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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 CO_2 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 N_2-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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3 ***Firmicutes* phylum.**
4

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14 ***Running title:*** *Firmicutes* contribute to denitrification
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20 ## Abstract

21 Rapid and transient changes in pH frequently occur in soil, impacting dissolved organic matter
22 (DOM) and other chemical attributes such as redox and oxygen conditions. Although we have
23 detailed knowledge on microbial adaptation to long-term pH changes, little is known about the
24 response of soil microbial communities to rapid pH change, nor how excess DOM might affect
25 key aspects of microbial N processing.

26 We used potassium hydroxide (KOH) to induce a range of soil pH changes likely to be observed
27 after livestock urine or urea fertilizer application to soil. We also focus on nitrate reductive
28 processes by incubating microcosms under anaerobic conditions for up to 48 hours. Soil pH was
29 elevated from 4.7 to 6.7, 8.3 or 8.8, and up to 240-fold higher DOM was mobilized by KOH
30 compared to the controls. This increased microbial metabolism but there was no correlation
31 between DOM concentrations and CO_2 respiration nor N-metabolism rates. Microbial
32 communities became dominated by *Firmicutes* bacteria within 16 hours, while few changes were
33 observed in the fungal communities. Changes in N-biogeochemistry were rapid and denitrification
34 enzyme activity (DEA) increased up to 25-fold with the highest rates occurring in microcosms at
35 pH 8.3 that had been incubated for 24-hour prior to measuring DEA. Nitrous oxide reductase was
36 inactive in the pH 4.7 controls but at pH 8.3 the reduction rates exceeded 3000 ng N_2-N g^{-1} h^{-1} in
37 the presence of native DOM. Evidence for dissimilatory nitrate reduction to ammonium and/or
38 organic matter mineralisation was observed with ammonium increasing to concentrations up to 10
39 times the original native soil concentrations while significant concentrations of nitrate were
40 utilised. Pure isolates from the microcosms were dominated by *Bacillus* spp. and exhibited varying
41 nitrate reductive potential.

42

43 ## Introduction

44 Soil pH has a strong influence over soil processes such as N-cycling as it impacts soil
45 chemistry, physics and biology. Denitrification is an anaerobic stepwise enzymatic process
46 whereby nitrate (NO_3^-) is reduced (via NO_2^-) to nitric oxide (NO), N_2O and finally molecular
47 nitrogen (N_2). Denitrification efficiency is primarily affected by soil pH because pH influences
48 carbon supply and associated metabolisms but also impacts the activity of denitrification enzymes
49 adapted to specific pH conditions and the function of N_2O reductase (N_2O-R) (Anderson et al.
50 2017; Baggs et al. 2010; Bakken et al. 2012b; Curtin et al. 2016; Liu et al. 2010b; Morkved et al.

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51 2007; Samad et al. 2016b; Schimel et al. 2005; Simek & Cooper 2002). Dissimilatory reduction
52 of NO_3^- to NH_4^+ (DNRA) is also an anaerobic process that reduces NO_3^- and variably contributes
53 to N_2O emissions depending on carbon availability (Giles et al. 2012; Rutting et al. 2011).. Again,
54 pH affects carbon supply, alters its accessibility, and thus influences the microbial response. Under
55 anaerobic conditions there is also a strong interplay between carbon availability and whether or
56 not microbes will utilise fermentation or metabolisms such as NO_3^- reduction (van den Berg et al.
57 2017a; van den Berg et al. 2017b). Although there is a reasonable mechanistic understanding of
58 how changing soil pH affects chemistry and physics, the literature is less robust concerning the
59 dynamics of biological response at molecular levels both phylogenetically and functionally.

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

71 In contrast to studies describing microbial community response to pH in long-term trials,
72 reports about community response to rapid, short-term pH change are sparse; yet microbial
73 communities in soils are often subjected to dynamic change. For example, rapid pH change after
74 animal urine deposition or in the vicinity of urea fertiliser prills. When urea ($CO(NH_2)_2$) is added
75 to the soil (as fertiliser or urine), it quickly undergoes hydrolysis in the presence of urease enzymes:

76

$$CO(NH_2)_2 + 3H_2O \longrightarrow 2NH_4^+ + 2OH^- + CO_2$$

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

82 reactions and through microbial metabolism (Clough et al. 2010; Hansen et al. 2014; Nowka et al.
83 2015) – suitable conditions for NO_3^- reductive processes. Our previous research using KOH or
84 $Ca(OH)_2$ as proxies for NH_4OH indicates that denitrification response becomes elevated very
85 shortly after pH and DOM increases (Anderson et al., 2017).

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

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

107

108 ## 2. Materials and methods

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

111

112 *2.1 Soil collection, pH adjustment and DEA assays*

113 The Wakanui silt-loam soil used in this study was sourced from no-till plots in a long-term field
114 trial (12 years) at Lincoln, Canterbury, New Zealand. The basic chemical characteristics of the soil
115 were: pH 5.6; total C, 27 g kg^{-1}; total N, 2.4 g kg^{-1}; NO_3-N, 20 mg kg^{-1} and NH_4-N, 4 mg kg^{-1}.
116 Further details about the soil and sampling site can be found in Curtin et al. (2016) and Anderson
117 et al. (2017). Soil samples were treated with four rates of KOH (base addition rates of 0, 6.0, 16.0
118 & 20.0 $cmol_c$ kg^{-1} soil). These treatments were selected based on results from previous experiments
119 (Anderson et al. 2017) with the 6.0 $cmol_c$ kg^{-1} and 16.0 $cmol_c$ kg^{-1} treatments representing
120 "low/moderate" and "upper limit" pH increases following animal urine deposition. The 20 $cmol_c$
121 kg^{-1} treatment represented an alkaline pH outlier, where DEA was expected to be minimal
122 (Anderson et al 2017).

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

129 Microcosms were prepared as described by Anderson et al. (2017). Briefly, 25 g (dry
130 weight equivalent) soil was placed in 250 mL bottles and KOH was added together with KCl to
131 balance electrical conductivity across treatments. The final solution volume in each microcosm
132 was adjusted to 25 mL. The bottles were evacuated (to -1 atm.) then flushed three times with N_2
133 (instrument grade, <0.001% O_2) over a 30 min period until O_2 was < 0.03%. The microcosms were
134 then incubated at 20°C on an orbital shaking platform (150 rpm) for 16, 24, 32, 40 or 48 h. After
135 each incubation the headspace was sampled for N_2O and CO_2.

136 The following sampling protocol was followed: For chemical analyses, 5 mL of slurry was
137 collected from four microcosms to determine dissolved CO_2 (acidified with 2 mL of 2M HCl to
138 dissolve any carbonates). The remaining slurry from these four microcosms was centrifuged (5min
139 at 20,000 rpm), and the supernatants filtered (<0.45 µm) then frozen (-20°C), pending pH, EC,
140 DOC, DON and NO_3^-/NH_4^+ analyses. From a further two microcosms, 1 mL aliquots of slurry
141 were taken for nucleic acid extraction and bacterial colony isolations.

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142 To determine DEA, 5 mL of water containing 50 mg NO_3-N kg^{-1} (Luo et al. 1996) was added to
143 the remaining six microcosms, and the anaerobic atmosphere was regenerated by evacuation and
144 flushing three times with N_2. To three of the microcosms a volume of scrubbed (Hyman & Arp
145 1987) acetylene (final ratio of 10% v/v) was added by syringe and allowed equilibrate (with
146 shaking) for 10 minutes before venting these microcosms to atmospheric pressure. No external C-
147 sources such as glucose were added. The microcosms were incubated at 20°C on a shaking
148 platform (150 rpm) and sampled hourly over a 4 hour period to measure headspace N_2O and CO_2.
149 Headspace gases removed were replaced with an equivalent volume of N_2.
150 At the end of the DEA assay, dissolved CO_2, pH, EC, DOC, DON and NO_3^-/NH_4^+ were
151 measured in the remaining soil slurry samples.
152
153 *2.2 Chemical Analysis and Gas Chemistry*
154 Analytical methods are described in Anderson et al. (2017). Briefly, concentrations of N_2O
155 and CO_2 were determined on a Shimadzu Corp. GC-17A gas chromatograph and the DEA value
156 (i.e. potential denitrification rate) and respiration rates were calculated from the linear relationship
157 between evolved N_2O or CO_2 and time. The extracts were analyzed for pH (ThermoScientific
158 Orion™ AquaPro™ pH combination electrode) and electrical conductivity (Eutech Instruments
159 PC510 conductivity meter). Dissolved organic C was determined using a Total Organic Carbon
160 Analyzer (Shimadzu TOC-$V_{CSH,}$ Shimadzu Corp, Japan). Total N was determined by persulfate
161 oxidation, as described by Cabrera and Beare (1993), and organic N was estimated by subtracting
162 mineral N (KCl extracted NH_4^+ and NO_3^- determined using an automated colorimeter) from total
163 N.
164
165 *2.3 Microbial population profiling via next generation sequencing*
166 A 1 mL aliquot of soil slurry was centrifuged at 14,000 rpm for 5 minutes. DNA from the
167 resulting pellet was extracted with the MoBio Powersoil DNA kit (Carlsbad, CA). The V3-V4
168 variable regions of the bacterial 16S rRNA was amplified with the 341f and 785r primer pair
169 (Klindworth et al. 2013). The fungal internal transcribed spacer 1 (ITS1) region was amplified
170 with NSI1a_mod (5'-GATTGAATGGCTTAGTGAGK-3') and 58A2R (5'-
171 AGTCCTGCGTTCTTCATCGAT-3'), both adapted from (Martin & Rygiewicz 2005). Primers
172 included the Illumina adapter sequences.

173 PCR amplifications contained ~10 ng DNA template, 10 nmol each primer, 1X mastermix,
174 and 0.5 U KAPA3G polymerase (Merck, Auckland, New Zealand), in a final volume of 20 µl.
175 Reactions were performed in duplicate. Cycling parameters were 94°C for 2 min; 30 cycles of
176 95°C for 30 s, 50°C for 30 s, 72°C for 30 s. Duplicate reactions were combined and purified with
177 AMPure XP beads (Agencourt, Beckman Coulter Life Sciences). Purified amplicons were
178 quantified by gel electrophoresis and UV absorbance (NanoDrop ND-1000). Amplicons were 2 x
179 300 bp paired end sequenced on an Illumina MiSeq platform (New Zealand Genomics Limited,
180 Auckland).

181

182 *2.4 Sequence processing and statistical analysis*

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

191 The phyloseq (McMurdie & Holmes 2013) and ggplot2 (Wickham 2009) packages within R (Team
192 2016) were used for the analysis and visualisation of data at phyla level. OTU tables were also
193 analysed using Primer 7 with PERMANOVA add-on (Primer-E Ltd, Plymouth, UK). Both rarefied
194 and non-rarefied data was analysed based on the work of McMurdie and Holmes (McMurdie &
195 Holmes 2014). Where data was not rarefied, samples were standardised by total and no statistical
196 inferences were made regarding differentially abundant species (OTUs), rather our conclusions
197 were based on assessing broader scale relative changes in the microbial communities only.
198 Relationships among microbial community profiles based on Bray-Curtis similarity matrices were
199 graphed using unconstrained non-metric multidimensional scaling (nMDS) ordinations using 250
200 restarts along with cluster analysis. Relationships observed among all OTUs were then statistically
201 tested using 2-factor permutational ANOVA within the Primer 7 package (PERMANOVA) with
202 estimated components of variation being a standard output of the analysis. Data was untransformed
203 unless otherwise stated, whereupon log(X+1) transformations were applied.

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

212

213 *2.5 Isolation of bacteria, N-use characterisation*

214 2.5.1 Isolation of nitrate reducing bacteria

215 Previous research by O'Callaghan et al. (2010) suggested that Firmicutes increased by substantial
216 amounts in conditions similar to those we investigated. Although only a small percentage of soil
217 bacterial diversity can be cultured, it is relatively straightforward to culture members of the
218 Firmicutes (among others); hence, in order to gain some appreciation for the N-processing
219 capabilities of culturable bacteria from these microcosms, we attempted to isolate NO_3^- reducers.
220 Ten μL sample of slurry from each pH adjusted microcosm was serially diluted (10 μL into 1mL
221 followed by three dilutions of 10 μL into 100 μL) and plated onto TSB or 1/10 diluted TSB
222 containing KNO_3 (30g L^{-1} + 0.5g L^{-1} KNO_3). Plates were incubated under anaerobic conditions
223 (Whitney jars with gas packs) at 24°C for 2 to 6 days.
224 Representative colonies of different morphology were selected and re-streaked onto the same
225 media and grown under anaerobic conditions at 24°C. Isolates were stored in 20% glycerol at -
226 80°C, with a selection of isolates identified by amplification and Sanger sequencing of the 16S
227 ribosomal RNA gene using the 27F and 1492R primers (Anderson et al. 2009).

228

229 2.5.2 Nitrate utilisation by bacterial isolates

230 Cells were grown aerobically overnight in TSB with KNO_3. 20mL cultures were then initiated
231 with 1/100 dilutions of the overnight cultures, and grown anaerobically (O_2 replaced with N_2) at
232 24°C. After 48 hours, gas samples were extracted with a gas-tight Hamilton syringe and analysed

233 for the presence of N_2O and CO_2 by gas chromatography (as outlined above) and 10 mL samples
234 of the bacterial cultures were removed for NO_3^- and NH_4^+ analysis (as outlined above).
235

236 3. Results

237 *3.1 Chemical characterisation of pH amended soils prior to DEA assays*

238 After 48 hours of anaerobic incubation, soil slurries with additions of 0, 6, 16 and 20 $cmol_c$
239 kg^{-1} KOH had average pH values of 4.7, 6.7, 8.3 and 8.8 respectively. During the 48 h incubation
240 EC reached ~6.4 mS cm^{-1} in all microcosms except the pH 6.7 microcosms which reached ~5.7
241 mS cm^{-1} (Table 1). DOC in the control microcosms increased from 37 to 63 mg kg^{-1} during the 48
242 h incubation. In the pH 6.7, 8.3 and 8.8 microcosms DOC increased ~11, 185 and 240-fold
243 respectively in response to KOH addition, with the majority of that change (> 75%) occurring
244 during the first 16 h of incubation. During the 48 h incubation DON in the control microcosms
245 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,
246 140 and 223-fold in response to KOH addition, respectively (Table 1). There was a strong
247 correlation between the amounts of DOC and DON solubilised at each pH value, irrespective of
248 incubation time ($R^2 = 0.98$) (Supplementary Figure S2).

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

256 In control microcosms, ammonium (NH_4^+) increased linearly over 48 hours with a slope of
257 0.09, starting from native soil concentrations of ~4 mg kg^{-1}. The NH_4^+ profiles in the pH 6.7, and
258 8.3 microcosms were similar (approximately linear) but with greater slopes of 0.28 and 0.79
259 respectively (R^2 values of 0.99 and 0.98). In the pH 8.8 microcosms, NH_4^+ quickly elevated to 30
260 mg kg^{-1} during the first 16 hours of incubation and remaining at that concentration until after 32
261 hours, when a further increase from 30 to 50 mg kg^{-1} occurred by 48 hours (Table 1). The native
262 soil nitrate (NO_3^-) concentration was ~20 mg kg^{-1}. This NO_3^- was almost completely reduced after
263 32-40 hours incubation in the control, pH 6.7 and pH 8.3 microcosms (Table 1). In the pH 8.8

264 microcosms, NO_3^- remained at ~20 mg kg^{-1} until after 32 hours incubation, dropping to ~6 mg kg^{-1}
265 during the following 16 hours.

266 The control microcosms had the highest concentrations of N_2O in the headspace, increasing
267 from ~1650 to 6800 µg kg^{-1} between 16 and 48 hours of incubation (Table 1). The pH 6.7
268 microcosms had ~850 µg kg^{-1} of N_2O in the headspace after 16 hours incubation, but by 24 hours
269 this had declined to < 15 µg kg^{-1}. Headspace N_2O in the pH 8.3 and 8.8 treatments was < 15 µg
270 kg^{-1} for all time points except 24 hours for the pH 8.3 treatment (125 µg N_2O kg^{-1} soil).

271

272 *3.2 DEA assays*

273 Microcosms incubated for 16 hours had the lowest DEA rates with 80, 540, 34 and 0.2 ng
274 N_2O g^{-1} h^{-1} recorded for the control, pH 6.7, 8.3 and 8.8 treatments respectively (Fig. 2A). DEA
275 remained low in the control microcosms with a maximum DEA rate of only 121 ng N_2O g^{-1} h^{-1}
276 after 24 hours incubation, declining to 85 ng N_2O g^{-1} h^{-1} in microcosms incubated for 48 hours
277 (Fig. 1). In contrast, after 24 hours incubation, DEA rates in the pH 6.7 and 8.3 microcosms were
278 8 and 25-fold higher respectively with a maximum of ~3000 ng N_2O g^{-1} h^{-1} produced (Fig. 1A).
279 DEA rates remained in the vicinity of 900 and 2000 ng N_2O g^{-1} h^{-1} for the pH 6.7 and 8.3
280 treatments, respectively, for microcosms incubated up to 40 hours. DEA rates greater than those
281 in the control were not observed in the pH 8.8 treatment until microcosms were incubated for at
282 least 48 hours (~65 ng N_2O g^{-1} h^{-1}).

283 Replicate microcosms without acetylene added were used to assess N_2O-R activity during
284 the DEA assays based on the $N_2O/(N_2O + N_2)$ ratio. These showed that N_2O reduction was absent
285 in the control microcosms while in the pH 6.7, 8.3 and 8.8 treatments between 56 and 100% of the
286 N_2O produced was reduced to N_2 depending on incubation time (Fig. 1B). Near complete N_2O
287 reduction was observed in the pH 8.3 treatment for microcosms incubated longer than 24 hours.
288 Depending on pH treatment and incubation time, N_2O production (and reduction) profiles during
289 the DEA assays were mirrored by decreases in NO_3^- with an estimated 30 to 80 % of the available
290 NO_3^- reduced. In the control and pH 6.7 microcosms there were no differences in the NH_4^+ before
291 and after the 4-hour DEA assay period, however decreases of up to 9 mg kg^{-1} occurred in the pH
292 8.3 microcosms after 32 hours incubation and in the 8.8 microcosms after all incubation times
293 (Supplementary Table ST1).

294 T-tests comparing CO_2 respiration indicated that acetylene addition depressed microbial
295 activity, but only in the pH 8.3 and 8.8 treatments ($P < 0.05$). It is acknowledged that lower soil
296 respiration in the presence of acetylene will reflect both the absence of any CO_2 derived from
297 metabolic N_2O reduction but possibly also general impediment of other anaerobic metabolisms. It
298 is unlikely that acetylene would have served as dominant carbon source during the 4 hour DEA
299 incubation given the excess DOC available and time required to adapt to using acetylene (Felber
300 et al. 2012; Groffman et al. 2006). There is evidence to suggest that DOC and DON declined during
301 the DEA assay period, especially in the pH 8.3 and 8.8 treatments but the results were highly
302 variable (Supplementary Table ST1).
303
304 *3.3 Microbial community adaptation to pH treatments during 48 hours incubation*
305 The total number of OTUs identified across all treatments and incubation times was 2258 for fungi
306 and 6429 for bacteria with an average of 84000 reads per sample. Sequences from this Targeted
307 Locus Study (LTS) project has been deposited at DDBJ/EMBL/GenBank under the accession
308 KCDA00000000. The version described in this paper is the first version, KCDA01000000. Fungal
309 ITS1 sequences have been deposited at DDBJ/EMBL/GenBank under the accession numbers
310 MH624180 - MH625694. Two factor tests using PERMANOVA [pH x incubation time] for all
311 OTUs supported the nMDS observations indicating significant interactions between time and pH
312 (P=0.001 for both bacteria and fungi) (Figure 2). Approximately ~90% of the total variation was
313 explained for the bacterial relationships whereas only ~43% was explained for the fungi. Of the
314 variation explained for the bacteria, up to 60% was attributed to pH, a further 15% to incubation
315 time, and 18% was attributed to the interaction between the two factors. The corresponding values
316 for the fungal communities were 28% of variation attributed to pH, 6.6% to incubation time and
317 8.9% to the interaction between the two factors (Figure 3).
318 The phylum level bacterial profile from the control (pH 4.7) microcosms after 16 h
319 incubation was made up of Acidobacteria (~7%), Actinobacteria (~12%), Bacteroidetes (~7%),
320 Proteobacteria (~20%), Firmicutes (~5%), Planctomycetes (~5%) and phyla that had abundances
321 of >5% including Verrucomicrobia, Chloroflexi, Gemmatimonadetes and Armatimonadetes. Up
322 to 35% of OTUs could not be classified (Supplementary Figure S3). After 24 hours incubation,
323 the bacterial communities were still >85% similar before there was a relative decrease in
324 Actinobacteria coupled with an increase in Acidobacteria and Bacteroidetes (Supplementary

325 Figure S3). A decrease in Actinobacteria was also seen over the incubation periods for all three
326 pH modifications along with changes in the proteobacterial populations with almost complete
327 disappearance of OTUs from the order Rhizobiales (Figure 4, Supplementary Figure S3).
328 After 16 and 24 hours incubation, the bacterial profiles in the pH 6.7 microcosms were
329 similar to those in the control microcosms with the exception of a larger proportion of Firmicutes
330 (~20% versus 5% in the controls) (Supplementary Figure 3). The increased representation of
331 Firmicutes was driven by an expansion of OTUs from the orders Bacilliales (OTU_4) and
332 Clostridiales (OTU_1, *Tepidibacter sp.*). These two OTUs then decreased later in the incubation,
333 partially displaced by other *Clostridium spp.* (OTUs 16 and 26) (Fig. 4B). Across the 32 to 48h
334 incubation period there was a large relative increase in OTUs from the phylum Bacteroidetes,
335 driven by *Flavobacterium spp.* (OTUs 62, 9, 80, 410, 37 and 41) (Fig. 4B).
336 In the pH 8.3 treatment after 16 hours incubation, there was a very high proportion of
337 Firmicutes (~40%) and a low representation of Actinobacteria and Bacteroidetes compared with
338 the pH 4.7 control microcosms. Across the incubation period, the dominance of Firmicutes
339 increased further, initially driven by the same Bacilliales and Clostridiales OTUs (4 and 1), but at
340 a much higher relative 'abundance' than observed in the pH 6.7 treatment. Their expansion
341 coincided with the highest DEA rates, with the average 'abundance' of OTU_4 being ~20% of
342 total at this time and OTU_1 being ~23% (Fig. 4C, Supplementary Figure S3). After 32 h these
343 two dominant OTUs were partially displaced by a cohort of other OTUs from the orders Bacilliales
344 and Clostridiales (OTUs 2, 75, 31, 25, 36, 12, 13, 26 and 109) (Fig. 3). At the genus level these
345 OTUs represented *Bacillus*, *Paenibacillus*, *Clostridium* and *Alkaliphilus* spp. (Fig. 3).
346 Communities in the 40 and 48h incubations shared the highest level of similarity (Figs. 2, 4C and
347 Supplementary Figure S3).
348 After 16 and 24 hours incubation, the bacterial profiles from the pH 8.8 treatment shared
349 >70% similarity with the samples from the control microcosms (Fig. 2). Over the incubation period
350 to 32 h, there was a large expansion in the relative percentage of the Firmicutes, initially driven
351 almost entirely by an increase in OTU_5 from the Bacilliales order (Fig 4D). By 48 h, this OTU
352 was partially displaced by a group of Bacilliales and Clostridiales OTUs that shared some
353 similarity with those observed in the pH 8.3 treatment (OTUs 1, 4, 156, 36, 12, 13, and 199). These
354 OTUs included the same genus level representatives - *Bacillus*, *Paenibacillus*, *Clostridium* and
355 *Alkaliphilus* spp. (Fig. 4D).

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356 Fungal communities shared >70% similarity across all samples regardless of pH and time
357 (Figure 3). Based on percentages alone, no discernible patterns could be observed for the fungal
358 dataset (Supplementary Figure S4), however, some OTUs were displaced depending on pH. For
359 example, Zygomycota OTU_3 was dominant in the control and pH 6.7 treatments but was partially
360 supplanted by another Zygomycota OTU from the order Mortierellales (*Mortierella sp.*) (Fig. 5).
361 An identical analysis of rarefied bacterial and fungal OTUs datasets is presented in
362 Supplementary Figures S5 to S10. A range of diversity measures have been presented in
363 Supplementary Figures S11 and S12. An nMDS plot from a preliminary experiment showing the
364 relationship between bacterial communities in the initial untreated (aerobic) field-moist soil and
365 the soil microcosms incubated under anaerobic conditions with various pH treatments is presented
366 in Supplementary Figure S13.
367
368 3.4 *Isolation of bacteria and N-use characterisation.*
369 On TSB-nitrate medium, plates were quickly dominated by fast growing colonies sharing
370 morphologies characteristic of motile or swarming bacteria. A total of 33 isolates were screened
371 for nitrate utilisation. Seven showed minimal growth and respiration in liquid culture, 18 showed
372 near complete utilisation of NO_3^- accompanied by production of both NH_4^+ and N_2O, and 5 showed
373 moderate utilisation of NO_3^- with low production of NH_4^+ and N_2O. One isolate showed production
374 of NH_4^+ with little or no use of NO_3^- or production of N_2O and 2 isolates exhibited respiration but
375 did not appear to utilise N. Of the 33 isolates, 22 were selected for identification by ribosomal 16S
376 gene DNA sequencing. All were from the Firmicutes phylum, of which three were *Paenibacillus*
377 *spp.* and 1 was a *Brevibacillus sp.* (all producing less NH_4^+), while the remainder were *Bacillus*
378 *spp.* (Supplementary Table ST2A).
379 From 1/10 diluted TSB-nitrate medium, 44 isolates were screened for nitrate utilisation. Of
380 these, 24 showed no or minimal growth in liquid culture over 48 hours incubation while 13 reduced
381 NO_3^- to close to zero with 4 of these generating significant amounts of NH_4^+. Another 3 reduced
382 NO_3^-, but to a lesser extent, while 4 isolates respired but did not appear to utilise N. Seventeen
383 isolates were identified by 16S gene sequencing. Six belonged to the *Bacillus* genus, three to
384 *Achromobacter*, six to *Acidovorax*, one to *Bosea*, and one to *Rhodanobacter* (Supplementary Table
385 ST2B).

386 All isolates except one of the *Bacillus sp.* had high NO_3^- utilisation compared to
387 uninoculated controls. The *Bacillus spp.* produced up to 536 mg NH_4^+ L^{-1}, while the *Acidovorax*
388 *spp.* produced < 45 mg NH_4^+ L^{-1}. One *Bacillus sp.* and two *Acidovorax sp.* produced < 100 mg
389 N_2O L^{-1}, while the remaining *Acidovorax* sp. had the highest N_2O production at 2840 mg L^{-1} . In
390 general, isolates that exhibited high use of NO_3^- coupled with production of NH_4^+ and N_2O
391 exhibited an average of ~2.5-fold higher CO_2 production (respiration) compared with isolates that
392 exhibited high NO_3^- use with little or no NH_4 and N_2O production. Relevant 16S sequences were
393 submitted to NCBI with accession numbers assigned between MH211426 and MH211463,
394 submission number SUB3915485 (Supplementary Table 2). Six organisms were selected for future
395 genome sequencing, 3 *Bacillus spp*. and 3 *Acidovorax spp*. (Supplementary Table ST2C).
396

397 **4. Discussion**

398 ***The effects of rapid pH change on soil chemistry and microbiology***

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

407 Over medium to long time scales (months to years), pH is known to be a dominant
408 environmental variable that shapes soil microbial communities (Lauber et al., 2009; Zhalina et al.,
409 2015). Changes in pH are also known to cause shifts in active organisms over short timescales
410 (Brenzinger et al. 2015). Although the strongest predictor for both bacterial and fungal community
411 change in these experiments was pH, community change may also be indirectly influenced by the
412 effect that pH has on DOM release. Theoretically, high levels of DOM released via increased pH
413 should benefit copiotrophs (*r*) over oligotrophs (*K*) (Fierer et al. 2007; Goldfarb et al. 2011), but
414 at the same time elevated pH is likely to alter cellular homeostasis, regulation of nutrient

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

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

428 Proteobacteria are abundant in high C soils (Fierer et al. 2007) with β and γ–Proteobacteria
429 considered important soil copiotrophs (Eilers et al. 2010) in conjunction with Firmicutes and
430 Actinobacteria (Zhalnina et al. 2015). Bacteroidetes and β-Proteobacteria are initial metabolisers
431 of labile soil-C (Padmanabhan et al. 2003) and increases in the abundance of these organisms have
432 been correlated with C mineralisation rates (Fierer et al. 2007). Our study is consistent with regard
433 to expansion of Bacteriodetes (*Flavobacteriales*), specifically in the control and pH 6.7
434 microcosms (~5.5-fold-increase in DOM with ~2-fold increase in respired CO_2), suggesting that
435 the microbial community does respond to higher concentrations of bioavailable C at pH values <7.

436 In general, we observed a decrease in α-Proteobacteria (specifically *Rhizobiales*),
437 Actinobacteria and Acidobacteria OTUs at all pH and DOM values, while a few β- and γ-
438 Proteobacteria OTUs increased. Our results suggest that the chemical changes induced by KOH
439 addition to soil are comparable to soils where pH and DOM are elevated due to higher urea inputs.
440 Niche differentiation occurs in soil where higher bovine density (and presumably urea inputs)
441 induces increases in pH and total organic carbon, with Actinobacteria, α-Proteobacteria and
442 Verrucomicrobia decreasing and Bacteriodetes increasing (Philippot et al. 2010; Philippot et al.
443 2009a; Philippot et al. 2009b).

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

452 Comparisons of OTU distributions in our study indicate that the dominant feature driving
453 sample dissimilarities was large increases in Firmicutes from the classes Bacilli and Clostridia.
454 Large expansion of Firmicutes, first dominated by *Bacillales* (up to 46%) and then followed by
455 *Clostridiales* (up to 53%), (along with large decreases in Proteobacteria) have been observed in
456 alkaline soil crusts (pH 8.5) that were rehydrated and incubated under dark anoxic conditions
457 (Angel & Conrad 2013). Our results are also concordant with O'Callaghan et al. (2010), who
458 observed a 38% increase in Firmicutes, and decreases in Proteobacteria (18%), Acidobacteria
459 (8%), Actinobacteria (5%), and Bacteriodetes (5%), in soil where pH rose to values of >8, two
460 days after bovine urine addition.

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

473 Relative changes in fungal populations in response to pH change were smaller than for
474 bacteria, with ~28% of fungal variation attributed to pH, versus ~57% for bacteria. Only ~6.6
475 percent of the variation in fungal communities could be attributed to incubation time. Fungal
476 communities are known to be less responsive to pH than bacteria (Lauber et al. 2009; Rousk et al.
477 2010a) and fungal abundance has been found to be negatively correlated with pH (Rousk et al.
478 2010b), but positively correlated with C and N additions (Banerjee et al. 2016). For example,
479 investigations of the response of fungal (and bacterial) communities to ovine urine (where pH
480 increased from ~3.5 up to 6.5 and DOC increased from ~0 up to ~ 2000 mg $kg^{-}1$) indicates no
481 fungal biomass change (Nunan et al. 2006; Williams et al. 2000), no significant correlation
482 between biomass and pH, NH_4^+ or NO_3^-, but weak correlation between biomass and DOC (Singh
483 et al. 2009). In pH 6.1 soils, Banerjee et al (2016) noted that although fungal biomass increased,
484 the richness, evenness, and diversity decreased within 4 days after organic matter and nutrient
485 addition leading to 'keystone' fungal species being favoured. Singh et al. (2009) suggest that
486 because fungi are capable of degrading complex organic carbon they are less responsive to short
487 term changes in nutritional availability.

488

489 ***4.2 Soil N processes and their relationship to microbiology following pH change***

490 Denitrification occurred in all treatments because NO_3^- was consumed and N_2O was
491 produced, however there were variations in the amount of time required for the original supply of
492 native NO_3^- to be consumed. Native NO_3^- was nearly completely utilized within the first 16 hours
493 in the control, pH 6.7 and pH 8.3 microcosms, but in the pH 8.8 microcosms, NO_3^- did not decline
494 until after 32 hours. When additional NO_3^- was added to measure denitrification rates, maximum
495 DEA occurred in microcosms that had been incubated for at least 24 hours.

496 For all treatments, DEA potential declined at incubation times greater than 24 hours. This
497 may have been due to extended periods (i.e. >24 hours) of low NO_3^- concentrations prior to the
498 DEA assays leading to a decline in NO_3^- linked translation of denitrification genes such as N_2O-R
499 and nitrate reductase (Moreno-Vivian et al. 1999; Zumft 1997). Alternatively, the microbial
500 communities that developed with increasing incubation times may have expressed different
501 denitrification phenotypes (Dorsch et al. 2012; Sanford et al. 2012).

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

512 In contrast to the KOH amended microcosms, the $N_2O/(N_2O + N_2)$ ratio in the control
513 microcosms equalled 1, indicating that this treatment did not have active N_2O-R. Liu et al. (2010a)
514 and Bakken et al. (2012a) have previously shown that production of functional N_2O-R depends on
515 the post-transcriptional pH being greater than 6.1, which is consistent with our results. In an
516 agricultural environment this raises interesting ecological questions, because urea hydrolysis
517 happens to elevate pH for several days which would immediately alleviate any post-transcriptional
518 interference of *nos*Z expression and allow rapid production of functional N_2O-R.

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

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

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

547

548 ***Potential for DNRA and fermentation in the microcosms.***

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

562 *Bacillus* species are well known as nitrate reducers and N_2O emitters, but many strains do
563 not produce N_2O after NO_3^-/NO_2^- reduction (Verbaendert et al. 2011). DNRA is known to occur
564 in a number of *Bacillus* species with varying concentrations of N_2O produced (Heylen & Keltjens
565 2012; Mania et al. 2014; Nakano et al. 1998; Sun et al. 2016). Although genome information is
566 not yet available, possible N-metabolisms for the predominantly *Bacillus* species isolated from the
567 microcosms in this study include denitrification, DNRA and possibly N_2 fixation. These isolates
568 produced an excess NH_4^+ and N_2O compared to uninoculated controls suggesting DNRA could be
569 the dominant metabolism. Given the concentration of NO_3^- available in the medium, additional N
570 is still required to support the concentrations of NH_4^+ and N_2O produced supporting the idea that
571 depolymerisation and ammonification of organic matter is also active.

572 The other main driver of treatment differences were organisms from the order Clostridiales.
573 Teidje (1998) describes obligate anaerobic DNRA capable *Clostridium* spp. (Caskey & Tiedje
574 1980; Keith et al. 1982). Clostridia are also well known for their fermentative metabolisms that
575 have been exploited for over 100 years (Moon et al. 2016; Wiegel et al. 2006), which combined
576 with the observations that there was no correlation between NO_3^- consumption and CO_2 production
577 adds support to the theory that metabolisms aside from denitrification/DNRA operate in these
578 microcosms, especially beyond 40 hours when Clostridiales start displacing Bacilliales.
579 Fermentation is also known to occur in *Bacillus* species, specifically the well-studied *B. subtilis*
580 (Ramos et al. 2000).

581 Recent research has shown that fermentative organisms (Clostridiales) influence the
582 competition between denitrifiers and DNRA bacteria through competition for fermentative C-
583 substrates (electron donors). Higher ratios between substrates and nitrate leads to a combination
584 of fermentation and DNRA (both fermentative and respiratory) with no denitrification. When the
585 ratio between substrates and nitrate lowers, denitrification takes a larger role until it eventually
586 out-competes both fermentation and DNRA (van den Berg et al. 2017a; van den Berg et al. 2017b).
587 Comparing these studies to our microcosms is problematic as the C-sources in our study are so
588 diverse. However, the geochemical evidence indicates that NO_3^- quickly declines while DOM
589 remains high which would lead to a higher substrate/nitrate ratio and thus DNRA and fermentation
590 taking a dominant role. This possibility is further supported by increases in NH_4^+ and CO_2
591 respiration rates beyond the peaks in DEA. There is also the possibility that DNRA has a more
592 significant role that we envisage and that DEA measurements reflect reduction of N_2O via ‘atypical

593 nosZ' (Giblin et al. 2013; Jones et al. 2013; Jones et al. 2011; Samad et al. 2016a; Sanford et al.
594 2012).
595

596 ***Implications for urea impacted soil***

597 The pH changes induced by our KOH additions in this study are representative of what
598 could be expected in the field under animal urine patches or in the vicinity of urea fertilizer prills
599 (Clough et al. 2010; O'Callaghan et al. 2010). Increases in electrical conductivity (EC) associated
600 with elevated pH were high at 6 mS cm^{-1}, but were not out of the ordinary when compared to other
601 studies investigating urine additions to soil, nor is the associated release of excess SOM (Curtin et
602 al. 2016; Haynes & Williams 1992). Recent research documenting N_2O emissions in urea-
603 amended saturated soils with elevated pH and declines in O_2 and redox changes, also suggest that
604 this work has direct relevance to what would be expected under field conditions (Hansen et al.
605 2014; Owens et al. 2017; Owens et al. 2016).

606 Given that there is experimental and field evidence for chemical conditions conducive to
607 denitrification in the days following urea application, this supports the idea that N-cycling in soils
608 should not be considered a sequential process, but instead is actually highly dynamic, with N-
609 processing dependent on resources available. Our work suggests that N-resources can be quickly
610 supplied from both organic and inorganic sources with the distinct possibility that significant N
611 could be lost as N_2 shortly after urea deposition. It is unknown what happens to the large excess
612 of SOM released at high pH that is not metabolised during these short-term incubations. We should
613 be aware that pulses of fresh C into soil can lead to loss of native C (priming) with some research
614 indicating that excess N-fertilisation (and possibly intensification) leads to soil C declines
615 (Kuzyakov et al. 2000; Mau et al. 2015).

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

622 understand short and long-term cascades of biological N-processing and the transient ecologies
623 driving N-transformations as soil conditions stabilise post urea addition.
624

625 ## Conclusions

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

635

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639
640

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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.

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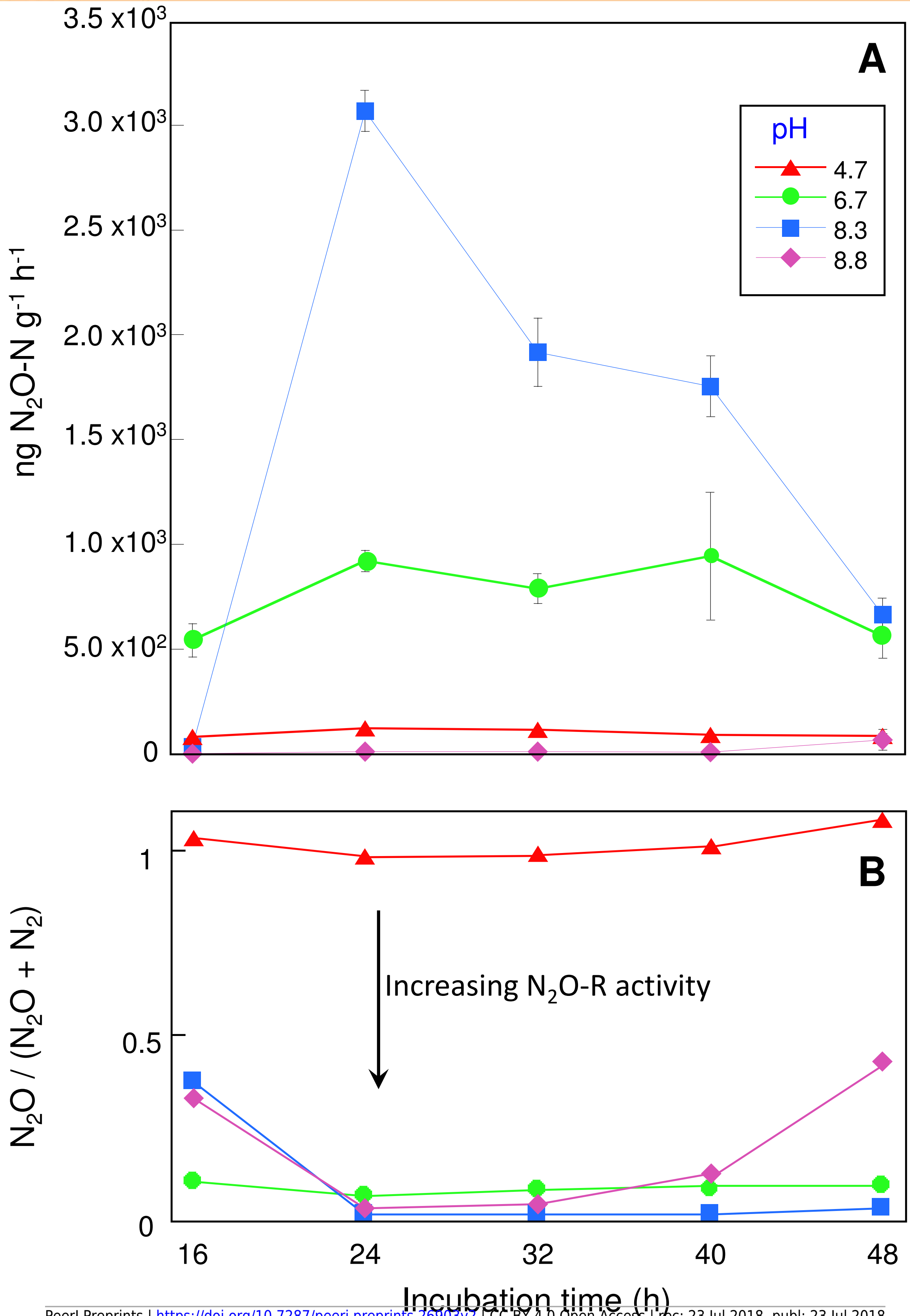

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Figure 2 (on next page)

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.

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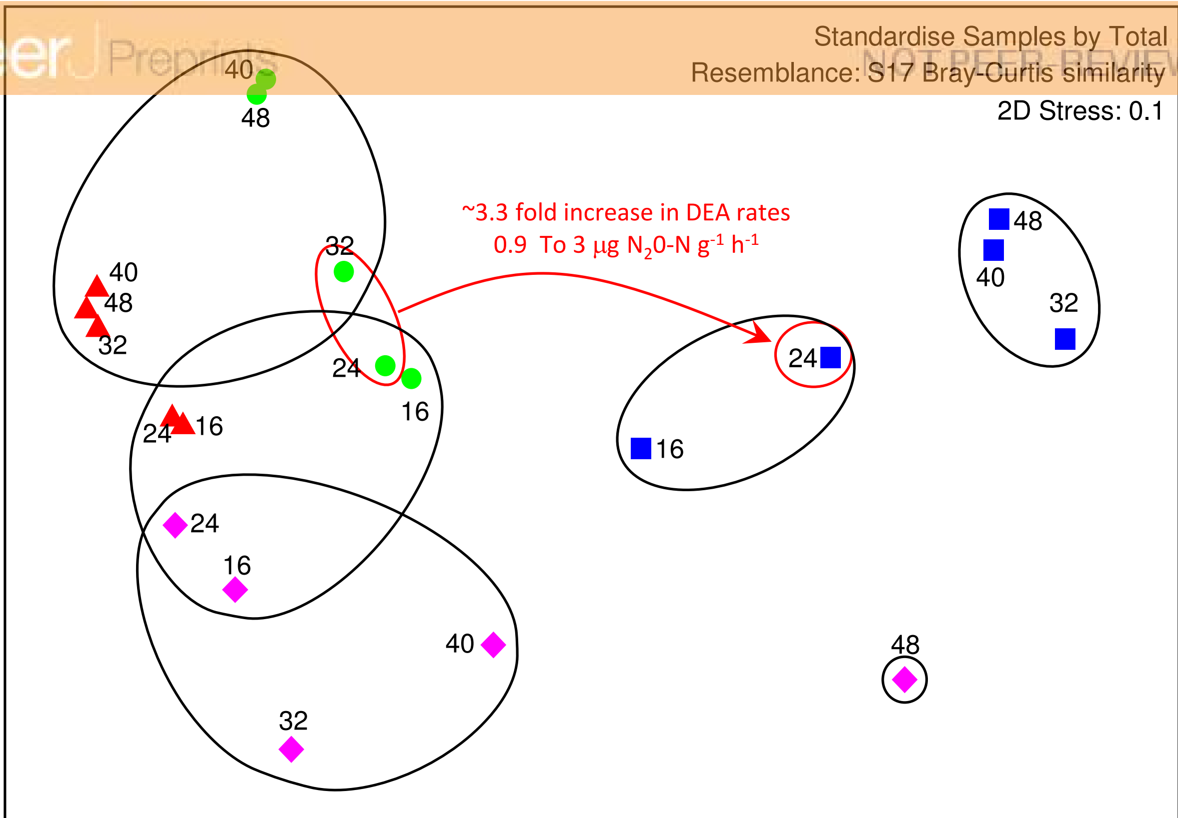

Source	Estimate	SD	% est. variation	*P*-values
S(pH)	1015.6	31.9	57.3	0.001
S(Time)	266.9	16.3	15.1	0.001
S(pHxTime)	321.9	17.9	18.2	0.001
V(Residual)	167.8	12.9	9.4	

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.

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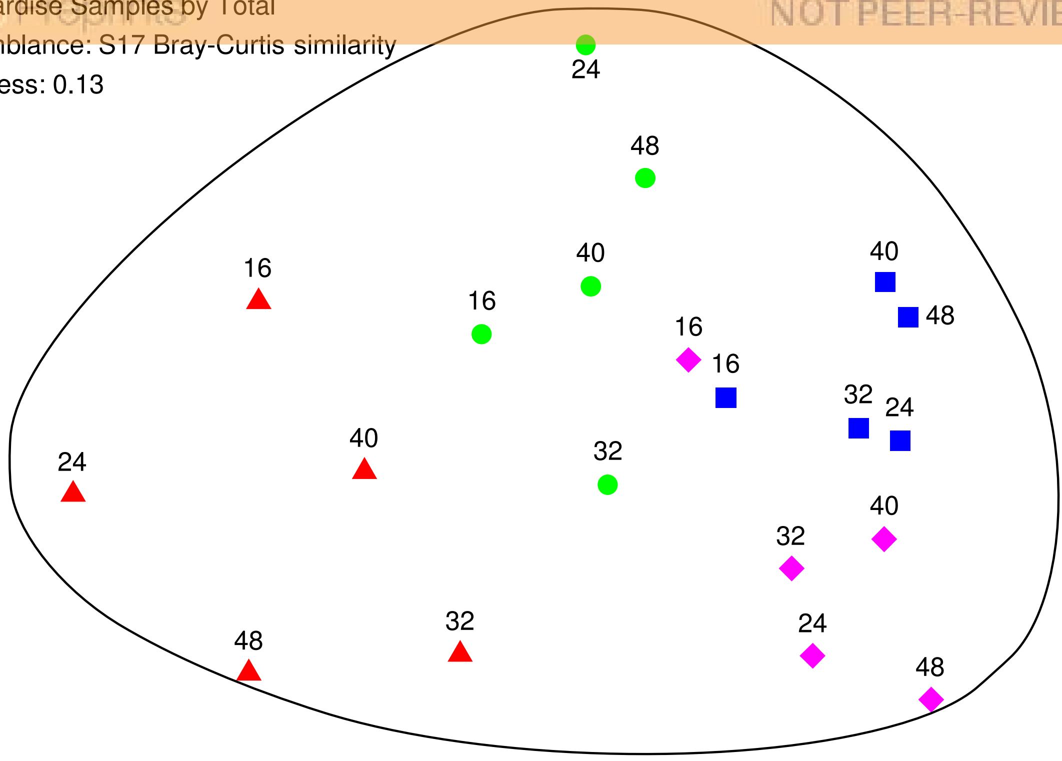


Source	Estimate	SD	% est. variation	*P*-values
S(pH)	97.9	9.9	27.8	0.001
S(Time)	23.2	4.8	6.6	0.001
S(pHxTime)	31.6	5.6	8.9	0.001
V(Residual)	199.9	14.1	56.7	

pH
4.7
6.7
8.3
8.8
Similarity
>70 %

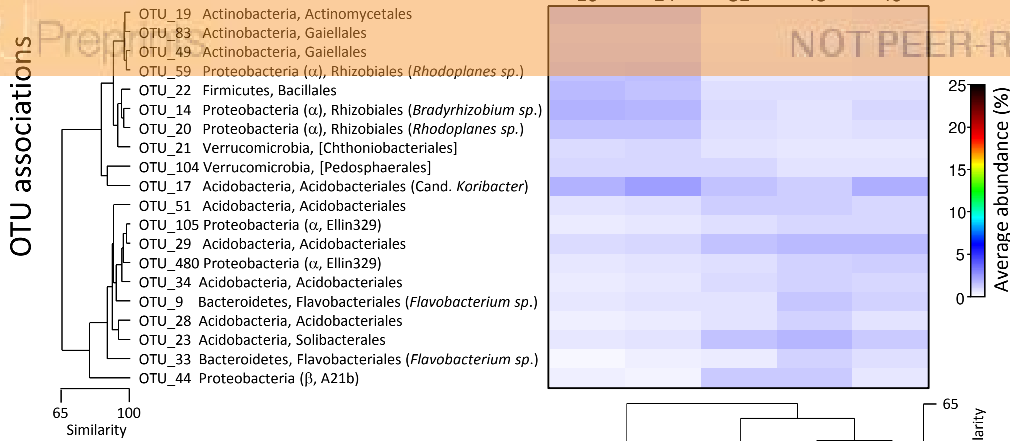
Figure 4(on next page)

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.

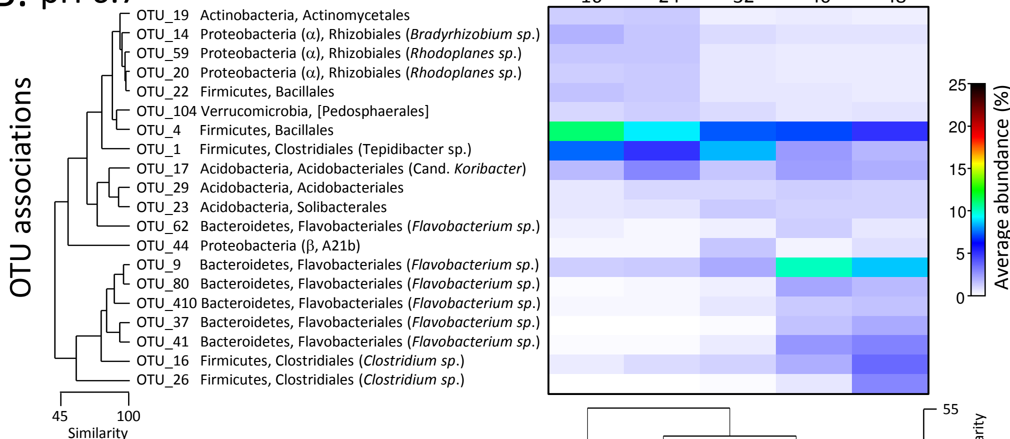
Control microcosms (A), pH 6.7 (B), pH 8.3 (C) and pH 8.8 (D). Samples for each pH treatment have been clustered via Bray Curtis similarity measures representing the entire set of OTUs while the 20 OTUs selected to represent major changes in each treatment have been clustered via an index of association. 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

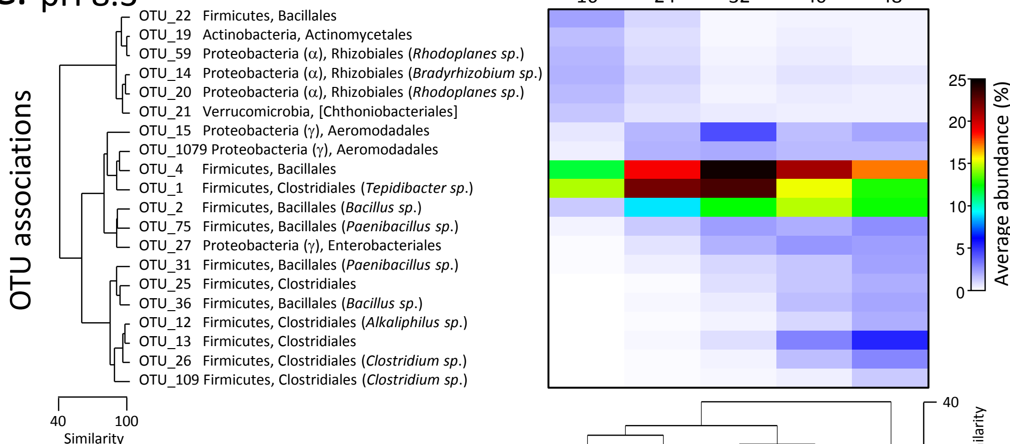
A. Control (pH 4.7)



B. pH 6.7



C. pH 8.3



D. pH 8.8

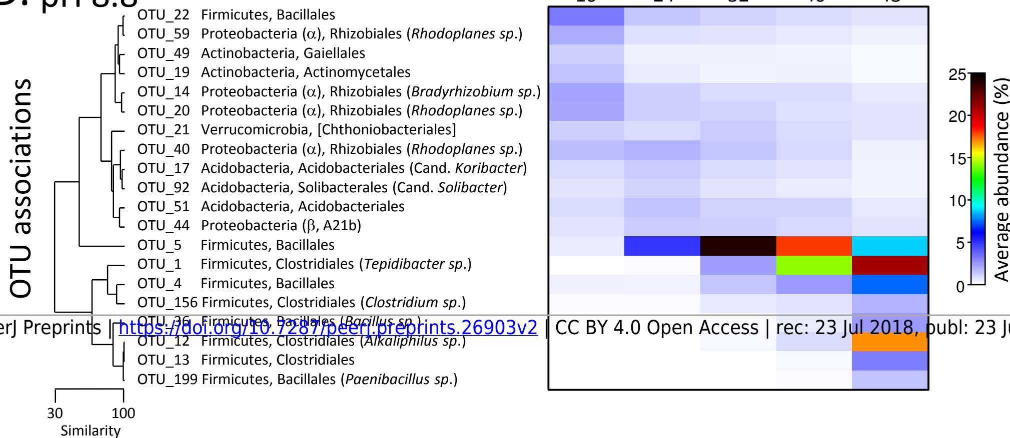


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Figure 5(on next page)

Matrix plot representingthe relative abundance and clustering of the 20 fungal OTUs with the highestcontribution 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.

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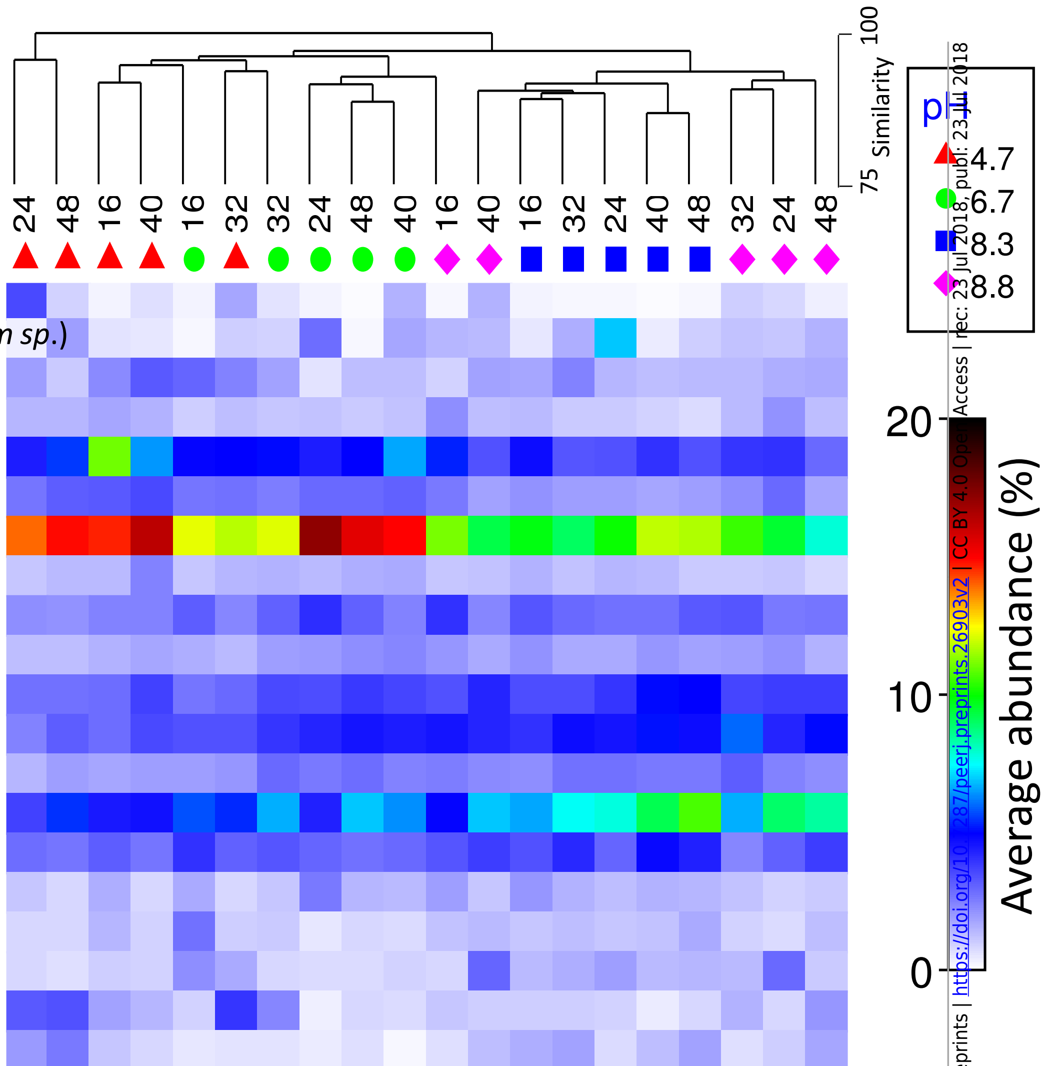

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Table 1(on next page)

N-chemistry trends, cumulative respired CO_2 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
NH_4^+	**16**	1.8	0.1	5.2	0.2	20.9	0.7	30.5	1.4
($mg\ kg^{-1}$)	**24**	2.5	0.2	8.2	0.4	27.4	1.4	31.0	0.7
*4**	**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
NO_3^-	**16**	5.4	1.0	0.2	0.1	13.0	0.3	18.3	0.5
($mg\ kg^{-1}$)	**24**	3.94	0.2	0.2	0.0	3.3	0.3	18.5	0.4
*20**	**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
N_2O	**16**	1651	75.9	849.3	323.8	11.0	3.0	3.2	4.4
($\mu g\ kg^{-1}$)	**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
CO_2	**16**	8.2	1.0	17.5	0.8	15.4	1.5	13.5	1.6
($mg\ kg^{-1}$)	**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	**16**	36.7	4.4	226.9	9.4	4846	106.0	5670	800.5
($mg\ kg^{-1}$)	**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	**16**	3.5	0.7	18.3	1.4	338.3	51.2	440.6	37.3
($mg\ kg^{-1}$)	**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	**16**	5.0	0.0	4.5	0.1	5.4	0.1	5.8	0.2
($mS\ cm^{-1}$)	**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 NH_4^+ and NO_3^- prior to experiments

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