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Chemical formation of hybrid di-nitrogen calls fungal codenitrification into question

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Removal of excess nitrogen (N) can best be achieved through denitrification processes that transform N in water and terrestrial ecosystems to di-nitrogen (N₂) gas. The greenhouse gas nitrous oxide (N₂O) is considered an intermediate or end-product in denitrification pathways. Both abiotic and biotic denitrification processes use a single N source to form N₂O. However, N₂ can be formed from two distinct N sources (known as hybrid N₂) through biologically mediated processes of anammox and codenitrification. We questioned if hybrid N₂ produced during fungal incubation at neutral pH could be attributed to abiotic nitrosation and if N₂O was consumed during N₂ formation. Experiments with gas chromatography indicated N₂ was formed in the presence of live and dead fungi and in the absence of fungi, while N₂O steadily increased. We used isotope pairing techniques and confirmed abiotic production of hybrid N₂ under both anoxic and 20% O₂ atmosphere conditions. Our findings question the assumptions that (1) N₂O is an intermediate required for N₂ formation, (2) production of N₂ and N₂O requires anaerobiosis, and (3) hybrid N₂ is evidence of codenitrification and/or anammox. The N cycle framework should include abiotic production of N₂.

The nitrogen (N) removal pathway known as denitrification is typically considered a biological process, where nitrate (NO₃⁻) or nitrite (NO₂⁻) is sequentially reduced by bacteria and archaea to nitric oxide (NO), nitrous oxide (N₂O) and finally inert di-nitrogen (N₂)¹. Incomplete denitrification results in emission of N₂O, an important greenhouse gas now playing a primary role in stratospheric ozone depletion². While prokaryotes are well known to denitrify³, new findings indicate denitrification by eukaryotes, such as fungi, may be widespread⁴⁻⁶. Unlike prokaryotes, fungi do not have the genes encoding nitrous oxide reductase, which reduces N₂O to N₂, so fungal denitrification terminates at N₂O^{7,8}. Denitrification occurs when a single N source is used to produce N₂O, such as NO₂⁻ or NO₃⁻. Codenitrification occurs when individual atoms of the N₂O or N₂ molecules are derived from two distinct N sources^{9,10}, resulting in hybrid N₂⁸. Formation of hybrid N₂ is widely reported as evidence of anammox¹¹ or codenitrification¹²⁻¹⁴. Previously, isotope pairing experiments revealed chemodenitrification^{15,16} and denitrification by the fungi *Bipolaris sorokiniana* used NO₂⁻ as the sole source for N₂O formation¹⁶. Fungal denitrification rates of NO₂⁻ to N₂O were similar under both anaerobic and microaerophilic conditions, contrary to classical denitrification, suggesting O₂ may not, in this case, be a strong regulator¹⁶.

Incubation experiments with pure cultures and soils report a number of fungi may play a significant role in soil N trace gas production^{5,6,17,18}. Fungi not only denitrify to N₂O but may also codenitrify to form N₂^{8,9}. When O₂ is not available, some fungi (*Fusarium oxysporum*) reportedly co-metabolise organic forms of N to reduce NO₂⁻ or NO₃⁻ and form hybrid N₂^{7,9,10}. This has been demonstrated in soils by tracing ²⁹N₂ and ³⁰N₂ following application of antibiotics to selectively inhibit bacteria or fungi^{12,14}. Codenitrification is widely viewed as an anaerobic, enzymatically-mediated nitrosation process requiring low (< -1) formal oxidation state of the nucleophilic N⁸. Figure 1 illustrates how anammox, like codenitrification, also forms hybrid N₂, although anammox uses two forms of inorganic N, NO₂⁻ and ammonium (NH₄⁺)^{11,19}.

The specific codenitrification pathway is unknown, but fungi reportedly reduce NO₂⁻ to NO using NO₂⁻ reductase (encoded by the *nirK* gene) and then reduce NO to N₂O using nitric oxide reductase (P450nor)⁷. The role of N₂O in the codenitrification process and in N₂ formation is not clear²⁰. While utilisation of N₂O to form N₂

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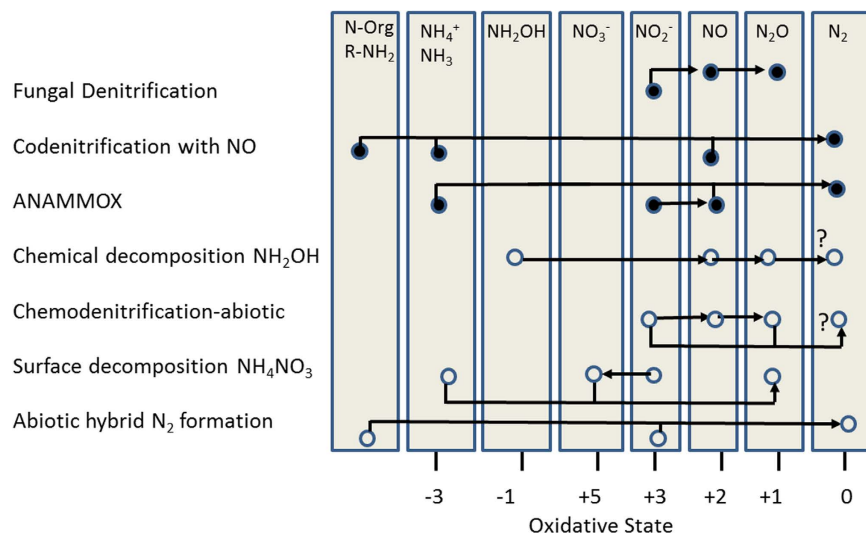


Figure 1. Schematic of codenitrification, anammox and known chemical denitrification pathways. Selected processes potentially leading to N₂O and N₂ formation, involved N compounds, their reaction pathways as well as their oxidation states are shown. Closed circles are biotic and open circles are abiotic reactions. Terms are defined as follows: N_{org}/R-NH₂, monomeric organically bound N forms; NH₄⁺, ammonium; NH₃, ammonia; NH₂OH, hydroxylamine; NO₂⁻, nitrite; NO₃⁻, nitrate; NO, nitric oxide; N₂O, nitrous oxide; N₂, molecular dinitrogen. The last process, abiotic N₂ formation, was observed in this study. Figure 1 is a truncated adaptation from Butterbach-Bahl *et al.*²³, which is licensed under a Creative Commons Attribution License 3.0 (<https://creativecommons.org/licenses/by/3.0/>).

has been suggested as a plausible codenitrification pathway⁸, reports of N₂O consumption during fungal production of N₂ (commonly observed during bacterial denitrification)²¹ are lacking. Potentially bypassing reduction of N₂O to form N₂, in addition to formation of hybrid N₂, sets codenitrification and anammox apart from classical denitrification²².

Laboratory studies commonly report evidence of fungal denitrification or codenitrification when pure cultures are incubated under anaerobic or microaerophilic conditions with sterile media, consisting of carbon, NO₂⁻ and mineral salts^{5,6,9,10,16}. However, reduced metals in the medium, such as Fe(II), could provide electrons required for abiotic reduction of NO₂⁻ to N₂O, commonly known as chemodenitrification^{15,23,24}. Chemodenitrification occurs through nitrosylation when reduced forms of inorganic N react with a metal centre to form N₂O in the absence of oxygen^{15,24}. Nitrosylation may also drive abiotic formation of N₂, given high concentrations of metal and NO₂⁻²⁵. Wullstein and Gilmour²⁵ mixed 10,000 ppm N as potassium nitrite (KNO₂) with 5,000 ppm ferrous sulfate in an abiotic, anoxic reactor and recovered 15% of added N as N₂ within 3 d. In this case, the N₂ formed would have been denitrified from a single N source, KNO₂. Alternatively, chemical formation of hybrid N₂ through nitrosation is often ignored by biologists. Abiotic nitrosation of organic matter by NO₂⁻ in soil was first suggested by Nelson and Bremner²⁶ when they recovered over 20% of added N (5 mmols NO₂⁻ g⁻¹ soil) as N₂ for sterile soil at neutral pH in helium (He) and heliox (20% O₂, 80% He) atmospheres. The isotopic composition of N₂ was not reported, but they indicated soil organic matter was an important factor²⁶. Trimmer and Prudy²⁷ showed that deep seawater samples amended with ¹⁵NO₂⁻ and ¹⁴NH₄ produced more ²⁹N₂ when organic N (allylthiourea) was added. They suggested an alternative metabolic pathway to anammox but did not address the possibility of abiotic ²⁹N₂ formation. Babbin *et al.*²⁸ also found organic N enhances anammox N₂ production. A common thread among chemical, anammox and fungal denitrification studies is NO₂⁻. Thus, NO₂⁻ is a pivot-point for divergence in biological and chemical N trace gas production²⁹ (Fig. 1).

Here, we pursue open questions raised by this early work regarding abiotic N trace gas production recently reviewed by Heil *et al.*³⁰. We aimed to investigate if previously unexplored sources of N₂O and N₂ could be contributing to reactive N removal and if N₂O was an intermediate in the abiotic N₂-production pathway. We questioned whether N₂ reportedly due to fungal codenitrification in pure culture experiments was formed abiotically in the presence and absence of O₂, given diverse inorganic and organic sources of N. New knowledge of abiotic N₂O and N₂ production would advance environmental N-removal research and applications and perhaps explain some mass balance discrepancies found in isotopic pairing studies^{27,28}.

To address these questions, we used a ubiquitous soil fungus, *Bipolaris sorokiniana* (Sacc.) Shoemaker [telemorph: *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur], as our model. Previously, we found that *B. sorokiniana* used NO₂⁻ as the sole source for denitrification to N₂O under anaerobic and microaerophilic conditions¹⁶. We also found NO₂⁻ as the sole source for chemodenitrification, which accounted for 6–8% of total N₂O production¹⁶. These results prompted further inquiry regarding fungal and chemical trace gas production of N₂. We aimed to more explicitly evaluate abiotic and biotic N₂O and N₂ production by comparing live fungi with fungal necromass incubated under strictly sterile conditions, with and without O₂. Pure culture experiments have demonstrated fungal and chemical N₂O production, but reports are lacking that indicate abiotic nitrosation of organic

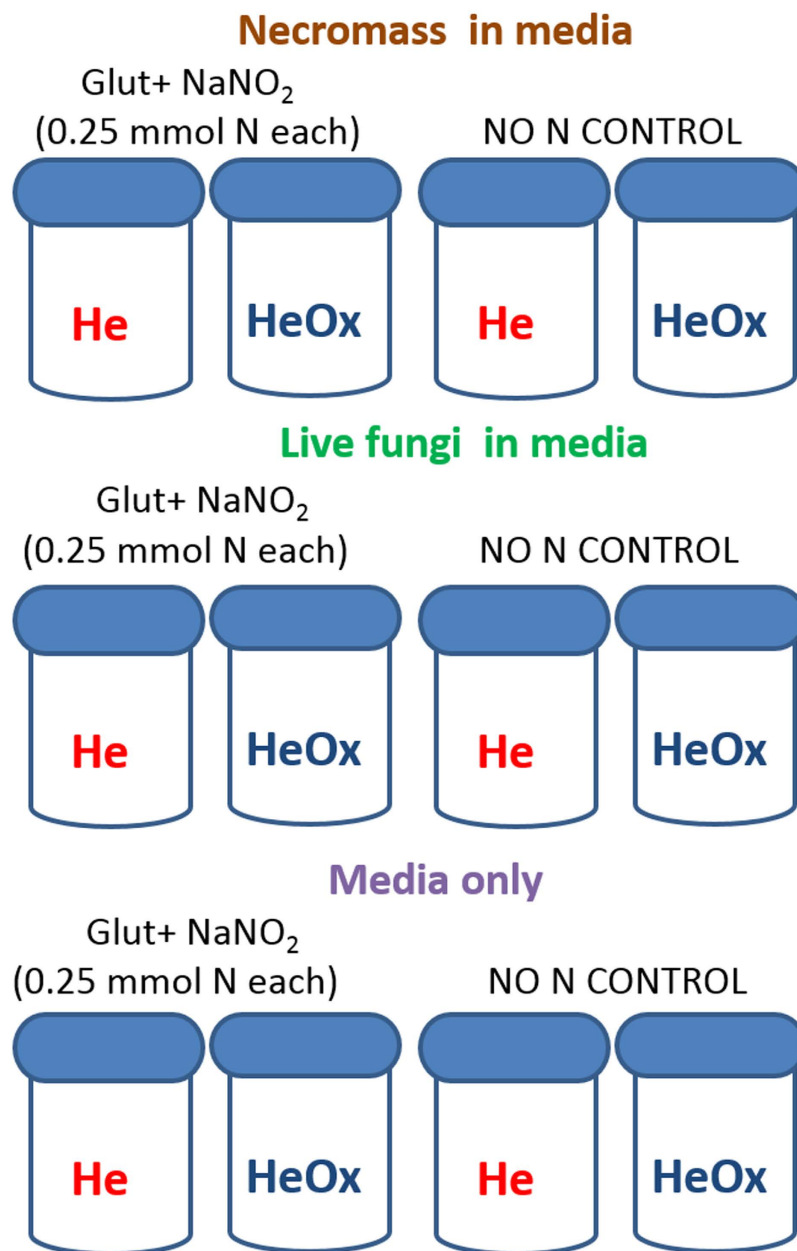


Figure 2. Experimental set-up to test how live fungi, necromass and media only incubated in an anoxic and 20% O₂ atmosphere affects production of N₂O, CO₂, O₂, and N₂ following addition of both organic and inorganic forms of N to pure cultures under aseptic conditions. Terms are defined as: Glut, glutamine; NaNO₂, sodium nitrite; He, helium; HeOx heliox.

compounds and formation of hybrid N₂. We include necromass in the design because amino acids and other nucleophilic compounds from necromass could potentially, in the absence of live fungi, react with NO₂⁻ to form N₂. Further, necromass would provide more surface area for chemical decomposition of NO₂⁻ to N₂O²⁴ (Fig. 1). This would serve as a test for abiotic formation of N₂O and N₂ in the presence of decomposing organic material.

We aimed to assess biotic and abiotic N₂O and N₂ production using established microbiological laboratory incubation methods (Fig. 2). We exposed live fungi and necromass to both inorganic and organic N sources [0.25 mmol N as sodium nitrite (NaNO₂) and 0.25 mmol N as glutamine (C₅H₁₀N₂O₃); concentration of 20 mmol L⁻¹ each] under two O₂ conditions (anaerobic and 20% O₂). For each treatment, there were replicate sets where N was not added to the media (No N control). We also included sterile media-only control vessels. Oxygen status was tightly controlled with airtight laboratory incubation vessels, where headspace was filled with either helium (anaerobic) or heliox (aerobic). Accumulation of headspace O₂, CO₂, N₂O, and N₂ were measured approximately every 6 h with a customised, robotic gas chromatography system²¹. Headspace O₂ was monitored to confirmed anaerobiosis was maintained during the 30 h incubation. Slopes of the linear increases in N₂O and N₂ in the headspace of each vessel were calculated to determine production rates. We used ANOVAs to test if

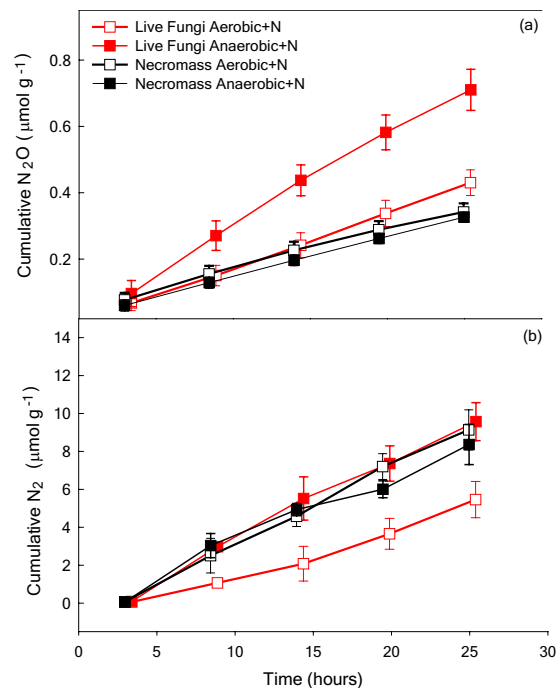


Figure 3. Kinetics of (a) N_2O and (b) N_2 production per g biomass over time following N addition at each time point as gases in the headspace accumulated. Average for each treatment are slightly staggered in time due to robotized measurement system, which is why comparisons were based on slopes over the entire incubation. Production rates of N_2O and N_2 were strongly affected by O_2 status [$\text{O}_2 \times$ fungal state (live or dead)]; $p < 0.001$ for those samples amended with N. Boxes represent average data; error bars, s.d.; $n = 4$.

production of N_2O and N_2 by live fungi was similar to necromass, and if both groups responded similarly to O_2 . Production rates were calculated in units of $\mu\text{mol N}$ as N_2O and N_2 g fungal biomass $^{-1}$ h $^{-1}$ and in units of $\mu\text{mol N}$ as N_2O and N_2 h $^{-1}$. We performed a second incubation (illustration of this incubation design not shown) using isotope pairing techniques to determine if $^{29}\text{N}_2\text{O}$ or $^{30}\text{N}_2\text{O}$ were produced abiotically from sterile medium amended with glutamine and NO_2^- in the presence or absence of O_2 . We used equal amounts of unlabelled glutamine and ^{15}N -labelled NO_2^- to achieve 0.5 mmol N and 1.0 mmol N, as well as a medium that was not amended with N.

Results and Discussion

We found both fungal states (live fungi and necromass) produced N_2O and N_2 but only for those samples amended with N. Linear production of N_2O and N_2 , following N amendment, occurred quickly under completely anaerobic conditions and in a 20% O_2 atmosphere (Fig. 3). Average [(standard deviation (SD))] rate of N_2O produced by necromass was 0.012 (<0.001) $\mu\text{mol N}_2\text{O}$ g biomass $^{-1}$ h $^{-1}$ under both aerobic and anaerobic conditions. Average N_2O production by live fungi was 0.017 (<0.001) $\mu\text{mol N}_2\text{O}$ g biomass $^{-1}$ h $^{-1}$ and 0.028 (0.003) $\mu\text{mol N}_2\text{O}$ g biomass $^{-1}$ h $^{-1}$, respectively, under aerobic and anaerobic conditions (Fig. 3a). Rates of N_2O production were significantly greater for live fungi incubated anaerobically ($\text{O}_2 \times$ fungal state interaction; $p < 0.001$), indicating biological production of N_2O in the absence of O_2 . In a 20% O_2 atmosphere, rates of N_2O production were similar to necromass. Our previous study indicated *B. sorokiniana* produced N_2O at similar rates when incubated anaerobically and in a 0.4% O_2 atmosphere¹⁶. Here, we used 20% O_2 , and we found no evidence of microbial denitrification under these high- O_2 conditions.

Figure 4a provides a basis of comparison for N_2O recovered in the headspace of sterile media using comparable units ($\mu\text{mol N}_2\text{O}$ h $^{-1}$). These data were not normalised per g of fungal biomass but illustrate accumulation of N_2O in sterile medium relative to necromass and live fungi. Nitrous oxide production rates were greater for necromass, as compared to media only (Fig. 4a). Necromass produced an average of 0.0034 $\mu\text{mol N}_2\text{O}$ h $^{-1}$ at both O_2 levels, as compared to 0.0011 $\mu\text{mol N}_2\text{O}$ h $^{-1}$ for sterile media. Greater N_2O for necromass suggests the presence of decaying biomass provided greater surface area for chemodenitrification²⁴ (Fig. 4a).

Rates of fungal N_2O production observed here are lower than other reports in the literature. Maeda *et al.*⁶ found fungi carrying the *nirK* gene produced from 0.1 to 3.2 $\mu\text{mol N}_2\text{O}$ g biomass $^{-1}$ h $^{-1}$, and Rohe *et al.*³¹ reported fungal N_2O production rates from 0.05 to 14 $\mu\text{mol N}_2\text{O}$ h $^{-1}$. In both cases, denitrification varied with fungal species and N amendment. Here, *B. sorokiniana* does not carry the *nirK* gene, which may influence NO_2^- reduction to NO. Data on chemical formation of N_2O under oxic (20% O_2) conditions are lacking, so these first results challenge the paradigm that chemodenitrification requires anoxia¹⁵. Contrary to chemostat studies, we included fungal necromass, which contributed to abiotic N_2O . Results call into question if all N_2O produced in pure culture experiments^{6,18,31} is enzymatically mediated if cultures include live and dead fungal or bacterial biomass.

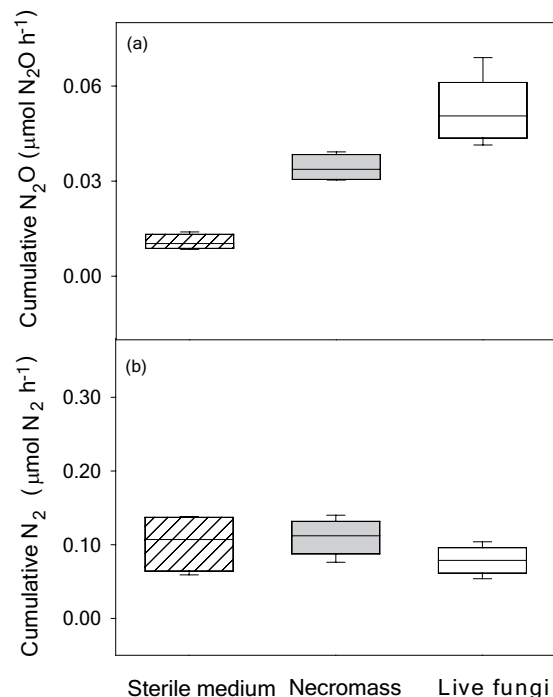


Figure 4. Abiotic and biotic contributions to rates of (a) N₂O and (b) N₂ accumulated per hour following N addition under both aerobic and anaerobic conditions for sterile medium, necromass and live fungi. Boxes represent the 90th percentile data; error bars, s.d.; n = 4. Median values are the lines horizontally bisecting each box.

We found no evidence of biological N₂ production in the presence or absence of necromass (Fig. 3b). Lowest rates of N₂ production were observed for live fungi incubated in a 20% O₂ atmosphere (O₂ × fungal state interaction; $p < 0.001$). Rates of N₂ produced in the headspace of live fungi were 0.243 (0.039) μmol N₂ g biomass⁻¹ h⁻¹ and 0.424 (0.049) μmol N₂ g biomass⁻¹ h⁻¹ under aerobic and anaerobic conditions, respectively. Rates of N₂ produced in the headspace of necromass were 0.415 (0.041) μmol N₂ g biomass⁻¹ h⁻¹ and 0.356 (0.047) μmol N₂ g biomass⁻¹ h⁻¹ under aerobic and anaerobic conditions, respectively. Our results indicate there is a strong abiotic component to measured rates of N₂ production and challenge the assumption that fungal N₂ production⁷ requires anoxic or microaerophilic conditions⁸.

Most of the N₂ accumulated in the headspace for live *B. sorokiniana* was commensurately found in the headspace of *B. sorokiniana* necromass (Fig. 3b), which points to chemical N₂ formation. We show chemical production of N₂ (μmol h⁻¹) for sterile medium relative to necromass and live fungi at both O₂ levels in Fig. 4b. Overall average rates of N₂ formation were 0.12 (0.039) μmol N₂ h⁻¹ for sterile medium, 0.11 (0.024) μmol N₂ h⁻¹ for necromass, and 0.08 (0.018) μmol N₂ h⁻¹ for live fungi. Others have reported much higher rates of anoxic chemical N₂ formation (7–32 μmol N₂ h⁻¹) at neutral pH when high concentrations of reduced metals were reacted with high concentrations of NO₂⁻²⁵ and when high NO₂⁻ solutions (20 M) were added to autoclaved soil²⁶, but these did not report if hybrid N₂ was formed or not. Comparable rates of abiotic N₂ production for necromass and sterile medium caused us to question if abiotic N₂, like N₂O, could result from nitrosylation of inorganic N only or from nitrosation to form hybrid N₂. Organic N has been found to increase biological production of ²⁹N₂ in oceanic studies³², but our results (Fig. 4b) indicated there may also be abiotic processes that contribute strongly to ²⁹N₂ production. Consequently, we determined if both inorganic and organic N were used to produce N₂ abiotically in a separate, isotope pairing experiment.

The headspace above sterile media incubated under oxic and anoxic conditions with ¹⁵N-labelled NaNO₂ and unlabelled C₃H₁₀N₂O₃ indicated abiotic, formation of hybrid N₂. Almost all (99.9%) of the N₂ produced was ²⁹N₂ (Table 1). The ²⁹N₂ production was proportional to the mass of added N and approximately 3–4% of the total N added was transformed to ²⁹N₂ under both oxic and anoxic conditions and at both levels of NO₂⁻ addition. Results demonstrate hybrid formation of N₂ is not necessarily enzymatically mediated and does not require anoxia. Formation of abiotic, hybrid N₂ by two distinct inorganic N molecules has not been ruled out here. Previous bodies of work that use formation of ²⁹N₂ as evidence of anammox and/or codenitrification^{12,14,32,33} need to be reviewed with respect to abiotic N₂ production.

Linear increases in cumulative N₂O and N₂ over time under aerobic and anaerobic conditions for both live fungi and necromass are shown in Fig. 3. These data suggest that N₂O was not consumed during the incubation and both gases were produced independently. This finding contrasts with bacterial denitrification²², where a sharp rise in microbial production of N₂O is followed by a rise in biological reduction of N₂O to N₂, and a drop in cumulative N₂O production²¹. *Bipolaris sorokiniana* denitrifies NO₂⁻ only and does not use glutamine to form N₂O¹⁶. If the pathway to N₂ were through the intermediate N₂O, we would expect not only N₂O consumption but

N addition (mmol N)	$^{29}\text{N}_2$ (^{14}N , ^{15}N) (μmol) aerobic	$^{30}\text{N}_2$ (^{15}N , ^{15}N) (μmol) aerobic	$^{29}\text{N}_2$ (^{14}N , ^{15}N) (μmol) anaerobic	$^{30}\text{N}_2$ (^{15}N , ^{15}N) (μmol) anaerobic
0	-0.012 (<0.001)	0.000 (0.000)	-0.005 (0.005)	<0.001 (<0.001)
0.5	16.274 (2.045)	0.001 (<0.001)	15.759 (0.995)	0.003 (0.002)
1.0	40.289 (2.318)	0.002 (<0.001)	31.025 (3.144)	0.007 (0.001)

Table 1. Average (SD) of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ recovered in the headspace following aerobic and anaerobic incubation of sterile media at three levels of N addition, where added N comprised 50% N from unlabelled $\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3$ and 50% N from labelled $^{15}\text{N}\text{-NaNO}_2$ ($n = 5$). Samples were incubated for >7 d prior to isotopic analyses. Values were adjusted according to helium or heliox blanks.

also accumulation of $^{30}\text{N}_2$ in the isotopic pairing experiment. Like anammox, our abiotic N_2 production results indicate N_2O formation was bypassed in the pathway to N_2 (Fig. 1).

We show compelling evidence that formation of $^{29}\text{N}_2$ does not result from solely biotic nitrosation but also abiotic nitrosation. Abiotic nitrosation occurred both in the presence and absence of O_2 , and abiotic N_2 was formed exclusively through hybridisation of inorganic and organic N sources and did not require the N_2O intermediary. Experimental protocols, including N concentrations, were in accordance with fungal denitrification^{6,16} and codenitrification¹⁰ studies but in the absence of soils and sediments. In soil, NO_2^- accumulates when excess free NH_3 inhibits bacterial NO_2^- oxidation, which is often a consequence of urea hydrolysis³⁰. Venterea *et al.*³⁴ recovered 3–60% of added urea N as NO_2^- , suggesting high soil NO_2^- is likely following urea addition (depending upon conditions and application rate). Based on these data³⁴, a 1-kg bovine urine addition to soil (N concentration of 0.4 mmol kg^{-1})³⁵ could result in up to 0.24 mmol NO_2^- . Here, we added 0.25 mmol NO_2^- to 10 ml of fungal culture, which is on the high end of this scale. While we would expect abiotic formation of hybrid N_2 via nitrosation of organic N to be more likely in grazed or fertilised agroecosystems, further N_2 investigations with soil and at lower NO_2^- levels are needed to bridge the gap between pure culture and environmental applications.

Other areas where NO_2^- could accumulate include laboratory incubations where antibiotic inhibitors are added to soil and used to partition N_2 and N_2O production into fungal and bacterial contributions^{12,14}. These data may be subject to artefacts if antibiotics repress NO_2^- oxidation, leading to NO_2^- accumulation and potential abiotic formation of N_2 and/or N_2O . Our report also calls into question if codenitrification alone accounts for N_2 and N_2O emissions following high doses of N as urea (approximately 9 mmol g^{-1} soil)¹³ and/or NO_2^- (166 mmol L^{-1})¹⁰. If abiotic, hybrid N_2 is formed under these conditions, our understanding of soil fungal codenitrification and N trace gas emissions would need to be re-examined. We offer new insight into chemodenitrification of NO_2^- to N_2 and present an alternative ‘ N_2O bypass pathway’. Finally, we conclude the potential for aerobic, abiotic removal of excess reactive N in terrestrial and aquatic ecosystems presents environmental mitigation research opportunities, particularly where transitory or chronic NO_2^- accumulation occurs.

Methods

Fungal incubations were conducted as described in Phillips *et al.* (2016) with the same culture ICMP 6809 isolated as a pathogen of *Hordeum distichon* in New Zealand. (https://scd.landcareresearch.co.nz/Specimen/ICMP_6809) GenBank: KU194490. We used laboratory incubations and controlled O_2 status to evaluate if rates of N_2O , N_2 and CO_2 production rates vary with aerobic conditions, as commonly reported for bacteria²⁰. Experimental design was similar to previous work¹⁶, with 4 replicates for each treatment (N with and without O_2 ; no N with or without O_2). Cultures were grown in medium similar to other fungal denitrification studies^{9,10,31}, consisting of 1% glucose, 0.2% peptone and inorganic salts^{5,36}. The only N source in this medium used for fungal growth was peptone. One day prior to the experiment, the growth medium was washed off cultures and replaced with the same medium that was identical except it was free of peptone and so contained no N. Using the peptone-free medium herein, two amendment solutions were prepared for fungal inoculation: (a) media without N and (b) 0.25 mmol N as NaNO_2 and 0.25 mmol N as $\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3$. Nitrogen concentration was $0.04 \text{ mmol N L}^{-1}$. Necromass was obtained by heating live *B. sorokiniana* at 60°C for 48 h. Cell death was confirmed by (a) microscopy and (b) lack of CO_2 respiration over a 24 h period under aerobic and anaerobic conditions. Approximately 10 ml live fungi or fungal necromass were blindly pipetted by a second independent scientist into 0.125 L serum bottles that were then amended with either media that included 0.25 mmol N as NaNO_2 and 0.25 mmol N as $\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3$ or media without N and mixed gently. Additional bottles containing sterile media solutions (with and without N) only and without fungi were also prepared. Oxygen status was controlled with airtight laboratory incubation vessels for all necromass, live fungi and media only samples. Headspace of each sample was evacuated and filled with either He (anaerobic) or heliox [aerobic (80% He, 20% O_2)] within 2 hr following inoculation^{18,20}. Headspace gases were quantified approximately every 6 h at 19°C using a robotic gas chromatograph (GC) fitted with electron capture and thermal conductivity detectors according to McMillan *et al.*²¹. Helium or heliox blank standards were included in each GC run. Cumulative production rates were calculated as slopes of the masses (μmol) of N_2O or N_2 measured over time (h) per g of fungal biomass. Vessels incubated in He remained anoxic with the exception of necromass, where we observed 0.05% O_2 . Vessels incubated in heliox remained above 19% O_2 with the exception of live fungi incubated without N, where 5% of the headspace O_2 was consumed. As reported previously, addition of NO_2^- inhibited O_2 consumption and CO_2 respiration by live fungi¹⁶. Fungal biomass was determined as the difference in mass with and without media after air-drying each vessel post-incubation¹⁶. For comparisons with sterile media, rates were also calculated as slopes of the masses (μmol) of N_2O and N_2 measured over time. Sterility was maintained and conditions remained constant, including pH (6.2–6.9). Sterility was tested at the end of gas sampling by pipetting $200 \mu\text{L}$ medium used in the experiment onto blood agar plates and incubating

under aerobic, anaerobic (AnaeroGen™, Thermo Scientific), and enriched CO₂ (3.5–9%; CO₂Gen™, Thermo Scientific) conditions. Further, medium was also pipetted onto yeast nutrient agar, brain heart infusion agar, and potato dextrose agar plates under aerobic conditions. No evidence of fungal or bacterial growth was observed following 3, 7, and 10 d incubations at 26 °C. Data were analysed to test for effects of O₂ and live fungi on either N₂O or N₂ production rates with a generalised linear model. We analysed only those samples amended with N because samples without N did not produce N₂O or N₂. Log transformations were employed when data did not meet the assumptions of normality. Treatments variances met assumptions of homoscedasticity. All interactions were tested and remained in the model if significant.

Di-nitrogen isotopes were evaluated in a separate experiment to assess sources of N used in abiotic production of N₂ by adding Na¹⁵NO₂ and C₅H₁₀N₂O₃ to sterile medium at neutral pH, as described above. We aimed to determine if N₂ would be produced through combination of ¹⁵NO₂ only (thus forming ³⁰N₂) or through combination of both C₅H₁₀N₂O₃ and ¹⁵NO₂ (thus forming the hybrid ²⁹N₂) for N-enriched medium relative to no-N medium only. In this experiment, we used five replicates at three levels of N: (a) no-N, (b) 0.25 mmol NaNO₂ and 0.25 mmol C₅H₁₀N₂O₃, and (c) 0.5 mmol NaNO₂ and 0.5 mmol C₅H₁₀N₂O₃. We used the same concentration of N in each N treatment but doubled the mass of N added. Vials were prepared under aerobic and anaerobic conditions as described previously and accompanied by He blanks. The aerobic experiment was conducted separately from the anaerobic experiment, which obviated testing for effects of O₂ on masses of ²⁹N₂ and ³⁰N₂. Headspace ²⁹N₂ and ³⁰N₂ were measured on a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta V, Thermo Scientific) in line with an automated gas bench interface (Thermo Gas Bench II). Precision of the isotopic analysis was <0.001atom%.

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Author Contributions

R.L.P., B.S. and G.G. designed the experiments, T.P. performed lab experiments, A.M.S.M. analysed chromatographic data, C.T. performed isotope pairing experiments, B.S.W. and G.G. cultured, and verified fungal isolates, and R.L.P. and B.S. wrote the manuscript.

Additional Information

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