

# Bacterial nitrite production oxidizes Fe(II) bioremediating acidic abandoned coal mine drainage

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**ABSTRACT** Passive remediation systems (PRSs) treating either acidic or neutral abandoned coal mine drainage (AMD) are colonized by bacteria that can bioremediate iron (Fe) through chemical cycling. Due to the low pH in acidic AMD, iron oxidation from soluble Fe(II) to precipitated Fe(III) is mainly directed by microbial oxidation. Less well described are biotic reactions that lead to iron remediation through abiotic secondary reactions. We describe here iron oxidation in acidic AMD that is mediated by the bacterial reduction of nitrate to nitrite followed by the geochemical oxidation of Fe(II). Within an acidic PRS, 4,560 bacteria cultured from the microbial community were screened for their ability to oxidize iron and to perform nitrate-dependent iron oxidation (NDFO). Iron oxidation in the culturable community was observed in every pond of the system, ranging from 2.1% to 11.4%, and NDFO was observed in every pond, ranging from 1.4% to 6.0% of the culturable bacteria. Five NDFO isolates were purified and identified as *Paraburkholderia* spp. One of our isolates, *Paraburkholderia* sp. AV18 was shown to drive NDFO through the bacterial production of nitrite that in turn chemically oxidizes Fe(II) (nitrate reduction-iron oxidation; NRIO). AV18 expressed nitrate reductase, *napA*, concurrent to nitrite production. Burkholderiales are found by 16S rRNA gene sequencing in every pond of the PRS. The frequency of NDFO metabolism in the culturable microbial community and abundance of Burkholderiales in the PRS suggest nitrite producers contribute to the bioremediation of iron in acidic AMD and may be an unharnessed opportunity to increase iron bioremediation in acidic conditions.

**IMPORTANCE** Our study sheds light on a poorly defined biogeochemical interaction, nitrate-dependent iron oxidation (NDFO), that has been described in several environments. We show that bacterial nitrate reduction produces nitrite, which can chemically oxidize ferrous iron, leading to insoluble ferric iron. We show that bacteria capable of the nitrate reduction-iron oxidation (NRIO) reactions are prevalent throughout multiple passive remediation systems that treat acidic coal mine drainage, indicating this may be a widespread mechanism for iron removal under acidic conditions. In acidic coal mine remediation, iron precipitation has been shown to be solely bacterially mediated, and NRIO provides a simple mechanism for aerobic oxidation of iron in these conditions.

**KEYWORDS** bioremediation, biogeochemical cycling, abandoned mine drainage (AMD), passive remediation systems (PRS), iron oxidation, nitrate reduction, nitrate-dependent iron oxidation (NDFO), nitrate reduction-iron oxidation (NRIO)

Abandoned coal mine drainage (AMD) is formed under acidic conditions as a self-propagating process that can last for tens of thousands of years (1–6). AMD can reach the ground surface at either circumneutral (from underground mines in areas with limestone in the soil) or acidic pH (from surface mines). Heavy metals found in AMD persist in the environment as non-biodegradable corrosive agents that decrease microbial soil activity, bioaccumulate in tissues, and are biomagnified along trophic

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levels (7–9). Heavy metals, like iron, perpetuate total mineral acidity, have undesirable aesthetic effects, and negatively impact human health at elevated levels (7–14).

Passive remediation systems (PRSs) for AMD are composed of various combinations of settling ponds, wetlands, limestone beds, and vertical flow ponds and are specifically designed for either circumneutral AMD or acidic AMD. The goal of PRSs is to increase pH in acidic AMD systems and precipitate metals close to the original contamination source in both acidic and circumneutral PRSs (15). Iron exists in multiple oxidation states; ferrous iron [Fe(II)] is the most common dissolved form of iron, and ferric iron [Fe(III)] is commonly found as a precipitate in circumneutral environments (16–18). Since the PRSs are open to the environment, they are naturally colonized by native microbial communities that impact remediation through their metabolic redox reactions, leading to the biogeochemical cycling of elements (19, 20). In acidic AMD, iron oxidation is bacterial-driven under aerobic, microaerophilic, or anaerobic conditions (17, 21–23).

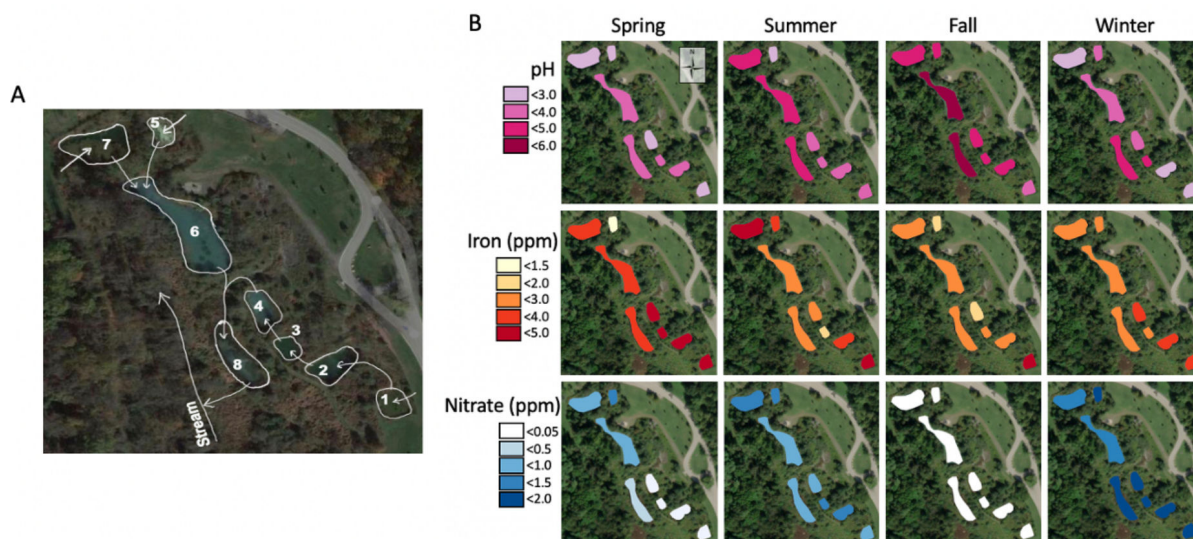
In addition to direct iron oxidation leading to iron bioremediation in acidic AMD, it could also occur via the cycling of multiple chemicals. Nitrate-dependent iron oxidation (NDFO) has been identified in phylogenetically diverse groups of bacteria in a variety of environments; however, the mechanisms remain obscure (24, 25). The mechanisms used to perform NDFO likely differ under different oxygen levels and pH values. These mechanisms can include biotic-only reactions or biotic–abiotic interactions that rely on enzymes and chemical intermediates (22). The distinction of NDFO mechanisms in different environmental conditions is further elucidated by the ability of acidic NDFO to occur under aerobic conditions as opposed to the strict anaerobic conditions required for neutral NDFO (22, 25, 26).

Our data suggest NDFO could play an important role in the overall effectiveness of bioremediation in acidic AMD. The extent and mechanism of NDFO contribution to microbial iron bioremediation is poorly understood in acidic AMD. Here, we evaluated the extent of direct biological iron oxidation and elucidated the potential for NDFO in the acidic Boyce Park PRS. From screening bacterial isolates in the acidic Boyce Park PRS located in Allegheny County, Pennsylvania, USA, bacteria capable of direct iron oxidation or acidic NDFO were detected in every pond of the system. We determined that the NDFO metabolism in our isolate of *Paraburkholderia* spp. is the result of bacterial nitrate reduction to nitrite that chemically oxidizes Fe(II) in a nitrate reduction-iron oxidation (NRIO) metabolism. Our research elucidates additional pathways that increase iron bioremediation in acidic AMD as well as characterizes the NDFO metabolism in acidic conditions.

## MATERIALS AND METHODS

### Sampling at the acidic Boyce Park PRS and Middle Branch PRS

The Boyce Park PRS was constructed in 2008 with six settling ponds (ponds 1, 2, 3, 4, 6, 7), one limestone bed (pond 5), and a wetland area (pond 8) at latitude 40° 27' 51.9984" N, longitude 79° 44' 56.0004" W to treat acidic AMD (Fig. 1A) (27). ArcGIS Pro software was used to visualize our data. The pH data were collected using a YSI Professional Plus Series handheld with YSI Quatro ISE-ISE-DO-COND 18E100032 probe (Xylem Inc., Yellow Spring, OH, USA). Iron in parts per million (PPM) was determined using a PerkinElmer NexION 300x Inductively Coupled Plasma Mass Spectrometry (ICP-MS) with PerkinElmer S10 Autosampler and the NexION 300x ICP-MS software. Nitrate and nitrite in PPM were determined using a Dionex ICS Series ICS-1100 Ultimate 3000 Diode Array Ion Chromatography (IC) (Thermo Fisher Scientific, Waltham, MA, USA) (28). One liter of mud-water slurry samples (containing approximately equal portions of sediment and water from the AMD PRS) was collected from all ponds of the system, a portion of the AMD was autoclaved at 121°C for 45 min, and a non-sterile mixed microbial community sample was maintained for metabolic testing (29). The Middle Branch PRS was constructed in 2001 with two settling ponds (ponds MB1, MB4), four vertical flow ponds (ponds MB2,



**FIG 1** Boyce Park acidic AMD PRS. (A) An aerial image of the Boyce Park PRS was taken from Google Maps to which arrows were added to indicate the flow of AMD through the system. Map data ©2022 Google. (B) Seasonal levels for pH (pink), iron (orange), and nitrate (blue) are indicated.

MB3, MB6, MB7), and wetland area (MB5) ponds, at 41° 20' 48.0012" N, longitude 77° 52' 3" W to treat acidic AMD (pH ~2.8) (Fig. S2) (27, 30).

### Metabolic capabilities of the culturable mixotrophic microbial community for iron oxidation and acidic NDFO in the Boyce Park PRS

To sample the metabolic capabilities of the culturable mixotrophic microbial community for iron oxidation and acidic NDFO, the slurry samples from each pond were diluted and plated for single colonies on R2A agar adjusted to pH 4.0 with HCl, and incubated at 30°C (31). Sterile AMD was confirmed to contain Fe(II) prior to the start of the experiment using the ferrozine assay (32, 33). For the iron oxidation screens, sterile AMD was supplemented with R2A to a final volume of 10%. For the acidic NDFO screens, sterile AMD was supplemented with R2A pH 4.0 to a final volume of 10% and with 5 mM NaNO<sub>3</sub>. The sterile AMD with 10% R2A or the sterile AMD with 10% R2A and 5 mM NaNO<sub>3</sub> were aliquoted into separate 96-well plates (200 µL per well). Individual colonies from the slurry plates that contained the culturable mixotrophic microbial community were individually inoculated into single wells of sterile 96-well plates with a sterile toothpick. Using one toothpick, a colony was inoculated into a well with 200 µL sterile AMD with 10% R2A to test for iron oxidation, immediately followed by inoculation into a separate single well containing 200 µL sterile AMD with 10% R2A and 5 mM NaNO<sub>3</sub> to screen for acidic NDFO. The 96-well plates were incubated for 7 days in a closed container with moistened paper towels in the bottom to keep media from drying out in the incubator (wet box). Sterile AMD was used as a negative control to confirm the levels of Fe(II) did not abiotically decrease, and as a qualitative comparison for positive screens for both iron oxidation and acidic NDFO. After incubation, isolates were assessed for iron oxidation and acidic NDFO using the ferrozine assay to detect Fe(II) by the development of a purple color and to detect Fe(II) oxidation by the lack of color development (34). The color development or lack of in each well was enumerated to quantify the metabolic frequency of iron oxidation and acidic NDFO within the culturable mixotrophic community. Five hundred seventy colonies from each pond, for a total of 4,560 bacteria, were screened for iron oxidation and acidic NDFO.

## Identification of NDFO isolates from acidic AMD

Five NDFO bacterial isolates (AV2, AV3, AV18, AV25, and AV26) capable of NDFO were single colony purified twice on R2A agar pH 4.0, and their NDFO phenotypes were confirmed in 10 mL liquid cultures, using the ferrozine assay to measure Fe(II) levels and the Griess assay to observe the production of nitrite (35). Bacterial isolates AV2, AV3, AV18, AV25, and AV26 were recovered from pond 1. DNA was extracted from AV2, AV3, AV18, AV25, and AV26 using the Quick-DNA Fungal/Bacterial Miniprep Plus Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. DNA extracted from AV2, AV3, and AV18 was sent for whole genome sequencing at SeqCenter ([www.seqcenter.com](http://www.seqcenter.com), SeqCenter, Pittsburgh, PA, USA) using their Illumina MiSeq 200 Mbp package. Sequences received for the forward and reverse reads were imported into KBase (kbase.us) (36). The forward and reverse reads were paired and assembled with SPAdes v.3.13.0, and the quality of the assembly and genome was determined with QUAST v.4.4 (37–41). CheckM v.1.0.18 was used to determine the completeness and contamination of the genome sequence using a set of marker genes (42). Annotation of whole genomes was carried out with Prokka v.1.14.5 (43). The nucleotide Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used to identify AV2, AV3, and AV18 by their 16S rRNA gene nucleic acid sequences (44). Bacterial identity was confirmed by the 97% nucleotide identity match as is standard for bacterial identification (45).

The 16S rRNA gene from AV25 and AV26 were PCR amplified using primers 27F (5' A GAGTTTGATCTMTGGCTCAG3') and 518R (5' GTATTACCGCGCTGCTGG3') at 4 mM MgCl<sub>2</sub> for both AV25 and AV26, and at an annealing temperature of 50°C for AV25 and 60°C for AV26 (46, 47). PCR products were cloned into pCR2.1 and transformed into chemically competent *Escherichia coli* Top10F' cells using the TOPO TA Cloning Kit and per the manufacturer's protocol (48). Clones were colony purified three times on Luria-Bertani (LB) plates containing 50 µg/L kanamycin, 40 mg/L X-Gal, and 100 mM isopropyl β-D-thiogalactopyranoside (IPTG). Plasmid DNA was extracted using a ZymoPURE Plasmid Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. Plasmid DNA was sent for sequencing at GeneWiz ([www.genewiz.com](http://www.genewiz.com), Azenta Life Sciences, South Plainfield, NJ, USA) using Sanger PCR-based sequencing with the GeneWiz M13R primer (5' CAGGAAACAGCTATGAC3'). Sequences were analyzed using 4Peaks v.1.8 (Nucleobytes, The Netherlands) and NCBI BLAST default settings (44).

## Characterization of NRIO

As the uncharacterized NDFO metabolism we observed was in phylogenetically related bacteria, all *Paraburkholderia* spp., the characterization of the NDFO metabolism was carried out in one isolate, AV18. Synthetic AMD was developed based on the average annual chemical levels within the Boyce Park system to minimize seasonal variations between water sampling (Table S1) (4, 49). AV18 was grown for 24 hours in R2A pH 4.0 broth, centrifuged to pellet bacteria, and resuspended in fresh R2A pH 4.0. The cells were diluted to a 1:10 ratio into synthetic AMD. NaNO<sub>3</sub> was added to a final concentration of 5 mM, and R2A was added to 10%. Samples of the culture were removed periodically (see Results), and the pH of the media was recorded with a Corning Model 440 pH meter 3-in-1 combo probe with RJ pH electrode (Corning Incorporated, Corning, NY, USA). Bacterial growth was measured at OD<sub>600</sub> concurrently using a Jenway Genova Plus spectrophotometer (Bibby Scientific Ltd., Stone, Staffs, UK). The ferrozine and Griess assays were used to measure Fe and nitrite levels, respectively (32, 33, 35).

The biotic interactions in the NRIO metabolism were determined by the addition of only 5 mM nitrate, only AV18, or both 5 mM nitrate and AV18 to sterile AMD. To rule out direct bacterial iron oxidation, the addition of AV18 to sterile AMD without nitrate was performed. A non-nitrate-reducing organism was included to determine if nitrite production must occur to see NRIO metabolism. AV18 was heat-killed by incubating at 65°C for 2 hours and confirmed to be heat-killed by plating an aliquot on R2A pH 4.0 agar plates concurrent to being added to sterile AMD and amended with nitrate to determine



if the cells must be alive and actively growing to reduce nitrate. Chemically driven nitrite iron oxidation was assessed by amending sterile AMD with 1 mM  $\text{NaNO}_2$ . To assess bacterially produced nitrite for iron oxidation, AV18 was grown for 24 hours in R2A, was qualitatively tested for the presence of nitrite with the Griess assay, then amended with nitrate and tested for nitrite production. After visual bacterial nitrite production, spent media were filter sterilized with a 0.2  $\mu\text{m}$  filter to remove cells, creating cell-free filtrate containing a metabolically produced  $\text{NO}_2$  byproduct. The sterile filtrate was added into sterilized AMD at 50% and 90% to measure changes in  $\text{Fe(II)}$ . Sterile AMD was used as a control. A two-factor analysis of variance with replication was performed to determine statistical significance using Microsoft Excel v.16.92.

### Design and optimization of novel *napA* and *rpoB* primer sets

Primers specific for AV18 were developed to determine the expression of the periplasmic nitrate reductase, *napA*, during nitrite production. Novel primer sets were developed as *Paraburkholderia* sp. is not a model organism with well-designed *napA* primer sets. This is the first known PCR amplification and target of this specific *napA* gene in this novel organism. KEGG was used to identify potential nitrate reduction genes in the Prokka v.1.14.5 genome annotation of AV18. A novel primer set for the control gene, *rpoB*, was also designed specifically for AV18. Primer sets were designed as nondegenerative with a length of 20–24 base pairs (bp) and a  $\Delta G$  no less than  $-6.0$  kcal/mol for hairpin loops, self-dimers, and hetero-dimers using the IDT oligo analyzer tool ([www.idtdna.com](http://www.idtdna.com)) (Table S2 and S3). Optimal PCR conditions were determined by testing the novel primer sets at 2 mM, 3 mM, and 4 mM  $\text{MgCl}_2$  and at annealing temperatures of 40°C, 50°C, or 60°C for a total of nine conditions using DNA extracted from AV18 as the template. Optimal conditions were determined by single bands of the expected band size on 5% acrylamide gels stained with ethidium bromide and visualized under UV (Fig. S1).

Sanger sequencing of the *napA* PCR product combined with a pairwise alignment to the annotated *napA* nucleotide sequence from the whole genome sequence was used to confirm the novel *napA* primer set (*napA\_F1* + *napA\_R1*) was targeting the correct gene sequence. The *napA\_F1* + *napA\_R1* PCR product was cloned into pCR2.1 and transformed into chemically competent *E. coli* Top10F' cells with the TOPO TA Cloning Kit and per the manufacturer's protocol (40). Transformants were selected and purified three times on LB agar with 50  $\mu\text{g/L}$  kanamycin, 40 mg/L X-Gal, and 100 mM IPTG. Plasmid DNA was purified from the clones with the ZymoPURE Plasmid Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instruction. The DNA sequence of the plasmid inserts was determined by Sanger sequencing using QuantumDye Terminator protocols (QuantumSeq, VH Bio, Gateshead, UK) and the universal M13 forward primer (5'-GTAAAAGGACGGCCAG3'). Sanger sequencing was carried out on an ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA). Sanger sequencing results were visualized with 4Peaks software v.1.8 (Nucleobytes, The Netherlands). The nucleic acid sequence of *napA* was aligned to the annotated whole genome nucleotide sequence using EMBOSS Needle v.6.6.0 to confirm amplification of the target gene was successful.

### Periplasmic nitrate reductase (*napA*) expression during nitrate reduction to nitrite

RNA was extracted from 0.25 mL of 5 mL log phase culture ( $\text{OD}_{600} = 0.500$ ) of AV18 to determine the expression of *napA*. A Griess assay was carried out on a 100  $\mu\text{L}$  portion of the culture to detect the bacterial production of nitrite. Nitrate was added to the culture to a final volume of 1 mM and at 5 min, 30 min, and 120 min post the addition of nitrate, RNA was extracted, and the Griess assay was conducted as above. RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The cDNA was used with our novel *napA* primers at an annealing temperature of 60°C and 2 mM  $\text{MgCl}_2$  and with our novel *rpoB* primers at an annealing temperature of

60°C and 4 mM MgCl<sub>2</sub> (50). Reverse transcriptase PCR (RT-PCR) products were visualized under UV light on a 5% acrylamide gel stained with ethidium bromide.

### Presence and abundance of *Paraburkholderia* sp. taxonomic relatives in two acidic AMD PRS at the order, family, and genus level

In a prior study (M. Valkanas, personal communication), two acidic AMD PRS systems (Boyce Park and Middle Branch) were used for a seasonal study to survey the composition of the mixed microbial community using sequences from the 16S rRNA genes in each season (four times per year) in replicates of two within each pond. These sequences can be found under NCBI BioProject accession number [PRJNA686480](#) (Fig. S1). For these sequences, DNA was extracted from slurry samples using the Qiagen DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The 16S rRNA gene V3 and V4 region was amplified using primers S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21(5'-GACTACHVGGGTATCTAATCC-3') on the Illumina MiSeq following the Illumina 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA) (51, 52).

In the data reported here, the sequences were downloaded from NCBI and were filtered and analyzed using dada2 v.1.16 in RStudio v.2021.09.01 (53). Analysis with dada2 was carried out for each individual pond within the systems and by pooling the sequences for all four seasons. In dada2, a parametric error model was used to determine the error rate, which was in turn used to dereplicate, filter, and trim the sequence reads. Forward reads had the last 30 bp removed, and reverse reads had the last 20 bp removed. All samples had the first 100 bp removed. Sequence reads were paired to construct an amplicon sequence variant (ASV) matrix that was denoised and had chimeras removed. Taxonomic 16S rRNA gene identity of the AMD PRS mixed bacterial community was assigned to individual ASVs in dada2 using the SILVA v.138.1 data set (54–56). Taxonomic 16S rRNA gene identity text files of the AMD PRS mixed bacterial community generated by dada2 were analyzed in Microsoft Excel v.16.9 to determine total bacterial abundance and abundance of Burkholderiales (order), Burkholderiaceae (family), and *Paraburkholderia*-*Burkholderia*-*Caballeronia* (genus) within the community.

## RESULTS

### Seasonal chemical data at Boyce Park PRS for pH, iron, nitrate, and nitrite

The acidic Boyce Park PRS was surveyed to determine its chemical and microbiological profile. Seasonal changes are seen for pH, iron, and nitrate (Fig. 1B). The pH at Boyce Park stays acidic <6.0 during all seasons tested. On average, iron enters the system above the Environmental Protection Agency (EPA) limits for drinking water (0.3 PPM) and freshwater aquatic organism exposure (1.0 PPM) (10, 57–60). Pond 1 of the Boyce Park system is consistently the most heavily contaminated pond within the system, at 3 PPM of iron. Although the amount of detectable iron from pond 1 decreases as it sequentially moves through ponds 2, 3, 4, and finally into pond 8, iron still exits the system above EPA limits at ≥2.0 PPM for aquatic life exposure and drinking water. Overall, the highest levels of iron were detected in the PRS in the spring, excluding pond 5, which was the lowest for all seasons. In fall, the levels of iron and nitrate in the PRS are overall lower compared to the other seasons. Levels of nitrate are below EPA limits for drinking water (44.3 PPM), and nitrite is below detectable levels by IC.

### Frequency of iron oxidation and NDFO within the culturable mixotrophic community in the acidic Boyce Park PRS

It has been shown that iron oxidation under acidic conditions in a PRS is mediated by bacteria (61). The metabolic frequency of direct iron oxidation and acidic NDFO in bacteria was determined for the culturable mixotrophic bacterial communities for all the ponds at the Boyce Park PRS by screening 570 colonies from each pond, for a total of 4,560 bacteria screened for the entire system (Fig. 2) (62–65). Of the 4,560 culturable

bacteria screened, iron oxidation metabolism and acidic NDFO metabolism were found in all ponds at Boyce Park. The lowest frequency of iron oxidation metabolism in the culturable mixotrophic community was found in ponds 4 and 8 at 2.1% (12/570) and the highest frequency of iron oxidation metabolism in pond 3 at 11.4% (65/570). The lowest frequency of NDFO metabolism in the culturable mixotrophic community was found in ponds 5 and 8 at 1.4% (8/570) and the highest frequency of NDFO metabolism in the cultured mixotrophic community in ponds 2 and 3 at 6.0% (34/570).

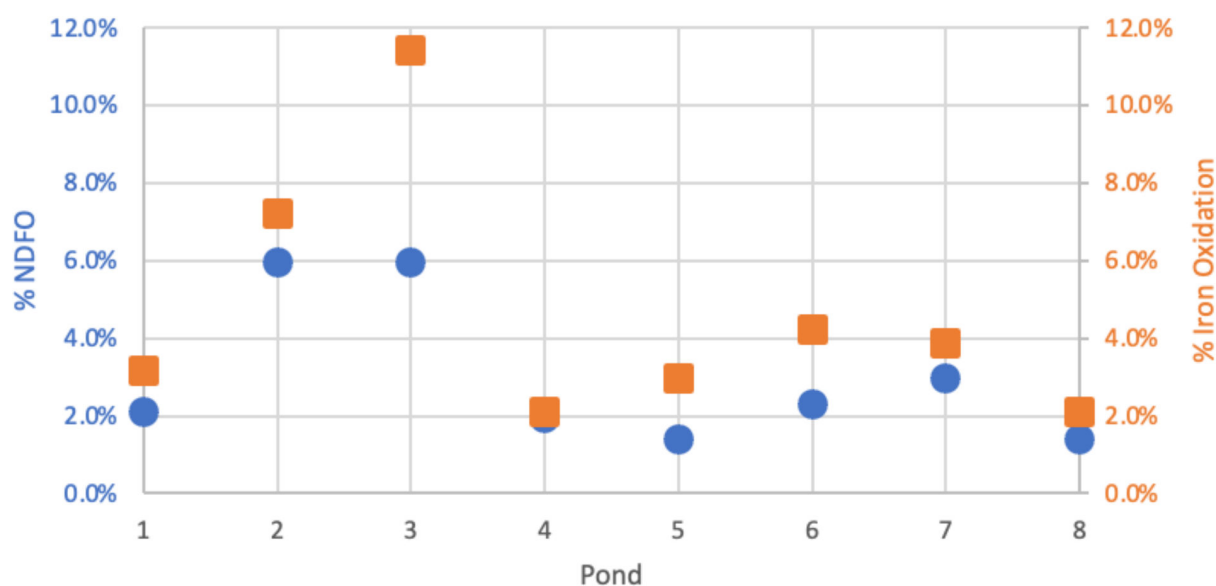
### Identification of isolates with NDFO phenotypes

Five NDFO isolates from the Boyce Park PRS were purified from NDFO screens to examine the NDFO mechanism. Whole genome sequencing was carried out for bacterial NDFO isolates AV2, AV3, and AV18. These isolates were identified as *Paraburkholderia* spp. with a percent identity of 99%, 100%, and 99%, respectively, to the known *Paraburkholderia acidipaludis* 16S rRNA gene. This is above the 97% identity routinely used for bacterial identification (Table 1) (45). We note that iron oxidase enzyme cytochromes, *cyc2* and *cyt572*, are not present in the annotated whole genome sequence for AV2, AV3, or AV18 (66–69). Isolates AV25 and AV26 were also identified as *Paraburkholderia* sp. via Sanger sequencing of their 16S rRNA gene and their percent identities of 98% to *Paraburkholderia acidipaludis*. As all NDFO isolates identified were *Paraburkholderia* spp., characterization of the NDFO mechanism was carried out in AV18.

### Characterization of acidic NDFO as bacterial nitrate reduction chemical iron oxidation

Characterization of iron oxidation and nitrate reduction in AV18 was quantified in sterile synthetic AMD supplemented with 10% R2A and  $\pm 5$  mM  $\text{NaNO}_3$  (Fig. 3; Table S1). The decrease in ferrous iron is only observed when 5 mM  $\text{NaNO}_3$  is added to the culture, nitrite is produced, there is concurrent bacterial growth (as measured by  $\text{OD}_{600}$ ), and the pH remains acidic. The oxidation of Fe(II) and production of nitrite are only observed when sterile AMD is inoculated with AV18 and supplemented with  $\text{NaNO}_3$  (Fig. 3A and B). As concurrent iron oxidation and nitrite production occur, bacterial growth is detectable, and the pH remains acidic (Fig. 3C and D).

Iron oxidation does not occur without the addition of both AV18 and nitrate (Fig. 4A and B). When a non-nitrate-reducing bacterium is used for inoculation, nitrite production



**FIG 2** Frequency of iron-oxidizing bacteria (orange square) and acidic NDFO bacteria (blue circle) was determined for all ponds at acidic Boyce Park. The frequency of bacteria with these metabolisms was determined in the culturable mixotrophic bacterial community.

TABLE 1 Genome characteristics for NDFO isolates AV2, AV3, and AV18

| Characteristic           | Value for isolate:                |                                  |                                   |
|--------------------------|-----------------------------------|----------------------------------|-----------------------------------|
|                          | AV2                               | AV3                              | AV18                              |
| Genome size              | 6.74 Mb                           | 6.64 Mb                          | 6.75 Mb                           |
| GC content               | 63%                               | 62%                              | 64%                               |
| Number of contigs        | 126                               | 95                               | 126                               |
| Largest contig           | 366,035 bp                        | 403,425 bp                       | 494,841 bp                        |
| Genome completeness      | 99.82%                            | 99.92%                           | 99.82                             |
| Contamination            | 0.96%                             | 1.34%                            | 0.96%                             |
| 16S rRNA gene % identity | 98.6% <i>Paraburkholderia</i> sp. | 100% <i>Paraburkholderia</i> sp. | 98.6% <i>Paraburkholderia</i> sp. |
| Order                    | Burkholderiales                   | Burkholderiales                  | Burkholderiales                   |
| Accession number         | <a href="#">SAMN40122491</a>      | <a href="#">SAMN40122492</a>     | <a href="#">SAMN40122493</a>      |

does not occur, and iron oxidation is not observed, indicating that the presence of bacterial cells alone does not cause the iron oxidation seen with nitrite producers (Fig. 4C and D). The addition of heat-killed AV18 cells to AMD with 5 mM NaNO<sub>3</sub> and R2A does not result in the production of nitrite or Fe(II) oxidation (Fig. 4E and F).

To determine the extent of abiotic Fe(II) oxidation, chemical grade NaNO<sub>2</sub> was added to sterile AMD, at a concentration of 1 mM nitrite, and Fe(II) levels were measured (Fig. 5A). Chemical grade NaNO<sub>2</sub> results in iron oxidation. Nitrite that was produced in an AV18 culture was filtered to remove the bacterial cells and added to AMD at a 1:1 ratio (50%) or a 1:9 ratio (90%) and measured for a decrease in Fe(II) in sterile AMD (Fig. 5B). In all cases of nitrite addition, no matter what the source of nitrite was, iron oxidation was observed.

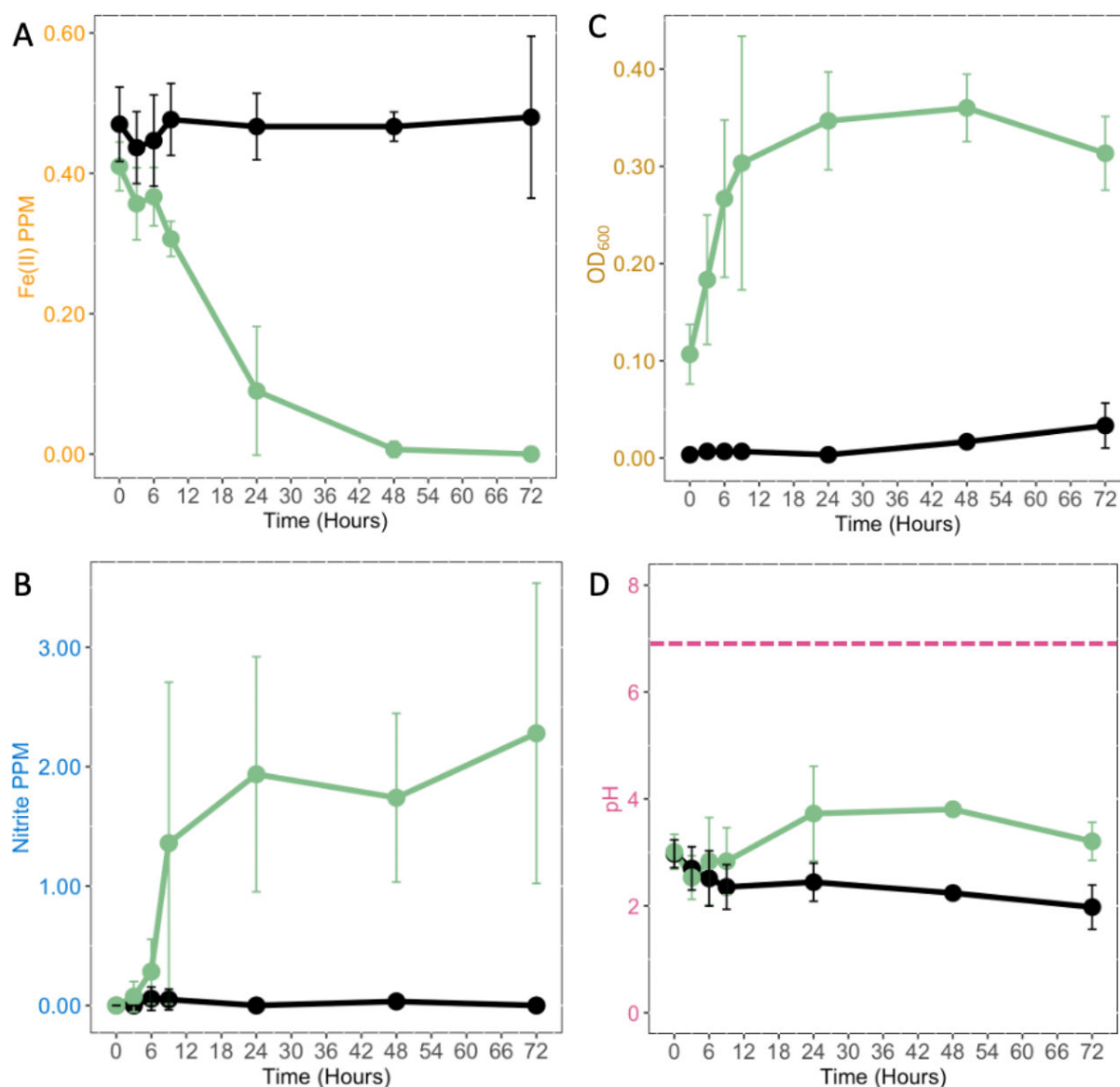
### *Paraburkholderia* sp. AV18 expresses *napA* during nitrate reduction to nitrite

The whole genome sequence of AV18 predicts a nitrate reductase (*napA*) gene. Primer sets were designed specifically for AV18 *napA*, using *rpoB* as a control. The primers were optimized using chromosomal DNA purified from AV18, yielding the predicted sized bands of 352 bp for *napA* and 208 bp for *rpoB* (Fig. S1). The EMBOSS Needle pairwise alignment of the DNA sequence of the *napA* PCR product and the annotated *napA* sequence from the whole genome sequencing resulted in a 100% identity and 100% alignment (352/352 bp). Cultures of AV18 qualitatively assessed using the Griess assay did not contain nitrite before nitrate was added to the media. Bacterially produced nitrite was present in the cultures at 30 min and 120 min (Fig. 6A). RT-PCR using our *napA* and *rpoB* primer sets show that *napA* is expressed by AV18 at 30 min and 120 min (Fig. 6B, lanes 4 and 5). Bands for *rpoB* are present at all time points.

### Presence and abundance of Burkholderiales in two acidic AMD PRS Boyce Park and Middle Branch

To determine the presence of *Paraburkholderia* sp. phylogenetic relatives in acidic AMD PRS, two PRSs (Boyce Park PRS and Middle Branch PRS) were examined. 16S rRNA gene sequences from our lab for each pond in the system are publicly available (accession number [PRJNA686480](#)) (27). The sequences were downloaded from NCBI and mined for phylogenetic relatives to *Paraburkholderia* sp. at the genus (*Paraburkholderia*), family (Burkholderiaceae), and order (Burkholderiales) level. Analysis of the 16S rRNA gene microbial community data from Boyce Park and Middle Branch PRSs was done using the RStudio dada2 pipeline to generate ASV tables that were analyzed in Microsoft Excel v.16.92 (Table S4). Burkholderiales (order level) are predicted in all ponds at Boyce Park and Middle Branch. The abundance of Burkholderiales at Boyce Park ranges from 2.8% to 9.7% (Fig. 7). The abundance of Burkholderiales at Middle Branch PRS ranges from 3.7% to 10.0%. At Boyce, sequencing was able to distinguish Burkholderiaceae in ponds 2, 6, 7, and 8, and *Paraburkholderia*-*Burkholderia*-*Caballeronia* were identified in pond 7. At



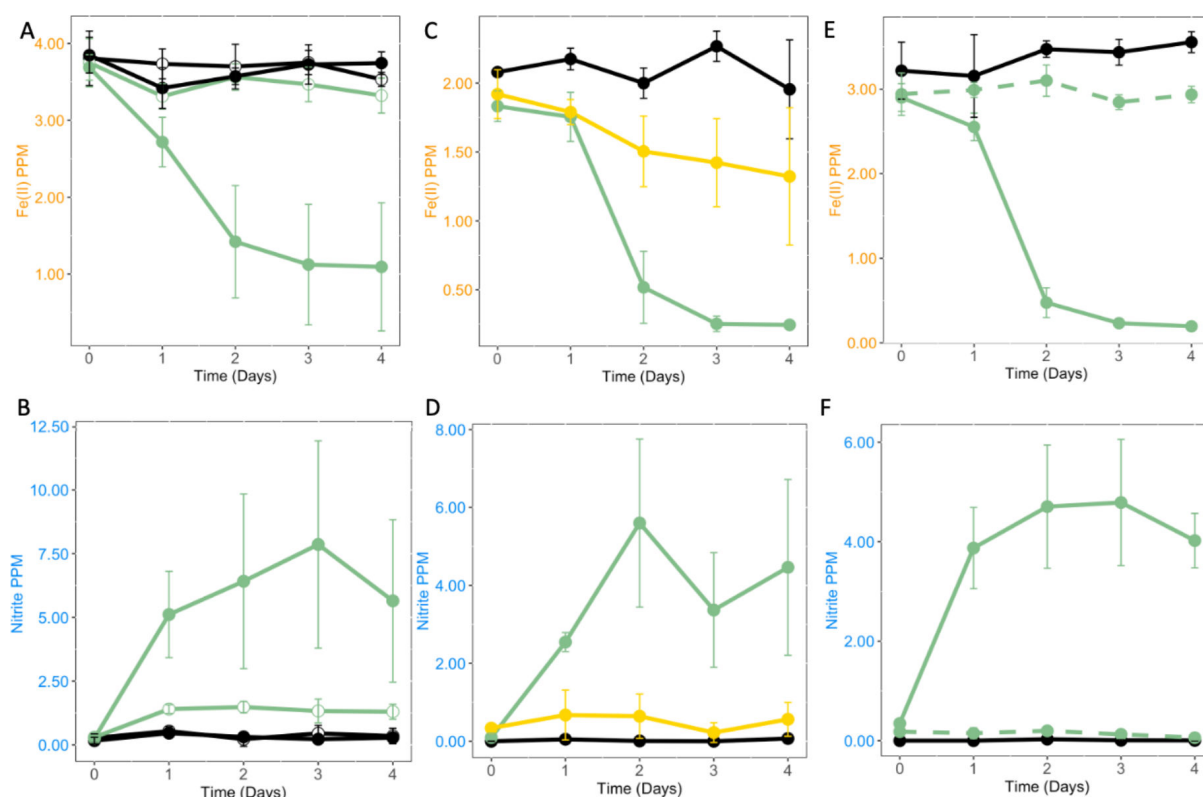


**FIG 3** As AV18 grows, it concurrently reduces nitrate and oxidizes iron while the pH remains acidic. Sterile AMD (black line) inoculated with *Paraburkholderia* sp. AV18 (green line) supplemented with 5 mM NaNO<sub>3</sub> (circle) and 10% R2A results in microbial nitrate reduction to nitrite that chemically oxidizes iron,  $n = 3$ . (A) Amount of ferrous iron measured in PPM over 72 hours (B) Amount of nitrite measured in PPM over 72 hours. (C) Bacterial growth determined by OD<sub>600</sub>. (D) The pH, the dashed pink line, shows neutral pH. There is a significant difference between samples that remained sterile and those inoculated with *Paraburkholderia* sp. AV18 overtime for Fe(II) ( $P < 0.00001$ ), NO<sub>2</sub> ( $P = 0.006$ ), and bacterial growth ( $P = 0.002$ ). The pH of the media for both inoculated and sterile AMD remained acidic with no significant change over time ( $P = 0.28$ ).

Middle Branch, sequencing was able to distinguish Burkholderiaceae in ponds MB2, MB3, MB4, MB5, and MB6, and *Paraburkholderia-Burkholderia-Caballeronia* were identified in pond MB4.

## DISCUSSION

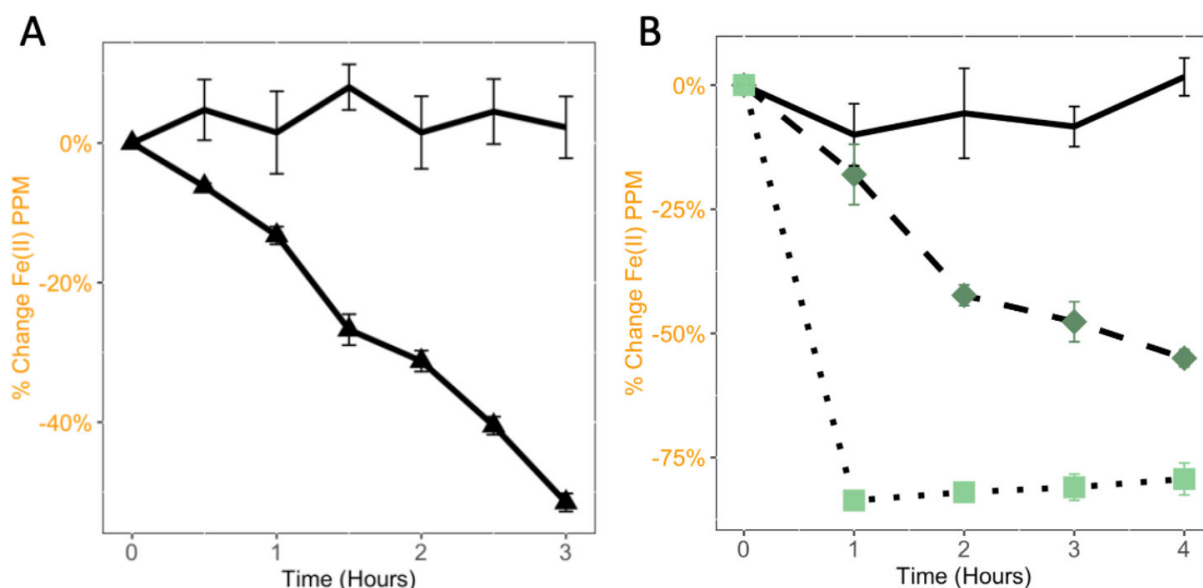
At a low pH ( $\leq 6$ ), iron oxidation is mainly microbially mediated as abiotic chemical iron oxidation is not thermodynamically favored under acidic conditions (23, 61). The Boyce Park PRS seasonally stays at a pH  $< 6$ , and despite levels of detectable iron decreasing from inflow into the system pond 1 at 4 PPM, it still exits the system above EPA limits at  $\geq 2$  PPM, leaving room for further system improvements (Fig. 1). Nitrate is detectable by IC at Boyce Park PRS, while nitrite is below detection levels for all seasons, which may



**FIG 4** The bacterial-mediated reduction of nitrate to nitrite causes iron oxidation in AMD ( $n = 4$ ). Sterile AMD is shown as a black line, AMD inoculated with *Paraburkholderia* sp. AV18 is shown as a green line, addition of nitrate is a closed circle, no addition of nitrate is an open circle, a non-nitrite-producing isolate is shown as a yellow line, and heat-killed *Paraburkholderia* sp. AV18 is shown by a green dashed line. (A) Iron oxidation is observed when *Paraburkholderia* sp. AV18 is inoculated into sterile AMD and supplemented with nitrate ( $P < 0.000001$ ). Direct bacterial iron oxidation by *Paraburkholderia* sp. AV18 does not occur ( $P = 0.08$ ). (B) Nitrite production is not observed without the addition of both *Paraburkholderia* sp. AV18 and nitrate ( $P = 0.0005$ ). (C) Iron oxidation does not occur to the same extent with the addition of a non-nitrite producer (recovered from the frequency screen) as AMD inoculated with *Paraburkholderia* sp. AV18 ( $P < 0.000001$ ). (D) Nitrate is not reduced to nitrite by the non-nitrite producer ( $P < 0.00001$ ). (E) Iron oxidation does not occur with the addition of heat-killed *Paraburkholderia* sp. AV18 to the same extent as live *Paraburkholderia* sp. AV18 ( $P < 0.00001$ ). The amount of Fe(II) from day 0 to day 4 does not result in a significant change when *Paraburkholderia* sp. AV18 is heat-killed ( $P = 0.33$ ). (F) Nitrite production does not occur when *Paraburkholderia* sp. AV18 is heat-killed before addition to the media ( $P < 0.000001$ ).

be due in part to the rapid chemical interaction of nitrite with Fe(II) (26, 70). Although biogeochemical interactions leading to chemical transformations between microbial-produced nitrite and environmental Fe(II) have been reported in freshwater sediments, coastal marine sediment, and paddy soils, this interaction has been underreported in acidic AMD (17, 22, 24, 70–73). Here, we demonstrate NDFO metabolism can significantly contribute to iron bioremediation and could be further leveraged to optimize bioremediation, reaching discharge goals.

From screening the culturable mixotrophic microbial population at Boyce Park PRS for both iron oxidation and NDFO metabolism, we found bacteria with both metabolisms present throughout the entire system with frequencies between 2.1%–11.4% and 1.4%–6.0%, respectively (Fig. 2). The finding of bacteria in the culturable mixotrophic community that contribute to NDFO in every pond at the Boyce Park system is important because we predict these microbes can be utilized to improve bioremediation. We used this culture-dependent approach of screening 4,569 individual bacterial colonies, in addition to molecular-based culture-independent methods, to better understand and characterize the microbial communities within the Boyce Park PRS (65). In the Boyce Park PRS, 16S rRNA gene sequencing of a total of 7,670 individual ASVs was reported, ranging from 680 to 1,196 ASVs per pond. Direct conclusions and comparisons between the number of microbes detected through molecular approaches and those that are culturable can be

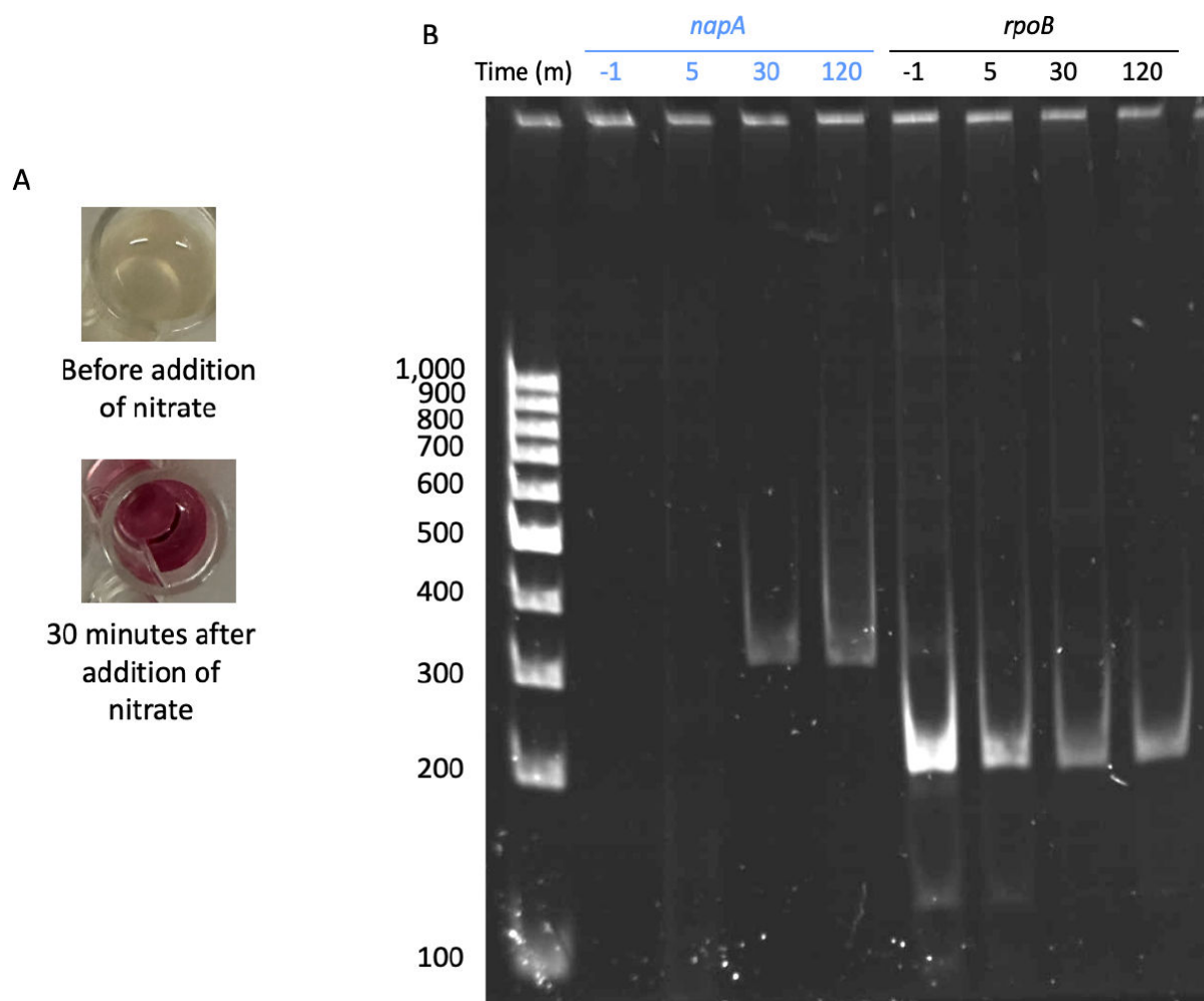


**FIG 5** The chemical oxidation of iron by nitrite was determined to occur both with chemical sodium nitrite and bacterial-produced sodium nitrite. Sterile AMD is the black line, AMD amended with laboratory sodium nitrite is shown with a black triangle marker, AMD amended with bacterial *Paraburkholderia* sp. AV18 produced nitrite at 50% is shown with a dark green diamond, and 90% is shown with a light green square. (A) After the addition of laboratory chemical sodium nitrite at time 0 min indicated by the arrow, abiotic chemical Fe(II) oxidation is detected. There was a significant change between sterile AMD with sodium nitrite by hour 3,  $n = 4$  ( $P < 0.00001$ ). (B) The addition of cell-free sterile nitrite produced by *Paraburkholderia* sp. AV18 to sterile AMD results in detectable iron oxidation. There is a significant difference between sterile AMD and AMD amended with cell-free biotically produced nitrite over time,  $n = 3$  ( $P < 0.00001$ ).

challenging to make as it has been estimated that less than 0.1% of microbes are culturable (62–65). Additionally, AMD PRS are generally considered low microbial diversity systems with low species richness and low complexity (3, 74). We do acknowledge the limitations of culture-dependent approaches and the selection bias that can occur based on culturing methods (63, 64). However, NRIO is seen with the *Paraburkholderia* spp. we cultured, and relatives of bacteria are present in both acidic PRS we examined. Future work in these systems can attempt to culture more diverse and novel organisms, including cultivation with iron, nitrate, and NDFO selection media, and with variations of cultivation temperatures (15°C, 22°C, etc.) to better mimic the *in situ* conditions of PRS located within Pennsylvania. There is a need for the cultivation of novel microorganisms from soil environments to better understand their metabolic roles in chemical cycling (65).

Acidic pH NDFO isolates were characterized to elucidate the distinction between neutral pH NDFO and acidic pH NDFO metabolisms (24). We characterized our acidic NDFO metabolism in bacterial isolates under aerobic conditions, in contrast to the NDFO at neutral pH that happens under strict anaerobic conditions (71, 75). Bacterial NDFO isolates AV2, AV3, AV18, AV25, and AV26 were all identified as *Paraburkholderia* sp. by their 16S rRNA gene (Table 1). *Paraburkholderia* sp. is a novel genus that split from Burkholderiales based on distinct *recA* clustering and isolation as nonpathogenic environmental bacteria (76–82). Some *Paraburkholderia* spp. have previously been shown to reduce nitrate to nitrite; iron oxidation has not previously been reported in *Paraburkholderia* spp. (76, 77, 79–84). *Paraburkholderia acidipaulis* was isolated from a Chinese water chestnut that had high sulfur concentrations in highly acidic soils, like the conditions found in AMD (80). The isolation of multiple NRIO *Paraburkholderia* spp. alludes to a strong presence of this bacteria within the PRS.

We characterized the NDFO mechanism at an acidic pH in AV18 as the biogeochemical interaction of nitrite produced biotically from bacterial nitrate reduction driving chemical Fe(II) oxidation (NRIO metabolism, Fig. 3 and 4 and 5B). AV18 did not directly oxidize iron (Fig. 4A), and its presence alone did not result in iron oxidation (Fig. 4E).



**FIG 6** Expression of *napA* occurs when nitrite production by AV18 is observed. (A) Observation of lack of nitrite before the addition of nitrate and the production of nitrite after the addition of nitrate at 30 min. (B) Lane 1 is GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA). Lanes 2–4 *napA* expression, lanes 5–8 *rpoB* control expression.

Rather, bacteria needed to be able to metabolically produce nitrite from nitrate as the first step. Then the bacterially produced nitrite can oxidize Fe(II) as the second step (Fig. 8). Furthermore, the addition of cell-free bacterially produced nitrite to sterile AMD (Fig. 5B) resulted in iron oxidation as measured by a decrease in Fe(II). In acidic AMD, the addition of nitrite leads to increased iron oxidation, unlike the example of bacterially mediated neutral NDFO reported in the literature (Fig. 5A) (71, 75). In neutral pH environments, bacterially driven NDFO does not include *Paraburkholderia* sp. or *Burkholderia* sp. (22, 24–26, 85). NRIO is most likely enzymatically driven by NapA, as *napA* is expressed during biotic nitrate reduction to nitrite (Fig. 6). Nitrite produced by NapA can react abiotically with dissolved Fe(II), leading to the oxidation and precipitation of Fe(III) (70, 72).

The presence of *Paraburkholderia* spp. phylogenetic relatives at the acidic Boyce Park PRS was determined by mining the microbial community 16S rRNA gene data in an approach to link cultivation results to *in situ* data (Fig. 7). Burkholderiales are present within all ponds of Boyce Park ranging from the lowest in pond 6 at 2.8% (33/1,179 ASVs) and highest in pond 8 at 9.7% (116/1,196 ASVs). At a second acidic PRS, Middle Branch PRS, located at latitude 41° 20' 48.0012" N, longitude 77° 52' 3" W, 65 miles north of state college in Renova, Pennsylvania, was also surveyed for its 16S rRNA community structure following the same methods within this paper (Fig. S1) (27). The AMD in the Middle

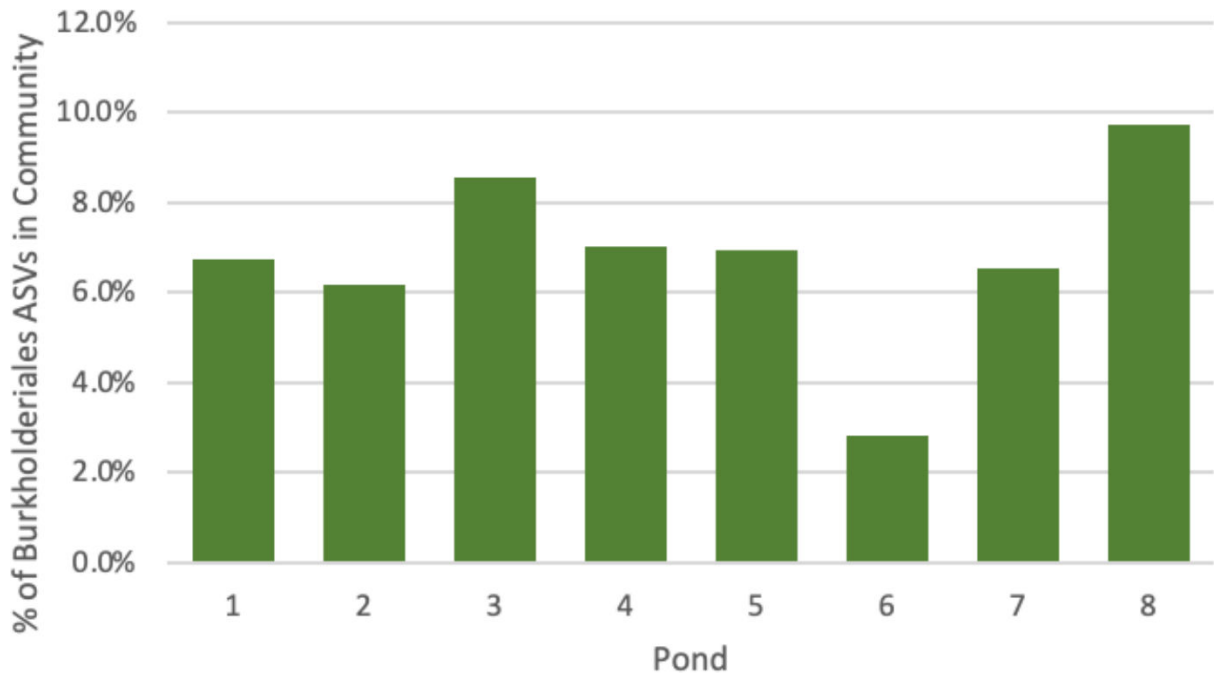


FIG 7 Presence and abundance of Burkholderiales compared to all bacteria within the Boyce Park acidic AMD PRS.

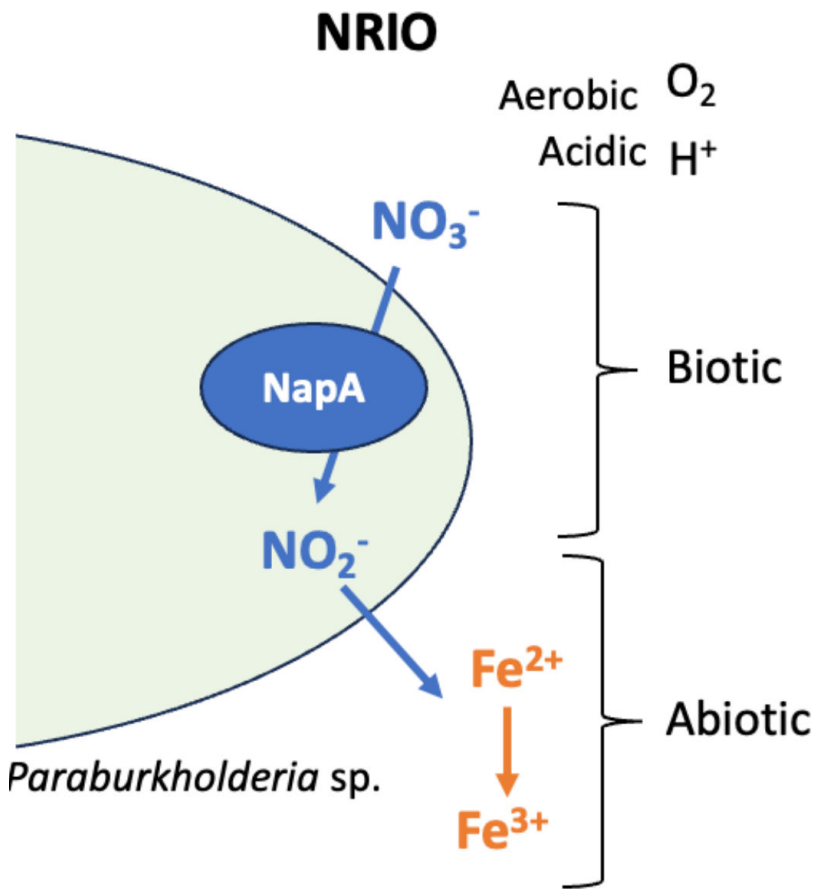


FIG 8 The biotic–abiotic biogeochemical interaction of nitrogen and iron by nitrate-reducing bacteria producing nitrite through NapA leads to chemical iron oxidation in acidic aerobic AMD under laboratory conditions.



Branch system is acidic at pH ~2.8 from both surface and underground source mixed together (27, 30). Burkholderiales are present within all ponds at Middle Branch, ranging from the lowest in pond MB3 at 3.7% (30/821 ASVs) and highest in pond MB4 at 10.0% (123/1,234 ASVs) (Fig. S2). The presence of Burkholderiales in two distinct acidic systems (Boyce Park and Middle Branch) alludes to the potential of bioremediation through nitrite production in multiple acidic systems. Additional research will determine if the relatives of *Paraburkholderia* spp. contain the same metabolic capabilities as AV18. We have used a combination of culture-dependent and culture-independent methods to better assess the microbial community and its potential impacts on iron remediation (62, 64).

As nitrate is present within Boyce Park PRS, we hypothesize that nitrate reducers may be an unharnessed microbial strategy to improve iron remediation in acidic AMD passive systems. We conclude that AV18 bioremediates iron by NRIO and suggest that NRIO by *Paraburkholderia* spp. and other nitrate reducers are actively impacting iron removal in acidic AMD (Fig. 8). We propose that promotion of nitrate reduction could be targeted as a strategy to meet discharge levels. AMD PRS can have limited organic matter available, which in turn limits microbial growth as compared to active systems (74). Our work shows the potential of biostimulation of the native nitrate-reducing microbial community could improve iron remediation within an acidic system. We have previously shown the potential for biostimulation of the native microbial community through natural carbon supplementation (86). As nitrate is already present within the system, driving NRIO bacteria to reduce nitrate to nitrite could decrease the concentration of soluble Fe(II) in the system, and may be a faster, more efficient approach for microbial iron bioremediation in acidic AMD or other acidic high iron environments.

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## AUTHOR CONTRIBUTIONS

Anna Vietmeier, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing | Michelle Valkanas, Conceptualization, Data curation, Investigation, Methodology, Resources, Visualization, Writing – review and editing | Natalie Lamagna, Data curation, Formal analysis, Methodology, Visualization, Writing – review and editing | Samuel Flett, Investigation, Writing – review and editing | Djuna Gulliver, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Visualization, Writing – review and editing | Nancy Trun, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review and editing

## DATA AVAILABILITY

Whole genome sequences were uploaded to NCBI (AV2 accession number [SAMN40122491](#), AV3 accession number [SAMN40122492](#), and AV18 accession number [SAMN40122493](#)). Nucleotide sequences of *napA* and *rpoB* from the whole genome sequence have been uploaded to NCBI GenBank under accession numbers BankIt2742455 *napA* [OR5574423](#) and BankIt2742466 *rpoB* [OR557424](#), alongside our novel primer sets for *napA* forward primer *napA\_F1* (5′AAGAGCGTAAAGAGCGTGTGCC3′) and reverse primer *napA\_R1* (5′CGTGTGTTGACGAGATACTGCG3′) and *rpoB* forward primer *rpoB\_F1* (5′CCCATCGTTACCAGTTCC3′) and reverse primer *rpoB\_R1* (5′ATTCCTTGATGTTGAATGCCG3′).

## ADDITIONAL FILES

The following material is available [online](#).

## Supplemental Material

**Supplemental material (AEM00405-25-s0001.pdf).** Tables A1 to A4; Fig. A1 and A2.

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