

A peer-reviewed version of this preprint was published in PeerJ on 12 December 2018.

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Anderson CR, Peterson ME, Frampton RA, Bulman SR, Keenan S, Curtin D. 2018. Rapid increases in soil pH solubilise organic matter, dramatically increase denitrification potential and strongly stimulate microorganisms from the *Firmicutes* phylum. PeerJ 6:e6090
<https://doi.org/10.7717/peerj.6090>

Rapid increases in soil pH solubilise organic matter, dramatically increase denitrification potential and strongly stimulate microorganisms from the *Firmicutes* phylum

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Rapid and transient changes in pH frequently occur in soil, impacting dissolved organic matter (DOM) and other chemical attributes such as redox and oxygen conditions. Although we have detailed knowledge on microbial adaptation to long-term pH changes, little is known about the response of soil microbial communities to rapid pH change, nor how excess DOM might affect key aspects of microbial N processing. We used potassium hydroxide (KOH) to induce a range of soil pH changes likely to be observed after livestock urine or urea fertilizer application to soil. We also focus on nitrate reductive processes by incubating microcosms under anaerobic conditions for up to 48 hours. Soil pH was elevated from 4.7 to 6.7, 8.3 or 8.8, and up to 240-fold higher DOM was mobilized by KOH compared to the controls. This increased microbial metabolism but there was no correlation between DOM concentrations and CO₂ respiration nor N-metabolism rates. Microbial communities became dominated by *Firmicutes* bacteria within 16 hours, while few changes were observed in the fungal communities. Changes in N-biogeochemistry were rapid and denitrification enzyme activity (DEA) increased up to 25-fold with the highest rates occurring in microcosms at pH 8.3 that had been incubated for 24-hour prior to measuring DEA. Nitrous oxide reductase was inactive in the pH 4.7 controls but at pH 8.3 the reduction rates exceeded 3000 ng N₂-N g⁻¹ h⁻¹ in the presence of native DOM. Evidence for dissimilatory nitrate reduction to ammonium and/or organic matter mineralisation was observed with ammonium increasing to concentrations up to 10 times the original native soil concentrations while significant concentrations of nitrate were utilised. Pure isolates from the microcosms were dominated by *Bacillus* spp. and exhibited varying nitrate reductive potential.

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Manuscript in preparation for PeerJ

Running title: *Firmicutes* contribute to denitrification

Abstract

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We used potassium hydroxide (KOH) to induce a range of soil pH changes likely to be observed after livestock urine or urea fertilizer application to soil. We also focus on nitrate reductive processes by incubating microcosms under anaerobic conditions for up to 48 hours. Soil pH was elevated from 4.7 to 6.7, 8.3 or 8.8, and up to 240-fold higher DOM was mobilized by KOH compared to the controls. This increased microbial metabolism but there was no correlation between DOM concentrations and CO₂ respiration nor N-metabolism rates. Microbial communities became dominated by *Firmicutes* bacteria within 16 hours, while few changes were observed in the fungal communities. Changes in N-biogeochemistry were rapid and denitrification enzyme activity (DEA) increased up to 25-fold with the highest rates occurring in microcosms at pH 8.3 that had been incubated for 24-hour prior to measuring DEA. Nitrous oxide reductase was inactive in the pH 4.7 controls but at pH 8.3 the reduction rates exceeded 3000 ng N₂-N g⁻¹ h⁻¹ in the presence of native DOM. Evidence for dissimilatory nitrate reduction to ammonium and/or organic matter mineralisation was observed with ammonium increasing to concentrations up to 10 times the original native soil concentrations while significant concentrations of nitrate were utilised. Pure isolates from the microcosms were dominated by *Bacillus* spp. and exhibited varying nitrate reductive potential.

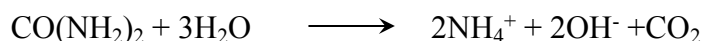
Introduction

Soil pH has a strong influence over soil processes such as N-cycling as it impacts soil chemistry, physics and biology. Denitrification is an anaerobic stepwise enzymatic process whereby nitrate (NO₃⁻) is reduced (via NO₂⁻) to nitric oxide (NO), N₂O and finally molecular nitrogen (N₂). Denitrification efficiency is primarily affected by soil pH because pH influences carbon supply and associated metabolisms but also impacts the activity of denitrification enzymes adapted to specific pH conditions and the function of N₂O reductase (N₂O-R) (Anderson et al. 2017; Baggs et al. 2010; Bakken et al. 2012b; Curtin et al. 2016; Liu et al. 2010b; Morkved et al.

2007; Samad et al. 2016b; Schimel et al. 2005; Simek & Cooper 2002). Dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) is also an anaerobic process that reduces NO_3^- and variably contributes to N_2O emissions depending on carbon availability (Giles et al. 2012; Rutting et al. 2011). Again, pH affects carbon supply, alters its accessibility, and thus influences the microbial response. Under anaerobic conditions there is also a strong interplay between carbon availability and whether or not microbes will utilise fermentation or metabolisms such as NO_3^- reduction (van den Berg et al. 2017a; van den Berg et al. 2017b). Although there is a reasonable mechanistic understanding of how changing soil pH affects chemistry and physics, the literature is less robust concerning the dynamics of biological response at molecular levels both phylogenetically and functionally.

The long-term effects of pH on microbial community structure and abundance have been studied at local to global scales, but only broad conclusions can be drawn (Fierer & Jackson 2006; Lauber et al. 2009). For example, at the localised scale in the Rothamsted Hoosefield acid strip, bacterial ‘richness’ has been shown to increase between pH 4 and 8, whereas changes in fungal populations were not pronounced (Rousk et al. 2010a); or within the Rothamsted Park Grass Experiment, soil pH was positively correlated with 14 of the 37 most abundant soil genera (Zhalnina et al. 2015). Correlations become more tenuous at larger scales with pH known to shape microbial communities, but this seems to be based more on functional genetic diversity than taxonomic classification. For example, at continental scales, Acidobacteria have been shown to be negatively correlated with pH (>4) but within phyla, the relative proportions of Acidobacteria subgroups exhibit opposing trends as pH increases (Lauber et al. 2009).

In contrast to studies describing microbial community response to pH in long-term trials, reports about community response to rapid, short-term pH change are sparse; yet microbial communities in soils are often subjected to dynamic change. For example, rapid pH change after animal urine deposition or in the vicinity of urea fertiliser prills. When urea ($\text{CO}(\text{NH}_2)_2$) is added to the soil (as fertiliser or urine), it quickly undergoes hydrolysis in the presence of urease enzymes:



The OH^- ions produced during this process cause substantial pH increases, to values >7.5 over the course of a few days, coupled with the pH mediated release of dissolved organic matter (DOM) (Clough et al. 2010; Curtin et al. 2016; O’Callaghan et al. 2010). During the first week post urine deposition, pH continues to rise (to values > pH 8), ammonium (NH_4^+) oxidation to NO_3^- (via NO_2^-) commences, while oxygen concentrations and redox conditions decrease via nitrification

reactions and through microbial metabolism (Clough et al. 2010; Hansen et al. 2014; Nowka et al. 2015) – suitable conditions for NO_3^- reductive processes. Our previous research using KOH or $\text{Ca}(\text{OH})_2$ as proxies for NH_4OH indicates that denitrification response becomes elevated very shortly after pH and DOM increases (Anderson et al., 2017).

In addition to allowing documentation of soil physicochemical response, urine patches represent a natural laboratory setting for investigating realistic microbial community structural and functional response to rapid pH change. A few studies have reported changes in soil microbial population structures in response to urine (or synthetic urine), but have not investigated the taxonomic profiles of these populations in detail (Rooney et al. 2006; Singh et al. 2009). In the study of Singh et al. (2009), addition of synthetic urine to soil was not associated with increases in microbial biomass C or N and the relative population structure of fungi did not change. Taxonomic changes observed by O’Callaghan et al. (2010) indicated that *Firmicutes* increased by 38% after urine addition while Proteobacteria decreased (18%), but fine resolution of taxonomic groups contributing to denitrification or any other N-cycling process was not achieved.

Using soil microcosms, we sought to simulate the effects of rapid soil pH change likely to occur under urine patches or around urea prills during the first 48 hours post urea deposition. We hypothesised that the microbial population would undergo major structural and physiological change in response to pH, increases in DOM and decreases in oxygen; with N-processing shifting to NO_3^- reductive processes. In order to change soil pH and isolate the denitrification processes without adding extra N (or C) via urea, we used potassium hydroxide as an NH_4OH proxy (see Anderson et al. 2017) and utilised anaerobic conditions. After different exposure times to four different pH treatments, the potential denitrification enzyme activity (DEA) of the microbial communities was assayed, amplicon sequencing was used to provide a detailed assessment of the changes in the microbial populations, and a collection of nitrate reducing bacteria were isolated from the microcosms.

2. Materials and methods

All aqueous solutions were prepared using ultrapure water from a MilliQ water system (18 MΩ-cm resistivity) and all chemicals used were ACS reagent grade, unless otherwise stated.

2.1 Soil collection, pH adjustment and DEA assays

The Wakanui silt-loam soil used in this study was sourced from no-till plots in a long-term field trial (12 years) at Lincoln, Canterbury, New Zealand. The basic chemical characteristics of the soil were: pH 5.6; total C, 27 g kg⁻¹; total N, 2.4 g kg⁻¹; NO₃-N, 20 mg kg⁻¹ and NH₄-N, 4 mg kg⁻¹. Further details about the soil and sampling site can be found in Curtin et al. (2016) and Anderson et al. (2017). Soil samples were treated with four rates of KOH (base addition rates of 0, 6.0, 16.0 & 20.0 cmol_c kg⁻¹ soil). These treatments were selected based on results from previous experiments (Anderson et al. 2017) with the 6.0 cmol_c kg⁻¹ and 16.0 cmol_c kg⁻¹ treatments representing “low/moderate” and “upper limit” pH increases following animal urine deposition. The 20 cmol_c kg⁻¹ treatment represented an alkaline pH outlier, where DEA was expected to be minimal (Anderson et al 2017).

A total of 240 soil microcosms were prepared, covering 4 KOH treatments and 5 incubation times. This provided twelve analytical replicate microcosms for each KOH rate x incubation time combination; where, four microcosms were designated for soil chemical analysis prior to DEA assays, two were designated for nucleic acid extraction and microbial culturing work (prior to DEA), and the remaining six microcosms were used for DEA assessments (two triplicate DEA assays, with or without acetylene) (Supplementary Figure S1).

Microcosms were prepared as described by Anderson et al. (2017). Briefly, 25 g (dry weight equivalent) soil was placed in 250 mL bottles and KOH was added together with KCl to balance electrical conductivity across treatments. The final solution volume in each microcosm was adjusted to 25 mL. The bottles were evacuated (to -1 atm.) then flushed three times with N₂ (instrument grade, <0.001% O₂) over a 30 min period until O₂ was < 0.03%. The microcosms were then incubated at 20°C on an orbital shaking platform (150 rpm) for 16, 24, 32, 40 or 48 h. After each incubation the headspace was sampled for N₂O and CO₂.

The following sampling protocol was followed: For chemical analyses, 5 mL of slurry was collected from four microcosms to determine dissolved CO₂ (acidified with 2 mL of 2M HCl to dissolve any carbonates). The remaining slurry from these four microcosms was centrifuged (5min at 20,000 rpm), and the supernatants filtered (<0.45 µm) then frozen (-20°C), pending pH, EC, DOC, DON and NO₃⁻/NH₄⁺ analyses. From a further two microcosms, 1 mL aliquots of slurry were taken for nucleic acid extraction and bacterial colony isolations.

To determine DEA, 5 mL of water containing 50 mg $\text{NO}_3\text{-N kg}^{-1}$ (Luo et al. 1996) was added to the remaining six microcosms, and the anaerobic atmosphere was regenerated by evacuation and flushing three times with N_2 . To three of the microcosms a volume of scrubbed (Hyman & Arp 1987) acetylene (final ratio of 10% v/v) was added by syringe and allowed equilibrate (with shaking) for 10 minutes before venting these microcosms to atmospheric pressure. No external C-sources such as glucose were added. The microcosms were incubated at 20°C on a shaking platform (150 rpm) and sampled hourly over a 4 hour period to measure headspace N_2O and CO_2 . Headspace gases removed were replaced with an equivalent volume of N_2 .

At the end of the DEA assay, dissolved CO_2 , pH, EC, DOC, DON and $\text{NO}_3^-/\text{NH}_4^+$ were measured in the remaining soil slurry samples.

2.2 Chemical Analysis and Gas Chemistry

Analytical methods are described in Anderson et al. (2017). Briefly, concentrations of N_2O and CO_2 were determined on a Shimadzu Corp. GC-17A gas chromatograph and the DEA value (i.e. potential denitrification rate) and respiration rates were calculated from the linear relationship between evolved N_2O or CO_2 and time. The extracts were analyzed for pH (ThermoScientific Orion™ AquaPro™ pH combination electrode) and electrical conductivity (Eutech Instruments PC510 conductivity meter). Dissolved organic C was determined using a Total Organic Carbon Analyzer (Shimadzu TOC-V_{CSH}, Shimadzu Corp, Japan). Total N was determined by persulfate oxidation, as described by Cabrera and Beare (1993), and organic N was estimated by subtracting mineral N (KCl extracted NH_4^+ and NO_3^- determined using an automated colorimeter) from total N.

2.3 Microbial population profiling via next generation sequencing

A 1 mL aliquot of soil slurry was centrifuged at 14,000 rpm for 5 minutes. DNA from the resulting pellet was extracted with the MoBio Powersoil DNA kit (Carlsbad, CA). The V3-V4 variable regions of the bacterial 16S rRNA was amplified with the 341f and 785r primer pair (Klindworth et al. 2013). The fungal internal transcribed spacer 1 (ITS1) region was amplified with NSI1a_mod (5'-GATTGAATGGCTTAGTGAGK-3') and 58A2R (5'-AGTCCTGCGTTCTTCATCGAT-3'), both adapted from (Martin & Rygielwicz 2005). Primers included the Illumina adapter sequences.

PCR amplifications contained ~10 ng DNA template, 10 nmol each primer, 1X mastermix, and 0.5 U KAPA3G polymerase (Merck, Auckland, New Zealand), in a final volume of 20 µl. Reactions were performed in duplicate. Cycling parameters were 94°C for 2 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s. Duplicate reactions were combined and purified with AMPure XP beads (Agencourt, Beckman Coulter Life Sciences). Purified amplicons were quantified by gel electrophoresis and UV absorbance (NanoDrop ND-1000). Amplicons were 2 x 300 bp paired end sequenced on an Illumina MiSeq platform (New Zealand Genomics Limited, Auckland).

2.4 Sequence processing and statistical analysis

USEARCH v8.0.1517 (Edgar 2013) was used to merge the paired end reads, filter chimeric sequences and cluster sequences at 97% similarity. An expected error of 1.0 was used for filtering. Singleton reads were discarded. The bacterial 16S OTUs were identified using the RDP Naïve Bayesian Classifier implemented in USEARCH against Greengenes (version 13_8) and fungal ITS OTUs against the UNITE reference dataset (Version 6, 04/07/2014, downloaded on 08/07/2014) (<http://www2.dpes.gu.se/project/unite/UNITE>) (Koljalg et al. 2013). Biom (OTU) tables were produced using biom-format (<http://biom-format.org/>) (McDonald et al. 2012) in USEARCH with rarefaction performed in phyloseq.

The phyloseq (McMurdie & Holmes 2013) and ggplot2 (Wickham 2009) packages within R (Team 2016) were used for the analysis and visualisation of data at phyla level. OTU tables were also analysed using Primer 7 with PERMANOVA add-on (Primer-E Ltd, Plymouth, UK). Both rarefied and non-rarefied data was analysed based on the work of McMurdie and Holmes (McMurdie & Holmes 2014). Where data was not rarefied, samples were standardised by total and no statistical inferences were made regarding differentially abundant species (OTUs), rather our conclusions were based on assessing broader scale relative changes in the microbial communities only. Relationships among microbial community profiles based on Bray-Curtis similarity matrices were graphed using unconstrained non-metric multidimensional scaling (nMDS) ordinations using 250 restarts along with cluster analysis. Relationships observed among all OTUs were then statistically tested using 2-factor permutational ANOVA within the Primer 7 package (PERMANOVA) with estimated components of variation being a standard output of the analysis. Data was untransformed unless otherwise stated, whereupon log(X+1) transformations were applied.

To understand gradients and group structures across treatments, matrix plots of standardised data were prepared using a reduced sample set of 20 OTUs, with the OTUs retained having the greatest contribution to total counts for the individual samples compared at each pH value, or in the case of fungi, across all pH values. Using reduced sample sets allows simplification of the matrix plots by removing those organisms accounting for a negligible proportion of the total number of OTUs. Samples in the matrix plots were clustered using Bray Curtis similarity (based on all OTUs) while the 20 OTUs presented were clustered according to similarity based on an index of association across samples tested.

2.5 Isolation of bacteria, N-use characterisation

2.5.1 Isolation of nitrate reducing bacteria

Previous research by O'Callaghan et al. (2010) suggested that Firmicutes increased by substantial amounts in conditions similar to those we investigated. Although only a small percentage of soil bacterial diversity can be cultured, it is relatively straightforward to culture members of the Firmicutes (among others); hence, in order to gain some appreciation for the N-processing capabilities of culturable bacteria from these microcosms, we attempted to isolate NO_3^- reducers. Ten μL sample of slurry from each pH adjusted microcosm was serially diluted (10 μL into 1mL followed by three dilutions of 10 μL into 100 μL) and plated onto TSB or 1/10 diluted TSB containing KNO_3 (30g L^{-1} + 0.5g L^{-1} KNO_3). Plates were incubated under anaerobic conditions (Whitney jars with gas packs) at 24°C for 2 to 6 days.

Representative colonies of different morphology were selected and re-streaked onto the same media and grown under anaerobic conditions at 24°C. Isolates were stored in 20% glycerol at -80°C, with a selection of isolates identified by amplification and Sanger sequencing of the 16S ribosomal RNA gene using the 27F and 1492R primers (Anderson et al. 2009).

2.5.2 Nitrate utilisation by bacterial isolates

Cells were grown aerobically overnight in TSB with KNO_3 . 20mL cultures were then initiated with 1/100 dilutions of the overnight cultures, and grown anaerobically (O_2 replaced with N_2) at 24°C. After 48 hours, gas samples were extracted with a gas-tight Hamilton syringe and analysed

for the presence of N_2O and CO_2 by gas chromatography (as outlined above) and 10 mL samples of the bacterial cultures were removed for NO_3^- and NH_4^+ analysis (as outlined above).

3. Results

3.1 Chemical characterisation of pH amended soils prior to DEA assays

After 48 hours of anaerobic incubation, soil slurries with additions of 0, 6, 16 and 20 cmol_c kg⁻¹ KOH had average pH values of 4.7, 6.7, 8.3 and 8.8 respectively. During the 48 h incubation EC reached ~6.4 mS cm⁻¹ in all microcosms except the pH 6.7 microcosms which reached ~5.7 mS cm⁻¹ (Table 1). DOC in the control microcosms increased from 37 to 63 mg kg⁻¹ during the 48 h incubation. In the pH 6.7, 8.3 and 8.8 microcosms DOC increased ~11, 185 and 240-fold respectively in response to KOH addition, with the majority of that change (> 75%) occurring during the first 16 h of incubation. During the 48 h incubation DON in the control microcosms increased from 2.4 to 7.4 mg kg⁻¹, while in the pH 6.7, 8.3 and 8.8 microcosms DON increased ~9, 140 and 223-fold in response to KOH addition, respectively (Table 1). There was a strong correlation between the amounts of DOC and DON solubilised at each pH value, irrespective of incubation time ($R^2 = 0.98$) (Supplementary Figure S2).

Higher respiration (CO_2 production) was associated with DOM increases but the amount of DOC (or DON) solubilised via pH change was not a good predictor of respiration. Respiration in the control and pH 6.7 microcosms generally followed an increasing trend for the first 32 hours before decreasing (Table 1). The pH 8.3 microcosms exhibited respiration rates that increased throughout the 48 h incubation period (maximum recorded rate of ~1600 ng $\text{CO}_2\text{-C g}^{-1} \text{ h}^{-1}$). Respiration in the pH 8.8 microcosms followed an opposing trend, declining slightly during the first 32 hours, followed by recovery.

In control microcosms, ammonium (NH_4^+) increased linearly over 48 hours with a slope of 0.09, starting from native soil concentrations of ~4 mg kg⁻¹. The NH_4^+ profiles in the pH 6.7, and 8.3 microcosms were similar (approximately linear) but with greater slopes of 0.28 and 0.79 respectively (R^2 values of 0.99 and 0.98). In the pH 8.8 microcosms, NH_4^+ quickly elevated to 30 mg kg⁻¹ during the first 16 hours of incubation and remaining at that concentration until after 32 hours, when a further increase from 30 to 50 mg kg⁻¹ occurred by 48 hours (Table 1). The native soil nitrate (NO_3^-) concentration was ~20 mg kg⁻¹. This NO_3^- was almost completely reduced after 32-40 hours incubation in the control, pH 6.7 and pH 8.3 microcosms (Table 1). In the pH 8.8

microcosms, NO_3^- remained at $\sim 20 \text{ mg kg}^{-1}$ until after 32 hours incubation, dropping to $\sim 6 \text{ mg kg}^{-1}$ during the following 16 hours.

The control microcosms had the highest concentrations of N_2O in the headspace, increasing from ~ 1650 to $6800 \text{ } \mu\text{g kg}^{-1}$ between 16 and 48 hours of incubation (Table 1). The pH 6.7 microcosms had $\sim 850 \text{ } \mu\text{g kg}^{-1}$ of N_2O in the headspace after 16 hours incubation, but by 24 hours this had declined to $< 15 \text{ } \mu\text{g kg}^{-1}$. Headspace N_2O in the pH 8.3 and 8.8 treatments was $< 15 \text{ } \mu\text{g kg}^{-1}$ for all time points except 24 hours for the pH 8.3 treatment ($125 \text{ } \mu\text{g N}_2\text{O kg}^{-1}$ soil).

3.2 DEA assays

Microcosms incubated for 16 hours had the lowest DEA rates with 80, 540, 34 and $0.2 \text{ ng N}_2\text{O g}^{-1} \text{ h}^{-1}$ recorded for the control, pH 6.7, 8.3 and 8.8 treatments respectively (Fig. 2A). DEA remained low in the control microcosms with a maximum DEA rate of only $121 \text{ ng N}_2\text{O g}^{-1} \text{ h}^{-1}$ after 24 hours incubation, declining to $85 \text{ ng N}_2\text{O g}^{-1} \text{ h}^{-1}$ in microcosms incubated for 48 hours (Fig. 1). In contrast, after 24 hours incubation, DEA rates in the pH 6.7 and 8.3 microcosms were 8 and 25-fold higher respectively with a maximum of $\sim 3000 \text{ ng N}_2\text{O g}^{-1} \text{ h}^{-1}$ produced (Fig. 1A). DEA rates remained in the vicinity of 900 and $2000 \text{ ng N}_2\text{O g}^{-1} \text{ h}^{-1}$ for the pH 6.7 and 8.3 treatments, respectively, for microcosms incubated up to 40 hours. DEA rates greater than those in the control were not observed in the pH 8.8 treatment until microcosms were incubated for at least 48 hours ($\sim 65 \text{ ng N}_2\text{O g}^{-1} \text{ h}^{-1}$).

Replicate microcosms without acetylene added were used to assess N_2O -R activity during the DEA assays based on the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio. These showed that N_2O reduction was absent in the control microcosms while in the pH 6.7, 8.3 and 8.8 treatments between 56 and 100% of the N_2O produced was reduced to N_2 depending on incubation time (Fig. 1B). Near complete N_2O reduction was observed in the pH 8.3 treatment for microcosms incubated longer than 24 hours. Depending on pH treatment and incubation time, N_2O production (and reduction) profiles during the DEA assays were mirrored by decreases in NO_3^- with an estimated 30 to 80 % of the available NO_3^- reduced. In the control and pH 6.7 microcosms there were no differences in the NH_4^+ before and after the 4-hour DEA assay period, however decreases of up to 9 mg kg^{-1} occurred in the pH 8.3 microcosms after 32 hours incubation and in the 8.8 microcosms after all incubation times (Supplementary Table ST1).

T-tests comparing CO₂ respiration indicated that acetylene addition depressed microbial activity, but only in the pH 8.3 and 8.8 treatments ($P < 0.05$). It is acknowledged that lower soil respiration in the presence of acetylene will reflect both the absence of any CO₂ derived from metabolic N₂O reduction but possibly also general impediment of other anaerobic metabolisms. It is unlikely that acetylene would have served as dominant carbon source during the 4 hour DEA incubation given the excess DOC available and time required to adapt to using acetylene (Felber et al. 2012; Groffman et al. 2006). There is evidence to suggest that DOC and DON declined during the DEA assay period, especially in the pH 8.3 and 8.8 treatments but the results were highly variable (Supplementary Table ST1).

3.3 Microbial community adaptation to pH treatments during 48 hours incubation

The total number of OTUs identified across all treatments and incubation times was 2258 for fungi and 6429 for bacteria with an average of 84000 reads per sample. Sequences from this Targeted Locus Study (LTS) project has been deposited at DDBJ/EMBL/GenBank under the accession KCDA00000000. The version described in this paper is the first version, KCDA01000000. Fungal ITS1 sequences have been deposited at DDBJ/EMBL/GenBank under the accession numbers MH624180 - MH625694. Two factor tests using PERMANOVA [pH x incubation time] for all OTUs supported the nMDS observations indicating significant interactions between time and pH ($P=0.001$ for both bacteria and fungi) (Figure 2). Approximately ~90% of the total variation was explained for the bacterial relationships whereas only ~43% was explained for the fungi. Of the variation explained for the bacteria, up to 60% was attributed to pH, a further 15% to incubation time, and 18% was attributed to the interaction between the two factors. The corresponding values for the fungal communities were 28% of variation attributed to pH, 6.6% to incubation time and 8.9% to the interaction between the two factors (Figure 3).

The phylum level bacterial profile from the control (pH 4.7) microcosms after 16 h incubation was made up of Acidobacteria (~7%), Actinobacteria (~12%), Bacteroidetes (~7%), Proteobacteria (~20%), Firmicutes (~5%), Planctomycetes (~5%) and phyla that had abundances of >5% including Verrucomicrobia, Chloroflexi, Gemmatimonadetes and Armatimonadetes. Up to 35% of OTUs could not be classified (Supplementary Figure S3). After 24 hours incubation, the bacterial communities were still >85% similar before there was a relative decrease in Actinobacteria coupled with an increase in Acidobacteria and Bacteroidetes (Supplementary

Figure S3). A decrease in Actinobacteria was also seen over the incubation periods for all three pH modifications along with changes in the proteobacterial populations with almost complete disappearance of OTUs from the order Rhizobiales (Figure 4, Supplementary Figure S3).

After 16 and 24 hours incubation, the bacterial profiles in the pH 6.7 microcosms were similar to those in the control microcosms with the exception of a larger proportion of Firmicutes (~20% versus 5% in the controls) (Supplementary Figure 3). The increased representation of Firmicutes was driven by an expansion of OTUs from the orders Bacilliales (OTU_4) and Clostridiales (OTU_1, *Tepidibacter sp.*). These two OTUs then decreased later in the incubation, partially displaced by other *Clostridium spp.* (OTUs 16 and 26) (Fig. 4B). Across the 32 to 48h incubation period there was a large relative increase in OTUs from the phylum Bacteroidetes, driven by *Flavobacterium spp.* (OTUs 62, 9, 80, 410, 37 and 41) (Fig. 4B).

In the pH 8.3 treatment after 16 hours incubation, there was a very high proportion of Firmicutes (~40%) and a low representation of Actinobacteria and Bacteroidetes compared with the pH 4.7 control microcosms. Across the incubation period, the dominance of Firmicutes increased further, initially driven by the same Bacilliales and Clostridiales OTUs (4 and 1), but at a much higher relative 'abundance' than observed in the pH 6.7 treatment. Their expansion coincided with the highest DEA rates, with the average 'abundance' of OTU_4 being ~20% of total at this time and OTU_1 being ~23% (Fig. 4C, Supplementary Figure S3). After 32 h these two dominant OTUs were partially displaced by a cohort of other OTUs from the orders Bacilliales and Clostridiales (OTUs 2, 75, 31, 25, 36, 12, 13, 26 and 109) (Fig. 3). At the genus level these OTUs represented *Bacillus*, *Paenibacillus*, *Clostridium* and *Alkaliphilus spp.* (Fig. 3). Communities in the 40 and 48h incubations shared the highest level of similarity (Figs. 2, 4C and Supplementary Figure S3).

After 16 and 24 hours incubation, the bacterial profiles from the pH 8.8 treatment shared >70% similarity with the samples from the control microcosms (Fig. 2). Over the incubation period to 32 h, there was a large expansion in the relative percentage of the Firmicutes, initially driven almost entirely by an increase in OTU_5 from the Bacilliales order (Fig 4D). By 48 h, this OTU was partially displaced by a group of Bacilliales and Clostridiales OTUs that shared some similarity with those observed in the pH 8.3 treatment (OTUs 1, 4, 156, 36, 12, 13, and 199). These OTUs included the same genus level representatives - *Bacillus*, *Paenibacillus*, *Clostridium* and *Alkaliphilus spp.* (Fig. 4D).

Fungal communities shared >70% similarity across all samples regardless of pH and time (Figure 3). Based on percentages alone, no discernible patterns could be observed for the fungal dataset (Supplementary Figure S4), however, some OTUs were displaced depending on pH. For example, Zygomycota OTU_3 was dominant in the control and pH 6.7 treatments but was partially supplanted by another Zygomycota OTU from the order Mortierellales (*Mortierella sp.*) (Fig. 5).

An identical analysis of rarefied bacterial and fungal OTUs datasets is presented in Supplementary Figures S5 to S10. A range of diversity measures have been presented in Supplementary Figures S11 and S12. An nMDS plot from a preliminary experiment showing the relationship between bacterial communities in the initial untreated (aerobic) field-moist soil and the soil microcosms incubated under anaerobic conditions with various pH treatments is presented in Supplementary Figure S13.

3.4 Isolation of bacteria and N-use characterisation.

On TSB-nitrate medium, plates were quickly dominated by fast growing colonies sharing morphologies characteristic of motile or swarming bacteria. A total of 33 isolates were screened for nitrate utilisation. Seven showed minimal growth and respiration in liquid culture, 18 showed near complete utilisation of NO_3^- accompanied by production of both NH_4^+ and N_2O , and 5 showed moderate utilisation of NO_3^- with low production of NH_4^+ and N_2O . One isolate showed production of NH_4^+ with little or no use of NO_3^- or production of N_2O and 2 isolates exhibited respiration but did not appear to utilise N. Of the 33 isolates, 22 were selected for identification by ribosomal 16S gene DNA sequencing. All were from the Firmicutes phylum, of which three were *Paenibacillus spp.* and 1 was a *Brevibacillus sp.* (all producing less NH_4^+), while the remainder were *Bacillus spp.* (Supplementary Table ST2A).

From 1/10 diluted TSB-nitrate medium, 44 isolates were screened for nitrate utilisation. Of these, 24 showed no or minimal growth in liquid culture over 48 hours incubation while 13 reduced NO_3^- to close to zero with 4 of these generating significant amounts of NH_4^+ . Another 3 reduced NO_3^- , but to a lesser extent, while 4 isolates respired but did not appear to utilise N. Seventeen isolates were identified by 16S gene sequencing. Six belonged to the *Bacillus* genus, three to *Achromobacter*, six to *Acidovorax*, one to *Bosea*, and one to *Rhodanobacter* (Supplementary Table ST2B).

All isolates except one of the *Bacillus* sp. had high NO_3^- utilisation compared to uninoculated controls. The *Bacillus* spp. produced up to 536 mg NH_4^+ L^{-1} , while the *Acidovorax* spp. produced < 45 mg NH_4^+ L^{-1} . One *Bacillus* sp. and two *Acidovorax* sp. produced < 100 mg N_2O L^{-1} , while the remaining *Acidovorax* sp. had the highest N_2O production at 2840 mg L^{-1} . In general, isolates that exhibited high use of NO_3^- coupled with production of NH_4^+ and N_2O exhibited an average of ~2.5-fold higher CO_2 production (respiration) compared with isolates that exhibited high NO_3^- use with little or no NH_4 and N_2O production. Relevant 16S sequences were submitted to NCBI with accession numbers assigned between MH211426 and MH211463, submission number SUB3915485 (Supplementary Table 2). Six organisms were selected for future genome sequencing, 3 *Bacillus* spp. and 3 *Acidovorax* spp. (Supplementary Table ST2C).

4. Discussion

The effects of rapid pH change on soil chemistry and microbiology

KOH additions and resulting pH elevation caused the concentration of DOM in soil microcosms to greatly increase (150-fold) compared to the controls. Added hydroxyl ions displaced negatively-charged organic molecules into solution. Previous work has shown that monovalent cations like K^+ (KOH) and NH_4^+ (NH_4OH – product of urea hydrolysis) are much more effective in solubilising organic matter than divalent cations such as Ca^{2+} ($\text{Ca}(\text{OH})_2$) (Curtin et al. 2016). The amount of DOM released at elevated pH in these experiments was concordant with our previous research using the same methodology (Anderson et al. 2017), as were the higher respiration rates.

Over medium to long time scales (months to years), pH is known to be a dominant environmental variable that shapes soil microbial communities (Lauber et al., 2009; Zhalina et al., 2015). Changes in pH are also known to cause shifts in active organisms over short timescales (Brenzinger et al. 2015). Although the strongest predictor for both bacterial and fungal community change in these experiments was pH, community change may also be indirectly influenced by the effect that pH has on DOM release. Theoretically, high levels of DOM released via increased pH should benefit copiotrophs (r) over oligotrophs (K) (Fierer et al. 2007; Goldfarb et al. 2011), but at the same time elevated pH is likely to alter cellular homeostasis, regulation of nutrient

availability, or other factors such as salinity, metal accessibility, or organic C characteristics (Lauber et al. 2009).

Previous analysis of the soil used in this study suggests that a mix of carbon sources are released as pH increases, of which 45% are bioavailable. These range from labile hexose and pentose sugars to more recalcitrant polyphenolic molecules (Curtin et al. 2016). The lack of proportionality between respiration rates and DOM released in this study suggests that higher amounts of bioavailable C did not lead to higher biomass, instead the microbial community and associated metabolic response has shifted toward more copiotrophic organisms. Addition of low molecular weight C compounds (glucose, citric acid, glycine) to soil has been previously observed to shift the structure of bacterial communities to more copiotrophic organisms (Eilers et al. 2010) with no strong correlations between respiration rates and community structure. Community changes and catabolic responses may be unlinked because some C-substrates are preferentially used without biomass changes (Devevre & Horwath 2000).

Proteobacteria are abundant in high C soils (Fierer et al. 2007) with β and γ -Proteobacteria considered important soil copiotrophs (Eilers et al. 2010) in conjunction with Firmicutes and Actinobacteria (Zhalnina et al. 2015). Bacteroidetes and β -Proteobacteria are initial metabolisers of labile soil-C (Padmanabhan et al. 2003) and increases in the abundance of these organisms have been correlated with C mineralisation rates (Fierer et al. 2007). Our study is consistent with regard to expansion of Bacteroidetes (*Flavobacteriales*), specifically in the control and pH 6.7 microcosms (~5.5-fold-increase in DOM with ~2-fold increase in respired CO₂), suggesting that the microbial community does respond to higher concentrations of bioavailable C at pH values <7.

In general, we observed a decrease in α -Proteobacteria (specifically *Rhizobiales*), Actinobacteria and Acidobacteria OTUs at all pH and DOM values, while a few β - and γ -Proteobacteria OTUs increased. Our results suggest that the chemical changes induced by KOH addition to soil are comparable to soils where pH and DOM are elevated due to higher urea inputs. Niche differentiation occurs in soil where higher bovine density (and presumably urea inputs) induces increases in pH and total organic carbon, with Actinobacteria, α -Proteobacteria and Verrucomicrobia decreasing and Bacteroidetes increasing (Philippot et al. 2010; Philippot et al. 2009a; Philippot et al. 2009b).

In the study by Fierer et al. (2007), abundance of Firmicutes could not be predicted by C-mineralisation (nor other measured soil parameters), while in the Park Grass experiment in the UK, total C and N and pH were negatively correlated with Firmicutes (Zhalnina et al. 2015). Our study differs from the literature with regard to the Firmicutes as they are the most responsive to pH and DOM increases. Our experimental conditions are quite different with the combined complexity of alkaline pH and anaerobic conditions likely playing a larger role than just DOM in defining niche differentiation and shaping microbial community structure (Banerjee et al. 2016; Husson 2013; Pett-Ridge & Firestone 2005).

Comparisons of OTU distributions in our study indicate that the dominant feature driving sample dissimilarities was large increases in Firmicutes from the classes Bacilli and Clostridia. Large expansion of Firmicutes, first dominated by *Bacillales* (up to 46%) and then followed by *Clostridiales* (up to 53%), (along with large decreases in Proteobacteria) have been observed in alkaline soil crusts (pH 8.5) that were rehydrated and incubated under dark anoxic conditions (Angel & Conrad 2013). Our results are also concordant with O'Callaghan et al. (2010), who observed a 38% increase in Firmicutes, and decreases in Proteobacteria (18%), Acidobacteria (8%), Actinobacteria (5%), and Bacterioidetes (5%), in soil where pH rose to values of >8, two days after bovine urine addition.

Genus level identifications from the Bacilli and Clostridia in our study included *Bacillus*, *Paenibacillus*, *Tepidibacter*, *Alkaliphilus* and *Clostridium*. Cultured examples of these organisms from the literature include facultative anaerobes (and obligate anaerobes) that are highly responsive to more recalcitrant C-sources, are either alkaliphilic or alkalotolerant, and show fermentative type metabolisms (Chen et al. 2006; Goldfarb et al. 2011; Lee et al. 2007; Slobodkin et al. 2003; Urios et al. 2004). *Bacillus* species and related genera can be found in a wide variety of habitats. *Bacillus* and *Paenibacillus* species can be considered as drivers of soil organic matter mineralisation, are frequently abundant in situations where C and N are not limited and are capable of degrading polymeric carbonaceous substances (Mandic-Mulec et al. 2015). Although there is some evidence to suggest that *Clostridium* are generally acid loving (Kuhner et al. 2000), the related clostridial OTUs (*Tepidibacter* and *Alkaliphilus*) identified in this study suggest a wider range of pH tolerance (Lee et al. 2007; Slobodkin et al. 2003; Urios et al. 2004).

Relative changes in fungal populations in response to pH change were smaller than for bacteria, with ~28% of fungal variation attributed to pH, versus ~57% for bacteria. Only ~6.6 percent of the variation in fungal communities could be attributed to incubation time. Fungal communities are known to be less responsive to pH than bacteria (Lauber et al. 2009; Rousk et al. 2010a) and fungal abundance has been found to be negatively correlated with pH (Rousk et al. 2010b), but positively correlated with C and N additions (Banerjee et al. 2016). For example, investigations of the response of fungal (and bacterial) communities to ovine urine (where pH increased from ~3.5 up to 6.5 and DOC increased from ~0 up to ~2000 mg kg⁻¹) indicates no fungal biomass change (Nunan et al. 2006; Williams et al. 2000), no significant correlation between biomass and pH, NH₄⁺ or NO₃⁻, but weak correlation between biomass and DOC (Singh et al. 2009). In pH 6.1 soils, Banerjee et al (2016) noted that although fungal biomass increased, the richness, evenness, and diversity decreased within 4 days after organic matter and nutrient addition leading to 'keystone' fungal species being favoured. Singh et al. (2009) suggest that because fungi are capable of degrading complex organic carbon they are less responsive to short term changes in nutritional availability.

4.2 Soil N processes and their relationship to microbiology following pH change

Denitrification occurred in all treatments because NO₃⁻ was consumed and N₂O was produced, however there were variations in the amount of time required for the original supply of native NO₃⁻ to be consumed. Native NO₃⁻ was nearly completely utilized within the first 16 hours in the control, pH 6.7 and pH 8.3 microcosms, but in the pH 8.8 microcosms, NO₃⁻ did not decline until after 32 hours. When additional NO₃⁻ was added to measure denitrification rates, maximum DEA occurred in microcosms that had been incubated for at least 24 hours.

For all treatments, DEA potential declined at incubation times greater than 24 hours. This may have been due to extended periods (i.e. >24 hours) of low NO₃⁻ concentrations prior to the DEA assays leading to a decline in NO₃⁻ linked translation of denitrification genes such as N₂O-R and nitrate reductase (Moreno-Vivian et al. 1999; Zumft 1997). Alternatively, the microbial communities that developed with increasing incubation times may have expressed different denitrification phenotypes (Dorsch et al. 2012; Sanford et al. 2012).

It also seemed that the relative metabolic contribution of denitrification declined over time as NO_3^- was utilized, giving the opportunity for other anaerobic metabolisms such as fermentation to have proportionally greater influence. For example, in the pH 8.3 treatment, the respiration rate in microcosms incubated for 24 hours was 40% lower than those incubated for 48 hours, yet at 24 hours denitrification rates were ~4.5-fold higher. *Bacillus* OTUs dominated at 24 hours where the lower respiration rates and higher denitrification was observed, but *Bacillus* was then displaced by a consortium of clostridial species after 48 hours. Given that *Clostridia* can be obligate or facultative anaerobes, we think that this species displacement is a response to the changing chemical conditions in the microcosms marked by nitrate depletion, elevated pH and sustained anaerobicity.

In contrast to the KOH amended microcosms, the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio in the control microcosms equalled 1, indicating that this treatment did not have active N_2O -R. Liu et al. (2010a) and Bakken et al. (2012a) have previously shown that production of functional N_2O -R depends on the post-transcriptional pH being greater than 6.1, which is consistent with our results. In an agricultural environment this raises interesting ecological questions, because urea hydrolysis happens to elevate pH for several days which would immediately alleviate any post-transcriptional interference of *nosZ* expression and allow rapid production of functional N_2O -R.

We observe full denitrification of NO_3^- to N_2 within 16 hours and maximum rates after 24 hours which indicates that suitable redox conditions for denitrification were established quickly in our microcosms and a corresponding rapid genetic and enzymatic response followed. The predominant electron acceptors in a weakly reducing environment are O_2 , NO_3^- and manganese oxide (MnO_2) (Uteau et al. 2015) with the threshold between oxic and anoxic soil lying somewhere between 300 and 400mV. These conditions develop in response to high soil moisture contents that slow down gas diffusion (e.g. post irrigation or flooding) and there are good correlations between N_2O flux and relative soil gas diffusivity (D_p/D_o) (Hansen et al. 2014; Owens et al. 2017; Owens et al. 2016). Biologically, low O_2 concentrations, or restricted diffusion of oxygen would trigger rapid induction of *de novo* denitrification enzyme synthesis depending on pH. *De novo* enzyme synthesis follows a sequential order, with nitrate reductase formed within 2-3 h, nitrite reductase between 4-12 h and N_2O -R between 24 and 42 h (Dendooven & Anderson 1994; Dendooven & Anderson 1995; Firestone & Tiedje 1979; Smith & Tiedje 1979). Recent investigations have

observed even earlier synthesis of N_2O -R than 24 – 42h, with peaks in gene transcripts for *nosZ* (and presumably translation of N_2O -R) occurring within <10 hours (Liu et al. 2014).

The denitrification trait is spread over a wide taxonomic range including bacteria, archaea and some eukaryotes (Zumft 1997). We observed large proliferations of Firmicutes in conjunction with peaks in DEA. Denitrification and/or reduction of nitrate/nitrite is common in cultured *Bacillus* spp. and they have been shown to be numerically important culturable members of denitrifying communities in agricultural soils (Jones et al. 2011; Verbaendert et al. 2011). The closely related *Paenibacillus* (pH 8.3 microcosms) are also capable of heterotrophic nitrification, dissimilatory NO_3^- reduction to NH_4^+ (DNRA), and full denitrification and grow optimally in neutral to alkaline pH conditions (Behrendt et al. 2010). Like *Paenibacillus*, some *Bacterioidetes* (as observed in the pH 6.7 microcosms) have N_2O -R and have been observed to fully denitrify NO_3^- to N_2 (Horn et al. 2005). To date, culture independent studies have not shown Firmicutes to be numerically important in denitrification, however, PCR primers and lysis techniques may not be effective for these bacteria, thereby artificially reducing their relative contribution (Verbaendert et al. 2011).

Potential for DNRA and fermentation in the microcosms.

After pH change, the microcosms had high DOC/NO_3^- ratios with no correlations evident between estimated DOC mineralisation and NO_3^- nor CO_2 respiration. There was also a mismatch between increasing NH_4^+ relative to consumed NO_3^- , especially in the pH 8.8 microcosms, suggesting that other anaerobic metabolisms were active aside from denitrification. DNRA is an energy yielding anaerobic process that is favoured in C-replete conditions when NO_3^- becomes limiting (C/NO_3^- ratio >12) (Giles et al. 2012; Rutting et al. 2011). Using the DNRA stoichiometry in equation [2] presented by Lam et al. (2009), if all available native NO_3^- in our microcosms (~20 mg kg^{-1} NO_3^- -N) was reduced via DNRA (i.e. ignoring NO_3^- also needed for denitrification) then approximately 26 mg kg^{-1} NH_4^+ could be produced, yet we observed up to 50 mg kg^{-1} . The significant amounts of additional N required to balance the N requirements in our experiments are likely to be derived from the ample supplies of DON and DOC in the microcosms that could undergo depolymerisation and ammonification (Burger & Jackson 2004; Rutting et al. 2011; Schimel & Bennett 2004).

Bacillus species are well known as nitrate reducers and N₂O emitters, but many strains do not produce N₂O after NO₃⁻/NO₂⁻ reduction (Verbaendert et al. 2011). DNRA is known to occur in a number of *Bacillus* species with varying concentrations of N₂O produced (Heylen & Keltjens 2012; Mania et al. 2014; Nakano et al. 1998; Sun et al. 2016). Although genome information is not yet available, possible N-metabolisms for the predominantly *Bacillus* species isolated from the microcosms in this study include denitrification, DNRA and possibly N₂ fixation. These isolates produced an excess NH₄⁺ and N₂O compared to uninoculated controls suggesting DNRA could be the dominant metabolism. Given the concentration of NO₃⁻ available in the medium, additional N is still required to support the concentrations of NH₄⁺ and N₂O produced supporting the idea that depolymerisation and ammonification of organic matter is also active.

The other main driver of treatment differences were organisms from the order Clostridiales. Teidje (1998) describes obligate anaerobic DNRA capable *Clostridium* spp. (Caskey & Tiedje 1980; Keith et al. 1982). Clostridia are also well known for their fermentative metabolisms that have been exploited for over 100 years (Moon et al. 2016; Wiegel et al. 2006), which combined with the observations that there was no correlation between NO₃⁻ consumption and CO₂ production adds support to the theory that metabolisms aside from denitrification/DNRA operate in these microcosms, especially beyond 40 hours when Clostridiales start displacing Bacilliales. Fermentation is also known to occur in *Bacillus* species, specifically the well-studied *B. subtilis* (Ramos et al. 2000).

Recent research has shown that fermentative organisms (Clostridiales) influence the competition between denitrifiers and DNRA bacteria through competition for fermentative C-substrates (electron donors). Higher ratios between substrates and nitrate leads to a combination of fermentation and DNRA (both fermentative and respiratory) with no denitrification. When the ratio between substrates and nitrate lowers, denitrification takes a larger role until it eventually out-competes both fermentation and DNRA (van den Berg et al. 2017a; van den Berg et al. 2017b). Comparing these studies to our microcosms is problematic as the C-sources in our study are so diverse. However, the geochemical evidence indicates that NO₃⁻ quickly declines while DOM remains high which would lead to a higher substrate/nitrate ratio and thus DNRA and fermentation taking a dominant role. This possibility is further supported by increases in NH₄⁺ and CO₂ respiration rates beyond the peaks in DEA. There is also the possibility that DNRA has a more significant role that we envisage and that DEA measurements reflect reduction of N₂O via 'atypical

nosZ' (Giblin et al. 2013; Jones et al. 2013; Jones et al. 2011; Samad et al. 2016a; Sanford et al. 2012).

Implications for urea impacted soil

The pH changes induced by our KOH additions in this study are representative of what could be expected in the field under animal urine patches or in the vicinity of urea fertilizer prills (Clough et al. 2010; O'Callaghan et al. 2010). Increases in electrical conductivity (EC) associated with elevated pH were high at 6 mS cm⁻¹, but were not out of the ordinary when compared to other studies investigating urine additions to soil, nor is the associated release of excess SOM (Curtin et al. 2016; Haynes & Williams 1992). Recent research documenting N₂O emissions in urea-amended saturated soils with elevated pH and declines in O₂ and redox changes, also suggest that this work has direct relevance to what would be expected under field conditions (Hansen et al. 2014; Owens et al. 2017; Owens et al. 2016).

Given that there is experimental and field evidence for chemical conditions conducive to denitrification in the days following urea application, this supports the idea that N-cycling in soils should not be considered a sequential process, but instead is actually highly dynamic, with N-processing dependent on resources available. Our work suggests that N-resources can be quickly supplied from both organic and inorganic sources with the distinct possibility that significant N could be lost as N₂ shortly after urea deposition. It is unknown what happens to the large excess of SOM released at high pH that is not metabolised during these short-term incubations. We should be aware that pulses of fresh C into soil can lead to loss of native C (priming) with some research indicating that excess N-fertilisation (and possibly intensification) leads to soil C declines (Kuzuyakov et al. 2000; Mau et al. 2015).

Although pH is a universal mechanism that selects for microbial communities, the response to pH will vary according to soil types. The microbial phenotypes expressed will also be dependent on the available soil C and N resources with multiple N-transformation pathways possible. For this particular soil, pH elevation and anoxia allowed Firmicutes bacteria to flourish and contribute to rapid processing of N resources. Investigations of the ratios between various N-metabolisms in these microcosms would require isotope labelling with more defined experiments also required to

understand short and long-term cascades of biological N-processing and the transient ecologies driving N-transformations as soil conditions stabilise post urea addition.

Conclusions

At soil pH values representing expected deviations induced by either urine or urea prills, we observed large increases in DOM, respiration and DEA potential within <24 hours of pH change. DEA potential was such that in order to balance the concentrations of NH_4^+ and N_2O concentrations produced there is a requirement for mineralisation of DOM to supplement the available NO_3^- resources. The microbial community structure changed dramatically in response to the new soil chemical regime, specifically moving towards a dominance of *Firmicutes* bacteria. The large increase in *Firmicutes* bacteria coincided with the highest DEA potential, while the cultured representatives of *Firmicutes* bacteria had inferred metabolisms that including denitrification and dissimilatory nitrate reduction to ammonia (DNRA).

Acknowledgements

This work was conducted as part of Plant & Food Research's Discovery Science initiatives. We also thank internal reviewers for their comments on the draft manuscript.

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Figure 1(on next page)

Denitrification enzyme activity assay results.

Sampling occurred at the end of each incubation period and prior to DEA assays with 4 replicate microcosms for each pH treatment and incubation time.

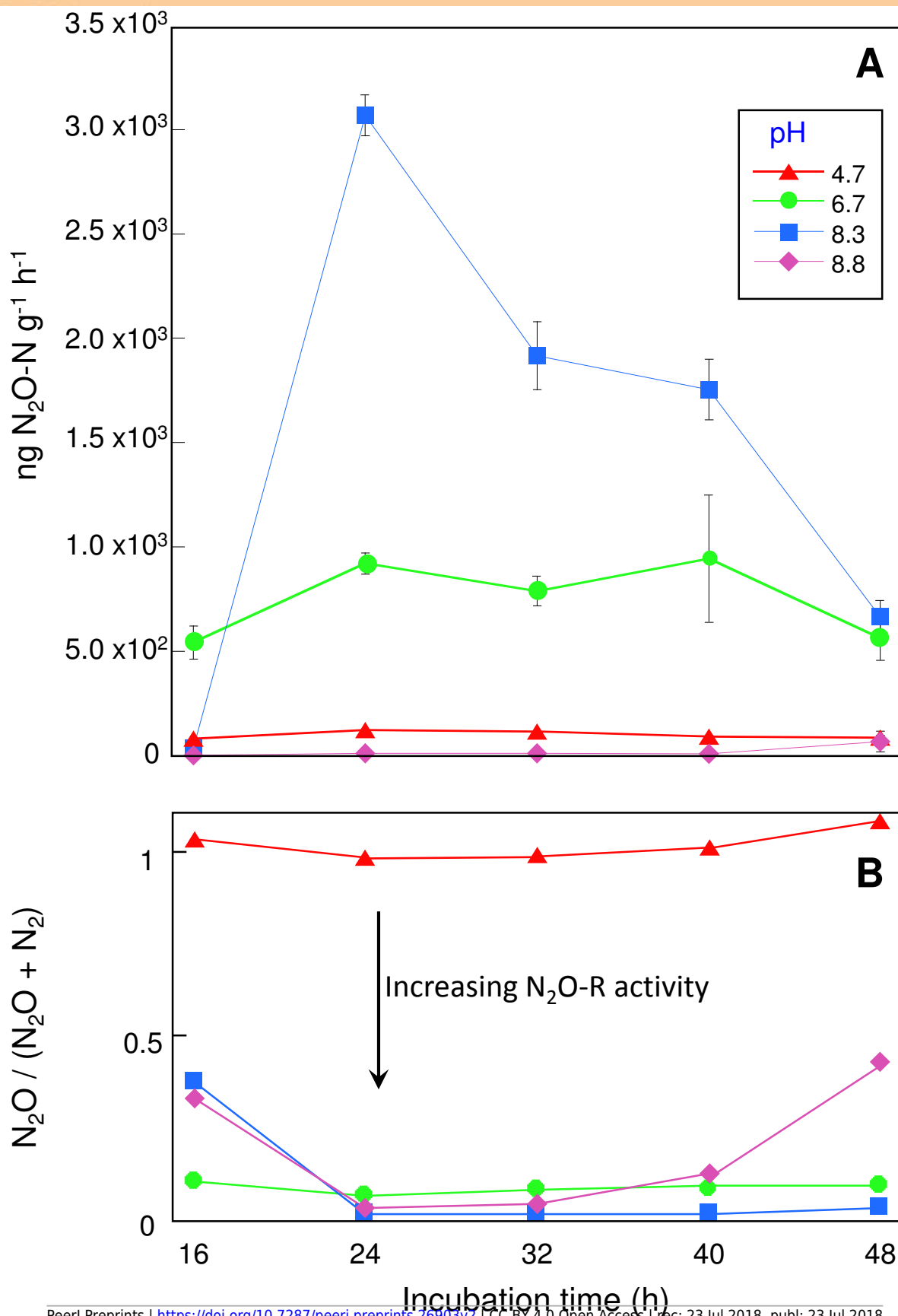
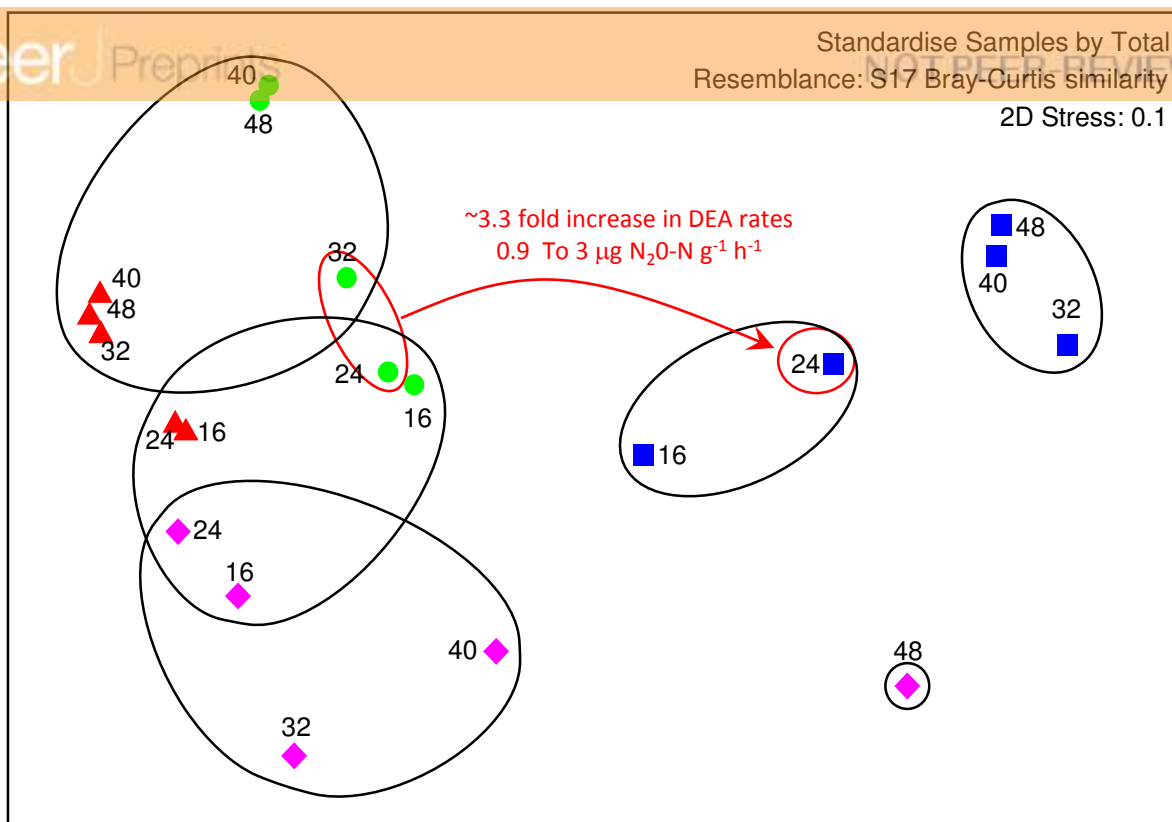


Figure 2(on next page)

nMDS ordination of bacterial OTUs identified using Illumina sequencing of the 16s rRNA gene.

OTU tables were compared using a Bray-Curtis similarity matrix with data standardised by total. The nMDS represents an unconstrained ordination of the Bray Curtis similarity measures from 250 restarts allowing examination of the broad relationships between microbial communities from each pH treatment and incubation time point. The 2D stress value of 0.1 indicates a good ordination with a low chance of a misleading interpretation. Bacterial communities among samples enclosed by black rings share >70% similarity, highlighting the level of similarity across both pH treatment and time. The estimated components of variation and interaction between sources of variation calculated via PERMANOVA are presented in the table below.

2D Stress: 0.1



Source	Estimate	SD	% est. variation	P-values
S(pH)	1015.6	31.9	57.3	0.001
S(Time)	266.9	16.3	15.1	0.001
S(pHxTime)	321.9	17.9	18.2	0.001
V(Residual)	167.8	12.9	9.4	

pH

- ▲ 4.7
- 6.7
- 8.3
- ◆ 8.8

Similarity

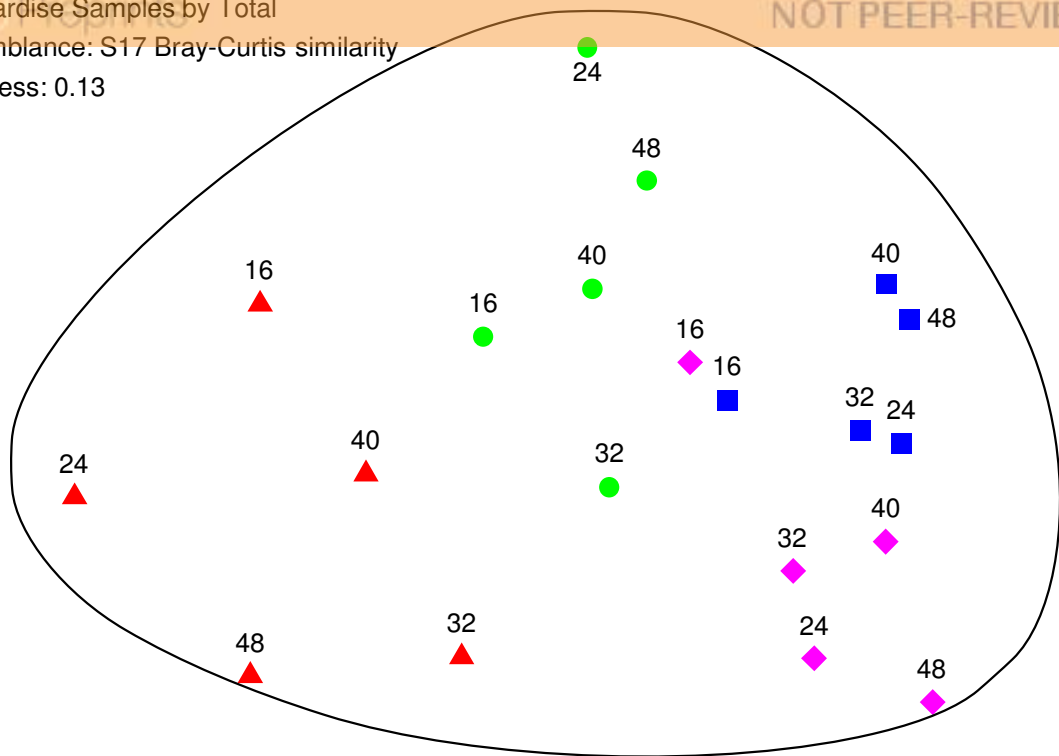
— >70 %

Figure 3 (on next page)

MDS ordination of fungal OTUs identified using Illumina sequencing of the fungal ITS1 region.

OTU tables were compared using a Bray-Curtis similarity matrix with data first $\log(X+1)$ normalized and standardised by total. The nMDS represents an unconstrained ordination of the Bray Curtis similarity measures from 250 restarts allowing examination of the broad relationships between microbial communities from each pH treatment and incubation time point. The 2D stress value of 0.14 indicates a good ordination with a moderate chance of a misleading interpretation. Fungal communities in samples enclosed by the black ring share >70% similarity, highlighting the high level of similarity in fungal communities with pH treatment and across time. The estimated components of variation and interaction between sources of variation are presented in the table below.

Standardise Samples by Total
 Resemblance: S17 Bray-Curtis similarity
 2D Stress: 0.13



Source	Estimate	SD	% est. variation	<i>P</i> -values
S(pH)	97.9	9.9	27.8	0.001
S(Time)	23.2	4.8	6.6	0.001
S(pHxTime)	31.6	5.6	8.9	0.001
V(Residual)	199.9	14.1	56.7	

pH

▲ 4.7

● 6.7

■ 8.3

◆ 8.8

Similarity

— >70 %

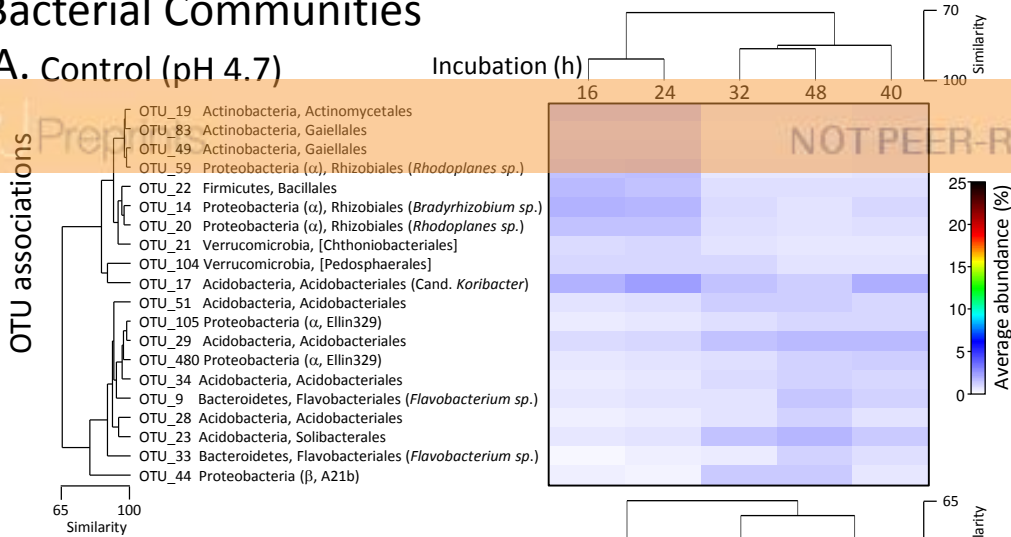
Figure 4 (on next page)

Matrix plots of the relative abundance and clustering of the 20 bacterial OTUs with the highest contribution to total sequence counts across incubation times for each pH treatment.

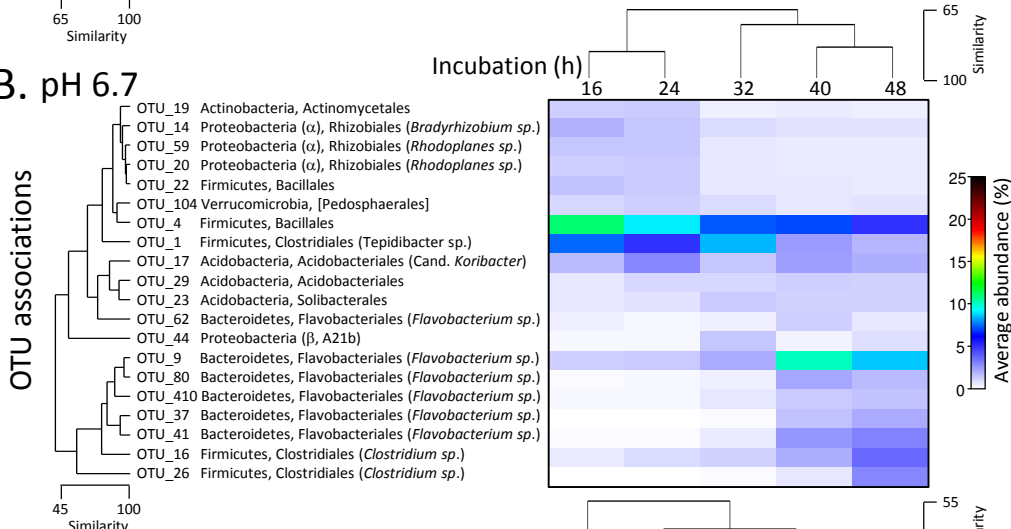
Control microcosms (A), pH 6.7 (B), pH 8.3 (C) and pH 8.8 (D). Samples for each pH treatment have been clustered via Bray Curtis similarity measures representing the entire set of OTUs while the 20 OTUs selected to represent major changes in each treatment have been clustered via an index of association. Similarity measures have been provided to enable comparison of changes as incubation time (h) increases. Average 'abundance' should be treated as a relative indication only. These 'abundances' represent data that is non-transformed and standardised-by-total.

Bacterial Communities

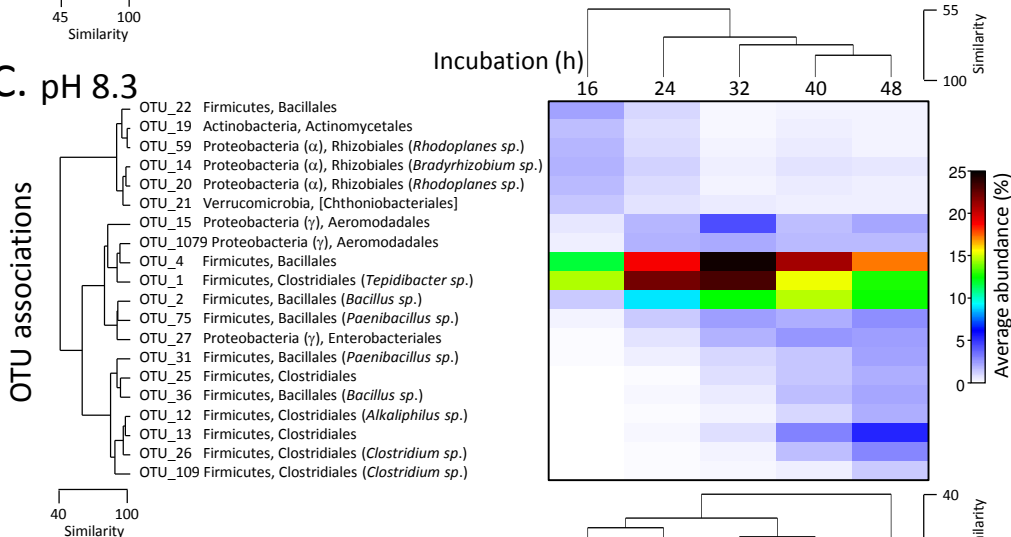
A. Control (pH 4.7)



B. pH 6.7



C. pH 8.3



D. pH 8.8

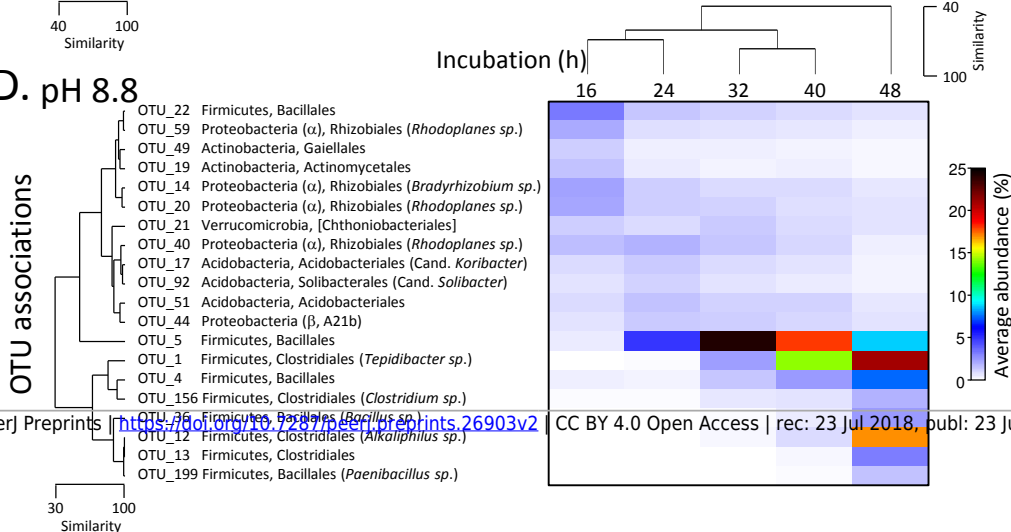
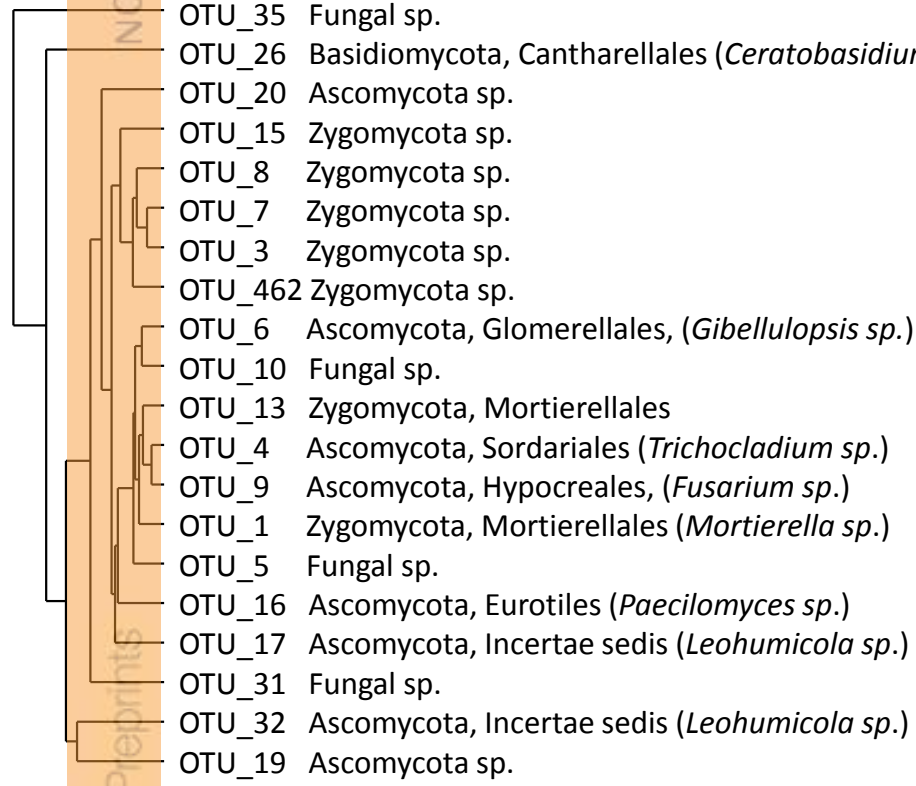


Figure 5(on next page)

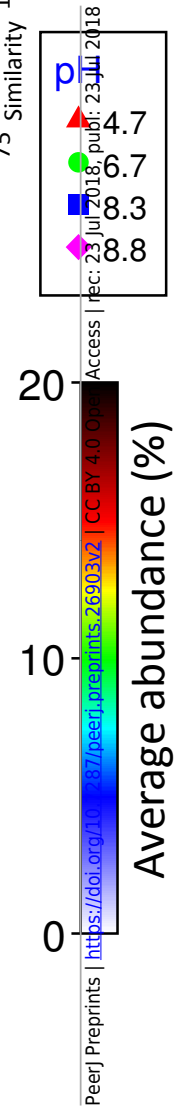
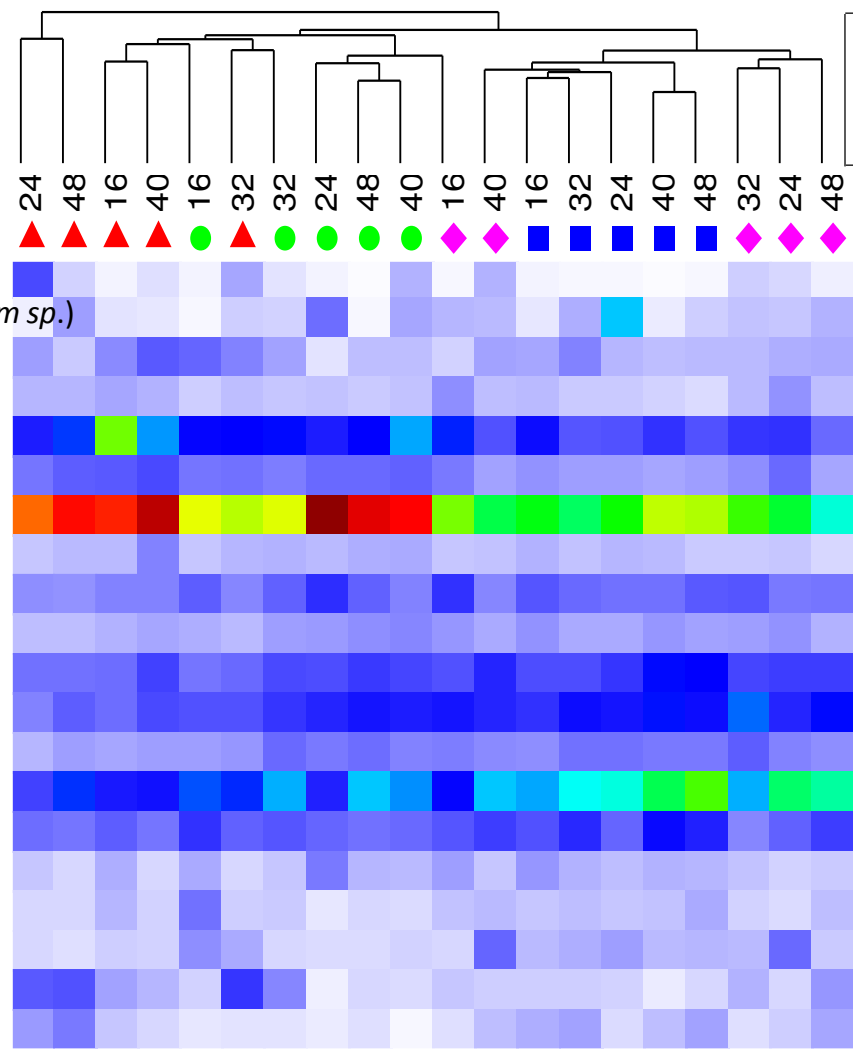
Matrix plot representing the relative abundance and clustering of the 20 fungal OTUs with the highest contribution to total sequence counts across incubation time and pH treatment.

Samples have been clustered via Bray Curtis similarity measures representing the entire set of OTUs, while the 20 OTUs presented have been clustered via an index of association. Average 'abundance' should be treated as a relative indication only. These 'abundances' represent data that is non-transformed and standardised-by-total.

Fungal communities



Incubation time (h)



55 Similarity 100

75 Similarity 100

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Table 1(on next page)

N-chemistry trends, cumulative respired CO₂ and EC data foreach pH treatment.

Sampling occurred at the end of each incubation period and prior to DEA assays with 4 replicate microcosms for each pH treatment and incubation time.

1 Table 1:

	Pre-DEA Incubation time (h)	pH [KOH addition - cmol _c kg ⁻¹]							
		4.7 [0]		6.7 [6]		8.3 [16]		8.8 [20]	
		Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD
NH₄⁺ (mg kg ⁻¹) 4*	16	1.8	0.1	5.2	0.2	20.9	0.7	30.5	1.4
	24	2.5	0.2	8.2	0.4	27.4	1.4	31.0	0.7
	32	3.0	0.2	10.1	0.1	35.5	3.0	32.1	2.0
	40	3.6	0.3	11.9	0.2	41.9	2.3	35.2	0.9
	48	5.0	0.6	14.4	0.9	45.2	3.0	50.4	1.0
NO₃⁻ (mg kg ⁻¹) 20*	16	5.4	1.0	0.2	0.1	13.0	0.3	18.3	0.5
	24	3.94	0.2	0.2	0.0	3.3	0.3	18.5	0.4
	32	4.7	0.6	0.3	0.1	4.2	0.5	18.8	1.1
	40	0.9	0.3	0.3	0.1	5.0	0.3	16.4	0.3
	48	0.2	0.1	0.3	0.1	4.8	0.4	7.4	1.1
N₂O (μg kg ⁻¹)	16	1651	75.9	849.3	323.8	11.0	3.0	3.2	4.4
	24	3587	1136	10.1	12.9	125.1	22.9	4.3	1.9
	32	4408	236.8	5.4	10.0	0.6	2.0	7.1	4.2
	40	5422	1748	9.8	15.1	0.8	1.1	9.6	2.6
	48	6810	503.5	13.0	14.4	2.6	6.6	14.0	16.8
CO₂ (mg kg ⁻¹)	16	8.2	1.0	17.5	0.8	15.4	1.5	13.5	1.6
	24	14.6	4.9	34.4	3.5	23.0	3.7	12.0	1.1
	32	25.5	2.3	56.6	14.6	34.0	8.6	8.2	1.1
	40	19.6	4.3	43.7	4.9	58.6	3.2	15.8	4.9
	48	21.1	1.8	49.3	4.9	76.8	17.5	39.2	14.2
DOC (mg kg ⁻¹)	16	36.7	4.4	226.9	9.4	4846	106.0	5670	800.5
	24	39.5	3.9	232.9	14.0	4767	322.8	5996	2038
	32	45.7	4.1	266.5	25.0	4608	147.6	5483	1029
	40	55.2	2.6	264.8	12.8	4543	232.5	5582	786.0
	48	62.9	4.2	301.8	12.5	4983	145.7	6493	498.5
DON (mg kg ⁻¹)	16	3.5	0.7	18.3	1.4	338.3	51.2	440.6	37.3
	24	5.6	0.5	15.8	3.5	323.4	22.4	490.8	57.1
	32	6.1	1.0	16.4	2.5	340.5	13.4	542.8	73.6
	40	7.3	0.3	18.0	1.4	367.3	41.4	462.8	185.8
	48	7.4	1.1	21.5	2.2	335.4	26.2	536.2	97.6
EC (mS cm ⁻¹)	16	5.0	0.0	4.5	0.1	5.4	0.1	5.8	0.2
	24	5.9	0.1	5.2	0.1	6.0	0.2	6.0	0.1
	32	6.4	0.0	5.6	0.1	6.2	0.1	6.1	0.1
	40	6.4	0.1	5.7	0.1	6.2	0.0	6.2	0.2
	48	6.4	0.1	5.7	0.1	6.4	0.2	6.4	0.1

2 *Native soil concentration of NH₄⁺ and NO₃⁻ prior to experiments