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A single *Thaumarchaeon* drives nitrification in deep oligotrophic Lake Constance

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Summary

Ammonia released during organic matter mineralization is converted during nitrification to nitrate. We followed spatiotemporal dynamics of the nitrifying microbial community in deep oligotrophic Lake Constance. Depth-dependent decrease of total ammonium (0.01–0.84 μM) indicated the hypolimnion as the major place of nitrification with ^{15}N-isotope dilution measurements indicating a threefold daily turnover of hypolimnetic total ammonium. This was mirrored by a strong increase of ammonia-oxidizing *Thaumarchaeota* towards the hypolimnion (13%–21% of bacterioplankton) throughout spring to autumn as revealed by amplicon sequencing and quantitative polymerase chain reaction. Ammonia-oxidizing bacteria were typically two orders of magnitude less abundant and completely ammonia-oxidizing (comammox) bacteria were not detected. Both, 16S rRNA gene and *amoA* (encoding ammonia monooxygenase subunit B) analyses identified only one major species-level operational taxonomic unit (OTU) of *Thaumarchaeota* (99% of all ammonia oxidizers in the hypolimnion), which was affiliated to *Nitrosopumilus* spp. The relative abundance distribution of the single *Thaumarchaeon* strongly correlated to an equally abundant *Chloroflexi* clade CL500-11 OTU and a *Nitrospira* OTU that was one order of magnitude less abundant. The latter dominated among recognized nitrite oxidizers. This extremely low diversity of nitrifiers shows how vulnerable the ecosystem process of nitrification may be in Lake Constance as Central Europe's third largest lake.

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Introduction

Microbially driven ammonia oxidation to nitrite is the rate limiting step in nitrification and as such an important part of the global nitrogen cycle (Jetten, 2008). Although nitrification does not directly change the inventory of inorganic N in freshwater ecosystems, it constitutes the only known biological source of nitrate and as such a critical link between mineralization of organic N and its eventual loss as N_2 by denitrification or anaerobic ammonia oxidation to the atmosphere (Jetten, 2008). The process of ammonia oxidation is known to be catalysed by three different microbial guilds. Two of these guilds, the ammonia-oxidizing bacteria (AOB; Bock and Wagner, 2013) and ammonia-oxidizing archaea (AOA; Pester *et al*., 2011; Alves *et al*., 2018) oxidize ammonia to nitrite and depend on nitrite oxidizing bacteria (NOB; Daims *et al*., 2016) to complete nitrification by further oxidation of nitrite to nitrate. The third guild oxidizes ammonia directly to nitrate and is therefore referred to as complete ammonia oxidizers (comammox; Daims *et al*., 2015; van Kessel *et al*., 2015). Most AOB belong to a monophyletic branch within the *Betaproteobacteria* represented by the genera *Nitrosomonas and Nitrosospira*, with the latter including the former genera *Nitrosolobus* and *Nitrosovibrio*, while the genus *Nitrosococcus* constitutes a separate branch within the *Gammaproteobacteria* (Head *et al*., 1993; Bock

and Wagner, 2013). Among the AOA, four major lineages represented by the two orders *Nitrosopumilales* and *Nitrososphaerales* and the two deep-branching *Candidatus* genera Nitrosotalea and Nitrosocaldus constitute together a major part of the diversity within the phylum *Thaumarchaeota* (Stieglmeier *et al*., 2014b; Lehtovirta-Morley *et al*., 2016; Qin *et al*., 2017). Based on a phylogenomic framework, it was recently proposed to re-integrate the *Thaumarchaeota* into the phylum *Crenarchaeota* (Parks *et al*., 2018), which awaits validation by the International Committee on Systematics of Prokaryotes. The comammox bacteria are currently described for the genus *Nitrospira* (phylum *Nitrospirae*) only (Daims *et al*., 2015; van Kessel *et al*., 2015), which is next to *Nitrospinae*, *Chloroflexi* and *Alpha-*, *Beta-* and *Gammaproteobacteria* one of the six phylogenetic lineages that harbours also strict NOB (Daims *et al*., 2016).

The presence and relative abundance of AOA and AOB have been extensively studied over the past decade in soils (e.g., Leininger *et al*., 2006; Prosser and Nicol, 2008; Pester *et al*., 2012; Hink *et al*., 2018), the ocean (e.g., Wuchter *et al*., 2006; Agogué *et al*., 2008; Tolar *et al*., 2013), or wastewater treatment plants (e.g., Limpiyakorn *et al*., 2011; Mußmann *et al*., 2011; Sauder *et al*., 2012; Pan *et al*., 2018). As a general trend, AOA typically outnumber AOB in soils and marine waters, while AOB prevail in wastewater treatment plants, albeit exceptions to this trend exist (Mußmann *et al*., 2011). How comammox fit into this picture is currently less clear because of their recent discovery. However, first studies indicated a prevailing abundance in oligotrophic habitats like groundwater wells (Pjevac *et al*., 2017) and the presence in soils, freshwater sediments and biofilms, marine coastal environments, as well as wastewater treatment plants (Daims *et al*., 2015; van Kessel *et al*., 2015; Gulay *et al*., 2016; Pjevac *et al*., 2017; Xia *et al*., 2018; Yu *et al*., 2018; Zheng *et al*., 2019). Based on the apparent half-saturation constants (K_m-values) for total ammonium (NH_3 + NH_4^+), cultured AOA seem to better adapted to lower (K_m-values: 133 nM–42 μM; Martens-Habbena *et al*., 2009; Kits *et al*., 2017) and cultured AOB rather to higher total ammonium concentrations (K_m-values: 30 μM–10 mM; Suzuki *et al*., 1974; Stehr *et al*., 1995; Martens-Habbena *et al*., 2009; Kits *et al*., 2017), which may explain their abundance ratios in the habitats mentioned above. The only determined K_m-value of comammox bacteria (0.65 μM, *Nitrospira inopinata*) places this guild rather into the group of microorganisms adapted to low total ammonium concentrations (Kits *et al*., 2017).

Also, the open water column of freshwater lakes has been studied over the past years, but mainly for AOA and AOB and typically without linking presence to nitrification activity. In general, the ratio of AOA to AOB decreased with increasing trophic state of the studied lakes, which would mirror the preference of AOB for increased inorganic nitrogen loading (Hou *et al*., 2013; Hugoni *et al*., 2013; Vissers *et al*., 2013a; Mukherjee *et al*., 2016; Okazaki and Nakano, 2016; Yang *et al*., 2016). In snapshot analyses of oligotrophic lakes, AOA typically outnumbered AOB and constituted preferentially in the deep oxygenated hypolimnion large bacterioplankton populations (Urbach *et al*., 2001; Callieri *et al*., 2016; Mukherjee *et al*., 2016). Whether this picture changes during the yearly phytoplankton succession of lakes and how comammox contribute to this functional group of microorganisms is not clear yet. Here, we followed the yearly bacterioplankton cycle of deep oligotrophic Lake Constance to identify spatiotemporal dynamics of the nitrifying microbial community and link this to nitrification activity.

Results

Active nitrification in the hypolimnion of upper Lake Constance

Depth profiles of total ammonium and nitrate as the primary substrate and product of nitrification, respectively, were followed throughout the year 2015. At all water depths, the concentrations of nitrate were at least one order of magnitude higher (28–57 μM) than of total ammonium (0.01–0.84 μM; Fig. 1). The water column was completely oxygenated throughout the year, irrespective of the temperature gradient build-up during spring, summer and autumn (Fig. 1). During the active phase of phytoplankton biomass production as indicated by chlorophyll a maxima in the epilimnion (spring to autumn), ammonium and nitrate showed opposing depth gradients (Fig. 1). While nitrate was increasing in concentration over depth, ammonium was decreasing. The latter indicated an ammonium sink in hypolimnetic waters through nitrification. This was corroborated by a gross nitrification estimate towards the end of the yearly phytoplankton succession using a ^{15}N-pool dilution method. Here, a nitrification rate of 3.2 μmol l^{-1} day^{-1} could be estimated for water sampled at 85 m depth on 6 October 2015 (Fig. S1), albeit based on a high variation of residual ^{15}N in individual water replicates after 3 days of incubation. This translated into a threefold turnover of the total ammonium pool per day. Incubations with 52 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) (13 October 2015, 85 m depth) as a selective inhibitor concentration of AOA but not AOB (Shen *et al*., 2013; Jung *et al*., 2014; Martens-Habbena *et al*., 2015) indicated an almost complete inhibition of nitrification (Fig. S1).

Seasonal bacterioplankton dynamics decrease with water depth

The phytoplankton reached maximum biomass concentrations of 1.6, 2.1 and 1.1 mg fresh weight per litre in April, June and September, respectively, which were

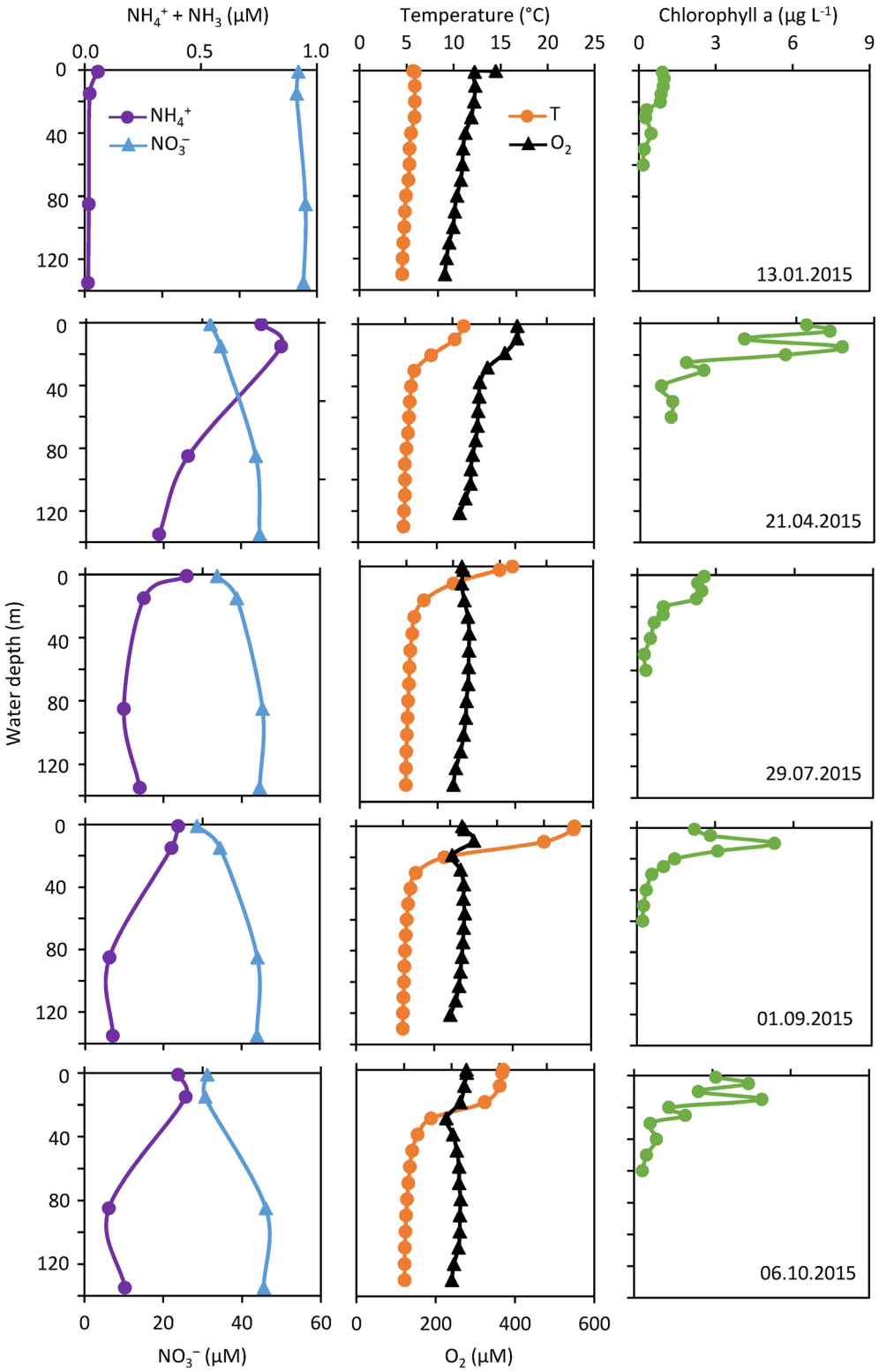

Fig. 1. Depth profiles of representative time points throughout the year 2015 illustrating the spatiotemporal variation of total ammonium (NH_3 + NH_4^+), nitrate, temperature, dissolved oxygen and chlorophyll a.

interrupted by phases of lower phytoplankton biomass concentrations between 0.1 and 0.7 mg fresh weight per litre. In winter, the phytoplankton biomass went down to 0.07 mg fresh weight per litre (Fig. S2). The phytoplankton itself was mainly composed of members of the *Centrales*, *Cryptophyceae*, *Dinnophyceae*, *Pennales*, *Chrysophyceae* and *Cyanophyceae* at varying relative contribution over the year. *Chlorophyceae* and *Conjugatophyceae* played a minor role (Fig. S2).

In parallel, the bacterioplankton community (including both bacteria and archaea) was analysed in representative water layers of the epilimnion (1 m), metalimnion (15–16 m) and hypolimnion (85 m) by 16S rRNA gene amplicon sequencing. The size fractions 0.1–5.0 μm and 5.0–30.0 μm were analysed, which we defined as planktonic and particle-associated bacterioplankton respectively. Bacterioplankton alpha diversity at a rarefied sequencing depth of 12 694 reads per sample was comparable throughout the water layers. The mean numbers of observed operational taxonomic units (OTUs) at an approximate species level (97% sequence identity) constituted for the planktonic size fraction 520 ± 73 (epilimnion), 517 ± 70 (metalimnion) and 594 ± 47 (hypolimnion) OTUs throughout the year (Fig. S3). While in the epi- and metalimnion a drop in alpha diversity was observable in spring (21 April 2015), which was significant in the epilimnion ($p < 0.05$), alpha diversity stayed stable in the hypolimnion (Fig. S3). A similar picture was observed for the particle-associated bacterioplankton with slightly higher average alpha diversity levels of 609 ± 127 (epilimnion), 628 ± 149 (metalimnion) and 805 ± 71 OTUs (hypolimnion) throughout the year. Significantly lowest alpha diversity levels in the epi- and metalimnion were observed on 10 March 2015. Alpha diversity in the hypolimnion was again stable throughout the year.

Differences in the dominating bacterioplankton community at the family level (represented by OTUs with > 1% relative abundance in at least one water sample) were visualized by non-metric multidimensional scaling (NMDS) plots. The NMDS analysis showed a separation of the hypolimnion from the epi- and metalimnion communities. In addition, the epi- and metalimnion communities showed a separation over the year, while the hypolimnion community was rather stable (Fig. 2). The observed differences were highly significant ($p < 0.001$) as determined by a permutational analysis of variance (PERMANOVA). For the planktonic size fraction, 40% of the variation was explained by water depth, 34% by the seasonal sampling, and 16% by the interaction of both. The latter corroborated that part of the observed seasonal changes were different among water depths. We also tested for differences in variability of the dominating bacterioplankton community among water samples, either attributed to seasonal sampling or water depth. Variability did not differ significantly among sampling dates ($F_{7,44} = 0.86$, $p = 0.55$). However, it did differ significantly between water depths ($F_{2,49} = 14.4$, $p < 0.001$). A post hoc Tukey's test revealed that this was due to a significantly different variability in the hypolimnion as compared with the epi- and metalimnion ($p < 0.001$), which reflected the less variable dominating bacterioplankton community in the hypolimnion as compared with epi- and metalimnion throughout the year (Fig. 2). In contrast to the planktonic size fraction, the PERMANOVA revealed that most of the variation (55%) in the dominating bacterioplankton community in the particle-associated fraction was explained by seasonal sampling and only 18% and 14% by water depth and the interaction of seasonal sampling and water depth respectively ($p < 0.001$). There was no significant difference in variability of the dominating bacterioplankton community in the particle-associated fraction according to seasonal sampling ($F_{7,44} = 0.54$, $p = 0.80$) or water depth ($F_{2,49} = 1.26$, $p = 0.29$).

Ammonia-oxidizing Thaumarchaota are a dominant bacterioplankton group in the hypolimnion

Bacterioplankton taxa at the family level that strongly influenced the observed differences in beta diversity were visualized by a NMDS bi-plot analysis (Fig. 2). Among families harbouring recognized nitrifiers, *Nitrosopumilaceae* (*Thaumarchaeota*) and *Nitrospiraceae* (*Nitrospirae*) clearly colocalized with hypolimnion and *Nitrosomonadaceae* (*Betaproteobacteria*) with metalimnion samples in the planktonic size fraction. A detailed inspection of their relative abundance distribution revealed that *Nitrosopumilaceae* predominated in the planktonic size fraction of the hypolimnion samples (85 m), with a relative abundance of 13%–21% (Fig. 3). Here, the *Nitrosopumilaceae* constituted the second most abundant bacterioplankton group after unclassified bacteria (Fig. S4). In the epilimnion (1 m) and metalimnion (15–16 m), *Nitrosopumilaceae* were generally of low relative abundance in the planktonic size fraction except in winter (13 January 2015; Fig. 3), when the water column was almost completely mixed (Fig. 1).

We verified this result by an independent quantitative polymerase chain reaction (qPCR) analysis. Based on the current knowledge of typically one *amoA* (encoding the beta subunit of ammonia monooxygenase) copy per genome in the family *Nitrosopumilaceae* and AOA in general (Hallam *et al*., 2006; Walker *et al*., 2010; Park *et al*., 2012; Spang *et al*., 2012; Bayer *et al*., 2015; Santoro *et al*., 2015; Kerou *et al*., 2016; Herbold *et al*., 2017), the total AOA relative abundance was determined from the ratio of thaumarchaeal *amoA* gene copies in relation to total bacterial and archaeal 16S rRNA gene copies. qPCR results mirrored exactly the spatiotemporal dynamics of the *Nitrosopumilaceae* population as determined by high throughput amplicon sequencing. Also

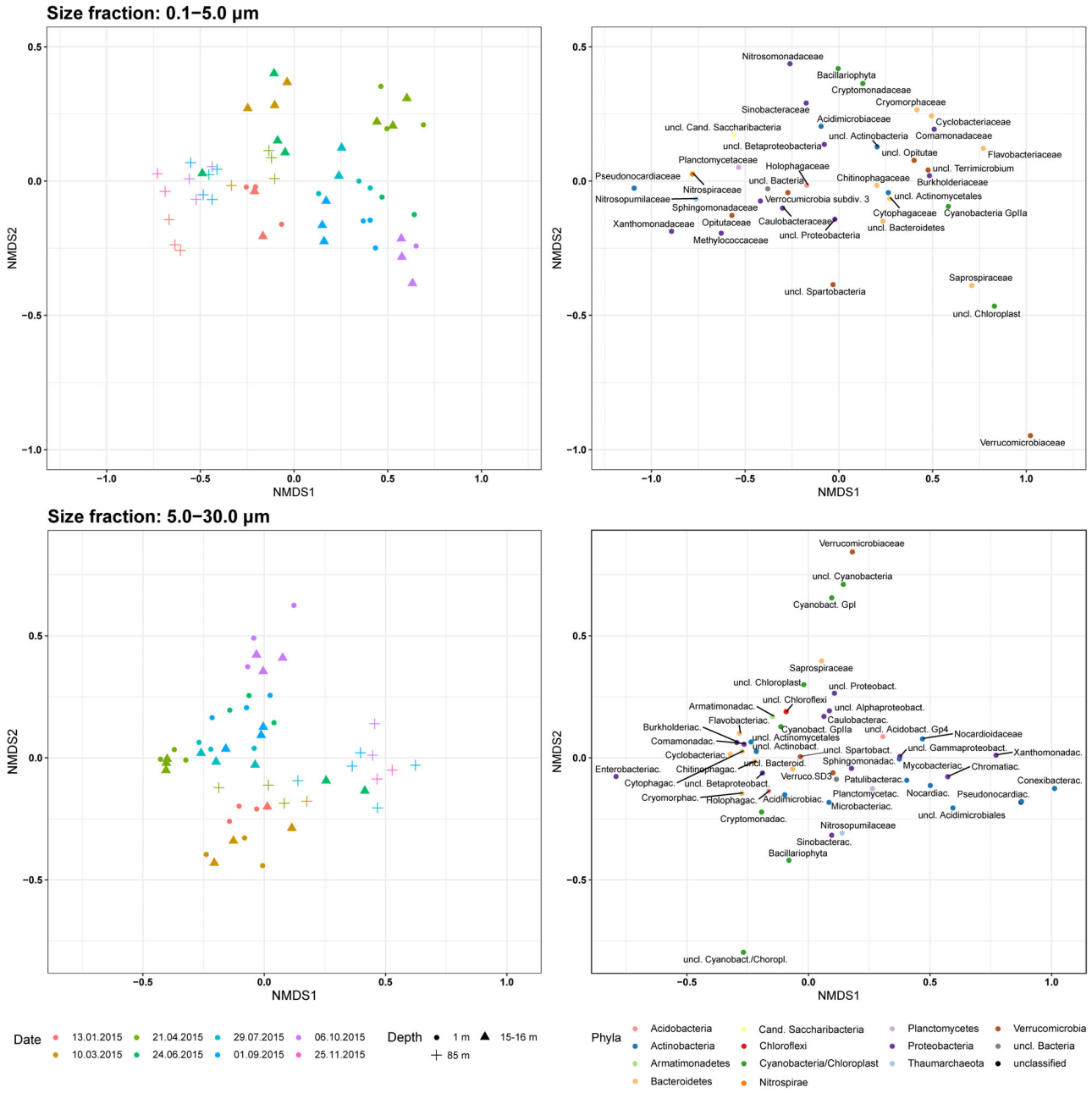

Fig. 2. Spatiotemporal dynamics in beta diversity of abundant bacterioplankton family-level taxa in Lake Constance throughout the year 2015. The planktonic (0.1–5.0 µm) and particle-associated (5.0–30.0 µm) size fraction are shown separately. The NMDS bi-plot analysis is based on Bray-Curtis distances and shows separation of bacterioplankton communities on the left and the corresponding separation of abundant bacterioplankton family-level taxa on the right side for the respective size fraction. Abundant bacterioplankton family-level taxa were defined as harbouring OTUs with > 1% relative abundance in the respective size fraction of at least one sequenced water sample.

here, total AOA predominated in the hypolimnion with relatively stable population sizes (8%–14% relative abundance). Only during the winter overturn, total AOA reached comparable population sizes throughout the water column (Fig. S5). Total AOA relative abundances estimated by qPCR were consistently slightly smaller than for *Nitrosopumilaceae* in the high-throughput amplicon analysis, which may reflect the different degree of coverage of the different universal 16S rRNA gene primer sets used in both analyses.

Interestingly, relative abundance dynamics of *Nitrospiraceae*, which consisted of members of the genus *Nitrospira* only (Table S1), followed closely those of the *Nitrosopumilaceae* being most abundant in the hypolimnion as well. However, *Nitrospiraceae* were generally one order of magnitude less abundant reaching at

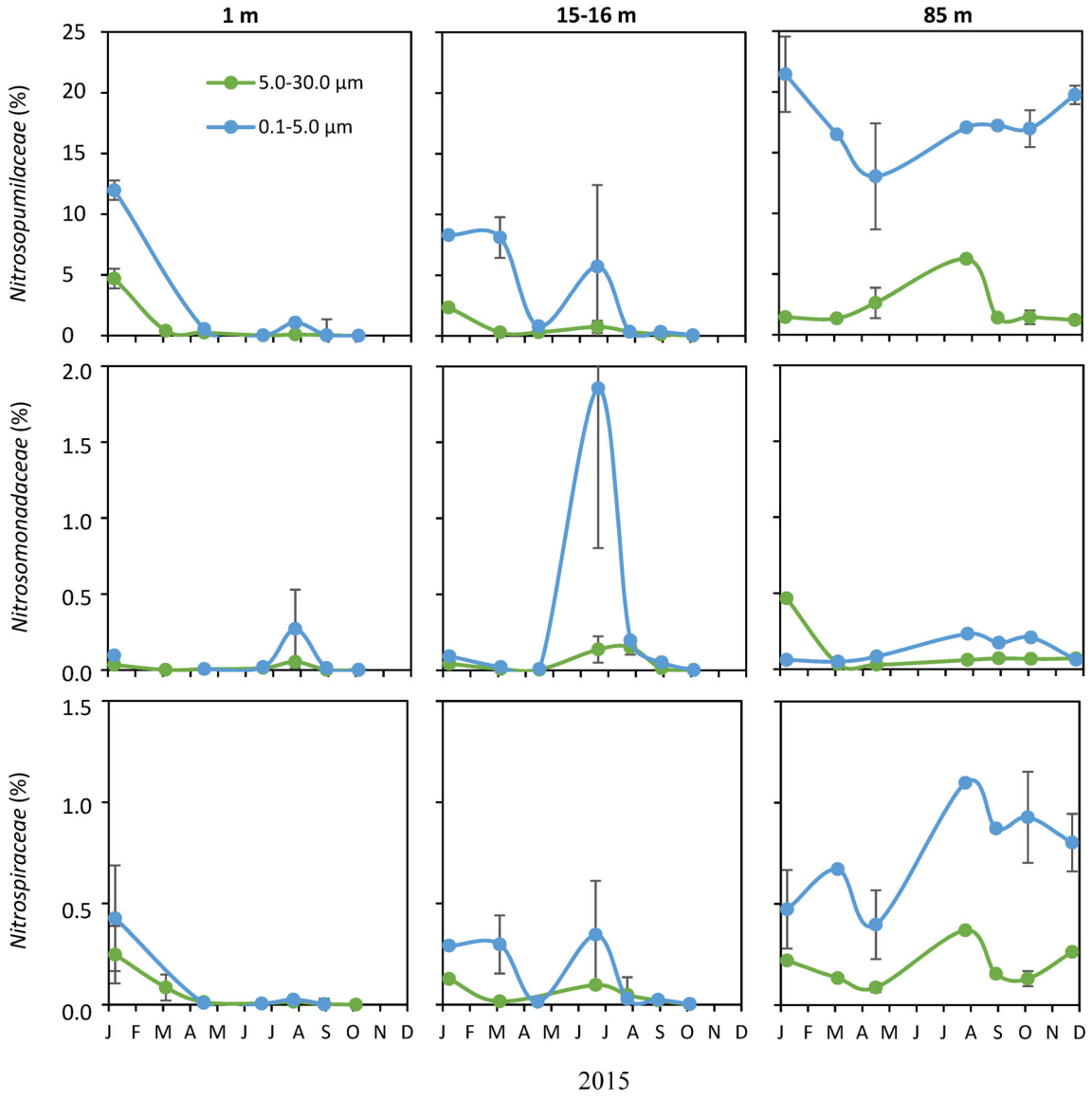

Fig. 3. Spatiotemporal dynamics of the sum of OTUs belonging to the *Nitrosopumilaceae*, *Nitrosomonadaceae* or *Nitrospiraceae* of the genus *Nitrospira* in the water column of Lake Constance throughout the year 2015. Error bars represent 1 SD (n = 3). In case error bars are not visible, symbols are either larger than error bars or the number of replicates was below 3.

maximum 1.1% (Fig. 3). In contrast, *Nitrosomonadaceae* relative abundance dynamics were clearly disconnected from those of *Nitrosopumilaceae* and typically two orders of magnitude less abundant (Fig. 3). The only exception was the planktonic size fraction in the metalimnion sampled on 24 June 2015, when *Nitrosomonadaceae* reached a maximum relative abundance of 1.9% (Fig. 3). Besides the *Nitrosopumilaceae* and *Nitrospiraceae*, other abundant family-level taxa that were specific to the planktonic size fraction of the hypolimnion bacterioplankton belonged to the phyla *Planctomycetes* (*Planctomycetaceae*), *Proteobacteria* (*Sphingomonadaceae*, *Xanthomonadaceae*, *Methylococcaceae*), *Verrucomicrobia* (*Opitutaceae*), *Actinobacteria* (*Pseudonoradiaceae*) and *Candidatus* Saccharibacteria (unclassified; Fig. 2, Fig. S4). None of these family-level taxa exceeded 3% relative abundance at any time point. The hypolimnion bacterioplankton was shaped in addition by abundant family-level taxa that showed similar or even higher relative abundances in the epi- and/or metalimnion. These family-level taxa belonged to the *Actinobacteria* (*Acidimicrobioaceae*, uncl. *Actinomycetales*, uncl. *Actinobacteria*), *Acidobacteria* (*Holophagaceae*), *Bacteroidetes* (*Chitinophagaceae*, *Cryomorphaceae*, uncl. *Bacteroidetes*), *Proteobacteria* (uncl. *Betaproteobacteria*, *Comamonadaceae*, uncl. *Proteobacteria*), *Verrucomicrobia*

(*Verrucomicrobia* SD3), *Cyanobacteria*/Chloroplasts (*Bacillariophyta*, *Cryptomonadaceae*, *Cyanobacteria* GpIIa) and unclassified bacteria. However, a large fraction of unclassified bacteria in the hypolimnion could be attributed to the CL500-11 lineage within the *Chloroflexi* (see network analysis below).

In the particle-associated size fraction, family-level taxa harbouring recognized nitrifiers did not colocalize with water samples from any particular depth. For example, abundant family-level taxa that specifically colocalized in the NMDS bi-plot with hypolimnion samples belonged rather to the phyla *Actinobacteria* (uncl. *Acidimicrobiales*, *Conexibacteraceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Patulibacteraceae*, *Pseudonocardiaceae*), *Acidobacteria* (unclassified *Acidobacteria* Gp4) and *Proteobacteria* (unclassified *Gammaproteobacteria*, *Xanthomonadaceae*; Fig. 2, Fig. S6). Among family-level taxa harbouring nitrifiers, only *Nitrosopumilaceae* reached abundant population sizes in the particle-associated size fraction. Again, they predominated in the hypolimnion but never exceeded 6% in relative abundance (Fig. 3).

Nitrifier alpha diversity is extremely low

Alpha diversity within the ammonia-oxidizing *Thaumarchaeota* was restricted to eight species-level OTUs (97% identity) with five OTUs being affiliated to the *Nitrosopumilaceae* and the remaining three affiliated to the *Nitrososphaeraceae* (Fig. S7, Table S1). Only one of these OTUs (16S-OTU3) dominated the overall thaumarchaeal population, while the remaining seven OTUs never exceeded a relative abundance of 0.11%. Phylogenetic analysis of 16S-OTU3 placed it within the *Nitrosopumilaceae* (Fig. 4) and direct sequence comparison identified *Candidatus* Nitrosopumilus oxyclinae as the most similar cultured relative (98.8% sequence identity). Since 16S-OTU3 predominated mainly in the hypolimnion, a microdiversity analysis of all thaumarchaeal amplicon reads was performed for this habitat. At 99% sequence identity, again only one overall dominating phylotype represented on average 95% $\pm$ 3% (range 87%–100%) of all thaumarchaeal reads. Even at the level of unique thaumarchaeal amplicon sequences, there was only one major phylotype that represented 59% $\pm$ 8% (range 47%–74%) of all reads, while each of the remaining unique sequence types never exceeded 5% of all thaumarchaeal reads (Fig. S8).

Parallel clone libraries targeting the thaumarchaeal *amoA* gene corroborated the 16S rRNA gene amplicon analysis. Clone libraries established from summer (19 August 2014, 40 clones) and winter (31 January 2015, 47 clones) samples, both spanning the meta- and hypolimnion, revealed just one thaumarchaeal *amoA*-OTU at 94% nucleotide sequence identity, which is clearly above the recommend species cut-off of 85% (Pester *et al*., 2012). The thaumarchaeal *amoA*-OTU was falling into the *Nitrosopumilales* cluster, subclade NP-γ-2.1 (Alves *et al*., 2018), with the *amoA* of *Candidatus* Nitrosomarinus catalina (*Nitrosopumilaceae*) being the most similar *amoA* gene (96.0% nucleic acid identity; Fig. 4). Within this species-level *amoA*-OTU, microdiversity at 99% *amoA* nucleic acid sequence identity revealed nine sequence types of which two represented the majority of clones (51% and 34% respectively) and three were represented by just one clone. Thaumarchaeal *amoA*-based T-RFLP analysis was used to extend the *amoA*-based analysis for the planktonic size fraction. Also here, only one major T-RF at 553 bp representing the single detected thaumarchaeal *amoA*-OTU was detected throughout all water depths and the yearly seasons of 2015 (Fig. S9).

As counterpart to ammonia-oxidizing *Thaumarchaeota*, we screened the 16S rRNA gene amplicon data set for the presence and relative abundance of bacterial ammonia oxidizer OTUs. Among the bacterial ammonia oxidizers, all OTUs belonged to the *Nitrosomonadaceae*: six OTUs were affiliated to the genus *Nitrosospira*, one OTU to the genus *Nitrosomonas*, and three OTUs could not be clearly affiliated to one of the two genera (Table S1, Fig. S7). Similar to the *Thaumarchaeota*, typically only one OTU (16S-OTU142) dominated the population of betaproteobacterial ammonia oxidizers (Table S1). It reached its highest relative abundance during the summer months in the planktonic size fraction with its maxima occurring throughout the water column at different sampling dates. Only in the particle-associated size fraction in the hypolimnion, a second OTU (16S-OTU598) was typically but not always more abundant than the 16S-OTU142 (Table S1). In all other samples, 16S-OTU598 was typically the second most abundant OTU. 16S-OTU142 could not be clearly affiliated to *Nitrosospira* or *Nitrosomonas* spp. (Fig. 4) but its 16S rRNA gene sequence was most similar to the 16S rRNA gene of *Nitrosospira briensis* (98.8% identity). 16S-OTU598 was falling into a cluster with *Nitrosomonas ureae* and *Nitrosomonas oligotropha* (Fig. 4) with its 16S rRNA gene sequence being most similar to the 16S rRNA gene of *Nitrosomonas ureae* (97.2% identity). None of the retrieved amplicon sequences was affiliated to known ammonia-oxidizers among the *Gammaproteobacteria* (genus *Nitrosococcus*).

Again, parallel clone libraries targeting betaproteobacterial *amoA* genes were established from summer (19 August 2014, 45 clones) and winter (31 January 2015, 46 clones) samples, both spanning the meta- and hypolimnion. Two betaproteobacterial *amoA* OTUs were detected at 91% nucleotide sequence identity, which were also stable at the recommended species cut-off of 80% nucleotide sequence identity (Purkhold *et al*., 2000). Ninety-one percentage of the clones were affiliated to betaproteobacterial *amoA*-OTU1 and 9% to betaproteobacterial *amoA*-OTU2, which mirrored well the 16S rRNA gene amplicon results. Both OTUs were

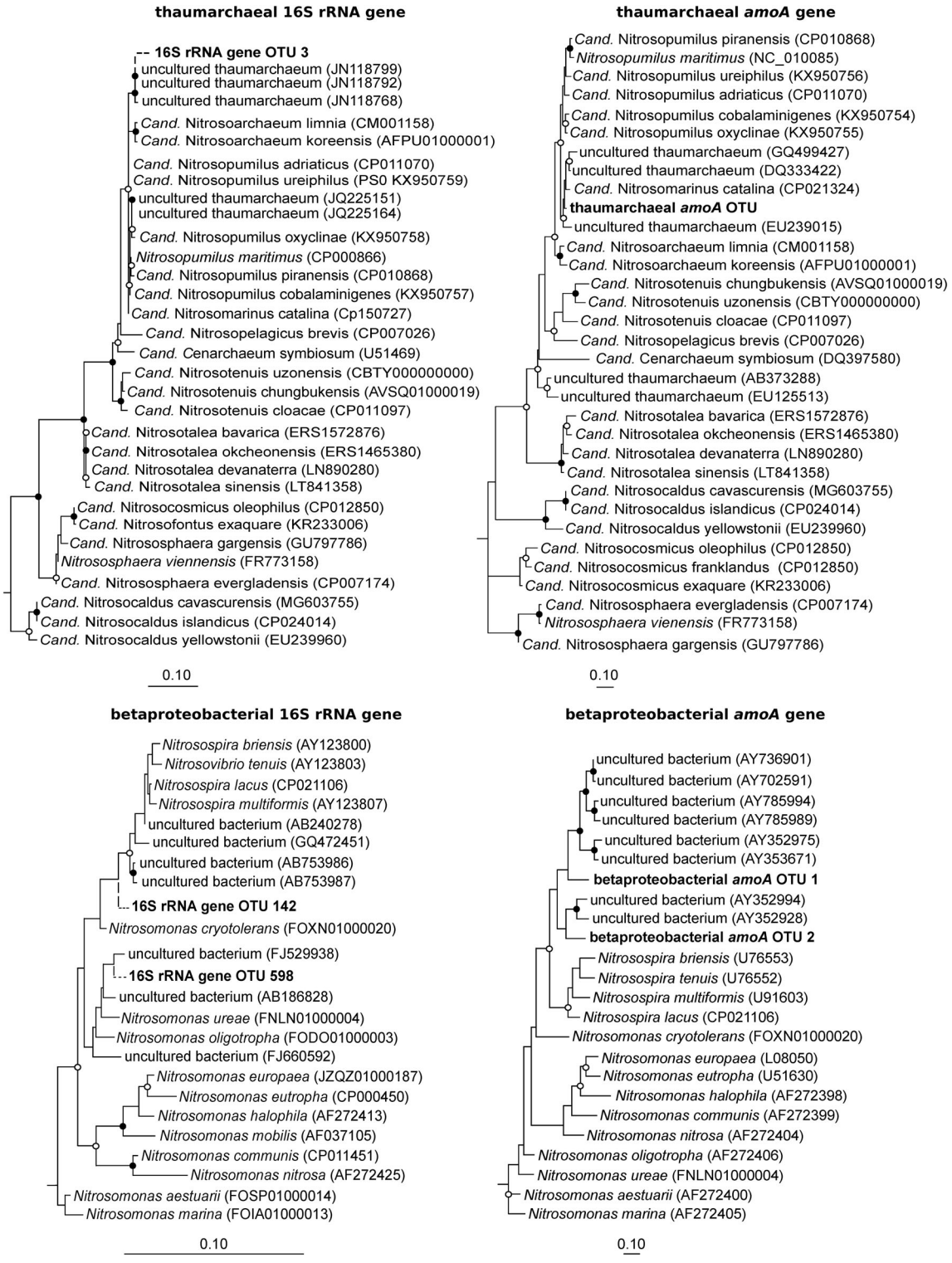

Fig. 4. Phylogenetic affiliation of dominant thaumarchaeal and betaproteobacterial 16S rRNA and *amoA* gene OTUs retrieved from the water column of Lake Constance. Maximum likelihood trees were inferred using the RAxML algorithm (Stamatakis, 2006). In the case of 16S rRNA gene trees, shorter amplicon reads were added using the Quick add parsimony tool as implemented in ARB (Ludwig *et al*., 2004) without changing the tree topology. This is indicated by dashed branches. Bootstrap support is indicated by closed (≥ 90%) and open (≥ 70%) circles at the respective branching points. The scale bar indicates 10% estimated sequence divergence.

falling into a cluster of exclusively environmental sequences, which formed a sister clade to *amoA* of *Nitrosospira* spp. (Fig. 4). There was little microdiversity at 99% nucleotide sequence identity with only four sequence types. The first sequence was identical to betaproteobacterial *amoA*-OTU1, while betaproteobacterial *amoA*-OTU2 was split into one major and two minor sequence types. Betaproteobacterial *amoA*-based T-RFLP analysis was used to extend the *amoA*-based analysis. In the planktonic size fraction, only one major T-RF at 134 bp representing betaproteobacterial *amoA*-OTU1 was detected throughout all water depths and the yearly seasons of 2015 (Fig. S9). Only occasionally, a second and very minor peak at 63 bp representing betaproteobacterial *amoA*-OTU2 was detected.

Comammox were absent from the water column

All four OTUs of the 16S rRNA gene amplicon dataset that affiliated to the *Nitrospiraceae* belonged to the genus *Nitrospira*. This genus includes mainly NOB in six distinct lineages (Pester *et al*., 2014; Daims *et al*., 2016) with lineage II harbouring in addition the recently described comammox bacteria (Daims *et al*., 2015; van Kessel *et al*., 2015). No OTUs clearly affiliated to NOB within the *Nitrospinae*, *Chloroflexi* or *Alpha*-, *Beta*- and *Gammaproteobacteria* (Daims *et al*., 2016) were detected. Again, only one OTU (16S-OTU86) dominated the overall *Nitrospira* population (Fig. 3), while the other three *Nitrospira* OTUs never exceeded a relative abundance of 0.04% (Table S1). The dominating 16S-OTU86 as well as two additional OTUs were affiliated to *Nitrospira* lineage II, while the remaining *Nitrospira* 16S-OTU2772 had no clear affiliation to one of the established lineages and was falling in between lineage III and lineage V (Fig. S10). To test whether the observed *Nitrospira* OTUs – especially those of lineage II – could be linked to comammox bacteria, we screened additional DNA extracts obtained from water sampled in summer 2016 (1 m and 85 m) and winter 2017 (1 m, 15 m, 85 m) for their respective *amoA* genes using comammox-*amoA* specific primers (Pjevac *et al*., 2017). Neither comammox *amoA* clade A nor clade B could be amplified from water samples of the planktonic and particle-associated size fraction.

CL500-11 Chloroflexi *strongly correlate to ammonia-oxidizing* Thaumarchaeota

Major identified nitrifiers were analysed for cocorrelating bacterioplankton 16S-OTUs by an association network analysis of the planktonic size fraction. Association networks closely mirrored the decreasing bacterioplankton family-level dynamics with depth (Fig. 2) and identified most relevant OTUs driving these dynamics. While there was a clear seasonal succession of eight OTU association networks characteristic for both the epi- and metalimnion, only two association networks were identified for the hypolimnion that were stable throughout the year (Fig. 5A). Thaumarchaeal 16S-OTU3 was central to the larger of the two hypolimnion networks and strongly

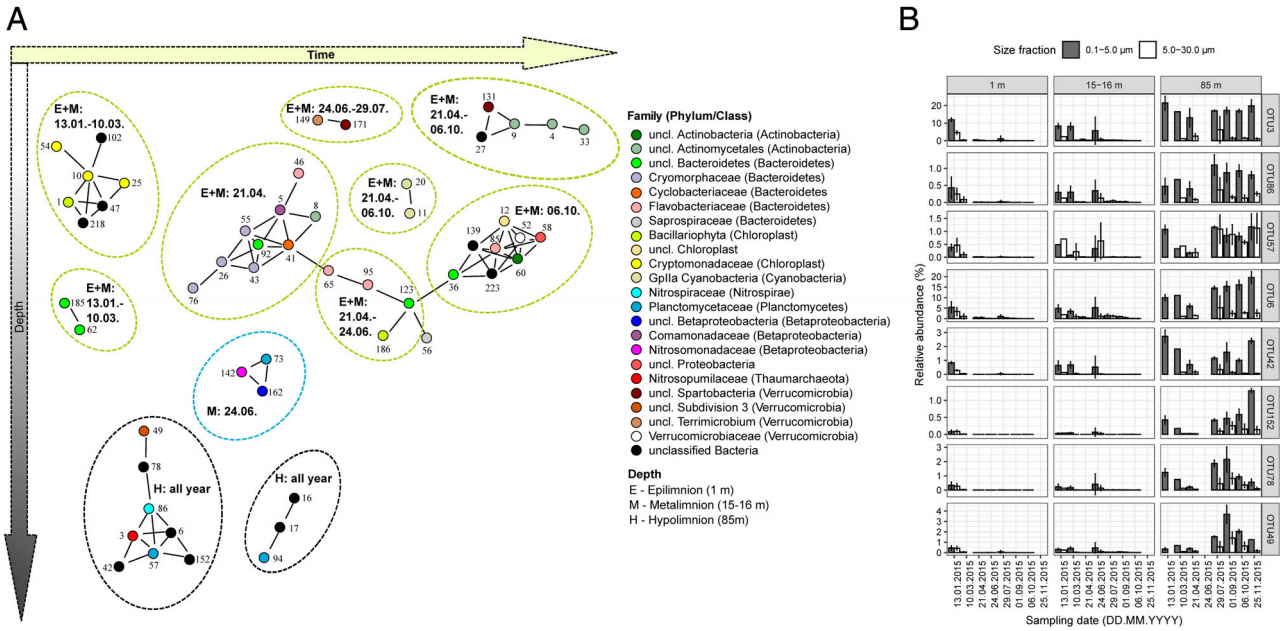

Fig. 5. A. Association network analysis visualizing spatiotemporal dynamics of abundant bacterioplankton OTUs strongly correlating in their relative abundance shifts ($r > 0.80$, FDR-corrected p-value < 0.05). The ID number of the respective OTUs is displayed besides representing circles. Maximum relative abundance occurrence in space and time is indicated next to each network and was extracted from Table S1.
B. Details of spatiotemporal relative abundance distribution of all OTUs associated with the network containing thaumarchaeal 16S-OTU3.

correlated in relative abundance dynamics to three other OTUs of high connectivity (≥ 4 edges): *Nitrospira* 16S-OTU86, unclassified *Planctomycetaceae* 16S-OTU57 and unclassified bacterium 16S-OTU6. While *Nitrospira* 16S-OTU86 and unclassified *Planctomycetaceae* 16S-OTU57 were generally one order of magnitude less abundant than thaumarchaeal 16S-OTU3, unclassified bacterium 16S-OTU6 was in the same abundance range (6%–19%; Fig. 5B). A detailed phylogenetic analysis identified 16S-OTU6 as member of the CL500-11 clade within the *Chloroflexi* and 16S-OTU57 as member of the plaI-D clade within the *Planctomycetaceae* (Fig. S10). Further phylogenetic analysis of hypolimnion network OTUs with low taxonomic resolution identified 16S-OTUs 16, 17 and 94 as members of the *Planctomycetes* freshwater clades CL500-52, CL500-3 and CL500-37; 16S-OTU42 as member of the *Ignavibacteria*; and 16S-OTUs 78 and 152 as members of the *Verrucomicrobia* (Fig. S10).

Discussion

A single species-level OTU of *Thaumarchaeota* constituted the overwhelmingly dominating fraction of microorganism known to catalyse ammonia oxidation (Lehtovirta-Morley, 2018) in the water column of Lake Constance. It strongly shaped a rather stable bacterioplankton community in the hypolimnion. Ammonia-oxidizing *Betaproteobacteria* were typically two orders of magnitude less abundant than *Thaumarchaeota*; and Commamox bacteria could not be detected at all. Our results compare well to other studies, which found a predominance of AOA in lakes of oligotrophic state (Hou *et al*., 2013; Hugoni *et al*., 2013; Vissers *et al*., 2013a; Mukherjee *et al*., 2016; Okazaki and Nakano, 2016; Yang *et al*., 2016) and a strong increase in their relative abundance towards the hypolimnion except in winter when the whole water column is equally cold and mixed (Urbach *et al*., 2001; Callieri *et al*., 2016; Mukherjee *et al*., 2016; Pollet *et al*., 2018). However, in other lakes with a predominating AOA population, alpha diversity of dominant AOA phylotypes was typically higher, for example, two in Lake Maggiore (Coci *et al*., 2015), five in Lake Lucerne (Vissers *et al*., 2013b) and up to 10 in Lake Redon (Auguet *et al*., 2012; Restrepo-Ortiz *et al*., 2014). In contrast, thaumarchaeal 16S-OTU3 constituted always between 98.6% and 99.5% of all identified ammonia-oxidizing microorganisms in the hypolimnion of Lake Constance (Table S1) with very limited microdiversity within this OTU (Fig. S8).

The observed continuous maintenance of the large population of this single Thaumarachaeon is very intriguing. The rather stable conditions in the hypolimnion as compared with the epi- and metalimnion (Fig. 1) certainly select for less variability in the bacterioplankton community as was also evident from the rather stable alpha and beta diversity in this water body in our study (Fig. 2, Fig. S3). Still, one would expect population breakdowns of the rather clonal thaumarchaeal population either by grazing, for example, by heterotropic or mixotrophic flagelates, or by viral attack that should allow for a more diverse ammonia-oxidizing thaumarchaeal community (Wetzel, 2001). Active grazing upon planktonic *Thaumarchaeota* was experimentally proven for marine heterotrophic nanoflagellates (De Corte *et al*., 2019) and mixotrophic freshwater flagellates (Ballen-Segura *et al*., 2017), with the latter even positively selecting for *Archaea*. Interestingly, freshwater *Actinobacteria* of the acII clade can escape nanoflagellate grazing by specific cell surface structures (S layer; Tarao *et al*., 2009). This crystalline protein or glycoprotein layer was also found in *Thaumarchaeota* (Stieglmeier *et al*., 2014a), thus potentially protecting them against specific flagellates as well. The recent description of metagenome-assembled contigs carrying both thaumarchaeal *amoC* and viral capsid genes in marine waters (Roux *et al*., 2016; Ahlgren *et al*., 2019), the presence of putative viral genes (Ahlgren *et al*., 2017) and proviruses (Krupovic *et al*., 2011; Abby *et al*., 2018) in the genomes of various *Thaumarchaeota* including the marine and planktonic *Candidatus* Nitrosomarinus catalina SPOT01, and the first isolation of viruses infecting *Nitrosopumilis* spp. (Kim *et al*., 2019) proved that thaumarchaeal populations are vulnerable to viral attack. However, the genome of *Candidatus N. catalina* SPOT01 also harboured genes for (genomic) DNA modification by phosphorothioation that may act in concert with an associated DNA restriction system for defence from foreign DNA (Dnd defence system), which was postulated to function as viral defence (Ahlgren *et al*., 2017). In addition, members of the *Nitrosphaerales* were shown to possess genes for a Clustered Regularly Interspaced Short Palindromic Repeats-dependent virus defence system (Stieglmeier *et al*., 2014a). Whether thaumarchaeal 16S-OTU3 is well adapted to resist flagellate grazing or viral attacks, rapidly recovers its populations after top-down control events, or is not lysed after viral infection as shown for viruses infecting *Nitrosopumilis* spp. (Kim *et al*., 2019) remains currently open. Alternatively and maybe serving as the most parsimonious explanation, the dilute nature of thaumarchaeal populations (10^3–10^5 cells ml^{-1}) in the hypolimnion of pre-alpine lakes (Callieri *et al*., 2016) may simply pose a physical barrier for efficient control by flagellate grazing or viral attack.

Most studied lakes including Lake Constance have in common that the dominating AOA populations were always affiliated to the *Nitrosopumilales* (formerly called Marine Group 1.1a; Auguet *et al*., 2012; Vissers *et al*., 2013b; Coci *et al*., 2015; Mukherjee *et al*., 2016), which

is a similar situation as observed in marine waters (Alves *et al*., 2018). However, a recent meta-analysis of published thaumarchaeal *amoA* sequences indicated that freshwater and marine AOA typically fall into different clades within the *Nitrosopumilales* (Alves *et al*., 2018). The *Nitrosopumilales* harbour cultured AOA with the lowest reported K_m-values for total ammonia. Those include *Nitrosopumilus maritimus* (K_m-value = 0.13 μM; Martens-Habbena *et al*., 2009) and *Candidatus* Nitrosoarchaeum koreensis (K_m-value = 0.61 μM; Park *et al*., 2010). In contrast, cultured members of the *Nitrososphaerales* have much higher K_m-values (6 μM), which are further exceeded by cultured AOB (30 μM–10 mM; Kits *et al*., 2017). Since total ammonium concentrations in Lake Constance water were always below 1 μM (in the hypolimnion below 0.5 μM, Fig. 1) and the detected thaumarchaeal 16S-OTU3 was closely related to Cand. *Nitrosopumilus oxyclinae* (Fig. 3), the observed pre-dominance of AOA affiliated to the *Nitrosopumilales* in Lake Constance and other oligotrophic lakes makes sense.

Based on the prevailing total ammonia concentrations in Lake Constance and the reported K_m-value of 0.84 μM total ammonia for *N. inopinata* (Kits *et al*., 2017), one could have expected the presence of comammox in Lake Constance waters as well. However, we found no indication of the latter. The reason may be founded in the hypothesis that comammox are typical K-strategists being rather adapted to slow growth but higher yield as based on the theory of optimal pathway length (Costa *et al*., 2006). Indeed, the comammox *N. inopinata* was shown to gain higher growth yields per mole ammonia at the drawback of lower maximum ammonia oxidation rates as compared with AOAs (Kits *et al*., 2017). Combined with the higher substrate affinity of *Nitrosopumilales*-AOA and the high turnover of the bacterioplankton biomass in the open water column of Lake Constance (1–13 days in the hypolimnion, Simon, 1988), comammox would probably not be able to sustain a stable population in Lake Constance waters. Therefore, the most parsimonious explanation for the ecological role of the detected *Nitrospira* OTUs is an involvement in nitrite oxidation to complement ammonia oxidation for nitrification. Nevertheless, the strong abundance discrepancy to the thaumarchaeal population (one order of magnitude lower, Fig. 3) indicates that additional nitrite oxidizing microorganisms must exist in Lake Constance waters.

The association network analysis revealed that 16S-OTU6, which affiliated to the CL500-11 clade within the *Chloroflexi* (Fig. S10), was of a similar relative abundance as thaumarchaeal 16S-OTU3 and that the spatio-temporal relative abundance distribution of both OTUs strongly correlated (Fig. 5). CL500-11 *Chloroflexi* have been identified as one of the dominating bacterioplankton lineages typical for the oxygenated hypolimnion of deep oligotrophic and mesotrophic lakes (Urbach *et al*., 2001; Okazaki *et al*., 2013; Denef *et al*., 2016; Okazaki and Nakano, 2016; Okazaki *et al*., 2017). Metagenome-assembled genomes of CL500-11 representatives from ultra-oligotrophic Lake Michigan encoded genes for DOM uptake and protein/peptide turnover, which were also expressed as shown in a parallel metatranscriptome analysis of hypolimnion waters (Denef *et al*., 2016). The involvement in protein/peptide turnover with subsequent release of the ammonia group from processed amino acids may explain the tight correlation of thaumarchaeal 16S-OTU3 to CL500-11 *Chloroflexi* OTU6 observed in our study. Alternatively, it is tempting to speculate that CL500-11 *Chloroflexi* may play a role in nitrite oxidation in the hypolimnion of Lake Constance. The *Chloroflexi* harbour *Nitrolancea hollandica* as a cultured nitrite oxidizer, which however was only distantly related to 16S-OTU6 (78% sequence identity; Fig. S10) and is characterized by a rather high K_m-value of 1 mM for nitrite (Sorokin *et al*., 2012; Sorokin *et al*., 2014). In addition, CL500-11 representatives showed typically a higher relative abundance than *Thaumarchaeota* across the hypolimnia of 10 Japanese lakes (Okazaki *et al*., 2017), which stands in contrast to the situation in Lake Constance (Fig. 5). Nevertheless, future studies should follow the hypothesis that the *Chloroflexi* may harbour additional nitrite oxidizers that are rather adapted to the extremely low nitrite concentrations prevailing in freshwaters.

Thaumarchaeal 16S-OTU3 correlated also strongly to *Planctomycetaceae* 16S-OTU57. Besides the *Thaumarchaeota* and *Chloroflexi*, the *Planctomycetes* freshwater clades CL500-3, CL500-15, CL500-37 and plaI-A were shown to constitute abundant bakterioplankton groups characteristic for oxygenated hypolimnion waters (Okazaki *et al*., 2017). In contrast, *Planctomycetaceae* 16S-OTU57 was affiliated to the *Planctomycetes* freshwater clade plaI-D (Fig. S10). Members of clade plaI-D were previously shown to correlate in relative abundance to *Thaumarchaeota* across 10 Japanese lakes (Okazaki *et al*., 2017). However, little can be said about their physiology at the moment.

A question remaining is whether the dominating thaumarchaeal species in Lake Constance is indeed involved in ammonia oxidation. Its large relative population size (Fig. 5), the detection of just one thaumarchaeal *amoA* OTU (Fig. 4), the concomitant decrease of total ammonia with increasing AOA population sizes from the epilimnion towards the hypolimnion (Figs. 1 and 5), and the measured active nitrification by ^{15}N-isotope dilution (Fig. S1) cumulatively indicate that this single thaumarchaeal species is indeed driving nitrification in the open water column. Depth-resolved nitrification rate measurements in oligotrophic Lake Superior showed that the increasing thaumarchaeal population with depth is paralleled with increasing nitrification rates (Small *et al*., 2013). Light-inhibited growth,

presence of reactive oxygen species, preferential predation by mixotrophic flagellates, and competition for NH_4^+ with photoautotrophs and heterotrophic microorganisms have been suggested as factors controlling low thaumarchaeal populations in the euphotic zone (Small *et al*., 2013; Tolar *et al*., 2016; Ballen-Segura *et al*., 2017; Horak *et al*., 2018). Future studies will have to show whether the dominating *Thaumarchaeon* in Lake Constance could also use urea instead of ammonia as a primary substrate for nitrification as observed in marine environments (Alonso-Sáez *et al*., 2012; Tolar *et al*., 2017). Since AOA are autotrophic microorganisms (Könneke *et al*., 2005; de la Torre *et al*., 2008; Hatzenpichler *et al*., 2008; Tourna *et al*., 2011) they also fix CO_2 and thus have an impact on primary production of an ecosystem. Indeed, *Thaumarchaeota* were shown to be responsible for 28% of dark CO_2-fixation in deep and oligotrophic Lake Maggiore (Callieri *et al*., 2014). A similar situation can be expected for Lake Constance. Considering that approximately 80% of the water body of Lake Constance is represented by the hypolimnion, the identified *Thaumarchaeon* may play an important role not only for nitrogen cycling but also for the carbon budget of this important drinking water reservoir.

Experimental procedures

Sampling procedure

Water samples were taken on regular cruises to the Lake Constance monitoring site of the University of Konstanz (47°45′27.19″ N 9°7′44.60″ E). The water depth at this site is c. 140 m. General characteristics of Lake Constance are described in the Supporting Information. The sampling period spanned the year 2015 encompassing one yearly plankton succession (Fig. S2). Composition and biomass of phytoplankton in the upper 20 m of the water column were determined weekly or bi-weekly using direct counting under an inverted microscope as described previously (Gaedke, 1998). For molecular analyses, three water depths were sampled in quadruplicates using a 10 l Niskin bottle: the epilimnion at 1 m, the metalimnion at 15–16 m and the hypolimnion at 85 m. On board, water samples were pre-filtered through a 70-μm and 30-μm mesh (Franz Eckert GmbH, Germany) to remove large detritus particles and eukaryotic organisms. Water samples were transported to the laboratory in light-tight (black) plastic containers. In the laboratory, they were stored according to the sampling water temperature at either 4°C or 15°C until further processing the same or the following day. Environmental parameters and gross nitrification were determined as described in Supporting Information.

16S rRNA gene amplicon sequencing and analysis

Water samples were filtered consecutively through a 5.0-μm and a 0.1-μm polycarbonate filter [Merck (formerly Millipore), Darmstadt, Germany] using a peristaltic pump to capture the respective size classes of microorganisms. For each water sample, 6 l were filtered through three filter sets in parallel, which were combined at the level of their size class for DNA extraction. After filtration, filters were frozen at −20°C. A phenol and bead-beating based DNA extraction was adapted from Loy and colleagues (2005) and is described in detail in Supporting Information. Bacterial and archaeal 16S rRNA genes were amplified from total DNA extracts according to the manual of the NEXTflex 16S V4 amplicon sequencing kit 2.0 (Bioo Scientific, Austin, TX). In brief, 5 ng of total extracted DNA were used as template for amplifying (10 cycles, annealing at 56°C) the V4 region of bacterial and archaeal 16S rRNA genes using the universal primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′; Caporaso *et al*., 2011). Both primers were elongated by oligonucleotide adaptors for subsequent introduction of barcodes and Illumina sequencing adaptors by a second PCR (12 cycles, annealing at 60°C). Amplicon sequencing was performed on the Illumina MiSeq platform using paired-end sequencing (2 × 300 bp) based on the MiSeq Reagents kit v3. Amplicon reads were processed in mothur v.1.39.5 (Schloss *et al*., 2009) including quality control, merging of paired-end reads, removal of unique singletons, de novo chimera filtering using UCHIME (Edgar *et al*., 2011), de novo clustering of OTUs at 97% sequence identity, and removal of OTUs with fewer than three reads to discriminate against sequencing artefacts. This resulted in 8.25 million high-quality paired-end reads forming 5780 species-level OTUs (97% identity). For microdiversity analysis, amplicon reads affiliated to *Thaumarchaeota* were also analysed at the level of unique sequence types and 99% sequence identity. All amplicon sequences were deposited at the Sequence Read Archive at National Center for Biotechnology Information (NCBI) under the bioproject number PRJNA464048. Phylogenetic analysis of representative 16S rRNA gene OTUs is detailed in Supporting Information.

All statistical analyses of 16S rRNA gene amplicons were performed using the program R, version 3.6.0. (R-Core-Team, 2019), using the R packages phyloseq 1.28.0 (McMurdie and Holmes, 2013), multcomp (Hothorn *et al*., 2008; Herberich *et al*., 2010), vegan 2.5.5; (Oksanen *et al*., 2013), igraph 1.2.4.1 (Csárdi and Nepusz, 2006) and ggplot2 3.2.0 (Wickham, 2009). Details are given in Supporting Information.

amoA *gene analysis*

DNA was extracted from water sampled at 14 m and 85 m (19 August 2014) and 15 m and 85 m (13 January 2015) using the sampling procedure and DNA extraction

protocol as described above. DNA extracts originating from 0.1 to 5.0 μm size fraction and from the same sampling date were pooled for amplifying thaumarchaeal and beta-proteobacterial *amoA* genes and subsequent cloning. Details are given in Supporting Information. All sequences were submitted to NCBI under the accession numbers MH780501-MH780654. Terminal restriction fragment length polymorphism (T-RFLP) analyses of thaumarchaeal and betaproteobacterial *amoA* PCR products were done as described previously (Pester *et al*., 2010). In brief: 7–10 ng of purified and 5-carboxyfluorescein (FAM)-labelled *amoA* PCR product were digested with the restriction enzyme *Hha*I (Thermo Fischer Scientific). An *in silico analysis* found this enzyme to discriminate well between the obtained *amoA* OTUs.

qPCR of thaumarchaeal *amoA* genes were done using the primer pair CamoA420F (5′-CGT ACT GGT AGG AAT GTC-3′; this study) and CamoA616R (5′-GCC ATC CAB CKR TAN GTC CA-3′; Tourna *et al*., 2008). Primer CamoA420F was designed in ARB (Ludwig *et al*., 2004) to specifically target thaumarchaeal *amoA* genes retrieved from the water column of Lake Constance. In parallel, the 16S rRNA genes of total Bacteria and Archaea were analysed using the universal primer pair 1389F (5′-TGY ACA CAC CGC CCG T-3′) and 1492R (5′-GGY TAC CTT GTT ACG ACT T-3′; Hausmann *et al*., 2016). Details for both qPCR assays are provided in Supporting Information.

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Conflict of Interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Mean relative abundance of all observed 16S rRNA gene OTUs at the approximate species-level (97% identity) across the spatio-temporal sampling regime of the water column in the year 2015. In addition, the resolved taxonomy and representative sequence are provided for each OTU. Provided as separate Excel file.
Data S1: Supporting information