Keywords: nitrite reductase (*nirK*, *nirS*), 16S rRNA, gene abundance, pH
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48 21 One sentence summary: Cultivation affected the overall size of the bacterial community,
49
50 22 while *nirS* containing denitrifiers showed a greater sensitivity to pH change than *nirK*
51
52 23 containing denitrifiers, with *nirS* showing a threshold pH of 4.7 for both abundance and
53
54 24 community structure.
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56

25 **Abstract**

26 Denitrification is a key process responsible for the majority of soil nitrous oxide (N₂O)
27 emissions but the influences of pH and cultivation on the soil denitrifier community remain
28 poorly understood. We hypothesised that the abundance and community structure of the total
29 bacterial community and bacterial denitrifiers would be pH sensitive and that *nirK* and *nirS*
30 containing denitrifiers would differ in their responses to change in pH and cultivation. We
31 investigated the effect of long-term pH adjusted soils (ranging from pH 4.2 to pH 6.6) under
32 different lengths of grass cultivation (one, two and three years of ley grass) on the general
33 bacterial and denitrifier functional communities using 16S rRNA, *nirK* and *nirS* genes as
34 markers. Denitrifier abundance increased with pH, and at pH below 4.7 there was a greater
35 loss in *nirS* abundance per unit drop in pH than soils above this threshold pH. All community
36 structures responded to changes in soil pH whilst cultivation only influenced the community
37 structure of *nirK*. These differences in denitrifier responses highlight the importance of
38 considering both *nirK* and *nirS* gene markers for estimating denitrifier activity. Identifying
39 such thresholds in response of the microbial community to changes in pH is essential to
40 understanding impacts of management or environmental change.

42 **Introduction**

43 Denitrification in agricultural soils is a major source of the greenhouse gas nitrous oxide
44 (N₂O) (IPCC 2014). An understanding of the controls on denitrifier abundance and
45 community composition is important to aid development of mitigation strategies for N₂O
46 emission from soil and it has been suggested that community composition is an important
47 control on pH-driven changes in denitrification rates (Dörsch *et al.* 2012). However, the link
48 between soil communities, both size and structure, and function remains one of the major
49 challenges in microbial ecology, in part because the response of denitrifiers to certain

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3 50 environmental variables, such as pH, remains poorly understood. The effects of pH on both
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5 51 soil conditions and microbial activity can be wide ranging. Soil pH can be correlated to
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7 52 concentrations of dissolved organic matter (Bárta *et al.*, 2010) through altering sorption of
8
9 53 dissolved organic matter components to soil molecules, affecting the formation of insoluble
10
11 54 organic molecules and the concentration of polyvalent cations which can create complexes
12
13 55 with organic molecules (Bárta *et al.* 2010). Changes in nutrient availability can alter the size
14
15 56 and composition of denitrifying communities affecting a soil's genetic capacity to denitrify.
16
17 57 In addition pH can directly affect denitrification through transcript (Liu *et al.* 2010),
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19 58 translation, transport and degradation of enzymes (Samad *et al.* 2016a).
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24 60 Denitrifying bacteria are phylogenetically diverse with genes encoding the copper containing
25
26 61 nitrite reductase (*nirK*) and the cd₁ type nitrite reductase (*nirS*) commonly used as markers.
27
28 62 Denitrifying bacteria possess either *nirK* or *nirS* with both genes exclusive within the genome
29
30 63 of denitrifiers (Rösch *et al.* 2002). These genes have been used to assess both denitrifier
31
32 64 abundance and community structure in response to environmental changes (e.g. Philippot
33
34 65 2006; Attard *et al.* 2011) and the *nirS*-to-*nirK* ratio may be related to a soil's N₂O sink
35
36 66 capacity (Jones *et al.* 2014). Denitrifier communities that possess these nitrite reductases can
37
38 67 be influenced by soil pH but reported responses vary reflecting the diverse influence pH can
39
40 68 have on functional communities; in one instance Enwall *et al.* (2010) found *nirS* community
41
42 69 structure to be affected by pH in a crop rotation while Dandie *et al.* (2011) found pH to affect
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44 70 *nirK* community structure in a gradient of land management from maize field to riparian
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46 71 zone. Recent studies have also found *nirK* and *nirS* community size to play an important role
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48 72 in determining potential rates of denitrification in grassland systems (Čuhel *et al.* 2010) and
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50 73 actual rates of denitrification in pasture soils (Samad *et al.* 2016a). pH driven differences in
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52 74 community response may be a consequence of soil conditions with pH affecting post
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3 75 transcriptional controls on the proteome of the denitrifying community where carbon was
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5 76 absent and pH affecting the transcription rates of denitrifiers where carbon was present, in 3
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7 77 different soils (Liu *et al.* 2010). Similarly Samad *et al.* (2016b) found carbon mineralization
8
9 78 to be linked to denitrification rates across 13 pasture soils with diverse pH's. Differences in
10
11 79 soils and agricultural practices between study systems and the range of pH over which
12
13 80 measurements are taken may help to account for the differences in findings. While pH is an
14
15 81 important control on denitrification, cultivation history may affect the size and structure of
16
17 82 the communities reacting to pH conditions. For example (Herold *et al.* 2012) found bacterial
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19 83 biomass to increase with the length of time soil had been left under ley grass. Consequently it
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21 84 is important to understand the effects of both pH and land management in the same soil type
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23 85 across a gradient of pH values
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29 87 Our objective here was to explore how the size and structure of the denitrifying bacterial
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31 88 community changed in arable soil of the same site managed long-term for pH and left for
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33 89 one, two and three years under ley grass. We hypothesised that (i) that there would be a
34
35 90 positive correlation between the gene copy numbers of both *nirS* and *nirK* with soil pH, (ii)
36
37 91 the size of both general bacterial and the *nirS* and *nirK* containing bacterial community
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39 92 would increase with increasing years of ley cultivation, and (iii) pH would have a stronger
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41 93 effect on the abundance and structure of both the 16S rRNA gene *nirS* and *nirK* containing
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43 94 bacterial communities in soil under the first year of ley grass compared to soils under the
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45 95 third year of ley grass.
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49

50 97 **Methods**

51
52 98 Soil was sampled from the long term (50 years) pH plots maintained by SRUC at the
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54 99 Craibstone Estate, UK; 57°11'N, 2°12'W. The soil at this site is a sandy loam, iron podsol as
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3 100 described in Herold *et al.* (2012). The site is sub-divided into 12 m² plots, maintained at
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5 101 seven different levels of pH which increase in increments of 0.5 pH units from pH 4.2 to pH
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7 102 7.5 (CaCl₂). Within each pH range there are 8 subplots each at different stage of an eight year
8
9 103 crop rotation, winter wheat, potatoes, spring barley, swedes, spring oat, followed by 3
10
11 104 successive years of ley grass with no re-sowing. Ley grass plots were sown with a mixture of
12
13 105 *Lolium perenne* (cv. Fornax, Elgon, Cooper), *Phleum Pratense* (cv. Comtal) and *Trifolium*
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15 106 *repens* (cv. Alice and grassland demand).
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20 108 For this study, soil was sampled from plots under they're 1st, 2nd and 3rd year ley grass for
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22 109 each of the pH areas. For each plot soil samples were taken randomly with a grass plot
23
24 110 sampler (Van Walt Ltd., Surrey, UK) to a depth of 10 cm in May 2008 and 2009 as described
25
26 111 in Herold *et al.* (2012). Soil was collected from each plot until a bulked sample of 500 g of
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28 112 soil was achieved. The soil was then sieved to ≤4 mm and stored at either 4 °C until chemical
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30 113 analysis and -80 °C until community analysis.
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35 115 Soil pH was determined from 10 g of soil from each plot and using 0.01 M CaCl₂. Dissolved
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37 116 organic C and inorganic N (NH₄⁺, NO₃⁻ and NO₂⁻) were extracted from 10 g of soil using 1 M
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39 117 KCl and quantified using a Skalar SAN^{plus} Segmented Flow analyser (Skalar Analytical B.V.,
40
41 118 The Netherlands). Soil water content was determined 10g of soil by oven drying at 60 °C
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43 119 until a constant weight was achieved.
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48 121 Total DNA was extracted from 1 g of frozen soil, taken from each plot. Samples were spiked
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50 122 with a mutated 16S rRNA gene which was used as an internal standard to control for
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52 123 differences in both extraction and PCR efficiency (Daniell *et al.* 2012) in a similar manner to
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54 124 the use of housekeeping genes in expression analysis (Pfaffl *et al.* 2002) and the addition of
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3 125 internal standards in lipid extractions (Schutter & Dick 2000). The DNA extraction was
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5 126 performed using a phenol-chloroform extraction (Deng *et al.* 2010). Gene copy numbers of
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7 127 *nirK* and *nirS* (Hallin *et al.* 2009), the 16S rRNA gene (Muyzer *et al.* 1993) and the internal
8
9 128 standard were quantified using relative real time PCR (Daniell *et al.* 2012) where the internal
10
11 129 standard controls for efficiency. The 16S rRNA gene, *nirK* and *nirS* community structures
12
13 130 were determined using T-RFLP (Braker *et al.* 1998; Griffiths *et al.* 2010; Gulden *et al.* 2015).

131

132 Genotyping was performed on an ABI 3730 capillary sequencer and data we processed using
133 GeneMapper[®] Software (Applied Biosystems, Paisley, UK). For T-RFLP analysis, relative
134 peak areas were calculated for each sample and peaks that contributed less than 1% of total
135 fluorescence were removed. The remaining peaks were Hellinger transformed to reduce the
136 effect of dominant peaks (Blackwood *et al.* 2003). The transformed relative abundance data
137 were analysed with Principal Component Analysis (PCA). For each gene and PC score from
138 each PCA, linear or non-linear (depending on the relationship between variables) regression
139 models were created. Soil pH, years of ley cultivation and calendar year were used as
140 independent variables. Model selection was performed forward-stepwise using the Akaike
141 Information Criterion with finite sample distribution and AICc. Parameter estimates are
142 quoted with standard errors in brackets. Principal Component Analyses and regression
143 models were fitted using Genstat 16th edition (VSN International Ltd., Hemel Hempstead,
144 UK).

145

146 **Results and discussion**

147 Analysis of bacterial communities across the Americas has shown that bacterial diversity
148 varies between ecosystems with differences largely related to pH (Fierer and Jackson 2006).
149 Similarly here, the general bacterial and denitrifier community structures showed a

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3 150 significant response to soil pH (Fig. 1b, d, f) and in all cases the greatest change in
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5 151 community structure per unit pH occurred in more acidic soils. Existing information about
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7 152 the effect of soil pH on the community structure of *nirK* or *nirS* containing bacteria is limited
8
9 153 and inconsistent, with studies finding *nirK*, *nirS* or both changing with pH in agricultural
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11 154 systems (Enwall *et al.* 2010; Dandie *et al.* 2011, Yin *et al.* 2015). In this study both *nirK* and
12
13 155 *nirS* community structures were influenced by soil pH. The scores from PCA analysis for
14
15 156 both *nirS* and *nirK* showed a polynomial relationship with pH (Fig. 1d, f) (Table 1), though
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17 157 changes in community structure appeared smoother in *nirK* than *nirS*. A study which
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19 158 compared over 600 microbial genomes has shown a significantly higher frequency of co-
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21 159 occurrence of *nirS* with both nitric oxide reductase (*nor*) and nitrous oxide reductase (*nosZ*)
22
23 160 than organisms that possess *nirK* (Graf *et al.* 2014). This could suggest that these organisms
24
25 161 are more likely to be capable of complete denitrification under suitable conditions than
26
27 162 denitrifiers that possess *nirK*. Hence, it could be proposed that the *nirS* community generally
28
29 163 translates more denitrification enzymes than the *nirK* community and potentially invests
30
31 164 more energy to maintain the process of denitrification. Interestingly, the reduction of N₂O to
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33 165 di-nitrogen gas (N₂) is impaired under acidic pH which is thought to be related to post-
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35 166 transcriptional inhibition of the nitrous oxide reductase enzyme (Bergaust *et al.* 2010, Liu *et*
36
37 167 *al.* 2014). It could be hypothesised that the more distinct *nirS* community at low pH is related
38
39 168 to a discrepancy of resources used in the assembly of denitrification enzymes, the small yield
40
41 169 of energy generated by enzymes with impaired functionality at acidic pH and potential post
42
43 170 transcriptional controls driving selection away from the *nirS* community structure found at
44
45 171 higher pH.

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52 173 Changes in the community structure of denitrifiers in response to pH may potentially explain
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54 174 observed variation in denitrification rates in soil (Šimek *et al.* 2002; Herold *et al.* 2012), and

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3 175 changes in the abundance of certain bacteria with pH have been found in soils where this pH
4
5 176 change has been linked to N₂O emissions (Samad *et al.* 2016a). Nonetheless denitrification
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7 177 rates are sometimes only indirectly linked to N₂O emission (Petersen *et al.* 2012) and a
8
9 178 correlation between gene abundance and activity measurements hindered by ecological or
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11 179 methodological factors (Rocca *et al.* 2015). Despite this microbial communities may account
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13 180 for variation in process rates that remain unexplained by environmental variables, with the
14
15 181 predictive strength of models explaining facultative processes expected to improve when the
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17 182 diversity of microbial communities are taken into account (Graham *et al.* 2016).

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22 184 Increasing soil pH had a limited but positive effect on 16S rRNA gene abundance. There was
23
24 185 also a difference in 16S rRNA gene abundance between soils under the first year of ley grass
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26 186 and soils under either the second or third year of ley grass. Neither *nirS* nor *nirK* gene copy
27
28 187 numbers were influenced by the age of the grass ley, but were both significantly influenced
29
30 188 by soil pH, increasing with pH across the pH gradient with two distinct slopes for *nirS* above
31
32 189 and below pH 4.7 (*nirK*; $P < 0.001$, *nirS* $> \text{pH } 4.7$; $P < 0.05$, *nirS* $< \text{pH } 4.7$; $P < 0.001$) (Fig.
33
34 190 1c, e; Table 1). Denitrification is a facultative anaerobic process therefore the presence or
35
36 191 absence of denitrifiers may not only be driven by the presence or absence of a particular
37
38 192 denitrification gene but also by the niche a microorganism occupies under aerobic conditions.
39
40 193 In this study it is likely the system was not selecting for the denitrification trait at the point
41
42 194 samples were taken. The moisture content of the soil was between 0.37 and 0.42 g water g
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44 195 soil⁻¹ from this the water filled pore space was estimated at between 54 and 64 %. While
45
46 196 denitrification will occur at this water content in smaller or less well connected pores it will,
47
48 197 in general, be occurring in competition with other aerobic processes (Bateman and Baggs,
49
50 198 2005). This suggests, that the bacterial community adapted to low pH in these soils contained
51
52 199 a limited number of organisms with *nirS*.

200

201 Other studies have also found differences in the abundance of *nirS* and *nirK* in response to
202 pH. Philippot *et al.* (2009) found correlations between *nirS*, but not *nirK* abundance, and soil
203 pH in grassland pasture. In cultured communities created from soil inocula the transcription
204 of *nirS* has been found to be sensitive to pH, with transcription limited at lower pH
205 (Brenzinger *et al.* 2015). Inhibition of the transcription of *nirS* at low pH will mean
206 organisms containing *nirS* will have a poor capacity to reduce NO_2^- to nitric oxide at lower
207 pHs. This makes its presence of limited competitive advantage to bacteria adapted to living in
208 low pH conditions and may result in its exclusion from these organisms, potentially
209 accounting for its far lower abundance in this study at pH's below 4.7. In contrast, *nirK*,
210 while sensitive to pH, did not show a threshold value. It has been suggested that *nirK* is
211 sensitive to soil organic C (Kandeler *et al.* 2006) which was proposed as a driver for
212 differences in *nirK* abundance in spruce forest soils of different pH (Bárta *et al.* 2010). In
213 contrast to that study, DOC in the Craibstone soil decreases linearly with pH ($R^2 = 0.54$, $P <$
214 0.001).

215

216 Highest DOC was found in soils with a pH of 4.7. For *nirK* containing bacteria a loss of
217 available DOC may have offset the enzyme regulatory benefits of higher pH's and resulted in
218 a smoother increase in gene copy numbers with pH. In contrast soil NO_3^- concentrations
219 increased linearly with pH ($R^2 = 0.24$, $P < 0.05$) and the $\text{DOC}:\text{NO}_3^-$ ratio also decreased
220 linearly with soil pH ($R^2 = 0.36$, $P < 0.001$). Total soil C, total soil N and copper
221 concentrations showed no differences between pH plots. Both the chemical changes and
222 differences in biological capacity to reduce NO_2^- (*nirS* and *nirK* abundance) between pH
223 plots are likely to affect the soils potential to denitrify. Herold *et al.* (2012) measured
224 potential denitrification rates in the same soils as this study and found them to be highest

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3 225 between pH 5.5 and 6.3 in 1st and 2nd year ley grass, which are the pHs where the highest
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5 226 abundance of *nirK* and *nirS* were found in this study. While direct correlations of potential
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7 227 denitrification and denitrifier abundance were not possible in this study, the abundance of
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9 228 *nirS* has been found to link to potential denitrification rates in soils between 5.52 and 7.67
10
11 229 (Čuhel *et al.* 2010).

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14
15 231 In addition to pH, the cultivation history of the plots also affected the microbial communities.
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17 232 The number of years plots had been under grass influenced the community structure of *nirK*
18
19 233 containing bacteria, while plots under their first year of ley grass had the lowest abundance of
20
21 234 the 16S rRNA gene (table 1.). The *nirK* community structure in the third year under ley grass
22
23 235 diverged from the community structure in plots under their 1st and 2nd years of ley grass. In
24
25 236 contrast, the *nirS* community structure showed no response to the number of years plots had
26
27 237 been under grass (Figure 1f). The planting of ley grass represents a significant disturbance
28
29 238 and disturbance rather than the crop present has been found to be an important control on
30
31 239 bacterial communities as factors such as nutrient dynamics can be altered (Smith *et al.* 2016).
32
33 240 An analysis of global patterns of microbial community structures has shown that both *nirS*
34
35 241 and *nirK* communities cannot be separated between disturbed and un-disturbed sites (Jones
36
37 242 and Hallin 2010). Assuming that the three years of ley grass form a disturbance gradient in
38
39 243 itself, the lack of response of the two communities in years one and two might not be
40
41 244 surprising, although smaller scale studies have found nitrite reducers to be affected by
42
43 245 disturbance (Kandeler *et al.* 2006, Smith *et al.* 2016). The habitat preference of *nirS* and *nirK*
44
45 246 is thought to be different with *nirS* better adapted to soil with higher water content (Petersen
46
47 247 *et al.*, 2012) and *nirK* sensitive to changes in concentrations of nitrate and carbon (Jones &
48
49 248 Hallin 2010). It has been shown that the diversity of *nirS* is largely clustered over a wide
50
51 249 range of habitats whereas *nirK* communities show greater habitat selectivity (Jones and
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3 250 Hallin 2010). This habitat selectivity could explain the divergence of the *nirK* community
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5 251 structure in the third year of ley grass but the underlying cause and process of community
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7 252 assembly is not clear.
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11 254 The plots most recently rotated to ley grass had lower 16S rRNA gene abundance ($p < 0.001$)
12
13 255 which were similar to values reported by Rousk *et al.* (2010) who investigated 16S rRNA
14
15 256 gene abundance under continuous winter wheat across a pH range (pH range 4-8). This
16
17 257 reflects the importance of time in the growth of a rhizosphere population. The gene copy
18
19 258 numbers of *nirS* and *nirK* did not vary with year since grass planting which could support the
20
21 259 hypothesis that soil type can override plant effects in arable systems (Graf *et al.* 2016). As a
22
23 260 consequence the *nirS* and *nirK* gene copy numbers decreased relative to 16SrRNA gene copy
24
25 261 numbers, reducing the soils genetic capacity to denitrify.
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31 263 Our study shows, that in an agricultural context, long term pH management of soils can affect
32
33 264 both denitrifier abundance and community structure. Our hypothesis that bacterial
34
35 265 communities respond differently to soil pH was confirmed with *nirS* being more sensitive
36
37 266 than *nirK* to pH below 4.7. While the years since ley grass establishment may influence the
38
39 267 size of the bacterial community, pH was a better predictor of denitrifier abundance and
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41 268 community structure. The different dynamics in denitrifier communities highlight the
42
43 269 importance of studying both *nirK* and *nirS* gene markers when considering denitrifier
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45 270 response to both cultivation and pH, as well as identifying thresholds in denitrifier response
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47 271 to environmental change.
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28 427 **Figure 1:** Relationship of bacterial and denitrifier gene copy number (estimated by relative
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30 428 real time PCR to correct for extraction efficiency and inhibition) and community structure
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32 429 (estimated by T-RFLP subjected to Principal Component Analysis) with soil pH and
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34 430 cultivation. Only significant linear or second polynomial regression models are shown (p
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36 431 <0.05). Figure a, c, e show gene abundance data for 16S rDNA, *nirK* and *nirS*, respectively;
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38 432 and figure b, d, f show community structure data for 16S rDNA, *nirK* and *nirS*, respectively.
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40 433 Please note that graphs differ in scale.

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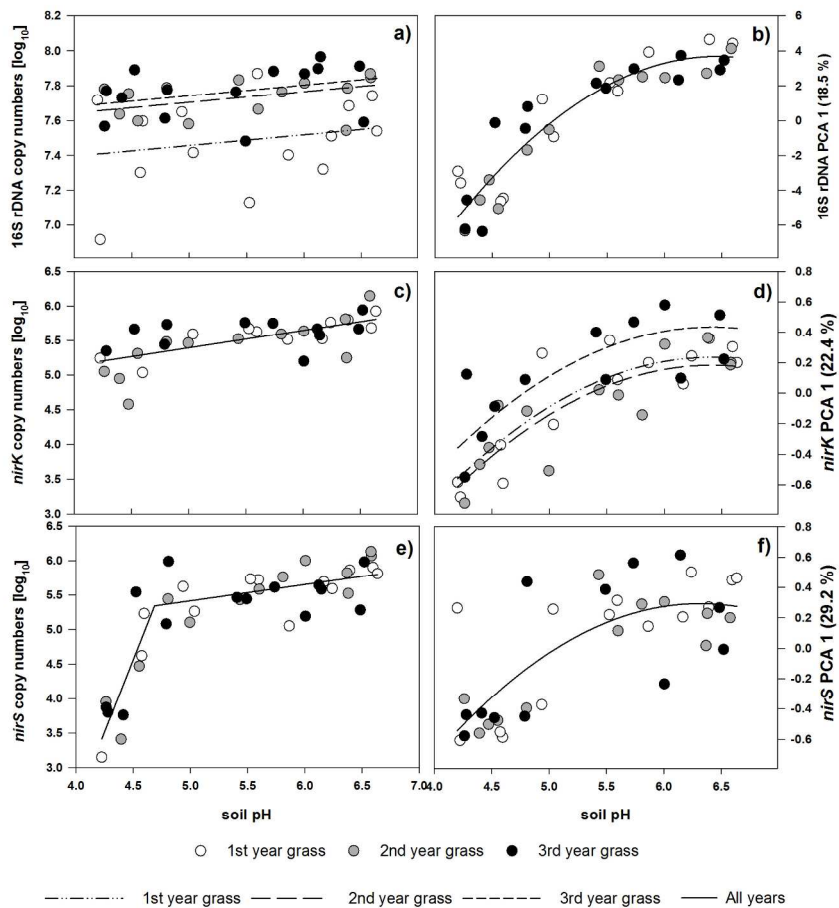
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48 436 **Table 1.** Parameters for the fitted regression models. Parameters were selected from
49
50 437 measured pH (linear and quadratic), ley grass cycle (years since cultivation), calendar year
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52 438 and any interactions. Principle component scores from principle component analysis using T-
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54 439 RFs of either *16s rRNA*, *nirS* and *nirK*, were used as the measure of community composition.

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3 440 Model selection was performed forward-stepwise using the Akaike Information Criterion
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5 441 with finite sample size correction (AICc). Note that for *nirS* gene abundance there are two
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7 442 sets of parameters for the split line regression and that the constant intercepts shown in
8
9 443 brackets have been derived for comparison with 16S and *nirK*. Asterisk indicate whether the
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11 444 parameter is significantly different from zero where ‘***’ is $p < 0.001$, ‘**’ is $p < 0.01$ and
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13 445 ‘*’ is $p < 0.05$. Entries with a ‘-’ symbol indicate parameters for a model term which was
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15 446 non-significant and not included in the regression
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Data Gene Abundance	All years				Year 2			Year 3		
	Intercept	Gradient linear	s.e.		Gradient linear	s.e.	significance	Gradient linear	s.e.	significance
16S rRNA	7.1	0.06	0.033	n.s.	0.25	0.068	***	0.29	0.068	***
<i>nirK</i>	4.1	0.25	0.051	***	-	-	-	-	-	-
<i>nirS</i> ¹ pH>4.7	(4.2)	0.24	0.102	*	-	-	-	-	-	-
pH<4.7	(-14.2)	4.17	0.766	***	-	-	-	-	-	-

Data Community Structure	All years						Year 2			Year 3		
	Intercept	Gradient linear	s.e.		Gradient quad.	s.e. significance	Gradient linear	s.e.	significance	Gradient linear	s.e.	significance
16S rRNA	-71.6	23.30	4.840	***	-1.80	0.451 ***	-	-	-	-	-	-
<i>nirK</i>	-6.4	2.08	0.664	**	-0.16	0.061 *	-0.05	0.075	n.s.	0.20	0.077	*
<i>nirS</i>	-7.2	2.36	0.983	*	-0.19	0.091 *	-	-	-	-	-	-



167x237mm (300 x 300 DPI)