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Nitrous oxide production and isotopomer composition by fungi isolated from salt marsh sediments

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The emissions of nitrous oxide (N_2O), a potent greenhouse gas and ozone-depleting agent, have been steadily increasing from coastal environments, such as salt marsh sediments, as a result of anthropogenic nutrient loading. Biotic processes, including nitrification and denitrification, are the largest sources of N_2O from salt marsh sediments. While it is assumed that the bulk of N_2O from salt marsh sediment is produced by nitrification and bacterial denitrification, recent reports suggest fungal denitrification may contribute significantly. In this study, four fungi capable of growth under sulfidic conditions were isolated from salt marsh sediments in North Inlet, South Carolina, USA. Fungal species included *Purpureocillium lilacinum, Trichoderma harzianum, Trichoderma virens*, and *Rhodotorula glutinis*, as determined by sequencing the18S and 28S rRNA genes. The isotopomer signatures of N_2O produced by these fungi were measured using isotope ratio mass spectrometry, which can be used to estimate the contribution of different sources of N_2O. Up to 22.8% of nitrite provided in growth media was converted to N_2O by fungal strains isolated from salt marsh sediments. The site preference (SP) of N_2O produced by salt marsh sediment fungi ranged from 7.5 $\pm$ 1.6‰ to 33.4 $\pm$ 1.2‰. These values are lower than the SP of N_2O from the model fungal denitrifier *Fusarium oxysporum* (37.1 $\pm$ 2.5‰), which is the SP typically used as an endmember in isotope mass balance considerations. The N_2O SP values we measured expand the range of N_2O SP used for isotope mass balances calculations to determine the relative contribution of fungi to N_2O production in salt marsh sediments.

KEYWORDS

nitrous oxide, fungi, stable isotop, site preference (SP), sulfidic, salt marsh, sediment, isotopomer

1 Introduction

Salt marshes represent one of the largest carbon sinks on Earth and effectively filter out excess nutrients, organic matter, and pollutants which would otherwise enter the ocean and potentially lead to coastal eutrophication, harmful algal blooms, and oxygen depletion (Vernberg, 1993; Teal and Howes, 2000; Burden et al., 2013). High amounts of primary production lead to the depletion of oxygen by aerobic respiration, allowing diverse anaerobic metabolisms to aid in the removal of nutrients, such as nitrate through denitrification (Kaplan et al., 1979; Kostka et al., 2002; Mcowen et al., 2017; Wu et al., 2021). Denitrification has been shown to impact water, sedimentary, and atmospheric chemistry by removing nitrate (NO_3^-) and producing nitrous oxide (N_2O) and N_2-gas (Philippot et al., 2013; Lecomte et al., 2018; Zheng et al., 2018). The recent increases in N_2O emissions from coastal environments is primarily due to the increases in anthropogenic N in riverine discharge (Murray et al., 2015; Martin et al., 2018; Al-Haj and Fulweiler, 2020; Tian et al., 2020). N_2O is of concern as it is a greenhouse gas with a warming potential 296 times higher than carbon dioxide (CO_2) and is an ozone-depleting agent (IPCC, 2014). Understanding N_2O sources in salt marshes and how the sources may change with climate change is, therefore, crucial. Multiple studies have indicated that anaerobic biogeochemical processes mediated by fungi are understudied in marine environments. This study focuses on marine fungal denitrifiers, which have been hypothesized to significantly contribute to salt marsh N_2O production (Gadd, 2006; Grossart et al., 2016; Wankel et al., 2017; Amend et al., 2019; Gutiérrez et al., 2020; Aldossari and Ishii, 2021).

Denitrification is a stepwise reaction where NO_3^- is reduced to nitrite (NO_2^-), nitric oxide (NO), N_2O, and dinitrogen gas (N_2) through a series of enzymatic reactions encoded by the following genes: respiratory nitrate reductase/periplasmic nitrate reductase (*narG*/*napA*), copper/iron containing nitrite reductase (*nirK*/*nirS*), nitric oxide reductase (*nor* in bacteria/*p450nor* in fungi), and nitrous oxide reductase (*nosZ*) (Nakahara et al., 1993; Zumft, 1997). Fungi differ from bacteria and archaea in that they lack *nosZ* (Shoun et al., 1992; Hayatsu et al., 2008). Fungal N_2O is thereby released into the environment where it can be consumed by N_2O-reducing bacteria or emitted to the atmosphere (Higgins et al., 2018).

$$NO_3^- \overset{narG/napA}{\rightarrow} NO_2^- \overset{nirK}{\rightarrow} NO \overset{p450nor}{\rightarrow} N_2O \; X \; N_2 \qquad \text{Eqn. 1)}$$

Since the turn of the century, terrestrial studies regarding N_2O emissions have shown that fungi, rather than bacteria, are a dominant source of soil N_2O production and account for up to 80% of released N_2O (Laughlin and Stevens, 2002; Ma et al., 2008). While bacterial and archaeal processes are considered the dominant source of N_2O from salt marshes, recent studies suggest fungal denitrification may contribute a greater share of N_2O emissions in coastal sediments than initially thought, similar to terrestrial counterparts (Wankel et al., 2017; Aldossari and Ishii, 2021). Recent advancements in sequencing technology and cultivation efforts have been instrumental in uncovering the estimated 10,000 species of undiscovered marine fungi, many of which may be capable of denitrification, a widespread trait amongst fungi (Jones, 2011; Maeda et al., 2015; Amend et al., 2019). Furthermore, recent studies have shown that the relative abundance of fungal denitrifiers is positively correlated with nutrient loading and is a significant N_2O source in estuaries (Kearns et al., 2019; Kim et al., 2020; Gao et al., 2022). The lack of fungal denitrifiers in recovering salt marshes has also been shown to limit bioavailable nitrogen (NO_3^-) removal, thus demonstrating the role denitrifying fungi play in salt marsh sediment biogeochemistry (Starr et al., 2022). These studies provide a basis to rethink salt marsh N_2O dynamics and study salt marsh fungi to determine the significance of fungi in acting as an N_2O source.

Isotopic approaches can be used to distinguish fungal N_2O production from other sources (Sutka et al., 2008; Rohe et al., 2014; Rohe et al., 2017; Wankel et al., 2017). N_2O is an asymmetric molecule where the centrally positioned alpha nitrogen (N^{α}) atom is bonded to the beta positioned nitrogen (N^{β}) and oxygen. Differences in isotopic fractionation between fungi, bacteria, and archaea produce changes in the positioning of light and heavy nitrogen atoms (^{14}N, ^{15}N) in the N_2O molecule (Sutka et al., 2006; Maeda et al., 2015). The intramolecular distribution of ^{15}N is defined by site preference (SP).

$$SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \qquad \text{Eqn. 2)}$$

N_2O SP is independent of both the initial isotopic composition of the substrate and changes with subsequent consumption (Toyoda et al., 2002; Sutka et al., 2006). Therefore, N_2O SP is thought to be only process-dependent and has been used as a tracer to identify the source of N_2O in terrestrial and marine environments (Butterbach-Bahl et al., 2013; Bourbonnais et al., 2017; Casciotti et al., 2018; Kelly et al., 2021). Fungal N_2O SP measured for the model denitrifying fungi *Fusarium oxysporum* (37.1 ± 2.5‰) has been used by multiple studies to estimate the contribution of fungi to N_2O production from soil and coastal sediments (Rohe et al., 2014; Wankel et al., 2017; Rohe et al., 2021; Su et al., 2021). However, studies have indicated that *F. oxysporum* is not a well-represented species in fungal communities where *Spartina* dominates, which is a common feature of salt marshes in Europe, the United States, and China (though invasive), questioning the use of this endmember in isotope mass balances in these environments (Parrondo et al., 1978; Newell et al., 1996; Buchan et al., 2002; Buchan et al., 2003; Torzilli et al., 2006; Ge et al., 2016). Furthermore, the N_2O SP of soil-isolated *F. oxysporum* may not be representative of salt marsh fungi, as saline environments are known to cause physiological responses

in fungi, with some studies suggesting fungal denitrification may be enhanced with at higher salinities (Thiem et al., 2018; Yu et al., 2019; Pérez-Llano et al., 2020; Aldossari and Ishii, 2021; Calabon et al., 2021; Jones et al., 2022). N_2O SP from salt marsh sediment fungi would therefore be more representative of fungi isolated from saline environments. A recent metabarcoding survey showed that fungi from the families *Teratosphearіaceae*, *Mycosphaerellaceae*, *Physalacriaceae*, and *Lasiosphaeriaceae* and from the orders Capnodiales and Rhytismatales dominated sediment mycobiomes in a New England salt marsh (Kearns et al., 2019). The same study found that the relative abundance of putative denitrifying fungi from the orders Sordariales and Hypocreales were the highest (Kearns et al., 2019). In this study, we isolated four N_2O-producing fungal strains from salt marsh sediments and measured their N_2O yield and SP.

2 Materials and methods

The first part of this study is designed to isolate N_2O-producing fungi from salt marsh sediments. Published studies on fungal N_2O production obtained their isolates either from a culture collection (e.g. Maeda et al., 2015) or from the environment using aerobic media (e.g. Jirout, 2015). To the best of our knowledge, we made the first attempt using anaerobic enrichment cultures to isolate fungi from salt marsh sediments, which typically become anoxic below just a few millimeters depth. Given previous reports on the presence of fungi in anoxic parts of salt marsh sediments (e.g. Kearns et al., 2019), we expect the use of anaerobic media will select for fungal lineages well adapted to anoxia. Previous studies on N_2O-producing fungi all used complex media including undefined components such as potato infusion and peptone. To select for fungi adapted to low nutrient supply, a defined mineral media recipe (detailed below) was included in our isolation efforts.

2.1 Aerobic media

Aerobic media were prepared with an artificial seawater base containing 20 g L^{-1} sodium chloride (Fisher Scientific), 3 g L^{-1} magnesium chloride hexahydrate (Fisher Scientific), 0.15 g L^{-1} calcium chloride (Fisher Scientific), 0.5 g L^{-1} potassium chloride (Fisher Scientific) in ultrapure water produced by Milli-Q® EQ 7000 Ultrapure Water Purification System (MilliporeSigma, Merck KGaA, Darmstadt, Germany). Mineral media included 10 mM 3-(N-Morpholino)-Propanesulfonic Acid (MOPS) buffer (pH = 7.2, from 1 M stock solution (209.26 g L^{-1} MOPS free acid from EMP Millipore Corp., 100 mL L^{-1} 5 M sodium hydroxide from Spectrum Chemical Mfg Corp.), 2 mM ammonium chloride (Fisher Scientific), 0.2 mM sodium sulfate (J.T. Baker), 0.146 mM dipotassium phosphate (J.T. Baker), and 0.0588 mM monopotassium phosphate (J.T. Baker), and supplemented with trace elements. The final concentration of trace elements included: 20 µM hydrochloric acid (VWR Chemicals), 7.5 µM ferrous ammonium sulfate (Fisher Scientific), 0.48 µM boric acid (Sigma Chemical Co.), 0.5 µM manganese chloride (Fisher Scientific), 6.8 µM cobalt sulfate (Sigma Chemical Co.), 1.0 µM nickel chloride (Acros Organics), 12 nM copper chloride (Acros Organics), 0.5 µM zinc sulfate (Sigma Chemical Co.), 0.15 µM sodium molybdate (Acros Organics), 25 nM metavanadate (Acros Organics), 9 µM sodium tungstate (Acros Organics), 23 nM sodium selenite (Sigma Chemical Co.). *Spartina Alterniflora* stems, collected from North Inlet salt marshes, were cleaned and chopped to about 3 mm in size, and included as a carbon substrate in vials (1% w v^{-1}). Complex media were prepared with the same recipe and two additional components, namely 2.5 g L^{-1} of yeast extract (Fluka BioChemika) and 2.5 g L^{-1} of peptone (Fluka BioChemika). Solid media were prepared by including 2% (w v^{-1}) agar (Thermo Scientific). After autoclave sterilization and cooling (20 minutes at 121°C), a mixture of penicillin-G sodium (Alfa Aesar) and streptomycin sulfate (Acros Organics) was added to reach a final concentration of 0.2 g L^{-1} to minimize bacterial growth.

2.2 Anaerobic media

The composition of anaerobic media used to isolate and maintain fungal cultures was identical to aerobic media with the following exceptions. Resazurin, 1 µg L^{-1}, (Acros Organics) was added as a redox indicator; 0.1 mM of sodium sulfide (Acros Organics) and 1 g L^{-1} of L-cysteine (Acros Organics) were included as reducing reagents. Liquid media were purged with ultra-high purity N_2 (Airgas) for 20 to 30 minutes, and 20-ml were dispensed into N_2-flushed 65-ml serum vials sealed with butyl rubber septa and aluminum crimps. After autoclave sterilization, each vial of anaerobic media was supplemented with a mixture of 0.2 g L^{-1} of penicillin-G sodium (Alfa Aesar), 0.2 g L^{-1}streptomycin sulfate (Acros Organics), and 10 µM sodium nitrite (Fisher Chemicals). For isotopic analysis (described below), the anaerobic media were amended with 100 µM NO_2^-. Anaerobic roll tubes for colony picking were prepared as described previously (Peng et al., 2018).

2.3 Isolation and maintenance of N_2O-producing fungal strains

Triplicate 30-cm sediment cores were taken from two sites, Clambank and Oyster Landing, at the North Inlet-Winyah Bay National Estuarine Research Reserve in Georgetown, South Carolina (33.35°N, 79.20°W) on June 17th, 2021 (Figure 1). The North Inlet salt marsh is dominated by *Spartina alterniflora* (Allen et al., 2014). The cores were put on ice during transportation from Georgetown to Columbia, South

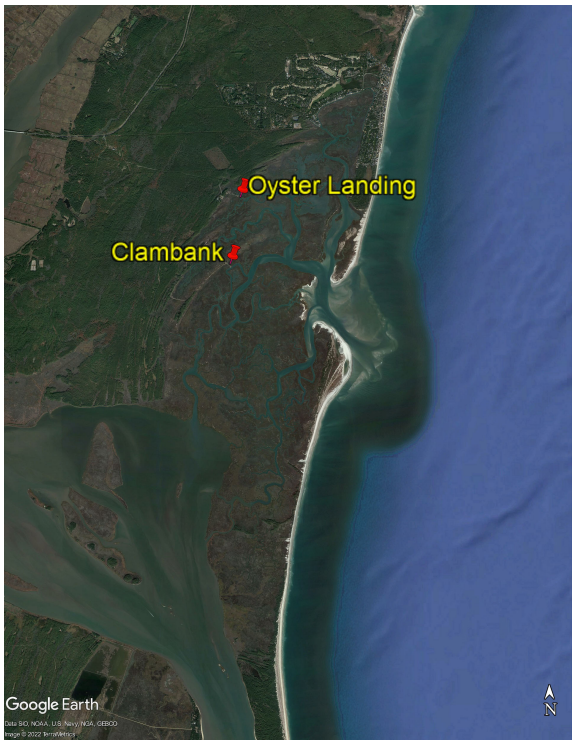

FIGURE 1
Samling locations (marked by pins) for salt marsh sediments at the North Inlet-Winyah Bay National Estuarine Research Reserve (33.35°N, 79.20°W). The image is produced using Google Earth Pro with data from SIO, NOAA, U.S. Navy, NGA, and GEBCO satellites.

Carolina, where initial enrichment culture inoculation occurred on the same day.

As marine fungi are typically hard to isolate (Edwards et al., 2017; Amend et al., 2019), we used the common plate dilution technique (Warcup, 1950) to isolate N_2O-producing fungi that are facultative anaerobes. Sediments from 1 cm and 10 cm depths were diluted in aerobic media and were used to streak agar plates. After three rounds of colony picking, isolated fungal cultures were inoculated into anaerobic media and screened for the ability to grow under sulfidic conditions and produce N_2O. Two fungal strains isolated using complex media and one fungal strain isolated using mineral media demonstrated high potential for N_2O production.

Anaerobic enrichment cultures of salt marsh sediment fungi were established in an Aldrich® AtmosBag (SKU Z555525) pre-flushed three times with ultra-high purity nitrogen (N_2). Approximately 0.1 g of sediment was placed into the serum vials containing anaerobic media using aseptic techniques. All enrichment cultures were screened for N_2O production in the headspace using a gas chromatograph (described below) equipped with an electron capture detector (ECD). N_2O-producing enrichment cultures were selected for fungal isolation using the anaerobic roll tube technique (Peng et al., 2018). One N_2O-producing fungal strain was isolated from anaerobic enrichment cultures. This was the first known case of anaerobic marine fungi isolated under sulfidic conditions.

Isolated fungal cultures were maintained in anaerobic batch cultures, which were transferred to fresh media every 7-10 days. When inoculating fresh media, 0.2 mL of media containing fungal cells were drawn from the inoculum culture using techniques that avoided oxygen contamination. For yeasts, the batch culture serving as the inoculum was thoroughly homogenized before inoculation. For filamentous fungi, we performed careful visual inspection to include similar amounts of filamentous fungi in each 0.2-ml inoculum to ensure replicate cultures had similar amount of initial biomass.

2.4 DNA extraction and rRNA gene sequencing

Fungal cells were harvested by centrifugation and DNA were extracted using the DNeasy Plant Pro kit (QIAGEN). The quantity and quality of the DNA were measured using a Nanodrop 2000C spectrophotometer (ThermoFisher Scientific).

The small (SSU) and large (LSU) subunits of the rRNA genes were amplified using the primers Fun18S1 (5'-CCATG CATGTCTAAGTWTAA-3') (Lord et al., 2002) and FR1 (5'-ANCCATTCAATCGGTANT-3') (Vainio and Hantula, 2000) targeting the V1 to V8 regions of the SSU rRNA gene and LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 targeting the D1 to D3 regions of the LSU rRNA gene (Tedersoo et al., 2015). The PCR reactions were performed using Phusion® high-fidelity DNA polymerase (New England BioLabs, M0530, Ipswich, MA). The thermal cycle started with 30 seconds at 98°C, followed by 30 cycles of 10 seconds at 98°C, 30 seconds at 60° C, and 30 seconds at 72°C. The final elongation at 72°C was 5 minutes long. The PCR products were gel purified using Zymo DNA Clean & Concentrator -5 following the manufacturer's protocol and sent to Etonbio (Research Triangle Park, North Carolina, USA) for Sanger sequencing.

SSU and LSU sequences were searched against the NCBI nt database using the web portal for blastn (Johnson et al., 2008). Sequences for phylogenetic analysis were retrieved from the NCBI GenBank. Sequences were aligned using MUSCLE and manually trimmed using MEGA version 11 (Edgar, 2004; Tamura et al., 2021). Maximum-likelihood trees were constructed using FastTree 2.1 with default settings (1,000 bootstrap replicates, Jukes-Cantor model) (Price et al., 2010). The tree was then imported to Interactive Tree of Life (iTOL v6.6) for visualization (Letunic and Bork, 2019).

2.5 Measurements of headspace gas composition

SRI Greenhouse Gas Monitoring Gas Chromatograph, model 8610-0040, equipped with an ECD and a flame ionized detector (FID) was used to measure N_2O and CO_2 headspace gas production. Prior to gas extraction, 5 mL of ultra-high purity N_2 gas was added to vials with a gas-tight syringe and a sterilized needle to avoid air contamination. A gas-tight syringe and a sterilized needle was used to extract 5 mL of headspace gas, which was manually injected into the sampling port on the instrument. Ultra-high purity N_2 gas was used as the carrier gas, maintained at 30 psi. The column oven temperature was set at 90 °C. The ECD and FID, coupled to a methanizer for CO_2, measurements, were set at 300 °C. N_2O standard curves were constructed using 5 ppm, 10 ppm, and 100 ppm N_2O (GASCO, Cal Gas Direct Incorporated, Huntington Beach, California, USA). CO_2 standard curves were constructed using 1%, 5%, and 10% CO_2 calibration standards from GASCO. Multiple reference samples served as controls, including uninoculated media and N_2O producing fungal cultures terminated with concentrated sodium hydroxide and purged with N_2.

2.6 N_2O isotopic analysis

Analysis of N_2O stable isotopes and isotopomers were made using an Elementar Americas Inc. isoprime precisION continuous flow, multicollector, isotope-ratio mass spectrometer (CF-MC-IRMS) equipped with a custom purge-and-trap and gas extraction systems, as described in Charoenpong et al. (2014). The CF-MC-IRMS has the necessary collector configuration for simultaneous determination of masses 30, 31 for the NO^+ fragment of N_2O (determination of $\delta^{15}N^{\alpha}$) and 44, 45, and 46 (determination of bulk $\delta^{15}N$ and $\delta^{18}O$). Cultures for isotopic analysis had a final NO_2^- concentration of 100 µM. A gas tight syringe was used to extract 3-mL to 5-mL headspace gas, which was then injected into the CF-MC-IRMS injection port. N_2O was purified in a purge and trap system under helium continuous flow (40 mL/min), CO_2 was chemically removed, and H_2O vapor was eliminated with both chemical and cryogenic traps. N_2O was cryofocused with two liquid N_2 traps and passed through a capillary GC column prior to IRMS analysis. These latter steps, including GC column backflushing to eliminate interferences in the SP determination, were nearly identical to what was described by McIlvin and Casciotti (2010). Helium flow was optimized to achieve quantitative extraction and reproducible results, even at low N_2O concentrations. N_2O concentrations in our samples were calculated from relative peak heights between the samples and a dilution series of pure N_2O gas mixtures (in N_2) of known N_2O concentrations (500 and 7500 ppm). The reproducibility of bulk $\delta^{15}N$ and $\delta^{18}O$ and SP as well as any instrumental drift were determined from measurements of an internal reference gas distributed through the analytical run. The measurements were calibrated from a four-point calibration correction using N_2O standards covering a large range of SP (-92.7‰ - 18.9‰), as well as bulk $\delta^{15}N$ and $\delta^{18}O$ composition calibrated by S. Toyoda (Tokyo Institute of Technology), and obtained from Joaquim Mohn (EMPA, Swiss Federal Laboratories for Materials Science & Technology). These standards were analyzed in duplicate for each run to quantify the scrambling effect, potential offset and iteratively solve for the different calibration parameters (Frame and Casciotti, 2010; Mohn et al., 2014). Correction for isobaric interference from ^{17}O was included in these procedures. Standard deviations for triplicate measurements of our N_2O standards were typically below 0.1‰ for $\delta^{15}N$-N_2Obulk, 0.2‰ for $\delta^{18}O$-N_2O and 1‰ for SP, which were comparable to values reported by Mohn et al. (2014).

3 Results

3.1 Phylogeny and classification

The four fungal strains we isolated and investigated in this study were *Purpureocillium lilacinum BL2022*, *Trichoderma virens XP2022*, *Trichoderma harzianum MB2022*, *and Rhodotourla glutinis MT2022*, as identified by blastn and phylogenetic analysis (Supplementary Figures S1, S2, and S3; Supplementary Table 1). *T. harzianum* and *T. virens* were closely related to terrestrial and marine derived species, such as *T. polysporum* and *T. citrinoviride*. *R. glutinis MT2022* was closely related to other species which have been isolated from both marine (*R. diobovata)* and terrestrial environments (*R. graminis* and *R. babjeavae*). *P. lilacinum* was closely related to terrestrially derived species but was distinct from *P.lilacinum CBS284.36*. *T. harzianum* was the only strain isolated using mineral media (Table 1), which did not contain yeast extract or peptone.

3.2 N_2O and CO_2 production from salt marsh sediment fungi

P. lilacinum produced the greatest amount of N_2O and CO_2 (22.8 ± 7.8 nmol of N_2O and 179 ± 24 µmol of CO_2, Figures 2A, E). *T. virens*, *T. harzianum*, and *R. glutinis*. produced an average of 17.4 ± 5.4 nmol, 4.45 ± 0.48 nmol, and 4.24 ± 1.57 nmol of N_2O (Figures 2B–D) and 39.0 ± 20.0, 5.00 ± 0.44, and 88.1 ± 37.5 µmol of CO_2 (Figures 2F–H), respectively. The production of N_2O by *P. lilacinum* did not plateau until the 22nd day. The production of N_2O by *T. virens* plateaued after the 16th day. The production of N_2O by *T. harzianum* and *R. glutinis* reached a maximum in only two to three days. N_2O yield for each fungal

TABLE 1 The mean and standard deviation (n = 3) of N_2O site preference (SP) produced by salt marsh sediment fungal strains from this study.

Species	Media type	Mean (‰)	SD (‰)
T. harzianum	Mineral	7.46	1.57
T. virens	Complex	30.56	2.09
P. lilacinum	Complex	31.00	1.31
R. glutinis	Complex	33.41	1.20

The composition of complex and mineral media was the same except that yeast extract and peptone were included in complex media.

strain was calculated using the average maximum N_2O produced, where N_2O yield is the fraction of NO_2^-N converted to N_2O-N (see Supplementary Information for additional details). *P.lilacinum, T. virens*, *T. harzianum*, and *R. glutinis* had respective yields of 22.8 ± 7.8%, 17.4 ± 5.4%, 4.45 ± 0.48%, and 4.24 ± 1.57%. There was a significant correlation between N_2O and CO_2 production ($p< 0.01$) for each replicate culture of the filamentous fungi *P. lilacinum*, *T. virens*, and *T. harzianum* (Figure 3). N_2O and CO_2 production by *R. glutinis* were not significantly correlated.

To further explore the effect of concentration on fungal N_2O production, the species with the highest cumulative N_2O production, *P. lilacinum*, was cultivated with 0 µM, 10 µM, 100 µM NO_2^- in triplicate. When grown without NO_2^-, *P. lilacinum* produced <1 nmol of N_2O (Figure 4). The addition of 100 µM to growth media resulted in a >10-fold increase in N_2O production for two of the three replicates when compared to the three replicates grown with 10 µM NO_2^- (285.0 ± 20.1 nmol and 22.8 ± 7.8 nmol of N_2O, respectively).

3.3 Site preference of N_2O from salt marsh sediment fungi

Site preference (SP) values of N_2O from salt marsh sediment fungi ranged from 7.49 ± 1.57‰ for *T. harzianum*, to 33.41 ± 1.20‰ for *R. glutinis* (Table 1), which were all lower than N_2O SP values (37.1 ± 2.5‰) measured from the model fungal denitrifier *F. oxysporum* (Sutka et al., 2008).

4 Discussion

4.1 N_2O production by salt marsh sediment fungi

We used both aerobic and anaerobic cultivation techniques to isolate N_2O-producing fungi from salt marsh sediments in North Inlet, South Carolina, USA. The aerobic technique is similar to previous efforts to isolate N_2O-producing fungi from

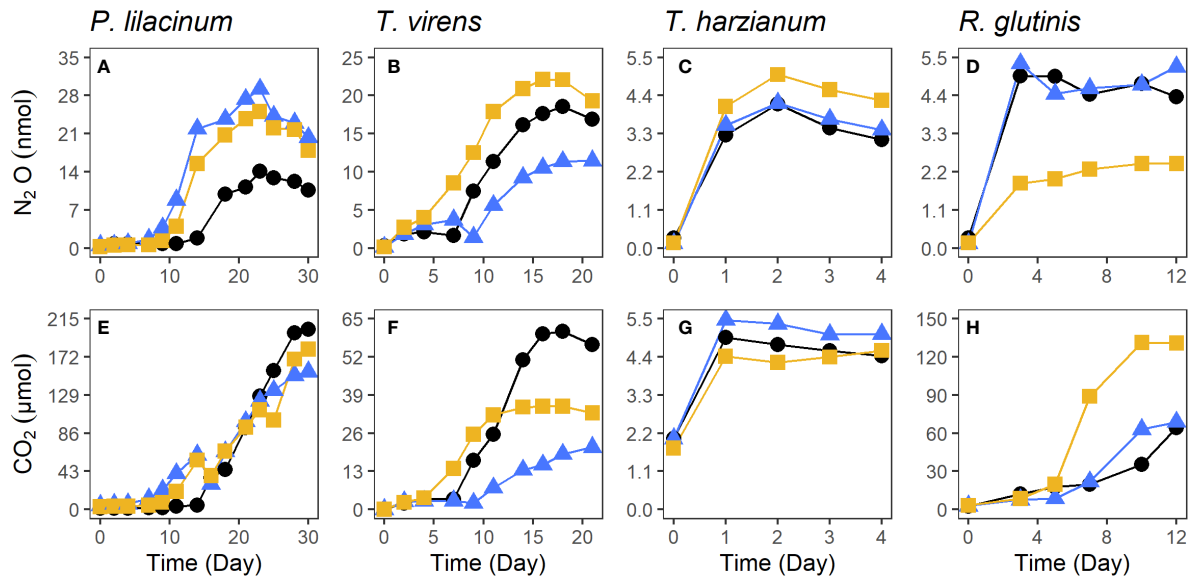

FIGURE 2
The production of N_2O and CO_2 by *P. lilacinum* **(A, E)**, *T. virens* **(B, F)**, *T. harzianum* **(C, G)**, and *R. glutinis* **(D, H)** isolated from salt marsh sediments. Each color and symbol combination represents one of the three replicates.

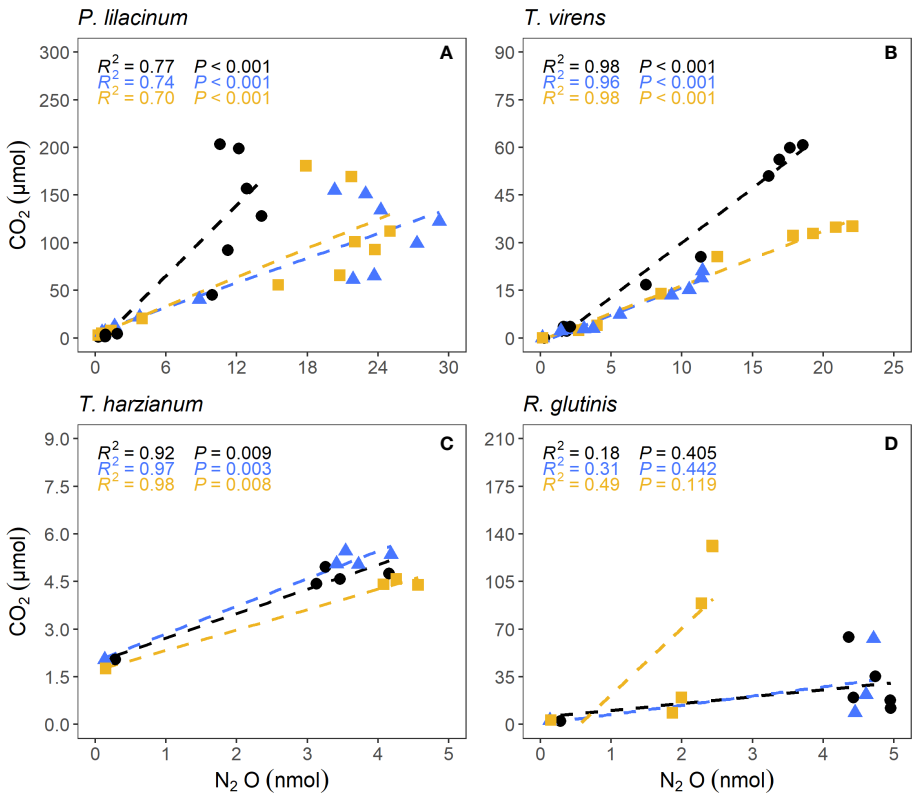

FIGURE 3
Correlation between N_2O and CO_2 production by salt marsh sediment fungi *P. lilacinum* **(A)**, *T. virens* **(B)**, *T. harzianum* **(C)**, and *R. glutinis* **(D)**. Each color represents one of the three replicates.

soil (Jirout et al., 2013; Mothapo et al., 2013) except that our media were prepared with a seawater base, which selected for fungi adapted to salinity in the range of 30 to 35 ppt. All filamentous species (*P. lilacinum*, *T. virens*, and *T. harzianum*) we isolated from salt marsh sediments have been isolated from soil and shown to produce N_2O (Lavrent'ev et al., 2008; Jirout, 2015; Maeda et al., 2015). A comparative genomics studies showed that all these three species possess the diagnostic gene for fungal denitrification, the cytochrome P450 nitric oxide reductase (*P450nor*) (Higgins et al., 2018). The positive correlations between N_2O and CO_2 production by filamentous fungi indicate respiratory denitrification is responsible for N_2O production. Variability in N_2O production, within individual strains' cultures, is likely due to small differences in the number of cells in the inoculum between triplicates. While this study was not intended as an exhaustive search for N_2O-producing fungi from salt marsh sediments, our results indicate that at least a subset of N_2O-producing fungi from terrestrial environments (Mothapo et al., 2015) are present in salt marsh sediments and have the potential to produce N_2O at high yields.

The ability to produce N_2O was widespread among the hundreds of strains tested by Maeda and colleagues (Maeda et al., 2015), but only one (Mucorales) of the 70 N_2O-producing strains in their study was not from the phylum Ascomycota. A survey of *P450nor* in over 700 fungal genomes also showed that this diagnostic gene for fungal denitrification was primarily found in Ascomycota (Higgins et al., 2018). While Ascomycota seem to be the main lineage capable of N_2O production, fungi from other phyla, particularly early diverging lineages, are poorly represented in these studies, partially due to their low abundance in terrestrial environments (Berbee et al., 2017). There is evidence that early diverging fungi play a more important role in marine environments than in terrestrial environments, and their potential to produce N_2O remains to be examined (Peng and Valentine, 2021).

To the best of our knowledge, this is the first study that used strictly anaerobic techniques to isolate fungi from salt marsh sediments, where oxygen is typically depleted a few millimeters below the surface (Peng et al., 2021). The only N_2O-producing

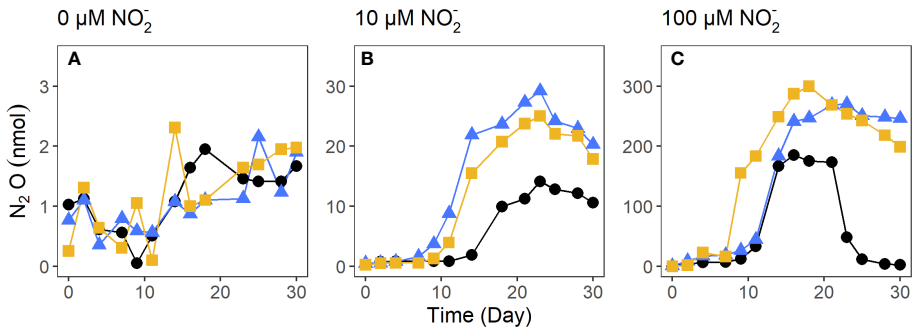

FIGURE 4
N_2O production by the salt marsh sediment fungi *P. lilacinum* cultivated with 0 μM **(A)**, 10 μM **(B)**, and 100 μM **(C)**. Each color and symbol combination represents one of the three replicates.

fungus isolated anaerobically was the basidiomycetous yeast *R. glutinis*, which is not known to possess *P450nor* in its genome (Higgins et al., 2018), but it does possess a fungal nitrite reductase (fungal *nirK*). The decoupling between N_2O and CO_2 production in *R. glutinis* cultures (Figure 3) suggests that N_2O production by *R. glutinis* may not be from respiratory denitrification. Instead, energy production by *R. glutinis*, an oleaginous yeast, was likely from fermentation (Yeeh, 1999; Xue et al., 2008). This could also explain the low N_2O yield by *R. glutinis* (3.25 ± 1.20%). While this is the first report of N_2O production by *R. glutinis* grown under sulfidic conditions, an unidentified *Rhodotorula* species grown under aerobic conditions produced N_2O after it reached stationary phase (Bleakley and Tiedje, 1982). Therefore, regardless of the mechanism of N_2O production by *R. glutinis*, it can contribute significantly to N_2O production from salt marsh sediments where the redox conditions fluctuate due to daily tides. A strong positive correlation was observed between N_2O production rate and the relative abundance of *Rhodotorula* in estuarine sediments in Xiamen, China (Su et al., 2021).

4.2 The influence of cultivation conditions on fungal N_2O production

Nearly all previous studies on N_2O production by fungi used media containing 10 mM of NO_2^- (Shoun et al., 1992; Mouton et al., 2012; Jirout et al., 2013; Mothapo et al., 2013; Maeda et al., 2015; Zou et al., 2021), which is orders of magnitude higher than the *in situ* NO_2^- concentrations in soil or marine sediments. It is known that different nutrient levels can cause different physiological responses in fungi, including changes which affect the ability of the cell to transport nutrients and degrade carbon (Ozcan and Johnston, 1999; Zaman et al., 2008). In this study we cultivated N_2O-producing fungi using sulfidic media containing NO_2^- at a level (10 μM) much closer to *in situ* conditions. Consequently, the total amounts of N_2O production in our study were lower than previously reported value, but the yield of N_2O from NO_2^- by salt marsh sediment fungi (up to 22.8 ± 7.8%) was comparable to some of the highest levels in previous studies (Mothapo et al., 2013; Jirout, 2015; Maeda et al., 2015). Furthermore, the salt marsh sediment fungi were cultivated under sulfidic conditions, demonstrating their relevance in N_2O production even at sulfidic depths of the sediments.

The ~10-fold increase in both N_2O and CO_2 production by *P. lilacinum* grown with 100 μM NO_2^- (compared to 10 μM) further supports the notion that respiratory reduction of NO_2^- was the primary mechanism for N_2O production, although we cannot rule out the possibility that secondary metabolisms also contribute to N_2O production (Higgins et al., 2018). Such a response by *P. lilacinum* implies that fungal N_2O production from salt marsh sediments will scale linearly with nutrient inputs from anthropogenic sources. A recent report demonstrated the drastic increase in N_2O production from salt marsh sediments under long-term fertilization (Peng et al., 2021), and fungi may have played a major role in the observed N_2O production by the bulk sediment community.

4.3 Site preference of fungi isolated from salt marsh sediment

Stable isotope mass balance has become a useful tool to determine the contribution of fungi to total N_2O production (Wankel et al., 2017; Rohe et al., 2021; Su et al., 2021). This approach constrains the contribution of different sources and sinks of N_2O by measuring N and O isotopes and isotopomers of N_2O from a complex system, which requires the knowledge of endmembers for each pathway (e.g. bacterial denitrification,

fungal denitrification). However, studies employing this method so far have relied primarily on the N_2O isotopomer signature determined for the model organism *F. oxysporum* (Sutka et al., 2008), which is not necessarily representative of N_2O produced by marine fungi from salt marsh sediments. A recent study using an isotope mass balance approach found that the site preference value and $\delta^{18}O$ of N_2O from estuarine sediments sometimes exceeded the values for the fungal endmember based on *F. oxysporum* (Su et al., 2021). We interpret this as additional evidence for the need to evaluate the N_2O isotopologue composition produced by fungi from salt marsh sediments.

A study on soil fungi showed that N_2O SP ranged from 15.8‰ - 37.1‰, depending on the species (Maeda et al., 2015). Yet, work by Maeda and colleagues (Maeda et al., 2015) has since not been used in any published isotope mass balances calculations. Our findings add to evidence presented by Maeda et al. (2015) that N_2O SP depends on the fungal isolate at the species and even strain level. We further present evidence that N_2O SP may differ based on growth conditions, even for fungi of the same species. N_2O SP values measured by Maeda et al. (2015) for several strains of *T. harzianum* ranged from 30 - 33.4‰. In contrast, the SP values of N_2O produced by the salt marsh sediment *T. harzianum* from this study (7.46 ± 1.57‰) was much lower, and was the lowest fungal N_2O SP reported to date. This may be attributed to two factors. Firstly, the *T. harzianum* isolated from salt marsh sediments in this study is the first and only N_2O-producing fungal culture grown on mineral media to the best of our knowledge. It has been shown that different organic nitrogen sources can impact N_2O emissions, though little is known about how N_2O SP values would be impacted (Pelster et al., 2012). Secondly, fungal culture media we prepared did not include any soluble sugars (e.g. dextrose), which was a staple ingredient in all previously published studies (Sutka et al., 2008; Rohe et al., 2014; Maeda et al., 2015). Instead, the stems of *Spartina alterniflora* (lignocellulose) was provided as the sole carbon source to *T. harzianum* grown on mineral media. It has recently been shown that the type of carbon substrates used to cultivate freshwater bacteria has a significant impact on the SP values of bacterial N_2O (Li et al., 2022). It is possible that this is true for N_2O-producing fungi as well, though future work is needed to verify.

Along with *T. harzianum*, other salt marsh sediment fungi in this study produced N_2O SP values lower than that from the model fungal denitrifier *F. oxysporum* (Sutka et al., 2008), indicating that it is inaccurate and oversimplifying to use the N_2O SP from one single strain as an endmember for stable isotope mass balance calculations. Many of the N_2O SP values measured from core incubations by Wankel and colleagues (Wankel et al., 2017) are within the range of the N_2O SP values reported in this study and by Maeda et al. (2015). Future work is needed to determine how carbon and nitrogen substrate types influence fungal N_2O SP values. While the range of fungal N_2O SP values overlaps with the N_2O SP range from abiotic N_2O production, abiotic denitrification is favored in high pH conditions with solid iron (III) or copper (II) catalysts (Zhu-Barker et al., 2015) and hence irrelevant or negligible in our cultures (pH buffered at 7.2) and in salt marsh sediments (typically < 7.5). Given the wide range of fungal N_2O SP values, the selection of endmember is therefore critical in studies using stable isotope mass balance calculations. The endmember selection can be informed by metabarcoding analysis of the fungal community in general (e.g. targeting the rRNA gene) and the functional genes for fungal denitrification (e.g. *nirK* gene) (Long et al., 2015; Chen et al., 2016; Maeda et al., 2017).

Data availability statement

The datasets presented in this study are deposited at NCBI GenBank with accession number PRJNA901534. The code to generate figures and calculations implemented in R is deposited at https://github.com/birchmaxwell/SaltMarshFungiN2O.

Author contributions

BL-M is a PhD student supervised by XP and AB. Samples were collected and maintained by BL-M, SL, SS, HB, and XP. BL-M, SS, HB, and JM conducted headspace gas analysis. BL-M and AB conducted IRMS analysis. XP and BL-M conducted sequencing analysis. BL-M conducted data analysis and led the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1098508/full#supplementary-material

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