Investigating the biological mechanism of N_2O emissions from arid southern Californian drylands

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ABSTRACT

Nitrous oxide (N_2O) is a powerful greenhouse gas, each molecule capable of warming the atmosphere 273 times more effectively than CO_2. Arid soils that have been rewetted by rainfall events can produce some of the highest instantaneous N_2O emission rates recorded globally. Recent work has shown that the majority of these emissions are biologically produced. While these emissions have classically been attributed to bacterial and fungal denitrification catalyzed by catabolic nitric oxide (NO) reductases (e.g. NOR), measured N_2O isotopic fingerprinting (site preference, SP) more closely matches flavohemoglobin enzymes involved in nitric oxide detoxification (e.g. Fhp). Analysis of the microbial community of the site demonstrates that fhp is significantly more phylogenetically abundant than nor. We hypothesize that NO detoxification pathways are responsible for the initial pulse of N_2O production after rainfall, with denitrification only becoming dominant after a few hours. N_2O production is only triggered once some critical saturation with the water is reached, suggesting that the soil community has to receive enough water to become anaerobic. Using coupled measurements of oxygen and N_2O concentration in soils, we show that N_2O production begins only once the added water depletes the soil of oxygen. Initial measurements of N_2O production from *Pseudomonas* synxantha, a bacterium isolated from soil, demonstrate clear differences in the timing and quantity of gas production following rewetting via the detoxification and denitrification pathways. We thus suggest that previously overlooked detoxification pathways may play key roles in observed biogeochemical events, as appears to be the case with soil N_2O emissions.

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Chapter 1: Background

IMPORTANCE

Microbial processes are largely absent from global Earth system models, yet their incorporation has been shown to significantly alter long-term predictions for ecosystem functions like carbon sequestration and organic matter turnover (1). In the context of the nitrogen cycle specifically, microbial life is important in controlling the flux of nitrogen into the atmosphere as the gases nitrous oxide (N_2O) and nitric oxide (NO), as well as its drawdown from atmospheric nitrogen gas (N_2) to ammonia (NH_3) (2, 3). N_2O is one of the most important greenhouse gases contributing to climate change, as an important ozone-depleting emission (4) and a significant contributor to radiative forcing (5). Soils are a key environment that hosts microorganisms contributing to these emissions: emissions of NO and N_2O from soil are similar in magnitude to fossil fuel emissions of NO_x (5, 6). However, the precise mechanisms producing N_2O within these microbes are not yet fully understood, and there is a focus on assimilatory or dissimilatory metabolic processes (2, 3, 7, 8). Specifically, the detoxification of NO that produces N_2O is not described, much less constrained, in any of these studies. A recent study performed on pure cultures of *Pseudomonas aeruginosa*, a bacterium that has the capacity for both denitrification and NO detoxification, has shown that NO detoxification is the dominant pathway for N_2O production when a system is rapidly shifted from aerobic to anoxic conditions (9), which happens frequently in the environment. This motivates the idea that NO detoxification pathways have been overlooked in studies of nitrogen biogeochemical cycling, and understanding their role in N_2O production can have important ramifications for improving our understanding of this important greenhouse gas.

INTRODUCTION

Nitrous oxide (N_2O) is a long-lived greenhouse gas implicated in both stratospheric ozone depletion and global warming (4, 5). N_2O is the third-most important contributor to current radiative forcing, with each molecule capable of warming 273 times more effectively than carbon dioxide (5, 8). Natural sources of N_2O currently dominate emissions (9.7 Tg N yr^{-1}), having a slightly higher flux than anthropogenic sources (7.3 Tg N yr^{-1}) (8). Of these natural sources, soils produce the highest flux of N_2O, with their outputs of nitric oxide (NO) and N_2O several times that of fossil fuel emissions of NO_x (6, 8). These soil N_2O emissions have been demonstrated to be primarily biologically produced (5, 10). The fluxes of nitrogen into and out of these microbial processes are not well parametrized, which hinders broader predictions of microbial activity at global scales (11). Such estimates would be essential for

including microbial processes in climate science models of the fluxes of these gases, which would improve our confidence in global biogeochemical model predictions (1).

Nitrogen biogeochemical cycling is heavily controlled and impacted by microbially catalyzed redox reactions (12). There are six distinct processes currently known that are commonly invoked to describe the role of microbial transformations in the nitrogen cycle: dinitrogen (N_2) gas fixation, ammonia (NH_3) assimilation, ammonification, nitrification, denitrification, and anaerobic ammonium oxidation (anammox) (3). The processes relevant to N_2O production are nitrification and denitrification. Nitrification occurs in aerobic conditions and describes the conversion of ammonium (NH_4^+) to nitrate (NO_3^-). Both nitric oxide (NO) and N_2O are produced as intermediates during this nitrification process (2, 3, 7). Denitrification occurs in anaerobic conditions and describes the reduction of NO_3^- to N_2, which also produces both NO and N_2O as intermediates (2, 3). The "hole-in-the-pipe" model originally described by Firestone and Davidson in 1989 (13) has been used to model the gaseous nitrogen emissions from soils by describing the processes of nitrification and denitrification as leaky pipes that lose NO and N_2O at some rate that is determined by soil moisture level (2). This idea relies on the concept that water in the soil affects the transport of gases, which determines the rates of oxygen diffusion into the soil and NO or N_2O escape out of the soil.

However, these attempts to account for biological N_2O production focus on reactions involved in microbial growth, neglecting a class of enzymes that produce N_2O during the detoxification of NO: flavohemoglobin proteins (e.g., Fhp/Hmp/Yhb – henceforth referred to as "Fhp") (14). In bacteria, these proteins are approximately four times more phylogenetically widespread than the enzymes used to produce N_2O in denitrification, nitric-oxide reductases (NORs) (9). While these Fhp enzymes are already known to be of interest in clinical settings due to the role of NO in immune systems (14), they have yet to be shown to be relevant to larger-scale phenomena in environmental settings. Recent work by Wang and Lonergan *et al.* (9) found distinct isotopic fingerprints for these two classes of enzymes, enabling accurate tracking of the sources of environmentally produced N_2O. These fingerprints are in the form of isotopic "Site Preference" (SP) values, which describe the enrichment of natural ^{15}N into the central (α) nitrogen position relative to the terminal (β) position (15). SP values reflect the reaction mechanism that produces the molecule of interest (16), so are likely conserved within classes of enzymes but distinct between them. SP measurements of N_2O produced by pure cultures of *Pseudomonas aeruginosa* reveal the Fhp SP to be 10.45 ± 2.17‰ and the NOR SP to be -2.60 ± 5.41‰ (9).

Subsequent experiments performed with pure cultures of *P. aeruginosa* revealed that NOR dominated in N_2O production during long-term anaerobic growth, while Fhp was responsible for N_2O emissions when cells experienced rapid, increased NO concentrations under anoxia, such as when a culture is quickly shifted from aerobic to anoxic conditions (9). Arid soils after rainfall represent an anoxic environmental condition in which NO concentrations may increase rapidly, as NO is transiently produced by a variety of microbial and abiotic processes (13, 17). A previous study involving an experimentally induced drought in a Norway spruce forest revealed a spike in N_2O emissions following soil re-wetting (18), which has been suggested to be a product of the accumulation of nutrients during the drought and reactivation of the microbes with the addition of water (2). An alternative hypothesis is that drought led to the accumulation of NO, which microbes in the

soil were subjected to in combination with hypoxia once the soil had been re-wetted, leading to N_2O production from Fhp proteins. A more recent study on soils in the southern California desert also found high N_2O fluxes following re-wetting. These N_2O pulses began within 15 minutes of wetting and had SP measurements of 12.8 ± 3.92‰ (10). These emissions have classically been attributed to a combination of bacterial and fungal denitrification (10), as fungal denitrification produces N_2O with a high SP (around 32‰) (9). However, an alternative hypothesis is that these high SP N_2O emissions are produced by Fhp. Fhp is both phylogenetically more abundant than NOR (9) and is, unlike NOR, expressed under aerobic conditions, as it is important for cell detoxification of reactive oxygen species (19, 20).

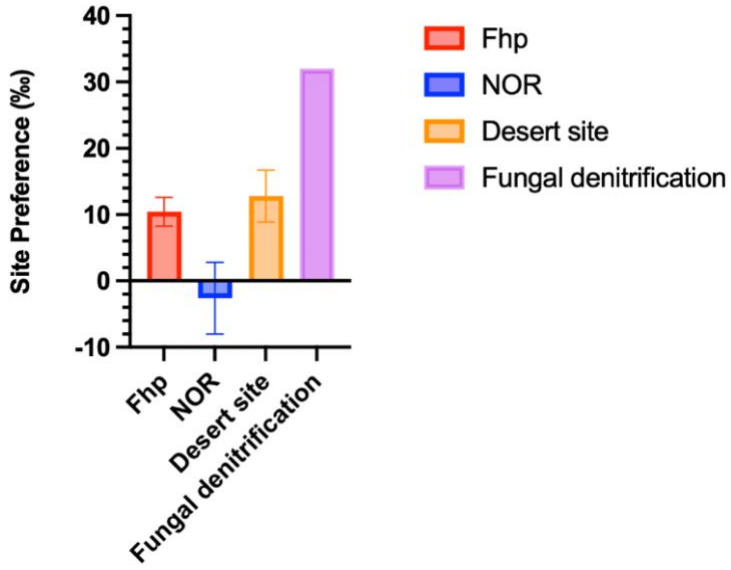

Figure 1. Site preference values of N_2O produced by the Fhp, NOR, and fungal denitrification enzymes alongside the measured N_2O SP from the southern Californian desert soil following rewetting. Data adapted from (9, 10).

We thus hypothesize that Fhp proteins have a significant role in environmental N_2O production when a soil system is rapidly shifted to anoxia, such as during the initial stages of re-wetting post-drought. However, if a system remains anoxic for an extended period, denitrification machinery will be expressed and become dominant in N_2O production, as it is both thermodynamically and kinetically favored in competing for NO (19). Consequently, we set out to identify specific conditions in which N_2O production is triggered in re-wetted soil and whether there is a distinction between the pulse of N_2O produced by Fhp in the detoxification of NO and that of NOR as it performs dissimilatory denitrification.

Chapter 2: Methodology

FIELD SAMPLING

Soil used in experiments. All soil was sampled from four sites (labeled 1 to 4) from the Boyd Deep Canyon Desert Research Center, part of the University of California Natural Reserve System. Since 1961, this site has received an average of 13.5 cm of rain per year, and the average maximum temperature of July, the hottest month, is 39.9 °C (21). Sampling was performed from soils underneath Creosote shrubs (*Larrea tridentata*) by Dr. Pete Homyak's group at UC Riverside. Further details regarding the sampling site can be found in their paper (22).

Field measurements of N_2O and proteins. In late August 2024, a re-wetting experiment was performed in the UC Boyd Deep Canyon Reserve. Four creosote shrubs were chosen as the site for this experiment. At the base of each shrub, two 20-cm diameter PVC collars were installed. 50 mL of NanoPure water was added to each collar and the collar was removed from one of the two rings at each site once the water had soaked into the ground. Samples of gas emissions and soil for proteomics were taken seven times during the experiment: 10 minutes prior to re-wetting, and 10, 20, 40, 60, 120 (2 hours), and 360 (3 hours) minutes after re-wetting. At each measurement, a cap was placed over the soil ten minutes before the measurement and then 25 mL of gas were removed from the chamber using a syringe and added to a pre-evacuated 20 mL Restek vial. As a backup, 1 L of gas was subsequently removed and added to a 1 L gas bag. About 2 to 5 grams of soil were removed from the parallel ring at each shrub and added to a prepared bead-beating cell lysis tube, which was kept on ice. An additional soil sample was taken from the parallel ring for measurement of soil moisture and ion content at the pre-wetting measurement, 40 minutes post-wetting, and 2 and 3 hours post-wetting.

DNA EXTRACTION AND 16S ANALYSIS

DNA extraction and amplification. DNA extractions were performed on all samples using the standard DNA extraction kit DNeasy PowerSoil Pro (Qiagen, Hilden, Germany) with the following modifications: About 250 mg of soil was added to each bead beat tube. Samples were vortexed for 10 minutes. Samples were eluted in 75 μL of the provided elution buffer. DNA concentrations in each sample were quantified using a NanoDrop. Sequences were amplified using 16S primers via PCR and sent for sequencing.

Illumina MiSeq sequencing of 16S rRNA gene. The V4-V5 region of the 16S rRNA gene was amplified using archaeal/bacterial primers with Illumina (San Diego, CA, USA) adapters on 5' end (515F 5'-TCGTCGGCAGCGTCAG ATGTGTATAAGAGACAG-GTGYCAGCMGCCGCGGTAA-3' and 926R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-CCGYCAATTYMTTT RAGTTT-3'). PCR reaction mix was set up in duplicate for each sample with Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs, Ipswich, MA, USA) in a 15 µL reaction volume according to manufacturer's directions with annealing conditions of 54 °C for 30 cycles. Duplicate PCR samples were then pooled and barcoded with Illumina Nextera XT index 2 primers that include unique 8-bp barcodes (P5 5'-AATGATACGGCGACCACCGAGATCTAC AC-XXXXXXXX-TCGTCGGCAGCGTC-3' and P7 5'-CAAGCAGAAGACGGCATACGAGA T-XXXXXXXX-GTCTCGTGGGCTCGG-3'). Amplification with barcoded primers used Q5 Hot Start PCR mixture but used 2.5 µL of product in 25 µL of total reaction volume, annealed at 66 °C, and cycled only 10 times. Products were purified using Millipore-Sigma (St. Louis, MO, USA) MultiScreen Plate MSNU03010 with vacuum manifold and quantified using ThermoFisher Scientific (Waltham, MA, USA) QuantIT PicoGreen dsDNA Assay Kit P11496 on the BioRad CFX96 Touch Real-Time PCR Detection System. Barcoded samples were combined in equimolar amounts into single tube and purified with Qiagen PCR Purification Kit 28104 before submission to Laragen (Culver City, CA) for 250 bp paired end sequencing on Illumina's MiSeq platform the addition of 15-20% PhiX.

Analysis of 16S rRNA gene sequence data. Sequence data was processed in DADA2 version 1.18 (23). Adapters were removed using cutadept (24). Raw sequences were trimmed to 260 bp for forward reads, and 180 bp for reverse reads based on quality of reads. Reads shorter than 260/180 were removed. Error rate was calculated using DADA2's algorithm. Reads were denoised and merged into ASVs, requiring a 12 bp overlap, and chimeras removed. Taxonomic identification for each representative sequence was assigned with the Silva-138 database at 100% identity (25).

GENE QUANTIFICATION

Primer design. MMSeqs2 was used on a database that includes 202,601 non-redundant prokaryotic genomes from lab isolates and metagenomically assembled genomes (1 representative per species at 96% ANI) to search for all protein homologs of Fhp and NorB from *Pseudomonas aeruginosa* (strain PA-14). Spurious hits were filtered out by examining the alignment score and coverage on a biplot (NorB: -logP

> 50, coverage > 0.9; Fhp: -logP > 25, coverage > 0.9). This resulted in 14,255 sequences for Fhp and 7,968 sequences for NorB. The corresponding nucleotide sequence from the original genome was then extracted for each homologous protein identified. A multiple sequence alignment (MSA) was then generated using MAFFT. A phylogenetic tree based on this MSA was also generated. This MSA and tree were imported into Python, where the module Phylo from the package Biopython was used to subset the sequences into smaller groups based on phylogenetic similarity. The SeqIO package was then used to create FASTA files of each subset. Each subset group with more than 200 sequences was then imported into Geneious Prime, where a consensus nucleotide sequence was generated and used to design primers for quantitative PCR (qPCR). Primer sets were generated to have annealing temperatures around 65 °C, have no more than 2 degenerate bases, and amplify a product between 150-200 bps. Four primer sets were generated for *fhp* and two were generated for *nor*.

***fhp* and *norB* gene abundance.** Fhp (K05916) and NorBC (K04561, K02305) were queried from AnnoTree, a functionally annotated database of > 27,000 bacterial and > 1,500 archaeal genomes (26). The default search parameters were used: 30% identity, 0.00001 E-value, 70% subject alignment, 70 query alignment. Results were subset by phylum, and the percentage of genomes from each phylum present at above 5% relative abundance in the 16S dataset containing each gene was calculated.

Quantitative PCR. Genomic DNA was extracted from WT *P. synxantha*, *P. synxantha Δfhp*, and *P. synxantha*. These were used as positive and negative controls for the general *fhp* and *norB* primers. DNA previously extracted from the soil was used in the qPCR experiment. qPCR was used to determine the true relative abundance of *fhp* and *nor* genes within the microbial community of the native soil. The 15-µL reactions consisted of SyberGreen, a primer pair, and template DNA at between 10 and 50 ng/µL. All reactions with soil DNA were performed in triplicate; all controls were performed in duplicate. Each experiment was run on the same 96-well plate with a Roche 480 LightCycler-II system. This work is ongoing.

LAB REWETTING EXPERIMENTS

General rewetting setup. Rewetting experiments were performed to determine N_2O production from soils in the laboratory under various conditions. 5 g of soil (dry weight) was placed in 50-mL Falcon tubes, which were capped but unsealed, with holes for each sensor used (gas production detector, oxygen probe, or both) and an additional small hole to prevent pressure buildup. N_2O production was measured continuously for 1-minute intervals every 5-15 minutes, depending on the number of

samples set up simultaneously. Gas measurements were performed by a Picarro G2508 analyzer (Picarro, Santa Clara, CA, USA), which uses cavity ring-down spectroscopy to measure the concentration of N_2O, CO_2, H_2O, NH_3, and CH_4. The inlet tube from which gas was vacuumed and measured was positioned 3 cm above the soil surface. The Picarro analyzer was connected to the eosMX multiplexer (Eosense, Darthmouth, NS, Canada) which enabled measuring from up to 11 samples per experiment. In some experiments, an oxygen microsensor (Unisense, Aarhus, Denmark) was added to continuously measure the oxygen concentration in the soil throughout the experiment. Measurements of oxygen concentration were taken every second. The tip of the O_2 microsensor was pushed through the soil to touch the bottom of the Falcon tube in every experiment. O_2 probe calibration was performed by creating a two-point calibration curve with ambient air and a sodium ascorbate solution for the 0 µM condition. 1.8 mL of NanoPure water was added to each tube for the rewetting.

***P. synxantha* mutant strain generation.** *P. synxantha* (*Ps*) 2-79 was the wild-type (WT) and parent strain of all genetic manipulations done in this study. Individual mutants of *Ps* flavohemoglobin protein/nitric oxide dioxygenase (Δ*fhp*) and nitric oxide reductase (Δ*norB*). Clean deletions were performed using allelic exchange as described for *Pseudonomas aeruginosa* (27), with slight modifications: deletion vectors were cloned into auxotrophic *Escherichia coli* WM3064, conjugation was performed on LB plates supplemented with diaminopimelic acid (DAP), and selection for *Ps* recombinants including the plasmid occurred on gentamycin (30 mg/mL) plates lacking DAP.

Table 1 Strains studied

Name	Strain description	Fhp?	Nor?
WT *Ps*	Wild-type *P. synxantha 2-79*	Yes	Yes
Δfhp	Deletion of flavohemoglobin protein (*fhp*) from WT *Ps*	No	Yes
ΔnorB	Deletion of nitric oxide reductase (*norB*) from WT *Ps*	Yes	No

Mutant experiments. Soil rewetting experiments were performed with autoclaved soil in the same quantities and with the same setup described above. *P. synxantha* strains (WT, *Δfhp*, *ΔnorB*) were grown in 5 mL LB at 30 °C shaking for about 16 hours prior to their use in these experiments. Cells were pelleted and resuspended in 1 mL of 1X PBS for an OD_{600} ~12. Each soil tube received about $5x10^8$ cells/g of soil suspended in 300 µL of 1xPBS buffer. The soil was well stirred to ensure it remained well-oxygenated and to ensure a relatively even distribution of bacteria in the soil.

QUANTITATIVE PROTEOMICS

Protein extraction. Soil was placed in a 15-mL Falcon tube containing ~4 g of glass beads and 3 mL of lysis buffer (50 mM Tris-Base, 10% SDS, 1 mM protease inhibitor, 1 mM dithiothreitol, and 20 mg/mL lysozyme) immediately after sampling. The tube was vortexed for 10 minutes at room temperature and then centrifuged at 15,000xg for 1 minute to extract cell contents. Proteins were stored at -80 °C until measured. To measure, cell extracts were run on an SDS-PAGE gel and then stained with Coomassie Blue. The gel band between 37 kD and 50 kD was cut out, and proteins were isolated.

Quantitative mass spectrometry. This procedure was performed by the Proteomics Exploration Laboratory (Caltech, Pasadena, CA, USA). LC–MS analysis of digested peptides was performed on an EASY-nLC 1200 (Thermo Fisher Scientific, San Jose, CA) coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanospray Flex ion source: 500 ng peptides of each sample were directly loaded onto an Aurora 25 cm × 75 µm ID, 1.6 µm C18 column (Ion Opticks) heated to 50 °C. The peptides were separated with a 2 h gradient at a flow rate of 350 nL/min as follows: 2 to 6% solvent B (7.5 min), 6 to 25% B (82.5 min), 25 to 40% B (30 min), 40 to 98% B (1 min), and held at 98% B (12 min). Solvent A consisted of 97.8% H2O, 2% ACN, and 0.2% formic acid, and solvent B consisted of 19.8% H2O, 80% ACN, and 0.2% formic acid. The Q Exactive HF was operated in data-dependent mode with Tune (version 2.8 SP1 build 2806) instrument control software; see SI Appendix for measurement parameters. Data analysis was performed using Thermo Proteome Discoverer 2.5 (Thermo Fisher Scientific, San Jose, CA) with a SEQUEST algorithm (PMID 24226387). The data were searched against a consensus sequence for *fhp* and *norB* generated with the Python AlignIO package in Biopython (Supplementary Table 1 contains protein sequences used).

RESULTS

fhp is more abundant than *norBC* within the native soil community.

The investigated soil was sampled from the University of California Boyd Deep Canyon Reserve, part of the arid Palm Desert region in southern California, USA. Previous studies in this region have shown that microbial N_2O producers in this region were capable of enduring extreme desiccation and heat to produce large N2O emissions once rewetted (10). Soil was sampled from beneath creosote bushes, which are known to be microbial activity hotspots in otherwise relatively inactive arid desert soils (Private correspondence, Dr. Pete Homyak). Sequencing of 16S rRNA gene amplicons from four sites revealed that the soil microbiome underneath creosote bushes across this field site had very similar community compositions. Communities contained roughly the same groups down to the genus level present at similar relative abundances within the community (Figure 1).

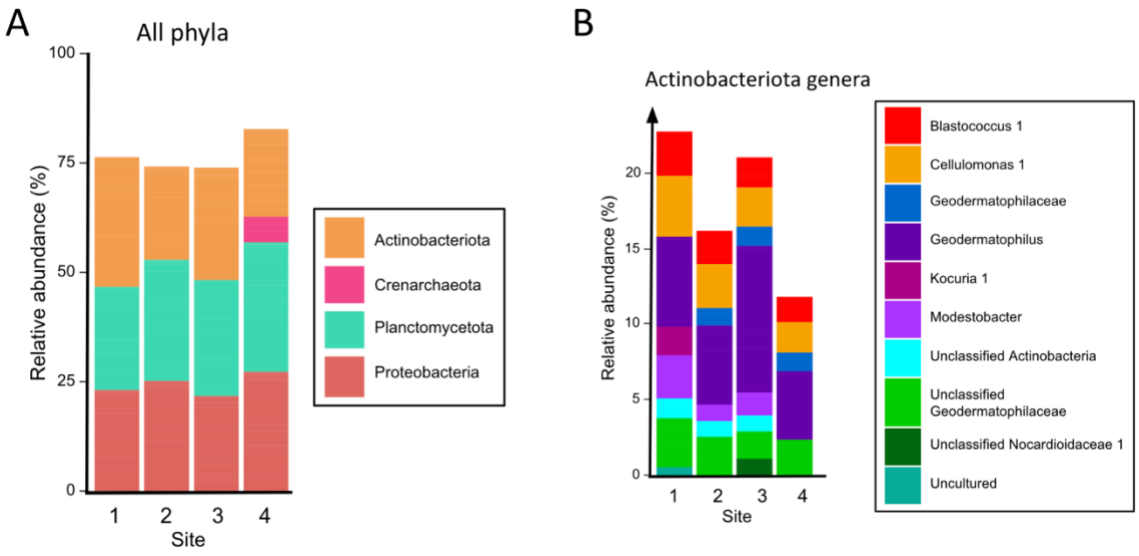

Figure 2. Relative abundance of 16S rRNA gene amplicons sequenced from the four soil sites. **A)** Relative abundance plotted at the phylum level, with phyla below 5% relative abundance omitted. **B)** One example phyla, Actinobacteriota, plotted at the genera level, demonstrating

the similarity remains down to the genus level.

Annotated genomes within the primary four phyla present at above 5% relative abundance within these sites were searched for *fhp* and *norBC* to determine the probable abundance of each gene within the soil community. *Fhp* is significantly more phylogenetically abundant among the Actinobacteriota *(*Actinomycetota*)*, Planctomycetota, and Proteobacteria *(*Pseudomonodota) phyla (Table 2).

Phylum	**Genomes containing *fhp***	**Genomes containing *norBC***
Actinobacteriota (Actinomycetota)	26.0%	0.1%
Planctomycetota	8.5%	1.1%
Proteobacteria (Pseudomonodota)	22.5%	14.0%
Crenarchaeota (Thermoproteota)	0%	0.5%

Table 2 Genomes containing *fhp* and *norBC* within relevant soil phyla from annotated genome database. Genes functionally annotated to be *fhp* are generally more abundant than *norBC*.

Quantitative PCR work is ongoing to determine the relative gene abundances of *fhp* and *norB* in the extracted soil DNA. Initial work to general standard curves using the designed generic *fhp* and *norB* primers are tentatively promising in specifically amplifying one gene product at quantities dependent on the initial concentration of DNA added (see Supplementary Figure 1).

N_2O production requires O_2 depletion from soil.

Wetting dry soils with deionized water reliably stimulated a consistent amount of N_2O emissions, but only when a sufficient quantity of water was added. Applying less than 1.4-mL of water to soils had resulted in no measurable N_2O production –

equivalent to not providing water at all. In contrast, when 1.8 mL or more water was added, a consistent amount of N_2O was produced. This suggests there is some critical state reached once a sufficient quantity of water is added. We hypothesized that enough water to make the soil system anaerobic was required to trigger N_2O production.

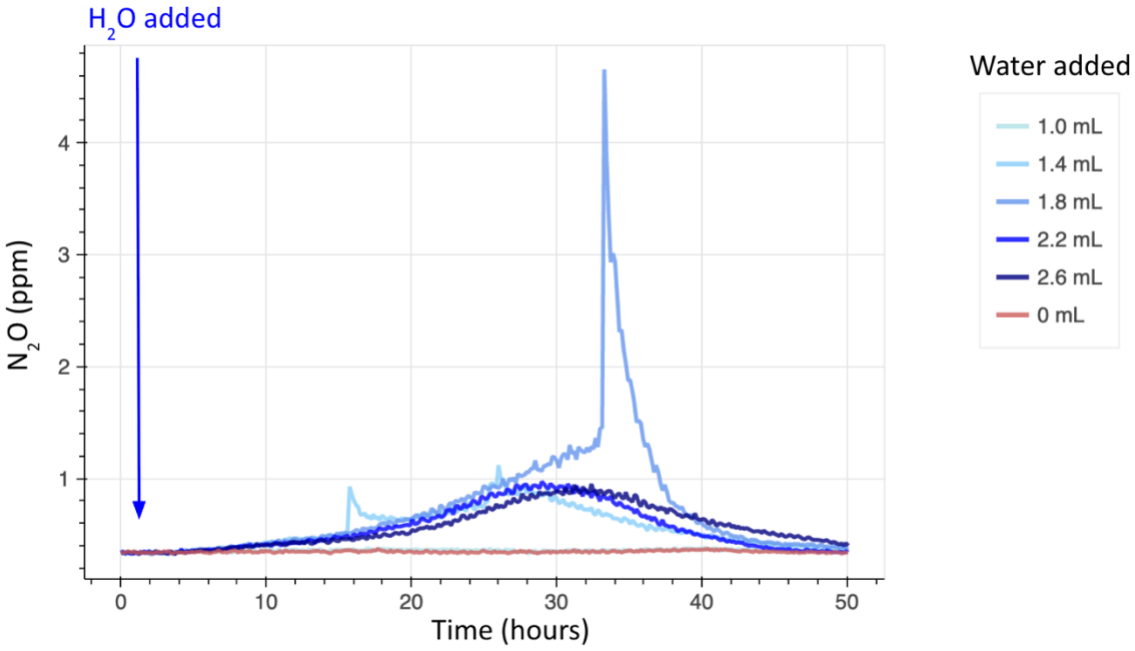

Figure 3. N_2O production following soil rewetting of native soil in laboratory conditions. Varying amounts of water, between 0 to 2.6 mL, were added. N_2O production only occurs when there is a critical quantity of wetting. Once this threshold is passed, roughly the same amount of N_2O is produced, regardless of whether additional water is added.

To test whether oxygen depletion occurs, we performed the same experiment with the addition of an O_2 microsensor to measure oxygen levels at the bottom of the Falcon tube containing the soil. O_2 concentrations dropped steadily to below measurable levels within two hours of wetting with 1.8 mL of water, showing that this quantity of water is sufficient to produce hypoxic conditions within the soil. N_2O production consistently only began once the oxygen level had dropped to this threshold. The microsensor measures an extremely rapid re-oxygenation of the soil that varies between experimental replicates. This is likely because oxygen enters the

pore spaces between soil particles at different times and fully re-oxygenates small regions within the soil even while most of the soil system is still hypoxic. It has also been noted that there is a sharp spike of N_2O production coinciding with this re-oxygenation spike. This is potentially due to the fact that water slows the gas release from the soil, so once an aerobic path has been created from the surface to the bottom of the soil, N_2O can suddenly escape more freely.

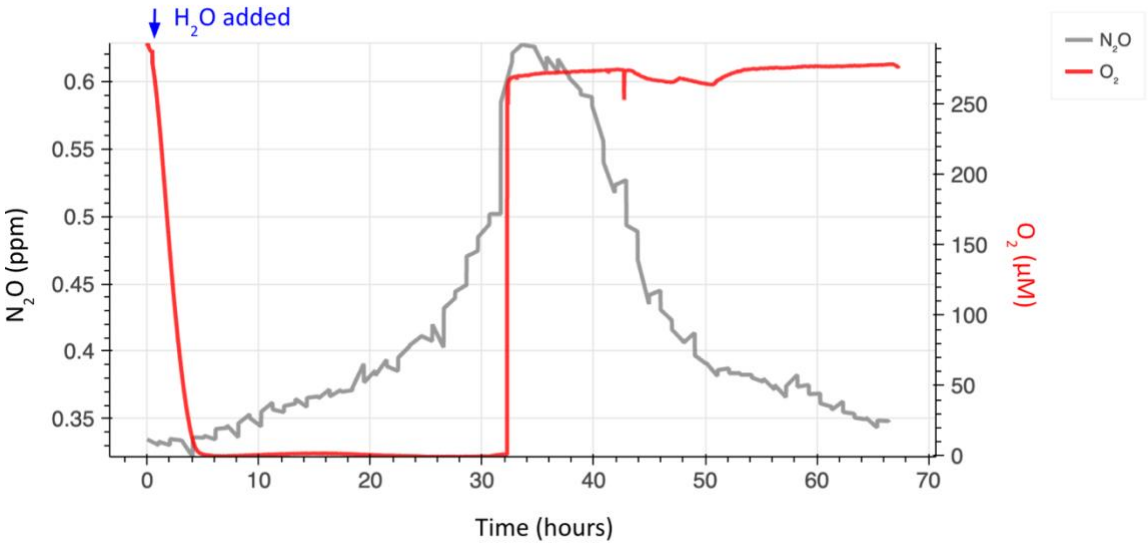

Figure 4. Representative curve of continuously measured N_2O production and corresponding O_2 levels in the soil. N_2O production begins once O_2 is depleted. (Replicate available in Supplemental Figure 2)

Mutant experiments reveal Fhp and NOR have distinct N_2O production profiles.

Experiments using mutant deletion strains of *Pseudomonas synxantha* were used to test whether the N_2O production of Fhp and NOR was different across time following rewetting. *P. synxantha* was chosen as a model organism as it is a genetically tractable native soil bacterium and contains both the NO detoxification and denitrification pathways. Wildtype, *Δfhp*, and *ΔnorB* strains of *P. synxantha* were grown in nutrient rich media aerobically overnight, and then washed and inoculated at high density into autoclaved soil.

These initial experiments reveal that there are distinct N_2O production regimes inhabited by each enzyme following rewetting: N_2O produced by NOR (demonstrated by the *Δfhp* strain) dominates emissions from the start of N_2O production, even relative to the wildtype strain. There is also a distinct N_2O production from Fhp, visible in the *Δnor* strain emissions. These experiments also reveal that autoclaved soil in the absence of bacteria does not produce N_2O following rewetting.

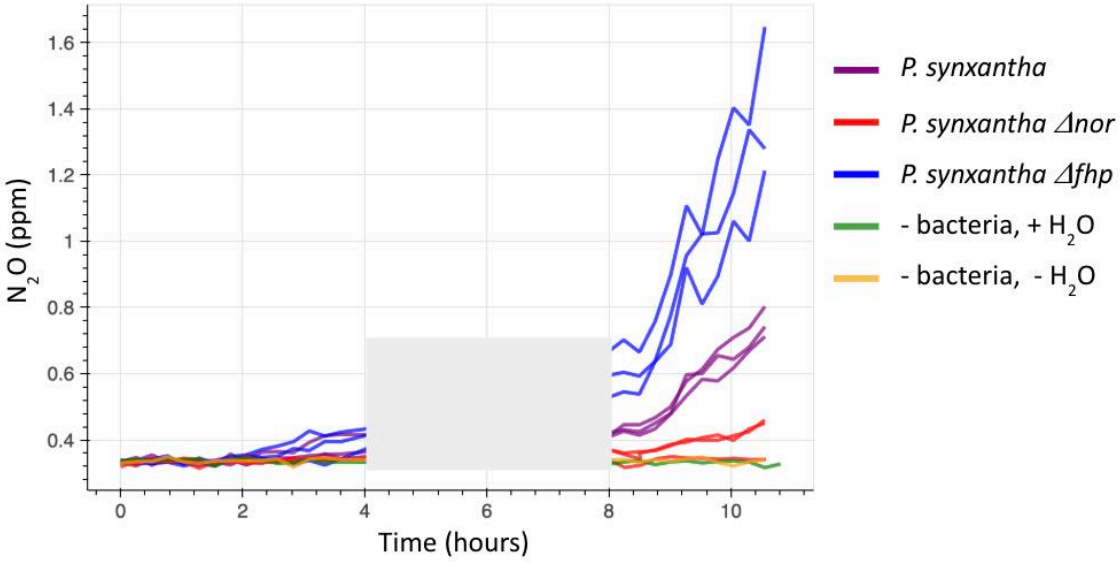

Figure 5. N_2O production following the rewetting of autoclaved soil inoculated with high densities of *P. synxantha* strains. These traces reveal there are distinct N_2O production curves from each enzyme, and the WT production appears to be a mix of the two. The grey box from 4 to 8 hours is the missing data from a power outage that occurred during this experiment.

Future experiments will focus on capturing the kinetics of N_2O production following rewetting at the initial stages of gas production, as this regime is where we expect Fhp to have important contributions. Additionally, O_2 concentration measurements will be taken in tandem to analyze under what levels of anoxia N_2O production is triggered by each enzyme.

Field measurements of N_2O SP.

Rewetting experiments were performed in the field at the Boyd Deep Canyon UC Reserve on August 29, 2024. For these experiments, a 20-cm collar was inserted into the soil at the base of four creosote bushes, that were subsequently re-wet with 50 mL of water. Gas samples for N_2O abundance and SP, as well as soil samples for quantitative proteomics, were taken at various times before and after re-wetting. Gas samples unfortunately leaked before they could be analyzed, preventing any data from being obtained. Proteomics data is still in progress, as the protocol for performing quantitative proteomics for Fhp and NOR from soil still requires more optimization.

Figure 6. Field sampling. **A)** Creosote bush with paired re-wetted sites at the base. **B)** Closer view of the paired sampling sites. The site with the collar was used for gas sampling while the open site was used for protein sampling. **C)** Gas sampling was performed by placing the cap over the collar and then removing gas with a needle and syringe.

Quantitative proteomics of Fhp/NOR over N_2O production curve.

Quantitative proteomics has previously been used to demonstrate when Fhp or NOR is being used by the cell, as the expression of these enzymes is translationally regulated (9). This work is ongoing, with the goal of quantifying the ratio of Fhp to NOR expressed by bacteria in the soil as N_2O production occurs after rewetting. We developed a method for protein extraction from soil that involves chemical and physical lysis of cells and subsequent extraction of cell contents. To increase the abundance of the proteins of interest, Fhp and NOR, within the sample, these total

proteins were run in a gel alongside a strain of *Escherichia coli* engineered to overexpress Fhp as a positive control (Supplemental Figure 3). The region around this band within the sample was cut out and subsequently analyzed via mass spectrometry for these two proteins. However, searching the resulting sequences for a list of all bacterial Fhp and NOR sequences had no results, as both proteins were below the limit of detection.

Two methods for improving this are being pursued. The first involves increasing the abundance of Fhp and NOR within the sample before performing the mass spectrometry. This can be done via a 2-dimensional gel, which first separates proteins by isoelectric point and then by mass. By running the sample alongside a positive control for both Fhp and NOR, such as an *E. coli* overexpression strain, the gel region around each protein of interest can be cut for subsequent quantitative proteomics analysis, increasing the concentration of these proteins within the analyzed sample. An alternative method is to limit the number of sequences the data is searched against, as searching against fewer sequences increases the detection limit. Consensus sequences for each protein were created by aligning all of the bacterial Fhp and NOR sequences available and the file will be searched against just these two sequences. If the consensus regions within these alignments are not sufficiently long, alignments will be made between smaller clades within each protein, similarly to how the consensus sequences for generating qPCR primers were created.

DISCUSSION

Toward the goal of understanding microbial inputs to N_2O emissions, it is key to understand the mechanisms involved in producing this key greenhouse gas under different conditions. Until this work, studies of N_2O generation from soil microorganisms have attributed all N_2O production to denitrification. However, our results suggest that in soils where organisms experience a sudden shift to anoxia, Fhp may be a relevant component in producing N_2O despite its thermodynamic disadvantages relative to NOR. This extends the work done by Wang and Lonergan *et al.* in evaluating the roles of Fhp and NOR in pure culture laboratory settings to at least one environmental condition – the southern Californian desert following rainfall – with the possibility of extending it to many other environments where drying and rewetting cycles are common.

This study shows that the previously established pattern of Fhp being more phylogenetically abundant than NOR holds true within soil communities as well – many more organisms contain the genetic potential for Fhp production relative to NOR. Previously obtained SP data also supports the idea that Fhp might be

responsible for the large fluxes of N_2O observed from arid southern California soils following rainfall, as these N_2O SP are much more positive than bacterial denitrification would produce.

Tracking both N_2O emissions and soil O_2 concentrations within samples during rewetting reveal that N_2O is only produced once oxygen is depleted, creating conditions where bacteria are shifted to anoxia and likely exposed to NO. Given that Fhp is expressed in the cell during aerobic conditions to combat reactive oxygen species toxicity (20, 28), but denitrification machinery is only expressed following O_2 depletion (19, 29), it is possible that in these rewetting conditions where cells are suddenly shifted to anoxia, Fhp is primed to produce the initial measured burst of N_2O.

Identifying the enzymatic source of this N_2O requires either SP measurements or direct measurements of the relative abundance of the two enzymes within the soil over the course of N_2O production rewetting, both measurements which are difficult to obtain. Future work involves potentially returning to the field site to try to capture larger volumes of gas from soils following rewetting for SP analysis. There are several ongoing avenues with the quantitative proteomics work that can potentially enable direct measurement of the relative abundance of these two proteins within the soil. As Fhp is present during aerobic conditions, we expect to see the ratio of Fhp to NOR enzymes decrease over the course of the N_2O production curve, as the anaerobic condition triggers denitrification expression. However, we expect that the initial burst of N_2O occurs when the dominant enzyme present is Fhp, resulting the observed high N_2O SP value measured in the field by Krichels *et al*.

Studying the causes of N_2O production following rewetting of very arid soils grows increasingly important as these conditions become more common around the world. Extreme weather events, chief among them drought and rain or flooding events, are expected to become more common as a result of anthropogenic climate change (30, 31). This study shows that such conditions trigger large pulses of N_2O, so understanding the precise mechanisms underpinning this microbial N_2O production is essential to being able to predict how soil N_2O emissions will change in the future. Given that natural soils represent the largest flux of N_2O to the atmosphere and the high radiative forcing potential of N_2O as a greenhouse gas, understanding these emissions will be essential for accurately predicting future climate change.

Within the field of biogeochemical cycles, this study also provides evidence that metabolic pathways not involved in catabolism or anabolism have been thus far overlooked. All known previous studies of N_2O emissions from soil attributed the gas production to denitrification or abiotic reactions. More broadly even, most studies of the contributions of soil microorganisms to biogeochemical cycles, highlight only dissimilatory or assimilatory metabolic reactions (12). However, this study provides a hint that this perspective may be limiting: there are some environmental conditions

in which other pathways, such as detoxification pathways, dominate the flux of certain components of biogeochemical pathways. If only the assimilatory or dissimilatory metabolic reactions are studied, such phenomena will be overlooked and misunderstood. While further work is needed to conclusively determine the relevance of Fhp and the NO detoxification pathway in N_2O production following the rewetting of arid soils, this study provides hints that such a pathway may have been overlooked in previous studies of environmental microbial N_2O emissions.

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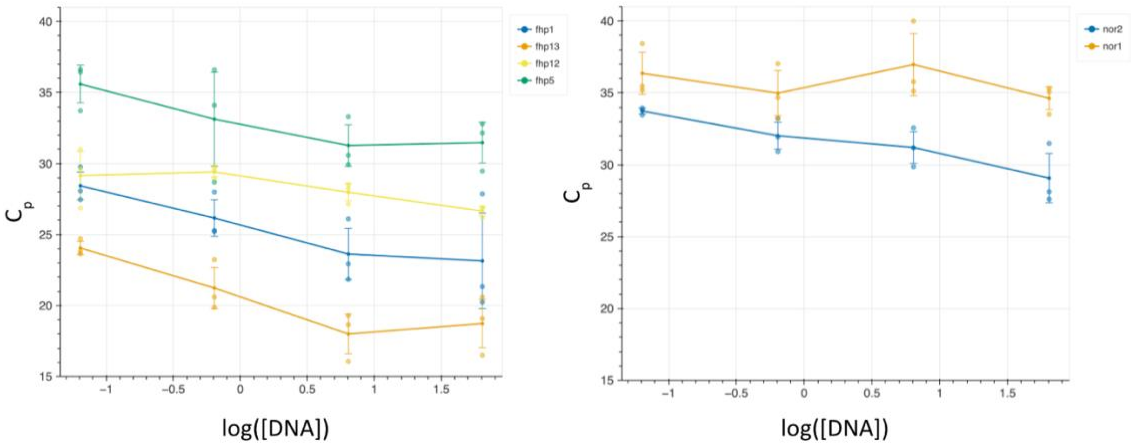
SUPPLEMENTARY MATERIALS

Supplemental Table 1. Consensus protein sequences used in search from proteomic data.

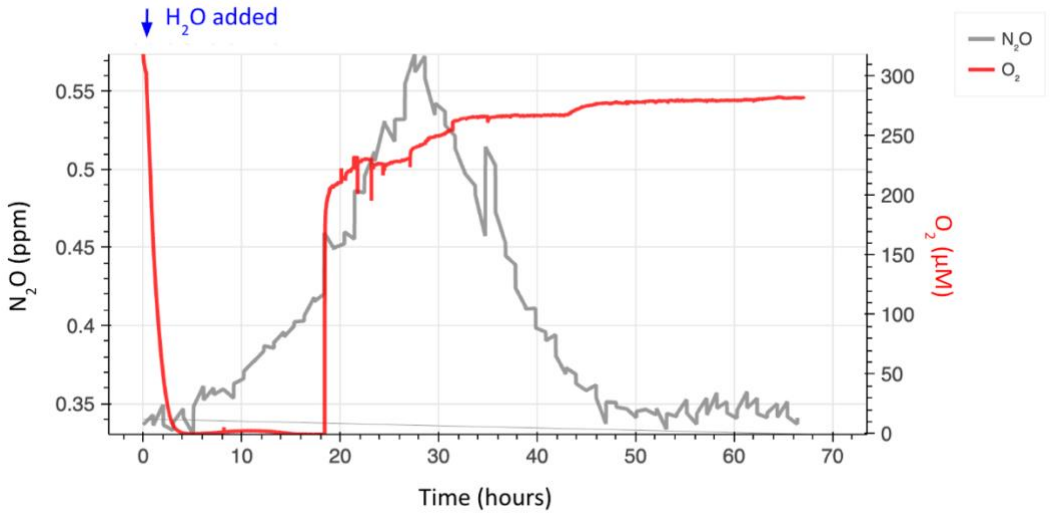
Fhp	MXXPXXYDLADADYAFLTLGWMTADAAYSRHPWHEQTAGRHXXXMXXXXXXXXXXXXXX XGTXXXGXXXXXXXDVPXXXXRXXPAGSVXTXXXXXXXXXEEXXXXXXXXXXXXXXXXXG LLYRXGXXXXXXXXXXXXXXXMXXXXXXXXXNPGSRXIAXELSVSYXXPXQXEAXTXXXXX PXXXPXXXXAPXLNWXXXGDGXXXXXXXXXCXXXXXXDSDHRAXXXXXXXXXXXXXXXXXX XXXXLXXXXXXXXPXXXXXXXPXXXXLXDXXXXXCXXXXPPLXXPXXXXXXXXLXXXXXX XXXQXAFFXXLXXXXXXXLTRXPXXTXLLALPQPVGXXXXXXXXXXXXXXXXMXXXXXXXXX GPPXXXAXLLLXXXXXXXXXXXXXXXXXXXXWWXDAXGXXXXXXXLXXXXVRXXXKXXXR KGVFXFLXXLAXXXXXAXXXXXXXXXXXXDXXXXXPXXXXXXXXXXXPPPSXHXXXPLITECG LRHALXRXXXXXXTXXXXXXXXSXXXXXXXXXFTXXPXXXXXXXXXXLQRXXXXXXXXSRR SGYAPDCXPGSSASCHLRAVDSRNPSLXXXGXGXXXXTTCIPNTSXXSGPHSPHGLLAGYGXCSR TSPSPSSRPPCARRLTDWPHHXXNFYXXXXXXXXFXXRXPEXLXXXXFXXXXXXXXXXXXXX XXXXNXXAXXXXXXXXXXQXXMKFXDGXXGGXXXXXXXQXXXXDIXPVFHRXVLXLALAX XXXXREGAXXXXAXXXXXXXXPAXXGXPXXXSXXXXGXDRXRAXSXXXPXXXDXXXDXXX XXXXXXXXVXXXXXXXXXXYXXXXXAXXXXXXXXXXXPXXXAXXXXVXXIXXXXRXXXK HXXXXXXAXXXXXXXXXXXXYXXVGXXXXXXXLXXXXXXXLGXXXXXXXXXXXTGXXXX SVSVXXXAXXXALDDXXXXARRLXXAXWXWXXAYGXXXAXXXXXXIXXXXCAPLXPXXEG XXXXXXMPXXXXXXVAYXKXGXTLXXXXXXXXXXXXXXEXXXXXXXXXXXGXXXSEGLGDG LGDGXGERLXXXXGEGWXXXGXXXRYXXXDXNXXEXXXXXRXFLXXXXXXXDRVVGRVDD RVVGRVDDRVVGRVDDTVVGXVENTVVXXXEXXXXXXGXXXXSXSFXLXPXXXXVNXXXXX PXXDXXSQXXXXXXNDXANXXXXXKXXXXDGXXLXXXSXXXAVLXXDXXSISPKVKLFTIET KKPHRIXPGQDYXXXXXTXXXXXGFXSXEXXXXXXXXXXXXXXXXXGXXXTGXXXXXXXRX YSLSDAPGXPYSLXXXXXXXXSXXXLXXXLXXXXXXXXYQVXXXXXALAXXXXXXXXXXQH GXRIXVSKXXXGXXRXXXGXXXXXXEXXAXXXXXXXXGXXXXXXXXXXXXXXXXDXXXXXXX XXPGAGAEGSRAGASRTAGAEAGADRTAGAGADRXXXXXXGXXXXXXSGXXXXXGVXXXXX XXXXXXXXXXXXXXXXXPIXGXXXXXGXVGSXNXLHXXXXDEGXKXGDXXXXXXPXGXXF XPELTFYXWXDLYGXXSXXPXLXXGNXTXXXXPXXXLQDLXXHXXXXXTTGVTGLTGXAGX XXXGGXGXXXGXXXXXXXXAXXXXRPGXXXXXGXXGXPPXXXXXXXLXNCSXGCQPXGXTP XXXMLXXIKXXXXXXXXXAAXXGXXXXXDPXXXXXXXXDXXXAXXXXXXXXXXTHSSFAQ KIMSMXXTKETAXXXXXPAXXXXXXXXXXXHIXXXXAHXXXXXXXXLXVXXHXXXGXXXA XXFXXXXGMFLPGXXXXXXXXXGGGGTGRXXXXLXXXXXXXXXXXGXXKLFXXXKKDXXX XXXVXXXXXXLXXXLCNXXYRXXXXXXPXPXXXXXXXXXXXXAGNXXGQPVXQSNXASSGW LXXXXXHXTGXXXXXXXXXXXXGGRHQEXVXXXXXXXAXXXXXXXAGSAFADXXXXXXPX XXDHTDXXGDVKAEXXXDXXXXXXXXXXXXLVXXXXXXXXXXXXXXAEXAEGGXCXGXVX VXXADXAXXXXXXXXXXXXXXXXXXXXXXXAAGIGXAXGXGXXXGAXXXXXXXXXXXXXX XXXXXDGXPEESXXXXGXYXXXAARRRRARPAAEDPGXXXXKFAXXXXXRPRHPGLSLWAA ALHAGHARSLARERCCGREHSLRGFRPGQLGGRXXXXXXXXXXXXXXXXXGXXXXXXXTXX XXXAXXXEKIGXXXXXXXXXXXXXXXFXWXXXGMXXXXXXLLXXXXXXXXYXXXGRXRSC GTWXGXCXXRRXGXXXXXXPXXXRRRXSXXSXSXXXFMLHSANHXXTTAPERTSDDYRARRS NXXGVRPXXLXXXXXXXXCIRDRLXGXVRLRSTMSYXGRTKLFXXVRGGYIPARAEDQALLGV VIXXXVXGTPGSRCEWDWVSGGSSXXXXXTTSSSARPRRWTPEPXXXEXNFXXXXXXXXVXPX XXXXXXALXXXXXXXXXXXXXXXXXXXXXXGXXXXXXXXXXXXXXPXXFNQAEPVRPVRLR YARAQREVQGDSRRSLLEQAEAEGLRPAHGCRQGICASCTCLLLSXXXXXXXXXXXXXXXXXX XXXXXXXXIXXXXXXXXXFXXSGXXXXXXAPCKXWXXXXXXXXXXXTRSRSRHGRHLXXXX XXXXXXXXXXXRXGXCGXCXXLPSXXXXGXVXYXXXPXXXRMRWYWLFRVIRMSSISSFSPR CSMVRXXXGXXLXXXXXXXXXXXXXXXXXXXXPXXXRRXXRXXXXXRSRTRDPSTPPPS
NOR B	MTDYMXXXXXXXXEKDRVNXXXXXXXGQARADNLPTSNAGTGKGLEVVXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXXXXXXXMXXXXXXXXXXXXXXXXIPXXX XXXXXXXXXXXXXXXXXNXXXXXXXXXXXXWVATIGGPPXXXXXXXXXXXCTARDSLAPIX XFRTSDKFETXXXXXXXXGXPDXXXXXXXXXXXXXXXXXFSXXXXXXXXQREEHMSHIVDIPX XXXXXXXXXAHAQTQRKGXXXXXXXNXXXXXXXXXXXXXXXXXFXXLXXXGXXXYXRXX XPPXPXXXXXXAXXXVXXXXXAXXGXTGXXXXXXTXXXIXXGQXXXXXXGXXXXGSXXGH GXYXXPDXXAXXLHXXXXSXXXXXXXXRSEXKDFXXXXXXSKDFAXXXXXGXXXXKXXXX XTXXXXXXXXDLPPXXRXXXXLXXVAXEXXXXXXXXXXXXXXXXNXXXXDXXXNRGXTXX XXXXXGXGXXXXXXXXXXXXIYAXXXXXXXYXXXXFXXXXXXXXXXFXPXXXXDLXXXAD GGMXPXXXXRXCGXTSNXXXXXGXXXXXXXAVDGLXXXXXPGXXXXXXXXXGDXXXXXXX XFFXWXXWXXXXXRXPAXVXGGXHXAXXXXGXXXXXXXXPQXXEGTTGXXXXXYXPNRPA AXXXXXXXXGXXXXXXXXXXLNMXRXYTXNWPXXPXXXNXPXXXXXXWSXXSXGWXXLX

	XGXGXXXXXXXXXSXXXDXGXXXXXHXXRXXXWEXXXXXXXQPAQAXRRRXXXFPXXXXX XXXXXXXXPXXXXXXXXDPXXXXXXXXXTXXXXXXXLTTXDLXNKGXXXVRXSQXXXXXY FXXXXXLFXXCACAVQXXXGXXXXXXXXEXDRLLGFDGXXXVFXGXXXALXXELXXXMXXP XPXXTCRNPGIXXXXRXXHXXXXXXWLLFGFMGATHYLIPEEAETELFSPKLGLITFWVFLVAG ALTIVGYLMVPYATLAAMTGNDLLPTMXWXXXXXXXXXXXCXXAXPXXRXGXXXEXPKXPX XXXXXXXXXDHXKQNAIFXRXXXXXTXSXXXXXVXXXXXXXXGIXXXXXXXGXXXXXXLAX XTXNXXXXXXXXXXXXXXXXQXGFFXXXXFWXGQPXXXQGXEXXXXXXXXXIVXXXXXXX XXXLGXXXXXXXXXPXXXXXLXXXXGXDGXXPXXXXGVGNXXAAPTXDPKXDPFDTHSSTSS XXXXXXXXXXXXTXGKXXXGXPXXAPLAFXXRGXXXXXHIFSXXXXXXXXXXXXXXXXXXX PXXXVGXXXXXXXXXXFXXXXXXXXXXWWVVHLWVEGXXEXXXXXXXAXXXXXXXXGXX XXIPDLEEXXXXXXXXXXXXXXXXXXXXXGXXGTXHHXYXXGGSGPAVIALGAXPXXXXXX GXXFSALEXXPXXXXXXXXXXAXXXXXXXXXXGXXXXXXGXEGXXXXXXXXXXXXXXXXXX GXXXSXXXXXXXXXAPTGSSHRRPGYXTXXXXXXXXXPXXXSSLXWXAHVPGRGQXXXXXX XXXXXXXGAGXXGFXLPDRHRPDRQPRARGHDGRVRHALCRGGTIAPDSRXXPXXXPGRXVX YYXXGXFXXTXXHXHRXAXXGXYXXXXLGLIXXXXXYHVPXCTRXXXXXXXXXXXRXXXXX XXXXQTXGXXXXXXXXXXLXXAXXXXXLLXXXXXXFXWXXXXGXXXMXXXXXXGLHEHR DRAIDRMAAPXGXXQXXXXXXXXXXXXXXXXGXWXXRXXXXXXXXXXXXXXPXXXWXXXX WFXXXXXWGAPRLRRGXXXRXXXXXXERPRGEVEFLVAQSRAGLDDFRFLXXPXGXXXXXXX XXXIXXXXXXXXXLXXXXXXXXXXXXXXPXXXRXXXXXXXSPXXEXXXXWXXTXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXPXIGLSGAVALSLVLFGGGGLGLWYGGQERVETSLRVGEE GRGKFLYAXXXXXXHXLDADGTXXXXXXXXXXXXXKPXXXGXGRWXRXLXXXXXXXWLRLE TQGKTQVGQLVAQNYXXMWXFSKYDIDFCCKGEKTIDEVCLKKGIDTEHLQERLGQXXXXXXX XXQDIDYXSWSLYLLANXXXXXXXXXLSFXXXXXXXXXXXXXLPPYXLXXXXXXXXXXXXXT LLFVWRFLXXXXXXXXXXXXXXXXXXXLXXLXXXXXXXXXXXXXXXXXXXXXXXXPAGEXXX LPSDGKXAXXXXXXXXXXXXXXXXXXXXXXXXXXMDRDGIAEMSCIAGVGGNVKKLVRTATS GRRIIVIDGCPLACSKACLXXHXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXRHLKHHMAME ETILFVAGRXYGYXXXXXXXXXXXXPXXXRXXXPPGRARGRRRXDVPDCCGAXXXXXXPXXE XXXXNWGGKXXXXXXXXXXXXXXXXXLXEADXXRXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXYKXAFSLLKEFXXDLLLHIHLENTILFPKAIAIEKGIVGNENLAFNSNN

Supplemental Figure 1. Quantitative PCR standard curve using primers created from consensus *fhp* and *norB* sequences. Genomic DNA from *P. synxantha* was used as a positive control.



Supplemental Figure 2. Replicate experiment recording N_2O production and O_2 concentration in rewetted native soil simultaneously.



Supplemental Figure 3. Proteins extracted from varying quantities of soil, run alongside proteins extracted from an *E. coli* strain overexpressing Fhp. The band at this height for the soil samples was then cut for quantitative proteomics analysis.