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DNA- and RNA-SIP Reveal *Nitrospira* spp. as Key Drivers of Nitrification in Groundwater-Fed Biofilters

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**ABSTRACT** Nitrification, the oxidative process converting ammonia to nitrite and nitrate, is driven by microbes and plays a central role in the global nitrogen cycle. Our earlier investigations based on 16S rRNA and *amoA* amplicon analysis, *amoA* quantitative PCR and metagenomics of groundwater-fed biofilters indicated a consistently high abundance of comammox *Nitrospira*. Here, we hypothesized that these nonclassical nitrifiers drive ammonia-N oxidation. Hence, we used DNA and RNA stable isotope probing (SIP) coupled with 16S rRNA amplicon sequencing to identify the active members in the biofilter community when subjected to a continuous supply of NH$_4^+$ or NO$_2^-$ in the presence of $^{13}$C-HCO$_3^-$ (labeled) or $^{12}$C-HCO$_3^-$ (unlabeled). Allylthiourea (ATU) and sodium chlorate were added to inhibit autotrophic ammonia- and nitrite-oxidizing bacteria, respectively. Our results confirmed that lineage II *Nitrospira* dominated ammonia oxidation in the biofilter community. A total of 78 (8 by RNA-SIP and 70 by DNA-SIP) and 96 (25 by RNA-SIP and 71 by DNA-SIP) *Nitrospira* phylotypes (at 99% 16S rRNA sequence similarity) were identified as complete ammonia- and nitrite-oxidizing, respectively. We also detected significant HCO$_3^-$ uptake by *Acidobacteria* subgroup10, *Pedomicrobium*, *Rhizobacter*, and *Acidovorax* under conditions that favored ammonia oxidation. Canonical *Nitrospira* alone drove nitrite oxidation in the biofilter community, and activity of archaeal ammonia-oxidizing taxa was not detected in the SIP fractions. This study provides the first *in situ* evidence of ammonia oxidation by comammox *Nitrospira* in an ecologically relevant complex microbiome.

**IMPORTANCE** With this study we provide the first *in situ* evidence of ecologically relevant ammonia oxidation by comammox *Nitrospira* in a complex microbiome and document an unexpectedly high H$_{13}$CO$_3^-$ uptake and growth of proteobacterial and acidobacterial taxa under ammonia selectivity. This finding raises the question of whether comammox *Nitrospira* is an equally important ammonia oxidizer in other environments.

**KEYWORDS** nitrification, comammox, *Nitrospira*, DNA SIP, RNA SIP

Nitrification, the stepwise oxidation of ammonia (NH$_3$) to nitrite (NO$_2^-$) and nitrate (NO$_3^-$), supplies the substrates for processes that initiate the loss of reactive nitrogen from the biosphere as N$_2$. Understanding the organisms and environmental controls that drive nitrification is important as it controls global homeostasis of the N cycle. In engineered environments, complete nitrification is often desired: this is essential when waters are prepared and distributed for human consumption. Residual NH$_3$ or NO$_2^-$—the result of incomplete nitrification—renders the water biologically unstable and unsafe for human consumption. Hence, biological systems for source...
water treatment are contingent on nitrifying prokaryotes. Based on evolutionarily
conserved taxonomic (small subunit, 16S rRNA) and functional (e.g., ammonia mono-
oxynase \([\text{amoA}]\) gene surveys, \textit{Nitrosomonas} (1–4), \textit{Nitrosoarchaeum}, and \textit{Ni-
trososphaera} have been identified as the abundant ammonia oxidizing prokaryotes (AOPs) and \textit{Nitrospira} (5, 6) as the abundant nitrite-oxidizing prokaryotes (NOPs) in drinking water treatment systems, consistent with the classical assumption of division of labor in the two nitrification steps.

Our previous studies on rapid gravity sand filters (RGSFs), used in potable water
preparation from groundwater, revealed nitrifying microbial communities in which \textit{Nitrospira} is far more abundant than \textit{Nitrosomonas} (7), with several \textit{Nitrospira} genomes containing genes for ammonia oxidation (8), and with an abundance of comammox (complete ammonia-oxidizing) \textit{amoA} over ammonia-oxidizing bacterial (AOB) \textit{amoA} genes (9). Together with the concurrent discovery of comammox \textit{Nitrospira} strains by others (10–12), this suggested that comammox \textit{Nitrospira} may drive ammonia oxidation in the examined groundwater-fed RGSFs. In addition, like \textit{Nitrospira}, several acidobac-
terial, and gamma- and alphaproteobacterial taxa were at consistently higher abun-
dance than \textit{Nitrosomonas}, raising questions about their potential role in nitrification, as \(\text{NH}_3\) is the primary growth substrate entering the filters (7, 13–15). Identifying the active ammonia- and nitrite-oxidizing organisms is essential not only for engineering pur-
poses, but also for understanding the niches and biodiversity of nitrifiers. There has
been a rapidly increasing documentation of global comammox \textit{Nitrospira} occurrence
across a myriad of habitats ranging from the subsurface, to soils, and sediments and
from groundwaters to source and residual water treatment plants, but apparently
excluding open oceanic waters (9, 16–19), with occasional abundances that exceed
those of canonical AOB (9, 20, 21). Nonetheless, it is yet to be shown whether
comammox \textit{Nitrospira} truly drives ammonia oxidation in open oligotrophic freshwater
and soil environments, their presumed preferred habitat based on genomic and
physiological evidence (22, 23).

Here, we sought to identify the active ammonia and nitrite oxidizers in a
groundwater-fed RGSF using RNA and DNA stable isotope probing (SIP) coupled to 16S
rRNA amplicon sequencing. Lab-scale columns packed with filter material from a
full-scale RGSF were fed with effluent water amended with \(\text{NH}_4^+\) or \(\text{NO}_2^-\) and with
\(^{13}\text{C}\)-labeled or unlabeled \(\text{HCO}_3^-\) for 15 days in the presence or absence of inhibitors of
autotrophic ammonia and nitrite oxidation (24). Our findings indicate that
\textit{Nitrospira} drives both ammonia and nitrite oxidation. In addition, several other taxa take up
substantial \(\text{HCO}_3^-\) and their DNA and RNA increase in relative abundance when
ammonia is the only provided energy source. This study provides the first \textit{in situ}
evidence of ammonia oxidation by comammox \textit{Nitrospira} in an ecologically relevant
complex microbiome.

RESULTS

Four different experimental treatments were designed to identify the microbes
involved in ammonia and nitrite oxidation (Table 1): (i) 71 \(\mu\text{M NH}_4^+\) (columns 1 and 2),
(ii) 71 \(\mu\text{M NH}_4^+\) and 100 \(\mu\text{M allylthiourea} (\text{ATU})\) (columns 3 and 4), (iii) 71 \(\mu\text{M NO}_2^-\)
(columns 5 and 6), and (iv) 71 \(\mu\text{M NH}_4^+\) and 1 \(\text{mM NaClO}_3\) (columns 7 and 8). All
columns were operated with an influent containing 100\% \(^{13}\text{C}\)-labeled or 100\% unlabeled
bicarbonate for 15 days.

In columns 1 (\(^{13}\text{C}\)-bicarbonate and 2 (bicarbonate), the full-scale conditions were mimicked,
with the aim to elucidate the complete \textit{in situ} food web related to nitrification. In
columns 3 (bicarbonate) and 4 (\(^{13}\text{C}\)-bicarbonate), \textit{ATU} was used to suppress bacterial ammonia
oxidation while feeding at the same \(\text{NH}_4^+\) loading as in columns 1 and 2 (25). Complete
inhibition of bacterial ammonia oxidation with \textit{ATU} has been observed at \textit{ATU}
concentrations of 8 to 86 \(\mu\text{M}\) (26), while archaeal ammonia oxidation is less sensitive to
\textit{ATU} (27). The mechanism of \textit{ATU} inhibition in AOB is proposed to be chelation of the
\(\text{Cu}^{2+}\) from the active site in the \textit{AMO} enzyme (24). To identify taxa associated with
nitrite oxidation, \(\text{NO}_2^-\) was fed to columns 5 (\(^{13}\text{C}\)-bicarbonate) and 6 (bicarbonate). In columns 8
Nitrospira Drives Nitrification in RGSFs

TABLE 1 Summary of experimental design, bulk 13C incorporation, substrate utilization and accumulation levels, and sequenced samples

<table>
<thead>
<tr>
<th>Run and column&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N source</th>
<th>C source (12C or 13C)</th>
<th>Inhibitor</th>
<th>13C/12C ratio&lt;sup&gt;c&lt;/sup&gt;</th>
<th>NH4&lt;sup&gt;+&lt;/sup&gt; removal (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NO3&lt;sup&gt;−&lt;/sup&gt; removal (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NO3&lt;sup&gt;−&lt;/sup&gt; accretion (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total DNA</th>
<th>Total RNA</th>
<th>Total SIP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Run 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column 1</td>
<td>NH4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>H13CO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td>279</td>
<td>99 ± 1</td>
<td>100 ± 0</td>
<td>88 ± 32</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Column 2</td>
<td>NH4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>HCO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td>98 ± 3</td>
<td>100 ± 0</td>
<td>82 ± 28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Column 3</td>
<td>NH4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>HCO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>ATU</td>
<td>19 ± 15</td>
<td>101 ± 2</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Column 4</td>
<td>NH4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>H13CO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>ATU</td>
<td>54</td>
<td>11 ± 15</td>
<td>99 ± 6</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Run 2</strong></td>
<td></td>
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<tr>
<td>Column 5</td>
<td>NO2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>H13CO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td>89</td>
<td>NA</td>
<td>88 ± 1</td>
<td>99 ± 34</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Column 6</td>
<td>NO2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>HCO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td>NA</td>
<td>92 ± 3</td>
<td>62 ± 36</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Column 7</td>
<td>NH4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>HCO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>ClO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>11 ± 5</td>
<td>70 ± 44</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Column 8</td>
<td>NH4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>H13CO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>ClO3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>63</td>
<td>6 ± 7</td>
<td>97 ± 28</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>Run 1 was initiated with inoculum 1, and run 2 was initiated with inoculum 2.
<sup>b</sup>Removal and accumulation rates were estimated from daily NH4<sup>+</sup>, NO2<sup>−</sup>, and NO3<sup>−</sup> measurements. NO3<sup>−</sup> removal was calculated based on ammonium removed (except for columns 5 and 6, where it was based on influent nitrite). NO3<sup>−</sup> accretion was calculated based on ammonium removed (except for columns 5 and 6, where it was based on nitrite removed). ND, differences between influent and effluent NO3<sup>−</sup> concentrations were not significant, and accretion could not be calculated. NA, not applicable.
<sup>c</sup>Bulk ratio in columns after 15 days, as determined by EA-IRMS.

(H13CO3<sup>−</sup>) and (HCO3<sup>−</sup>) were used to inhibit nitrite oxidation under NH4<sup>+</sup> feeding, with the aim to identify the taxa solely associated with NH4<sup>+</sup> oxidation (25). Chlorate is commonly used as a selective inhibitor for nitrite oxidation, as it is reduced by reverse activity of the nitrite oxidoreductase to the toxic chlorite (ClO2<sup>−</sup>) (28, 29).

**Physiological activity.** In the 71 μM NH4<sup>+</sup>-fed treatments, complete NH4<sup>+</sup> removal (99%) was observed without inhibitor addition, while NH4<sup>+</sup> removal ranged from 11% to 19% with ATU amendment. (Table 1; see Fig. S3A in the supplemental material). Inhibitor addition also significantly reduced overall 13C incorporation. Columns fed with NH4<sup>+</sup>, NH4<sup>+</sup>-ATU, and NO3<sup>−</sup> all had similarly high degrees of NO3<sup>−</sup> removal ranging from 88% to 100%. In the 1 mM ClO3<sup>−</sup>-amended columns, NH4<sup>+</sup> removal was severely inhibited (6% to 11%); removal of formed NO2<sup>−</sup> continued (from 70% to 54%), although accumulation of NO3<sup>−</sup> could not be detected. Nitrogen mass balances, based on influent and effluent NH4<sup>+</sup>, NO2<sup>−</sup>, and NO3<sup>−</sup> concentrations closed for most experimental runs, minimizing the possibility of additional nitrogen cycling; N loss was only observed in the ATU-supplemented columns (columns 3 and 4) with ongoing treatment (Table 1; Fig. S3B and C).

**Detection of 13C-labeled taxa from DNA- and RNA-SIP.** DNA and RNA, extracted from column samples taken at the end of the experiments, were subjected to equilibrium density centrifugation, gradient fractionation, and 16S rRNA gene amplification. A total of 147 and 65 gradient fractions from DNA-SIP and RNA-SIP were sequenced using the illumina Miseq and 454 pyrosequencing platforms, respectively (see Fig. S1a and b in the supplemental material). Operational taxonomic units (OTUs) were defined at 99% similarity, to minimize the effect of microdiversity, as similarities of 98.7% and lower represent the taxonomic levels of species, genus, and higher (30).

We first examined the incorporation of 13C in OTUs in all treatments by comparing replicate columns with H13CO3<sup>−</sup> versus H12CO3<sup>−</sup> amendment. In DNA-SIP, where all SIP fractions were sequenced, we calculated the average shift in buoyant density of each OTU based on its relative sequence abundance and buoyant density in all fractions (see equation 2 in Text S1 in the supplemental material). As only selected fractions were sequenced in RNA-SIP, the mean buoyant density of each OTU in treatments with H13CO3<sup>−</sup> versus H12CO3<sup>−</sup> amendment was calculated using the standard deviation of the RNA distribution across the buoyant density gradient (Fig. S1C), as described by Zemb et al. (31). The buoyant density shift of each OTU was then determined from the calculated mean buoyant density in the replicate columns of each treatment.

Among all detected OTUs (3,364,425), 4,075 and 5,045 in the NH4<sup>+</sup> treatment, 4,133 and 5,155 in the NH4<sup>+</sup> plus ATU treatment, 4,183 and 706 in the NO2<sup>−</sup> treatment, and 44,916 and 52 in the NH4<sup>+</sup>-ClO3<sup>−</sup>-fed treatment showed a buoyant density shift (after...
filter 1; see Fig. S2 in the supplemental material) in the DNA- and RNA-SIP experiments, respectively. Only those OTUs that belonged to genera that contained OTUs that were 13C labeled by both RNA and DNA-SIP were retained (filter 2; Fig. S2). A bootstrap resampling of labeled OTUs within each genus was then used to estimate taxon-specific 90% confidence intervals (CIs) for the buoyant density shift of a labeled genus (filter 3; Text S1 and Fig. S2).

Hence, after the 3rd filter step, 676 (57% DNA and 43% RNA of the total 13C-labeled OTUs, NH4+ -fed treatment), 735 (67% DNA and 33% RNA, NH4+ -ATU-fed treatment), 529 (65% DNA and 35% RNA, NO2− treatment), and 43 (83% DNA and 16% RNA, NH4+ -ClO3−-fed treatment) OTUs were retained as significantly labeled. The fractional 13C uptake of labeled OTUs was calculated by dividing the DNA and RNA buoyant density shift for each OTU by the total observed buoyant density shift for DNA and RNA, respectively. The abundances of labeled OTUs in the total community were estimated based on total (i.e., nonfractionated) DNA and rRNA extracts collected on day 15 (see Fig. S4A to D in the supplemental material).

In the NH4+ -only fed treatment, 13C-labeled OTUs affiliated with 17 genera of the Alpha-, Beta-, and Gammaproteobacteria, Nitrospira, Actinobacteria, Latescibacteria, and Acidobacteria (Fig. S4A). Among them, the genus Nitrospira had the highest fraction of 13C uptake (32% and 1.1% for DNA-SIP and RNA-SIP, respectively) and highest relative abundance in the total DNA (26%). Nitrosomonas OTUs were also labeled but displayed low levels of 13C uptake (0.07% and 0.8% for DNA-SIP and RNA-SIP, respectively) and were at low abundance (0.15% and 0.18% in total DNA and RNA, respectively). Labeled rRNA, an approximation of metabolic activity, was distributed evenly between 5 different 13C-labeled genera, including Woodsholea, Blastocatella, subgroup 10 Acidobacteria, Pedobacterium, and Sphingomonas (Fig. S4A). Although ammonia oxidation was severely inhibited in the NH4+ -ATU-fed column (Fig. 1), OTUs in 15 genera incorporated 13C. These were identical to labeled OTUs in the NH4+ -fed treatment, with the exception of OM27, Rhizobacter, Variovorax and uncultured representatives of the order Xanthomonadales, which were not labeled in the presence of ATU (Fig. S4B). Azospira incorporated H13CO3− only in the presence of ATU. In the NH4+ -ATU-fed treatment, Nitrospira (10% DNA-SIP, 4.7% RNA-SIP), Pseudomonas (3% DNA-SIP, 1% RNA-SIP), Methylobacterium (2.7% DNA-SIP, 2.6% RNA-SIP), and Blastocatella (2.6% DNA-SIP, 3.1% RNA-SIP), incorporated the highest fraction of label, while Sphingomonas (1.8%) and Woodsholea (1.4%) were dominant in the total DNA pool (Fig. S4B). In the NH4+ -ClO3−-amended columns, where both ammonia and nitrite oxidation were suppressed, OM27 (2.7% DNA, 2.6% RNA) and Woodsholea were the only taxa that assimilated significant amounts of HCO3− (Fig. S4C).

In the NO2−-fed columns, 12 genera, belonging to Alphaproteobacteria (10% of H13CO3− uptake in DNA-SIP, 23% of H13CO3− uptake in RNA-SIP), Deltaproteobacteria (1% DNA-SIP, 21% RNA-SIP), and Gammaproteobacteria (6% DNA-SIP, 11% RNA-SIP), Nitrospira (71% DNA-SIP, 15% RNA-SIP), Actinobacteria (3% DNA-SIP, 7% RNA-SIP), Latescibacteria (0.5% DNA-SIP, 5% RNA-SIP), and Acidobacteria (6% DNA-SIP, 11% RNA-SIP), were labeled (after filter 3; Fig. S4D). Nitrospira had the highest number of labeled OTUs (a total of 96) and was responsible for the majority of the H13CO3− uptake. After application of filters 4 through 6, only Nitrospira OTUs were retained and hence identified as the sole nitrite oxidizers.

**Hypothesis: Nitrospira is an active ammonia oxidizer.** H13CO3− was incorporated by Nitrospira in treatments fed with NH4+, NH4+ -ATU, and NO2− (after filter 3, Fig. S4A to D). The observed labeling in individual treatments does not indicate whether labeled Nitrospira OTUs are capable of ammonia oxidation because both ammonia and nitrite oxidation occur in NH4+ treatment. We therefore performed a binary comparison between labeled Nitrospira OTUs detected in the NH4+ - versus NO2−-fed treatments (Fig. 1a). We assume that the labeled Nitrospira OTUs in NH4+ -fed treatment would include both comammox and nitrite-oxidizing Nitrospira, while the NO2−-fed treatment would exclude comammox Nitrospira based on observation that comammox Nitrospira
growth is not supported by oxidation of environmental nitrite in the absence of ammonia (11).

Heat maps of labeled Nitrospira OTUs (Fig. 1b) reveal that 8 (24%) and 70 (51%) OTUs are uniquely labeled in NH$_4^+$-fed treatment at the RNA and DNA levels, respectively.
indicating that several ammonia-oxidizing *Nitrospira* strains are actively assimilating H\(^{13}\)CO\(_3^-\) or H\(^{11}\)O\(_2\)\(^-\). A large number of labeled *Nitrospira* OTUs are unique to the NH\(_4^+\)-fed column, and few *Nitrospira* OTUs are shared between the NH\(_4^+\)- and NO\(_2^-\)-fed columns, suggesting that most comammox *Nitrospira* do not readily switch from ammonia oxidation to nitrite oxidation. A large number of OTUs were also uniquely labeled in the NO\(_2^-\)-amended columns (25 in RNA and 66 in DNA), which suggests that, in the NH\(_4^+\)-fed treatment, the produced NO\(_2^-\) was not sufficient to achieve labeling of nitrite-oxidizing *Nitrospira* due to complete nitrification by comammox *Nitrospira*.

Based on their \(^{13}\)C labeling in NH\(_4^+\)- and NO\(_2^-\)-fed treatments, 78 (8 in RNA-SIP and 70 in DNA-SIP) and 96 (25 in RNA-SIP and 71 in DNA-SIP) *Nitrospira* OTUs were identified as complete ammonia and nitrite oxidizing, respectively (Fig. 1c). All labeled *Nitrospira* OTUs belonged to lineage II, which comprises both comammox and non-comammox types. No clear branching between comammox and nitrite-oxidizing phyotypes was observed from the tree topology.

**High \(^{13}\)CO\(_3^-\)** incorporation and growth by other bacteria. In our previous 16S rRNA amplicon-based analysis of the same and related RGSF communities, members of the Rhizobiales (Alphaproteobacteria), and Acidobacteria were consistently more abundant than *Nitrosomonas*, where NH\(_4^+\) is thought to be the largest source of energy available for microbial growth (7). We observed that some of these taxa incorporated \(^{13}\)HCO\(_3^-\) in both RNA-SIP and DNA-SIP (after filter 6; Fig. 2, left). In addition, several OTUs displayed higher buoyant density shifts than *Nitrosomonas* in NH\(_4^+\)-fed columns (Fig. S4A). Finally, these OTUs also increased in relative abundance in both DNA and RNA over the course of the experiment (Fig. 3c).
Only *Nitrospira* was associated with both ammonia and nitrite oxidation (after filter 6; Fig. 2). Among the 13HCO3/11002-incorporating taxa in the presence of NH4/H11001, subgroup 10 Acidobacteria, *Nitrospira*, *Pedomicrobium* (Alphaproteobacteria), *Rhizobacter*, and *Acidovorax* (Betaproteobacteria) displayed higher shifts in relative RNA and DNA abundance compared to *Nitrosomonas* (Fig. 3c). With the exception of *Pseudomonas* (see Fig. S5A in the supplemental material), 13C-labeled genera in SIP columns (Fig. S4A to D) differed from the dominant genera in the feed water, itself the effluent from the full-scale biofilter. Hence, invasion from feed water communities did not cause the increased relative abundance of subgroup 10 Acidobacteria, *Pedomicibrium*, *Rhizobacter*, or *Acidovorax*.

A metagenome, obtained from the same parent biofilter within the same year (2013 [8]) was further examined for its content of genes that are not canonical AOB amoA.
canonical AOA amoA, or comammox \textit{Nitrospira} amoA, nor methanotrophic pmoA (8) (see Fig. S6 in the supplemental material) but showed homology with genes encoding AMO protein family fragments from putative heterotrophic nitrifiers (PF05145 and IPR017516 from the Pfam and InterPro databases, respectively). Twenty-nine unique amoA gene fragments matching the PF05145 model were aligned with reference putative heterotrophic amoA gene fragments (see Fig. S7 in the supplemental material). However, no amoB or amoC genes were found on any of the contigs carrying these atypical putative amoA gene fragments (Fig. S7); furthermore, no similar gene synteny was detected between the other genes of PF05145 amoA-containing contigs and our metagenome amoA-containing contigs (Fig. S7).

The genus \textit{Nitrospira} was the only taxon associated with nitrite oxidation (Fig. 2, right).

**DISCUSSION**

Stable isotope probing has previously been used to identify active nitrifiers in sediments (32–34) and soils (35–38). In most studies, either DNA-SIP (35) or RNA-SIP (39) is applied individually, yet both are important to identify key catalysts (38). While DNA-SIP detects isotope incorporation into dividing cells, RNA-SIP detects active potentially slow- or nongrowing cells (40). By coupling SIP with next-generation sequencing (NGS), we improved taxonomic resolution and differentiated phylotypes in taxa with high microdiversity.

Here, we examined the assimilation of H13CO3− coupled to nitrification in a RGSF using both RNA- and DNA-SIP. OTUs incorporating 13C isotope label in the different treatments were unambiguously identified as those displaying significant buoyant density shifts between the H12CO3− and the H13CO3− replicates and were detected at high phylogenetic resolution (>99% pairwise identity [30]). A total of 200 gradient fractions were processed with sample size equalization.

Our results provide the first \textit{in situ} physiological evidence of ecologically relevant NH4+ oxidation by comammox \textit{Nitrospira} in any environment. Other reports of \textit{in situ} activity are inferred from bulk observations (ammonium removal when comammox \textit{Nitrospira} bacteria are more abundant than AOB or AOA [20, 41]) or from comammox-specific amoA transcript analysis (42). Our former observations that \textit{Nitrospira} was more abundant than \textit{Nitrosomonas} and the discovery that \textit{Nitrospira} harbors the complete nitrification pathway in a full-scale RGSF microbiome (8, 10, 11) are thus directly linked to the ammonia-oxidizing activity of \textit{Nitrospira} in this environment. \textit{Nitrospira} was the only genus incorporating H13CO3− in both NH4+-fed and NO2−-fed treatments, indicating that \textit{Nitrospira} is the only genus oxidizing both environmental NH4+ and NO2− in this system.

Ammonia- and nitrite-oxidizing phylotypes of \textit{Nitrospira} were compared phylogenetically; the resulting 16S rRNA tree topology shows no clear evolutionary separation of comammox and canonical \textit{Nitrospira}. This is in line with previous studies that show that comammox \textit{Nitrospira} bacteria are not evolutionarily distant from known canonical \textit{Nitrospira} (>99% 16S rRNA nucleotide identity) (10). Furthermore, our phylogenetic analysis shows that the labeled—both ammonia-oxidizing and nitrite-oxidizing—\textit{Nitrospira} OTUs branch within \textit{Nitrospira} sublineage II, as reported in previous studies (8, 10–12). We did not identify the amoA clade affiliation of the active comammox \textit{Nitrospira} phylotypes in this study, although separate investigations on this and related RGSFs have indicated a dominance of amoA clade B over clade A comammox \textit{Nitrospira} (9, 23).

It remains unclear whether comammox \textit{Nitrospira} can switch between modes of ammonia and nitrite oxidation. However, the large numbers of \textit{Nitrospira} phylotypes that were exclusively labeled in the NH4+- versus NO2−-fed columns, respectively, suggests that comammox \textit{Nitrospira} may not prefer to oxidize external NO2− alone, in agreement with observations in \textit{"Candidatus Nitrospira inopinata"} (10, 11).

Although ClO3− is a well-known competitive inhibitor of nitrite oxidoreductase (43), strong inhibition of both ammonia and nitrite oxidation was observed in NH4+-ClO3−-
fed columns. We expect that the inhibition of nitrite oxidation in comammox *Nitrospira* would be caused by ClO$_3^-$ reduction to the toxic ClO$_2^-$, which would negatively affect overall metabolism in these organisms, including ammonia oxidation. Hence, it appears that the inhibitory effect of ClO$_3^-$ on ammonia oxidation provides preliminary support for the contribution of comammox *Nitrospira* to ammonia oxidation, as we observed before (25). In addition, ClO$_2^-$ may contribute to inhibition of other ammonia oxidizers as observed before (44). PTIO, an NO-chelating compound, has also been documented as a potent inhibitor of ammonia oxidation in *Candidatus Nitrospira inopinata*, although its selectivity is unclear (45). ATU significantly suppressed ammonia oxidation in NH$_4^+$-ATU-fed treatments, although the taxa assimilating $^{13}$HCO$_3^-$ did not change significantly compared to the NH$_4^+$-fed treatment, excluding *Azospira*. This similarity in labeled taxa in the ATU-fed treatment may indicate that the ammonia monooxygenase (AMO) of the major ammonia oxidizers in this environment may be less sensitive to ATU than AOB at the given concentrations, as previously observed for AOA (46).

No archaean taxa were $^{13}$C labeled in any of the columns, although archaean ammonia oxidizers (AOAs) are present, albeit at much lower abundance than *Nitrospira* (100- to 1,000-fold), in the RGSF used in this study (8, 13, 47). Columns were fed 71 $\mu$M NH$_4^+$ to mimic full-scale conditions (14), while bottom layers of the full-scale biofilter receive very low ammonium concentrations due to removal in the top layers (14, 15). The absence of AOAs in the $^{13}$C-labeled taxa may be due to their low initial abundances or the elevated NH$_4^+$ concentrations applied during the experiment, as AOAs may thrive better under conditions of reduced energy supply consistent with their elevated abundance at bottom layers of the examined RGSF (48–51), even though a recently isolated comammox strain of *Candidatus Nitrospira inopinata* displayed higher NH$_4^+$ affinity than many of the characterized AOAs (22).

After strong filters were applied to remove heterotrophic OTUs, we retain the following taxa with substantial $^{13}$CO$_3^-$ incorporation in the NH$_4^+$-fed treatments: Acidobacteria subgroup 10, Pedamicrobium, Rhizobacter, and Acidovorax; these taxa also show a greater shift in relative abundance in DNA and RNA during the experiment than *Nitrosomonas* and *Nitrospira* OTUs (Fig. 3c; see Fig. S8 in the supplemental material). Most heterotrophic microbes can engage in CO$_2$ assimilation via carboxylation reactions (52, 53). However, CO$_2$ assimilation via anaplerotic metabolism (54) typically results in only 3 to 8% of the cellular carbon assimilated by heterotrophs, which would be insufficient for label detection by DNA-SIP (52, 55). Thus, heterotrophic carbon assimilation would not explain the greater extents of $^{13}$CO$_3^-$ incorporation (higher density shifts; see Fig. 2 and see Table S1, parts A and B, in the supplemental material) relative to *Nitrosomonas* OTUs, known ammonia oxidizers. Furthermore, the cellular mass and activity supported by cross-feeding decay products from autotrophs (56) would be significantly less than the chemolithoautotrophic biomass and activity itself. Thus, the observations of $^{13}$C-labeled genera with higher buoyant density shifts and higher shifts in DNA and RNA abundance shifts (Fig. 2 and 3) compared to *Nitrosomonas* and *Nitrospira* are difficult to explain by cross-feeding alone.

Can a plausible explanation for the high $^{13}$CO$_3^-$ assimilation of these taxa be nitrification? An earlier metagenome from the same parent material revealed the presence of putative *amoA* genes (Table S1, part C) that could not be classified as *amoA* from canonical AOB, canonical AOAs, or comammox *Nitrospira* (8) (Fig. S6), yet were phylogenetically related to PF05145, purported to contain AMOA-encoding genes in heterotrophic bacteria (57). In addition, the phylogeny of 10/30 of these aberrant *amoA* genes indicated their presence, among others, in *Hyphomicrobiaceae* and Comamonadaceae. Of the highly labeled taxa (in both RNA-SIP, and DNA-SIP) in the NH$_4^+$-fed treatment, Pedamicrobium, and Acidovorax (but not Acidobacteria subgroup 10 or Rhizobacter) belong to the *Hyphomicrobiaceae* and Comamonadaceae. While it is tempting to speculate that we have identified novel ammonia-oxidizing bacteria, we were unable to identify additional *amo* genes that would constitute a complete *amo* operon on any of the metagenomic contigs. In addition, recent doubt has been cast on the assignment of PF05145 as encoding a putative ammonia monooxygenase (58), and
careful physiological or genomic evidence of heterotrophic nitrification remains elusive (59). On the other hand, some of the acidobacterial metagenome-assembled genomes (MAGs) that were retrieved from the studied RGSF metagenome contained CO₂ fixation pathways (i.e., CG15 encoded a near-complete reductive tricarboxylic acid [rTCA] pathway [8]).

The second step of nitrification, the oxidation of nitrite to nitrate, is known to be performed by nitrite-oxidizing chemolithoautotrophs such as Nitrotoga, Nitrospina, Nitrobacter, Nitrolancea, and Nitrospira (60–62), which use nitrite oxidoreductase (NXR) as the key enzyme. The known autotrophic nitrite oxidizer Nitrospira was identified as the only active nitrite oxidizer in the studied system.

In summary, comammox Nitrospira and Nitrosomonas are the chemolithoautotrophic drivers of ammonia oxidation in the groundwater-fed biofilter, and comammox Nitrospira make the greatest contribution. The fundamental niche of comammox Nitrospira, however, remains poorly defined. While kinetics and modeling suggest that these bacteria thrive in environments with low ammonium concentrations (22, 63), as observed in this study, they are equally abundant in some environments with higher ammonium content, such as fertilized soils and wastewater treatment systems (64). AOs did not contribute significantly to nitrification, and Nitrospira bacteria were the only nitrite oxidizers identified in this environment. Hence, we provide the first in situ evidence of ecologically relevant ammonia oxidation by comammox Nitrospira in a complex microbiome and document an unexpectedly high H¹³CO₃⁻ uptake and growth of proteobacterial and acidobacterial taxa under ammonium selectivity.

MATERIALS AND METHODS

Sampling sites and procedure. Filter material samples were collected from a rapid gravity sand filter (biofilter) at the Islevbro waterworks (Rødovre, Denmark) in May 2013. The influent and effluent water quality is reported elsewhere (13, 14, 65). Filter material was collected from three random horizontal locations of the biofilter using a hand-pushed core sampler. From the extracted filter material core, the top 10 cm was aseptically segregated on site and stored on ice for further use. A portion was frozen on-site in liquid nitrogen for RNA extraction.

Column experiments and stable isotope labeling. Experiments were conducted using a continuous-flow lab-scale system consisting of glass columns (2.6 cm diameter, 6 cm long) filled with parent filter material (26.5 cm³) as described previously (14). Effluent water from the investigated waterworks was used as the medium in all experiments to avoid interference of other autotrophic processes and approximate full-scale conditions.

The experimental design consisted of 4 treatments applied to columns fed with ¹³C-labeled or unlabeled HCO₃⁻ (at 1 mM). The influent and effluent pHs of all treatments were 7.5 to 7.6. The experiments were organized in two phases of 4 columns each; filter material was sampled for DNA and RNA extraction just before the onset of each experimental phase. In the 4 treatments, the influent waters were spiked with (i) NH₄⁺ (NH₄Cl at 1 mg/liter N [71 μM]; Sigma-Aldrich, 254134), (ii) NH₄⁺ and ATU (N-allylthiourea at 100 μM; Merck Chemicals, 808158), (iii) NO₂⁻ (NaNO₂ at 1 mg/liter N [71 μM]; Sigma-Aldrich, S2252), or (iv) NH₄⁺ and ClO₃⁻ (KClO₃ at 1 mM; 99%, Sigma-Aldrich, 12634) (Table 1)(25). The applied flow rates (40 ml/h) and influent (NH₄⁺ or NO₂⁻) concentrations were set to match the volumetric NH₄⁺-N loading rates (approximately 1.5 g N/m³/h) experienced by the full-scale parent biofilter (14). Test and control columns were operated for 15 days with continuous feeding to allow sufficient ¹³C label incorporation. Further details are given in Text S1.

Analytical methods. Column effluents were sampled daily, filtered (0.2-μm-pore cutoff), frozen and analyzed colorimetrically for NH₄⁺ and NO₂⁻ as described in Tatari et al. (25). Colorimetric analysis of ammonium in samples containing ATU underestimated the NH₄⁺ concentration (25), and thus NH₄⁺ in these samples was quantified by flow injection analysis (66). NO₂⁻ was quantified by ion chromatography ( Dionex, ICS 1500) with a device fitted with a guard column ( Dionex, AG 22) and an analytical column ( Dionex, Ion Pac AS22). NH₄⁺ removal (%) was calculated by subtracting effluent from influent NH₄⁺ concentration and normalizing for the influent NH₄⁺ concentration. NO₂⁻ removal (%) was calculated as the difference between produced NO₃⁻ concentration and effluent NO₂⁻ concentration, after correcting for trace NO₃⁻ present in the water (ca. 0.3 μM NO₃⁻) and normalization for the produced NO₂⁻ concentration. The NO₂⁻ produced by ammonia oxidation was estimated as the difference between influent and effluent NH₄⁺ concentrations. NO₃⁻ accumulation (%) was calculated from the difference between the effluent and influent NO₃⁻ concentrations, normalized for the produced NO₃⁻ concentration. The NO₃⁻ produced was estimated as the difference between influent and effluent NH₄⁺ (or NO₂⁻) in the case of columns 5 and 6 concentrations.

Nucleic acid extraction and SIP. Filter material samples collected from the full-scale biofilter and the sacrificed columns were subject to DNA and RNA extraction. Genomic DNA was extracted from 0.5 g of drained filter material using the MP FastDNA Spin kit (MP Biomedicals, LLC, Solon, OH) according to manufacturer’s instructions. The concentration and purity of extracted DNA were checked by spectro-
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Density gradient ultracentrifugation of DNA isolated from columns and full scale was performed according to Neufeld et al. (69). Briefly, 1.6 μg of DNA in CeCl₃ with a final density of approximately 1.725 g/ml was subject to ultracentrifugation at 44,800 rpm for 44 h at 20°C in a Beckman VTi 65.2 rotor (70). Gradients were fractionated into 250-μl fractions, density was determined by refractometry, and DNA was recovered by precipitation with PEG. DNA concentration was determined by a Picogreen high-sensitivity double-stranded DNA (dsDNA) quantification kit (Invitrogen). 

PCRs amplification and tag sequencing. RNA samples purified from density gradient fractions and from sacrificed column experiments and full-scale biofilters were reverse transcribed using reverse primer 1492R with the Sensiscript reverse transcription (RT) kit (Qiagen) according to the manufacturer’s protocol. Ten nanograms of cDNA or DNA from direct DNA extracts (Table 1) was used to amplify the V3 and V4 regions of bacterial 16S rRNA genes using the Phusion (Finnzymes, Finland) and 16S rRNA gene-targeted (tDNA) modified universal primers PRK341F and PRK806R (70). PCR was performed as described in reference 7. All fractions (a total of 145 [Fig. S1A]) from DNA-SIP and selected fractions (a total of 62 [Fig. S1B]) from RNA-SIP experiments were sequenced on an Illumina MiSeq and GS FLX pyrosequencing platforms, respectively. Pyrosequencing was applied in a two-region 454 run on a 70-75 GS PicoTiterPlate using a Titanium kit (71); paired-end 16S RNA amplicon sequencing was done on the Illumina MiSeq platform with MiSeq reagent kit v3 (2 × 301 bp; Illumina). All sequencing was performed at the National High-Throughput DNA Sequencing Center (Copenhagen, Denmark).

Bioinformatic and statistical analysis. All bioinformatic and statistical analyses are described in detail in Text S1. Briefly, raw 454 sequence data from RNA-SIP samples were quality-checked (denoised) with Ampliconnoise (71) and chimeras were removed with UCHIME (72) using default settings. Raw MiSeq Illumina sequence data from DNA-SIP samples were quality-controlled with MOTHUR (73), and chimeras were removed with UCHIME (72) using a reference data set. Sequence libraries were combined and trimmed to 418 bp. All further sequence analyses were performed in QIIME 1.9.1 (74).

A total of six filter steps were applied to identify ammonia- and nitrite-oxidizing phylotypes (Fig. S2). Detailed steps are described in Text S1. OTUs incorporating H¹⁴CO₃⁻ were determined by the following: (i) filter 1, comparing the mean buoyant density of each OTU in columns with and without ¹⁴C amendment (31); (ii) filter 2, identifying OTUs affiliated with genera that are present in both DNA and RNA SIP; and (iii) filter 3, selecting OTUs with buoyant density shifts higher than genus-specific 90% CIs for buoyant density shifts. The remaining filter steps were applied to assess ammonia and nitrite oxidizing phylotypes. Cross-feeders and taxa performing heterotrophic CO₂ assimilation (i.e., carboxylation) were largely removed by (iv) filter 4, excluding OTUs with lower buoyant density shift than the maximum buoyant density shift value of labeled Nitrosomonas and Nitrospira OTUs, respectively, (v) filter 5, selecting the genera that contained OTUs in both RNA and DNA-SIP, and (vi) filter 6, comparing the labeled OTUs between treatments (NH₄⁺ fed, NH₄⁺-ATU fed, NO₂⁻ fed, or NH₄⁺-ClO₃⁻ fed). To identify ammonia-oxidizing phylotypes, labeled OTUs in all treatments, excluding the one fed only with NH₄⁺, were removed from the labeled OTU library of NH₄⁺-fed treatment. To identify nitrite-oxidizing phylotypes, labeled OTUs in the treatment fed with ClO₃⁻ were removed from the labeled OTU library of the only-NO₂⁻-fed treatment.

As an additional step, detected genera were ranked according to the increase in their relative abundance in both total DNA and RNA from the beginning (day 0) to the end (day 15) of the experimental runs.

Data availability. R codes for all bioinformatics and statistics, including the detection of labeled OTUs in DNA- and RNA-SIP, can be found in https://github.com/ardagulay.

All sequence data have been deposited at NCBI GenBank under Biosample accession numbers from SAMN12227610 to SAMN12227705.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01870-19.

TEXT S1, PDF file, 0.2 MB.

FIG S1, PDF file, 0.6 MB.

FIG S2, PDF file, 0.2 MB.

FIG S3, PDF file, 0.2 MB.

FIG S4, PDF file, 1.1 MB.

FIG S5, PDF file, 0.4 MB.

FIG S6, PDF file, 0.1 MB.
REFERENCES


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