Propagation of viral genomes by replicating ammonia-oxidising archaea during soil nitrification

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Abstract

Ammonia-oxidising archaea (AOA) are a ubiquitous component of microbial communities and dominate the first stage of nitrification in some soils. While we are beginning to understand soil virus dynamics, we have no knowledge of the composition or activity of those infecting nitrifiers or their potential to influence processes. This study aimed to characterise viruses having infected autotrophic AOA in two nitrifying soils of contrasting pH by following transfer of assimilated CO_2-derived ^{13}C from host to virus via DNA stable-isotope probing and metagenomic analysis. Incorporation of ^{13}C into low GC mol% AOA and virus genomes increased DNA buoyant density in CsCl gradients but resulted in co-migration with dominant non-enriched high GC mol% genomes, reducing sequencing depth and contig assembly. We therefore developed a hybrid approach where AOA and virus genomes were assembled from low buoyant density DNA with subsequent mapping of ^{13}C isotopically enriched high buoyant density DNA reads to identify activity of AOA. Metagenome-assembled genomes were different between the two soils and represented a broad diversity of active populations. Sixty-four AOA-infecting viral operational taxonomic units (vOTUs) were identified with no clear relatedness to previously characterised prokaryote viruses. These vOTUs were also distinct between soils, with 42% enriched in ^{13}C derived from hosts. The majority were predicted as capable of lysogeny and auxiliary metabolic genes included an AOA-specific multicopper oxidase suggesting infection may augment copper uptake essential for central metabolic functioning. These findings indicate virus infection of AOA may be a frequent process during nitrification with potential to influence host physiology and activity.

Main text

38 Microbially mediated oxidation of ammonia to nitrate during nitrification is a central component of the global nitrogen (N) cycle. It is also responsible for major losses of
40 applied fertiliser N in soil, generating atmospheric pollution via direct and indirect production of nitrous oxide (N_2O) as well as nitrate (NO_3^-) pollution of groundwater [1].
42 Autotrophic ammonia-oxidising archaea (AOA) of the class *Nitrososphaeria* are a ubiquitous component of soil microbial communities and often dominate ammonia
44 oxidation and nitrification-associated N_2O emissions when ammonia is supplied at low rates via organic matter mineralisation [2], slow-release fertilisers [3] or in acidic soils
46 [4]. Integrated temperate viruses (proviruses) and other virus-associated protein-encoding genes are found in most AOA genomes suggesting frequent interaction (see
48 Supplementary Text). While viruses infecting marine AOA have been characterised through metagenomic approaches [5] and cultivation [6], those infecting soil AOA or
50 other nitrifier groups are currently uncharacterised.

Virus infection can influence biogeochemical cycling by augmenting host
52 activity or causing cell mortality and subsequent release of nutrients [7]. Recent advances have demonstrated that soil virus communities are dynamic in a wide range
54 of soils [e.g. 8, 9] and augmenting virus loads modulate C and N fluxes [10, 11]. Nevertheless, identifying active interactions with specific populations or functional
56 groups in soil remains challenging due to structural complexity and the vast diversity of hosts and viruses. Recent use of stable-isotope approaches has investigated whole
58 community host-virus dynamics [12, 13] or interactions between individual host-virus populations specific to a functional process and substrate [14]. The aim of this study
60 was to utilise the latter approach with $^{13}CO_2$-based DNA-SIP to focus on nitrification-associated interactions and to test the hypothesis that viruses are a dynamic
62 component of soil AOA activity.

64 *Hybrid analysis of GC mol% fractionation and ^{13}C-DNA-SIP for identifying active AOA hosts and viruses*

66 Microcosms were established (see Supplementary Text) with two sandy loam soils of contrasting pH (4.5 or 7.5), which were characterised previously as containing distinct 68 AOA communities [15]. As ammonia from hydrolysed urea is used by AOA in these and other soils [1, 3], nitrification was fuelled by addition of urea (two pulses of 100 µg 70 N g^{-1} soil (dry weight)) to stimulate AOA growth and potential enrichment of AOA-infecting viruses. Microcosms were incubated at 25°C for 30 days (Supplementary 72 Figure 1) with a 5% ^{12}C- or ^{13}C-CO_2 headspace to target viruses of autotrophs via transfer of assimilated carbon. Extracted DNA was subjected to isopycnic 74 centrifugation in CsCl gradients, in which DNA migrates as a function of GC mol% and isotopic enrichment, before fractionation and analysis of genomic DNA of different 76 buoyant densities [14]. Quantification of AOA genomic DNA distribution across CsCl gradients demonstrated growth of AOA populations (Figure 1A; Supplementary Figure 78 2) and genomic DNA in high buoyant density (HBD) fractions (>1.719 g ml^{-1}) was then pooled for each replicate microcosm and metagenomes sequenced (Supplementary 80 Table 1). While ^{13}C-incorporation clearly separated DNA from growing vs non-growing AOA populations, ^{13}C-enriched AOA genomes (average contig GC mol% content of 82 38%) co-migrated within the CsCl gradient with more abundant community ^{12}C genomes with higher GC mol% (>57%), reducing AOA host and virus sequencing 84 depth, contig assembly and binning. Consequently, only one medium-quality AOA metagenome-assembled genome (MAG) was recovered from HBD DNA and no AOA-86 associated virus contigs were identified amongst 290 viral operational taxonomic units (vOTUs) ≥10 kb (Figure 1B).

88 To facilitate analysis of low GC mol% genomes while also identifying activity, we developed a hybrid approach combining metagenomic analysis of low buoyant 90 density (LBD) ^{12}C DNA with read-mapping of sequenced HBD DNA (Figure 1; Supplementary Figure 2). The AOA and virus genomes were assembled from 92 metagenomic libraries of ^{12}C LBD DNA only (<1.699 g ml^{-1}) representing both dormant and active populations. Individual sequence reads from HBD DNA libraries from both 94 ^{12}C- and ^{13}C-incubations were then mapped to identify activity with a significantly greater relative abundance in ^{13}C-incubated samples demonstrating isotope 96 incorporation. For taxonomically defined contigs of hosts or host-linked viruses, in comparison to HBD, assemblies from LBD libraries increased the relative proportion 98 of contigs from the AOA-containing *Thermoproteota* phylum (GTDB taxonomy; *Nitrososphaerota* in ICNP taxonomy and *Thaumarchaeota* in NCBI taxonomy) and 100 *Thermoproteota*-infecting virus contigs from 0.3 to 2.8% and 0 to 22.3%, respectively (Supplementary Figure 3). In LBD metagenomes, 123 medium- and high-quality 102 MAGs from both soils were assigned taxonomy (Supplementary Table 2) including nine AOA MAGs representing six genera (GTDB classification [16]) within the 104 *Nitrososphaerales* and *Nitrosopumilales* orders and one nitrite-oxidising bacterium (NOB) *Nitrobacter* MAG (MAG 15) (Figure 1B, Supplementary Figure 4), contributing 106 to the oxidation of nitrite (NO_2^-) to NO_3^- during the second step of canonical nitrification. Mapping of HBD sequence reads revealed that eight of the ten MAGs were derived 108 from active autotrophic populations (Figure 1C). Consistent with previous studies characterising the distribution of soil AOA from different taxa [17], this included four 110 *Nitrosotalea* genus populations (MAGs 9, 61, 77 and 107) from pH 4.5 soil and three *Nitrososphaera* genus populations (MAGs 12, 52 and 96) from pH 7.5 soil. No 112 evidence of autotrophic growth was observed for populations represented by a further two MAGs within the *Nitrososphaerales*. Both are from genera with no cultivated

114 representatives with MAG 112 from genus TA-21 (*amoA*-gene lineage NS-β-2 [17])
and MAG 63 (*amoA*-gene lineage NS-δ-2.1) representing a novel genus (designated
116 here as CS63). The latter belongs to the *amoA* gene-defined NS-δ lineage that while
widely found in soil, is rarely implicated in contributing to ammonia oxidation. Although
118 these two MAGs may simply be from AOA populations not active under the incubation
conditions used, they are also consistent with microorganisms possessing
120 physiologies distinct from cultivated AOA.

122 *AOA viruses*

After predicting contigs of virus origin using established bioinformatic tools, those from
124 AOA-infecting viruses were identified with a custom database of hallmark genes from
AOA proviruses and analysis of other shared homologues (see Supplementary Text).
126 Specifically, proviruses were identified in 184 (87%) of 212 AOA genome sequences
downloaded from GTDB. Capsid, terminase, portal and integrase protein sequences
128 from these proviruses were subsequently used as search queries. While previous
analysis of 12 total soil metagenome or virome libraries from the same soil samples
130 did not enable identification of any AOA-associated virus contigs (average of 152
million quality filtered reads and 1,947 contigs ≥10 kb per library) [14], this targeted
132 approach using LBD DNA from nitrifying microcosms yielded 64 contigs predicted as
derived from AOA-infecting viruses and each representing a unique vOTU. In addition,
134 viruses infecting other nitrifier populations were identified with five and three vOTUs
derived from viruses infecting ammonia-oxidising bacteria (AOB) and NOB,
136 respectively. Virus and host genome GC mol% correlate [18] and AOA-infecting virus
contigs possessed a similar average content of 36.3% which was considerably lower
138 than averages of 46.6% and 67.0% of all viral contigs recovered from LBD and HBD

metagenomes, respectively. As with their hosts, AOA virus communities were distinct
140 between the two pH soils (Figure 2A) as also observed previously for total virus communities [19] with 29 (45%) and 34 (53%) vOTUs significantly more relatively
142 abundant in pH 4.5 and 7.5 LBD libraries compared to the other soil, respectively. Comparison of the relative abundance of HDB sequence reads from both ^{12}C and ^{13}C
144 libraries revealed that 27 contigs from both soils (42%) were enriched in ^{13}C and thus confirming infection during incubation and transfer of C from AOA hosts to viruses.

146 Soil AOA viruses were distinct from characterised archaeal and bacterial viruses. Gene-sharing network analysis using vConTACT v2.0 [20] did not identify any
148 associations with reference sequences or recently described viruses of marine AOA [6] and genome-wide sequence comparisons [21] placed 60 of the 64 soil AOA viral
150 contigs within a unique proteomic grouping (Figure 2B). The majority were predicted as capable of lysogeny with 55 encoding an integrase. While it can be difficult to
152 determine virulent versus temperate capability from partial genomes, only 6.8 and 8.9% of all virus contigs from LBD and HBD metagenomes possessed a predicted
154 integrase. Although suggesting a higher proportion of AOA viruses may be temperate, it should be noted that this could be a consequence of using AOA provirus-associated
156 genes to identify viral contigs, biasing towards identification of those from temperate viruses. These results also highlight that while DNA-SIP of total metagenomes
158 enables identification of ^{13}C incorporation into virus genomes, it is potentially limited in facilitating confident bioinformatic prediction of free temperate virus particles versus
160 proviruses present from a previous infection of a currently active host. While the integration status of a temperate virus can be predicted bioinformatically, using
162 targeted “viromes” metagenomes of filtered virus particles will provide greater confidence of identifying genomes from free virus particles.

164 Eleven contigs contained large terminase (TerL) sub-unit gene sequences and were similar to those recovered from other predicted AOA virus or provirus genomes
166 only (e-value $<10^{-5}$) (Figure 2C). In addition to viral hallmark genes encoding portal, endonuclease, tail and capsid proteins, contig 053596 contained a type 1 multicopper
168 oxidase (MCO1) homologue found exclusively in AOA genomes [22] (Figure 2D). Copper (Cu) is essential for ammonia oxidation and electron transport in AOA and
170 MCO1 is upregulated under Cu limitation and potentially involved in Cu sequestration from the environment [23]. This virus-encoded MCO1 homologue therefore represents
172 an auxiliary metabolic gene that may contribute to a core process in AOA physiology.

174 *Summary*

These results from two soils indicate that virus infection of AOA during nitrification is
176 likely a common and dynamic process, with the potential to influence nitrification rates in soil. Although viruses were only identified from contigs representing partial viral
178 genomes, analysis of individual marker genes and genome-wide similarity comparisons revealed that those infecting AOA are highly divergent from known
180 viruses. While one *Nitrobacter* MAG was obtained and both AOB and NOB viruses were also predicted, the hybrid approach developed in this study targeted low GC
182 mol% DNA and was specifically tailored for the analysis of AOA populations and their viruses. A broader analysis of all viruses infecting nitrifying prokaryotic populations
184 would therefore likely require a more comprehensive sequencing effort of all fractions throughout the CsCl gradient using quantitative SIP metagenomics [24] or alternative
186 approaches, such as virus-targeted metagenomic viromes.

188 *Data availability*

Metagenome sequence data are deposited in NCBI's GenBank under BioProject accession nos. PRJNA621418-PRJNA621429 (^{13}C data) and PRJNA868779 (^{12}C data) with MAGs available under accession numbers SAMN30436125-SAMN30436246.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

SL, GWN and CH conceived and designed the experiment with additional input from ETS. SL performed all laboratory work and analysed the data with input from GWN in

phylogenetic analyses. SL, GWN and CH drafted the manuscript text and figures with all authors contributing to the final paper.

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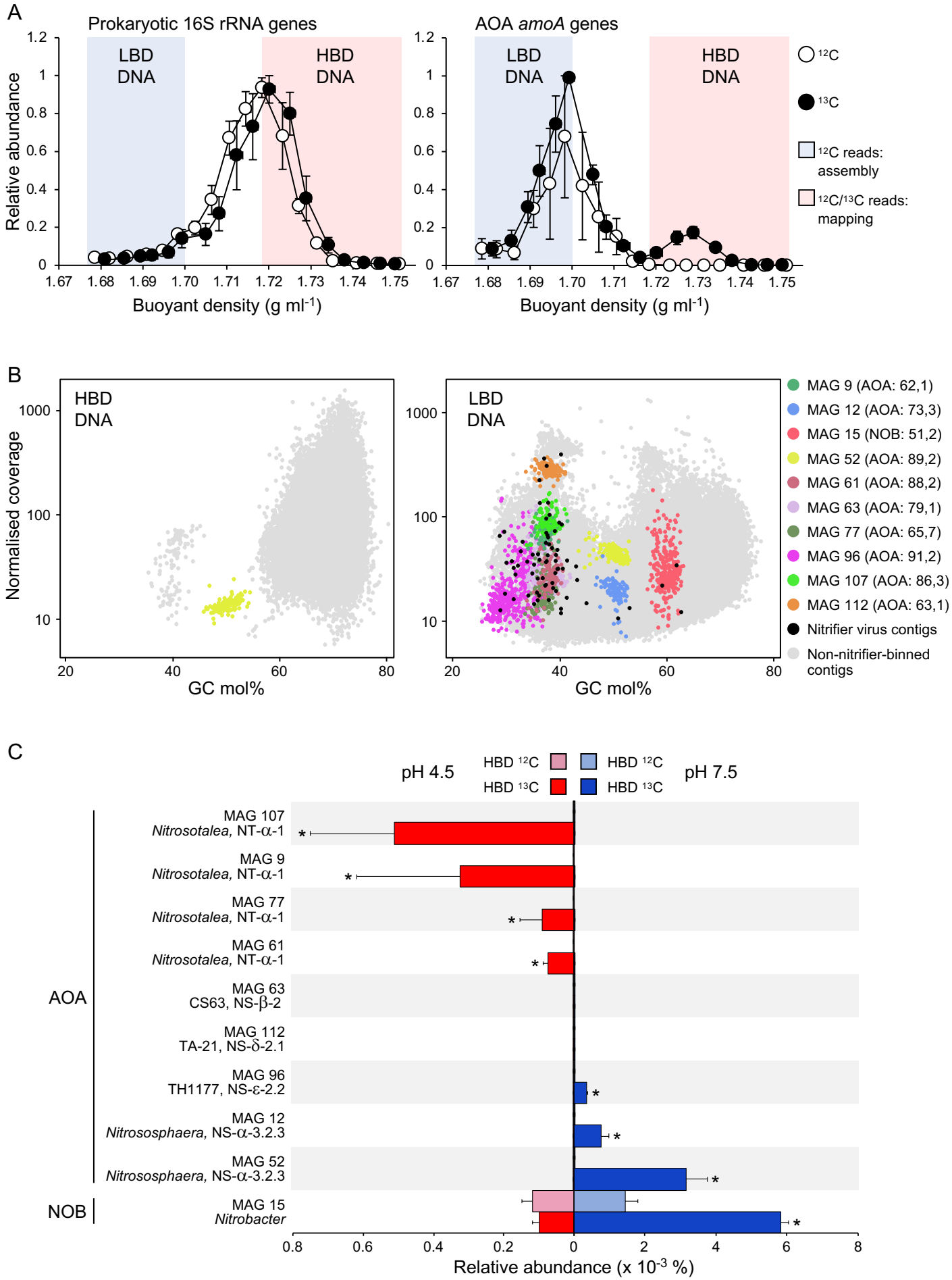
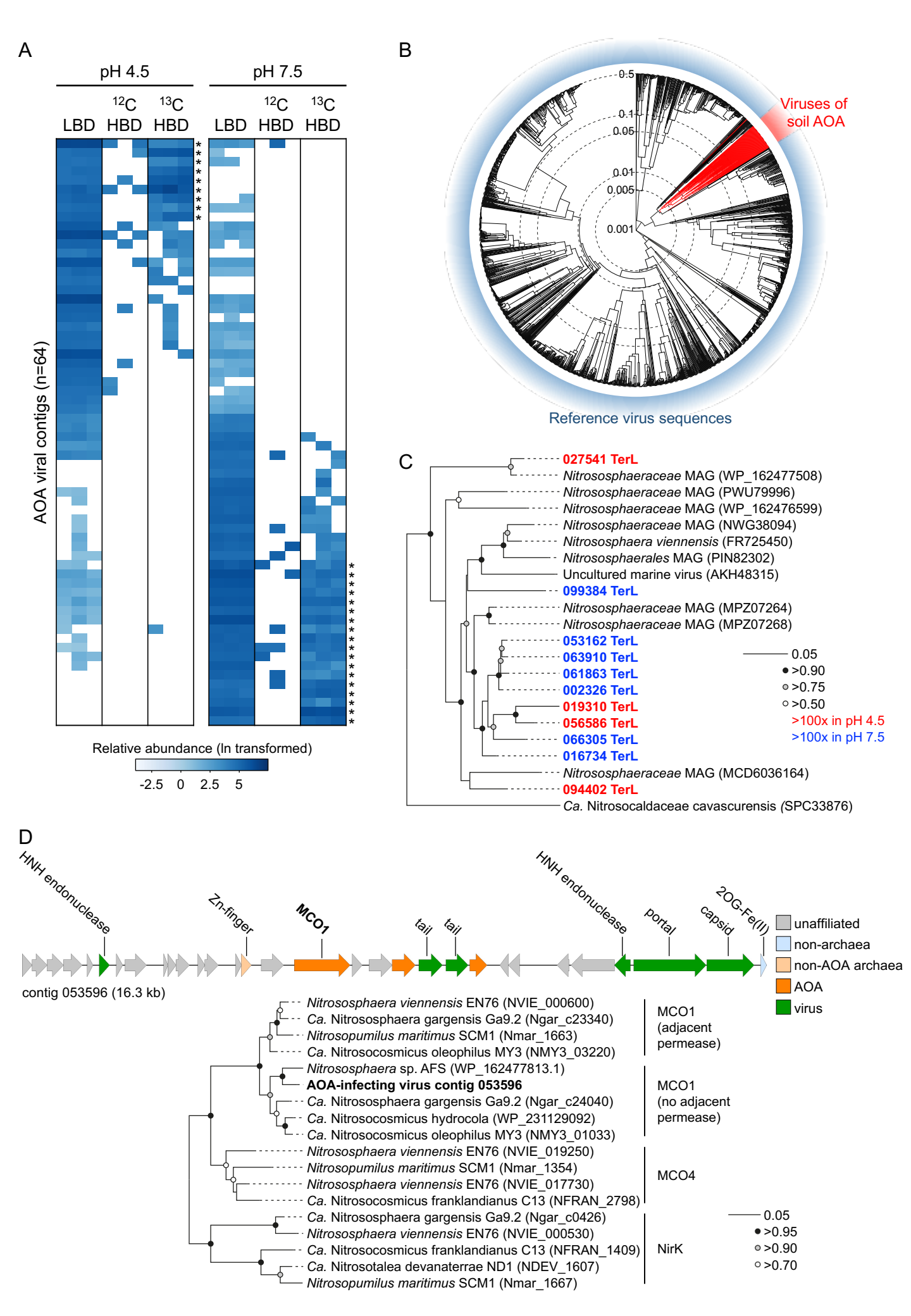
290 **Figure legends**

Figure 1. Contigs and MAGs from low GC mol% DNA and *in situ* growth of AOA in pH
292 4.5 and 7.5 soils determined from hybrid analysis of ^{12}C- and ^{13}C-enriched DNA. **A** Distribution of total prokaryotic and AOA genomes in CsCl gradients determined from
294 the relative abundance of 16S rRNA genes and *amoA* genes, respectively. Example profiles are shown for pH 4.5 soil only (see Supplementary Figure 2 for pH 7.5
296 profiles). Vertical error bars are the standard error of the mean relative abundance and horizontal bars (mostly smaller than the symbol size) the standard error of the mean
298 buoyant density of individual fractions from three independent CsCl gradients, each representing an individual microcosm. Genomic DNA in fractions highlighted in blue
300 or pink areas were pooled for each replicate microcosm for metagenomic sequencing. **B** Distribution of assembled contigs (≥5 kb) from metagenomic libraries prepared from
302 LBD or HBD DNA in sequence coverage vs. GC mol% plots. Contigs binned into ten medium- and high-quality nitrifier MAGs and predicted viruses of nitrifiers (≥10 kb) are
304 highlighted, with number in parenthesis beside each MAG identifier giving estimated completeness and contamination (%). **C** Mean and standard error of the relative
306 proportion of metagenomic reads from ^{12}C- and ^{13}C-enriched HBD DNA mapped onto contigs of nine AOA and one *Nitrobacter* MAGs. A significantly greater relative
308 proportion of reads in ^{13}C-derived libraries are indicated with * (p <0.05, two-sample Student's t-test or Welsch's t-test when variances were not homogenous). GTDB
310 genus [16] and *amoA* gene lineage-affiliation [17] are given.

312 **Figure 2.** Identification and determination of activity of viruses infecting AOA in pH 4.5 and 7.5 soils using a hybrid analysis of ^{12}C- and ^{13}C-enriched DNA. **A** Heatmap
314 showing the relative abundance of 64 vOTUs identified in LBD DNA libraries from pH 4.5 and 7.5 soil (≥1× coverage, ≥75% contig breadth). To identify activity, reads from

316 ^{12}C- and ^{13}C-enriched HBD DNA were mapped onto LBD-derived contigs with a significantly greater relative proportion of reads in ^{13}C-derived libraries indicated with
318 * (p <0.05, two-sample Student's t-test or Welsch's t-test when variances were not homogenous). **B** Proteomic tree showing genome-wide sequence similarities between
320 AOA viral contigs and 1,981 curated virus genomes. Values at dotted circles represent a distance metric based on normalized tBLASTx scores plotted on a log scale. **C**
322 Maximum likelihood phylogenetic analysis of derived large sub-unit terminase (TerL) protein sequences (336 unambiguously aligned positions, LG substitution model)
324 identified in this and other metagenomic studies or in proviruses of cultivated AOA. NCBI accession numbers are given in parenthesis. Circles at nodes represent
326 percentage bootstrap support from 1000 replicates, scale bar denotes an estimated 0.05 changes per position and colour-coding describes the comparative relative
328 abundance in each soil. **D** Genetic map of AOA-infecting virus-derived contig 053596 containing a type 1 multicopper oxidase (MCO1) gene. Maximum likelihood
330 phylogenetic tree describes placement of MCO1 within the AOA MCO/NirK family of cultivated AOA following designations of Kerou *et al.* [22] (206 unambiguously aligned
332 positions, LG substitution model, invariant and gamma distributed sites). NCBI accession numbers are given in parenthesis. Circles at nodes represent percentage
334 bootstrap support from 1000 replicates and the scale bar denotes an estimated 0.05 changes per position.


A
Prokaryotic 16S rRNA genes
AOA amoA genes
LBD DNA
HBD DNA
Relative abundance
Buoyant density (g ml-1)
12C
13C
12C reads: assembly
12C/13C reads: mapping
B
HBD DNA
LBD DNA
Normalised coverage
GC mol%
MAG 9 (AOA: 62,1)
MAG 12 (AOA: 73,3)
MAG 15 (NOB: 51,2)
MAG 52 (AOA: 89,2)
MAG 61 (AOA: 88,2)
MAG 63 (AOA: 79,1)
MAG 77 (AOA: 65,7)
MAG 96 (AOA: 91,2)
MAG 107 (AOA: 86,3)
MAG 112 (AOA: 63,1)
Nitrifier virus contigs
Non-nitrifier-binned contigs
C
pH 4.5
pH 7.5
HBD 12C
HBD 13C
AOA
NOB
MAG 107 Nitrosotalea, NT-α-1
MAG 9 Nitrosotalea, NT-α-1
MAG 77 Nitrosotalea, NT-α-1
MAG 61 Nitrosotalea, NT-α-1
MAG 63 CS63, NS-β-2
MAG 112 TA-21, NS-δ-2.1
MAG 96 TH1177, NS-ε-2.2
MAG 12 Nitrososphaera, NS-α-3.2.3
MAG 52 Nitrososphaera, NS-α-3.2.3
MAG 15 Nitrobacter
Relative abundance (x 10-3 %)


A
pH 4.5
pH 7.5
12C HBD
13C HBD
LBD
AOA viral contigs (n=64)
Relative abundance (ln transformed)
-2.5 0 2.5 5
B
Viruses of soil AOA
Reference virus sequences
0.5
0.1
0.05
0.01
0.005
0.001
C
027541 TerL
Nitrososphaeraceae MAG (WP_162477508)
Nitrososphaeraceae MAG (PWU79996)
Nitrososphaeraceae MAG (WP_162476599)
Nitrososphaeraceae MAG (NWG38094)
Nitrososphaera viennensis (FR725450)
Nitrososphaerales MAG (PIN82302)
Uncultured marine virus (AKH48315)
099384 TerL
Nitrososphaeraceae MAG (MPZ07264)
Nitrososphaeraceae MAG (MPZ07268)
053162 TerL
063910 TerL
061863 TerL
002326 TerL
019310 TerL
056586 TerL
066305 TerL
016734 TerL
Nitrososphaeraceae MAG (MCD6036164)
094402 TerL
Ca. Nitrosocaldaceae cavascurensis (SPC33876)
0.05
>0.90
>0.75
>0.50
>100x in pH 4.5
>100x in pH 7.5
D
HNH endonuclease
Zn-finger
MCO1
tail
tail
HNH endonuclease
portal
capsid
2OG-Fe(II)
contig 053596 (16.3 kb)
unaffiliated
non-archaea
non-AOA archaea
AOA
virus
Nitrosophaera viennensis EN76 (NVIE_000600)
Ca. Nitrososphaera gargensis Ga9.2 (Ngar_c23340)
Nitrosopumilus maritimus SCM1 (Nmar_1663)
Ca. Nitrosocosmicus oleophilus MY3 (NMY3_03220)
Nitrosophaera sp. AFS (WP_162477813.1)
AOA-infecting virus contig 053596
Ca. Nitrososphaera gargensis Ga9.2 (Ngar_c24040)
Ca. Nitrosocosmicus hydrocola (WP_231129092)
Ca. Nitrosocosmicus oleophilus MY3 (NMY3_01033)
Nitrosophaera viennensis EN76 (NVIE_019250)
Nitrosopumilus maritimus SCM1 (Nmar_1354)
Nitrosophaera viennensis EN76 (NVIE_017730)
Ca. Nitrosocosmicus franklandianus C13 (NFRAN_2798)
Ca. Nitrososphaera gargensis Ga9.2 (Ngar_c0426)
Nitrosophaera viennensis EN76 (NVIE_000530)
Ca. Nitrosocosmicus franklandianus C13 (NFRAN_1409)
Ca. Nitrosotalea devanaterrae ND1 (NDEV_1607)
Nitrosopumilus maritimus SCM1 (Nmar_1667)
MCO1 (adjacent permease)
MCO1 (no adjacent permease)
MCO4
NirK
0.05
>0.95
>0.90
>0.70