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# An increased ratio of fungi to bacteria indicates greater potential for $N_2O$ production in a grazed grassland exposed to elevated $CO_2$

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ABSTRACT

Nitrous oxide ($N_2O$) is an important greenhouse gas and emissions of $N_2O$ have been shown to increase under elevated $CO_2$ ($eCO_2$) resulting in a positive feedback on climate change. $CO_2$-driven increases under grassland have often been associated with greater $N_2O$ emitted during denitrification. We examined the soils from a Free Air Carbon Dioxide Enrichment (FACE) experiment on grassland on the west coast of the North Island of New Zealand that had received long-term exposure to elevated $CO_2$. Importantly, the grassland was grazed thus representing much of the world's grassland situation and providing data for a land use that has not been well studied. We conducted soil incubations where a fungicide and bactericide were used to isolate the contribution of bacteria and fungi to potential $N_2O$ production using denitrification enzyme activity (DEA). We found greater gene abundance of fungi under $eCO_2$ and reduced bacterial gene abundance. $N_2O$ DEA was dominated by fungi in both ambient and elevated $CO_2$. Total potential $N_2O$ emissions were 49% higher under $eCO_2$ entirely due to greater emissions from the fungal component. An increasing fungal contribution to $N_2O$ emissions presents a challenge to mitigation as, to date, mitigations have largely been targeted at bacteria.

## 1. Introduction

Nitrous oxide ($N_2O$) is the third most important anthropogenic greenhouse gas and has now become the most damaging stratospheric ozone depleting substrate making it a major form of nitrogen pollution (Davidson and Kanter, 2014). Any change in $N_2O$ emissions in response to climate change drivers are important as this could result in a positive or negative feedback on greenhouse gas forcing (van Groenigen et al., 2011).

Grasslands cover 37% of the ice-free surface of the globe (O'Mara, 2012) and experimental evidence suggests that they will produce an increasing amount of $N_2O$ as the $CO_2$ concentration rises (Baggs et al., 2003b; Kammann et al., 2008; Cantarel et al., 2012). The importance of denitrification in this $CO_2$ effect on grasslands has been consistently identified. For example, Cantarel et al. (2012), in an upland grassland, and Baggs et al. (2003a) in a heavily fertilized lowland grassland, saw increased $N_2O$ produced from denitrification in response to elevated $CO_2$ ($eCO_2$). Regan et al. (2011) reported higher $N_2O$ emissions under $eCO_2$ in a German meadow after 10 years of $CO_2$ enrichment and suggested these increased $N_2O$ emissions might be caused by a higher proportion of $N_2O$ producing rather than $N_2O$ consuming denitrifiers at $eCO_2$.

Studies of denitrification have focused largely on bacterial activity although fungal denitrification has been shown to be very active in grassland soils (Laughlin and Stevens, 2002). A fungal component to denitrification would be important for $N_2O$ production as fungi generally lack an $N_2O$ reductase and so the gaseous emission is $N_2O$ rather than $N_2$ (Baggs, 2011). As one of the most common responses to $eCO_2$ is an increasing fungal:bacterial microbial biomass ratio in the soil (Rillig et al., 1999; Kandeler et al., 2008; Guenet et al., 2012; Hayden et al., 2012) resulting from greater carbon (C) inputs to the rhizosphere (Rillig et al., 1999; Drigo and Kowalchuk, 2013) we might expect that a greater proportion of soil processes could be catalyzed by fungi under $eCO_2$ including, perhaps, $N_2O$ production. Nitrous oxide production by fungi has long been identified in grasslands (Laughlin and Stevens, 2002) and has now become widely recognized as a component of $N_2O$ production that was previously thought to be dominated by bacteria (Chen et al., 2014; Maeda et al., 2015).

In this paper we examined soil samples from a long-running Free Air Carbon Dioxide Enrichment (FACE) experiment on grassland grazed by

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sheep (Newton et al., 2014) and asked a) is there potential for higher $N_2O$ emissions under $eCO_2$ and b) if so, does a change in the fungal contribution to denitrification play a role? We were encouraged to examine these questions by previous studies on this experiment that have suggested higher $N_2O$ emissions under $eCO_2$ related to a fungal source (Rütting et al., 2010) and a reduction in the activity of $N_2O$ reductase (expression of *nosZ* genes) in $eCO_2$ soil (Zhong et al., 2015) that could result in greater $N_2O$ relative to $N_2$ production from denitrification.

## 2. Materials and methods

### 2.1. The New Zealand FACE experiment

Soil was collected from the New Zealand FACE experiment which is located at 40°14′S, and 175°16′ E where the long-term (since 1945) mean annual temperature was 12.9 °C and annual rainfall 870 mm. The experiment was established on a pasture that had not been resown for several decades and contained a mix of C3 and C4 grasses, legumes and forbs (Edwards et al., 2001a). The soil was a Mollic Psammaquent – soil properties and changes in soil properties over time can be found in Ross et al. (2013). Fertiliser (phosphorus, potassium and sulfur) was added (Newton et al., 2010) but not nitrogen (N) fertiliser – the N input coming from nitrogen fixation by legumes which is typical of low input systems in temperate regions (Lüscher et al., 2014). The experiment had six 12 m diameter areas described as rings. Three of the rings were enriched with $CO_2$ using FACE technology (Edwards et al., 2001b) and three were left at ambient $CO_2$.

Enrichment started in October 1997 and was continuous for each day of the year until December 2011 with a target concentration of 475 ppm $CO_2$; there was then a hiatus while the facility was refurbished and enrichment recommenced in October 2013 with a target concentration of 500 ppm. The rings were irrigated occasionally during the summer period (December-February) post-2013. The rings were periodically grazed by adult sheep, details of the grazing protocol and actual grazing times can be found in Newton et al. (2014).

### 2.2. Soil sampling

Soil samples were collected from each ring on 11 November 2013 (one month after $CO_2$ enrichment restarted) and 13 March 2015. Twenty soil cores were collected in each ring to a depth of 75 mm using a 25 mm diameter soil corer and were then bulked. Fresh composite samples were subsampled for soil moisture determination, pH and inorganic N content (see Zhong et al., 2015 for methods) and denitrification potential, and a further subsample stored at −80 °C for DNA analysis.

### 2.3. Denitrification and $N_2O$

Denitrification potential was measured from denitrification enzyme activity (DEA). We conducted a soil incubation according to the protocol described by Patra et al. (2006) and Marusenko et al. (2013). Three sub-samples (12 g dry soil equivalent) from each soil sample were placed into 240 ml specimen bottles, then 7 ml of solution containing $KNO_3$ (50 μg $NO_3^-$N $g^{-1}$ dry soil), glucose (0.5 mg C $g^{-1}$ dry soil) and glutamic acid (0.5 mg C $g^{-1}$ dry soil) were added. Additional distilled water was provided to achieve 100% saturation and therefore ideal conditions for denitrification. Three treatments were applied: (I) cycloheximide ($C_{15}H_{23}NO_4$; a fungicide) at 1.5 mg $g^{-1}$ in solution, (II) streptomycin sulphate ($C_{42}H_{84}N_{14}O_{36}S_3$; a bactericide) at 3.0 mg $g^{-1}$ in solution (Castaldi and Smith, 1998; Laughlin and Stevens, 2002), and (III) a no-inhibitor control. Note that archaea were not targeted in these treatments.

The headspace air of the bottles was replaced by nitrogen gas to provide anaerobic conditions and $C_2H_2$ (10% v/v) was added to inhibit $N_2O$ reductase activity. The bottles were then sealed with a lid containing a rubber septum for gas sample collection and incubated at 28 °C for 48 h with constant agitation (180 rpm) in an orbital shaker (Lab-Line 3527; Boston, USA). During incubation, 12 ml gas samples were taken at 0, 24 and 48 h by syringe and injected into pre-evacuated 6 ml glass vials. The $N_2O$ concentration of the gas samples was analysed by gas chromatography (Kelliher et al., 2012) at the National Centre for Nitrous Oxide Measurements, Lincoln University, Christchurch, New Zealand. The $N_2O$ produced was calculated from a linear regression of the three sampling times (0, 24 and 48 h). An alternative approach using only the 0 and 48 h samplings gave very similar results indicating the effects of any non-linearity were minor.

Gas samples collected from bottles incubated with $C_2H_2$ were assumed to represent total denitrification activity ($N_2O + N_2$) and those without $C_2H_2$ the $N_2O$ fraction only. The difference in $N_2O$ concentration between the two samples was then the $N_2$ production potential. Treatment (I) (the fungicide treatment) was assumed to give values for bacterial denitrification enzyme activity (BDEA); treatment (II) (the bactericide treatment) to give the fungal component (FDEA) and treatment (III) (the no-inhibitor treatment) to give total denitrification enzyme activity (TDEA).

### 2.4. Gene abundance

Soil DNA was extracted from 0.5 g of the previously frozen and stored soil using a FastDNA™ Kit for Soil (QBIOgene, Irvine, CA, USA) following the manufacturer's instructions and stored at −20 °C until required for further analysis.

The abundance of bacteria and fungi was quantified in triplicate by real-time PCR using a LightCycler™ 480 II (Roche, Vienna, Austria). The real time PCR mixture contained 5 ng of soil DNA, 2 pmol of primers and 10 × SYBR Green iCycler iQ mixture (Roche Applied Science, Mannheim, Germany) in a total of 20 μl reaction volume. The primer details for bacteria were 341F 5′- CCT ACG GGA GGC AGC AG −3′ and 534R 5′- ATT ACC GCG GCT GCT GGC A −3′ (López-Gutiérrez et al., 2004) and the thermal cycle conditions were steps of 10 min at 95 °C; 35 cycles of PCR were performed as follows: 20 s at 95 °C, 15 s at 55 °C and 30 s at 72 °C. A final 5-min extension step completed the protocol. The primer details for fungi were FU18S1 5′-GGAAACTCACCAGGTCC AGA- 3′ derived from Nu-SSU-1196 and Nu-SSU-1536 5′-ATTGCAA TGCYCTATCCCCA-3′ (Borneman and Hartin, 2000) and the thermal cycle conditions were steps of 10 min at 95 °C; there were 40 cycles of PCR: 20 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C with a final 5-min extension step.

### 2.5. Statistical analysis

The effects of $CO_2$ treatment and time were analysed by a mixed effects model ($CO_2$*time + (1|block/ring)) where $CO_2$, time and their interaction were fixed effects and ring nested within block was the random effect. To account for the small sample size (a total of 12 observations) a permutation test was used to obtain P-values using 999 iterations for each fixed term in the above model. The method is an extension of permutation tests for random effects in linear mixed models (Lee and Braun, 2012). The analysis was conducted using R version 3.4.1 packages 'lme4' and 'predictmeans' (R Core Writing Team, 2017).

## 3. Results

Gravimetric soil moisture was lower at the second sampling but not different between $CO_2$ treatments (P = 0.234) (ambient 29.1% (s.e.m. 1.47), $eCO_2$ 30.5% (1.38) at the first sampling; ambient 13.3% (1.14), $eCO_2$ 16.9% (1.55) at the second sampling). We measured pH at the second sampling and this was the same for both treatments (ambient 6.3 (s.e.m. 0.03), $eCO_2$ 6.3 (0.02)). There was no $CO_2$ effect on soil