Article:
Herold, M.B., Giles, M.E., Alexander, C.J. et al. (2 more authors) (2018) Variable response of nirK and nirS containing denitrifier communities to long term pH manipulation and cultivation. Fems Microbiology Letters, 365 (7). fny035. ISSN 0378-1097

https://doi.org/10.1093/femsle/fny035

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Journal:	*FEMS Microbiology Letters*
Manuscript ID	FEMSLE-17-11-0644.R1
Manuscript Type:	Research Letter
Date Submitted by the Author:	n/a
Complete List of Authors:	Herold, Miriam; University of Aberdeen, SBS; The James Hutton Institute, Ecological Sciences Giles, Maddy; The James Hutton Institute, Ecological Sciences Alexander, Colin; Biomathematics and Statistics Scotland, The James Hutton Institute Baggs, Elizabeth; University of Edinburgh, The Royal (Dick) School of Veterinary Studies Daniell, Tim; The James Hutton Institute, Ecological Sciences; The University of Sheffield, Department of Animal and Plant Sciences
Keywords:	nitrite reductase (nirK, nirS), community dynamics, gene abundance, pH, 16S rRNA
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Variable response of *nirK* and *nirS* containing denitrifier communities to long term pH manipulation and cultivation

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Running title: pH and cultivation effects on soil denitrifier community

Keywords: nitrite reductase (*nirK*, *nirS*), 16S rRNA, gene abundance, pH

One sentence summary: Cultivation affected the overall size of the bacterial community, while *nirS* containing denitrifiers showed a greater sensitivity to pH change than *nirK* containing denitrifiers, with *nirS* showing a threshold pH of 4.7 for both abundance and community structure.

Abstract

Denitrification is a key process responsible for the majority of soil nitrous oxide (N_2O) emissions but the influences of pH and cultivation on the soil denitrifier community remain poorly understood. We hypothesised that the abundance and community structure of the total bacterial community and bacterial denitrifiers would be pH sensitive and that *nirK* and *nirS* containing denitrifiers would differ in their responses to change in pH and cultivation. We investigated the effect of long-term pH adjusted soils (ranging from pH 4.2 to pH 6.6) under different lengths of grass cultivation (one, two and three years of ley grass) on the general bacterial and denitrifier functional communities using 16S rRNA, *nirK* and *nirS* genes as markers. Denitrifier abundance increased with pH, and at pH below 4.7 there was a greater loss in *nirS* abundance per unit drop in pH than soils above this threshold pH. All community structures responded to changes in soil pH whilst cultivation only influenced the community structure of *nirK*. These differences in denitrifier responses highlight the importance of considering both *nirK* and *nirS* gene markers for estimating denitrifier activity. Identifying such thresholds in response of the microbial community to changes in pH is essential to understanding impacts of management or environmental change.

Introduction

Denitrification in agricultural soils is a major source of the greenhouse gas nitrous oxide (N_2O) (IPCC 2014). An understanding of the controls on denitrifier abundance and community composition is important to aid development of mitigation strategies for N_2O emission from soil and it has been suggested that community composition is an important control on pH-driven changes in denitrification rates (Dörsch *et al*. 2012). However, the link between soil communities, both size and structure, and function remains one of the major challenges in microbial ecology, in part because the response of denitrifiers to certain

environmental variables, such as pH, remains poorly understood. The effects of pH on both soil conditions and microbial activity can be wide ranging. Soil pH can be correlated to concentrations of dissolved organic matter (Bárta *et al.*, 2010) through altering sorption of dissolved organic matter components to soil molecules, affecting the formation of insoluble organic molecules and the concentration of polyvalent cations which can create complexes with organic molecules (Bárta *et al.* 2010). Changes in nutrient availability can alter the size and composition of denitrifying communities affecting a soil's genetic capacity to denitrify. In addition pH can directly affect denitrification through transcript (Liu *et al*. 2010), translation, transport and degradation of enzymes (Samad *et al*. 2016a).

Denitrifying bacteria are phylogenetically diverse with genes encoding the copper containing nitrite reductase (*nirK*) and the cd_1 type nitrite reductase (*nirS*) commonly used as markers. Denitrifying bacteria possess either *nirK* or *nirS* with both genes exclusive within the genome of denitrifiers (Rösch *et al*. 2002). These genes have been used to assess both denitrifier abundance and community structure in response to environmental changes (e.g. Philippot 2006; Attard *et al*. 2011) and the *nirS*-to-*nirK* ratio may be related to a soil's N_2O sink capacity (Jones *et al*. 2014). Denitrifier communities that possess these nitrite reductases can be influenced by soil pH but reported responses vary reflecting the diverse influence pH can have on functional communities; in one instance Enwall *et al*. (2010) found *nirS* community structure to be affected by pH in a crop rotation while Dandie *et al*. (2011) found pH to affect *nirK* community structure in a gradient of land management from maize field to riparian zone. Recent studies have also found *nirK* and *nirS* community size to play an important role in determining potential rates of denitrification in grassland systems (Čuhel *et al.* 2010) and actual rates of denitrification in pasture soils (Samad *et al*. 2016a). pH driven differences in community response may be a consequence of soil conditions with pH affecting post

transcriptional controls on the proteome of the denitrifying community where carbon was absent and pH affecting the transcription rates of denitrifiers where carbon was present, in 3 different soils (Liu *et al.* 2010). Similarly Samad *et al*. (2016b) found carbon mineralization to be linked to denitrification rates across 13 pasture soils with diverse pH's. Differences in soils and agricultural practices between study systems and the range of pH over which measurements are taken may help to account for the differences in findings. While pH is an important control on denitrification, cultivation history may affect the size and structure of the communities reacting to pH conditions. For example (Herold *et al.* 2012) found bacterial biomass to increase with the length of time soil had been left under ley grass. Consequently it is important to understand the effects of both pH and land management in the same soil type across a gradient of pH values

Our objective here was to explore how the size and structure of the denitrifying bacterial community changed in arable soil of the same site managed long-term for pH and left for one, two and three years under ley grass. We hypothesised that (i) that there would be a positive correlation between the gene copy numbers of both *nirS* and *nirK* with soil pH, (ii) the size of both general bacterial and the *nirS* and *nirK* containing bacterial community would increase with increasing years of ley cultivation, and (iii) pH would have a stronger effect on the abundance and structure of both the 16S rRNA gene *nirS* and *nirK* containing bacterial communities in soil under the first year of ley grass compared to soils under the third year of ley grass.

Methods

Soil was sampled from the long term (50 years) pH plots maintained by SRUC at the Craibstone Estate, UK; 57°11´N, 2°12´W. The soil at this site is a sandy loam, iron podsol as

described in Herold *et al*. (2012). The site is sub-divided into 12 m^2 plots, maintained at seven different levels of pH which increase in increments of 0.5 pH units from pH 4.2 to pH 7.5 ($CaCl_2$). Within each pH range there are 8 subplots each at different stage of an eight year crop rotation, winter wheat, potatoes, spring barley, swedes, spring oat, followed by 3 successive years of ley grass with no re-sowing. Ley grass plots were sown with a mixture of *Lolium perenne* (cv. Fornax, Elgon, Cooper), *Phleum Pratense* (cv. Comtal) and *Trifolium repens* (cv. Alice and grassland demand).

For this study, soil was sampled from plots under they're 1st, 2nd and 3rd year ley grass for each of the pH areas. For each plot soil samples were taken randomly with a grass plot sampler (Van Walt Ltd., Surrey, UK) to a depth of 10 cm in May 2008 and 2009 as described in Herold *et al.* (2012). Soil was collected from each plot until a bulked sample of 500 g of soil was achieved. The soil was then sieved to ≤4 mm and stored at either 4 °C until chemical analysis and -80 °C until community analysis.

Soil pH was determined from 10 g of soil from each plot and using 0.01 M $CaCl_2$. Dissolved organic C and inorganic N (NH_4^+, NO_3^- and NO_2^-) were extracted from 10 g of soil using 1 M KCl and quantified using a Skalar SAN^{plus} Segmented Flow analyser (Skalar Analytical B.V., The Netherlands). Soil water content was determined 10g of soil by oven drying at 60 °C until a constant weight was achieved.

Total DNA was extracted from 1 g of frozen soil, taken from each plot. Samples were spiked with a mutated 16S rRNA gene which was used as an internal standard to control for differences in both extraction and PCR efficiency (Daniell *et al*. 2012) in a similar manner to the use of housekeeping genes in expression analysis (Pfaffl *et al.* 2002) and the addition of

internal standards in lipid extractions (Schutter & Dick 2000). The DNA extraction was performed using a phenol-chloroform extraction (Deng *et al*. 2010). Gene copy numbers of *nirK* and *nirS* (Hallin *et al*. 2009), the 16S rRNA gene (Muyzer *et al*. 1993) and the internal standard were quantified using relative real time PCR (Daniell *et al*. 2012) where the internal standard controls for efficiency. The 16S rRNA gene, *nirK* and *nirS* community structures were determined using T-RFLP (Braker *et al*. 1998; Griffiths *et al*. 2010; Gulden *et al.* 2015).

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

Results and discussion

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

significant response to soil pH (Fig. 1b, d, f) and in all cases the greatest change in community structure per unit pH occurred in more acidic soils. Existing information about the effect of soil pH on the community structure of *nirK* or *nirS* containing bacteria is limited and inconsistent, with studies finding *nirK*, *nirS* or both changing with pH in agricultural systems (Enwall *et al*. 2010; Dandie *et al*. 2011, Yin *et al*. 2015). In this study both *nirK* and *nirS* community structures were influenced by soil pH. The scores from PCA analysis for both *nirS* and *nirK* showed a polynomial relationship with pH (Fig. 1d, f) (Table 1), though changes in community structure appeared smoother in *nirK* than *nirS*. A study which compared over 600 microbial genomes has shown a significantly higher frequency of co-occurrence of *nirS* with both nitric oxide reductase (*nor*) and nitrous oxide reductase (*nosZ*) than organisms that possess *nirK* (Graf *et al*. 2014). This could suggest that these organisms are more likely to be capable of complete denitrification under suitable conditions than denitrifiers that possess *nirK*. Hence, it could be proposed that the *nirS* community generally translates more denitrification enzymes than the *nirK* community and potentially invests more energy to maintain the process of denitrification. Interestingly, the reduction of N_2O to di-nitrogen gas (N_2) is impaired under acidic pH which is thought to be related to post-transcriptional inhibition of the nitrous oxide reductase enzyme (Bergaust *et al*. 2010, Liu *et al*. 2014). It could be hypothesised that the more distinct *nirS* community at low pH is related to a discrepancy of resources used in the assembly of denitrification enzymes, the small yield of energy generated by enzymes with impaired functionality at acidic pH and potential post transcriptional controls driving selection away from the *nirS* community structure found at higher pH.

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

changes in the abundance of certain bacteria with pH have been found in soils where this pH change has been linked to N_2O emissions (Samad *et al*. 2016a). Nonetheless denitrification rates are sometimes only indirectly linked to N_2O emission (Petersen *et al*. 2012) and a correlation between gene abundance and activity measurements hindered by ecological or methodological factors (Rocca *et al*. 2015). Despite this microbial communities may account for variation in process rates that remain unexplained by environmental variables, with the predictive strength of models explaining facultative processes expected to improve when the diversity of microbial communities are taken into account (Graham *et al.* 2016).

Increasing soil pH had a limited but positive effect on 16S rRNA gene abundance. There was also a difference in 16S rRNA gene abundance between soils under the first year of ley grass and soils under either the second or third year of ley grass. Neither *nirS* nor *nirK* gene copy numbers were influenced by the age of the grass ley, but were both significantly influenced by soil pH, increasing with pH across the pH gradient with two distinct slopes for *nirS* above and below pH 4.7 (*nirK*; $P < 0.001$, *nirS* > pH 4.7; $P < 0.05$, *nirS* < pH 4.7; $P < 0.001$) (Fig. 1c, e; Table 1). Denitrification is a facultative anaerobic process therefore the presence or absence of denitrifiers may not only be driven by the presence or absence of a particular denitrification gene but also by the niche a microorganism occupies under aerobic conditions. In this study it is likely the system was not selecting for the denitrification trait at the point samples were taken. The moisture content of the soil was between 0.37 and 0.42 g water g soil^{-1} from this the water filled pore space was estimated at between 54 and 64 %. While denitrification will occur at this water content in smaller or less well connected pores it will, in general, be occurring in competition with other aerobic processes (Bateman and Baggs, 2005). This suggests, that the bacterial community adapted to low pH in these soils contained a limited number of organisms with *nirS*.

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

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

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

In addition to pH, the cultivation history of the plots also affected the microbial communities. The number of years plots had been under grass influenced the community structure of *nirK* containing bacteria, while plots under their first year of ley grass had the lowest abundance of the 16S rRNA gene (table 1.). The *nirK* community structure in the third year under ley grass diverged from the community structure in plots under their 1st and 2nd years of ley grass. In contrast, the *nirS* community structure showed no response to the number of years plots had been under grass (Figure 1f). The planting of ley grass represents a significant disturbance and disturbance rather than the crop present has been found to be an important control on bacterial communities as factors such as nutrient dynamics can be altered (Smith *et al.* 2016). An analysis of global patterns of microbial community structures has shown that both *nirS* and *nirK* communities cannot be separated between disturbed and un-disturbed sites (Jones and Hallin 2010). Assuming that the three years of ley grass form a disturbance gradient in itself, the lack of response of the two communities in years one and two might not be surprising, although smaller scale studies have found nitrite reducers to be affected by disturbance (Kandeler *et al.* 2006, Smith *et al.* 2016). The habitat preference of *nirS* and *nirK* is thought to be different with *nirS* better adapted to soil with higher water content (Petersen *et al*., 2012) and *nirK* sensitive to changes in concentrations of nitrate and carbon (Jones & Hallin 2010). It has been shown that the diversity of *nirS* is largely clustered over a wide range of habitats whereas *nirK* communities show greater habitat selectivity (Jones and

Hallin 2010). This habitat selectivity could explain the divergence of the *nirK* community structure in the third year of ley grass but the underlying cause and process of community assembly is not clear.

The plots most recently rotated to ley grass had lower 16S rRNA gene abundance ($p < 0.001$) which were similar to values reported by Rousk *et al*. (2010) who investigated 16S rRNA gene abundance under continuous winter wheat across a pH range (pH range 4-8). This reflects the importance of time in the growth of a rhizosphere population. The gene copy numbers of *nirS* and *nirK* did not vary with year since grass planting which could support the hypothesis that soil type can override plant effects in arable systems (Graf *et al*. 2016). As a consequence the *nirS* and *nirK* gene copy numbers decreased relative to 16SrRNA gene copy numbers, reducing the soils genetic capacity to denitrify.

Our study shows, that in an agricultural context, long term pH management of soils can affect both denitrifier abundance and community structure. Our hypothesis that bacterial communities respond differently to soil pH was confirmed with *nirS* being more sensitive than *nirK* to pH below 4.7. While the years since ley grass establishment may influence the size of the bacterial community, pH was a better predictor of denitrifier abundance and community structure. The different dynamics in denitrifier communities highlight the importance of studying both *nirK* and *nirS* gene markers when considering denitrifier response to both cultivation and pH, as well as identifying thresholds in denitrifier response to environmental change.

Acknowledgements

MBH received a joint studentship from the University of Aberdeen and the James Hutton Institute partly funded by the Rural and Environment Science and Analytical Services Division of the Scottish Government. TJD is partly funded by the HEFCE N8 Agrifood programme. EMB was supported by an Advanced Research Fellowship awarded by the Natural Environment Research Council. We are grateful to the SRUC who granted field site access. This paper was written by MBH who was supervised by EMB and TJD. Statistical help was provided by CJA. Final versions of the paper were revised by MEG.

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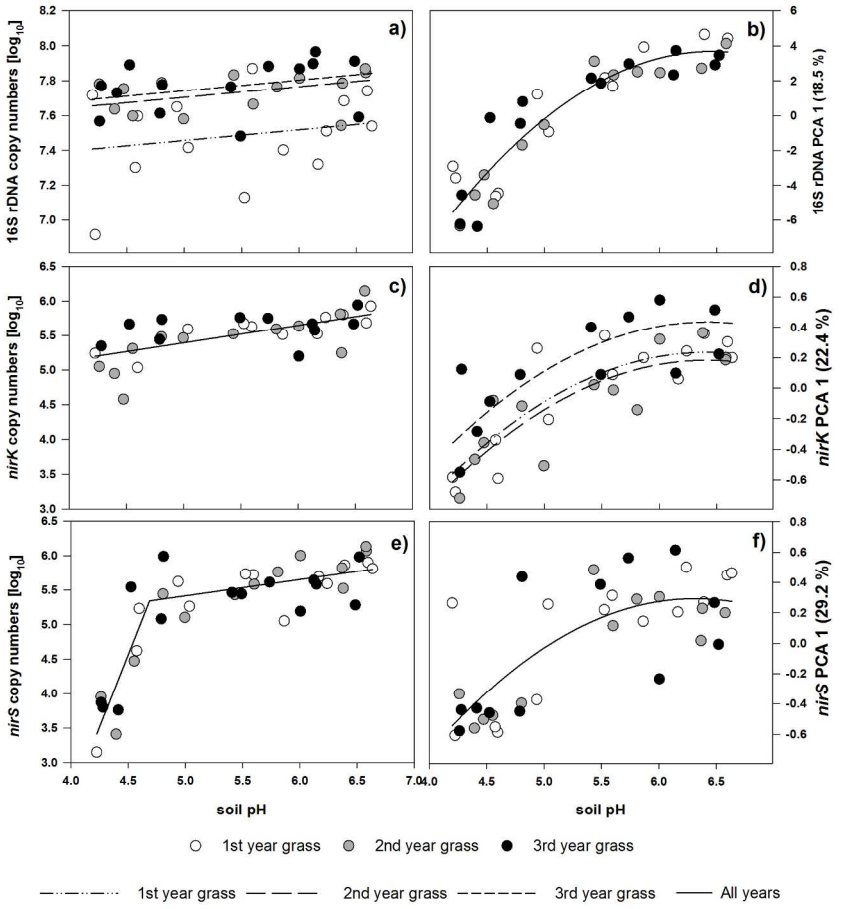
Figure 1: Relationship of bacterial and denitrifier gene copy number (estimated by relative real time PCR to correct for extraction efficiency and inhibition) and community structure (estimated by T-RFLP subjected to Principal Component Analysis) with soil pH and cultivation. Only significant linear or second polynomial regression models are shown (p <0.05). Figure a, c, e show gene abundance data for 16S rDNA, *nirK* and *nirS*, respectively; and figure b, d, f show community structure data for 16S rDNA, *nirK* and *nirS*, respectively. Please note that graphs differ in scale.

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

Model selection was performed forward-stepwise using the Akaike Information Criterion with finite sample size correction (AICc). Note that for *nirS* gene abundance there are two sets of parameters for the split line regression and that the constant intercepts shown in brackets have been derived for comparison with 16S and *nirK*. Asterisk indicate whether the parameter is significantly different from zero where '***' is $p < 0.001$, '**' is $p < 0.01$ and '*' is $p < 0.05$. Entries with a '-' symbol indicate parameters for a model term which was non-significant and not included in the regression

Data	All years				Year 2			Year 3		
Gene Abundance	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	***
nirK	4.1	0.25	0.051	***	-	-	-	-	-	-
nirS[1] **pH>4.7**	(4.2)	0.24	0.102	*	-	-	-	-	-	-
pH<4.7	(-14.2)	4.17	0.766	***	-	-	-	-	-	-

Data	All years						Year 2			Year 3		
Community Structure	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 ***	-	-	-	-	-	-
nirK	-6.4	2.08	0.664	**	-0.16	0.061 *	-0.05	0.075	n.s.	0.20	0.077	*
nirS	-7.2	2.36	0.983	*	-0.19	0.091 *	-	-	-	-	-	-



167x237mm (300 x 300 DPI)