# A robust nitrifying community in a bioreactor at 50°C opens up the path for thermophilic nitrogen removal

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**Abstract**

The increasing production of nitrogen-containing fertilizers is crucial to meet the global food demand, yet, high losses of reactive nitrogen associated with the food production/consumption chain progressively deteriorate the natural environment. Currently, mesophilic nitrogen-removing microbes eliminate nitrogen from wastewaters. Although thermophilic nitrifiers have been separately enriched from natural environments, no bioreactors are described that enchain these processes for the treatment of nitrogen in hot wastewaters. Samples from composting facilities were used as inoculum for the batch-wise enrichment of thermophilic nitrifiers (350 days). Subsequently, the enrichments were transferred to a bioreactor to obtain a stable, high-rate nitrifying process (560 days). The community contained up to 17% ammoni-oxidizing archaea (AOA) closely related to "*Candidatus* Nitrososphaera gargensis", and 25% nitrite-oxidizing bacteria (NOB) related to *Nitrospira calida*. Incorporation of $^{13}$C-derived bicarbonate into the respective characteristic membrane lipids during nitrification supported their activity as autotrophs. Specific activities up to 198±10 and 894±81 mg N $g^{-1}$ VSS $day^{-1}$ for AOA and NOB were measured, where NOB were 33% more sensitive to free ammonia. The NOB were extremely sensitive to free nitrous acid, while the AOA could only be inhibited by high nitrite concentrations, independent of the free nitrous acid concentration. The observed difference in product/substrate inhibition could facilitate the development of NOB inhibition strategies to achieve more cost-effective processes such as deammonification. This study describes the enrichment of autotrophic thermophilic nitrifiers from a nutrient-rich environment and the successful operation of a thermophilic nitrifying bioreactor for the first time, facilitating opportunities for thermophilic nitrogen removal biotechnology.

## Introduction

The increased combustion of fossil fuels and extensive production of nitrogen-containing fertilizers and industrial products lead to accumulation of reactive nitrogen in many natural ecosystems, causing a worldwide environmental problem (Galloway *et al.* 2014). Just as biodiversity loss and climate change, the aforementioned anthropogenic distortion of the nitrogen cycle has by far exceeded the safety bounderies of our planet (Steffen *et al.* 2015). Nitrification, the two-step microbe-mediated aerobic oxidation of ammonia to nitrate, plays a key role in the transformation of reactive nitrogen necessary to restore the imbalanced nitrogen cycle. Ammonia-oxidizing bacteria (AOB) and archaea (AOA) catalyze the first step, i.e., the oxidation of ammonia ($NH_3$) to nitrite ($NO_2^-$), while the successive oxidation to nitrate ($NO_3^-$), is carried out by nitrite-oxidizing bacteria (NOB).

Most AOB grow optimally at temperatures between 25 and 30°C (Ward *et al.* 2011), with a maximum reported growing temperature of 55°C (Lebedeva *et al.* 2005). The recent discovery of "*Candidatus* Nitrosocaldus yellowstonii", an archaeon that grows up to 74°C however broadened the phylogenetic spectrum of ammonia oxidizers active at high temperatures (de la Torre *et al.* 2008). Two other moderately thermophilic (46°C) AOA "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler *et al.* 2008) and "*Candidatus* Nitrosotenuis uzonensis" (Lebedeva *et al.* 2013) have been enriched from Russian hot springs. Thermophilic ammonia oxidation is fuelling hydrothermal and geothermal life. Many archaeal ammonia monooxygenase subunit A (*amoA*) genes have been detected in high-temperature habitats such as deep-sea hydrothermal vents (Baker *et al.* 2012, Wang *et al.* 2009), subsurface thermal springs (Spear *et al.* 2007, Weidler *et al.* 2008) and terrestrial hot springs (Dodsworth *et al.* 2011, Reigstad *et al.* 2008). In addition to these oligotrophic ecosystems, the *amoA* gene was also measured in nutrient-rich high-temperature engineered environments such as petroleum reservoirs (Li *et al.* 2011) and composting facilities (Zeng *et*

78 *al.* 2011). Although many archaeal *amoA* genes were detected in thermophilic environments,
79 only three enrichments were described so far ("*Candidatus* Nitrosocaldus. yellowstonii",
80 "*Candidatus* Nitrososphaera gargensis" and "*Candidatus* Nitrosotenius uzonensis ").

81 Regarding thermophilic nitrite oxidation, it appears that *Nitrospira* spp. are the most dominant
82 $NO_2^-$ oxidizers up to 60°C. *Nitrospira calida* was isolated from a microbial mat of a terrestrial
83 geothermal spring and maximally oxidizes $NO_2^-$ at 46-52°C (Lebedeva *et al.* 2011). Thus far,
84 other detected/enriched NOB from geothermal springs are all closely related with *Nitrospira*
85 *calida* (Edwards *et al.* 2013, Marks *et al.* 2012).

86 Thermophilic microorganisms played a crucial role during the evolution of life on our planet
87 (Nisbet and Sleep 2001). Despite of the partnership between AOA and *Nitrospira* spp. that
88 potentially pioneered in ancestral nitrification (Vlaeminck *et al.* 2011), until now,
89 thermophilic nitrifiers were always separately enriched/studied in batch cultures. Enchained
90 ammonia and nitrite oxidation under thermophilic conditions has not yet been described,
91 neither in long-term batch flask enrichments nor in bioreactors. Besides for the recently
92 described *Nitrolancea hollandica* (Sorokin *et al.* 2014), all reported substrate/product
93 inhibitions levels for the described thermophilic nitrogen–converting organisms are relatively
94 low (Hatzenpichler *et al.* 2008, Lebedeva *et al.* 2011), making them rather unsuitable for
95 robust biotechnological applications. Lopez-Vazquez *et al.* (2014) recently reported nitrifying
96 activity up to 50°C in mesophilic sludge from an industrial wastewater treatment plant after
97 temperature shocking of mesophilic biomass (34°C) in short term batch activity assays.
98 However, the observations by Courtens *et al.* (2014a) pointed out that prolonged exposure (48
99 hours) of mesophilic biomass to a temperature shock can lead to a complete loss of nitrifying
100 activity. To our knowledge, there is no description of nitrification at 50°C or above for an
101 extended period of time. Long-term thermophilic aerobic bioreactor studies focused mainly
102 on the oxidation of organics. The major nitrogen removal mechanisms in those systems were

assumed to be ammonia stripping (65 ± 14%) and nitrogen assimilation into biomass (14 ± 4) (Abeynayaka and Visvanathan 2011a, Abeynayaka and Visvanathan 2011b, Kurian *et al.* 2005, Yi *et al.* 2003). As neither nitrite nor nitrate was ever measured in these bioreactors at 45-60°C, there is no evidence that nitrification took place in these thermophilic systems. Until now, only a few bioreactor studies focused on the long-term establishment of enchained thermophilic ammonia and nitrite oxidation, yet, reaching no more than 42°C (Courtens *et al.* 2014a, Shore *et al.* 2012).

This study describes the enrichment of autotrophic thermophilic nitrifiers from compost and the successful operation of a thermophilic nitrifying bioreactor with high biotechnological potential. We demonstrate that autotrophic AOA and NOB serve as key players in the microbial community of the thermophilic nitrifying bioreactor. We also provide a phylogenetic, physiological and morphological characterization of this unique nitrifying consortium.

## Materials and methods

### *Inoculum and batch enrichments*

Different aerobic compost facilities were sampled during the thermophilic stage (50-70°C): digested organic waste (a), green waste (b), cow manure (c) and a mix of rabbit manure/green waste (d). A 'compost extract' was prepared by shaking 20 g of compost in 200 mL water with glass beads (12 h). The extract was used as inoculum (25 vol%) for enrichment incubations (50°C) in a buffered medium (pH 7) with final concentrations of 0.929 g $KH_2PO_4$ $L^{-1}$, 1.622 g $K_2HPO_4$ $L^{-1}$ and 0.5 g $NaHCO_3$ $L^{-1}$ with $(NH_4)_2SO_4$ or $NaNO_2$ as the only substrate (20 mg N $L^{-1}$). All incubations were provided with two different packing materials, Kaldness K1 and polyurethane foam, to allow for both floccular as well as biofilm growth.

### *Reactor set-up and operation*

The compost enrichments showing both $NH_3$ and $NO_2^-$ oxidation (b, d) were transferred to a bioreactor. The reactor vessel (2 L, diameter 12 cm) was jacketed, allowing temperature control at 50°C with a circulating thermostatic water bath. The reactor was operated in a sequencing batch feeding/withdrawal mode. The 3-h cycle consisted of a 150-min aerobic reaction period, a 10-min feeding period at the beginning of the cycle, a 15-min settling period, a 5-min decanting period and a 10-min idle period. The bioreactor was fed with a synthetic medium consisting of $(NH_4)_2SO_4$ (10-140 mg N $L^{-1}$), $NaNO_2$ (0-50 mg N $L^{-1}$), 9 g $NaHCO_3$ $g^{-1}$ N, $KH_2PO4$ (10 mg P $L^{-1}$), NaCl (1.2 g $L^{-1}$) and 0.1 mL $L^{-1}$ trace element solution (Kuai and Verstraete 1998) dissolved in tap water. A flow rate of 3.4 ± 0.2 L $d^{-1}$ resulted in a hydraulic retention time of 14 ± 0.7 h. Any transient $NH_4^+$/$NO_2^-$ build-up was immediately corrected by adjusting the nitrogen loading, preventing accumulation of free ammonia (FA) or free nitrous acid (FNA). The reactor pH was controlled between pH 6.8 and 7.2 by a dosage of 0.1 M NaOH/HCl. The dissolved oxygen was controlled at 3.6 ± 0.2 mg $L^-$

[1] with air pumps providing aeration through a diffuser stone at a superficial air flow rate of 1.33 $m^3$ $m^{-2}$ $h^{-1}$.

***Physiological characterization***

Physiological characterization along with inhibition tests were performed in ex-situ batch activity measurements in 96-well plates with a working volume of 250 µL. Plates were incubated in a MB100-4A Thermoshaker (Hangzhou Allsheng Instruments, Hangzhou, China) at 50°C and 600 rpm, containing a buffer solution with a final concentration of 500 mg P $L^{-1}$ ($KH_2PO_4$/$K_2HPO4$), 500 mg $NaHCO_3$ $L^{-1}$, 0.1 mL $L^{-1}$ trace element solution (Kuai and Verstraete 1998) and $(NH_4)_2SO_4$ or $NaNO_2$.

Operational parameters in the batch tests varied according to the investigated parameter. pH, temperature and substrate concentrations were measured in all tests. From these, FA/FNA concentrations were calculated based on their chemical equilibrium (Anthonisen *et al.* 1976). The effects of the different parameters can only be separated from each other by a combination of different tests as presented in **Table S1** for ammonia oxidation. A similar strategy was applied for separation of nitrite and FNA effects on nitrite oxidation (**Table S2**). All treatments were performed in sextuple, and liquid samples (2 µL) were taken over time for $NH_4^+$ and $NO_2^-$ analysis. Protein measurements enabled the calculation of specific rates that were converted to volatile suspended solids (VSS) based on the average protein content of the thermophilic sludge (32.7% protein $VSS^{-1}$).

***High-throughput DNA sequencing, phylogenetic analysis and qPCR***

Biomass samples of the reactor were collected over time, and total DNA was extracted as described previously (Courtens *et al.* 2014b). Prokaryotic biodiversity was analyzed using pair-end high-throughput sequencing (MiSeq Illumina platform) of the regions V5-V6 of the 16S rRNA gene, using the primers 807F and 1050R previously described (Bohorquez *et al.*

2012). Libraries for barcoding sequencing were constructed as previously described (Camarinha-Silva *et al.* 2014). The sequences were analyzed, obtaining 189358 total reads of 240 nucleotides in length. After a quality filter, 153611 total operational taxonomic units (OTUs) were obtained and clustered into 155 unique taxa (Camarinha-Silva *et al.* 2014). Forward and reverse reads were aligned manually, allowing zero mismatch (**Dataset S1**). Sequencing depth was rarefied to the minimum, obtaining 18191 OTUs per sample. The 163 unique taxa were taxonomically annotated manually (**Dataset S1**). The vegan and phyloseq packages in R were used to plot the rarefaction curves and normalize to the minimum sequencing depth respectively. Phylogenetic analyses were performed with MEGA5 (Tamura *et al.* 2011) using the neighbor-joining method with Jukes-Cantor correction and pairwise deletion of gaps/missing data. In total 1000 bootstrap replications were performed to test for branch robustness. The heat map was generated using gplots and RColorBrewer packages. The SYBR Green assay (Power SyBr Green, Applied Biosystems) was used to quantify the 16S rRNA of *Nitrospira* spp. (Dionisi *et al.* 2002) and the functional archaeal amoA gene (Tourna *et al.* 2008).

***Electron microscopy***

For electron microscopy, biofilm material from three different sampling points in the bioreactor was fixated and embedded in SPURR as described by Spieck and Lipski (2011). The ultrathin sections were observed using a transmission electron microscope (model JEM 100C or LEO-906E, Zeiss, Jena, Germany).

***Stable isotope probing: membrane lipids***

Reactor biomass was incubated (50°C, 100 rpm) in 120 mL gas-tight serum flasks containing 20 mL phosphate buffer (pH 7) with final concentrations of 750 mg P $L^{-1}$ ($KH_2PO4$/ $K_2HPO4$), 1 g $NaH^{13}CO_3$ $L^{-1}$ and $NH_4^+$ or $NO_2^-$ as the sole nitrogen source. Liquid samples (2

189 μL) were taken over time for $NH_4^+$ and $NO_2^-$ analysis. pH was adjusted through the addition
190 of HCl or $NaH^{13}CO_3$. Biomass from three parallel incubations with $NH_4^+$ (harvested at day 0,
191 49 and 85) served for alkyl iodides analysis, while biomass from five parallel incubations
192 with $NO_2^-$ (harvested at day 0, 3, 7, 14 and 21) served for PLFA analysis. The sampling points
193 were determined based on the relative abundance of the AOA/NOB, the oxidation rates and
194 the sensitivity of the respective biomarker analysis.

195 *Alkyl iodides analysis*

196 Biomass was subjected to acid hydrolysis by refluxing for 3 h with 5% HCl in MeOH. The
197 resulting extract was separated using $Al_2O_3$ chromatography. Hexane:DCM (9:1) and
198 DCM:methanol (1:1) as eluents, yielding an apolar and polar fraction. An aliquot of the polar
199 fraction was analyzed for tetraether lipids using HPLC/MS (Schouten *et al.* 2007). The
200 remaining polar fractions were subjected to chemical treatment to release the biphytanyl
201 chains from the tetraether lipids (Lengger *et al.* 2014). The stable carbon isotopic composition
202 of the released biphytanes was analyzed in replicate using an Agilent 6800 GC coupled to a
203 Thermo Fisher Delta V isotope ratio monitoring mass spectrometer (Thermo Fisher Scientific,
204 Waltham, MA, USA) (Lengger *et al.* 2014).

205 *Phospholipid fatty acid analysis*

206 Extraction and derivatization of PLFAs for compound specific $^{13}C$ analysis was adapted from
207 Huygens *et al.* (2011). Identification of 11-methyl C16:0 was based on the retention time and
208 comparison with published mass spectra (Lipski *et al.* 2001) using the mass fragments m/z
209 185 and m/z 213 resulting from cleavage of the molecule at both sides of the methyl-branch,
210 as these are diagnostic fragments of 11-methyl-branched FAME. Isotopic enrichment was
211 assessed using the m/z 74/(74 + 76) ratio of the methyl acetate ion fragment.

212 ***Chemical analyses***

213 $NH_4^+$ (Nessler method) and volatile suspended solids (VSS) were measured according to
214 standard methods. $NO_2^-$ and $NO_3^-$ were determined on a 930 Compact Ion Chromatograph
215 (Metrohm, Herisau, Switzerland) equipped with a conductivity detector. Dissolved oxygen
216 (DO) and pH levels were measured with an Oxymax COS22D probe (Endress Hauser,
217 Reinach, Switzerland) and a Dulcotest pH-electrode PHEP 112 SE (Prominent, Heidelberg,
218 Germany), respectively. In the batch activity tests, $NH_4^+$ and $NO_2^-$ concentrations were
219 determined spectrophotometrically with the Berthelot and Montgomery reaction.
220 Measurements were obtained using a Tecan infinite plate reader (Tecan, Männedorf,
221 Switzerland), and biomass was quantified through protein concentrations. To determine the
222 protein concentration, the method developed by Lowry was used with bovine serum albumin
223 (BSA) as the standard.

## Results

### *Thermophilic batch enrichments*

Samples from four composting facilities served as inocula for the batch-wise enrichment of thermophilic (50°C) nitrifying communities. The different origin of the organic fractions and different compost process parameters (temperature, pH) resulted in different nitrogen compound distributions in the four compost solutions. The green waste (a) and rabbit manure/green waste mixture (b) exclusively contained oxidized forms of nitrogen ($NO_2^-$/$NO_3^-$), while the digested organic waste (c) and cow manure (d) only contained $NH_4^+$ (**Table S3**). This distinction was reflected in the observed thermophilic nitrifying activity. First $NH_3$ and $NO_2^-$ oxidation was observed after approximately 100 days of incubation. Samples (a) and (b) showed both $NH_4^+$ and $NO_2^-$ oxidation, while samples (c) and (d) only showed $NO_2^-$ oxidation. After one year of incubation and several dilution steps, two highly active nitrite oxidizing and two enchained ammonia and nitrite oxidizing enrichment communities were obtained (**Fig. S1**).

### *Bioreactor performance*

The enrichments showing complete nitrification were pooled and served as inoculum for the bioreactor at 50°C. Initial volumetric nitrification rates were low (4.7±2.6 mg N $L^{-1}$ $d^{-1}$). However, after two months of operation, a clear exponential increase in nitrifying activity was observed in the reactor reaching volumetric $NH_3$ and $NO_2^-$ oxidation rates of 126±7 and 189±17 mg N $L^{-1}$ $d^{-1}$, respectively (**Fig. 1**). After this first stage, due to a technical failure, the community was challenged by a temperature drop to 30°C and a subsequent shock at pH 11 (days 235-238), leading to an initial loss of ammonia oxidation activity. However, the reactor re-stabilized successfully, reaching nitrification rates higher than 200 mg $NH_4^+$-N $L^{-1}$ $d^{-1}$ (**Fig. 1**). Practically all the removed $NH_4^+$-N was recovered as $NO_3^-$-N (93±4%), confirming that

nitrification was the main process involved. The biomass predominantly appeared as an orange to brownish biofilm on the packing material and wall of the reactor vessel.

***Phylogeny and morphology***

The thermophilic nitrifying microbial community was analyzed once in the first stage (day 150, data not shown) and once during the re-stabilization period of the reactor (days 245-387). Illumina sequencing identified one unique sequence (OTU7) of archaea closely related to the AOA "*Candidatus* Nitrososphaera gargensis" Ga9.2 (99% similarity) (**Fig. 2**), while no known AOB could be detected. For nitrite oxidation, several different sequences closely related to *Nitrospira* spp. were identified. OTU1, 99% similar to *Nitrospira calida* Ns10 (**Fig. 3**), was the most abundant *Nitrospira* sequence in the *Nitrospira*-community (98±2%) and the only *Nitrospira*-related OTU that strongly increased in abundance over time (**Fig. S2**). Both Illumina sequencing as qPCR analyses showed a considerable increase in abundance of *Nitrospira*-related NOB and archaeal ammonia oxidizers over 6 months of operation (**Fig S3**). The higher relative abundance of NOB (±25% vs. ±10%) in this community might have been a result from the influent feeding strategy in which, besides ammonium, nitrite was provided over most of the experiment to prevent limitation in NOB growth in case ammonia oxidation would attenuate (**Fig. 1**). Beside the core nitrifiers (OTU1 and OTU7), the most abundant OTU's (>1%) at the end of the experiment appeared to be, among others, OTU2 and OTU3 (Meiothermus), OTU4 (*Thermomonas*), OTU5 and OTU8 (*Armatimonadetes*), OTU9 (*Ignavibacterium)* and OTU11 (*Proteobacterium*) (**Fig. S4**).

The presence of the described nitrifiers in the biofilm of the thermophilic reactor was morphologically confirmed through transmission electron microscopy (TEM) (**Table S4**). Cells of *Nitrospira* spp. were characterized by a spiral-shaped morphology with a pleomorphic cell appearance, a wide periplasmic space and a granular cell interior (Ehrich *et*

*al.* 1995)(**Table S4**). Small, very electron-dense, spherical cells with thick cell wall with visible fimbriae and pili were observed, which were similar to the "*Candidatus* Nitrososphaera gargensis" AOA characterised by Hatzenpitcher et al. (2008) (**Table S4**). As the putative AOA cells were observed closely to the *Nitrospira* cells, this suggest the role of the former cells as ammonia oxidizers. .

***Carbon incorporation***

The autotrophic nature of the AOA and NOB during nitrification was investigated by incorporation of $^{13}$C-derived bicarbonate into the characteristic membrane lipids during two sets of incubations, one with $NH_4^+$ and another with $NO_2^-$. Isotopic analysis of the biphytane moieties of the characteristic archaeal membrane lipids, glycerol dibiphytanyl glycerol tetraether lipids (GDGTs), was performed for AOA. The GDGTs were dominated by crenarchaeol, in agreement with culture studies of "*Candidatus* Nitrososphaera gargensis" (Pitcher *et al.* 2010). The two biphytanes released showed considerable enrichment in $^{13}$C compared to the start of the incubation, pointing at AOA autotrophy (**Fig. 4)**. The activity of NOB was determined by assessing the incorporation of $^{13}$C-labeled bicarbonate into 11-methyl C16:0, a specific biomarker for moderately thermophilic *Nitrospira* (Lipski *et al.* 2001, Spieck and Lipski 2011). The isotopic label was incorporated in the 11-methyl C16:0 phospholipid fatty acid biomarker after a lag-time of 3 days at the rate of 0.3% day$^{-1}$ during the 21 days of incubation. Interestingly, both for AOA and NOB, the $^{13}$C enrichment (%) appeared to be linear with the total amount of nitrogen oxidized (**Fig. 4**), demonstrating that the autotrophic carbon assimilation by AOA and NOB occurred concurrently with the $NH_3$ and $NO_2^-$ oxidation. Furthermore, the partnership between "*Candidatus* Nitrososphaera gargensis" and *Nitrospira calida* was confirmed, as a 26% $^{13}$C enrichment was measured for the *Nitrospira* biomarker at the end of the incubation fed with $NH_4^+$.

296

297 ***Physiological characterization***

298 The thermophilic biomass showed specific nitrifying rates up to 198±10 and 894±81 mg N $g^{-1}$
299 VSS $day^{-1}$, for $NH_3$ and $NO_2^-$ oxidation, respectively. Taken into account an average relative
300 abundance of 10% AOA and 25% NOB and the simplified assumption that total protein was
301 equally distributed among all organisms in the culture, these rates result in a specific AOA
302 and NOB rate of 18±1 and 33±3 µg N $mg^{-1}$ protein $h^{-1}$, respectively. With respect to the
303 development of biotechnological applications and effective process control strategies, it is
304 important to distinguish the inhibitory effects of $NH_4^+$ from those of free ammonia (FA) and
305 $NO_2^-$ from those of free nitrous acid (FNA). The thermophilic $NH_3$ and $NO_2^-$ oxidizers were
306 both sensitive to FA, while insusceptible to $NH_4^+$. Ammonia oxidation was not inhibited up to
307 300 mg $NH_4^+$-N $L^{-1}$ for the batch activity series with low FA, while it was inhibited for the
308 series tested at a higher FA, resulting in an $IC_{50}$ of 7.5 mg $NH_3$-N $L^{-1}$ (**Fig. 5A**). Interestingly,
309 no complete inhibition of ammonia oxidation was observed up to 80 mg $NH_3$-N $L^{-1}$ (**Fig. S5**).
310 A stable FA ammonia inhibition of 64±5% was measured from 8.5 mg $NH_3$-N $L^{-1}$ onwards
311 (**Fig. S5**). Nitrite oxidation was slightly more sensitive for FA with an $IC_{50}$ of 5.0 mg $NH_3$-N
312 $L^{-1}$ (**Fig. 5B**). Regarding $NO_2^-$/FNA inhibition, ammonia oxidizers were clearly inhibited by
313 $NO_2^-$ and not by FNA. Both the series with high and low FNA gave the same inhibition
314 response with increasing $NO_2^-$ concentrations (**Fig. 5C**). Sensitivity was, however, very low,
315 characterized with an $IC_{50}$ of 2117 mg $NO_2^-$-N $L^{-1}$. In contrast, the NOB were extremely
316 sensitive to FNA and not to $NO_2^-$ with an $IC_{50}$ of 0.0010 mg $HNO_2$-N $L^{-1}$ (**Fig. 5D**). Lowering
317 FNA while applying the same $NO_2^-$ concentrations namely eliminated the inhibitory effect.
318 Nitrate inhibition of nitrite oxidation was also observed ($IC_{50}$ 360 mg $NO_3^-$-N $L^{-1}$) (**Fig. S6**).

319 Thermophilic $NH_3$ oxidation showed a pH optimum at pH 7, maintaining >70% of its activity

within the tested pH range (pH 6-8) (**Fig. 6**). Although the bioreactor was controlled between pH 6.8-7.2, it showed increasing $NO_2^-$ oxidation at lower pH, given low FNA concentrations (**Fig. 6**). Ammonia oxidation showed a broad temperature optimum (45-55°C), while nitrite oxidation showed a clear optimal activity at the reactor temperature (50°C). Thermophilic $NH_3$ oxidation could be inhibited by the conventional nitrification inhibitor ATU with an $IC_{50/100}$ of 3.5/8.8 mM and the AOA-specific inhibitor carboxy-PTIO with an $IC_{50/100}$ of 63/117 μM (**Fig. S7**).

## Discussion

In this study, the enrichment of coupled autotrophic thermophilic ammonia- and nitrite-oxidizers from compost was achieved followed by the successful operation of a thermophilic nitrifying bioreactor, opening up opportunities for nitrogen removal in warm wastewater.

The thermophilic nitrifying community in the bioreactor consisted of an AOA and NOB closely related to "*Candidatus* Nitrososphaera gargensis" and *Nitrospira calida*. Interestingly, the same co-existence was found in nature as both were originally isolated from the Garga hot spring (Russia) (Hatzenpichler *et al.* 2008, Lebedeva *et al.* 2011). In contrast to the oligotrophic nature of these geothermal springs, this study enriched nitrifiers from aerobic compost, a nutrient-rich high-temperature anthropogenic environment. Although many archaeal *amoA* genes (Maeda *et al.* 2011, Zeng *et al.* 2011) and even "*Candidatus* Nitrososphaera gargensis"–like sequences (Oishi *et al.* 2012, Yamamoto *et al.* 2011) were detected during composting processes, so far, no autotrophic thermophilic nitrifiers were enriched from compost. Only a heterotrophic AOB growing at 50°C related to *Bacillus halodurans* was isolated previously from animal waste composting (Shimaya and Hashimoto 2011). As the two compost piles that originally contained appreciable nitrate levels developed thermophilic ammonia oxidation, while all compost yielded nitrite oxidation, the presence of nitrate can lead to a smart selection of compost type for further studies focusing on thermophilic AOA. The presence of the described core nitrifiers in the bioreactor was, furthermore, linked with their activity and functionality. Incorporation of $^{13}C$ labeled bicarbonate was observed into crenarchaeol and 11-methyl C16:0, characteristic membrane lipids for "*Candidatus* Nitrososphaera gargensis" (Pitcher *et al.* 2010) and *Nitrospira* (Lipski *et al.* 2001), respectively. Although the carbon assimilation confirmed the autotrophic activity of the studied nitrifiers, it does not exclude the presence of other, unknown autotrophic or heterotrophic nitrifiers. An abundant cell type, embedded in a dense biofilm structure could

not be identified. Together with the observed delay/heterogeneity of the AOA presence over time (**Fig. S3)**, this could suggest that an uncharacterized ammonia oxidizing organism was also present, as was recently observed in reactors with low dissolved oxygen concentrations (Fitzgerald *et al.* 2015). The linearity of the nitrogen oxidation and the $^{13}C$ enrichment in the stable isotope experiment (**Fig. 4**), however, suggest that "*Candidatus* Nitrososphaera gargensis" and *Nitrospira calida* were important thermophilic nitrifiers in the biomass community.

The physiological characterization revealed that the specific oxidation rates of both AOA (18±1 µg N $mg^{-1}$ protein $h^{-1}$) and NOB (33±3 µg N $mg^{-1}$ protein $h^{-1}$) were in the same order of magnitude as related nitrifiers. In particular, the specific rates for AOA range from 11 to 24 µg N $mg^{-1}$ protein $h^{-1}$ (Kim *et al.* 2012), while reported rates for *Nitrospira* spp. range between 16 and 42 µg N $mg^{-1}$ protein $h^{-1}$ (Nowka *et al.* 2015). Interesting differences in substrate/product tolerances were observed. Until now, data concerning $NH_4^+/NH_3$ inhibition on (thermophilic) AOA has been limited attributing the inhibitory effect to $NH_4^+$ without excluding FA inhibition. However, with respect to biotechnological applications and the development of effective process control strategies, this distinction can be of great importance and was determined in this study. The "*Candidatus* Nitrososphaera gargensis"–like AOA in the thermophilic nitrifying bioreactor appeared to be insensitive to $NH_4^+$, and could maximally be inhibited by 63±5% from 8.5 mg $NH_3$-N $L^{-1}$ (**Fig. 5, Fig. S5**). At a neutral pH and a temperature of 50°C, this inhibition corresponds to a $NH_4^+$ concentration of 300 mg $NH_4^+$-N $L^{-1}$. This concentration is 7 times higher than the inhibitory $NH_4^+$ concentration reported for "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler *et al.* 2008). The higher FA tolerance could be attributed the fact that the AOA in this study originated from nutrient-rich compost in contrast with oligotrophic geothermal springs. Indeed, the AOA detected in cattle manure compost by Oishi et al. (2012) (Oishi *et al.* 2012) also showed a higher

377 tolerance towards media with a higher $NH_4^+$ concentration. The thermophilic NOB in the
378 bioreactor of our study were also sensitive to FA and insensitive to $NH_4^+$, but the higher
379 sensitivity ($IC_{50}$ of 5.0 mg $NH_3$-N $L^{-1}$) could allow a selective NOB inhibition based on FA.
380 Furthermore, the AOA were insensitive to FNA, while the NOB were extremely sensitive to
381 FNA ($IC_{50}$ of 0.0010 mg $HNO_2$-N $L^{-1}$). Both the insensitivity of AOA for FNA and the high
382 sensitivity of NOB for FA and FNA suggest that a selective NOB inhibition could be easily
383 established in the described thermophilic nitrifying community, enabling the development of
384 more cost-effective nitrogen removal processes, such as nitritation/denitritation or
385 deammonification.

386 Until now, the main thermophilic nitrogen removal mechanism was assumed to be ammonia
387 stripping and nitrogen assimilation into biomass (Abeynayaka and Visvanathan 2011b).
388 Development of thermophilic biotechnology for nitrogen removal is necessary, as ammonia
389 stripping transfers the problem to the gas phase, and no sufficient nitrogen removal can be
390 reached based on assimilation. Besides eliminating cooling requirements, thermophilic
391 nitrogen removal also lowers sludge production and confers better settling properties
392 (Suvilampi and Rintala 2003). These advantages apply not only to warm wastewaters but also
393 to wastewaters on sites with excess heat available. A few lab-scale studies have explored the
394 potential of thermophilic nitrification for wastewater treatment, but achieved no more than
395 40-42.5°C (Courtens *et al.* 2014a, Shore *et al.* 2012). Thus far, this is the first study
396 describing a thermophilic nitrifying bioreactor at 50°C. Although challenges such as the effect
397 of carbon on the autotrophic/heterotrophic competition and the coupling of nitrification with a
398 reductive nitrogen removal process (denitrification, anammox) have to be addressed to enable
399 implementation, this study paves the way for thermophilic nitrogen removal.

400

## Conflict of Interest

The authors declare no conflict of interest.

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608

609

610 **Figure legends**

611 **Figure 1.** Nitrification performance (mg N $L^{-1}$ $d^{-1}$) and nitrogen loss (%), i.e., the amount of
612 removed $NH_4^+$-N not recovered as $NO_2^-$-N or $NO_3^-$-N, in the thermophilic bioreactor (50°C)
613 inoculated with thermophilic nitrifying batch enrichments from compost samples. The white
614 and black triangles indicate the sampling for high-throughput DNA sequencing and
615 transmission electron microscopy, respectively.

616 **Figure 2.** Phylogenetic relationships between the archaeal 16S rRNA gene sequence (OTU7)
617 of the thermophilic nitrifying reactor biomass and all described AOA cultures or isolates, as
618 well as relevant environmental clone sequences. OTU7 belongs to the group 1.1b of
619 Thaumarchaeota (formerly Crenarchaeota).

620 **Figure 3.** Phylogenetic relationships between the most dominant *Nitrospira* 16S rRNA gene
621 sequences of the thermophilic nitrifying reactor biomass (OTU1) and all described *Nitrospira*
622 cultures or isolates, as well as relevant environmental clone sequences.

623 **Figure 4.** Relationship between the absolute amount of nitrogen oxidized and the $^{13}C$
624 incorporation in characteristic biomarkers: the biphytane moieties of the glycerol dibiphytanyl
625 glycerol tetraether lipids (GDGTs), more specifically crenarchaeol, as a biomarker for
626 "Candidatus Nitrososphaera gargensis" and the 11 methyl C16:0 as a specific phospholipid
627 fatty acid biomarker of *Nitrospira* spp. Data points represent the average replicate extractions
628 (n=3), error bars represent the standard error.

629 **Figure 5.** Effect of ammonium/FA (free ammonia) and nitrite/FNA (free nitrous acid) levels
630 on thermophilic ammonia (A and C) and nitrite (B and D) oxidation. Each panel represents
631 two complementary batch activity experiments (filled and empty circles) with full lines
632 depicting the remaining activity, while the dotted lines display the corresponding FA/FNA

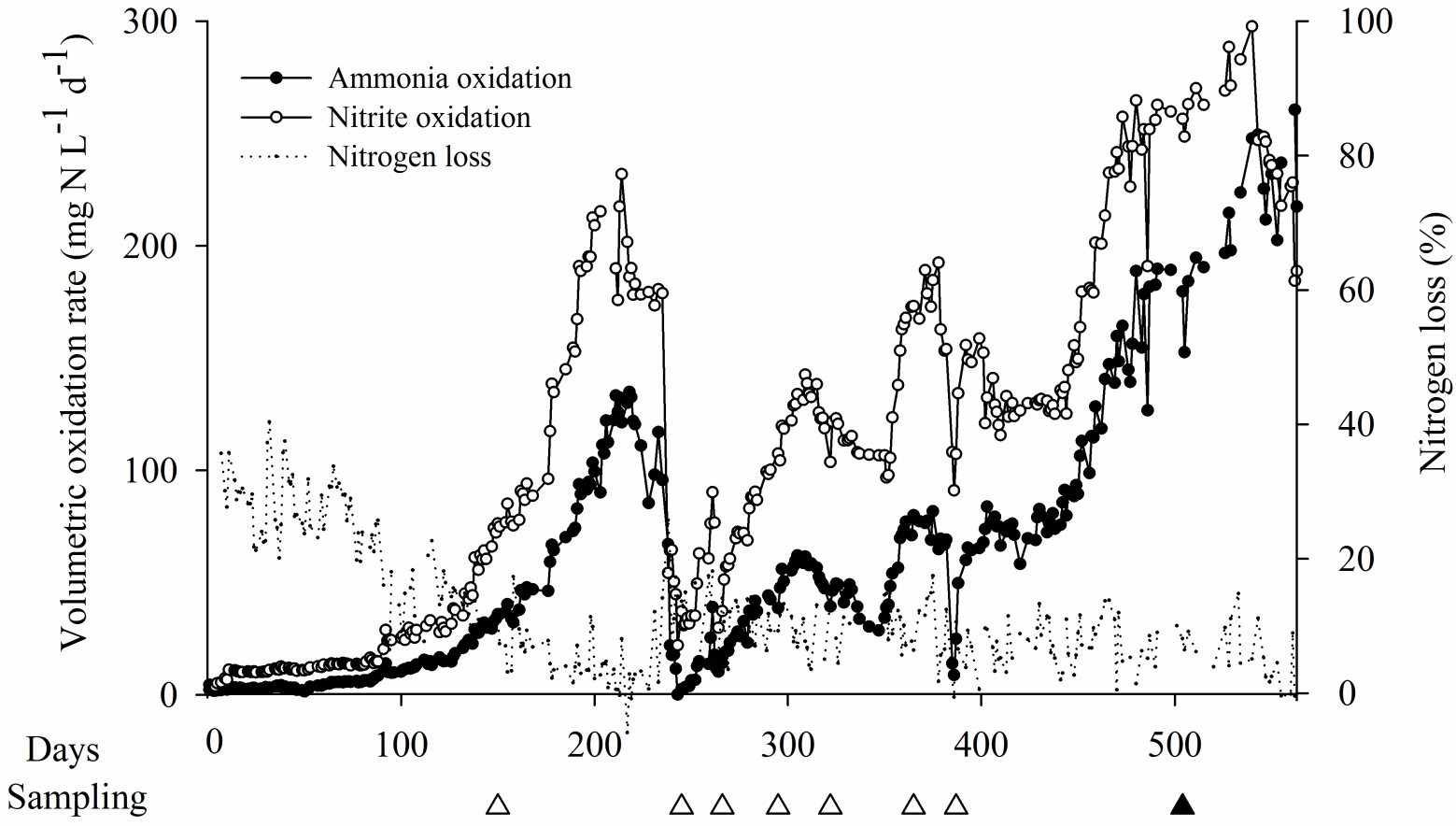
633 levels per test. Data points represent the average replicate tests (n=6), error bars represent the
634 standard error.

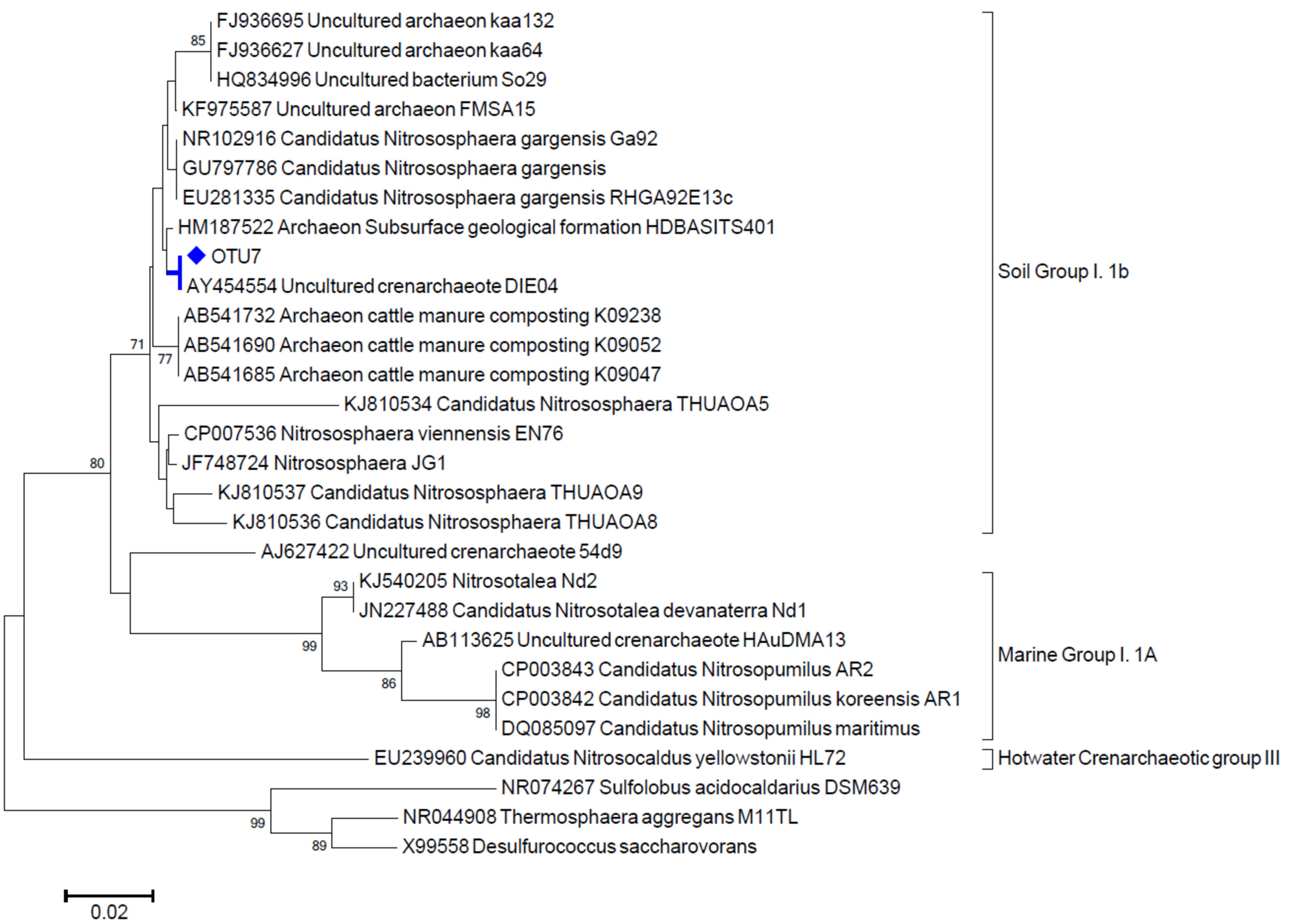
635 **Figure 6**. Effect of pH and temperature on thermophilic ammonia (A and B) and nitrite (C
636 and D) oxidation. Data points represent the average replicate tests (n=6), error bars represent
637 the standard error.

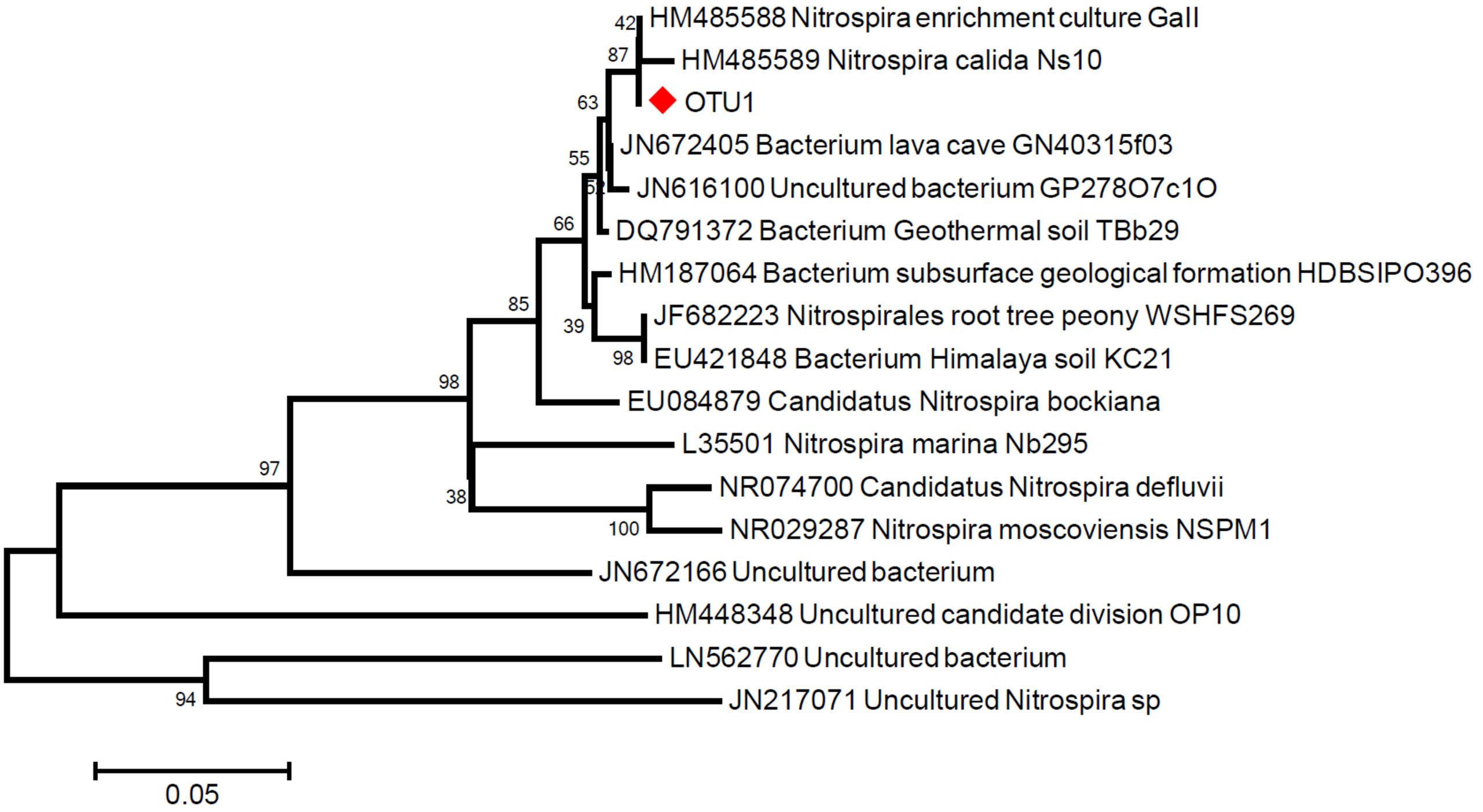
638

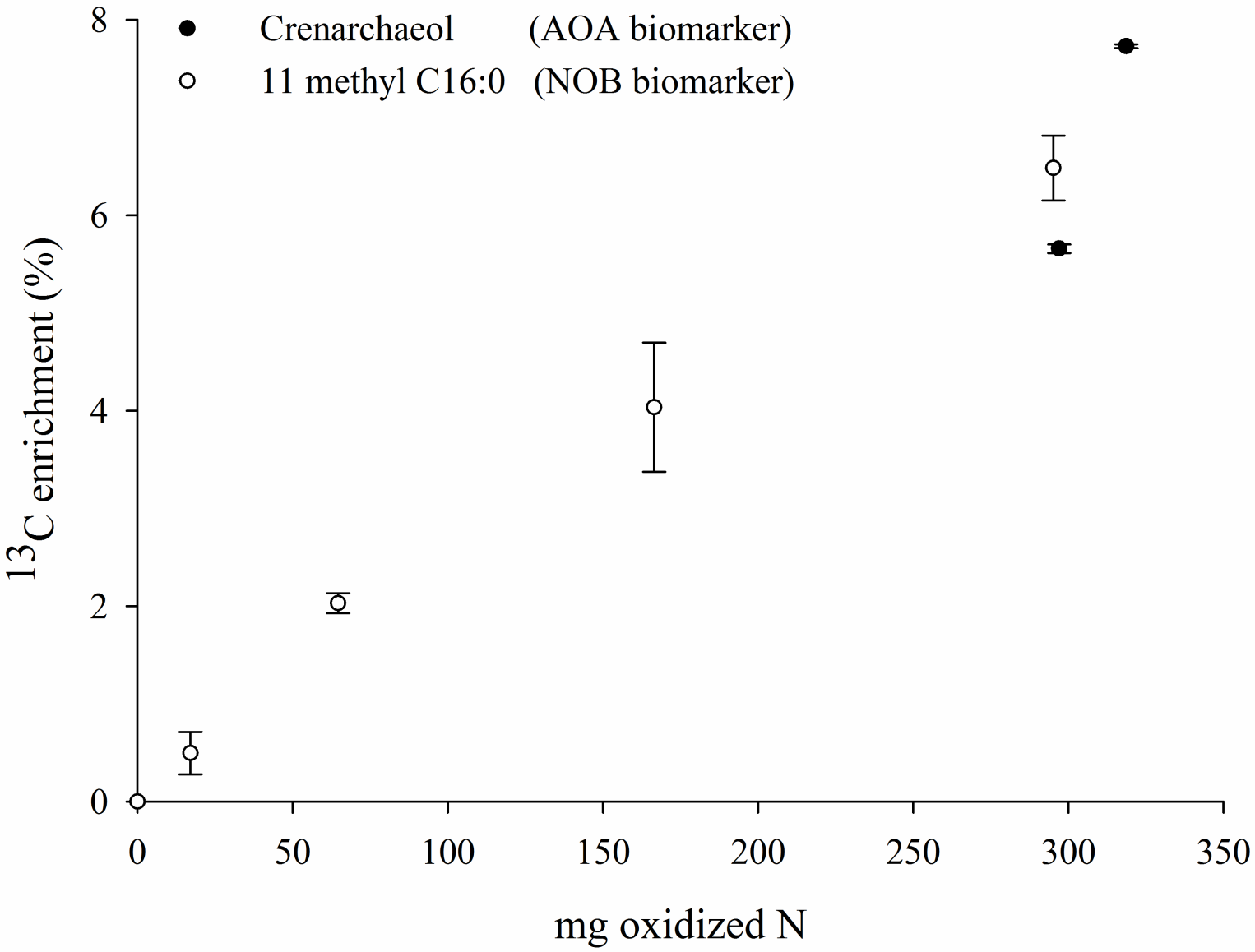
Influent (mg N $L^{-1}$)

| | | | | | | |
|---|---|---|---|---|---|---|
| $NH_4^+$ | 19 ± 2 | 37 ± 11 | 32 ± 25 | 34 ± 9 | 66 ± 25 | 123 ± 20 |
| $NO_2^-$ | 20 ± 3 | 35 ± 14 | 35 ± 18 | 46 ± 8 | 38 ± 9 | - |

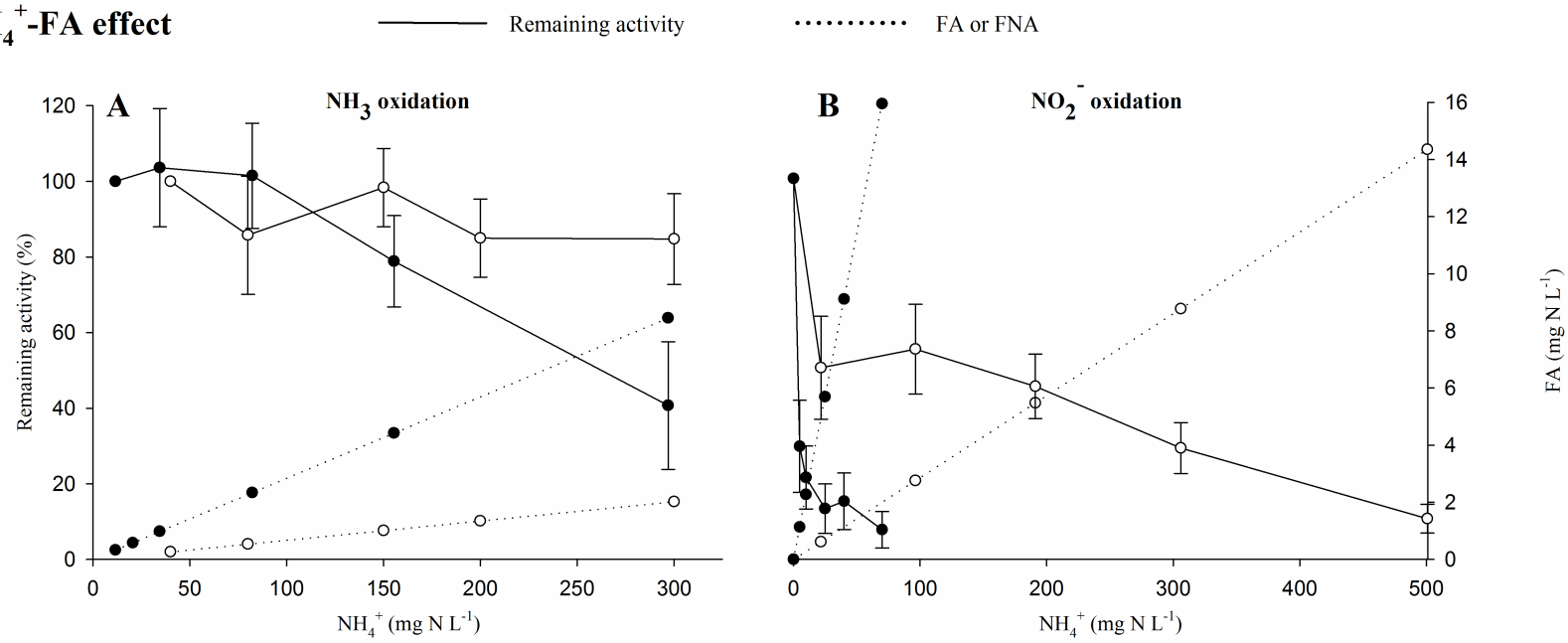



FJ936695 Uncultured archaeon kaa132
85
FJ936627 Uncultured archaeon kaa64
HQ834996 Uncultured bacterium So29
KF975587 Uncultured archaeon FMSA15
NR102916 Candidatus Nitrososphaera gargensis Ga92
GU797786 Candidatus Nitrososphaera gargensis
EU281335 Candidatus Nitrososphaera gargensis RHGA92E13c
HM187522 Archaeon Subsurface geological formation HDBASITS401
OTU7
AY454554 Uncultured crenarchaeote DIE04
AB541732 Archaeon cattle manure composting K09238
71
AB541690 Archaeon cattle manure composting K09052
77
AB541685 Archaeon cattle manure composting K09047
KJ810534 Candidatus Nitrososphaera THUAOA5
CP007536 Nitrososphaera viennensis EN76
80
JF748724 Nitrososphaera JG1
KJ810537 Candidatus Nitrososphaera THUAOA9
KJ810536 Candidatus Nitrososphaera THUAOA8
AJ627422 Uncultured crenarchaeote 54d9
93
KJ540205 Nitrosotalea Nd2
JN227488 Candidatus Nitrosotalea devanaterra Nd1
99
AB113625 Uncultured crenarchaeote HAuDMA13
86
CP003843 Candidatus Nitrosopumilus AR2
98
CP003842 Candidatus Nitrosopumilus koreensis AR1
DQ085097 Candidatus Nitrosopumilus maritimus
EU239960 Candidatus Nitrosocaldus yellowstonii HL72
NR074267 Sulfolobus acidocaldarius DSM639
99
NR044908 Thermosphaera aggregans M11TL
89
X99558 Desulfurococcus saccharovorans
Soil Group I. 1b
Marine Group I. 1A
Hotwater Crenarchaeotic group III
0.02

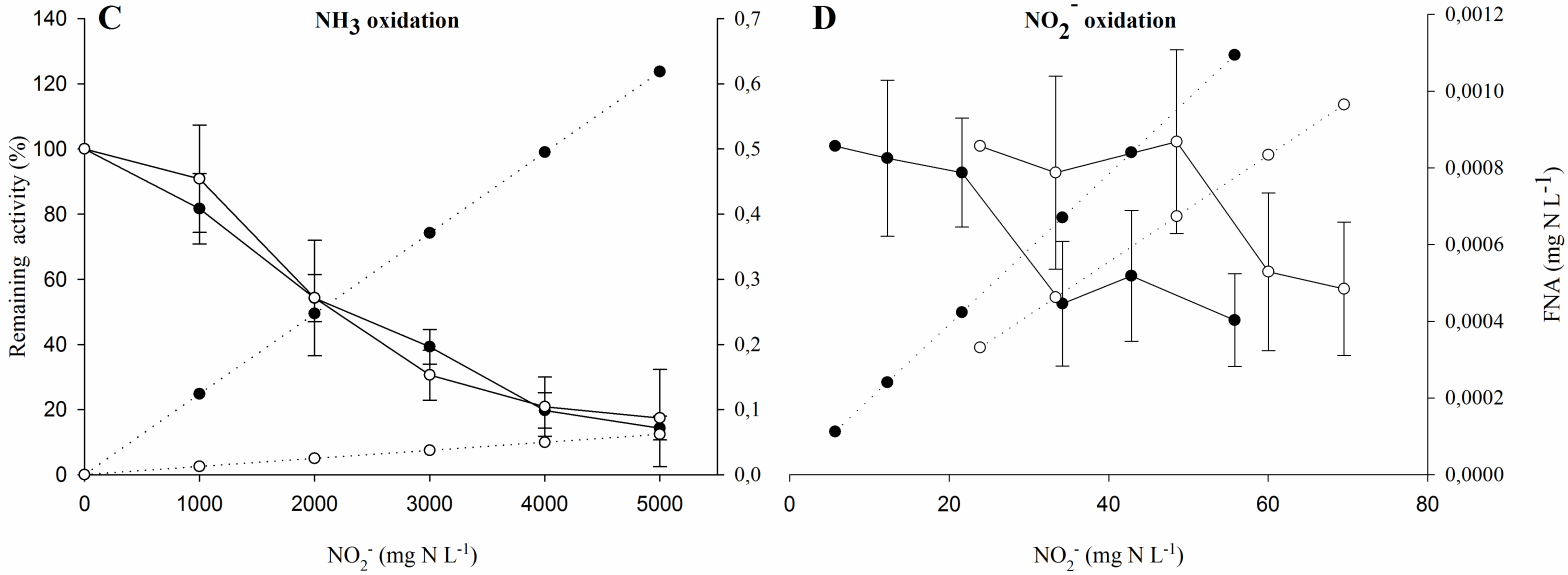

HM485588 Nitrospira enrichment culture GaII
HM485589 Nitrospira calida Ns10
OTU1
JN672405 Bacterium lava cave GN40315f03
JN616100 Uncultured bacterium GP278O7c1O
DQ791372 Bacterium Geothermal soil TBb29
HM187064 Bacterium subsurface geological formation HDBSIPO396
JF682223 Nitrospirales root tree peony WSHFS269
EU421848 Bacterium Himalaya soil KC21
EU084879 Candidatus Nitrospira bockiana
L35501 Nitrospira marina Nb295
NR074700 Candidatus Nitrospira defluvii
NR029287 Nitrospira moscoviensis NSPM1
JN672166 Uncultured bacterium
HM448348 Uncultured candidate division OP10
LN562770 Uncultured bacterium
JN217071 Uncultured Nitrospira sp
42
87
63
55
52
66
85
39
98
98
97
38
100
94
0.05

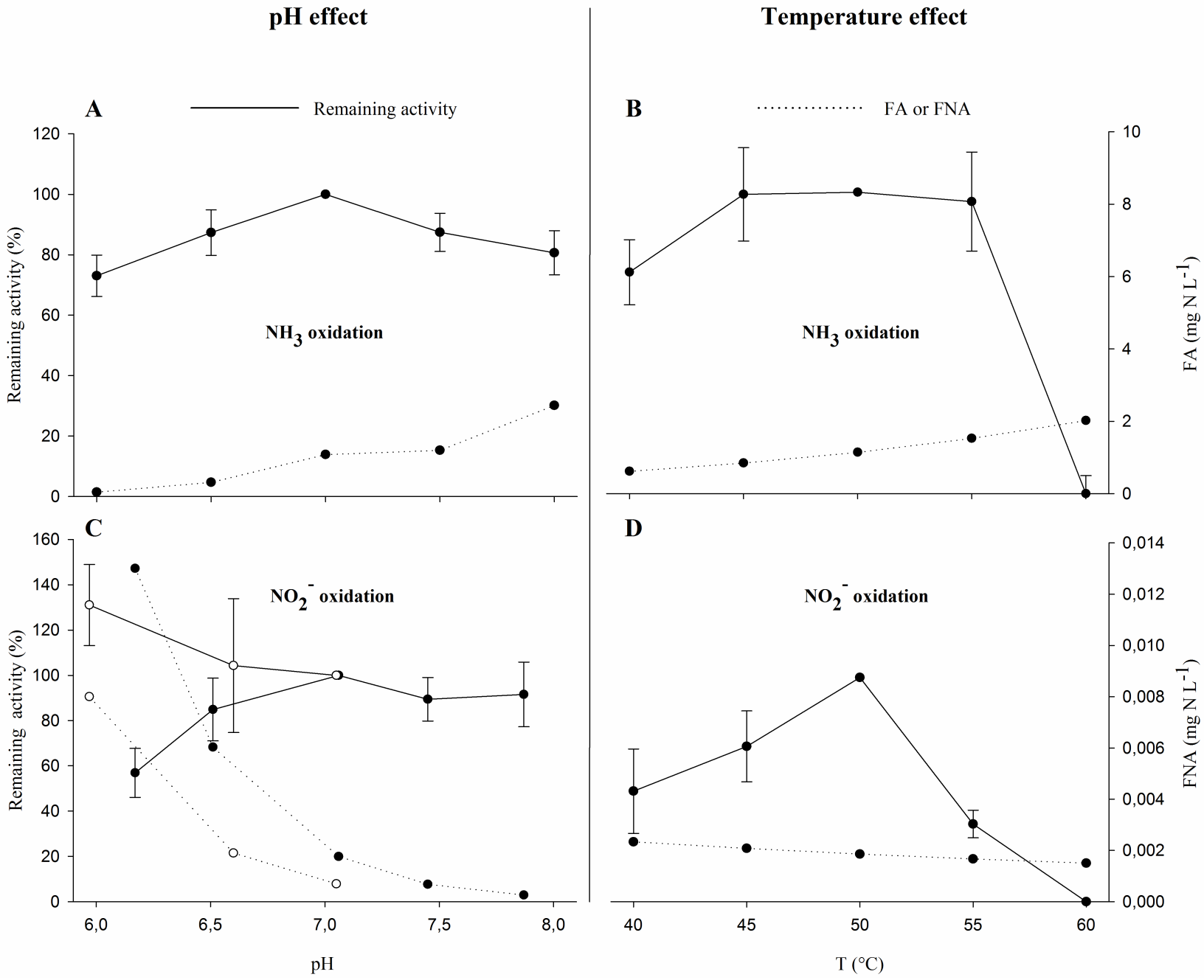

Crenarchaeol (AOA biomarker)
11 methyl C16:0 (NOB biomarker)
$^{13}$C enrichment (%)
0
2
4
6
8
0
50
100
150
200
250
300
350
mg oxidized N

## $NH_4^+$-FA effect




## $NO_2^-$-FNA effect




pH effect
Temperature effect
A
Remaining activity
B
FA or FNA
Remaining activity (%)
NH3 oxidation
FA (mg N L-1)
C
D
NO2- oxidation
FNA (mg N L-1)
pH
T (°C)