Reduction of nitrate to nitrite by microbes under oxic conditions

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ABSTRACT

Human activities have greatly increased the input of nitrate to natural and managed ecosystems, but the fate of excess soil nitrate is still unclear. Many studies assume that dissimilatory reduction of nitrate to nitrite is an anaerobic process, but this first step of denitrification can occur in some bacteria at oxygen concentrations that are high enough to repress downstream reduction of nitrite to gaseous products. Here, we examine whether dissimilatory reduction of nitrate under aerobic conditions is an additional, underappreciated fate of nitrate in soil. Aerobic nitrate reduction occurred in soils when provided with both nitrate and a carbon source, with the greatest nitrite accumulation in the wetland sites. The addition of a nitrification inhibitor did not significantly reduce aerobic nitrate reduction activity, nor did an assimilation repressor. Average nitrite production in soils with added carbon, nitrate, and nitrification inhibitor ranged from 7.5-50% of added N-nitrate in a five-hour incubation. Bacteria capable of aerobic nitrate reduction were readily isolated from these soils, comprising approximately 35% of the isolates retrieved. Sequencing 16S rDNA of these isolates revealed both gram-negative and gram-positive bacteria, with the majority being gram-negative proteobacteria. In six of the isolates, onset of nitrate reduction occurred at 45-86% of atmospheric oxygen concentrations. Reduction of nitrate under aerobic and semi-aerobic conditions did not result in significant enhancements in carbon dioxide production or total electron flow rate to electron acceptors. The genomes of these six isolates were sequenced and targeted RT-qPCR revealed a wide diversity of regulatory controls on the nitrate reductase(s). The results suggest that aerobic nitrate reduction can occur in diverse bacteria, have multiple types of physiological controls, and can occur independently of the gas-forming reactions of denitrification. Thus, it is an unappreciated fate of nitrate in soil.

55 *Keywords:* Aerobic nitrate reduction; Bacterial genome; Dissimilatory nitrate reductase; Nitrite
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1. Introduction

Anthropogenic nitrogen (N) production has led to new challenges in understanding how excess N cycles in natural and managed ecosystems (Schlesinger, 2009; Fowler et al., 2013). A particular problem is that excess nitrate can potentially be transferred from soil to small streams and shallow groundwater, to rivers, and ultimately to coastal marine ecosystems where it fuels eutrophication (Carpenter et al., 1998). This flow from soil to aquatic ecosystems can be mitigated to some extent, since nitrate can be assimilated by plants and bacteria or reduced to either ammonium (DNRA) or gaseous end products through microbial dissimilatory processes. Microbial denitrification, the stepwise reduction of nitrate to dinitrogen via nitrite, nitric oxide, and nitrous oxide, can potentially alleviate the negative effects of nitrate leaching from soils.

Denitrification is frequently viewed as a tightly coupled pathway whose activity is inversely correlated with oxygen (O_2) levels, and thus has been studied mostly in soils with very low O_2 concentrations. The typical denitrifier is a facultative anaerobe that preferentially utilizes O_2 as an electron acceptor and restricts electron flow towards N-oxides as long as O_2 is available (Shapleigh, 2012; Bergaust et al., 2011). However, studies on model denitrifiers have shown that different steps in the pathway can be differentially controlled by O_2 , and the reason that O_2 quenches the process may lie as much with chemical constraints as with physiological necessities (Martinez-Espinosa et al., 2011; Shapleigh, 2011; Bateman and Baggs, 2005). For example, the rapid chemical reaction of NO with O_2 may be the reason why the production and reduction of NO is the part of the pathway most tightly regulated in the presence of O_2 (Bergaust et al., 2008; Hartsock and Shapleigh, 2010; Watmough et al., 1999). In contrast, nitrate and nitrite do not readily react with O_2 , and thus reduction of nitrate to nitrite is adjusted to best fit physiological

necessities (Dalsgaard et al., 2014; Hartsock and Shapleigh, 2011; Sears et al., 2000; Martinez-Espinosa et al., 2011).

Given that the multiple enzymes in the denitrification pathway do not necessarily have tightly linked expression, it is reasonable to postulate that specific steps in the pathway function independently. More specifically, there is no reason to assume that the first step in the pathway is obligatorily linked to any of the remaining three steps, and thus, this step could occur under less stringent O₂ control resulting in an underappreciated source of nitrite. For example, Su et al. (2011) and Isobe et al. (2012) showed circumstantially that nitrite production in acidic soils is necessary to balance the budget of the chemically converted nitrous acid (HONO) compound found in the atmosphere (Isobe et al., 2012; Su et al., 2011). Oswald et al. (2013) provided evidence that neutral-to-basic soils in arid and arable areas show relatively high emission fluxes of HONO and NO in nitrite rich soils, accounting for up to 50% of the reactive N release from soil (Oswald et al., 2013). Chemodenitrification of nitrite has also resulted in the production of gaseous N-oxides, with the reactive free radical NO as the major product (Thorn and Mikita, 2000; Davidson, 1992; Stuvén et al., 1992). As chemodenitrification occurs when soil pH is <5 (Chalk and Smith, 1983), this process is likely significant in soils of the northeastern USA (Kulkarni et al., 2013). Another possible abiotic fate of nitrite is the binding to soil via the so-called nitrosation process (Smith and Tiedje, 1979; Matocha et al., 2012). This fate could explain nitrate fixation onto soil organic matter that has been suggested as a sink for added anthropogenic N in some forest ecosystems (Davidson et al., 2003).

The production of nitrite from nitrate in soils under oxic conditions has been shown by a few other studies. Previous work by Carter et al. (1995) has described heterotrophic bacterial isolates from soil capable of nitrate reduction in the presence of atmospheric oxygen, which has

been referred to as aerobic nitrate reduction (ANR) and will be the term used here (Carter et al., 1995). Also, Vega-Jarquin et al. (2008) demonstrated ANR in soil from an alkaline lake bed in Mexico when incubated in 100% atmospheric O₂ and in the presence of acetylene, a nitrification inhibitor (Vega-Jarquin et al., 2008).

There is still limited understanding of the bacteria capable of performing ANR. Here, we examine ANR in a range of soil types using an oxidized carbon source, succinate, with the addition of nitrification and nitrate assimilation inhibitors in order to provide a more detailed understanding of the eco-physiological context of ANR, including the determination of the O₂ concentration at the onset of nitrate reduction in bacterial isolates demonstrating the ANR phenotype. With these bacterial isolates we also investigated the diversity of regulation of the dissimilatory nitrate reductase(s), which include the membrane-associated nitrate reductase (Nar) and a periplasmic enzyme (Nap). The results of this work suggests that ANR is a common, yet underappreciated trait, and this pathway may make a significant but still unclear contribution to the soil nitrogen cycle.

2. Methods

2.1. Experimental sites and soils

Soils were collected in September 2010 from four sites near Ithaca, NY (Table 1). The soil at the agricultural field site and second-growth forest site at McGowan Farm are classified as an Alfisol (mesic Glossoboric Hapludalf; USDA Classification) derived from sandy outwash with a fine loamy texture (Beatty and Stone, 1986; Castrillo et al., 2007). The agricultural field site and second-growth forest site at Smith Woods are Alfisol (mesic Psammentic Hapludalf; USDA classification) with a sandy texture (Beatty and Stone, 1986; Marks et al., 1999). Two

forested wetland sites, Sapsucker Woods and Stewart Park, were also sampled. In both sites, trees occur on elevated hummocks surrounded by water-filled pits, which are 0-40 cm deep. A fragipan soil horizon about 1 m deep prevents drainage and maintains a high water table. The soil at the Sapsucker Woods site is classified as an Inceptisol (mesic Mollic Endoaquept; USDA Classification), which has a fine-loamy texture and is non acidic (Miller et al., 2004). Soil at the Stewart Park site is also classified as an Inceptisol (mesic Fluvaquentic Endoaquept; USDA Classification). At both of the forested wetland sites soils were collected separately from the hummocks and pits. In July 2011 samples were taken from a forested site at the Hubbard Brook Experimental Forest (HBEF) within the White Mountain National Forest, West Thornton, NH. Soils at this second-growth forest site are classified as Spodosol (Typic Haplorthod; USDA Classification) derived from granitic glacial till with sandy-loam to loamy-sand texture (Adams et al., 2008).

At each of the sites described above, soil samples (roughly 200 g each) were collected from three randomized locations from the 0-10 cm depth interval. Samples were stored for <60 minutes in plastic bags before being assayed. Soil pH was measured with a glass electrode in a 1:5 soil-water slurry. Soil moisture content was calculated as $((\text{wet weight} - \text{dry weight}) / \text{wet weight}) \times 100$.

<Insert Table 1 here>

2.2. Assay for ANR

Analysis of ANR activity began within 60 minutes of soil collection. Soil (0.5 g fresh-weight) was added to a 1 ml solution in a 1.5 ml microcentrifuge tube. The solutions added to the soils were: 1) deionized water (W), 2) deionized water with 0.03 mg N-KNO₃ g⁻¹ fresh-weight

soil (W+N), 3) Sistrom's medium (Lueking et al., 1978; SIS); 4) SIS plus 0.03 mg N-KNO₃ g⁻¹ fresh-weight soil (SIS+N); 5) SIS+N and 300 mM dicyandiamide (DCD) (SIS+N+DCD), and 6) SIS+N and 113 mM ammonium sulfate (SIS+N+NH₄). Succinate, a relatively oxidized carbon source found in soils, was the carbon source in the Sistrom's medium and was used to provide the approximate redox level to cell biomass (McKinlay and Harwood, 2011), where Carter et al. (1995) previously used a reduced carbon source of butyrate to demonstrate ANR. DCD was used as a nitrification inhibitor (Giltrap et al., 2010; Davies and Williams, 1995) and excess ammonium sulfate was used to ensure nitrite production was not a consequence of the assimilatory nitrate reductase (Giltrap et al., 2010; Vega-Jarquin et al., 2008).

All incubations were done in triplicate and agitated continuously by vortex under atmospheric conditions for up to five hours. Nitrite accumulation was measured after 30 minutes, 60 minutes, three hours, and five hours of incubation. Nitrite was determined by adding 50 µl of the soil slurry to 900 µl phosphate buffer and 1 mL of Griess reagents A and B (Roussel-Delif et al., 2005). Intensity of colorimetric change was measured on the spectrophotometer at an absorbance of 540 nm.

2.3. Classification and identification of isolates

Bacterial isolates were isolated under well-aerated, heterotrophic conditions from the soils collected from the various sites. Bacteria capable of ANR were isolated as described in Roco et al. (*in press*). In brief, isolates from soil were incubated aerobically for 24 hours at 30°C in a 96-well plate on Sistrom's agar medium supplemented with 113 mM (NH₄)₂SO₄ and 10 mM KNO₃ before being tested for nitrite production (using 20 µl of the Griess reagents). Approximately 50 isolates capable of nitrite production under these conditions were selected for

16S rDNA gene sequence using the 8F and 1492R universal primers and a previously described PCR protocol (Roco et al. *in press*). Amplicons were sent for Sanger sequencing at the Cornell University Core Laboratory Center.

Isolates capable of ANR were also tested for growth in an anaerobic jar on Sistrom's medium with 10 mM KNO₃. Anoxic conditions were maintained using the GasPak™ system as described by the manufacturer (Becton, Dickinson and Company). Six isolates capable of ANR were chosen for full genome sequencing based on phenotypic traits. Details of their sequencing and annotation can be found elsewhere (Roco et al., 2016).

2.4. Gas and nitrite measurements

The six bacterial isolates were raised from frozen stocks and pre-cultured at 30°C under aerobic conditions in Sistrom's medium with no amended nitrate. These cultures were used as inocula for two sets of incubation experiments, which were performed in triplicate at 30°C in 120 mL glass vials with 50 mL liquid Sistrom's medium. The experiments were set up to assess: 1) apparent critical [O₂] threshold for ANR; and 2) the impact of ANR on apparent growth rates and electron (e⁻)-flow (isolates 2, 5 and 16 only). Since both O₂ and N-oxides produced were monitored, it was possible to estimate the e⁻-flow rate (V_{e⁻} to O₂ and N-oxides, μmol vial⁻¹ h⁻¹) for each time increment between two samplings. For the first experiments examining the O₂ threshold of ANR, aerobic pre-cultures of each of the six isolates were transferred to vials to initial OD₆₀₀ ≤ 0.035, starting at ambient [O₂] and containing either 1 or 2 mM KNO₃. The set-up for the second experiment exploring the impact of ANR on growth rate was similar to the first experiment, except that nitrate was added approximately 2.5 hours into the incubations to assess whether this had any apparent effect on growth and electron flow rates. After inoculation into

sealed vials, cultures were stirred continuously at 500-700 rpm to ensure complete dispersal of cells and proper gas exchange between liquid and headspace. We used a robotic incubation system (Molstad et al., 2007) to monitor headspace gases O₂ and CO₂ every ~0.5-1.5 hours). The combination of headspace and liquid measurements produced detailed datasets so the concentrations of O₂, CO₂ and N-oxides could be accurately calculated for each sampling. This facilitated assessment of respiration rates and e⁻-flow to each terminal electron acceptor (as described in Bergaust et al., 2010; Hassan et al., 2014).

Nitrite measurements were carried out separately throughout the incubations by injecting 10 µL of withdrawn liquid culture into a purged vessel containing 1% w/v sodium iodide (NaI) in acetic acid, reducing the injected nitrite to NO (MacArthur et al., 2007). N₂ was bubbled continuously through the purge vessel to maintain anaerobic conditions and was also the carrier gas transporting NO to the connected chemiluminescence detector (Nitric Oxide Analyzer NOA 280i, General Electric).

2.5. RNA extraction and RT-qPCR

Genome sequencing of the six isolates allowed for specific primer design for each of the nitrate reductase genes within each isolate. Further details of growth conditions, harvesting times and real time quantitative PCR protocols can be found elsewhere (Roco et al. *in press*). In brief, isolates were grown in flasks in triplicate with either a foam or rubber stopper, for aerobic or hypoxic conditions, respectively, in Sistro's medium with or without 10 mM KNO₃ and cells were harvested during various growth stages based on detectable nitrite levels. The expression of *nar* and *nap* was estimated relative to their respective standard curves (log-linear regression of threshold cycle (*C_T*) value against log copy number). A list of the specific primers used, their

annealing temperature, and qPCR efficiencies of the standard curves can be found in Table S1. Real-time qPCRs were set up in triplicate and the results are given as mRNA copies μg^{-1} total RNA.

3. Results

3.1. ANR activity in soil

An initial assay was undertaken as a means to identify soils in which ANR occurred. Soil with additions of water, water plus nitrate, or Sistrom's medium produced only trace amounts of nitrite (Fig. 1). In contrast, additions of nitrate plus Sistrom's medium led to nitrite production in nearly all the soils examined, with nitrite being detected as early as 30 minutes after incubations began (data not shown). The average accumulation of nitrite after 5 hours of incubation in samples with nitrate plus Sistrom's medium was 2 to 14 $\mu\text{g N-NO}_2 \text{ g}^{-1}$ fresh-weight soil (Fig. 1), which was 7.5-50% of the 28 $\mu\text{g N-NO}_3$ added per gram of fresh soil. Additions of either DCD or excess ammonium along with nitrate plus Sistrom's medium did not significantly change the amount of nitrite accumulation ($p \leq 0.05$, two-way ANOVA) beyond that exhibited by soil with added nitrate and Sistrom's medium alone.

The greatest rates of ANR occurred in the forested wetland sites when both carbon and nitrate was present (Fig. 1). Notably, in the wetland sites, the amount of nitrite accumulation differed significantly between the pits and hummocks ($p \leq 0.05$, two-way ANOVA)(Fig. S1). The Stewart Park hummock soil was the only site that had significant nitrite accumulation in the absence of added nitrate when incubated with a carbon source (Fig. 1, Fig. S1). The hummock at Sapsucker Woods only showed nitrite accumulation when incubated with carbon, nitrate and added ammonium (Fig. S1). Nitrite production from the pits required both carbon and nitrate,

and the Stewart Park pit showed the most noteworthy increase (Fig. 1). At both sites hummocks were significantly drier than the pits, with a soil moisture content 30-50% lower, and pH 0.6-0.8 units lower (Table 1).

<Insert Figure 1 here>

3.2. Isolates with ANR phenotype

A total of 250 bacterial strains were isolated under aerated, heterotrophic conditions from the various soils. Approximately 90 of the 250 isolates exhibited ANR and were grouped based on production of relatively low ($\text{mg N-NO}_2 \text{ g}^{-1} \text{ soil} \geq 0.0013$), medium ($\text{mg N-NO}_2 \text{ g}^{-1} \text{ soil} \geq 0.009$), or high ($\text{mg N-NO}_2 \text{ g}^{-1} \text{ soil} \geq 0.018$) levels of nitrite during aerobic growth. Notably, most of the isolates had less nitrite production in well-mixed liquid than on solid agar. In general, soils with low ANR activity yielded isolates with a low level of nitrite production, as bacterial isolates from the three forested sites yielded the highest proportion of low nitrite producing isolates compared to the other sites (Fig. S2). In contrast, soils with the greatest ANR activity produced about equal numbers of low and high nitrite producing isolates (Fig. S2). Among the 90 isolates, only about 30% were able to grow with nitrate as the sole electron acceptor (data not shown). Phylogenetic characterization of the isolates revealed that Actinobacteria, Firmicutes, and alpha, beta and gamma Proteobacteria. Gammaproteobacteria constituted 42% of the total isolates, with pseudomonads as the majority, constituting 31% of the gammaproteobacteria.

To gain more insight into the diversity of ANR phenotypes, six strains were examined for detailed timing of nitrite production in relation to O_2 concentration (Fig. 2). Two isolates (5, 16) had rapid net nitrite production rates (initial $v_{\text{NO}_2^-}$) when O_2 was 57 to 86% of the ambient level. Two isolates (2, 28) had lower initial $v_{\text{NO}_2^-}$ when O_2 was 35 to 66% of ambient. Finally, two

isolates (21, 26) had low initial $v_{\text{NO}_2^-}$ when O_2 was 60 to 84% of ambient. Isolates 2, 5, 16, and 28 did not reduce nitrite further, and thus nearly all of the added nitrate-N was recovered as nitrite. Isolates 21 and 26 accumulated nitrite only transiently, consistent with their abilities to grow as denitrifiers.

Further measurements in isolates 2, 5 and 16 indicated there was no change in CO_2 production or total electron flow (V_{e^-} to O_2 or N-oxides, $\mu\text{mol vial}^{-1} \text{ h}^{-1}$) with the addition of nitrate to the medium during aerobic growth (data not shown). The complete denitrifiers, 21 and 26, were not assessed as they can reduce the nitrite which would confound the results, and isolate 28 was a very slow grower and omitted for practical purposes.

< Insert Figure 2 here >

3.3. *Nap* and *Nar* genes

We sequenced the genomes of six isolates capable of ANR to examine the gene content for nitrate reductase. The genomes were compared to previously published genomes in the Department of Energy (DOE) Integrated Microbial Genomes (IMG) public database. Genome analysis revealed that isolates 21 and 26 had the genes necessary for complete denitrification (Fig. S3). Isolate 21 is related to *Pseudomonas nitroreducens* and has two clusters of genes encoding membrane bound Nar. The two respiratory nitrate reductase alpha chain subunits (NarG) share 71% identity, indicating they are not paralogous. NarG in cluster 1 (locus tag UB43_11370) has significant sequence similarity with proteins from *Pseudomonas aeruginosa* (90% identity). NarG from cluster 2 (locus tag UB43_19730) is only 70% identical to NarG from *Pseudomonas aeruginosa* but about 80% identical with Nar from various *Halomonas* species, another γ -proteobacterium but a member of the family Halomonadaceae. Isolate 26, classified as

a member of the genus *Ralstonia*, contains a single nitrate reductase cluster that encodes a Nap-type protein. This protein has high identity to orthologs in other *Ralstonia*. However, many *Ralstonia* usually have one or two copies of the genes encoding the Nar type enzyme. At this time, there is only one other *Ralstonia* strain like isolate 26 in having Nap but no Nar in the DOE IMG public database, making this particular arrangement rare among members of this genus.

Isolates 2 and 5 are both pseudomonads and have nitrate reductase as their only dissimilatory N-oxide reductase. Interestingly, both strains have gene clusters for both Nar and Nap type nitrate reductases. Examination of the genomes of 728 other pseudomonads in the DOE IMG database (as of 05/14/2015) found only one other strain with both Nar and Nap that lacked all other downstream denitrification N-oxide reductases, making this arrangement rare in this dataset. Nearly all the sequenced pseudomonad strains contained Nar and <30% contained both Nar and Nap. The orthologous clusters in isolates 2 and 5 are identical in gene content and organization. These strains are not siblings, however, as 492 genes in isolate 5, out of a predicted total of ~5000 genes, do not have obvious orthologs, defined as >30% identity, to isolate 2. Likewise, 643 genes in isolate 2, out of a total of ~5300 genes, are not found in isolate 5. However, conserved regions are highly similar, with DNA identity of about 88%.

Isolate 16, a member of the Burkholderiaceae family, contains a Nar encoding gene cluster. This cluster is identical in organization with most orthologs from other *Ralstonia* strains. The regions of the genome flanking the Nar encoding gene clusters are not conserved among the strains, however. For example, the eight genes downstream of this cluster are present in other *Ralstonia* strains but not with the same organization as in isolate 16. Isolate 28, a member of the order Actinomycetales, is the only gram positive among the characterized isolates and has a Nar

type nitrate reductase. Nar are fairly common among the order Actinomycetales and the predicted Nar proteins from 28 are most closely related to proteins from members of this order.

3.4. Expression of nitrate reductase(s) in the isolates

In order to determine whether the dissimilatory nitrate reductase genes displayed similar or contrasting patterns of expression among the six isolates, qPCR was performed on RNA isolated from cells growing under various conditions. Results from the nitrate reductase RT-qPCR are summarized in Table 2. The two complete denitrifiers, isolates 21 and 26, were found to have dissimilar patterns of nitrate reductase expression. Only one of the two *nar* clusters in isolate 21, cluster 1, showed changes in copy number, with an increase in expression during oxygen limitation in combination with NO_3^- (AE_HighOD_ NO_3), and the highest expression in the anoxic treatment with nitrate (AN_HighOD_ NO_3). The other *nar* cluster showed virtually no transcription under any conditions. The *nap* in isolate 26 showed significant transcription under all conditions. Cells from low-density oxic conditions showed the lowest expressions, but this increased in higher density cultures, regardless of the presence of nitrate (AE_HighOD). Expression in the high-density cultures was equivalent to that seen in cells isolated under denitrifying conditions.

Isolates 2 and 5 had dissimilar expression patterns despite their high sequence similarities. *nar* in isolate 5 was controlled by the presence of nitrate, regardless of O_2 , showing high transcription in both AE_LowOD_ NO_3 and AE_HighOD_ NO_3 . Comparatively, *nap* had low background expression levels in isolate 5 under all conditions and increased as $[\text{O}_2]$ decreased in nitrate supplemented medium (AE_HighOD_ NO_3). The expression of *nap* in isolate 2 also occurred at high cell density but did not require added nitrate (AE_HighOD). *nar* in this

isolate had a comparatively low level of transcription under all conditions, only showing a limited increase in expression during oxygen limitation in NO_3^- amended medium (AE_HighOD_ NO_3).

Strain 16 showed a clear increase in *nar* expression in response to oxygen limitation, especially in the presence of NO_3^- (AE_HighOD_ NO_3). Transcription of *nar* was relatively low for isolate 28 under all conditions. However, this gene cluster showed a modest increase in copy number in late exponential phase with the presence of nitrate (AE_HighOD_ NO_3).

<Insert Table 2 here>

4. Discussion

We examined ANR in a range of soils in the northeastern USA, and our results suggest that ANR is an underappreciated sink for nitrate in soil. Despite being mostly ignored in soil science and soil ecological work, a few studies have quantified ANR in soils (Russow et al., 2009; Isobe et al., 2012; Müller et al., 2006) and report nitrite production rates of $<1 \mu\text{g g}^{-1} \text{hr}^{-1}$. Here, we found rates from 0.5 to $2.8 \mu\text{g N-NO}_2 \text{g}^{-1} \text{hr}^{-1}$, which resulted in the reduction of 7.5-50% of the added nitrate. Our study advances the understanding of ANR in natural ecosystems for at least two reasons. The first is that we measured ANR within a few hours of soil collection, suggesting that enzymes capable of the process are pre-existing in soils. Secondly, we characterized several bacteria able to perform ANR and found a diversity of physiological controls on the process. This implies that ANR is more diverse than previously suspected.

The highest rate of nitrite production in all of the soils occurred with additions of nitrate plus carbon, i.e. Sistrom's medium (Fig. 1), suggesting that ANR is a heterotrophic process associated with respiration. Also, because all of the soils exhibited nitrite production with the

addition of a nitrification inhibitor, we are confident that the process was not simply a consequence of nitrification, but rather due to a different group of bacteria. The highest rate of nitrite production occurred in the soils from the forested wetland sites, but the contrast of nitrite production between the pits and hummocks suggest different contributing processes. Nitrification may be a major contributor of nitrite production in the hummocks at these forested wetland sites. This might explain the finding at Sapsucker Woods, where the hummocks experienced nitrite production only when excess ammonium was available (Fig. S1). The fact that nitrite was produced in the hummocks at Stewart Park with carbon alone also suggests an addition of nitrate via nitrification (Fig. 1, Fig. S1). In contrast, the pit soils from both Stewart Park and Sapsucker Woods suggests nearly all the nitrite production occurred via ANR, as no nitrite was detected without the addition of both carbon and nitrate (Fig. 1). Soils in forested wetlands generally have high levels of available organic carbon (Rosenblatt et al., 2001), which is consistent with the notion that heterotrophic metabolism helps to sustain ANR.

Isolation of bacteria capable of ANR from these soils proved surprisingly easy. However, the percentage of isolates with this phenotype is likely inflated due to the isolation procedure, and is not likely representative of the entire soil community. Taxonomic identification of the ANR strains revealed a diversity of genera. This is similar to what is seen with denitrification (Bakken et al., 2012) and indicates these processes are not restricted to particular taxonomic groups but can be adapted to fit the physiological needs of many organisms. While ANR has been reported previously (Carter et al., 1995; Vega-Jarquin et al. 2008), this is the first study to accurately measure the [O₂] levels at the onset of nitrite production. This is an important parameter to measure to ensure nitrite production can occur at high O₂ saturation, particularly since we were not able to measure O₂ saturation during soil incubations or initial isolate

characterization. The fact that the onset of nitrite production occurred between 45-86% atmospheric O₂ (Fig. 2) in the six isolates indicates the isolate screen was effective at identifying bacteria capable of reducing nitrate at nearly saturated O₂. It is noteworthy that this [O₂] is much higher than the onset of nitrite reduction in some model denitrifiers, which has been reported at ≤1.5% atmospheric [O₂], showing that particular steps in the denitrification pathway have very different O₂ thresholds (Bakken et al., 2012; Bergaust et al., 2011; Bergaust et al., 2008).

Although the ANR phenotype was common in our soils and the isolates were representative of common soil genotypes, the nitrate reductase genotypes of isolates 2, 5 and 26 were relatively unique compared to full genomes available at the DOE IMG database (19,000 strains as of June 2014). This demonstrates there is a wide diversity of nitrate reductase arrangements in bacteria and particular assemblages are not restricted to particular taxonomic groups or physiological processes. It should be noted, though, that the occurrence of nitrate reductase is not rare, since 51% of the isolates in the DOE IMG database as of 06/2014 possessed the genes encoding a dissimilatory nitrate reductase. Moreover, surveys of denitrification genes in the environment reveal that *nar* and *nap* are more prevalent than the genes involved in the production and consumption of NO and N₂O, suggesting that soils have a high potential for nitrate reduction (Henry et al., 2006; Bru et al., 2011).

This common occurrence of the ANR phenotype in the environment brings into question its physiological role to bacteria. This is of particular interest in the four sequenced isolates that lacked a dissimilatory nitrite reductase, thereby limiting any physiological benefit of N-oxide reduction to the reduction of nitrate to nitrite. Nitrate reduction to nitrite has a $\Delta G'_0$ of -161 kJ/mol, making it possible to use this reaction to generate ATP. This is sufficient to support growth under hypoxic conditions, but this does not explain why the strains characterized here

express nitrate reductases under high [O₂] (Nishimura et al., 2007). In a previous study investigating ANR, it was suggested that this trait might be used to dissipate excess electrons (Carter et al., 1995). However, a more oxidized carbon source was used in this study, so it is unlikely the disposal of excess electrons is the reason why ANR is utilized by the isolates described here. None of the four isolates lacking nitrite reductase in this study showed evidence of growth under anoxic conditions nor were respiration rates under oxic conditions with and without added nitrate, measured by CO₂ production and electron flow, significantly different in isolates 2, 5 and 16. These results imply no physiological benefit in the co-respiration of O₂ and nitrate, a result seemingly at odds with the ready isolation of bacteria with this phenotype from soil. Defining the physiological role of these proteins likely await more detailed physiological investigations under diverse growth conditions.

In addition to the diversity of nitrate reductase types found in these isolates, a range of regulatory patterns was also observed. For example, despite the fact that the two isolates with both Nar and Nap, isolates 2 and 5, had very similar genotypes they demonstrated very different *nar* and *nap* expression patterns. Isolate 5 had consistently much higher levels of *nar* and *nap* expression than isolate 2, which correlated with higher rates of nitrite production. This diversity in expression provides another demonstration that these nitrate reductases are being regulated to best support the metabolic needs of the cell even if the rationale for these regulatory patterns are not obvious.

We have developed an isolation protocol for bacteria capable of ANR, demonstrating the common occurrence of this phenotype in soil, and have seen that ANR is not ammonium dependent, but is carbon and nitrate dependent and oxygen independent. We have also demonstrated that the bacterial isolates capable of ANR show an intricate phenotypic landscape,

indicating new types of complexity within the denitrifying community. Our working hypothesis is that ANR is a common, yet underappreciated trait, and that this pathway has the potential to make a significant contribution to the soil nitrogen cycle. In terms of future understanding of denitrification, it will be important to recognize that this first step cannot be assumed to be under tight O₂ control, as there is a wide disparity of O₂ concentrations at the onset of nitrate reduction, which, importantly, is higher than the onset of nitrite reduction. Without an adequate assessment of aerobic nitrate reduction our understanding of factors controlling nitrate flux in soils remains incomplete.

Acknowledgements

We thank Trever Smith and Yao Wong for their contributions to strain isolation. This work was supported by the U.S. National Science Foundation (NSF) grant DEB #1311335 and by the Norwegian Research Council (FRIMEDBIO: 231282). Partial funding support was provided by Cornell University Programs in Biogeochemistry and Environmental Biocomplexity and Cross-Scale Biogeochemistry and Climate, both of which were and are supported by NSF and the Atkinson Center for a Sustainable Future. The authors report no conflict of interest.

Appendix. Supplementary data

Supplementary data related to this article can be found in the online version, at <insert URL here>.

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Table 1

Sites from which soil was collected for ANR assay and bacterial isolation. Soil pH and soil moisture were measured on the day of soil collection.

Site name	pH	Soil moist. (%)	Vegetation/ Soil classification
McGowan agriculture	4.89	29	Corn / Mesic Glossoboric Hapludalf
McGowan forest	5.44	27	Mixed hardwood forest / Mesic Glossoboric Hapludalf
Smith agriculture	4.55	18	Corn / Mesic Psammentic Hapludalf
Smith forest	4.05	33	Hardwood-Hemlock forest / Mesic Psammentic Hapludalf
Hubbard Brook forest	3.76	52	Northern hardwood forest / Typic Haplorthod
Stewart wetland pit	4.65	54	Red maple swamp /
hummock	4.05	35	Mesic Fluvaquentic Endoaquept
Sapsucker wetland pit	3.85	68	Maple-Alder-Hemlock swamp /
hummock	3.08	48	Mesic Mollic Endoaquept

Table 2

Quantitative RT-qPCR of each dissimilatory nitrate reductase from the six genome sequenced isolates. Values are mean + S.D. Copy numbers are given per μg total RNA over five growth conditions^a and each gene was run in triplicate.

Growth conditions	Isolate 21		Isolate 26	
	narI	narII	nap	
AE_LowOD	0.1 ± 0.04	0.02 ± 0.02	0.8 ± 0.2	
AE_LowOD_NO ₃	0.04 ± 0.01	0.02 ± 0.01	1.0 ± 0.2	
AE_HighOD	0.1 ± 0.01	0.1 ± 0.02	4.6 ± 0.2	
AE_HighOD_NO ₃	0.3 ± 0.03	4.5 ± 1.0	5.7 ± 0.4	
AN_HighOD_NO ₃	0.1 ± 0.01	24 ± 4.0	3.0 ± 0.3	

Growth conditions	Isolate 5		Isolate 2	
	nar	nap	nar	nap
AE_LowOD	0.6 ± 0.2	2.6 ± 0.4	0.1 ± 0.05	0.03 ± 0.01
AE_LowOD_NO ₃	42 ± 2.1	7.4 ± 1.4	0.3 ± 0.03	0.03 ± 0.01
AE_HighOD	6.6 ± 1.9	8.9 ± 0.6	0.4 ± 0.1	0.2 ± 0.01
AE_HighOD_NO ₃	57 ± 21	27 ± 9.2	1.0 ± 0.1	0.3 ± 0.04
AN_HighOD_NO ₃	N/A	N/A	N/A	N/A

640		Isolate 16	Isolate 28
641	Growth conditions	nar	nar
642	AE_LowOD	0.3 + 0.04	0.05 + 0.008
643	AE_LowOD_NO ₃	0.3 + 0.06	0.05 + 0.008
644	AE_HighOD	1.9 + 0.16	0.08 + 0.01
645	AE_HighOD_NO ₃	25 + 2.0	0.1 + 0.01
646	AN_HighOD_NO ₃	N/A	N/A
647			

648 ^a The five growth conditions were: 1) AE_LowOD = Aerobic, low OD₆₀₀ (<0.4), no NO₃; 2)
649 AE_LowOD_NO₃ = Aerobic low OD, 10 mM KNO₃; no nitrite production detected; 3)
650 AE_HighOD = Aerobic, high OD₆₀₀ (>0.8), no NO₃; 4) AE_HighOD_NO₃ = Aerobic, high OD,
651 10 mM KNO₃, nitrite detected; and 5) AN_HighOD_NO₃ = Anaerobic, high OD₆₀₀ (>0.8), 10
652 mM KNO₃, no nitrite detected.

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Figure captions

Fig. 1. ANR activity in selected soils from the Northeast US. Average nitrite accumulation of soils incubated in triplicate in different medium after five hours of incubation. Soils were incubated in the following medium: 1) deionized water (W), 2) deionized water with 0.03 mg N-KNO₃ g⁻¹ fresh-weight soil (W+N), 3) Sistrom's medium (SIS); 4) SIS plus 0.03 mg N-KNO₃ g⁻¹ fresh-weight soil (SIS+N); 5) SIS+N and 300 mM dicyandiamide (DCD) (SIS+N+DCD), and 6) SIS+N and 113 mM ammonium sulfate (SIS+N+NH₄). The last two medium are not shown as they were not significantly different in nitrite accumulation compared to the SIS+N treatment. The nine sites are as followed: McGowan Farm agricultural field (MA); McGowan Farm forest (MF); Smith Woods agricultural field (SA); Smith Woods forest (SF); Hubbard Brook Experimental Forest (HBEF); Stewart Park pits (SPP); Stewart Park hummocks (SPH); Sapsucker Woods pits (SWP); and Sapsucker Woods hummocks (SWH).

Fig. 2. Oxygen concentration and nitrite measurements in six isolates. [O₂] in the liquid (% of ambient, assuming 100% at first measurement) and specific net rate of NO₂⁻ accumulation (v_{NO2}-μmol mg⁻¹ dw h⁻¹) in isolates 2, 5, 16, 21, 26 and 28 in Sistrom's medium with 1 or 2 mM (isolate 28 only) initial NO₃⁻ under initial atmospheric conditions (21% [O₂]). 1 mM initial NO₃⁻ equals 50 μmol NO₃⁻-N, and thus there is a potential accumulation of 50 μmol NO₂⁻-N vial⁻¹. The decline in oxygen is due to respiration and dilution by sampling. [O₂] is given as estimated

679 average values for each mid-time increment of nitrite measurements. Specific net rates of NO_2^-
680 production were estimated based on inferred dry mass (mg) per vial for each mid-time increment
681 of nitrite measurements, assuming a conversion factor of $0.4 \text{ mg ml}^{-1} \text{ OD}_{600}^{-1}$. Isolates 5, 16 and
682 28 reduced nearly all the available NO_3^- to NO_2^- , isolates 21 and 26 reduced nitrite further to N_2
683 (not shown), and in the isolate 2 cultures, approximately 16% of the available NO_3^- was
684 recovered as NO_2^- at the end of the experiment. Solid black line and open squares: $[\text{O}_2]$ (% of
685 ambient); dashed line, no symbol: $\text{NO}_2^- \text{ } \mu\text{mol vial}^{-1}$; grey diamonds: $v_{\text{NO}_2^-}$ ($\mu\text{mol mg}^{-1} \text{ dw h}^{-1}$).
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Fig. 1

Fig. 2