Soil Chemistry and Microbiome Determine N₂O Emission Potential in Soils

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Abstract

Microbial nitrogen (N) transformations in soil, notably denitrification, result in the production of the potent greenhouse and ozone depleting gas nitrous oxide (N_2O). Soil chemistry and microbiome composition impact N_2O emission potential but the relative importance of these factors as determinants of N_2O emission in denitrifying systems is rarely tested. In addition, previous linkages between microbiome composition and N_2O emission potential rarely demonstrate causality. Here, we determined the relative impact of microbiome composition (i.e. soil extracted cells) and chemistry (i.e. water extractable chemicals) on N_2O emission potential utilizing an anoxic cell based assay system. Cells and chemistry for assays were sourced from soils with contrasting $\text{N}_2\text{O}/\text{N}_2\text{O}+\text{N}_2$ ratios, combined in various combinations and denitrification gas production was measured in response to nitrate addition. Average directionless effects of cell and chemical extract on $\text{N}_2\text{O}/\text{N}_2\text{O}+\text{N}_2$ (Cell: $\Delta 0.16$, Chemical extract: $\Delta 0.22$) and total N_2O hypothetically emitted (Cell: $\Delta 2.62 \mu\text{mol-N}$, Chemical extract: $\Delta 4.14 \mu\text{mol-N}$) indicated chemistry is the most important determinant of N_2O emissions. Independent pH differences of just 0.6 points impacted $\text{N}_2\text{O}/\text{N}_2\text{O}+\text{N}_2$ on par with independent chemical extract differences, supporting the dominance of this variable in previous studies. However, impacts on overall N_2O hypothetically emitted were smaller suggesting that soil pH manipulation may not necessarily be a successful approach to mitigate emissions over a fixed time period. In addition, we observed increased N_2O accumulation and emission potential at the end of incubations concomitant with predicted decreases in carbon availability suggesting that carbon limitation increases N_2O emission transiently with the magnitude of emission dependent on the both chemical and microbiome controls.

36 Introduction

37 Nitrous oxide (N₂O) is a potent greenhouse gas and ozone depleter accounting for around
 38 6.2 percent of worldwide greenhouse gas emissions on a CO₂ mass equivalence basis
 39 (Intergovernmental Panel on Climate Change, 2013). Around 45% percent of this is
 40 anthropogenically produced, mostly (60%) in agricultural settings via soil based N
 41 transformations (Syakila and Kroeze, 2011). Denitrification, the anaerobic microbial
 42 reduction of N compounds (NO₃⁻ → NO₂⁻ → NO → N₂O → N₂), is considered a major pathway
 43 of anthropogenic N₂O production (Bouwman *et al.*, 2013). Soil conditions (e.g. O₂
 44 concentration (Zumft, 1997; Smith and Tiedje, 1979; Firestone *et al.*, 1979) and pH (Simek
 45 and Cooper, 2002; Liu *et al.*, 2014; Čuhel and Šimek, 2011)), can affect the ratio of the
 46 major gaseous end products of this process (N₂O & N₂) and overall process rates resulting
 47 in higher or lower N₂O emissions to the atmosphere. Therefore, understanding the soil
 48 factors that favour low N₂O emission in the presence of available soil N is of great
 49 importance to manipulating agricultural systems towards reduced N₂O production in the
 50 future.

51 Conceptually, factors affecting soil N₂O emission potential can be separated into three
 52 areas: distal controls which act in the long term to determine denitrifier microbiome
 53 composition, the genetic and regulatory potential of the microbiome itself, and the
 54 immediate scale impact of proximal controls which may be transduced through the
 55 denitrifiers present (Wallenstein *et al.*, 2006). Proximal factors such as O₂, pH and
 56 temperature are easily isolated as independent variables, making them ideal experimental
 57 targets. In contrast, microbiome impacts are difficult to isolate due to confounding by soil
 58 chemical and physical factors, likely distal controls. As such, they are more poorly
 59 understood. Studies are often suggestive (Graf *et al.*, 2014) or correlative (Samad *et al.*,
 60 2016; Jones *et al.*, 2014; Philippot *et al.*, 2009; Čuhel *et al.*, 2010; Morales *et al.*, 2010) and
 61 it is often unclear whether microbiome features described are the true driver of an N₂O
 62 emission outcome. The issue is exacerbated when co-variance is observed with variables
 63 such as pH, which are known to affect both N₂O/N₂ emission ratios and changes in
 64 microbiome composition (Samad *et al.*, 2016; Philippot *et al.*, 2009).

65 Attempts have been made to control “all” variables relevant to denitrification within soils to
 66 isolate microbiome based effects, however, this may not account for the effect of physical
 67 differences between the soils and certainly doesn’t for unknown and unaccounted variables
 68 impacting denitrification gas kinetics at the time of experimentation (Cavigelli and

69 Robertson, 2000; Holtan-Hartwig *et al.*, 2000). A solution to such problems may be the
 70 extraction of whole microbiomes from soils. Though probably biased in the portion of soil
 71 communities extracted e.g. (Nadeem *et al.*, 2013; Holmsgaard *et al.*, 2011), this method has
 72 demonstrated that communities from different soils or the same soil under different long
 73 term pH treatments will show contrasting N₂O emission responses to the same pH
 74 conditions (Dörsch *et al.*, 2012; Liu *et al.*, 2014).

75 Despite an increasing focus on microbiome impacts, the relative impact of proximal effects
 76 vs. microbiome composition on N₂O emission from denitrification is still poorly
 77 understood. In practice, should management of soil to control N₂O emissions be targeted
 78 towards proximal effects, or is the long term selection of certain denitrifier community
 79 biomes (distal control) more important?

80 Here, we incubated soil extracted cells in chemical extracts from pairs of soils with
 81 contrasting N₂O/N₂O+N₂ emission ratios in all potential combinations with the aim of
 82 identifying whether microbiome composition (extracted cell origin) or proximal control
 83 (extracted chemical environment) in general was the most important determinant of the
 84 contrasting N₂O/N₂O+N₂ ratios and total N₂O emission in our model system and soils in
 85 general. We hypothesized chemical differences (especially pH) would be the dominant
 86 effector while microbiome composition effects would weaker but still detectable. Soil cell
 87 extraction allowed treatment of microbial communities as independent transferable units
 88 while extraction of soil chemistry ensured that whatever water-extractable components of
 89 the soil were present (e.g. dissolved carbon) reflected the parent soil in the produced
 90 incubation media. This is in contrast to traditional lab-based analyses which typically use a
 91 single simple carbon source.

92

93 **Methods**

94 **2.1 Soil sampling**

95 Soils were re-sampled from New Zealand South Island pasture farms (Karangarua,
96 Makarora, Tapawera, Fairlie-Geraldine, Woodend, Rae's Junction) previously sampled in
97 Highton et al. (2020). Sampling took place from 21st to 23rd of March, 2018. Soils were
98 selected based on contrasting pH and N₂O hypothetically emitted (%) identified in Highton
99 et al. (2020). Multiple soil cores (10cm length, 2.5cm diameter) were sampled along a 7.5m
100 transect evenly at distances of 0, 2.5, 5 and 7.5m using a foot-operated auger until ~3kg of
101 soil was collected. Repeated cores at each distance were carried out in 4 perpendicular rows
102 up to 6 cores across. Pooled site cores were stored field moist on ice in partially open
103 ziplock bags during transport and at 4°C in the lab. Grass, insects, worms and large roots
104 were removed and cores were sieved at 2mm. Sieved soils were stirred rigorously with a
105 metal spoon to homogenize. Soils underwent a 36hr period without temperature control
106 during transport to the Norwegian University of Life Sciences (NMBU, Ås, Akershus,
107 Norway).

108 **2.2 Soil pH**

109 Soil pH was measured using both CaCl₂ (10mM) and ddH₂O extractants as in Highton et al.
110 (2020). Values were measured using an Orion 2 star pH meter (ThermoFisher Scientific,
111 Waltham, Massachusetts, USA) with an Orion Ross Sure Flow Electrode (ThermoFisher
112 Scientific), allowing up to 5 minutes for readings to stabilize.

113 **2.3 Anoxic soil incubations**

114 Anoxic soil incubations were carried out to determine soil denitrification gas kinetics and
115 N₂O emission potential. Incubations were prepared as in Highton et al. (2020) excluding
116 overnight storage and oxic preincubation. Briefly, 3mM NH₄NO₃ was amended to soils by a
117 flooding and draining procedure. Twenty grams dry weight equivalent of soil were weighed
118 into triplicate 120ml serum vials per soil. Vials were crimp sealed with butyl rubber septa
119 and made anoxic by repeated evacuation and helium flushing,

120 Soil vials were incubated at 20°C in a temperature controlled water bath. Headspace gases
121 (1ml) were sampled every 4hrs via an automated robotic gas sampling system (Molstad *et*
122 *al.*, 2007, 2016). Gases (O₂, CO₂, NO, N₂O and N₂) were quantified in real time using a
123 coupled Agilent 7890A gas chromatograph (GC) equipped with an ECD, TCD, FID, and

124 chemiluminescence NO_x analyser (Model 200A, Advanced Pollution Instrumentation, San
125 Diego, USA). An equal volume of helium is returned to the vials by back pumping ensuring
126 consistent vial pressure. Dilution of headspace gases is accounted for later through back
127 calculation. Gas concentrations were calibrated using premixed standard gases supplied by
128 AGA industrial gases (Oslo, Akershus, Norway). The overall system and its improvements
129 are described in detail in (Molstad *et al.*, 2007, 2016).

130 **2.4 Cell based assay**

131 A soil extracted cell based assay (CBA) was developed to determine the relative
132 importance of microbiome composition and soil chemistry on N₂O emission potential (see
133 emission potential metrics 2.7). Extraction of soil components allowed them to be treated as
134 independent experimental units. Soil chemistry and cells were extracted separately from
135 soils with similar native pH and contrasting N₂O emission potential: Karangarua, a low
136 N₂O emitting soil (N₂O hypo emit ratio = 0.26, pH = 5.75) and Rae's Junction, a high N₂O
137 emitting soil (N₂O hypo emit ratio = 0.92, pH = 5.6). Extracted cells and chemistry were
138 combined in 4 possible combinations to give the standard treatments: High emitting cells
139 (HEC) + high emitting extract (HEE), high emitting cells (HEC) + low emitting extract
140 (LEE), low emitting cells (LEC) + high emitting extract (HEE), low emitting cells (LEC) +
141 low emitting extract (LEE). Standard treatments were carried out in triplicate vials.
142 Minimum duplicate 3mM glutamate amended controls of each treatment were produced to
143 understand the impact of carbon limitation. Duplicate chemical extract free control
144 incubations containing just extracted cells and milliQ were prepared to test the baseline
145 activity of extracted cells. Occasional replication in duplicate was necessitated by limited
146 vial space in the automated incubator/gas sampler. Cell negative controls were prepared to
147 confirm the sterility of chemical extracts and to quantify the elution of any N₂ and O₂
148 remaining in the extract media after He flushing. Full treatment contents and replication is
149 detailed in Table S1. Hereafter this initial cell based assay is referred to as CBA-int to
150 differentiate it from the CBA using alternate pH soils (section 2.5)

151 **2.4.1 Chemical extract media preparation**

152 Water extractable organic carbon (WEOC) extraction was based on a previous protocol
153 (Guigue *et al.*, 2014). Air-dried soil was combined with milliQ H₂O at a 1:3 ratio (170g:
154 510ml) in 1L Schott bottles. Bottles were shaken lengthways on an orbital shaker at
155 120rpm for 1hr. Coarse particles were allowed to settle out for 5 minutes and supernatant
156 was poured into 250ml polycarbonate Nalgene centrifuge tubes (ThermoFisher). Fine

157 particles were removed by successive centrifugation (pelleting) and filtration steps:
 158 centrifugation at 4600G for 20minutes using JXN-26 high-speed centrifuge with JS-7.5
 159 swing out rotor (Beckman Coulter, Brea, California, USA), filtration using 500ml Sterafil
 160 Filter Holders (Merck, Burlington, MA, USA) loaded with 1.2µm glass-fibre pre filters
 161 (Merck) and 0.45µm cellulose filters (Merck), syringe filtration using sterile 0.22µm mixed
 162 cellulose ester filters (Merck). Filter sterilized Na-glutamate solution was added to a
 163 portion of the chemical extract solution from each soil to give a final concentration of 3mM
 164 once diluted in final treatment vials. An equivalent volume of milliQ H₂O was added to the
 165 rest of the extract to account for dilution. Standard extracts, glutamate amended extracts
 166 and milliQ for carbon free controls were buffered to pH 6 using 20mM Na-phosphate
 167 buffer, as this was the closest value to parent soil pH H₂O (Rae's Junction= 5.60,
 168 Karangarua = 5.75) within the bufferable range. Extracts and milliQ were re-filtered at
 169 0.22µm to ensure sterility after pH and carbon manipulation. 22.5ml of solution was added
 170 to autoclaved 120ml glass serum vials containing magnetic stir bars. Vials were crimp
 171 sealed with butyl rubber septa + aluminium cap. Anoxia was induced through 8 repeated
 172 cycles of vacuum evacuation and helium filling with continuous magnetic stirring at
 173 360rpm. Vials were stored at 8°C until inoculation and incubation.

174 **2.4.2 Cell extraction by low speed centrifugation**

175 The cell extraction procedure was modified from (Lindahl and Bakken, 1995) with cell
 176 separation on the basis of sedimentation rate using low speed centrifugation. Cell
 177 extractions were performed on the same day they would be used, using optimized
 178 conditions determined in an earlier test extraction yielding approximate cell extraction
 179 efficiencies for each soil. Twenty g of field moist soil was blended with 200ml of milliQ
 180 H₂O in a two speed Waring blender (Waring, Stamford, Connecticut, USA) on high for
 181 3x1min with 5min intermittent cooling on ice between each blending run. Coarse particles
 182 were allowed to settle for 5min before supernatant was poured off into sterile falcon tubes
 183 up to the 35ml mark (equivalent to 8cm centrifugation distance). Tubes were centrifuged at
 184 1000G for 10minutes with 4°C cooling on a benchtop Mega star 1.6R centrifuge with a TX-
 185 150 swing out rotor (VWR, Radnor, Pennsylvania, US) to sediment out non-cellular debris.
 186 Cell containing supernatant was recovered into additional falcon tubes and centrifuged at
 187 10,000G for 20 minutes with 4°C cooling to pellet cells using an Avanti JXN-30 highspeed
 188 centrifuge with JA 14.50 fixed angle rotor (Beckman Coulter). Supernatant was removed
 189 without disturbing the cell pellet. Cells were washed/resuspended with 40ml milliQ H₂O,
 190 re-pelleted and supernatant was removed. Cells were re-suspended and pooled to a final

191 stock concentration of 6.25×10^8 cells ml^{-1} based on predictions from previously performed
192 cell extraction and cell counts from the same soils.

193 **2.4.3 Cell counts**

194 2ml cell extract solution was collected for cell quantification at the time of initial blending
195 and after washed cell re-suspension in milliQ H_2O . Samples were amended gluteraldehyde
196 to give a 1.5% fixation solution and stored at 4°C for at least 2hrs to allow fixation. Cell
197 counts were carried out using SYBR Green staining and epifluorescence microscopy
198 (Noble and Fuhrman, 1998). Cell solutions were diluted 200 fold, and 6ml was vacuum
199 filtered through $0.2\mu\text{m}$ Anodisc 25 diameter filters (Whatman, Maidstone, UK). SYBR
200 Green I (Molecular Probes, Eugene, Oregon, Texas) was diluted 2.5×10^{-3} to a working
201 solution. Filters were placed on a $100\mu\text{L}$ drop of solution and allowed to stain for 20 min in
202 the dark. Filters were oven dried at 60°C . Duplicate filters per sample were prepared. Filters
203 were mounted onto glass slides with an antifade mounting solution consisting of 50%
204 glycerol, 50% phosphate buffered saline (0.05M Na_2HPO_4 , 0.85% NaCl, pH 7.5) and 0.1 %
205 p-phenylenediamine. Cells were counted by epifluorescence microscopy.

206 **2.4.4 Inoculation and incubation**

207 All vials used during incubations were placed in a 20°C waterbath to equilibrate. Headspace
208 overpressure was removed by water filled syringe. All vials were amended with 0.5ml He-
209 flushed NH_4NO_3 solution to give a 3mM final concentration. 2ml helium washed
210 concentrated cells from the appropriate soil were added to give a total of $\sim 5 \times 10^7$ cells ml^{-1}
211 in each standard, glutamate amended and carbon negative treatment. 2ml of dummy He
212 flushed milliQ H_2O was added to make up the volume in cell free chemical extract controls.
213 Vials were magnetically stirred at 360rpm. Headspace gases were sampled and measured
214 every 4hrs using the robotic autosampler gas chromatographs described above under anoxic
215 soil incubations (2.3)

216 **2.5 Cell based assay with alternate pH soils**

217 The cell based assay experiment was repeated using soils with contrasting pH and N_2O
218 hypothetically emitted ratio to test the impact of cells and chemical extract within the
219 context of added pH complexity (Here-after referred to as CBA-pH). Rae's Junction was
220 used as a high N_2O hypo emitting low pH (native pH = 5.60, ratio = 0.92) soil, as in CBA-
221 int, while Tapawera was used as the higher pH lower high N_2O hypothetically emitted (%)
222 soil (native pH 6.58, ratio = 0.68). Again, Rae's Junction chemical extracts were buffered

223 to pH 6. Tapawera chemical extracts were buffered closer to the native soil pH at 6.6.
 224 Triplicate standard treatments and their pHs were: HEC + HEE (6), HEC + LEE (6.6), LEC
 225 + HEE (6), LEC + LEE (6.6). Minimum duplicate alternative pH controls were produced
 226 for each treatment in which the pH of the treatment chemical extract media was switched to
 227 the opposite pH. This allowed determination of the independent effects of pH and chemical
 228 extract. Duplicate carbon negative controls and cell negative controls were carried out as in
 229 CBA-int but glutamate amended treatments were not included. Full treatment contents and
 230 replication is detailed in Table S1.

231 **2.6 Nitrate and nitrite quantification**

232 Nitrate + nitrite ($\text{NO}_3^- + \text{NO}_2^-$) measurements were performed on soil chemical extracts
 233 before incubation media preparation using a previously described chemiluminescent
 234 detection method ((Braman and Hendrix, 1989; Lim *et al.*, 2018). This allowed accurate
 235 adjustment to a 3mM NO_3^- concentration in the cell based assay media. 10 μL of chemical
 236 extract was injected into a sealed glass piping system containing heated (95°C) vanadium
 237 chloride solution (50mM VCl_3 , 1M HCl). VCl_3 reacts rapidly with NO_3^- and NO_2^- at high
 238 temperature to produce NO gas. Produced NO is transported via an N_2 carrier stream to a
 239 Sievers Nitric Oxide Analyzer 280i system (GE Analytical Instruments, Boulder, CO,
 240 USA). Cell based assay sample NO_2^- concentrations during incubations were quantified
 241 using the same chemiluminescence detection system, however, a separate reaction crucible
 242 containing NaI (1% w/v NaI in 50% acetic acid, room temperature) was used to specifically
 243 target NO_2^- . Signal peak areas were calibrated using 10 μL injections of a 10-fold KNO_3 or
 244 KNO_2 dilution series (1mM to 0.001mM). A single rep from each CBA treatment was
 245 sampled every ~24hrs (0.15ml) for immediate quantification of accumulated NO_2^- .

246 **2.7 N_2O emission potential**

247 Soil and CBA treatment N_2O emission potential was evaluated based on two time-
 248 integrated measures: N_2O hypothetically emitted (from here on referred to as N_2O emitted)
 249 and N_2O hypothetically emitted ratio (from here on referred to as N_2O ratio). Both
 250 measures were developed to account for periods of net N_2O reconsumption from vial
 251 headspace which would not occur in an open system and is therefore not indicative of N_2O
 252 emission potential. N_2O hypothetically emitted is calculated as the sum of net positive N_2O
 253 accumulations between each sampling point over the course of the incubation + N_2O lost
 254 due to sampling dilution. N_2O hypothetically emitted ratios are calculated as N_2O

hypothetically emitted/(N₂O hypothetically emitted + N₂O emission prevented) where the N₂O emissions prevented term is the total N₂ finally accumulated in the vial + losses to sampling and leaks - N₂ derived from reduction of headspace accumulated N₂O. This formula can also be applied to soil incubations which include only a single N₂O accumulation peak and the resulting value is almost equivalent to the N₂O hypothetically emitted (%) term previously utilized in Highton et al. (2020), differing only in use of cumulative N₂O (zeroed, sampling dilution and leakage accounted for) in calculations rather than the previously used actual in vial quantities.

Differences in these measures of N₂O emission potential between treatments were evaluated based on non-overlapping 95% confidence intervals. Independent variable (cell origin, chemical extract origin, pH) effects on N₂O emitted or ratio were calculated by comparison of relevant treatments and with a specific predicted direction of effect in mind. LE cells, chemical extracts and higher pH (6.6) were expected to decrease N₂O emitted and ratios while HE cells, chemical extracts and lower pH (6.0) were expected to increase N₂O emitted and ratios. Expected directions of effect were denoted with a positive value and unexpected with a negative value. When averaged, effects were maintained as positive or negative values unless stated that the effect size given was directionless.

2.8 Microbiome composition

DNA was extracted from cell stock and parent soil for each soil to determine extraction bias and community differences between separate cell extracts. For soils, parent soil was collected at the start of the cell extraction protocol and stored at -80°C until DNA extraction of duplicate 0.25g replicates using the DNeasy powerlyzer powersoil extraction kit (Qiagen, Hilden, Germany). Duplicate 5ml cell stock aliquots were harvested just prior to inoculation of cell based assay treatments and frozen at -80°C until cell pelleting and DNA extraction.

16S amplicon sequencing of samples was carried out on illumina hiseq using Version 4_13 of the Earth Microbiome Project standard protocol (Caporaso *et al.*, 2012). Sequences are available in the NCBI Sequence Read Archive under the BioProject ID PRJNA678002. Sequence quality control and ASV (Amplicon sequence variant) picking was carried out in R version 3.6.1 (R Core Team, 2016) using the dada2 pipeline version 1.12.1 (Callahan *et al.*, 2016). Taxonomy was assigned using the SILVA database (version 132) (Quast *et al.*, 2013) and the RDP (Ribosomal Database Project) bayesian classifier (Wang *et al.*, 2007).

287 Sample sequence reads were rarefied 10 times to a depth of 11500 sequences using
288 phyloseq package functions (McMurdie and Holmes, 2013). Independent rarefactions were
289 combined and normalised to the number of rarefactions. Fractional ASV counts were
290 rounded to integers.

291 **2.8.1 Beta diversity and ASV sharing**

292

293 All beta diversity and ASV sharing plots were generated using ggplot2 version 3.2.1
294 (Ginestet, 2011) and adjusted with the ggpubr (Kassambara, 2020) and forcats (Wickham,
295 2020) packages unless otherwise stated. The phyloseq package (McMurdie and Holmes,
296 2013) was used to calculate and display community composition dissimilarity, the mean
297 number of shared and unique ASVs, and the relative abundance of organisms at the phylum
298 rank with the additional usage of the dplyr (Wickham *et al.*, 2019) and Rmisc (Hope, 2013)
299 packages. Community composition dissimilarity patterns were confirmed using vegan
300 package (Dixon, 2003) ANOSIM and ADONIS tests.

301

302 The fold change of ASV abundance differences between extracted cells and soil samples,
303 and its accompanying p-value was generated with the use of the edgeR (Robinson *et al.*,
304 2009) package to identify significantly changing ASVs with an exact test. P-values were
305 adjusted based on Benjamini-Hochberg p value correction and ASVs were only displayed if
306 their false discover rate (FDR) was below 0.1. ASV Genus taxonomy was only labelled if
307 abundance differed more than 5-fold with a p-value $< 1 \times 10^{-4}$.

308

309

310 **Results**

311 **3.1 Soil and cell based incubations have distinct gas accumulation patterns** 312 **but relative emission potential is conserved**

313 Denitrification gas (NO , N_2O , N_2) kinetics were compared between soil and cell based
314 incubations to determine whether the cell based system accurately modeled the trends
315 observed using soils. Soil incubations (Figure 1A, Figure S1) displayed a single N_2O
316 accumulation and depletion curve. N_2O ratios were determined by the sequentiality of N_2O
317 production and reduction steps as previously described in Highton et al. (2020). In the most
318 extreme cases, close to all added N was accumulated as N_2O before high rate N_2
319 production/ N_2O reduction was initiated, predicting high emissions from an in situ
320 (unsealed) environment.

321 Gas accumulation patterns in cell-based incubations were inconsistent with soil incubations.
322 Most treatments experienced an initial lag phase in denitrification product accumulation
323 and CO_2 accumulation (Figure S2). Only +glutamate treatments completed processing of
324 added N (Figure S3B) during the experimental timeframe. Early N_2O accumulation was
325 very low while major differences in N_2O accumulation, and thus N_2O ratio, occurred later
326 in the incubation when total N turnover rates and N_2O reduction (N_2 production) rates
327 suddenly dropped (Figure S3, Figure S4). Late drops in N_2O reduction rate were usually
328 greater than drops in N_2O production rates, resulting in increased N_2O accumulation.

329 Despite distinct gas accumulation patterns, soil and cell based assays sustained relative
330 rankings based on N_2O ratios (Figure 2, Rae's Junction>Tapawera>Karangarua). Gas
331 production profiles were not completely consistent between separate cell based assay runs
332 as evidenced by the repeated Rae's Junction based incubations (Figure 1, Rae's Junction vs.
333 2-Rae's Junction), however, this variation did not greatly impact N_2O ratios and relative
334 ranking of incubations (Figure 2).

3.2 Both chemistry and microbiome determine N₂O emission potential

We compared N₂O ratios and N₂O accumulation in a CBA (CBA-int) seeded with cells and chemical extracts from soils with similar native pH (5.6, 5.75) to determine whether microbiome (cells) or chemical factors (extracts) were the most important determinant of N₂O emission potential in the absence of pH effects. Both cell and chemical extract origin affected N₂O ratio and N₂O emitted resulting in a gradient: HEC+HEE>LEC+HEE≈HEC+LEE>LEC+LEE (Figure 3A, B). Cell and chemical extract origin had similar impacts on N₂O ratio but chemical extract origin was the most important determinant of overall emissions, with on average 60% greater impact (Table 1, CBA-int).

To account for the role of pH, soils with differing N₂O ratio and pH were also compared (CBA-pH). pH of the treatment was coupled to the soil chemical extract (HE extracts: 6.0, LE extracts: 6.6). Again, both cell and chemical extract origin (including coupled pH) affected N₂O ratio and N₂O emitted resulting in a gradient: LEC+HEE>HEC+HEE>HEC+LEE>LEC+LEE (Figure 3C, D) but chemical extract was the most important determinant of both N₂O ratio and emissions (Table 1, CBA-pH). Patterns were largely determined by the unexpected emission patterns of LE cells which had very high emission potential in the presence of HE extracts yet low emission potential in the presence of LE extracts. **Negative** emission potential difference values (Table 1, CBA-pH) indicate the unexpected **increase** in emission potential using LE cells in the presence of HE extract.

3.3 pH has an outsized impact on low emitting cells

pH switched control treatments (HEE 6.0→6.6, LEE 6.6→6.0) revealed high N₂O ratio in the LEC+HEE treatment was largely a response to the low pH of the HE extracts; LE cell N₂O ratios were much more sensitive to independent pH change than HE cells (Table 2). We accounted for these strong impacts on LE cells by examination of the overall assay at pH 6.6, revealing a similar trend to the CBA-int assay: equal impact of cell and chemical extract origin on ratio (average change of 0.13 points), greater impact of chemical extract on total N₂O emissions (average change cell= 0.37μmol-N, chemical extract=4.19, Table S2, overall). However, it should be noted that independent impact of HE extracts still lead to unexpectedly high absolute N₂O emissions from the LE+HE treatment at pH 6.6 due to rate effects of the from the HE extract (Table S2, overall).

366 Comparison of independent pH, and chemical extract origin effects revealed an additional
367 two notable pH related phenomena:

- 368 1) Low pH drove large increases in N₂O ratio (average change 0.11 points), on par
369 with independent chemical extract effects (Figure 4A), yet only minor changes in
370 total N₂O emissions (average 1.30 μ mol-N, Figure 4B) due to the contrasting
371 impact of pH on N turnover rates and N₂O ratios. In one instance pH increase to 6.6
372 actually increased total emissions (Table 2, 6 HEC + HEE).
- 373 2) Low pH and HE extract acted synergistically to increase LE cell emission potential
374 i.e. Switching pH and chemical extract of 6.6 LEC + LEE treatment to 6 and HE
375 extracts lead to a greater increase in N₂O ratio and N₂O emitted than would be
376 predicted by independent changes in pH or extract alone (Table 2). A much weaker
377 positive synergistic effect (reduction in N₂O ratio and total N₂O) of LE extracts and
378 LE pH (higher-6.6) on HE cells was also indicated (Table 2).

379 **3.4 Carbon/starvation effect**

380 We hypothesized that sudden changes in N turnover (especially N₂ production) and
381 emissions during the cell based incubations were linked to shifts in carbon availability. +C
382 (3mM Na-glutamate) controls were included for each swap treatment in CBA-int to
383 determine whether any of the observed differences in treatments were caused by changes in
384 C availability. Divergence of gas accumulation rates in +C controls compared with standard
385 treatments indicated that all treatments became carbon limited during the course of the
386 incubation (Figure 5). Further, carbon amended controls did not experience the late
387 incubation decreases in N₂ production rate, or the associated increased N₂O accumulation,
388 seen in -C treatments suggesting these features may result from C limitation. Predicted
389 actual total N gas and CO₂ production rates typically dropped during the transition to the
390 lower N₂ rate period also supporting increasing C limitation (Figure S2). CO₂ rate drops
391 during this time period were often definitive and of high magnitude but were less obvious
392 for some incubations: HEC + LEE, 6 HEC + HEE, 6.6 LEC + LEE. We carried out a
393 further analysis separating the impact of cell and chemical extracts during the carbon non-
394 limited and limited periods of the incubation (Supplemental document S1, Figure S5)

395 **3.5 Microbiome analysis**

396 To assess if extracted cells were representative of soil microbiomes, and to compare
397 differences in microbiomes across soils we used 16S rRNA amplicon sequencing and

398 processed results into amplicon sequence variants (ASVs). Microbiome differences were
 399 primarily associated to soil origin (ANOSIM: $R^2 = 0.72$, $p < 0.001$, Figure 6A) with
 400 extracted cells clustering alongside their original soils. However, small but significant
 401 changes were detected between extracted cells and soils (ANOSIM: $R^2 = 0.34$,
 402 $p = 0.003$). While both extracted cells and soils shared a large proportion (mean 50 %
 403 with a standard deviation of 12 %) of their total ASVs (Figure 6B), extracted cells
 404 consistently recovered a larger number of ASVs (Wilcox, $W = 16$, $p = 0.029$). This bias in
 405 ASV detection was reflected at the phylum level (Figure 6C) where Firmicutes were more
 406 represented in the soils compared to extract. It also highlighted differences between soils.
 407 To identify specific organisms enriched in either soils or extracted cells ASVs with
 408 differential abundance between sample type were detected using an exact test (Figure 6D).
 409 ASV's in the Bacillaceae family were significantly enriched in all soils relative to extracted
 410 cells but otherwise no consistent extraction bias was observable.

411

412 Discussion

413 4.1 Relevance of model to soils

414 The cell based assay approach allowed causal linkage of microbiome composition and
 415 chemistry to N₂O emission potential. However, as with any model system, applicability to
 416 the initial environment studied must be present. Conserved soil rankings based on N₂O
 417 ratios implied general relevance of the system to soils (Figure 2), however, a number of
 418 kinetic dissimilarities from soils resulted in different absolute N₂O ratios, which must be
 419 considered.

- 420 1) An initial lag phase in which cell based assay incubations accumulated only very
 421 low concentrations of CO₂ and denitrification products NO₂⁻, NO, N₂O, N₂ (Figure
 422 S3, Figure S4). This could hypothetically be caused by an initial lack of sufficient
 423 denitrifier cell density or a stress response to the cell extraction procedure. Lag or at
 424 least very low early denitrification activity and CO₂ production is also observable in
 425 some previous soil-extracted cell based experiments, though the cause is unclear
 426 (Nadeem *et al.*, 2013; Brenzinger *et al.*, 2015; Dörsch *et al.*, 2012).
- 427 2) Low N₂O accumulation during the early incubation period (Figure S3, Figure S4).
 428 This occurred in most CBA treatments, notably excluding those containing
 429 Tapawera cells, and resulted in lowered N₂O ratios relative to parent soils. Low
 430 N₂O accumulation could be a feature of extracted cell based incubations in the
 431 presence of easily utilizable carbon as indicated by very low N₂O accumulation in
 432 the present carbon amended cell based assay treatments (Figure S3B) and a number
 433 of previous extracted cell based studies (Dörsch *et al.*, 2012; Brenzinger *et al.*,
 434 2015).
- 435 3) A secondary period of high N₂O accumulation/reduced N₂ production rates in cell
 436 based incubations. Evidence discussed below (4.4) suggests this was most likely a
 437 result of carbon limitation and utilization of less energetically favourable carbon
 438 sources.

439 In addition to the explanations given above, the kinetic dissimilarities between soils and
 440 cell based incubations are potentially explained by a variety of differences in experimental
 441 conditions. Soil and cell incubations most likely differed in cell density and numbers,
 442 microbiome composition (due to any biases inherent in the extraction procedure
 443 (Holmsgaard *et al.*, 2011; Nadeem *et al.*, 2013)), carbon availability and type (the soluble

444 water extractable component of soil C is usually only around 1% of total soil C and no
 445 attempt was made to match carbon concentration in incubations to soils (Gregorich *et al.*,
 446 2003; Guigue *et al.*, 2014)), time of soil in storage (due differences in when separate
 447 incubation experiments were carried out), and notably, physical differences including,
 448 presence/absence of soil particles, water content and stirring. Water can slow gas diffusion
 449 by 4 orders of magnitude (Heincke and Kaupenjohann, 1999), initially leading to gas
 450 retention (Clough *et al.*, 2005) while soil heterogeneity might limit or enhance local carbon
 451 availability (Parkin, 1987; Kuzyakov and Blagodatskaya, 2015).

452 Relevance to the soils is further dependent on extracted microbiomes accurately
 453 representing soil microbiomes. During any soil cell extraction method, only a portion of
 454 soil cells are extracted (Lindahl and Bakken, 1995) leaving the possibility for biases in
 455 composition of the community extracted. For example, Nycodenz based extractions have
 456 previously been shown to result in reduced microbiome diversity and bias towards or
 457 against certain bacterial phyla compared to parent soils (Holmsgaard *et al.*, 2011). Different
 458 dispersal methods may also recover metabolically distinct communities (strongly attached
 459 vs. loosely attached cells) with different N₂O emission potentials (Nadeem *et al.*, 2013).

460 Our own investigations revealed high similarity between parent soil and extracted cell
 461 microbiomes at a DNA level (Figure 6A). Unfortunately, we are unable to completely
 462 confirm this DNA represented viable cells rather than dead or free floating DNA which
 463 passed through the cell extraction procedure. Further, our investigations consistently
 464 identified a high number of unique ASVs in extracted cells and total observed richness
 465 above that captured from soils. The reason for this is unlikely to be resolved without further
 466 empirical evidence but could be due to the larger soil pool and concentration steps used for
 467 cell extraction vs. direct soil DNA extractions, movement of species out of rare biosphere
 468 in response to the cell extraction protocol disturbance, removal of DNA sorbing soil
 469 particles which otherwise inhibit recovery of DNA during extraction (Paulin *et al.*, 2013),
 470 dilution of soil pcr/sequencing inhibitors, or increased relative abundance of rarer species
 471 due to destruction of abundant organisms during cell extraction. Irrespective of the above
 472 limitations, the extracted microbiomes from separate soils will with certainty represent
 473 distinct microbiomes from one another, while the conserved relative ranking of N₂O hypo
 474 emit ratios between soil and cell based assays indicate representivity at a functional level
 475 (Figure 2).

476 **4.2 Proximal vs. microbiome effects**

Cell origin impacted both N₂O ratio and emissions (Table 1), indicating a strong role for microbiome composition in mediating N₂O emission potential. Previous extracted cell based studies support this claim (Dörsch *et al.*, 2012; Nadeem *et al.*, 2013; Liu *et al.*, 2014) but have typically focused on understanding soil community responses to pH and provide little evaluation of overall impact of community differences compared to other chemical controls. In contrast, another soil based study previously found minimal impact of distal control (implied microbiome composition) on N₂O ratio but significant impact on total emissions (rate/ enzyme activity) (Čuhel and Šimek, 2011). Here, the directionless effect size of cell origin effects on N₂O ratio and emissions across both CBAs were not minor, on average only 22 and 37% lower than chemical effects. Therefore, microbiome composition should be considered an important determinant of N₂O emission potential.

Directional analyses (i.e. LE cells and chemical extracts are expected to decrease N₂O emission potential and HE cells/extracts vice versa) supported the notion that specific microbiomes and chemical backgrounds can be predictably generalized as lower or higher emitting. In the absence of pH effects (CBA-int or CBA-pH at pH 6.6) LE cells and chemical extracts predictably lowered total emissions and ratios while HE cells and chemical extracts increased them (Table 1, Table S2). Excepting a single case in which HE extracts increased total emissions due to an increased N turnover rate (Table S2, LE cells + HE extract). Such communities or chemical backgrounds might hypothetically be selected for in farms soils to reduce N₂O emissions. Generalizations might also be applied about the relative importance of microbiome and chemical backgrounds. In the absence of pH effects (CBA-int or CBA-pH at pH 6.6) cell and chemical extracts had a similar average impact on N₂O ratios but chemical extracts had a greater impact on total emissions due to rate effects (Table 1, Table S2).

Contrastingly, our assays also supported specific less predictable interactions between certain cells, chemical backgrounds and pH that broke the above generalizations. Tapawera LE cells were particularly sensitive to lower pH (Table 2) and especially so in the HE chemical background, showing the highest ratios and total emissions of any treatment (Table 1) in CBA-pH. Our ultimate interpretation is that some generalisations can be made about what is a “good” (low N₂O emitting) denitrifying community and chemical background but that unpredictable specific effects may occur, especially when cells are denitrifying below their typical pH.

An important caveat of all the above interpretations is our inability to completely confirm that cell origin effects were only the result of community composition effects. Extracted cells clearly displayed some lesser but notable activity when incubated in just H₂O (Figure S3C, Figure S4C) indicating some carbon pool associated with the cells (lysed cells, adherent carbon, stored carbon). Differences in this carbon availability between different cell extractions could potentially influence the denitrification kinetics within the main treatments, especially rates. Cell + H₂O controls demonstrate similar gas accumulation rates across both cell types in CBA-int (Figure S3C) indicating that, most likely, cell associated carbon should have little observable impact on treatment differences. However, this cannot be claimed for CBA-pH where gas accumulation rates were clearly lower in HE cell + H₂O controls (Figure S4C).

4.3 pH effects

pH differences of just 0.6 points could account for similar changes in N₂O ratio as differences in chemical extract during CBA-pH (Figure 4A). This is consistent with denitrification literature which commonly identifies pH as a major driving factor of differences in N₂O/N₂ emission ratios between soils (Simek and Cooper, 2002; Čuhel and Šimek, 2011; Liu *et al.*, 2014). In contrast, N₂O emissions were much less susceptible to pH change compared with chemical extract origin due to the conflicting effects of pH on N₂O ratio and denitrification rates, which are also previously noted (Šimek *et al.*, 2002). In one case, lowering the pH actually resulted in increased N₂O emissions, therefore, this evidence supports the view that pH manipulation of soil is not necessarily a successful approach to reduce overall N₂O emissions over a fixed time period. Further, we noted the unideal scenario in which decreasing the pH experienced by higher pH adapted cells had a significant negative impact on N₂O ratio, while increasing the pH experienced by lower pH adapted cells had only a minor positive impact on N₂O ratio. In essence, it may be easier for pH change to cause detrimental effects than repair them. Although our pH system may be not be ideal to test this effect. Due to the buffer system used, the low pH soil was already above its natural pH under the low pH treatment.

4.4 Differential stages in N₂O production: the role of carbon

The timing of sudden decreases in CO₂ production and overall denitrification rates (Figure S2), combined with the lack of late N₂O accumulation from glutamate amended controls (Figure 5) suggest carbon limitation caused the increased N₂O accumulation and reduced

541 N₂ rate observed in the later period of the cell based incubations. If simple carbon limitation
 542 was occurring, it is expected that drops in CO₂ production and denitrification rates would
 543 wane gradually over time as carbon concentrations reduced, however, the drops in CO₂ and
 544 denitrification rates were often well defined and rapid. Therefore, we suggest the sudden
 545 transitions in rates are the result of exhaustion of a more labile carbon pool and initiation,
 546 or maintenance, of consumption of a more recalcitrant carbon pool. Soil extracted carbon is
 547 typically quantified in these two separate pools with separate consumption rate constants
 548 assigned to the consumption of each pool e.g. (Bowen *et al.*, 2009; Guigue *et al.*, 2014;
 549 Kalbitz *et al.*, 2003). The multiple (greater than two) N₂ rate switches observable in some
 550 incubations (Figure S3A, LEC + HEE, Figure S6, 6 LEC + LEE extended) suggest effects
 551 to denitrification rates could be through greater than two distinct carbon pools of
 552 consecutively reduced energy availability.

553 Alternatively, denitrification rates may be sustained by consumption of energy storage
 554 molecules during the reduced N₂ rate period. Increased N₂O accumulation was previously
 555 shown in monocultures of *Alcaligenes faecalis* during carbon limitation and co-occurred
 556 with consumption of energy storage molecules (Schalk-Otte *et al.*, 2000). This was
 557 attributed to competition for limited electrons between N₂O reductase and the previous
 558 denitrification reductases. Under this mechanism, differing N-reductase electron carrier
 559 affinities or regulatory mechanisms create an uneven distribution of electrons to the
 560 separate denitrification steps (Pan *et al.*, 2013; Ribera-Guardia *et al.*, 2014; Wang *et al.*,
 561 2018; Schalk-Otte *et al.*, 2000). Earlier N-reductases are thought to outcompete N₂O
 562 reductase resulting in N₂O accumulation during limited electron supply. Electron supply
 563 can be limited due to substrate availability but also carbon oxidation rates (Pan *et al.*, 2013)
 564 which depend on the substrate being utilized (Ribera-Guardia *et al.*, 2014) and presumably
 565 the organism carrying out the oxidation.

566 Electron competition is consistent with concurrent drops in CO₂ production, N turnover
 567 rates and uneven rebalancing of N₂O production/reduction in the present study, whether
 568 this is during consumption of energy storage molecules or more recalcitrant carbon.
 569 However, it is unclear how this mechanism should proceed in a complex community of
 570 denitrifiers as competition for electrons is only hypothetically viable when N₂O production
 571 and reduction proceed within the same organism. This is not necessarily a valid assumption
 572 in a complex denitrifying community where multiple species of denitrifiers could specialize
 573 in separate steps of the process due to the modularity of denitrification genes (Graf *et al.*,

2014; Roco *et al.*, 2017; Lycus *et al.*, 2017). Electron competition between N-reductases has been tested in complex communities (Pan *et al.*, 2013; Ribera-Guardia *et al.*, 2014; Wang *et al.*, 2018) and in some cases it was assumed that denitrification was carried out by complete denitrifiers based on the genera of the dominant microbes within the culture (Pan *et al.*, 2013; Wang *et al.*, 2018). In depth sequencing of metagenomes and metatranscriptomes with genome reconstruction would be necessary to actually resolve the modularity of active denitrifiers within the present system since phylogeny is usually considered a poor predictor of denitrification genetic potential (Jones *et al.*, 2008).

A point of confusion, possibly contradicting the above interpretations, is that cell + H₂O treatments also demonstrated the distinct denitrification rate changes which we have attributed to carbon limitation (Figure S3C, Figure S4C). This either means the carbon limitation hypothesis and associated interpretations are wrong or that these incubations begun with a non or initially less limiting availability of carbon. Cells were washed multiple times during extraction to remove carbon from the suspension solution. It is therefore most likely that the utilized carbon sources in these treatments is derived from lysed cellular constituents, cell adherent carbon, insoluble carbon or stored carbon.

4.5 Conclusion

These investigations provide causal evidence for microbiome composition effects on N₂O emission potential, but these were on average still weaker than chemical effects. Differences in cell based assay gas accumulation kinetics reduce the general applicability of this system to soils but also serendipitously provide evidence that carbon limitation or switching to more recalcitrant carbon sources can lead to increased N₂O emissions. Investigations into the effects of pH corroborate the large body of research suggesting that this is a particularly important determinant of soil N₂O emission ratios but also suggest that its impact on total N₂O emissions over a fixed time period could be minor compared to other soil variables. Ultimately, we add to the mounting evidence that microbiome composition needs to be considered during soil manipulations aimed at reducing N₂O emissions.

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758

759 **Figure Legends**

760 **Figure 1.** Comparison of parent soils (A) and equivalent unswapped cell based assay
761 treatments (CBA) from CBA-int (B) and CBA-pH (C) reveal contrasting gas accumulation
762 patterns. Headspace gases NO (blue), N₂O (orange), N₂ (black) were quantified every 4hrs
763 from triplicate (dots, squares, triangles) 3mM NH₄NO₃ amended anoxic incubations. Note
764 separate scales between treatments to highlight relative gas accumulation

765 **Figure 2.** Relative ranking of parent soil N₂O ratios is maintained in equivalent CBA
766 treatments but lower on an absolute scale. N₂O ratios summarise the N₂O emission
767 potential from 90hr CBA anoxic incubations amended with 3mM NH₄NO₃ and are
768 calculated as N₂O/N₂O+N₂ at the end of a CBA incubation, where periods of net negative
769 N₂O accumulation are ignored to account for multiple gas peaks. Equivalent CBA
770 treatments include both cells and chemical extracts derived from the parent soil. Results
771 from triplicate vials per treatment are displayed with 95% confidence intervals.

772 **Figure 3.** Cell and chemical extract origin impact CBA N₂O ratios and N₂O emitted (μmol-
773 N per vial). Standard swap treatments from CBA-int (A,B) or CBA-pH (C,D). N₂O ratios
774 and N₂O emitted summarise the N₂O emission potential from 90hr CBA anoxic incubations
775 amended with 3mM NH₄NO₃ and are calculated as N₂O/N₂O+N₂ and total N₂O
776 accumulated at the end of a CBA incubation, where periods of net negative N₂O
777 accumulation are ignored to account for multiple gas peaks. Results from triplicate vials per
778 treatment are displayed with 95% confidence intervals. pH of CBA-pH chemical extracts
779 were buffered at two levels and are labeled accordingly.

780 **Figure 4.** Comparison of independent pH and chemical extract origin changes indicates
781 similar impact of pH and chemical extract on N₂O ratios (A) but minor impact of pH on
782 N₂O emitted (B). Each symbol compares the change in N₂O emission potential from 1 of 4
783 CBA-pH baseline treatments. Filled symbols indicate non-overlapping 95% confidence
784 intervals for alternative pH or chemical extract changes to the same baseline treatment.
785 Positive values indicate variable change had expected direction of effect on N₂O ratio or
786 emissions i.e. higher pH and LE extracts are expected to decrease N₂O ratio and emissions,
787 lower pH and HE extracts vice versa.

788 **Figure 5.** Carbon limitation associated with increased N₂O accumulation and reduced N₂
 789 accumulation in CBA-int incubations. Standard treatments (dots), 3mM glutamate amended
 790 treatments (squares). Headspace gases NO (blue), N₂O (orange), N₂ (black) were quantified
 791 every 4hrs from 3mM NH₄NO₃ amended anoxic extracted cell and chemistry based
 792 incubations. Average gas accumulation from triplicate (standard treatments) or minimum
 793 duplicate (glutamate amended treatments) vials per treatment are presented. Note separate
 794 scales between treatments to highlight relative gas accumulation.

795 **Figure 6** Extraction bias in microbial communities. Community differences due to cell
 796 extraction are shown using NMDS (A), zeta-diversity (B), and community abundance (C-
 797 D). NMDS shows community dissimilarity (Bray-Curtis), where colours represent origin
 798 soil and shapes extraction source (soil or extracted cells). B depicts shared and unique
 799 ASVs between soil and cell extracted sequences. C depicts differences in phylum level
 800 relative abundance between soil and cell DNA extraction sources. D depicts fold changes in
 801 specific ASVs between soil and cell DNA extraction sources, calculated by dividing ASV
 802 abundance from soil communities, by those from extracted cells. ASV's with significant
 803 changes are labelled by genera.

804 Tables

805 **Table 1.** Differences in treatment emission potential indicating strength of cell and chemical extract origin effects for CBA-int and CBA-pH

CBA-int					CBA-pH				
Treatment	N ₂ O hypo emit ratio	95% CI	N ₂ O hypo emit (μmol-N)	95% CI	Treatment	N ₂ O hypo emit ratio	95% CI	N ₂ O hypo emit (μmol-N)	95% CI
HE cells + HE extract	0.39	0.37, 0.42	6.34	4.43, 8.25	HE cells + HE extract (6)	0.41	0.38, 0.44	4.77	4.04, 5.5
HE cells + LE extract	0.18	0.17, 0.19	2.32	1.8, 2.84	HE cells + LE extract (6.6)	0.32	0.21, 0.43	2.91	2.22, 3.58
LE cells + HE extract	0.19	0.18, 0.21	3.49	2.76, 4.22	LE cells + HE extract (6)	0.57	0.54, 0.61	9.61	8.49, 10.73
LE cells + LE extract	0.07	0.05, 0.1	1.25	0.9, 1.61	LE cells + LE extract (6.6)	0.12	0.11, 0.14	1.19	1.01, 1.36
Cell effect	Differences		Differences		Cell effect	Differences		Differences	
HE, HE vs LE, HE	0.20	0.18, 0.22	2.85	1.19, 4.52	HE, HE vs LE, HE	-0.16*	-0.19, -0.13	-4.85*	-5.77, -3.92
HE, LE vs LE, LE	0.10	0.08, 0.12	1.07*	0.64, 1.49	HE, LE vs LE, LE	0.19*	0.09, 0.3	1.72*	1.08, 2.35
Average cell effect	0.15		1.96		Average cell effect	0.02		-1.56	
Av Directionless cell effect	0.15		1.96		Av Directionless cell effect	0.18		3.28	
Extract effect	Differences		Differences		Extract effect	Differences		Differences	
HE, HE vs HE, LE	0.21	0.19, 0.24	4.02	2.26, 5.78	HE, HE vs HE, LE	0.09*	-0.01, 0.2	1.86*	1.22, 2.51
LE, HE vs LE, LE	0.12	0.1, 0.14	2.23*	1.62, 2.84	LE, HE vs LE, LE	0.45*	0.42, 0.48	8.43*	7.34, 9.51
Average extract effect	0.17		3.13		Average extract effect	0.27		5.14	
Av Directionless extract effect	0.17		3.13		Av Directionless extract effect	0.27		5.14	

807 Emission potential differences are expressed relative to the HE extract or cells. Positive values indicate reduced N₂O emission potential when comparatively LE
808 extracts or cells were used.

809 Average cell effects are calculated using signed difference values while average directionless cell effects are calculated using absolute values

810 * Indicates difference values have non-overlapping confidence intervals with the appropriate comparison. Direct comparison of cell vs. chemical extract
811 difference values should be compared relative to the equivalent baseline treatment i.e. HE, HE vs. LE, HE compared with HE, HE vs. HE, LE.

Table 2. CBA-pH: Difference in N₂O hypo emit (ratio) associated with independent treatment difference in pH or chemical extract relative to a baseline sample

Difference in N ₂ O hypo emit ratio for treatments varying in:							
Baseline sample	Extract	95% CI	pH	95% CI	Extract + pH actual	95% CI	Extract + pH predicted (sum independent extract and pH differences)
6 HEC + HEE	-0.03	-0.08, 0.01	-0.03	-0.33, 0.28	-0.09	-0.2, 0.01	-0.06
6.6 HEC + LEE	0.07	-0.21, 0.08	0.06	-0.03, 0.15	0.09	-0.01, 0.2	0.13
6 LEC + HEE	-0.33	-0.52, -0.15	-0.26	-0.29, -0.23	-0.45	-0.48, -0.42	-0.59
6.6 LEC + LEE	0.19	0.17, 0.21	0.12	-0.13, 0.36	0.45	0.42, 0.48	0.31

Difference in N ₂ O hypo emit (μmol-N) for treatments varying in:							
Baseline sample	Extract	95% CI	pH	95% CI	Extract + pH actual	95% CI	Extract + pH predicted (sum independent extract and pH differences)
6 HE + HE	-1.74	-2.52, -0.96	0.98	-2.31, 4.26	-1.86	-2.49, -1.23	-0.76
6.6 HE + LE	2.84	-0.73, 6.4	0.12	-0.88, 0.65	1.86	1.23, 2.49	2.96
6 LE + HE	-7.17*	-8.37, -5.99	-2.88*	-3.85, -1.93	-8.42	-9.51, -7.34	-10.06
6.6 LE + LE	5.54*	5.12, 5.96	1.25*	-1.54, 4.04	8.42	7.34, 9.51	6.78

Negative values indicate a lower N₂O ratio or hypothetical emissions relative to the baseline sample

* Indicates pH and chemical extract difference values have non overlapping confidence intervals for equivalent baseline samples (same row)

A

B

C

A **B**

HEC + HEE Glutamate Amended **HEC + LEE Glutamate Amended** **LEC + HEE Glutamate Amended** **LEC + LEE Glutamate Amended**

A

B

C

D