Fungal denitrification: Bipolaris sorokiniana exclusively denitrifies inorganic nitrogen in the presence and absence of oxygen

R Phillips
G Grelet
A McMillan
B Song
Virginia Institute of Marine Science
B Weir

See next page for additional authors

Follow this and additional works at: https://scholarworks.wm.edu/vimsarticles

Part of the Aquaculture and Fisheries Commons

Recommended Citation
Phillips, R; Grelet, G; McMillan, A; Song, B; Weir, B; and Et al., "Fungal denitrification: Bipolaris sorokiniana exclusively denitrifies inorganic nitrogen in the presence and absence of oxygen" (2016). VIMS Articles. 815.
https://scholarworks.wm.edu/vimsarticles/815

This Article is brought to you for free and open access by W&M ScholarWorks. It has been accepted for inclusion in VIMS Articles by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
Authors
R Phillips, G Grelet, A McMillan, B Song, B Weir, and Et al.

This article is available at W&M ScholarWorks: https://scholarworks.wm.edu/vimsarticles/815
Fungal denitrification: *Bipolaris sorokiniana* exclusively denitrifies inorganic nitrogen in the presence and absence of oxygen

Rebecca Phillips¹,*,†, Gwen Grelet¹, Andrew McMillan¹, Bongkeun Song², Bevan Weir¹, Thilak Palmada¹ and Craig Tobias³

¹Landcare Research, Gerald Street, Lincoln, Canterbury 7608, New Zealand, ²Department of Biological Sciences, Virginia Institute of Marine Science, Gloucester Point, VA 23062, USA and ³Department of Marine Sciences, University of Connecticut, Groton, CT 06269, USA

*Corresponding author: Landcare Research, Gerald Street, Lincoln, Canterbury 7608, New Zealand. Tel: +64-3-3219873; E-mail: phillipsr@landcareresearch.co.nz

**One sentence summary:** Inorganic nitrogen addition alters fungal denitrification and respiration in the presence and absence of oxygen.

**ABSTRACT**

Fungi may play an important role in the production of the greenhouse gas nitrous oxide (N₂O). *Bipolaris sorokiniana* is a ubiquitous saprobe found in soils worldwide, yet denitrification by this fungal strain has not previously been reported. We aimed to test if *B. sorokiniana* would produce N₂O and CO₂ in the presence of organic and inorganic forms of nitrogen (N) under microaerobic and anaerobic conditions. Nitrogen source (organic-N, inorganic-N, no-N control) significantly affected N₂O and CO₂ production both in the presence and absence of oxygen, which contrasts with bacterial denitrification. Inorganic N addition increased denitrification of N₂O (from 0 to 0.3 µg N₂O-N h⁻¹ g⁻¹ biomass) and reduced respiration of CO₂ (from 0.1 to 0.02 mg CO₂ h⁻¹ g⁻¹ biomass). Isotope analyses indicated that nitrite, rather than ammonium or glutamine, was transformed to N₂O. Results suggest the source of N may play a larger role in fungal N₂O production than oxygen status.

**Keywords:** nitrite; glutamine; nitrous oxide; carbon dioxide; organic nitrogen; co-denitrification

**INTRODUCTION**

Fungal denitrification may contribute more to soil emissions of the greenhouse gas nitrous oxide (N₂O) than bacterial denitrification (Laughlin and Stevens 2002; Chen, Motiapo and Shi 2015), but physicochemical factors potentially altering fungal nitrogen (N) cycling require investigation. While many soil bacteria contain the N₂O reductase gene (*nosZ*) (required for N₂O to N₂ conversion), this gene is not found in fungi. Nonetheless, N₂ production by soil fungi has been reported (Shoun et al. 2012; Long et al. 2013). Some fungi, particularly for genera in the Hypocreales order (such as *Fusarium*), are capable of producing N₂O (Maeda et al. 2015) under anaerobic (Zumft 1997; Morozkina and Kurakov 2007; Shoun et al. 2012) or microaerobic conditions (Zhou et al. 2001; Morozkina and Kurakov 2007; Takaya 2009). Two pathways of fungal denitrification have been reported: (a) classical denitrification of nitrate or nitrite when oxygen is limited and insufficient to support aerobic respiration and (b) hybrid formation of N₂O, where two N sources are combined (Spott, Russow et al. 2007).
and Stange 2011). It is not clear if induction of anaerobiosis or changes in N source (Takaya 2002; Wei et al. 2014) cause a shift from one pathway to another. Hybrid N₂O and/or N₂ formation (also referred to as co-denitrification) has been observed in the presence of inorganic and organic N (Su, Takaya and Shoun 2004; Spott, Russow and Stange 2011; Long et al. 2013) but may also occur in the presence of two inorganic forms of N (Spott, Russow and Stange 2011). Data are lacking that indicate how inorganic and organic sources of N affect fungal denitrification and co-denitrification to N₂O pre- and postinduction of anaerobiosis. Addition of two N sources (one enriched with ¹⁵N) would allow us to determine if N₂O formation could be attributed to denitrification or co-denitrification.

While a number of fungal strains have been tested for denitrification potential (Maeda et al. 2015), many strains, such as Bipolaris sorokiniana, have not been tested. Bipolaris sorokiniana is the asexual form (anamorph) of the fungus Cochliobolus sativus, a common plant pathogen in the order Pleosporales with a wide range of plant hosts. It is particularly common on cereals in the Poaceae family and is widely known as the causal agent for the common root rot. It can also survive as thick-walled conidia or as saprotrophic mycelium in soil or crop debris. Geographic distribution of B. sorokiniana is worldwide, particularly near mid-latitudes, in grasslands and agricultural fields (Kumar et al. 2002). It is not known if this soil fungus will denitrify inorganic or organic forms of N and if aerobic respiration and denitrification co-occur under controlled conditions. While a large body of research has reported bacterial N₂O and CO₂ production under microaerobic and anaerobic conditions (Butterbach-Bahl et al. 2013), few reports are available indicating if rates of fungal CO₂ and N₂O production vary with inorganic and/or organic N. It is unclear if fungi incubated with O₂ will respond to induction of anaerobiosis in a manner similar to denitrifying bacteria, with increased rates of N₂O production occurring within hours of induction (Firestone and Tiedje 1979; Smith and Tiedje 1979). The goal of this research was to test how N source (organic or inorganic) influences production of N₂O and CO₂ by B. sorokiniana pre- and post anaerobiosis. Results add to the emerging body of knowledge regarding controls on fungal denitrification and co-denitrification processes.

MATERIALS AND METHODS
We used pure culture of B. sorokiniana (Sacc.) Shoemaker [teleomorph: C. sativus (S. Ito & Kurib.) Drechsler ex Dastur] to test how organic N [glutamine (C₅H₇O₂N₃)] and inorganic N [ammonium sulfate (NH₄)₂SO₄] and sodium nitrate (NaNO₃) sources might influence fungal production of N₂O and CO₂ under sterile conditions. We controlled O₂ status with air-tight laboratory incubation vessels to evaluate rates of N₂O and CO₂ production pre and post anaerobiosis on the same samples (Firestone and Tiedje 1979; Smith and Tiedje 1979; Zhou et al. 2001; Butterbach-Bahl et al. 2013). We obtained isolate ICMP 6809 from the ICMP culture collection (http://www.landcareresearch.co.nz/resources/collections/icmp) in its anamorphic state. It was grown under aerobic conditions in the same media previously used by Rohe et al. (2014a,b) and Shoun et al. (1992), containing 1% glucose, 0.2% peptone, 0.02% MgSO₄·7 H₂O, 2 ppm CaCl₂·6 H₂O, 2 ppm FeSO₄·7 H₂O and 0.01 mol potassium phosphate (pH 7.4). This media, which contained N as peptone, was used only for fungal growth. Another media, where the N source was omitted, was used for the incubations and referred to here as no-N media. After 7 days, cultures were washed, drained and stored in a reduced volume of the no-N media. Using the no-N media, four solutions were prepared for fungal inoculation: (a) no-N media (b) 0.5 mmols N as C₅H₇O₂N₃ (c) 0.25 mmols N as Na¹⁵NO₃ (99.5 atom%); Cambridge Isotope Laboratory, Andover, MA) and 0.25 mmols N as (NH₄)₂SO₄, or (d) 0.25 mmols N as Na¹⁵NO₃ and 0.25 mmols N as C₅H₇O₂N₃. The Na¹⁵NO₃ was used to determine if these fungi would preferentially use NO₃⁻ to form N₂O (thus forming ¹⁵N₂O) or if other sources of N would be utilized.

Approximately 10 ml of fungal biomass was transferred to 125 ml serum bottles, and four replicate bottles were inoculated with 1 ml of each sterile solution and 1 ml of no-N media. Additional bottles containing media solutions without fungi were also prepared. All bottles were sealed and the headspace evacuated and flushed with ultrapure helium, and then injected with O₂ to achieve 0.4% O₂ headspace before setting up on a robotic gas chromatograph (GC) fitted with electron capture and thermal conductivity detectors (Phillips et al. 2014; McMillan et al. 2015). Bottles were placed on the GC and measured every 6 hours for N₂O, CO₂ and O₂ at 19°C. Following this 48-h incubation experiment, bottles were then evacuated and flushed three times to create an anaerobic headspace for the second experiment, where the only difference between experiments was headspace O₂ concentration. Sterility was maintained and conditions remained constant during both incubations, including pH (ranged from 6.2 to 6.9). Data were normalized to the corresponding dry weight of fungal biomass in each bottle. The N isotopes for N₂O gas in the headspace of each sample at the end of the incubations were measured on a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta V, Thermo Scientific, Waltham, MA) in line with an automated gas bench interface (Thermo Gas Bench II) to determine if ¹⁵N₂O or ¹⁴N₂O were present. Precision of the isotopic analysis was <1 atom%.

Data were analysed for effects of N source treatment on N₂O and CO₂ production rates for microaerobic and anaerobic experiments separately with a generalized linear model and means were compared with Tukey’s test.

RESULTS AND DISCUSSION
Table 1 and Fig. 1 show effects of treatment on CO₂ and N₂O production for the microaerobic incubations were similar to effects of treatment for the anaerobic incubations. Fungi inoculated with only organic-N (C₅H₇O₂N₃) or no N media only linearly produced CO₂ (1–2 mg CO₂ h⁻¹ g⁻¹) but not N₂O. Fungi inoculated with inorganic-N (Na¹⁵NO₃ or (NH₄)₂SO₄) or inorganic-N plus organic-N (Na¹⁵NO₃ and C₅H₇O₂N₃) linearly produced N₂O (0.2–0.3 µg N₂O-N h⁻¹ g⁻¹) but an order of magnitude less CO₂. The mass ¹⁵N₂O comprised 94.7%–97.3% of all N₂O in the headspace, yielding an equivalent ¹⁵N of 98 to 99 (±0.3 atom%). This enrichment was effectively equivalent to enrichment of the ¹⁵NO₃ used in the experiment. The lack of headspace ¹⁴N₂O indicated hybrid formation of N₂O by B. sorokiniana did not occur under any of these conditions, and N₂O was the only N form used to form N₂O. Headspace O₂ (Fig. 2) over the aerobic time course indicated rapid declines for the no-N media and organic-N treatments but not the inorganic-N treatments. Minimal rates of CO₂ respiration (Table 1) in the presence of inorganic-N were consistent with lack of O₂ utilization by B. sorokiniana (Fig. 2). We also observed chemodenitrification (6%–8% of total biologically produced N₂O) for media amended with inorganic-N only (van Cleemput 1998; Kampschreur et al. 2011; Jones et al. 2015).
Table 1. Rates of N$_2$O and CO$_2$ produced during (1) aerobic and (2) anaerobic incubation experiments by treatment per gram of fungal biomass. Average rates followed by different letters for each experiment represent significant differences between treatments. The same letters indicate no differences between treatments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Mean ng N$_2$O-N h$^{-1}$ g$^{-1}$ biomass</th>
<th>Std. Dev.</th>
<th>Mean μg CO$_2$ h$^{-1}$ g$^{-1}$ biomass</th>
<th>Std. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>No N</td>
<td>0.51$^a$</td>
<td>0.26</td>
<td>896.45$^a$</td>
<td>206.08</td>
</tr>
<tr>
<td></td>
<td>Organic N</td>
<td>0.29$^a$</td>
<td>0.11</td>
<td>728.46$^a$</td>
<td>148.66</td>
</tr>
<tr>
<td></td>
<td>Inorganic N</td>
<td>311.30$^b$</td>
<td>18.98</td>
<td>6.17$^b$</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>Inorganic N + organic N</td>
<td>211.79$^c$</td>
<td>14.44</td>
<td>7.87$^b$</td>
<td>1.48</td>
</tr>
<tr>
<td>Aerobic</td>
<td>No N</td>
<td>0.57$^a$</td>
<td>0.23</td>
<td>1179.89$^a$</td>
<td>262.62</td>
</tr>
<tr>
<td></td>
<td>Organic N</td>
<td>0.32$^a$</td>
<td>0.23</td>
<td>988.19$^a$</td>
<td>222.95</td>
</tr>
<tr>
<td></td>
<td>Inorganic N</td>
<td>354.55$^b$</td>
<td>26.00</td>
<td>18.26$^b$</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>Inorganic N + organic N</td>
<td>306.39$^c$</td>
<td>28.61</td>
<td>31.52$^b$</td>
<td>4.03</td>
</tr>
</tbody>
</table>

Evidence of chemodenitrification, where N$_2$O was produced under sterile conditions, may be due to reactions between NO$_2^-$ and reduced metals in the media (van Cleemput 1998; Kampschreur et al. 2011). We chose the media for this experiment because it is commonly used for fungal denitrification investigations (Shoun et al. 1992; Rohe et al. 2014a,b). Kampschreur et al. (2011) used a chemostat to demonstrate that NO$_2^-$ to NO is an equilibrium-based reaction, where emissions of NO and N$_2$O were coupled to iron oxidation under anoxic conditions. A recent review by Medinets et al. (2015) argued that NO$_2^-$, a precursor of NO, is central to processes associated with chemodenitrification. Additional research is needed to determine the reactants involved in this specific media and the prevalence of chemodenitrification in an oxygenated headspace.

Evidence of biological denitrification coupled with reduced CO$_2$ respiration in the presence of inorganic-N may be due to B. sorokiniana preferentially denitrifying as a defence against accumulation of NO$_2^-$ (Geets, Boon and Verstraete 2006; Clark et al. 2012). Fungi are capable of converting NO$_2^-$ to NO, the precursor to soil N$_2$O emissions (Russow, Stange and Nueue 2009), through expression of the NO$_2^-$ reductase gene (nirK), and NO is a regulator of O$_2$ consumption in eukaryotes (Thomas et al.
2011). We suspect that some NO$\text{\textsubscript{2}}$ was converted to NO (both abiotic and biotic), which inhibited mitochondrial respiration (Thomas et al., 2001; Medinet et al. 2015) and therefore CO$\text{\textsubscript{2}}$ production, as shown in Fig. 1 and Table 1. One way of discerning the importance of NO$\text{\textsubscript{2}}$ reduction to NO and N$_2$O by B. sorokiniana would be to identify nirK gene expression in conjunction with NO measurements. If B. sorokiniana does not reduce NO$\text{\textsubscript{2}}$ to NO, then chemical transformation of NO$\text{\textsubscript{2}}$ may be the source of NO (van Cleemput 1998; Kampschreur et al. 2011). In either event, there is a need to identify chemical versus biological sources of NO, the precursor to N$_2$O (Russow, Stange and Nueve 2009).

Denitrification in soils and sediments is referred to as a key-stone ecosystem service with positive water quality implications. However, when N$_2$O is the final end product of denitrification, there are negative impact on stratospheric ozone and radiative forcing in the troposphere (Erisman et al. 2013). Here, B. sorokiniana, a ubiquitous soil fungus, demonstrated the capacity to transform dissolved NO$\text{\textsubscript{2}}$ to gaseous N$_2$O at the expense of aerobic respiration, a more energetically favourable pathway. Denitrification and aerobic respiration for B. sorokiniana were closely linked via NO$\text{\textsubscript{2}}$. Additional investigations into alternative respiration pathways and effects of inorganic N on C assimilation are warranted, particularly with respect to O$_2$ status. Here, we focused on how inorganic and organic N sources influence pathways to N$_2$O production before and after onset of anaerobiosis (Firestone and Tiedje 1979; Smith and Tiedje 1979; Zhou et al. 2001).

Future work should be designed with sample in parallel to test how O$_2$ and N interact to affect fungal denitrification phenotype and genotype. Our results suggest that fungal denitrification may not be driven by anaerobiosis but instead by form of N; however, additional strains need to be tested to determine if this is widespread among soil fungi or unique to B. sorokiniana.

ACKNOWLEDGEMENTS

The authors are grateful to Veronica Rolleston, Trish McLeanachan and Sujatha Senananae for contributing their technical expertise and laboratory facilities. We also thank Mikki Eken for editorial review and Fiona Carswell, Sue Scheele, Chris Phillips, and David Whitehead at Landcare Research for their continued support.

**REFERENCES**


Rohe L, Anderson T-H, Braker G et al. Dual isotope and isotopomer signatures of nitrous oxide from fungal

**FUNDING**

This work was funded by a US Department of Agriculture grant [2014-67019021614]; New Zealand’s Ministry of Business, Innovation and Employment; and New Zealand Ecosystems and Global Change Fund.

**Conflict of interest.** None declared.