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

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We used potassium hydroxide (KOH) to induce rapid soil pH changes and then observed microbial community change over 48 hours in anaerobic conditions before measuring denitrification enzyme activity (DEA). Soil pH was elevated from 4.7 to 6.7, 8.3 or 8.8, straddling the range of localized pH changes likely to be observed in soil after deposition of livestock urine or urea fertiliser. Up to 240-fold higher dissolved organic matter (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 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 (N_2O-R) was inactive in the pH 4.7 controls but at pH 8.3 the reduction rates exceeded of 3000 ng N_2-N g^{-1} h^{-1} in the presence of native DOM. Evidence for DNRA and/or organic matter mineralisation was observed with ammonium (NH_4^+) increasing to concentrations up to 10 times the original native soil concentrations while significant concentrations of nitrate (NO_3^-) were utilised. Pure isolates from the microcosms were predominantly *Bacillus* spp. and exhibited varying NO_3^- reductive potential.

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1 # Rapid increase in soil pH solubilises organic matter, dramatically increases
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3 *Firmicutes* phylum.
4

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31 ## Abstract

32 We used potassium hydroxide (KOH) to induce rapid soil pH changes and then observed microbial
33 community change over 48 hours in anaerobic conditions before measuring denitrification enzyme
34 activity (DEA). Soil pH was elevated from 4.7 to 6.7, 8.3 or 8.8, straddling the range of localized
35 pH changes likely to be observed in soil after deposition of livestock urine or urea fertiliser. Up to
36 240-fold higher dissolved organic matter (DOM) was mobilized by KOH compared to the controls.
37 This increased microbial metabolism but there was no correlation between DOM concentrations
38 and CO_2 respiration nor N-metabolism rates. Microbial communities became dominated by
39 *Firmicutes* bacteria within 16 hours, while few changes were observed in the fungal communities.
40 Changes in N-biogeochemistry were rapid and DEA increased up to 25-fold with the highest rates
41 occurring in microcosms at pH 8.3 that had been incubated for 24-hour prior to measuring DEA.
42 Nitrous oxide reductase (N_2O-R) was inactive in the pH 4.7 controls but at pH 8.3 the reduction
43 rates exceeded of 3000 ng N_2-N g^{-1} h^{-1} in the presence of native DOM. Evidence for DNRA and/or
44 organic matter mineralisation was observed with ammonium (NH_4^+) increasing to concentrations
45 up to 10 times the original native soil concentrations while significant concentrations of nitrate
46 (NO_3^-) were utilised. Pure isolates from the microcosms were predominantly *Bacillus* spp. and
47 exhibited varying NO_3^- reductive potential.

48

49 ## Introduction

50 Denitrification in agricultural environments results in nitrous oxide (N_2O) emission which
51 contributes to stratospheric ozone depletion and is a powerful greenhouse gas (Forster et al. 2007;
52 Ravishankara et al. 2009; Su et al. 2011). Denitrification is a stepwise enzymatic process whereby
53 NO_3^- is reduced (via NO_2^-) to nitric oxide (NO), N_2O and finally molecular nitrogen (N_2). The
54 efficiency of denitrification is affected by soil pH, which influences soil physicochemical
55 conditions, and functional genetic potential of the soil microbial communities (Morkved et al.
56 2007; Samad et al. 2016; Schimel et al. 2005). Soil pH affects carbon supply and metabolism, the
57 activity of denitrification enzymes adapted to specific pH conditions and the function of N_2O
58 reductase (N_2O-R) (Anderson et al. 2017; Baggs et al. 2010; Bakken et al. 2012; Curtin et al. 2016;
59 Liu et al. 2010; Morkved et al. 2007; Schimel et al. 2005; Šimek & Cooper 2002).

60 The long-term effects of pH on microbial community structure and abundance have been
61 studied at local to global scales (Fierer & Jackson 2006; Lauber et al. 2009). At the localised scale,

62 bacterial 'richness' has been shown to increase between pH 4 and pH 8 in the Rothamsted
63 Hoosefield acid strip, whereas fungal changes were not pronounced (Rousk et al. 2010a). Changes
64 were particularly noted in Acidobacteria and, to a lesser degree, Proteobacteria. Within the
65 Rothamsted Park Grass Experiment, soil pH was positively correlated with 14 of the 37 most
66 abundant genera (taxa) including *Flavobacterium* and *Paenibacillus* (Zhalnina et al. 2015), and
67 more broadly with Proteobacteria and Gemmatimonadetes. At the larger continental scale,
68 Acidobacteria have been shown to be negatively correlated with pH (>4), whereas Actinobacteria
69 and Bacteroidetes increase significantly as pH increases (Lauber et al. 2009). Within phyla in the
70 same study, the relative proportions of Acidobacteria subgroups exhibited opposing trends when
71 pH increased, while within the Alphaproteobacteria, the Sphingomonadales increased as other
72 subgroups decreased as pH was raised.

73 In contrast to studies describing microbial community response to pH in long-term trials,
74 reports about community response to short-term, rapid pH change are sparse, yet it is important to
75 understand these dynamics. For example, rapid pH change is common in soil environments
76 affected by animal urine deposition or in the vicinity of urea fertiliser prills. When urea
77 ($CO(NH_2)_2$) is added to the soil (as fertiliser or urine), it undergoes hydrolysis in the presence of
78 urease enzymes:

79

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

80 The OH^- ions produced during this process cause substantial pH increases, to values >7.5
81 over the course of a few days, coupled with the pH mediated release of dissolved organic matter
82 (DOM) (Clough et al. 2010; Curtin et al. 2016; O'Callaghan et al. 2010). During the first week
83 post urine deposition, pH continues to rise (to values > pH 8), ammonium (NH_4^+) oxidation to
84 NO_3^- (via NO_2^-) commences and oxygen concentrations and redox conditions decrease via
85 nitrification reactions and through microbial metabolism (Clough et al. 2010; Hansen et al. 2014;
86 Nowka et al. 2015). Our previous research using KOH or $Ca(OH)_2$ as proxies for NH_4OH indicates
87 that denitrification response also becomes elevated very shortly after pH and DOM increases
88 (Anderson et al., 2017).

89 In addition to allowing documentation of soil physicochemical response, urine patches
90 represent a natural laboratory setting for investigating microbial community structural and
91 functional response to rapid pH change. A few studies have reported changes in soil microbial
92 population structures in response to urine (or synthetic urine), but have not investigated the

93 taxonomic profiles of these microbial populations in detail (Rooney et al. 2006; Singh et al. 2009).
94 In the study of Singh et al. (2009), addition of synthetic urine to soil was not associated with
95 increases in microbial biomass C or N and the relative population structure of fungi did not change.
96 Taxonomic changes observed by O'Callaghan et al. (2010) indicated that *Firmicutes* increased by
97 38% while Proteobacteria decreased (18%) after urine addition to soil, but fine resolution of
98 taxonomic groups was not achieved.

99 In this work we sought to simulate the effects of rapid soil pH change likely to occur under
100 urine patches or around urea prills during the first 48 hours post urea deposition. We hypothesised
101 that the microbial population would undergo major structural and physiological change in response
102 to pH, and increases in DOM. In order to change soil pH without adding extra N (or C) via urea,
103 we used potassium hydroxide as an NH_4OH proxy (see Anderson et al. 2017). After different
104 exposure times to 4 different pH treatments, the potential denitrification enzyme activity (DEA)
105 of the microbial communities was assayed, amplicon sequencing was used to investigate changes
106 in the microbial populations, and a collection of nitrate reducing bacteria were isolated from the
107 microcosms.

108

109 ## 2. Materials and methods

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

112

113 *2.1 Soil collection, pH adjustment and DEA assays*

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

122 kg^{-1} treatment represented an alkaline pH outlier, where DEA was expected to be minimal
123 (Anderson et al 2017).

124 A total of 240 soil microcosms were prepared, covering 4 KOH treatments and 5 incubation
125 times. This provided twelve analytical replicate microcosms for each KOH rate x incubation time
126 combination; where, four microcosms were designated for soil chemical analysis prior to DEA
127 assays, two were designated for nucleic acid extraction and microbial culturing work (prior to
128 DEA), and the remaining six microcosms were used for DEA assessments (two triplicate DEA
129 assays, with or without acetylene) (Fig. 1).

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

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

143 To determine DEA, 5 mL of water containing 50 mg NO_3-N kg^{-1} (Luo et al. 1996) was
144 added to the remaining six microcosms, and the anaerobic atmosphere was regenerated by flushing
145 three times with N_2. Prior to venting microcosms to atmospheric pressure after each flushing, 10%
146 v/v purified acetylene (Hyman & Arp 1987) was added to 3 of the microcosms. 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*

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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 tables were produced
189 using biom-format (http://biom-format.org/) (McDonald et al. 2012).
190 The phyloseq (McMurdie & Holmes 2013) and ggplot2 (Wickham 2009) packages within R (Team
191 2016) were used for the analysis and visualisation of data. OTU tables were also analysed using
192 Primer 7 with PERMANOVA add-on (Primer-E Ltd, Plymouth, UK). Relationships among
193 microbial community profiles based on Bray-Curtis similarity matrices were graphed using
194 unconstrained non-metric multidimensional scaling (nMDS) ordinations using 250 restarts along
195 with cluster analysis. Relationships were then statistically tested using 2-factor permutational
196 ANOVA (PERMANOVA) and matrix plots were based on the 20 OTUs with the highest
197 contribution to sample similarity/dissimilarity at each pH value, or in the case of fungi, across all
198 pH values.

199 *2.5 Isolation of bacteria, N-use characterisation*

200 2.5.1 Isolation of nitrate reducing bacteria

201 Ten μL sample of slurry from each pH adjusted microcosm was serially diluted (10 μL into 1mL
202 followed by three dilutions of 10 μL into 100 μL) and plated onto TSB or 1/10 diluted TSB
203 containing KNO_3 (30g L^{-1} + 0.5g L^{-1} KNO_3). Plates were incubated under anaerobic conditions
204 (Whitney jars with gas packs) at 24°C for 2 to 6 days.
205 Representative colonies of different morphology were selected and re-streaked onto the same
206 media and grown under anaerobic conditions at 24°C. Isolates were stored in 20% glycerol at -
207 80°C, with a selection of isolates identified by amplification and Sanger sequencing of the 16S
208 ribosomal RNA gene using the 27F and 1492R primers (Anderson et al. 2009).
209

210 2.5.2 Nitrate utilisation by bacterial isolates

211 Cells were grown aerobically overnight in TSB with KNO_3. 20mL cultures were then initiated
212 with 1/100 dilutions of the overnight cultures, and grown anaerobically (O_2 replaced with N_2) at

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213 24°C. After 48 hours, gas samples were extracted with a gas-tight Hamilton syringe and analysed
214 for the presence of N_2O and CO_2 by gas chromatography (as outlined above) and 10 mL samples
215 of the bacterial cultures were removed for NO_3^- and NH_4^+ analysis (as outlined above).
216

217 ## 3. Results

218 *3.1 Chemical characterisation of pH amended soils prior to DEA assays*

219 After 48 hours of anaerobic incubation, soil slurries with additions of 0, 6, 16 and 20 $cmol_c$
220 kg^{-1} KOH had average pH values of 4.7, 6.7, 8.3 and 8.8 respectively. During the 48 h incubation
221 EC reached ~6.4 mS cm^{-1} in all microcosms except the pH 6.7 microcosms which reached ~5.7
222 mS cm^{-1}. DOC in the control microcosms increased from 37 to 63 mg kg^{-1} during the 48 h
223 incubation. In the pH 6.7, 8.3 and 8.8 microcosms DOC increased ~11, 185 and 240-fold
224 respectively in response to KOH addition, with the majority of that change (> 75%) occurring
225 during the first 16 h of incubation. During the 48 h incubation DON in the control microcosms
226 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,
227 140 and 223-fold in response to KOH addition, respectively (Table 1). There was a strong
228 correlation between the amounts of DOC and DON solubilised at each pH value, irrespective of
229 incubation time ($R^2 = 0.98$) (Supplementary Figure S1).

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

237 In control microcosms, ammonium (NH_4^+) increased linearly over 48 hours with a slope of
238 0.09, starting from native soil concentrations of ~4 mg kg^{-1}. The NH_4^+ profiles in the pH 6.7, and
239 8.3 microcosms were similar (approximately linear) but with greater slopes of 0.28 and 0.79
240 respectively (R^2 values of 0.99 and 0.98). In the pH 8.8 microcosms, NH_4^+ quickly elevated to 30
241 mg kg^{-1} during the first 16 hours of incubation and remaining at that concentration until after 32
242 hours, when a further increase from 30 to 50 mg kg^{-1} occurred by 48 hours (Table 1). The native
243 soil nitrate (NO_3^-) concentration was ~20 mg kg^{-1}. This NO_3^- was almost completely reduced after

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244 32-40 hours incubation in the control, pH 6.7 and pH 8.3 microcosms (Table 1). In the pH 8.8
245 microcosms, NO_3^- remained at ~20 mg kg^{-1} until after 32 hours incubation, dropping to ~6 mg kg^{-1}
246 during the following 16 hours.

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

252

253 *3.2 DEA assays*

254 Microcosms incubated for 16 hours had the lowest DEA rates with 80, 540, 34 and 0.2 ng
255 N_2O g^{-1} h^{-1} recorded for the control, pH 6.7, 8.3 and 8.8 treatments respectively (Fig. 2A). DEA
256 remained low in the control microcosms with a maximum DEA rate of only 121 ng N_2O g^{-1} h^{-1}
257 after 24 hours incubation, declining to 85 ng N_2O g^{-1} h^{-1} in microcosms incubated for 48 hours
258 (Fig. 1). In contrast, after 24 hours incubation, DEA rates in the pH 6.7 and 8.3 microcosms were
259 8 and 25-fold higher respectively with a maximum of ~3000 ng N_2O g^{-1} h^{-1} produced (Fig. 2A).
260 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
261 treatments, respectively, for microcosms incubated up to 40 hours. DEA rates greater than those
262 in the control were not observed in the pH 8.8 treatment until microcosms were incubated for at
263 least 48 hours (~65 ng N_2O g^{-1} h^{-1}).

264 Replicate microcosms without acetylene added were used to assess N_2O-R activity during
265 the DEA assays based on the $N_2O/(N_2O + N_2)$ ratio. These showed that N_2O reduction was absent
266 in the control microcosms while in the pH 6.7, 8.3 and 8.8 treatments between 56 and 100% of the
267 N_2O produced was reduced to N_2 depending on incubation time (Fig. 2B). Near complete N_2O
268 reduction was observed in the pH 8.3 treatment for microcosms incubated longer than 24 hours.
269 Depending on pH treatment and incubation time, N_2O production (and reduction) profiles during
270 the DEA assays were mirrored by decreases in NO_3^- with an estimated 30 to 80 % of the available
271 NO_3^- reduced. In the control and pH 6.7 microcosms there were no differences in the NH_4^+ before
272 and after the 4-hour DEA assay period, however decreases of up to 9 mg kg^{-1} occurred in the pH

273 8.3 microcosms after 32 hours incubation and in the 8.8 microcosms after all incubation times
274 (Supplementary Table 1).

275 T-tests comparing CO_2 respiration indicated that acetylene addition depressed microbial
276 activity, but only in the pH 8.3 and 8.8 treatments ($P < 0.05$). It is acknowledged that lower soil
277 respiration in the presence of acetylene will reflect both the absence of any CO_2 derived from
278 metabolic N_2O reduction but possibly also general impediment of other anaerobic metabolisms. It
279 is unlikely that acetylene would have served as dominant carbon source during the 4 hour DEA
280 incubation given the excess DOC available and time required to adapt to using acetylene (Felber
281 et al. 2012; Groffman et al. 2006). There is evidence to suggest that DOC and DON declined during
282 the DEA assay period, especially in the pH 8.3 and 8.8 treatments but the results were highly
283 variable (Supplementary Table 1).
284

285 *3.3 Microbial community adaptation to pH treatments during 48 hours incubation*

286 The total number of OTUs identified across all treatments and incubation times was 2258
287 for fungi and 6429 for bacteria. Two factor tests using PERMANOVA [pH x incubation time] and
288 resulting estimated components of variation supported nMDS observations with significant two-
289 way interactions between time and pH (P=0.001 for both bacteria and fungi) (Figures 3 and 6).
290 Approximately ~90% of the total variation was explained for the bacterial relationships whereas
291 only ~43% was explained for the fungi. Of the variation explained for the bacteria, up to 60% was
292 attributed to pH, a further 15% to incubation time, and 18% was attributed to the interaction
293 between the two factors. The corresponding values for the fungal communities were 28, 6.6 and
294 8.9% for incubation time, pH and the interaction between the two factors respectively. (Figures 3
295 and 6).

296 The phylum level bacterial profile from the control (pH 4.7) microcosms after 16 h
297 incubation was made up of Acidobacteria (~7%), Actinobacteria (~12%), Bacteroidetes (~7%),
298 Proteobacteria (~20%), Firmicutes (~5%), Planctomycetes (~5%) and phyla that had abundances
299 of >5% including Verrucomicrobia, Chloroflexi, Gemmatimonadetes and Armatimonadetes. Up
300 to 35% of OTUs could not be classified (Fig. 4). After 24 hours incubation, the communities were
301 still >85% similar (Figures 4 and 5) before there was a relative decrease in Actinobacteria coupled
302 with an increase in Acidobacteria and Bacteroidetes (Fig. 5). A decrease in Actinobacteria was
303 also seen over the incubation periods for all three pH modifications along with changes in the

304 proteobacterial populations with almost complete disappearance of OTUs from the order
305 Rhizobiales (Figures 4 and 5).
306 After 16 and 24 hours incubation, the bacterial profiles in the pH 6.7 microcosms were
307 similar to those in the control microcosms with the exception of a larger proportion of Firmicutes
308 (~20% versus 5% in the controls) (Fig. 4). The increased representation of Firmicutes was driven
309 by an expansion of OTUs from the orders Bacilliales (OTU_4) and Clostridiales (OTU_1,
310 *Tepidibacter sp.*). These two OTUs then decreased later in the incubation, partially displaced by
311 other *Clostridium spp.* (OTUs 16 and 26) (Fig. 5). Across the 32 to 48h incubation period there
312 was a large relative increase in OTUs from the phylum Bacteroidetes, driven by *Flavobacterium*
313 *spp.* (OTUs 62, 9, 80, 410, 37 and 41) (Fig. 5).
314 In the pH 8.3 treatment after 16 hours incubation, there was a very high proportion of
315 Firmicutes (~40%) and a low representation of Actinobacteria and Bacteroidetes compared with
316 the pH 4.7 control microcosms. Across the incubation period, the dominance of Firmicutes
317 increased further, initially driven by the same Bacilliales and Clostridiales OTUs (4 and 1), but at
318 a much higher relative 'abundance' than observed in the pH 6.7 treatment. Their expansion
319 coincided with the highest DEA rates, with the average 'abundance' of OTU_4 being ~20% of
320 total at this time and OTU_1 being ~23% (Figs. 4 and 5). After 32 h these two dominant OTUs
321 were partially displaced by a cohort of other OTUs from the orders Bacilliales and Clostridiales
322 (OTUs 2, 75, 31, 25, 36, 12, 13, 26 and 109) (Fig. 5). At the genus level these OTUs represented
323 *Bacillus*, *Paenibacillus*, *Clostridium* and *Alkaliphilus* spp. (Fig. 5). Communities in the 40 and 48h
324 incubations shared the highest level of similarity (Figs. 3, 4 and 5).
325 After 16 and 24 hours incubation, the bacterial profiles from the pH 8.8 treatment shared
326 >70% similarity with the samples from the control microcosms (Fig. 3). Over the incubation period
327 to 32 h, there was a large expansion in the relative percentage of the Firmicutes, initially driven
328 almost entirely by an increase in OTU_5 from the Bacilliales order (Fig 5). By 48 h, this OTU was
329 partially displaced by a group of Bacilliales and Clostridiales OTUs that shared some similarity
330 with those observed in the pH 8.3 treatment (OTUs 1, 4, 156, 36, 12, 13, and 199). These OTUs
331 included the same genus level representatives - *Bacillus*, *Paenibacillus*, *Clostridium* and
332 *Alkaliphilus* spp. (Fig. 5).
333 Fungal communities shared >70% similarity across all samples regardless of pH and time
334 (Figure 6). Based on percentages alone, no discernible patterns could be observed for the fungal

335 dataset (Fig. 7), however, some OTUs were displaced depending on pH. For example, Zygomycota
336 OTU_3 was dominant in the control and pH 6.7 treatments but was partially supplanted by another
337 Zygomycota OTU from the order Mortierellales (*Mortierella sp.*) (Fig. 8).
338
339 3.4 *Isolation of bacteria and N-use characterisation.*
340 On TSB-nitrate medium, plates were quickly dominated by fast growing colonies sharing
341 morphologies characteristic of motile or swarming bacteria. A total of 33 isolates were screened
342 for nitrate utilisation. Seven showed minimal growth and respiration in liquid culture, 18 showed
343 near complete utilisation of NO_3^- accompanied by production of both NH_4^+ and N_2O, and 5 showed
344 moderate utilisation of NO_3^- with low production of NH_4^+ and N_2O. One isolate showed production
345 of NH_4^+ with little or no use of NO_3^- or production of N_2O and 2 isolates exhibited respiration but
346 did not appear to utilise N. Of the 33 isolates, 22 were selected for identification by ribosomal 16S
347 gene DNA sequencing. All were from the Firmicutes phylum, of which three were *Paenibacillus*
348 *spp.* and 1 was a *Brevibacillus sp.* (all producing less NH_4^+), while the remainder were *Bacillus*
349 *spp.* (Supplementary Table 2A).
350 From 1/10 diluted TSB-nitrate medium, 44 isolates were screened for nitrate utilisation. Of
351 these, 24 showed no or minimal growth in liquid culture over 48 hours incubation while 13 reduced
352 NO_3^- to close to zero with 4 of these generating significant amounts of NH_4^+. Another 3 reduced
353 NO_3^-, but to a lesser extent, while 4 isolates respired but did not appear to utilise N. Seventeen
354 isolates were identified by 16S gene sequencing. Six belonged to the *Bacillus* genus, three to
355 *Achromobacter*, six to *Acidovorax*, one to *Bosea*, and one to *Rhodanobacter* (Supplementary Table
356 2B).
357 All isolates except one of the *Bacillus sp.* had high NO_3^- utilisation compared to
358 uninoculated controls. The *Bacillus spp.* produced up to 536 mg NH_4^+ L^{-1}, while the *Acidovorax*
359 *spp.* produced < 45 mg NH_4^+ L^{-1}. One *Bacillus sp.* and two *Acidovorax sp.* produced < 100 mg
360 N_2O L^{-1}, while the remaining *Acidovorax* sp. had the highest N_2O production at 2840 mg L^{-1} . In
361 general, isolates that exhibited high use of NO_3^- coupled with production of NH_4^+ and N_2O
362 exhibited an average of ~2.5-fold higher CO_2 production (respiration) compared with isolates that
363 exhibited high NO_3^- use with little or no NH_4 and N_2O production. Relevant 16S sequences were
364 submitted to NCBI with accession numbers assigned between MH211426 and MH211463,

365 submission number SUB3915485 (Supplementary Table 2). Six organisms were selected for future
366 genome sequencing, 3 *Bacillus spp*. and 3 *Acidovorax spp*. (Supplementary Table 2C).
367

368 4. Discussion

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

370 KOH additions and resulting pH elevation caused the concentration of DOM in soil
371 microcosms to greatly increase (150-fold) compared to the controls. Added hydroxyl ions
372 displaced negatively-charged organic molecules into solution. Previous work showed that KOH is
373 much more effective in solubilising organic matter than $Ca(OH)_2$ (Curtin et al. 2016). The amount
374 of DOM released at elevated pH in these experiments was concordant with our previous research
375 using the same methodology (Anderson et al. 2017), as were the higher respiration rates.

376 Over medium to long time scales (months to years), pH is known to be a dominant
377 environmental variable that shapes soil microbial communities (Lauber et al., 2009; Zhalina et al.,
378 2015). Changes in pH are also known to cause shifts in active organisms over short timescales
379 (Brenzinger et al. 2015). Although the strongest predictor for both bacterial and fungal community
380 change in these experiments was pH, community change may also be indirectly influenced by the
381 effect that pH has on DOM release. Theoretically, high levels of DOM released via increased pH
382 should benefit copiotrophs (*r*) over oligotrophs (*K*) (Fierer et al. 2007; Goldfarb et al. 2011), but
383 at the same time elevated pH is likely to alter cellular homeostasis, regulation of nutrient
384 availability, or other factors such as salinity, metal accessibility, or organic C characteristics
385 (Lauber et al. 2009).

386 Previous analysis of the soil used in this study suggests that a mix of carbon sources are
387 released as pH increases, of which 45% are bioavailable. These range from labile hexose and
388 pentose sugars to more recalcitrant polyphenolic molecules (Curtin et al. 2016). The lack of
389 proportionality between respiration rates and DOM released in this study suggests that higher
390 amounts of bioavailable C did not lead to higher biomass, instead the microbial community and
391 associated metabolic response has shifted toward more copiotrophic organisms. Addition of low
392 molecular weight C compounds (glucose, citric acid, glycine) to soil has been previously observed
393 to shift the structure of bacterial communities to more copiotrophic organisms (Eilers et al. 2010)
394 with no strong correlations between respiration rates and community structure. Community

395 changes and catabolic responses may be unlinked because some C-substrates are preferentially
396 used without biomass changes (Devevre & Horwath 2000).

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

405 In general, we observed a decrease in α-Proteobacteria (specifically *Rhizobiales*),
406 Actinobacteria and Acidobacteria OTUs at all pH and DOM values, while a few β- and γ-
407 Proteobacteria OTUs increased. Our results suggest that the chemical changes induced by KOH
408 addition to soil are comparable to soils where pH and DOM are elevated due to higher urea inputs.
409 Niche differentiation occurs in soil where higher bovine density (and presumably urea inputs)
410 induces increases in pH and total organic carbon, with Actinobacteria, α-Proteobacteria and
411 Verrucomicrobia decreasing and Bacteriodetes increasing (Philippot et al. 2010; Philippot et al.
412 2009a; Philippot et al. 2009b).

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

421 Comparisons of OTU distributions in our study indicate that the dominant feature driving
422 sample dissimilarities was large increases in Firmicutes from the classes Bacilli and Clostridia.
423 Large expansion of Firmicutes, first dominated by *Bacillales* (up to 46%) and then followed by
424 *Clostridiales* (up to 53%), (along with large decreases in Proteobacteria) have been observed in

425 alkaline soil crusts (pH 8.5) that were rehydrated and incubated under dark anoxic conditions
426 (Angel & Conrad 2013). Our results are also concordant with O'Callaghan et al. (2010), who
427 observed a 38% increase in Firmicutes, and decreases in Proteobacteria (18%), Acidobacteria
428 (8%), Actinobacteria (5%), and Bacteriodetes (5%), in soil where pH rose to values of >8, two
429 days after bovine urine addition.

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

442 Relative changes in fungal populations in response to pH change were smaller than for
443 bacteria, with ~28% of fungal variation attributed to pH, versus ~57% for bacteria. Only ~6.6
444 percent of the variation in fungal communities could be attributed to incubation time. Fungal
445 communities are known to be less responsive to pH than bacteria (Lauber et al. 2009; Rousk et al.
446 2010a) and fungal abundance has been found to be negatively correlated with pH (Rousk et al.
447 2010b), but positively correlated with C and N additions (Banerjee et al. 2016). For example,
448 investigations of the response of fungal (and bacterial) communities to ovine urine (where pH
449 increased from ~3.5 up to 6.5 and DOC increased from ~0 up to ~ 2000 mg $kg^{-}1$) indicates no
450 fungal biomass change (Nunan et al. 2006; Williams et al. 2000), no significant correlation
451 between biomass and pH, NH_4^+ or NO_3^-, but weak correlation between biomass and DOC (Singh
452 et al. 2009). In pH 6.1 soils, Banerjee et al (2016) noted that although fungal biomass increased,
453 the richness, evenness, and diversity decreased within 4 days after organic matter and nutrient
454 addition leading to 'keystone' fungal species being favoured. Singh et al. (2009) suggest that

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455 because fungi are capable of degrading complex organic carbon they are less responsive to short
456 term changes in nutritional availability.

457

458 ***4.2 Soil N cycling and its relationship to microbiology following pH change***

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

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

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

480 In contrast to the KOH amended microcosms, the $N_2O/(N_2O + N_2)$ ratio in the control
481 microcosms equalled 1, indicating that this treatment did not have active N_2O-R. Liu et al. (2010)
482 and Bakken et al. (2012) have previously shown that production of functional N_2O-R depends on
483 the post-transcriptional pH being greater than 6.1, which is consistent with our results. In an
484 agricultural environment this raises interesting ecological questions, because urea hydrolysis

485 happens to elevate pH for several days which would immediately alleviate any post-transcriptional
486 interference of *nos*Z expression and allow rapid production of functional N_2O-R.

487 We observe full denitrification of NO_3^- to N_2 within 16 hours and maximum rates after 24
488 hours which indicates that suitable redox conditions for denitrification were established quickly in
489 our microcosms and a corresponding rapid genetic and enzymatic response followed. The
490 predominant electron acceptors in a weakly reducing environment are O_2, NO_3^- and manganese
491 oxide (MnO_2) (Uteau et al. 2015) with the threshold between oxic and anoxic soil lying somewhere
492 between 300 and 400mV. These conditions develop in response to high soil moisture contents that
493 slow down gas diffusion (e.g. post irrigation or flooding) and there are good correlations between
494 N_2O flux and relative soil gas diffusivity (D_p/D_O) (Hansen et al. 2014; Owens et al. 2017; Owens
495 et al. 2016). Biologically, low O_2 concentrations, or restricted diffusion of oxygen would trigger
496 rapid induction of *de novo* denitrification enzyme synthesis depending on pH. *De novo* enzyme
497 synthesis follows a sequential order, with nitrate reductase formed within 2-3 h, nitrite reductase
498 between 4-12 h and N_2O-R between 24 and 42 h (Dendooven & Anderson 1994; Dendooven &
499 Anderson 1995; Firestone & Tiedje 1979; Smith & Tiedje 1979). Recent investigations have
500 observed even earlier synthesis of N_2O-R than 24 – 42h, with peaks in gene transcripts for *nos*Z
501 (and presumably translation of N_2O-R) occurring within <10 hours (Liu et al. 2014).

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

515

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

517 After pH change, the microcosms had high DOC/NO_3^- ratios with no correlations evident
518 between estimated DOC mineralisation and NO_3^- nor CO_2 respiration. There was also a mismatch
519 between increasing NH_4^+ relative to consumed NO_3^-, suggesting that other anaerobic metabolisms
520 were active aside from denitrification. DNRA is an energy yielding anaerobic process that is
521 favoured in C-replete conditions when NO_3^- becomes limiting (C/NO_3^- ratio >12) (Giles et al.
522 2012; Rutting et al. 2011). Using the DNRA stoichiometry in equation [2] presented by Lam et al.
523 (2009), if all available native NO_3^- in our microcosms (~20 mg kg^{-1} NO_3^--N) was reduced via
524 DNRA (i.e. ignoring NO_3^- also needed for denitrification) then approximately 26 mg kg^{-1} NH_4^+
525 could be produced, yet we observed up to 50 mg kg^{-1}. The significant amounts of additional N
526 required to balance the N requirements in our experiments are likely to be derived from the ample
527 supplies of DON and DOC in the microcosms that could undergo depolymerisation and
528 ammonification (Burger & Jackson 2004; Rutting et al. 2011; Schimel & Bennett 2004).

529 *Bacillus* species are well known as nitrate reducers and N_2O emitters, but many strains do
530 not produce N_2O after NO_3^-/NO_2^- reduction (Verbaendert et al. 2011). DNRA is known to occur
531 in a number of *Bacillus* species with varying concentrations of N_2O produced (Heylen & Keltjens
532 2012; Mania et al. 2014; Nakano et al. 1998; Sun et al. 2016). Although genome information is
533 not yet available, possible N-metabolisms for the predominantly *Bacillus* species isolated from the
534 microcosms in this study include denitrification, DNRA and possibly N_2 fixation. These isolates
535 produced an excess NH_4^+ and N_2O compared to uninoculated controls suggesting DNRA could be
536 the dominant metabolism. Given the concentration of NO_3^- available in the medium, additional N
537 is still required to support the concentrations of NH_4^+ and N_2O produced supporting the idea that
538 depolymerisation and ammonification of organic matter is also active.

539 The other main driver of treatment differences were organisms from the family
540 Clostridiales. Teidje (1998) describes obligate anaerobic DNRA capable *Clostridium* spp. (Caskey
541 & Tiedje 1980; Keith et al. 1982). Clostridia are also well known for their fermentative
542 metabolisms that have been exploited for over 100 years (Moon et al. 2016; Wiegel et al. 2006),
543 which combined with the observations that there was no correlation between NO_3^- consumption
544 and CO_2 production adds support to the theory that metabolisms aside from denitrification/DNRA

545 operate in these microcosms, especially beyond 40 hours when Clostridiales start displacing
546 Bacilliales. Fermentation is also known to occur in *Bacillus* species, specifically the well-studied
547 *B. subtilis* (Ramos et al. 2000).

548 Recent research has shown that fermentative organisms (Clostridiales) influence the
549 competition between denitrifiers and DNRA bacteria through competition for fermentative C-
550 substrates (electron donors). Higher ratios between substrates and nitrate leads to a combination
551 of fermentation and DNRA (both fermentative and respiratory) with no denitrification. When the
552 ratio between substrates and nitrate lowers, denitrification takes a larger role until it eventually
553 out-competes both fermentation and DNRA (van den Berg et al. 2017a; Van den Berg et al. 2017b).
554 Comparing these studies to our microcosms is problematic as the C-sources in our study are so
555 diverse. However, the geochemical evidence indicates that NO_3^- quickly declines while DOM
556 remains high which would lead to a higher substrate/nitrate ratio and thus DNRA and fermentation
557 taking a dominant role. This possibility is further supported by increases in NH_4^+ and CO_2
558 respiration rates beyond the peaks in DEA. There is also the possibility that DNRA has a more
559 significant role that we envisage and that DEA measurements reflect reduction of N_2O via 'atypical
560 nosZ' (Giblin et al. 2013; Jones et al. 2013; Jones et al. 2011; Samad et al. 2016; Sanford et al.
561 2012).

562

563 ***Implications for urea impacted soil***

564 The pH changes induced by our KOH additions in this study are representative of what
565 could be expected in the field under animal urine patches or in the vicinity of urea fertilizer prills
566 (Clough et al. 2010; O'Callaghan et al. 2010). Increases in electrical conductivity (EC) associated
567 with elevated pH were high at 6 mS cm^{-1}, but were not out of the ordinary when compared to other
568 studies investigating urine additions to soil, nor is the associated release of excess SOM (Curtin et
569 al. 2016; Haynes & Williams 1992). Recent research documenting N_2O emissions in urea-
570 amended saturated soils with elevated pH and declines in O_2 and redox changes, also suggest that
571 this work has direct relevance to what would be expected under field conditions (Hansen et al.
572 2014; Owens et al. 2017; Owens et al. 2016).

573 Given that there is experimental and field evidence for chemical conditions conducive to
574 denitrification in the days following urea application, this supports the idea that N-cycling in soils

575 should not be considered a sequential process, but instead is actually highly dynamic, with N-
576 processing dependent on resources available. Our work suggests that N-resources can be quickly
577 supplied from both organic and inorganic sources with the distinct possibility that significant N
578 could be lost as N_2 shortly after urea deposition. It is unknown what happens to the large excess
579 of SOM released at high pH that is not metabolised during these short-term incubations. We should
580 be aware that pulses of fresh C into soil can lead to loss of native C (priming) with some research
581 indicating that excess N-fertilisation (and possibly intensification) leads to soil C declines
582 (Kuzyakov et al. 2000; Mau et al. 2015).

583 Although pH is a universal mechanism that selects for microbial communities, the response
584 to pH will vary according to soil types. The microbial phenotypes expressed will also be dependent
585 on the available soil C and N resources with multiple N-transformation pathways possible. For this
586 particular soil, pH elevation and anoxia allowed Firmicutes bacteria to flourish and contribute to
587 rapid processing of N resources. Investigations of the ratios between various N-metabolisms in
588 these microcosms would require isotope labelling with more defined experiments also required to
589 understand short and long-term cascades of biological N-processing and the transient ecologies
590 driving N-transformations as soil conditions stabilise post urea addition.

591

592 **Conclusions**

593 At pH values representing expected deviations induced by either urine or urea prills we have
594 observed:

595 • Large increases in DOM and respiration associated with pH change.
596 • Large increases in DEA potential within <24 hours of pH change.
597 • A requirement for mineralisation of DOM to supply adequate N to supplement NO_3^-
598 resources and therefore balance NH_4^+ and N_2O concentrations produced.
599 • Concomitant shifts in microbial community structure, specifically a dominance of
600 *Firmicutes* bacteria when DEA potential is highest.
601 • Cultured representatives of Firmicutes bacteria with inferred metabolisms including
602 denitrification and dissimilatory nitrate reduction to ammonia (DNRA).

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606
607
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Figure 1 (on next page)

Microcosm treatment structure

There was a total of 240 microcosms, 60 for each of the four KOH treatment, giving 12 microcosms for each of the five incubation times. Microcosms were destructively sampled. At each time point, 4 microcosms were used for chemical characterisation, and 2 were used as inoculums for isolation of denitrifying microorganisms and DNA extraction to determine microbial community structure. The remaining 6 microcosms at each time point were used for the denitrification enzyme activity (DEA) assay, 3 with acetylene added to block nitrous oxide reductase activity and 3 without acetylene to gauge the activity of the nitrous oxide reductase.

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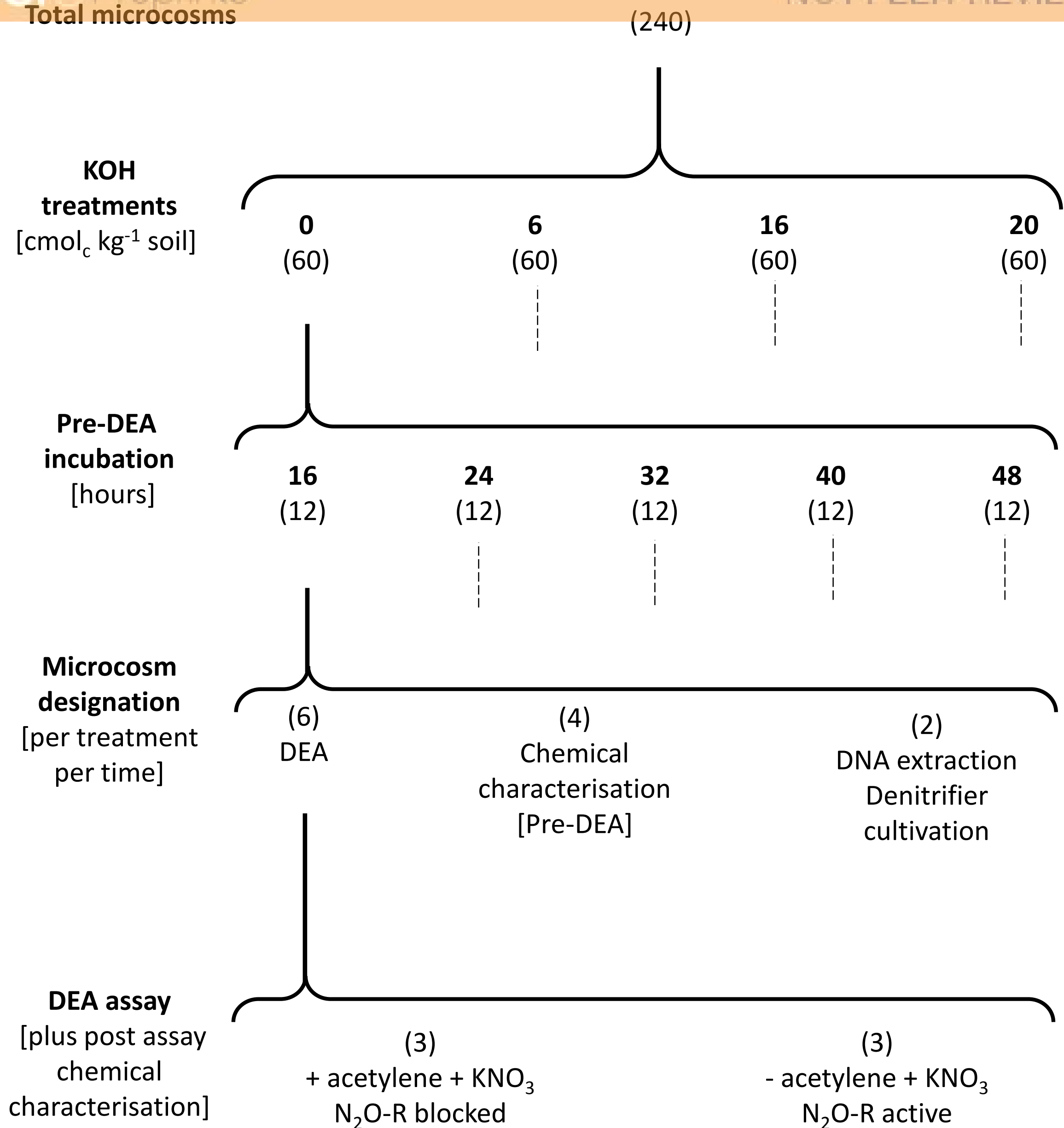

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Figure 2 (on next page)

Denitrification enzyme activity assay results

A) Nitrous oxide (N_2O) emission in microcosms with acetylene added to block nitrous oxide reductase (N_2O-R), error bars represent the standard deviation, n=3 for each time point. B) The ratio of $N_2O/(N_2O + N_2)$ to gauge the activity of the nitrous oxide reductase using the microcosms with and without acetylene added. A ratio of 1 means that N_2O-R is inactive with none of the N_2O produced then further reduced to N_2.

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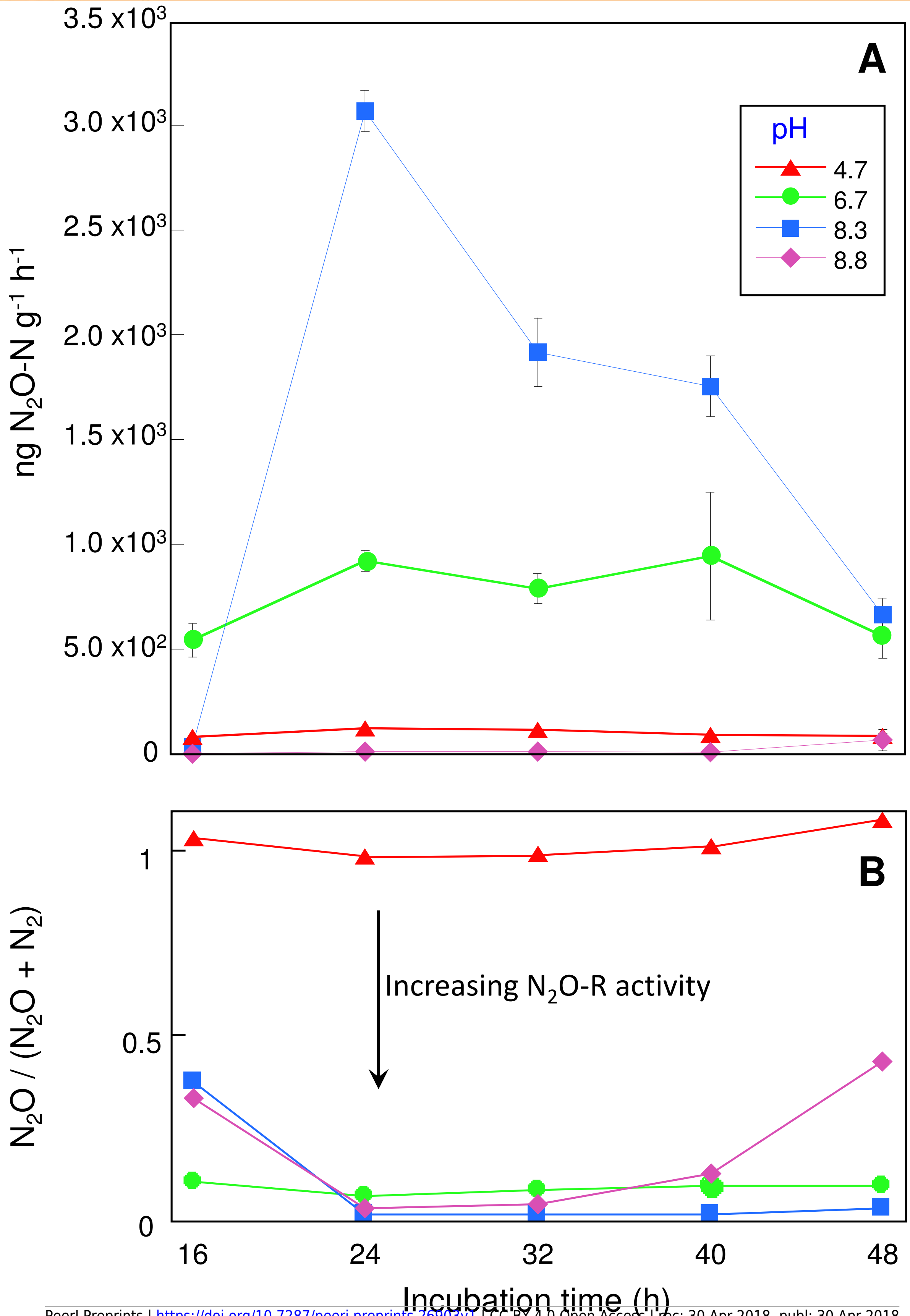

Figure 3(on next page)

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. Samples enclosed by black rings share >70% similarity. The estimated components of variation and interaction between sources of variation are presented in the table below.

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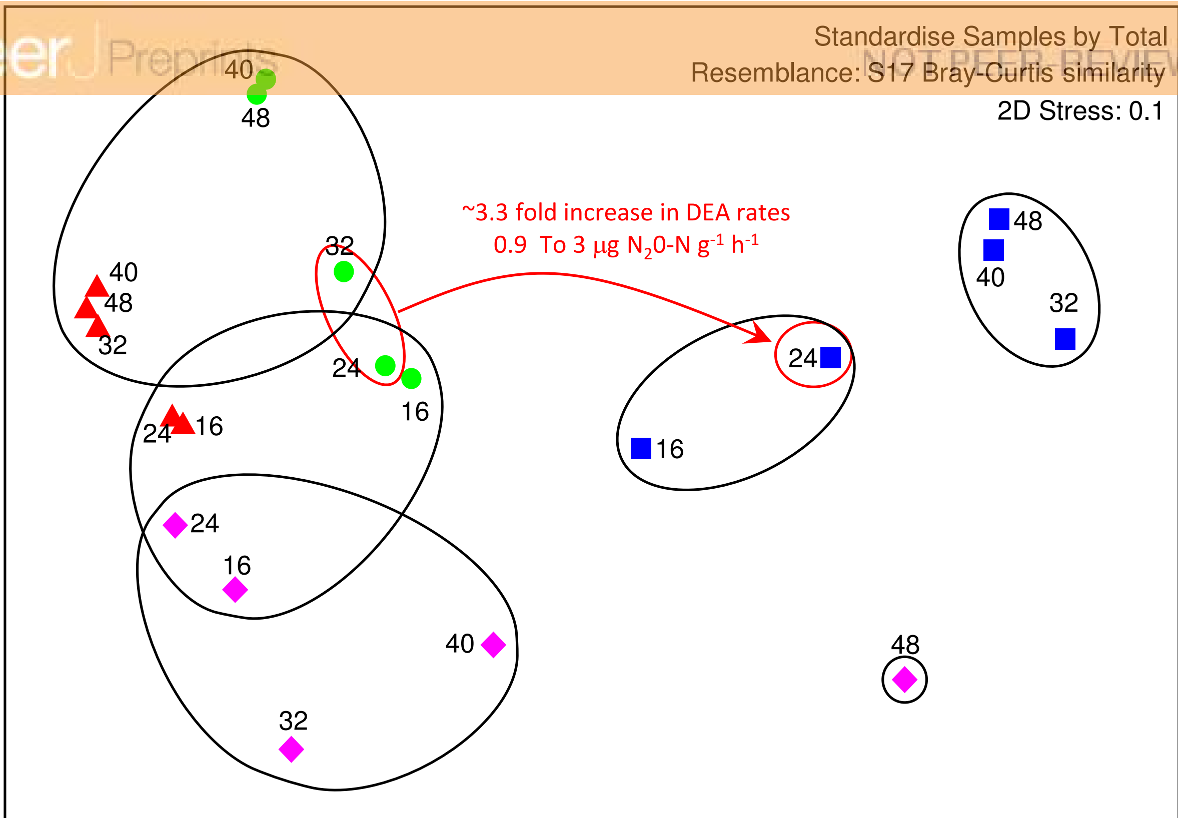

Source	Estimate	SD	% est. variation	*P*-values
S(pH)	1015.6	31.9	57.3	0.001
S(Time)	266.9	16.3	15.1	0.001
S(pHxTime)	321.9	17.9	18.2	0.001
V(Residual)	167.8	12.9	9.4	

pH
4.7
6.7
8.3
8.8

Similarity
——— >70 %

Figure 4

(on next page)

Phylum level bacterial community structure

A representation of the relative abundance of bacteria at phylum level with incubation time for each pH treatment.

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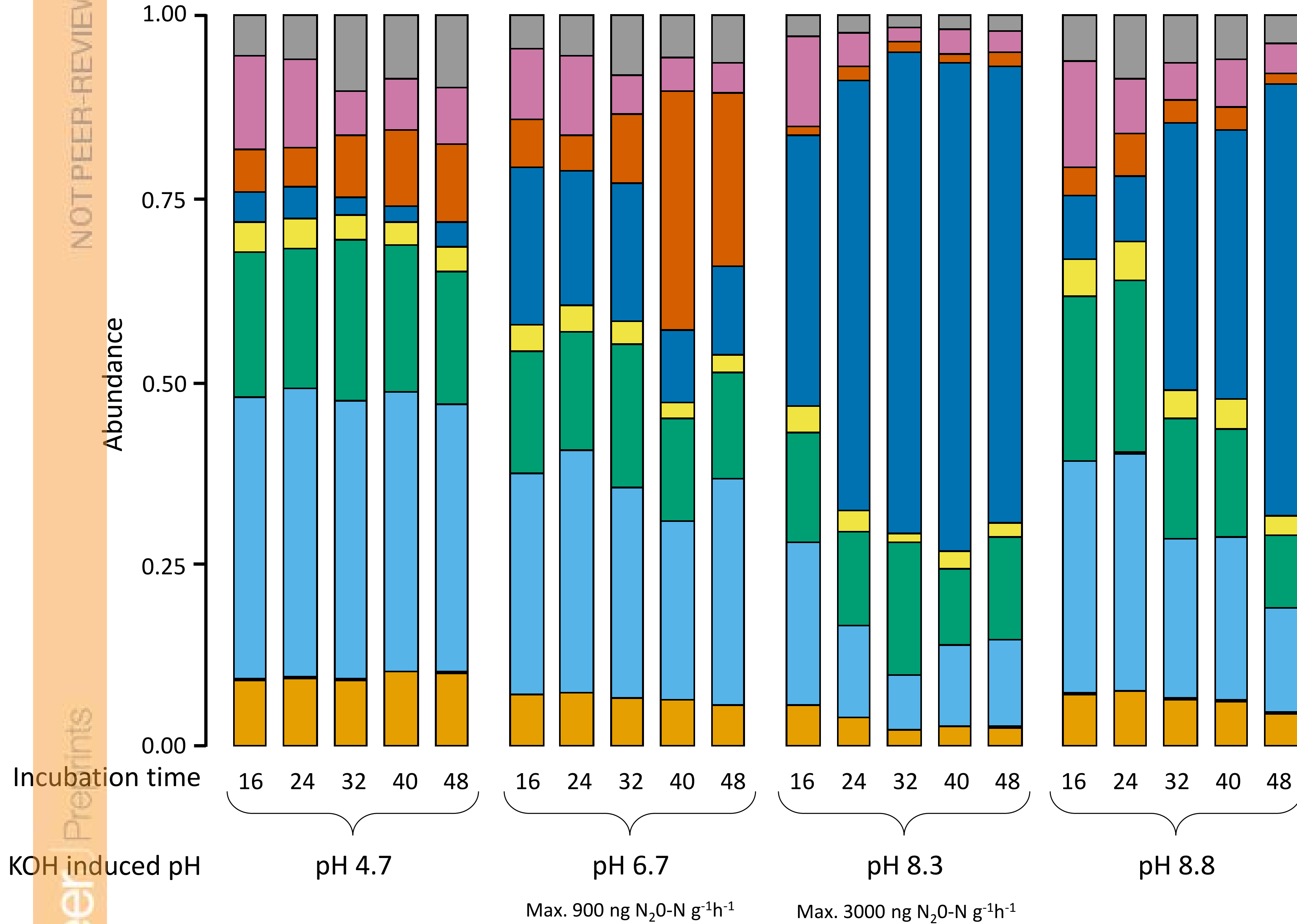

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Figure 5 (on next page)

Bacterial matrix plots

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

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Bacterial Communites
Control (pH 4.7)

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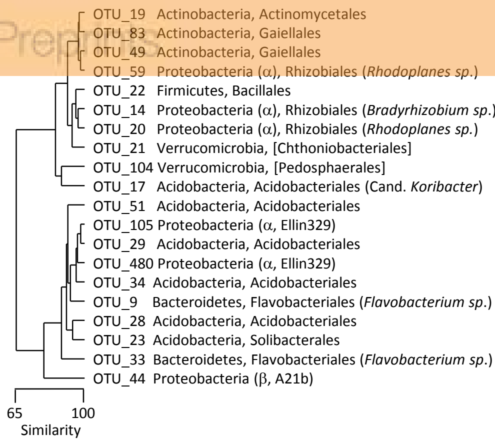
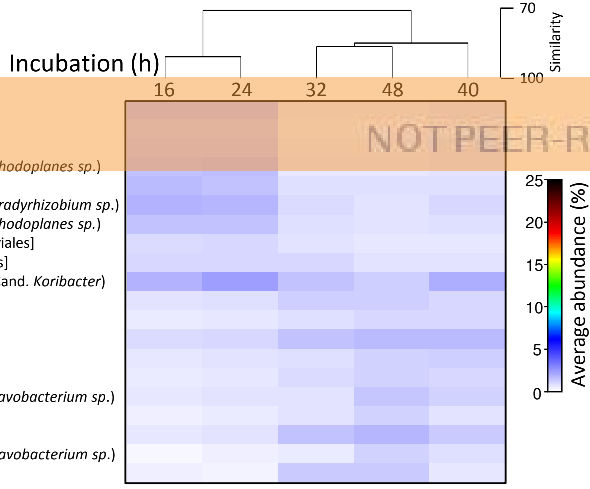
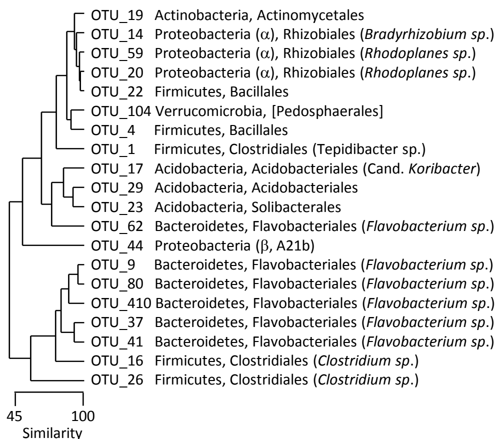
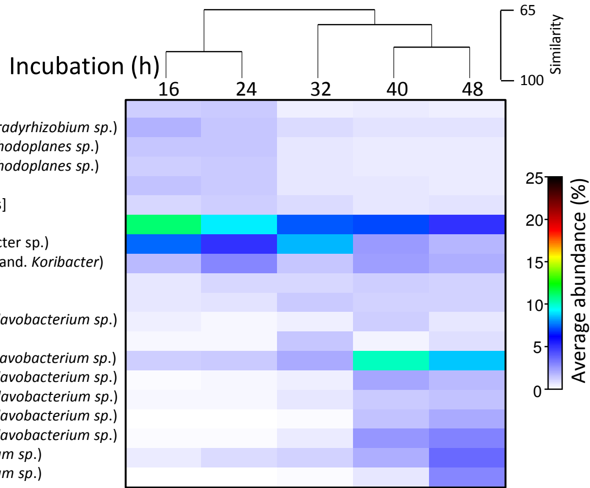


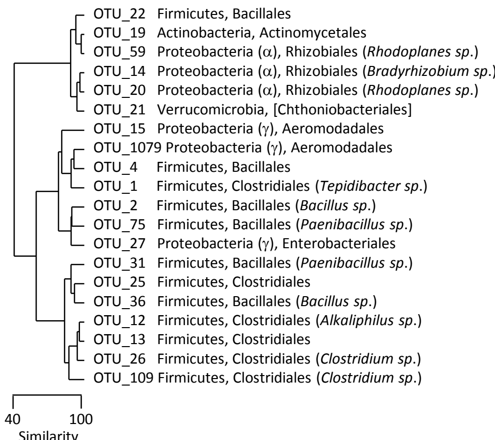


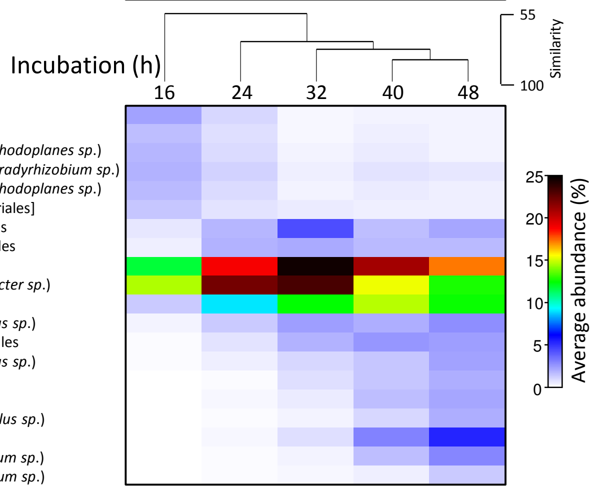
pH 6.7



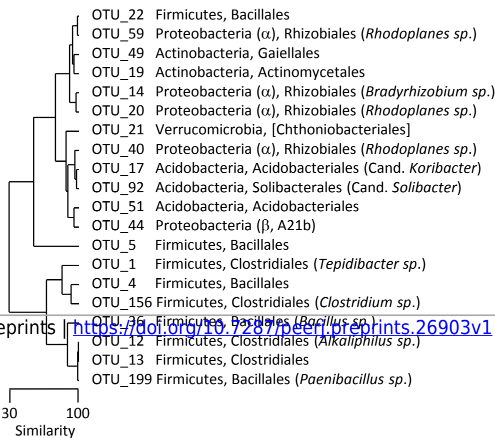


pH 8.3





pH 8.8



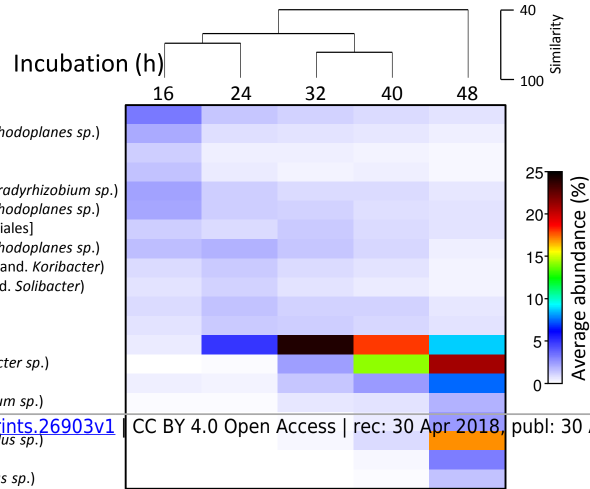


Figure 6(on next page)

nMDS ordination of fungal OTUs identified using Illumina sequencing of the fungal ITS region.

OTU tables were compared using a Bray-Curtis similarity matrix with data first 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. Samples enclosed by the black ring share >70% similarity. The estimated components of variation and interaction between sources of variation are presented in the table below.

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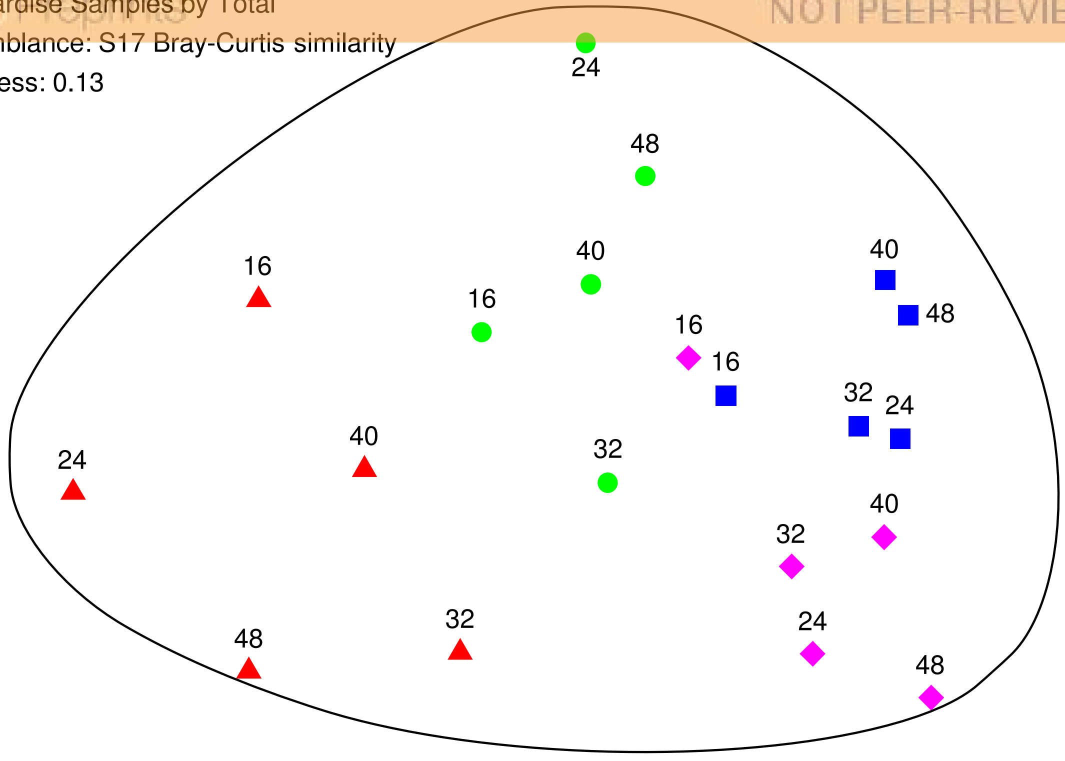

Source	Estimate	SD	% est. variation	*P*-values
S(pH)	97.9	9.9	27.8	0.001
S(Time)	23.2	4.8	6.6	0.001
S(pHxTime)	31.6	5.6	8.9	0.001
V(Residual)	199.9	14.1	56.7	

pH
4.7
6.7
8.3
8.8

Similarity
>70 %

Figure 7(on next page)

Fungal phylum level structure

A representation of the change in Phylum level fungal community structure and relative abundance with incubation time for each pH treatment.

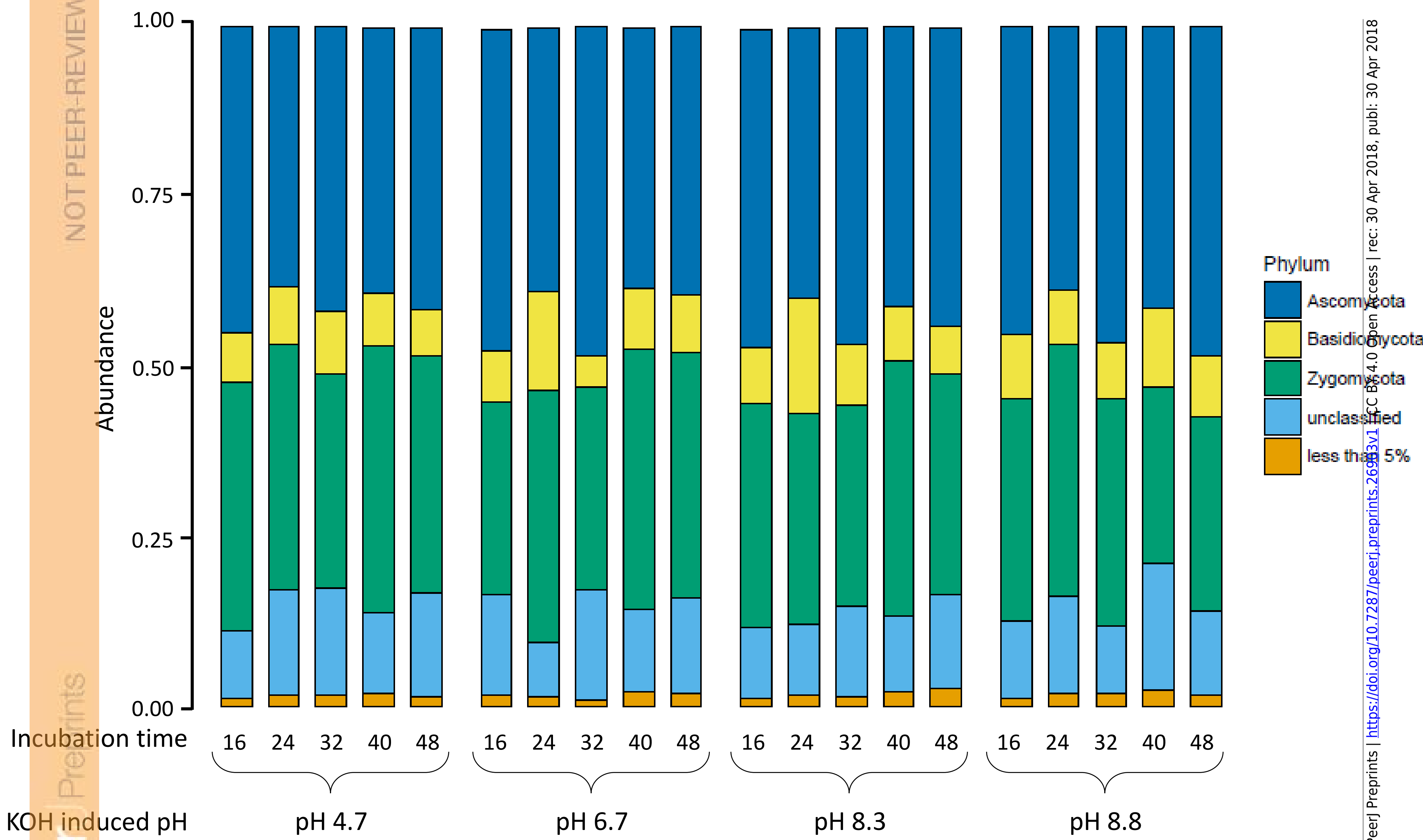
1.00
0.75
0.50
0.25
0.00
Abundance
Incubation time
16
24
32
40
48
KOH induced pH
pH 4.7
pH 6.7
pH 8.3
pH 8.8
Phylum
Ascomycota
Basidiomycota
Zygomycota
unclassified
less than 5%

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Figure 8 (on next page)

Matrix plot for fungi

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

Fungal communities

OTU_35 Fungal sp.
OTU_26 Basidiomycota, Cantharellales (*Ceratobasidium sp.*)
OTU_20 Ascomycota sp.
OTU_15 Zygomycota sp.
OTU_8 Zygomycota sp.
OTU_7 Zygomycota sp.
OTU_3 Zygomycota sp.
OTU_462 Zygomycota sp.
OTU_6 Ascomycota, Glomerellales, (*Gibellulopsis sp.*)
OTU_10 Fungal sp.
OTU_13 Zygomycota, Mortierellales
OTU_4 Ascomycota, Sordariales (*Trichocladium sp.*)
OTU_9 Ascomycota, Hypocreales, (*Fusarium sp.*)
OTU_1 Zygomycota, Mortierellales (*Mortierella sp.*)
OTU_5 Fungal sp.
OTU_16 Ascomycota, Eurotiles (*Paecilomyces sp.*)
OTU_17 Ascomycota, Incertae sedis (*Leohumicola sp.*)
OTU_31 Fungal sp.
OTU_32 Ascomycota, Incertae sedis (*Leohumicola sp.*)
OTU_19 Ascomycota sp.

55 100
Similarity

Incubation time (h)

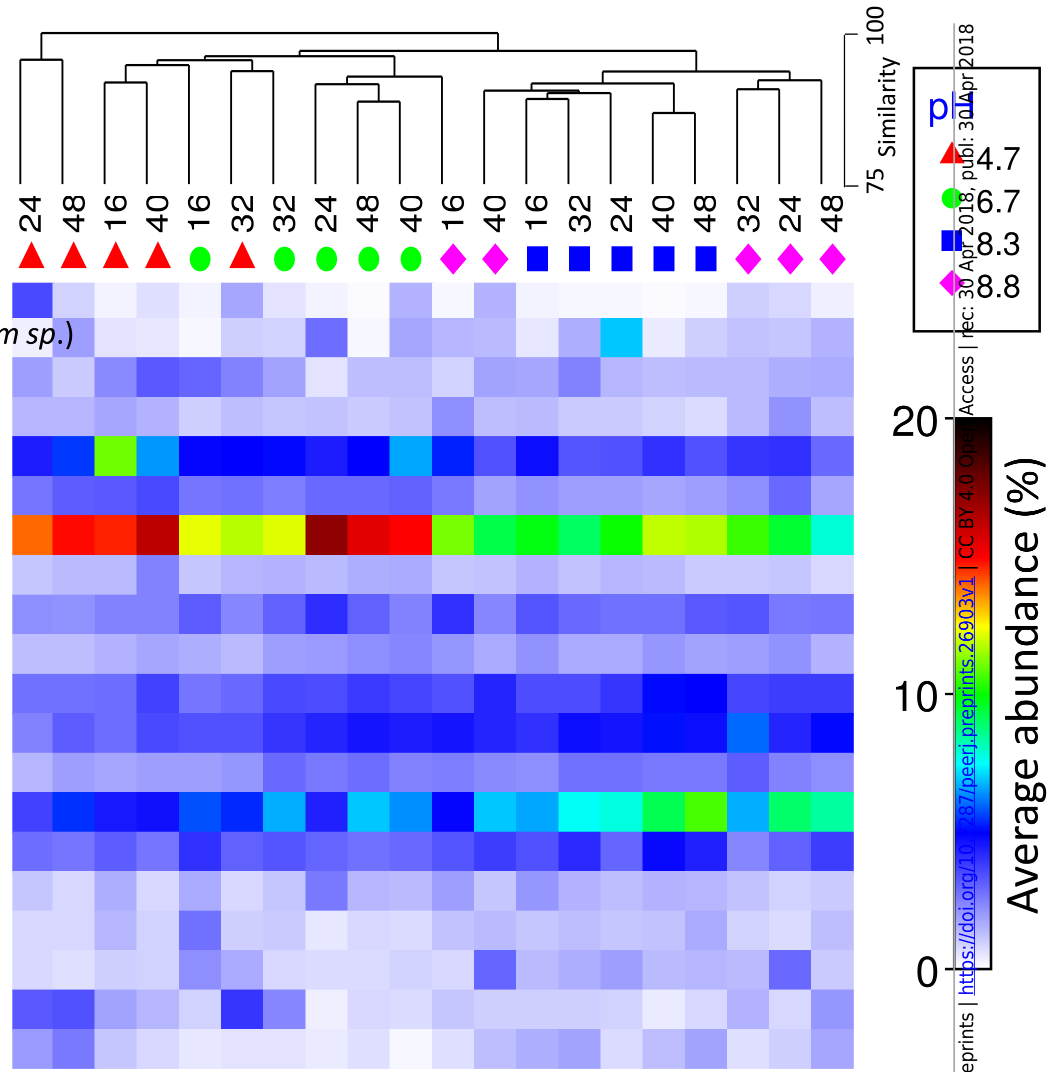

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Table 1 (on next page)

N-chemistry and cumulative respired CO_2 for each pH treatment.

These results represent the chemical conditions in the microcosms prior to the DEA assays. Sampling occurred at the end of each incubation period.The $cmol_c$ kg^{-1} additions of KOH are represented in square brackets beside the pH value induced. Average (Ave.) and standard deviations (SD) for each measure are presented.

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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^+ (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
NO_3^- (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
N_2O (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
CO_2 (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

2 *Native soil concentration prior to experiments

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