Differential contribution of nitrifying prokaryotes to groundwater nitrification

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Abstract

The ecophysiology of complete ammonia-oxidizing bacteria (CMX) of the genus *Nitrospira* and their widespread occurrence in groundwater suggests that CMX bacteria have a competitive advantage over ammonia-oxidizing bacteria (AOB) and archaea (AOA) in these environments. However, the specific contribution of their activity to nitrification processes has remained unclear. We aimed to disentangle the contribution of CMX, AOA and AOB to nitrification and to identify the environmental drivers of their niche differentiation at different levels of ammonium and oxygen in oligotrophic carbonate rock aquifers. CMX *amoA* genes accounted on average for 16 to 75% of the total groundwater *amoA* genes detected. Nitrification rates were positively correlated to CMX clade A associated phylotypes and AOB affiliated with *Nitrosomonas ureae*. Short-term incubations amended with the nitrification inhibitors allylthiourea and chlorate suggested that AOB contributed a large fraction to overall ammonia oxidation, while metaproteomics analysis confirmed an active role of CMX in both ammonia and nitrite oxidation. Ecophysiological niche differentiation of CMX clades A and B, AOB and AOA was linked to their requirements for ammonium, oxygen tolerance, and metabolic versatility. Our results demonstrate that despite numerical predominance of CMX, the first step of nitrification in oligotrophic groundwater appears to be primarily governed by AOB. Higher growth yields at lower ammonia turnover rates and energy derived from nitrite oxidation most likely enable CMX to maintain consistently high populations.

32 ## Introduction

33 Nitrification is a globally relevant process of the nitrogen cycle mediated by a polyphyletic
34 group of microorganisms [1]. The discovery of complete ammonia oxidizers (comammox
35 bacteria, CMX) challenged the paradigm of a strict division of labor in the process of
36 nitrification [2]. While ammonia oxidation is carried out by ammonia-oxidizing bacteria (AOB)
37 and archaea (AOA) [3] and nitrite oxidation by nitrite-oxidizing bacteria (NOB) [2], CMX of the
38 genus *Nitrospira* can perform complete oxidation of ammonia (NH_3) to nitrate (NO_3^-) in one
39 cell [4, 5]. CMX have been frequently reported from engineered environments such as drinking
40 water or wastewater treatment facilities [6–8] and from natural environments like soils [9, 10],
41 surface freshwater [11, 12] and groundwater [13, 14]. However, our knowledge about the role
42 and relevance of CMX for nitrification activity in natural environments is still scarce.

43 In most environments, CMX will compete with canonical nitrifiers for either ammonia (NH_3) or
44 nitrite (NO_2^-) or both, raising the question of the actual contribution of CMX to nitrification
45 processes and of the factors that control their co-occurrence with AOB, AOA, and NOB.
46 Recent investigations consider affinity to NH_3, growth rates, growth yields, and metabolic
47 versatility as key factors that determine the niche differentiation and competition between
48 CMX, AOB, and AOA [2, 15]. Since CMX exhibit higher affinity to NH_3 and oxygen than
49 terrestrial AOB [15, 16] along with higher growth yields compared to AOB and AOA [2], CMX
50 might have a competitive advantage over AOB in environments that are limited in NH_3 and
51 ammonium (NH_4^+) such as oligotrophic groundwater. Particular lineages of AOB and AOA
52 exhibit adaptation to NH_4^+ limited conditions [17–20] and have previously been reported from
53 groundwater environments [21–23]. As such, oligotrophic groundwater represents an
54 excellent microbial habitat to study the co-occurrence of CMX, AOB and AOA and their
55 contribution to nitrification activity.

56 Based on the phylogeny of the ammonia monooxygenase (Amo), the key enzyme of ammonia
57 oxidation, CMX *Nitrospira* are divided into two clades, A and B [2, 4]. Metagenomic studies
58 uncovering the phylogeny and metabolic features of CMX proposed an ecophysiological niche

differentiation between the two clades in natural habitats [4, 21, 24–26]. Those findings are linked to genomic variability in nitrogen uptake and alternative energy metabolism [25, 26], and several studies suggest a more prominent role of clade A in nitrification. So far, CMX enrichments and isolates comprise only clade A representatives [27]. ^{13}C stable isotope probing confirmed growth of CMX clade A with NH_4^+ during incubations of wetland coast sediments [28], and CMX clade A were highly abundant in salt marsh sediments showing strong potential for nitrification [29]. However, the ecophysiological niche differentiation between the clades has rarely been studied in the context of their co-occurrence and competition with canonical nitrifiers.

Previous studies from karstic carbonate rock aquifers of the Hainich Critical Zone Exploratory in central Germany pointed to strong links between *in situ* nitrification and chemolithoautotrophic carbon fixation [30, 31]. In addition, genome resolved metagenomics revealed an important role of CMX *Nitrospira* in the nitrogen and carbon cycle in this oligotrophic habitat [21]. The aquifers are characterized by a high spatial heterogeneity of NH_4^+ and NO_3^- concentrations across an oxygen gradient, linked to differences in groundwater flow regime and distance to groundwater recharge areas [28, 29, 30]. These settings are ideal for the investigation of the ecophysiological niche differentiation of CMX clade A and B, AOB and AOA.

We combined ^{15}N-based nitrification rate measurements with selective nitrification inhibitors and genome-resolved metagenomics and metaproteomics to assess the contributions of CMX *Nitrospira*, AOB and AOA to overall groundwater nitrification in these aquifers and to follow the composition of the groundwater nitrifier populations across the hydrochemical gradients. We hypothesized that (i) CMX act as key ammonia oxidizers in these oligotrophic groundwater habitats and account for a large fraction of overall nitrification activity. We further hypothesized that (ii) distribution patterns of ammonia oxidizers across gradients of NH_4^+ and oxygen availability are linked to their ecophysiological niche differentiation, indicated by differences in encoded metabolic potentials.

Materials and methods

Study site characteristics and sampling of groundwater

Groundwater was obtained from karstic limestone aquifers at the Hainich Critical Zone Exploratory (CZE) in Thuringia (Germany) within the framework of the Collaborative Research Center (CRC) AquaDiva [34]. Further details about location and geological setting are reported elsewhere [33, 34]. Sampling wells H14, H32, H42, H43, H52 and H53 at varying depths provide access to oxygen-deficient (less than 15 µM DO) groundwater from an upper mudstone-dominated low-flow aquifer (HTU) and wells H13, H31, H41 and H51 (mean values 154 to 672 µM DO) provide access to oxic groundwater from a lower fast-flow limestone-dominated karstified aquifer (HTL) [32, 34] (Figure 1A, Figure 1B). The main recharge zone for groundwater is located at the hilltop of the transect near wells H13 and H14. Groundwater collected from regular monthly sampling between 2018 to 2021 was obtained using submersible motor pumps (Grundfos, Denmark). Hydrochemical parameters (pH, T, TOC, DOC) were determined as described in [32, 33]. NH_4^+ (sum of NH_3 and NH_4^+), NO_2^- and NO_3^- concentrations were measured with colorimetric methods [35, 36] from groundwater filtered through 0.2 µm-pore size sterile polyvinylidine fluoride filters. . Concentrations of free NH_3 were calculated based on pH and NH_4^+ concentrations (pKa NH_4^+/NH_3 = 9.73 at 10°C). Groundwater for molecular analysis was collected in sterile 10 L fluorinated polyethylene containers and filtered through 0.2 µm polycarbonate filters (Nuclepore, Whatman). Additional groundwater samples for metaproteomic analysis were collected from wells H14, H41, H43 and H52 in January 2019 and filtered through 0.2 µm pore size filters (Merck Millipore, Germany). All filters were immediately frozen on dry ice and stored at -80 °C until extraction.

^{15}N-isotope labelling and rate measurements

Groundwater was collected in sterile 0.5 L glass bottles and filled from the bottom using sterile tubing. Bottles were overfilled with three volumes exchanges before they were capped with silicone septa without headspace to minimize alterations of *in situ* oxygen concentrations. At each sampling campaign, groundwater was collected in triplicate bottles for each well along

113 with one control and kept at 4°C until further processing within 2 hours. Prior to the ^{15}N
114 labelling, 10 ml of each sampling bottle was removed for analysis of inorganic nitrogen
115 concentrations and pH, and replaced with N_2. Bottles containing anoxic groundwater were
116 amended with 1 % sterile oxygen (4 ml 100% oxygen) to stimulate nitrification under oxygen-
117 deficient conditions. Groundwater of control bottles was sterile filtered through 0.2 µm filters
118 (Supor, Pall Corporation, USA). Sterile filtered ^{15}N-labeled ammonium sulfate solution
119 ($(^{15}NH_4)_2SO_4$) (98 at%, Cambridge Isotope Laboratories, Tewksbury) as the substrate source
120 for nitrifiers was added to all 0.5 L bottles to a final concentration of 50 µM, at a natural
121 background of 0.5 to 55 µM NH_4^+. Nitrification rate measurements were conducted according
122 to Overholt *et al.* [21] and detailed descriptions are provided in Supplementary Methods.

123 **Incubation experiments in the presence of nitrification inhibitors**

124 The ammonia and nitrite oxidation activity of CMX, and the nitrite oxidation activity of NOB
125 were selectively inhibited by 1 mM potassium chlorate ($KClO_3$) [7], and 100 µM allylthiourea
126 (ATU) was used to inhibit the ammonia oxidation activity of CMX and AOB [7, 37]. Nitrification
127 rate measurements with ^{15}N tracer were carried out over five days as described above.
128 Groundwater from well H41 was collected in February 2020 and amended with 50 µM NH_4^+
129 as $(^{15}NH_4)_2SO_4$ and one of the nitrification inhibitors along with untreated controls.

130 Groundwater for mesocosm experiments was collected in March and May 2021 and was filled
131 in sterile 2 L glass bottles without headspace to assess potential growth of ammonia oxidizers
132 with NH_4^+ in the presence of nitrification inhibitors at the same concentrations as before.
133 Groundwater from oxic well H41 (10 µM NH_4^+ *in situ*) was adjusted to 10 and 50 µM NH_4^+, and
134 groundwater from hypoxic well H53 (36 µM NH_4^+ *in situ*) was used without additional
135 supplement. All samples were incubated for 15 to 25 days at 15°C without agitation in the
136 dark. Subsamples for nitrogen compound analysis were taken at the onset and every two to
137 three days until the end of the incubation to monitor changes of the nitrogen chemistry
138 (Supplementary Methods). Incubation of well H41 with an initially 10 µM NH_4^+ was adjusted

139 again to 15 µM NH_4^+ after 15 days. After incubation, the groundwater was filtered on 0.2 µm
140 polycarbonate filters and stored at -80°C for later extraction of nucleic acids.

141 Nucleic acid extraction, cDNA-synthesis and quantitative PCR

142 Genomic DNA and total RNA of microbial biomass from the 0.2 µm filters were extracted using
143 the DNeasy PowerSoil kit (Qiagen, Germany) and the RNeasy PowerWater kit (Qiagen,
144 Germany), respectively, according to the manufacturer's specifications. RNA was further
145 processed with the TURBO DNA-free kit (Thermo Fischer Scientific, Germany) to remove
146 remnant DNA in the samples before cDNA synthesis [38]. Reverse transcription was
147 performed using the GoScript Reverse Transcription System (Promega, USA). Gene and
148 transcript abundances of *amoA* from CMX *Nitrospira*, AOB, AOA and of *nxrB* from *Nitrospira*-
149 like NOB were determined using a CFX96 qPCR cycler (Bio-Rad, USA). Primer pairs for
150 quantification were comamoAF/ comamoAR for CMX *amoA* [11], amoA-1F/ amoA-2R for AOB
151 *amoA* [39], Arch-amoAF/ Arch-amoAR for AOA *amoA* [40] and nxrB169f/ nxrB638r for
152 *Nitrospira*-like *nxrB* [41]. More details are provided in the Supplementary Methods. For testing
153 of potential correlations between CMX *amoA* and Nitrospira *nxrB* genes, we assumed on
154 average one copy of *amoA* and 1.5 copies of *nxrB* per cell. Calculation of the correction factor
155 1.5 was based on the analysis of putative canonical *Nitrospira*-affiliated MAGs from the
156 Hainich groundwater metagenome, which harbored 1 to 2 *nxrB* gene copies per genome [21]

157 Amplicon sequencing of *amoA* and 16S rRNA genes and sequence analysis

158 Amplicon libraries of CMX, AOB and AOA *amoA* genes were created using the NEBNext Ultra
159 DNA Library Prep Kit for Illumina (New England Biolabs, MA) according to the manufacturer's
160 guideline. The same primers as for qPCR were used for CMX and AOB *amoA*, while primers
161 Arch-amoA-104F/ Arch-amoA-616Rmod [42, 43] were chosen for sequencing of AOA *amoA*
162 genes due to shorter fragment length (Supplementary Methods). Sequencing was conducted
163 on an Illumina MiSeq using v3 chemistry. After trimming primer sequences, raw reads were
164 first quality filtered with DADA2 1.22.0 [44] and further processed with Mothur v.1.46.1 [45]

165 (Supplemental Methods). OTUs were assigned at 95% sequence identity for CMX, 95% for
166 AOB [46] and 96% for AOA [42] (see Dataset S3).

167 Amplicon libraries of the bacterial 16S rRNA gene were constructed as described elsewhere
168 [47] to assess the relative abundance of nitrite oxidizers present in the groundwater, including
169 non-*Nitrospira* NOB. Raw sequence reads were processed using DADA2 1.22.0. Taxonomy
170 was assessed using the SILVA reference data base release 138.1 [48]. Sequence analysis
171 was performed using the R package phyloseq [49].

172 Inferring metabolic potential from metagenome assembled genomes of CMX 173 *Nitrospira* and other nitrifiers

174 To assess metabolic capabilities of the two CMX clades, genomic features of metagenome
175 assembled genomes (MAGs) were investigated. Groundwater sampling for metagenomic
176 sequencing and generation of MAGs were described in detail in [21, 50]. Here, Hainich
177 groundwater MAGs, which were taxonomically classified as *Nitrospira* on genus level and 243
178 *Nitrospira* reference genomes downloaded from NCBI (July 2021) were aligned and screened
179 for both *amoA* and *nxrB* genes with HMMER in Anvi'o v7.1 [21] using custom AmoA and NxrB
180 databases containing sequences from the FunGene repository [51].

181 After sorting good quality MAGs (> 90 % completeness, except *N. inopinata* with 42.25 %)
182 and dereplication, 35 CMX *Nitrospira* genomes were kept for final analysis. The affiliation of
183 those genomes to CMX clade A and clade B was achieved by alignment of protein sequences
184 of bacterial single copy core genes detected with default 'Bacteria_71' hmm profile using
185 MUSCLE [52] in Anvi'o and subsequent construction of a maximum likelihood phylogenetic
186 tree using FastTree with 1000 bootstrap iterations. The tree was then processed with iTOL for
187 visualization [53]. The genomic repertoire of representative groundwater nitrifier MAGs was
188 inferred by KEGG orthology (KO) previously generated using kofamscan [21] (Supplementary
189 Methods). Relative transcriptional activity of each investigated MAG was extracted from
190 Overholt *et al.* [21], who determined the transcriptomic coverages for each open reading frame

191 from each MAG from previously generated metatranscriptomic libraries of the groundwater
192 [30].

193 Metaproteomic analysis

194 Proteins were extracted from filters using a phenol-chloroform based protocol as previously
195 described [54]. Proteins were subjected to SDS polyacrylamide gel electrophoresis,
196 subsequently in-gel tryptic cleavage and LC-MS/MS analysis was performed as previously
197 described [55] (Supplemental Methods). Translated amino acid sequences from all genes of
198 the MAGs obtained from the metagenomic datasets [21, 50] were used as a reference
199 database for protein identification. Taxonomic and functional annotations of identified peptides
200 were transferred from the metagenomic dataset [21, 50]. Mass spectrometry proteomics data
201 have been deposited into the ProteomeXchange Consortium via the PRIDE [30] partner
202 repository.

203 Statistical analysis

204 All statistical analyses were conducted using R 4.12 (R Core Team, 2020) and packages FSA
205 [57], ComplexHeatmap [58] and vegan [59]. Analysis details are explained in the
206 Supplementary Methods. To analyze communities of CMX, AOB and AOA together, the
207 absolute *amoA* read abundances of OTUs > 2% of all reads for each ammonia oxidizer
208 community were determined as followed: The percentage of each OTU was calculated from
209 the relative abundance obtained by *amoA* amplicon sequencing and was multiplied by the
210 total *amoA* counts from corresponding samples measured with qPCR.

211 # Results

212 ## Nitrification activity and distribution of nitrifiers across the different
213 groundwater wells

214 Nitrification rates in groundwater varied over two orders of magnitude ranging from 0.54 – 136
215 nmol N L^{-1} d^{-1} across the wells (Figure 1C). We observed the highest rates at oxic well H41
216 with 93 ± 24 nmol N L^{-1} d^{-1} and high activity at hypoxic well H52 with 58 ± 4.0 nmol N L^{-1} d^{-1}.
217 Rates were positively correlated with NH_4^+ concentrations in the groundwater (r_s = 0.67, p <
218 0.05), which were on average below 40 µM and significantly higher in the hypoxic wells (Figure
219 1B). NO_3^- concentrations ranged from 1.7 to 38 µM in hypoxic and from 80 to 384 µM in oxic
220 groundwater and showed no correlation with the rates across the transect.

221 CMX *amoA* genes accounted for 16 ± 7.2 to 75 ± 12.3 % of the total groundwater *amoA* genes
222 detected (mean values per well, see Dataset S2). Proportions of CMX *amoA* and AOB *amoA*
223 genes increased substantially to maxima of 95% and 34% at wells with higher NH_4^+
224 concentrations (H41, H51, H52, H53), and gene abundances were positively correlated to
225 NH_4^+ (r_s = 0.48, p < 0.01; r_s = 0.44, p < 0.01), respectively (Figure 1E, Figure 2D). In contrast,
226 AOA dominated the ammonia-oxidizing community by up to 90% in wells with lower NH_4^+
227 concentrations located closer to the main recharge area (H13 and H14). Oxic well H41 and
228 anoxic well H53 harbored the highest absolute *amoA* gene and transcript abundances of CMX
229 and AOB (Figure 1D, Figure S 1A). Nitrification rates were strongly correlated with AOB *amoA*
230 gene abundances (r_s = 0.87, p < 0.001) (Figure 2D), and AOB also had the highest *amoA*
231 transcript/ gene ratios across all wells (Figure S 1B).

232 Across all wells, abundances of CMX *amoA* genes and *Nitrospira nxrB* genes encoding nitrite
233 oxidoreductase were positively correlated to each other (r_s = 0.72, p < 0.001) (Figure S 1D).
234 Assuming one *amoA* and 1.5 *nxrB* copies per genome (see Dataset S4), CMX accounted for
235 18 to 44 % and 48 to 70 % of the *Nitrospira* population at oxic well H41 and hypoxic well H52,
236 respectively (Figure S 1C), which exhibited the highest observed nitrification rates (Figure 1C).

237 Furthermore, CMX and *Nitrosomonadaceae* had the highest relative abundance of peptides
238 at wells H41 and H52 (Figure S 2). Out of the *Nitrosomonadaceae* affiliated peptides, 88.3%
239 were assigned to *Nitrosomonas*.

240 The ammonia oxidizer community composition showed formation of clusters depending on
241 local groundwater chemistry and the location in the transect (Figure 2A). The CMX community
242 was dominated by three OTUs affiliated to clade A and six OTUs affiliated to clade B, of which
243 clade A OTUs were closely related to *Ca.* Nitrospira nitrificans (Figure 2C). These clade A
244 OTUs increased in abundance at wells H41, H51, H52 and H53 and accounted for up to 50%
245 of the CMX community (Figure 2B). CMX clade A affiliated OTUs were also positively
246 correlated to the nitrification rates (r_s = 0.72 – 0.88, p < 0.05), while only two low abundant
247 clade B affiliated OTUs correlated with the rates (Figure 2D). The AOB community was mainly
248 dominated by AOB OTU1 affiliated to *Nitrosomonas ureae*, which was the only AOB phylotype
249 showing a strong correlation with nitrification rates (r_s = 0.88, p < 0.01). The most dominant
250 AOA OTU1 related to *Nitrosoarchaeum koreense* accounted for an average of 70% of all AOA
251 across the groundwater sites (Figure S 3). Clustering based on the correlation with
252 hydrochemical parameters suggested similar ecological preferences of (i) AOA and some
253 clade B affiliated CMX OTUs, and (ii) *Nitrosomonas ureae* affiliated AOB OTU1 and CMX
254 clade A affiliated OTUs (Figure 2D). Among potential nitrite oxidizers, *Nitrospira* sp. were
255 consistently present in all the samples, while 16S rRNA gene sequences affiliated with
256 *Nitrospinaceae*, *Cand*. Nitrotoga, and *Nitrobacter* occurred only occasionally, and relative
257 abundances of these groups were at least one order of magnitude lower than those of
258 *Nitrospira* (Figure S 4).

259 Metabolic capabilities of CMX *Nitrospira* clades and AOB

260 We analyzed nine CMX affiliated metagenome assembled genomes (MAGs) from the Hainich
261 groundwater [21], which were phylogenomically affiliated to two CMX clade A MAGs and
262 seven clade B MAGs (Figure 3A). Both clade A MAGs (H51-bin291-1 and H41-bin216-1) were
263 closely related to *Ca.* Nitrospira nitrificans on the genome level and showed 99% amino acid

264 sequence identity to the AmoA of CMX OTU5 (Figure 2C). AmoA sequences of Clade B
265 representative MAGs showed closest relation to AmoA of CMX OTU2, OTU3, OTU8 and
266 OTU9.

267 Genes encoding essential components for ammonia oxidation, nitrite oxidation and urea
268 degradation were shared in MAGs representing both CMX clades (Figure 3B) and most of
269 these genes were highly transcribed in the groundwater. Metaproteomic analysis revealed the
270 presence of peptides related to AmoA, AmoB, AmoC, Hao and NxrA of *Nitrospira*,
271 demonstrating that nitrification-related proteins of CMX *Nitrospira* populations were formed *in*
272 *situ*. We found different genomic repertoires between CMX clade A and clade B affiliated
273 *Nitrospira* MAGs containing genes for sulfur and alternative energy metabolism such as
274 oxidation of formate and hydrogen, nitrogen transporter types and cellular regulation of
275 reactive oxygen species. CMX clade B MAGs exclusively harbored genes involved in
276 conversion of bicarbonate to carbon dioxide, oxidation of thiosulfate via the *sox* pathway,
277 dissimilatory sulfate reduction, as well as genes encoding two superoxide dismutases (SOD1/
278 SOD2) and a formate transporter (*focA*). Transporters for cellular import of urea as well as for
279 NO_2^-/NO_3^- were present in representative genomes from both CMX clade A and clade B. We
280 found that clade B MAGs encoded Amt-type NH_4^+ transporters, while clade A MAGs employed
281 Rh-type NH_4^+ transporters. MAGs from both clades harbored genes for ATP citrate lyase (*acl*),
282 which were transcriptionally active, indicating an active role in carbon fixation.

283 Compared to the CMX and AOA affiliated MAGs, transcription of *amo* genes was more than
284 100 times higher for AOB affiliated MAG H51-bin202-1 (Figure S 4). The AmoA sequence of
285 this MAG had 93% sequence identity to the AmoA of the dominant, *N. ureae* affiliated AOB-
286 OTU1 obtained from amplicon sequencing in this study (Figure S 5), suggesting that both
287 approaches probably identified the same population of AOB. This MAG employed Rh-type
288 NH_4^+ transporters as did representatives of CMX clade A, while AOA affiliated MAGs had Amt-
289 type NH_4^+ transporters as representatives of CMX clade B (Figure S 4).

290 **Contributions of ammonia-oxidizing groups to ammonia oxidation activity**

291 Short-term incubations in the presence of the nitrite oxidation inhibitor chlorate did not affect
292 the ammonia oxidation rates (74.5 ± 9.4 nmol N L^{-1} d^{-1}) compared to the rates in controls
293 without inhibitor (75.9 ± 3.7 nmol N L^{-1} d^{-1}) (Figure 4A). Chlorate is supposed to inhibit nitrite
294 oxidation of NOB, and both ammonia oxidation and nitrite oxidation by CMX, as nitrite
295 oxidoreductase catalyzes the formation of toxic chlorite from chlorate [7]. In the presence of
296 the ammonia oxidation inhibitor allylthiourea, ammonia oxidation rates were not significantly
297 different from zero. Allylthiourea is supposed to block ammonia oxidation, as it inhibits the
298 ammonia monooxygenases of AOB as well as of CMX but affects those of AOA much less at
299 the 100 µM concentrations used in this study [37, 60].

300 Long-term incubations (15-28 days) were performed to further investigate the potential growth
301 and shifts in community composition of CMX, AOB, AOA and *Nitrospira*-affiliated NOB under
302 the inhibitor treatments, using oxic groundwater from well H41 (condition 1: oxic/ 10 µM NH_4^+;
303 condition 2: oxic/ 50 µM NH_4^+) and hypoxic groundwater from H53 (condition 3: hypoxic/
304 without supplement). Ammonia oxidation was always completely inhibited in the presence of
305 allylthiourea, regardless of groundwater source or initial NH_4^+ concentrations (Figure S 6).
306 NH_4^+ was only oxidized in incubations without inhibitor and in the chlorate treatment, while
307 nitrite oxidation was only measured in incubations without inhibitor. Condition 3 without
308 inhibitor showed complete consumption of NH_4^+ after 13 days, while it took 19 and 25 days
309 under condition 1 and 2, respectively.

310 In incubations without inhibitor, CMX increased about two-fold (6.5 x10^5 and 2.5 x 10^6 *amoA*
311 L^{-1} after 25 and 20 days of incubation) compared to initial groundwater (2.8 x 10^5 and 1.44 x
312 10^6 *amoA* L^{-1}) under condition 1 and 2, respectively, while we did not detect growth of CMX
313 under condition 3 (Figure 4B). CMX *amoA* gene abundance also increased in the allylthiourea
314 treatment under condition 1 but decreased in the chlorate treatments. *Nitrospira*-affiliated NOB
315 targeted using *nxrB* genes had similar growth patterns across condition and treatment types
316 as CMX, and CMX accounted for an estimated 14 to 63 % of the *Nitrospira*-affiliated NOB

population. AOB *amoA* abundance increased by one to two orders of magnitude in all untreated and chlorate treatments of the different conditions, while they decreased in the presence of allylthiourea. AOA *amoA* gene abundance showed only minor variations during all incubation settings. CMX OTU1 affiliated to CMX clade A (Figure 4C) and AOB-OTU1 related to *Nitrosomonas ureae* (Figure 4D) increased in abundance after incubations without inhibitor. These two OTUs were already dominant in the ammonia oxidizer communities of the original groundwater.

324 ## Discussion

325 We present evidence that despite numerical predominance of CMX bacteria, they contribute
326 only a small fraction to ammonia oxidation in the studied oligotrophic groundwater. Instead,
327 ammonia oxidation in this system appeared to be largely driven by AOB. Ranging from 0.54
328 up to 136 nmol N L^{-1} d^{-1}, the measured nitrification rates (ammonia oxidation + nitrite oxidation)
329 matched those from oligotrophic lakes and open marine environments including oxygen
330 minimum zones [61–64], but were lower than in rivers, and estuaries [65, 66] (Table S1). The
331 observed nitrification rates did not reflect the spatial heterogeneity of groundwater NO_3^-
332 concentrations, suggesting that surface-derived inputs mask the effects of *in situ* NO_3
333 formation on groundwater NO_3^- loads in these carbonate-rock aquifers.

334 The oligotrophic conditions in the groundwater appeared to select for ammonia oxidizers with
335 the highest reported NH_3 affinities of their respective group. As the three groups differ in their
336 NH_3 affinity, with 0.049 to 0.083 µM for *Ca.* Nitrospira inopinata [15] (corresponding to 11 to
337 19 µM NH_4^+ at pH 7.3), 0.3 to 4 µM for the *Nitrosomonas oligotropha* lineage [15, 20]
338 (corresponding to 55 to 829 µM NH_4^+ at pH 7.3), and < 0.01 µM for AOA closely related to
339 *Nitrosoarchaeum koreensis* [15] (corresponding to < 2.3 µM NH_4^+ at pH 7.3), fluctuating
340 resource availability at overall limiting conditions may have enabled their coexistence.
341 Previous metagenomics analysis work at this study site identified CMX as key nitrifiers and
342 demonstrated that measured groundwater carbon fixation rates could fully be accounted for
343 by carbon fixation associated to nitrification, assuming nitrification and carbon fixation kinetics
344 of CMX *Nitrospira* [21]. Similarly, in our study, CMX accounted for up to 80% of the ammonia
345 oxidizer community at the groundwater well showing the highest nitrification activity,
346 supporting our original assumption that CMX were the most competitive ammonia-oxidizing
347 group. Our inhibitor-based approach strongly suggested that ammonia oxidation appeared to
348 be largely driven by AOB, even though CMX outnumbered AOB by a factor of 10 in the
349 samples used for rate measurements.

350 Treatment with 1 mM chlorate in nitrification assays was expected to inhibit the nitrite oxidation
351 activity of canonical nitrite oxidizers as well as both steps of nitrification in CMX, since
352 reduction of chlorate to toxic chlorite from the activity of nitrite oxidoreductase would eventually
353 also inhibit ammonia oxidation in the same cell [7]. Ammonia oxidation was not affected in the
354 chlorate treatment, but was completely inhibited in the treatment with allylthiourea, which
355 specifically targets the ammonia monooxygenase and acts as a reversible inhibitor preventing
356 the oxidation of NH_4^+ [60]. At a concentration of below 160 µM, it selectively inhibits the activity
357 of AOB and CMX but not of AOA [37]. Consequently, this inhibitor approach strongly
358 suggested that AOB were primarily responsible for the highest observed ammonia oxidation
359 activity at well H41. A strong bias of our results by potential residual activity of CMX in the
360 presence of chlorate appears unlikely. Although *Nitrospira* is capable of detoxifying hazardous
361 chlorite by chlorite dismutases [67], the chlorate concentrations used here obviously resulted
362 in growth inhibition and cell death, which is in line with previous observations in wetland
363 sediment mesocosms [28]. Similarly, it appears unlikely that our results were caused by
364 excessive growth of AOB, since nitrification rates were linear across these five-days
365 incubations. A negligible contribution of AOA to ammonia oxidation was indicated by no
366 detectable activity in the presence of allylthiourea.

367 We cannot completely rule out that activity by AOB was enhanced in the chlorate treatment
368 due to absence of competition with CMX. This is one of the pitfalls associated with inhibition
369 experiments and could have led to an overestimation of the contribution of AOB in our case.
370 In fact, increased activity and growth of AOB in soil mesocosms where AOA were inhibited
371 was recently demonstrated by Zhao *et al*. [68]. Our transcriptional and metaproteomics-based
372 findings indicate a very active role of AOB in ammonia oxidation, supporting a substantial
373 contribution of this group to ammonia oxidation rates. Ammonium concentrations at well H41,
374 which had the highest nitrification activity, were around 10 µM NH_4^+ *in situ* and 50 µM were
375 used for rate measurements, corresponding to 0.04 or 0.22 µM free NH_3, respectively. The
376 NH_3 affinities and per cell ammonia oxidation rates differ between AOB and CMX [15]. It

377 remains unclear to what extend competition for NH_4^+ would restrict their ammonia oxidation
378 rates under the given ammonia-limited conditions. These conditions presumably affect AOB
379 more than CMX, and if this would result in increased activity of AOB when CMX were
380 suppressed due to inhibition. According to Vilardi *et al*., CMX and AOB can fill independent
381 niches in the nitrifying community, which would reduce potential ammonia oxidation
382 competition between both groups [69]. More detailed knowledge of the exact ammonia
383 oxidation kinetics of the groundwater AOB and CMX representatives is needed to further
384 disentangle the mechanisms of their coexistence.

385 Despite the presumably small contribution of CMX to ammonia oxidation rates, we obtained
386 strong evidence of their active role in overall nitrification. CMX expressed genes and formed
387 peptides involved in both ammonia and nitrite oxidation *in situ*, which was confirmed by growth
388 of CMX in the presence of NH_4^+ in long-term incubations. However, it remains unclear if the
389 major energy source to sustain cell metabolism originated from complete ammonia oxidation
390 or rather from nitrite oxidation. In fact, a recent subsurface study detected transcriptional
391 activity in association with nitrite oxidation but not ammonia oxidation for a CMX-related MAG
392 [70], while CMX *Nitrospira* in water from rapid sand filters did not prefer to oxidize external
393 nitrite alone [7]. As we found NO_2^- transporters in representative groundwater MAGs from both
394 CMX clades, the cells may also be capable of taking up the NO_2^- formed by AOB, supporting
395 their primary role as nitrite oxidizers along with *Nitrosomonas ureae* and *Nitrosospira* sp.
396 affiliated AOB as key ammonia oxidizers.

397 AOB with faster ammonia oxidation rates than CMX [3, 15] did not outcompete CMX for NH_4^+
398 but simply processed a larger fraction of the available NH_4^+, thereby driving the ammonia
399 oxidation rates and providing the resulting NO_2^- to both CMX and canonical NOB. Overall, the
400 genus *Nitrospira* clearly dominated the NOB community, as NOB affiliated with
401 *Nitrospinaceae*, *Cand*. Nitrotoga, and *Nitrobacter* occurred only occasionally and were one or
402 two orders of magnitude less abundant than *Nitrospira*. The question remains unclear, why
403 CMX which accounted for more than 50% of the *Nitrospira* population were apparently favored

404 as nitrite oxidizers over canonical nitrite-oxidizing *Nitrospira* in the groundwater. Physiological
405 studies of *Ca.* Nitrospira inopinata demonstrated a much lower affinity to external NO_2^-
406 compared to canonical *Nitrospira* [15], a trait which is, however, not shared among all CMX
407 *Nitrospira* [71]. A recent microcosm-based study of drinking water biofilter media suggested
408 that CMX outcompete canonical NOB in natural nitrifier communities from nitrogen-limited
409 environments over time [69]. However, co-occurrence of CMX and canonical *Nitrospira*
410 appears to be common across a broad range of terrestrial and aquatic habitats [27], and their
411 respective roles in nitrification might be highly variable.

412 In contrast to our findings, CMX *Nitrospira* were identified as the driver of the two steps of
413 nitrification in groundwater-fed rapid gravity sand filters (RGSF) [7, 72]. Moreover, they made
414 a similar contribution as AOB to ammonia oxidation in wetland soils [28]. At low NH_4^+
415 concentrations of 71 µM and 1.1 µmol g^{-1}, CMX also dominated the ammonia oxidizer
416 communities in the RGSF [7] and in coastal wetland sediments [28] , respectively. However,
417 our results demonstrate that high abundance of CMX in an environment does not necessarily
418 imply that they also control the complete nitrification process. The competitive advantage of
419 CMX has been associated with a biofilm-associated lifestyle such as in RGSF. However,
420 division of labor in nitrification may remain the preferred strategy in the planktonic communities
421 of groundwater environments at low NH_4^+ concentrations.

422 Distribution patterns of ammonia oxidizers across the groundwater wells suggested different
423 niche preferences for AOB and CMX clade A compared to AOA and some clade B affiliated
424 CMX. Both AOA and CMX clade B dominated in groundwater closer to the major recharge
425 area with lower NH_4^+ availability. AOA can be the most abundant ammonia oxidizers in soils
426 [73] and several studies reported CMX clade B in soil habitats [9, 74]. Consequently, transfer
427 from soils to shallow groundwater could result in the observed high fractions of AOA and CMX
428 clade B in the ammonia oxidizer communities of the respective wells [47]. In turn, deeper and
429 more distant groundwater favored both AOB and clade A affiliated CMX under slightly
430 increased availability of NH_4^+ and lower DO concentrations.

431 The observed ecophysiological niche differentiation of CMX clade A and clade B across
432 different groundwater conditions was not reflected by differences in central nitrogen
433 metabolism of representative MAGs [21, 30] and metaproteomic data from the groundwater.
434 Genes encoding enzymes of ammonia and nitrite oxidation were highly transcribed and
435 respective peptides represented both CMX clades. However, differences in the encoded NH_4^+
436 transporters could be linked to different ecological preferences. Rh-type NH_4^+ transporters in
437 CMX clade A convey a better adaptation to higher NH_4^+ concentrations than Amt-type
438 transporters. As Rh-type transporters have higher uptake capacity but lower NH_3 affinity than
439 Amt-type transporters [25] and are also encoded in AOB [75], CMX clade A might have a
440 competitive advantage against clade B in the groundwater when more NH_4^+ is available. Along
441 with NH_4^+ uptake efficiency, also oxygen tolerance appeared to drive the distribution patterns
442 of the two clades, as CMX clade B but not clade A encode two types of superoxide dismutases
443 (SOD1, SOD2) [25, 26]. Finally, given the broader metabolic versatility of clade B including
444 the potential for formate and thiosulfate utilization, CMX clade B representatives might be less
445 strongly tied to energy generation by nitrification compared to the clade A representatives.

446 In conclusion, our findings point to a strong contribution of AOB to ammonia oxidation activity
447 in oligotrophic groundwater, despite numerical predominance of CMX. Given the limitations of
448 differential inhibitor assays, the exact contribution of CMX to ammonia oxidation *in situ*
449 remains unclear. Conditions of low NH_4^+ availability and varying oxygen concentrations
450 appeared to generally support coexistence of CMX, AOB and AOA with representatives
451 closely related to those with the highest NH_4^+ affinities in their respective groups. Our results
452 suggest that high substrate affinity, high growth yields, and a potentially major role in nitrite
453 oxidation enable CMX *Nitrospira* to maintain consistently high populations in the groundwater.
454 We further propose niche differentiation of CMX clade A and B with a more important role in
455 nitrification for clade A and a higher metabolic versatility, including the use of alternative
456 electron donors, for clade B.

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675 ## Data availability

676 Sequence data generated in this study was submitted to the European Nucleotide Sequence
677 Archive with project number PRJEB57321 for *amoA* genes under accessions ERS13665998
678 to ERS13665900, and with project number PRJEB63087 for bacterial 16S rRNA genes under
679 accessions ERS15585018 to ERS15584991.

680 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
681 Consortium via the PRIDE [76] partner repository with the dataset identifier PXD039573.

682 Datasets containing the calculation of the groundwater nitrification rates, gene abundances,
683 hydrochemical measurements and *amoA* OTU sequences will be released upon publication
684 of the manuscript.

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Author contributions

MH and MK designed the study. MK conducted all molecular work, incubation experiments and data analysis. BT and LB performed IRMS measurements and analysis. NC and WO performed metagenomic data analysis. MT, NJ and MvB conducted metaproteomic data analysis. MK and MH wrote the manuscript with contributions of all authors.

Declaration of competing interest

The authors declare that there was no financial or personal conflict of interest in the preparation of this study.

Figures

Figure 1

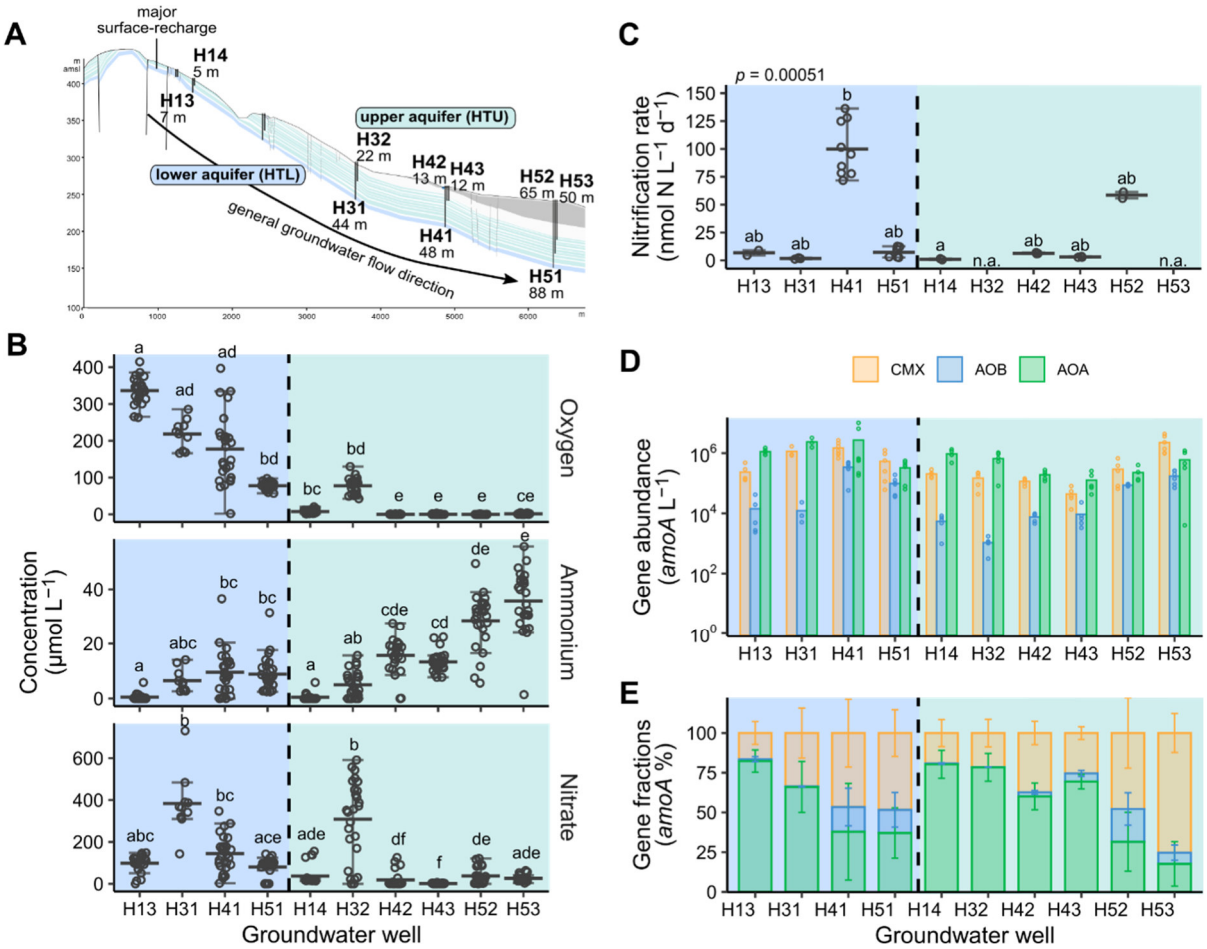


Figure 1: **A)** Schematic cross-section of the Hainich groundwater monitoring transect showing two superimposed aquifer assemblages accessible by 10 wells with various depths. **B)** Overview of the concentrations of dissolved oxygen, ammonium, and nitrate from oxic (blue) and hypoxic/ anoxic (green) groundwater wells (n = 24 per well). Single dots represent one measurement, the crossbar indicates the mean of all values, and the error bars show the variation from the mean. Outliers are beyond the error bars. Lowercase letter code indicates significant differences based on Dunn's multiple comparison test ($p < 0.05$). **C)** Nitrification rates assessed by ^{15}N based labeling of groundwater from five days incubation close to in situ conditions (n ≥ 3 per well; n.d. = not detected; n.a. = not available). **D)** Gene abundances of *amoA* per L and **E)** fractions of *amoA* genes in groundwater from different groups of ammonia-oxidizing prokaryotes in 10 groundwater wells (n ≥ 3).

Figure 2

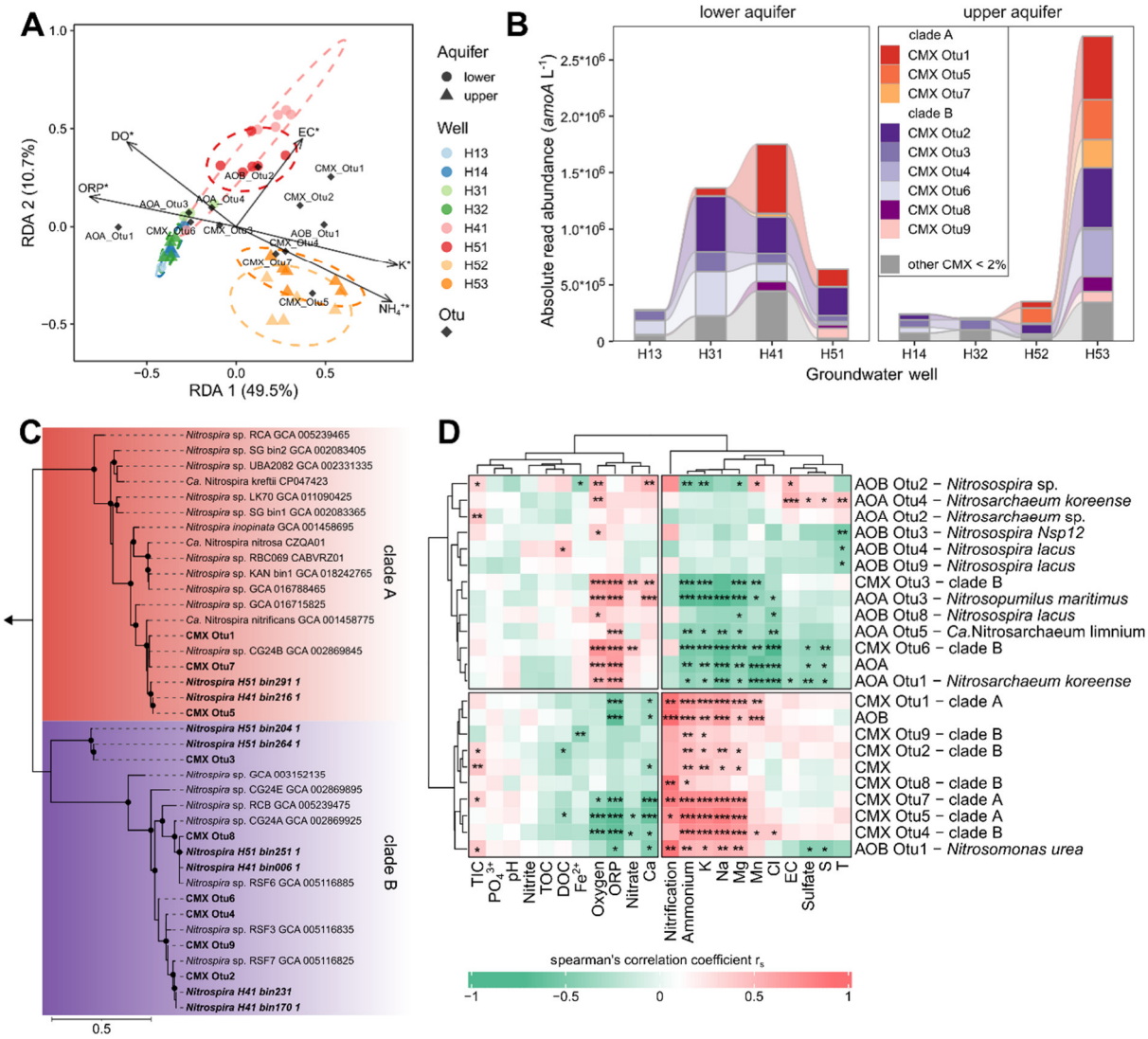
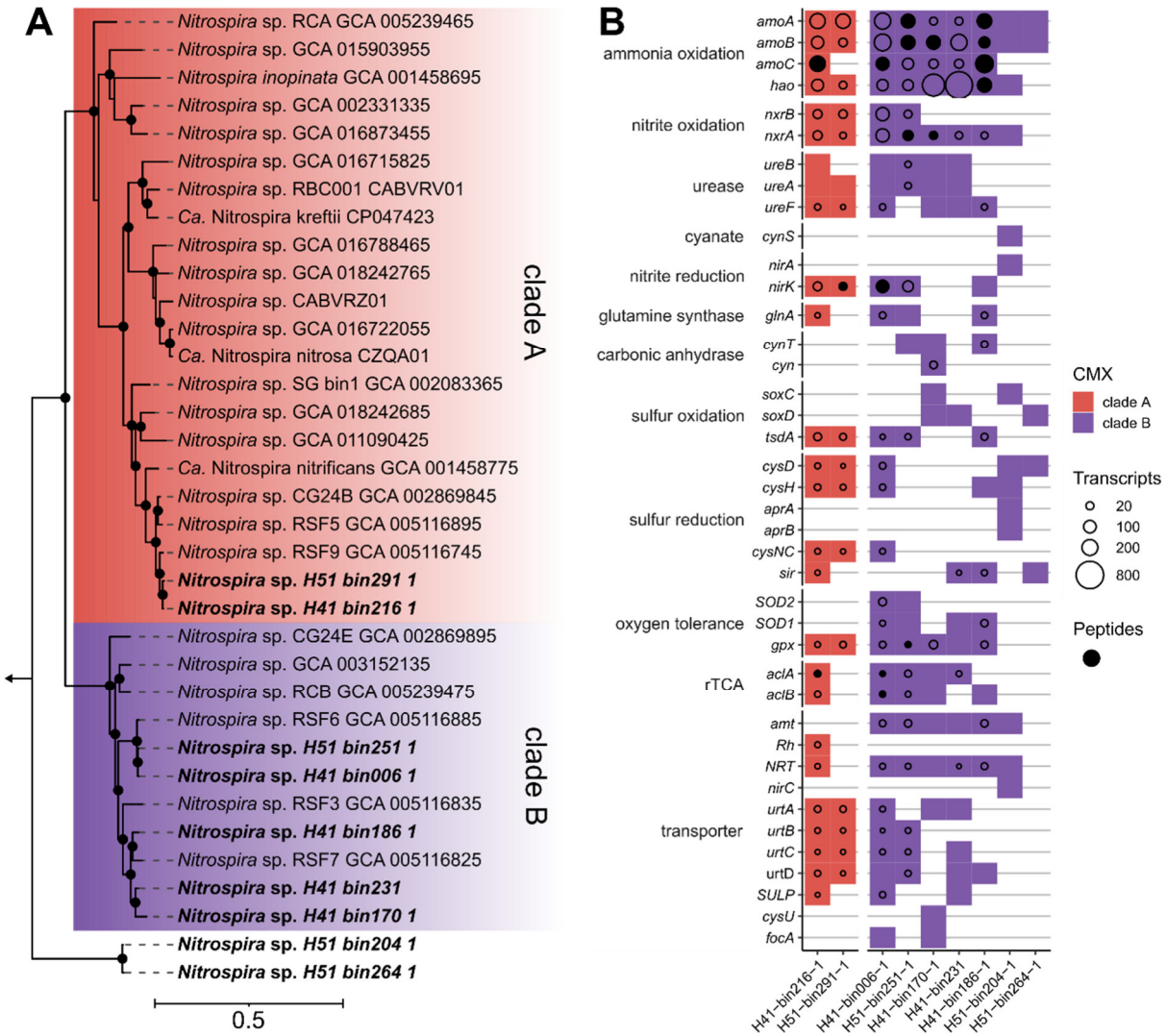


Figure 2: **A)** Redundancy analysis (RDA) demonstrates association between distribution of ammonia-oxidizing community (relative *amoA* read abundances of CMX, AOB and AOA OTUs which account for < 2% sequence reads of each community were normalized to *amoA* gene abundances to generate absolute *amoA* read abundances) and significant hydrochemical parameters fitting ($p < 0.05$) with nitrifier community distribution across eight groundwater wells (n ≥ 3). Dotted ellipses show the confidence of clusters assuming a multivariant normal distribution. **B)** Community composition of CMX *Nitrospira* showing dominant OTUs (< 2% of sequence reads) affiliated to clade A and clade B across eight groundwater wells (n ≥ 3 per well). Each bar displays the mean absolute *amoA* read abundance of OTUs. **C)** Maximum likelihood phylogenetic tree of deduced AmoA amino acid sequences of CMX *Nitrospira* depicting evolutionary relationship between reference genomes, and sequences of OTUs and MAGs from Hainich groundwater. The tree was constructed using the JTT substitution model with gamma distribution and 1000 bootstrap iterations. Bootstrap support values greater than 75% are denoted with black dots. An AmoA sequence from *Nitrosomonas oligotropha* served as outgroup indicated by the arrow. **D)** Pairwise correlations between total *amoA* gene abundances of CMX, AOB and AOA, absolute *amoA* read abundances of OTUs and groundwater hydrochemical parameters (TIC = total inorganic carbon, TOC = total organic carbon, DOC = dissolved organic carbon, ORP = redox potential, EC = electrical conductivity) using Spearman's rank correlation. Hierarchical clustering is based on Euclidean distance. Color code of correlation coefficient r_s displays strength of association using following significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

748 **Figure 3**

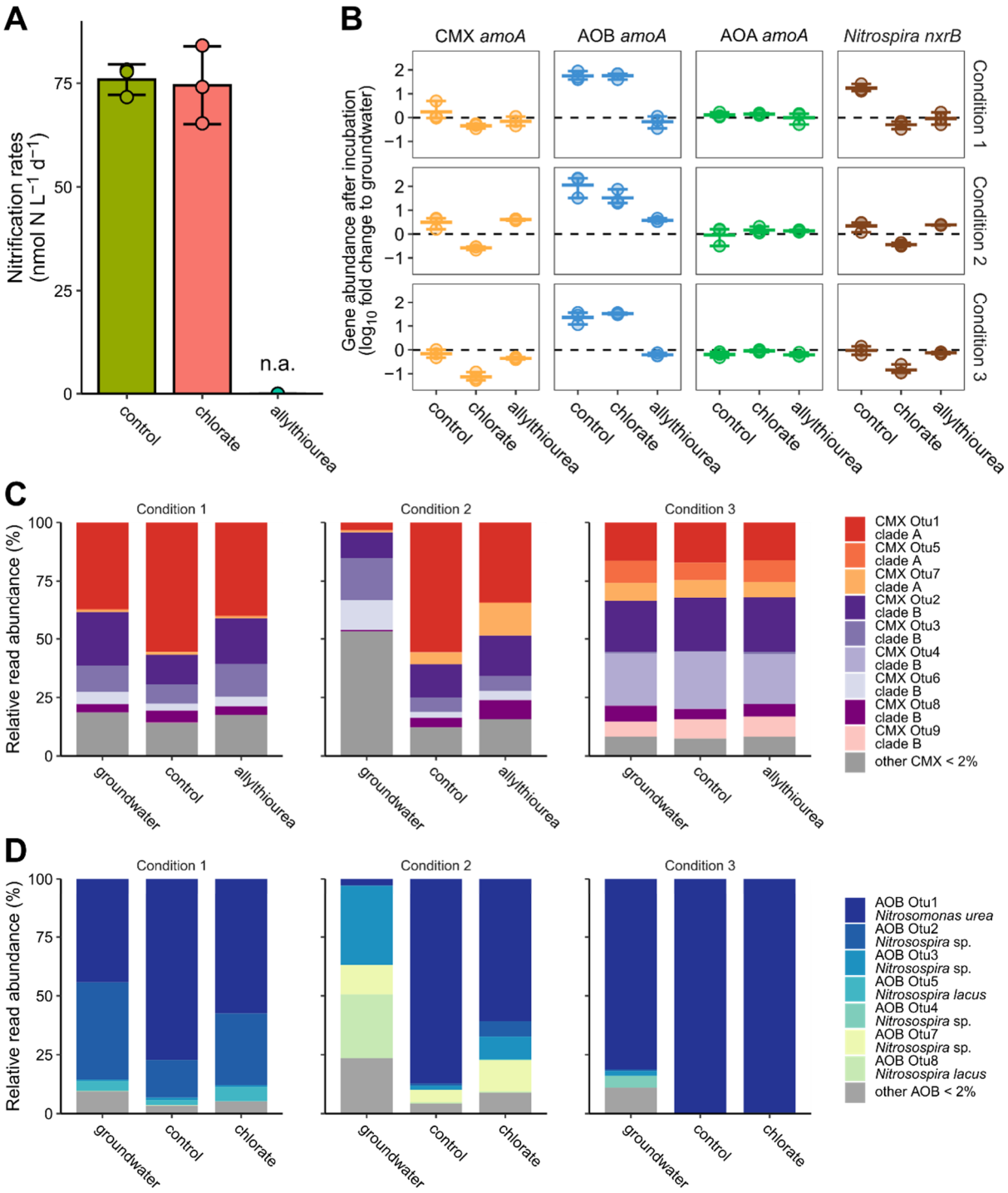


749

750 Figure 3: **A)** Maximum likelihood tree of bacterial core protein sequences of CMX *Nitrospira* from nine
751 MAGs originating from Hainich groundwater depicting the evolutionary relationship to reference
752 genomes. The tree was constructed using the JTT substitution model with gamma distribution and 1000
753 bootstrap iterations. Bootstrap support values greater than 75% are shown as black dots. AmoA
754 sequences from *Nitrosomonas oligotropha* and *Nitrosomonas europaea* served as an outgroup
755 indicated by the arrow. **B)** Metabolic capacities of nine groundwater CMX *Nitrospira* MAGs inferring
756 genomic potential for nitrogen and sulfur energy metabolism, and corresponding transporter types as
757 well as protective systems against radical oxygen species, and genes involved in carbon fixation (rTCA
758 = reductive tricarboxylic acid cycle). Sizes of circles display the transcript coverage of each gene
759 mapped from groundwater metatranscriptome. If more than one gene was present, maximum
760 transcriptional coverage is shown. Filled circles indicate peptides from metaproteomic data matching
761 for displayed genes. rTCA = reverse tricarboxylic acid cycle.

762

763 **Figure 4**



764

765 Figure 4: Groundwater incubations from wells H41 and H53 supplemented with different NH_4^+
766 concentrations and nitrification inhibitors across 5 days (short-term) and 25 to 35 days (long-term). **A)**
767 Nitrification rates from short-term incubation of oxic groundwater supplemented with 50 µM NH_4^+ and
768 selective inhibition with chlorate and allylthiourea (n.d. = not detected). Dots depict single rates of each
769 replicate. Crossbar shows the average of three replicates and error bars show the variation between
770 rates. **B)** Fold-change of *amoA* genes from ammonia oxidizers and *nxrB* genes from *Nitrospira*-like
771 nitrite oxidizers after long-term incubation of groundwater. Single points represent one replicate and
772 crossbar shows the average. Error bars display variance between conditions (n = 3). Condition 1: oxic
773 groundwater supplemented with 10 µM NH_4^+. Condition 2: oxic groundwater supplemented with 50 µM
774 NH_4^+. Condition 3: hypoxic groundwater without additional NH_4^+. **C)** Composition of CMX *Nitrospira*

clade A and clade B affiliated OTUs of groundwater and after long-term incubation (n = 3). Bars show the mean relative *amoA* read abundance of OTUs representing > 2%. **D)** Composition of bacterial ammonia-oxidizing community and taxonomic affiliation of OTUs.