Received: 11 November 2023 | Accepted: 8 March 2024

DOI: 10.1111/1462-2920.16616

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY

Out of sight, but not out of season: Nitrifier distributions and population dynamics in a large oligotrophic lake

Logan M. Peoples[1] | Miranda H. Seixas[1] | Kate A. Evans[1] | Evan M. Bilbrey[1,2] | John R. Ranieri[1] | Tyler H. Tappenbeck[1] | John E. Dore[3] | Adam Baumann[1] | Matthew J. Church[1]

[1]Flathead Lake Biological Station, University of Montana, Polson, Montana, USA

[2]Department of Biological Sciences, Idaho State University, Pocatello, Idaho, USA

[3]Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, Montana, USA

Correspondence
Matthew J. Church, Flathead Lake Biological Station, University of Montana, Polson, MT, USA.
Email: matt.church@flbs.umt.edu

Funding information
National Science Foundation, Grant/Award Numbers: 1950963, 1951002

Abstract

Nitrification is an important control on the form and distribution of nitrogen in freshwater ecosystems. However, the seasonality of nitrogen pools and the diversity of organisms catalyzing this process have not been well documented in oligotrophic lakes. Here, we show that nitrogen pools and nitrifying organisms in Flathead Lake are temporally and vertically dynamic, with nitrifiers displaying specific preferences depending on the season. While the ammonia-oxidizing bacteria (AOB) Nitrosomonadaceae and nitrite-oxidizing bacteria (NOB) *Nitrotoga* dominate at depth in the summer, the ammonia-oxidizing archaea (AOA) Nitrososphaerota and NOB Nitrospirota become abundant in the winter. Given clear seasonality in ammonium, with higher concentrations during the summer, we hypothesize that the succession between these two nitrifying groups may be due to nitrogen affinity, with AOB more competitive when ammonia concentrations are higher and AOA when they are lower. Nitrifiers in Flathead Lake share more than 99% average nucleotide identity with those reported in other North American lakes but are distinct from those in Europe and Asia, indicating a role for geographic isolation as a factor controlling speciation among nitrifiers. Our study shows there are seasonal shifts in nitrogen pools and nitrifying populations, highlighting the dynamic spatial and temporal nature of nitrogen cycling in freshwater ecosystems.

INTRODUCTION

Nitrification, the biological oxidation of ammonia (NH_3) to nitrate (NO_3^-), is a key component of the nitrogen (N) cycle (Kowalchuk & Stephen, 2001; Prosser, 1990). Nitrification is a major control of the form, distribution, and abundance of nitrogen in lakes (Finlay et al., 2007; Small et al., 2013; Sterner et al., 2007). While some bacteria can perform complete oxidation of ammonia (Daims et al., 2015; van Kessel et al., 2015), this process is typically completed in two steps by chemolithoautotrophic microorganisms: ammonia-oxidizing archaea (AOA) or bacteria (AOB) first oxidize NH_3 to nitrite (NO_2^-), followed by the oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB; e.g., Dworkin & Gutnick, 2012; Könneke et al., 2005). Nitrifiers present in freshwater habitats can include the AOA Nitrososphaerota (commonly known as Thaumarchaeota), AOB of the genera *Nitrosomonas* and *Nitrosospira* within the Nitrosomonadaceae, and NOB of the phylum Nitrospirota, genus *Nitrobacter*, and genus *Nitrotoga* (e.g., Alfreider et al., 2018; Boddicker & Mosier, 2018; Cabello-Yeves et al., 2018, 2020; Hayden & Beman, 2014; Herber et al., 2019; Klotz et al., 2022; Lantz et al., 2021; Podowski et al., 2022; Ngugi et al., 2023). These lineages can represent large (>30%) proportions of lake and ocean prokaryotic communities (e.g., Francis et al., 2005; Karner et al., 2001;

Ngugi et al., 2023), highlighting the importance of these organisms to both N and carbon cycles.

Nitrifying organisms are subject to control by physical, chemical, and biological factors (Daims et al., 2016; Hatzenpichler, 2012). In both lakes and oceans, rates of nitrification and abundances of nitrifiers tend to be greatest in the lower euphotic zone and dark hypolimnetic waters (e.g., Karner et al., 2001; Klotz et al., 2022; Ngugi et al., 2023; Small et al., 2013), in part owing to the photosensitive nature of these microorganisms (e.g., French et al., 2012; Horak et al., 2018; Merbt et al., 2012; Olson, 1981). AOA and AOB communities coexist, but they often exhibit niche separation in natural environments. Prior studies examining whether AOA or AOB predominate in a given environment have documented trophic status, ammonium (NH_4^+) concentration, temperature, light, and pH as correlating with AOA or AOB abundances (e.g., Bollmann et al., 2014; Bouskill et al., 2011, 2012; Fernàndez-Guerra & Casamayor, 2012; Herrmann et al., 2009; Mosier & Francis, 2008; Mukherjee et al., 2016; Nicol et al., 2008; Podowski et al., 2022; Prosser & Nicol, 2012; Vissers et al., 2013a). One of the major controls on the distribution of different clades of nitrifiers is NH_4^+ availability. AOA are typically thought to outcompete AOB in oligotrophic conditions because of their higher affinity for ammonia (e.g., French et al., 2012, 2021; Hink et al., 2018; Jung et al., 2022; Martens-Habbena et al., 2009). Geographic separation can also impact nitrifier diversity, as recent studies have shown high abundances and similar ecotypes of AOA within widespread European lakes (Ngugi et al., 2023) while different nitrifier populations are present in the Great Lakes of North America (Podowski et al., 2022). However, the diversity of nitrifiers in lakes and their distributions across depths and seasons are not well described.

Here, we explored the temporal dynamics of major fixed N pools and the organisms catalysing nitrification in Flathead Lake, Montana, USA, one of the largest natural freshwater lakes in the western United States. Flathead Lake is oligotrophic and persistently oxic (normally >8 mg L^{-1}) with a short hydrologic residence time (~2.2 years; Ellis et al., 2011). Total N concentrations are typically ~8 μM while total phosphorus (P) levels are ~0.1 μM, leading to elevated N:P stoichiometric ratios (Elser et al., 2022). The microorganisms in Flathead Lake appear highly seasonal, with the onset of stratification leading to clear changes in community composition and diversity (Evans et al., 2024). Here, we describe the seasonality and distribution of inorganic N pools and nitrifying organisms in this largely pristine, oligotrophic lake. We find that N and nitrifying organisms are temporally and vertically dynamic, with diverse bacteria and archaea displaying specific preferences depending on the time of year.

EXPERIMENTAL PROCEDURES

Sampling was conducted at the long-term monitoring site Midlake Deep (MLD; 47.867 N, 114.067 W; maximum lake depth, 113 m) in Flathead Lake from aboard the research vessel *Jessie B*. Sampling was performed 15× per year at near-monthly intervals over five years (2014–2019) as part of the Flathead Monitoring Program (FMP). Vertically resolved temperature and oxygen measurements were obtained with a Hydrolab DS5 (OTT HydroMet, Sheffield, UK). The R (R Core Team, 2020) package akima (v0.6-2.3; Akima et al., 2016) was used to interpolate temperature between depths and time points. The mixed layer depth was calculated as the depth where temperature differed by 0.5°C from the average temperature between 0.5 and 3.5 m (Evans et al., 2024). Photosynthetically active radiation (PAR; 400–700 nm) was measured using an underwater spherical quantum sensor (LI-193). Water samples were collected via electric winch using an opaque 3.2 L Van Dorn water sampler affixed to a stainless steel wire cable. Samples were stored on ice in dark coolers during transport back to the laboratory.

Nutrients

NH_4^+ and nitrate + nitrite (NO_3^- and NO_2^-; here referred to as NO_x^-) concentrations were measured at three depths (5, 50, and 90 m) between 2014 and 2019 at MLD. During 2018 and 2019, NO_x^- concentrations were measured at higher vertical resolution in the upper 30 m from 12 depth profiles. Samples were filtered through MilliQ- and lake water-rinsed, 47 mm diameter, 0.45 μm pore size mixed cellulose ester filters and frozen at −20°C until analysis on an Astoria A2 segmented flow analyser (Astoria-Pacific, OR, USA). NH_4^+ concentrations were quantified colorimetrically as indophenol blue via reaction with alkaline phenol and hypochlorite (Standard Methods 4500-NH3 G, Baird et al., 2017). For NO_x determinations, NO_3^- was converted to NO_2^- via cadmium reduction, and total $NO_3^- + NO_2^-$ quantified colorimetrically via Greiss chemistry azo dye formation (Standard Methods 4500-NO3 E, Baird et al., 2017; Strickland & Parsons, 1968). The lower detection limit for NO_x^- and NH_4^+ was 0.11 μM N. N concentrations below this value are reported as the limit of detection.

Estimates of NH_4^+ and NO_x^- entering Flathead Lake from the Flathead River, the major source of water to the lake, were obtained at Sportsman's Bridge at near-monthly intervals during 2014–2019. This location sits ~4 km from where the mouth of the river enters the lake. Water was collected from ~1m depth upstream of the bridge using a 3.2 L Van Dorn water sampler. Sample processing and N concentration measurements were performed as described above. To estimate the flux of NH_4^+ and NO_x^- entering the lake, we used the

R packages rloadest and loadflex (Appling et al., 2015; Runkel et al., 2004). Daily mean discharge was estimated based on measurements in Columbia Falls by the US Geological Survey (USGS 12363000; https://waterdata.usgs.gov/nwis). A regression model and the composite method were used with the commands loadReg2, loadComp, and predictSolute.

Sample collection and extraction for DNA analyses

Water collection and DNA extraction of samples from MLD during 2017–2018 were performed as previously described (Evans et al., 2024; Peoples et al., 2023). Briefly, approximately 1 L was serially filtered through both a 25 mm diameter, 3 μm pore size GTTP polycarbonate filter (EMD Millipore, MA, USA) and a 25 mm diameter, 0.2 μm pore size polyethersulphone filter (SUPOR, Pall Co., NY, USA). DNA from the <3.0 to >0.2 μm fraction was extracted using a MasterPure DNA purification kit (Lucigen, WI, USA). Samples were stored at −80°C prior to subsequent analysis.

16S rRNA gene amplicon sequencing

PCR amplification and sequencing of 16S rRNA gene amplicons were performed as previously described (Evans et al., 2024). Briefly, the 16S rRNA gene V4–V5 region was amplified using the primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT) using reaction conditions previously recommended (Parada et al., 2016). Triplicate PCR reactions were pooled and sequenced at the University of Montana Genomics Core on an Illumina MiSeq. Sequence data were processed using the QIIME2 platform (Bolyen et al., 2019). Sequences were denoised, chimeras were removed, and amplified sequence variants (ASVs) were identified using DADA2 (Callahan et al., 2016). Sequences were classified against the SILVA 138 database (Quast et al., 2013). Further processing was performed using phyloseq (McMurdie & Holmes, 2013) in R. Sequences related to chloroplasts and eukaryotes were removed. Lineages known to perform nitrification were specifically identified, including those related to the phylum Nitrososphaerota (named Crenarchaeota in this version of SILVA), phylum Nitrospirota, family Nitrosomonadaceae, and family Gallionellaceae. Relative abundance pie charts were created using the R package scatterpie v0.2.1 (Yu & Xu, 2023).

Shotgun metagenomic sequencing

To document the microbial diversity and potential for nitrification in Flathead Lake, metagenomic sequencing was conducted from samples collected at MLD during the year 2018 at depths ranging from 5 to 90 m. Sequencing, processing, and annotation were performed as previously described (Peoples et al., 2023). Briefly, raw reads were quality trimmed using Trimmomatic v0.39 (Bolger et al., 2014) and assembled with MEGAHIT v1.2.9 (Li et al., 2015). Open reading frames were identified with Prodigal V2.6.3 (Hyatt et al., 2010) and functional annotation was performed using GhostKOALA (Kanehisa et al., 2016). Ammonia monooxygenase alpha subunit (*amoA*) gene phylogenetic trees were created by sequence alignment using muscle (Edgar, 2004), constructed using FastTree (Price et al., 2010), and visualized using iTOL (Letunic & Bork, 2021). Metagenome-assembled genomes were obtained using MetaBAT 2 v2.11.1 (Kang et al., 2019) and the size and quality of each genome bin were evaluated using CheckM v1.0.13 (Parks et al., 2015). Genomes were taxonomically classified with GTDB-tk v1.6.0 (Chaumeil et al., 2019) using KBase (Arkin et al., 2018). Whole-genome trees were created using concatenated single-copy marker genes identified and aligned using CheckM, constructed using FastTree, and displayed using iTOL. Average nucleotide identity (ANI) comparisons were performed using OrthoANI (Yoon et al., 2017). The relative representation of each bin within each metagenome was estimated by read recruitment using Bowtie 2 v2.3.5.1 (Langmead & Salzberg, 2012) and SAMtools v1.10 (Li et al., 2009) to obtain the number of reads mapped per kilobase million reads (RPKM). Clinker was used to compare gene operons (Gilchrist & Chooi, 2021).

Droplet digital PCR

To quantify the abundances of ammonia-oxidizing organisms in Flathead Lake, we performed droplet digital PCR (ddPCR) to amplify the *amoA* gene using the QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Inc., CA, USA). Using shotgun metagenomics, we identified three abundant *amoA* gene sequences that clustered into three separate clades: one member of the archaeal phylum Nitrososphaerota and two members of the bacterial family Nitrosomonadaceae. ddPCR assays were run using the EvaGreen Digital PCR Supermix with Droplet Generation Oil for EvaGreen (Bio-Rad Laboratories). PCR reactions (20 μL final volume) consisted of 1× Supermix, 0.5 μM forward primer, 0.5 μM reverse primer, and 2 μL DNA. Droplets were generated using a QX200™ Bio-Rad Droplet Generator and the droplet-PCR mixture cycled on a Bio-Rad 1000 Touch™ Thermal Cycler. The Arch-amoAFA and Arch-amoAR primer set described by Beman et al. (2008) was used to amplify archaeal *amoA* genes. Reaction cycling conditions included 95°C for 4 min followed by 35 cycles at 95°C for 30 s, 56°C for 45 s, and 72°C for 60 s. For AOB *amoA* sequences, primers were designed based on metagenomic lake sequences modified from those described in Rotthauwe et al. (1997). Primers were named based on the genus-level clades they amplified: clade VFJL01 (F 5′-GGGTTTCTACTGGTGGT-3′, R 5′-CCCCTCAGCAAATCCTTCCTC-3′) and BJGV01 (F 5′-GGGCTTCTACTGGTGGT-3′, R 5′-CCCCTCTGGAAAGCCTTCTTC-3′). Primer specificity was checked in silico using Primer-BLAST (Ye et al., 2012) against representative sequences related to the Nitrosomonadaceae. While the VFJL01 primer pair appears highly specific to this clade, the BJGV01 primer pair is also identical to sequences belonging to other divergent members of the Nitrosomonadaceae (Figure A1). Therefore, we urge caution in applying these primers to lakes without first evaluating *amoA* gene diversity. PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, with one final hold at 72°C for 7 min. No template control (NTC) reactions were included for each set of reactions. Results were analysed in QuantaSoft Analysis Pro v1.0.596.

RESULTS

NH_4^+ and NO_x^- concentrations were measured monthly at three depths (5, 50, and 90 m) over 5 years (2014–2019) in Flathead Lake. N pools varied seasonally (Figure 1**)**. NH_4^+ concentrations began to increase at all depths in May, reaching maximum concentrations in June and July. Concentrations were greatest at 50 and 90 m, peaking near 0.75 μM. The timing and concentrations of NH_4^+ and NO_x^- are consistent with riverine input being a major source of N to the lake (Figure A2). By August, NH_4^+ had declined to <0.15 μM at all depths. Concentrations of NO_x^- were ~3.5 μM throughout the water column during the winter when the lake was mixed. As the lake stratified, NO_x^- decreased in the epilimnion (5 m), falling below detection in August, September, and October. High-resolution sampling in the upper 30 m showed that NO_x^- rapidly accumulated below the mixed layer (Figure A3). In the hypolimnetic waters (50 and 90 m), NO_x^- accumulated throughout the summer and fall (July–October), reaching concentrations as high as 7.5 μM. As the mixed layer deepened in the winter, NO_x^- concentrations subsequently increased at the surface and decreased at depth, consistent with mixing-driven homogenisation of the water column.

To identify the distributions and abundances of nitrifiers across water depths and seasons, we used shotgun metagenomics, 16S rRNA gene amplicon sequencing, ddPCR amplification of *amoA* genes, and binning of metagenome-assembled genomes (MAGs) from samples obtained in the year 2018. To initially identify lineages capable of performing ammonia oxidation, the first step in nitrification, metagenomes were searched for *amoA* genes. We identified three dominant, unique *amoA* genes; two related to the bacterial family Nitrosomonadaceae (similar to *Nitrosospira*) and one related to the archaeal phylum Nitrososphaerota (alternatively called Thaumarchaeota). These sequences are identical to (Nitrososphaerota, VJFL01 Nitrosomonadaceae) or share >99.8% identity (BJGV01 Nitrosomonadaceae) with those from the Great Lakes (Figure A1; Podowski et al., 2022). Identification of these lineages in the amplicon 16S rRNA gene dataset revealed both groups showed clear seasonal and vertical dynamics. Generally, the family Nitrosomonadaceae represented ~2% of the Flathead Lake prokaryotic community while the Nitrososphaerota composed less than 1% (Figure 2). Relative abundances of both the AOB and AOA were greatest in the hypolimnion; however, deep winter mixing appeared to vertically homogenize the relative abundances of both AOB and AOA. At depth (50 and 90 m), Nitrosomonadaceae increased in relative abundance in July and remained elevated throughout the summer before decreasing in December and January, at least in part due to dilution by mixing with shallower waters. Nitrosomonadaceae remained present in the shallower lake waters prior to the onset of stratification in May and June. In contrast, Nitrososphaerota represented a lower fraction of the summer community but increased in relative abundance in the winter (November–March). While the Nitrososphaerota was represented by only one dominant ASV, the family Nitrosomonadaceae was composed primarily of two ASVs but also included others at lower percentages (Figure A4). This suggests there could be other, low-abundance members of the Nitrosomonadaceae within Flathead Lake.

For additional insight into the diversity of ammonia-oxidizing organisms, we performed metagenome-assembled genome binning. Because of the high similarity between distinct ecotypes of nitrifiers and the apparent importance of auxiliary genes, we did not dereplicate genomes between samples (e.g., Evans & Denef, 2020). Average nucleotide identity comparisons and genome phylogenies revealed multiple near-identical MAGs (suggesting they are the same organism at different depths and time points) from three apparent ammonia-oxidizing organisms in Flathead Lake: two related to the Nitrosomonadaceae and one related to the *Nitrosarchaeum* within the Nitrososphaerota (Figure 3; Table S1). The two Nitrosomonadaceae species were identified as members of the genus-level clades VFJL01 (five representative MAGs) and BJGV01 (four representative MAGs) based on whole-genome GTDB-tk annotation. These clades were previously designated as NspGL2b and NspGL1, respectively, in the Great Lakes (Podowski et al., 2022). We continue to use these labels here as ecotype designations where applicable. No *amoA* genes were binned within the VFJL01 genomes (Figure A5). Podowski et al. (2022) also reported difficulty binning *amo* and other nitrification-related genes,

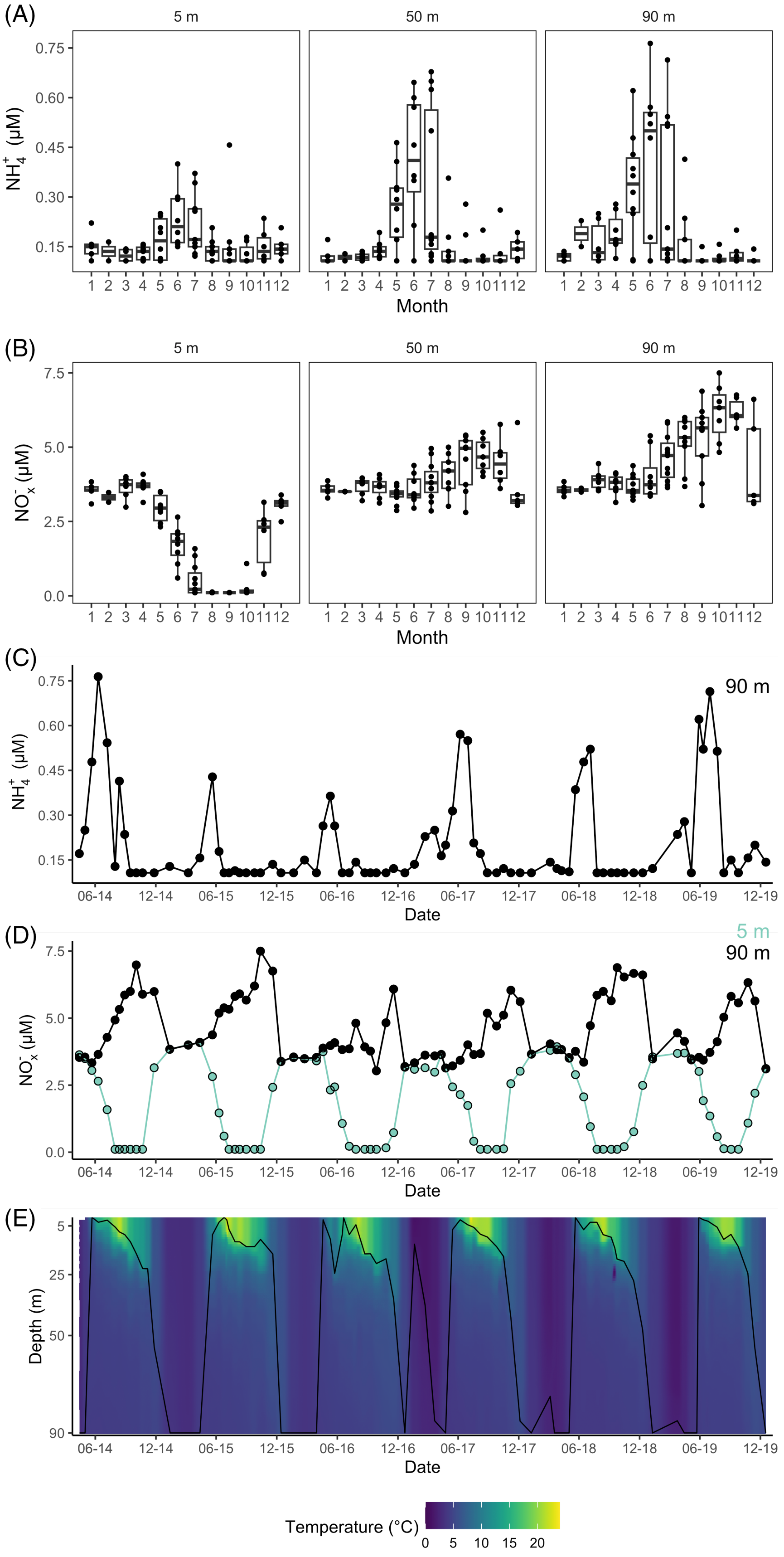


FIGURE 1 Concentrations of NH_4^+, NO_x^- (nitrate + nitrite), and temperature show seasonal variability in Flathead Lake (2014–2019). (A, B) NH_4^+ and NO_x^- concentrations binned monthly (January = 1 to December = 12). (C, D) NH_4^+ (90 m) and NO_x^- (5 m, blue; 90 m, black) concentrations at discrete depths over time. (E) Vertically-resolved temperature profiles over time. The black line represents the depth of the mixed layer.

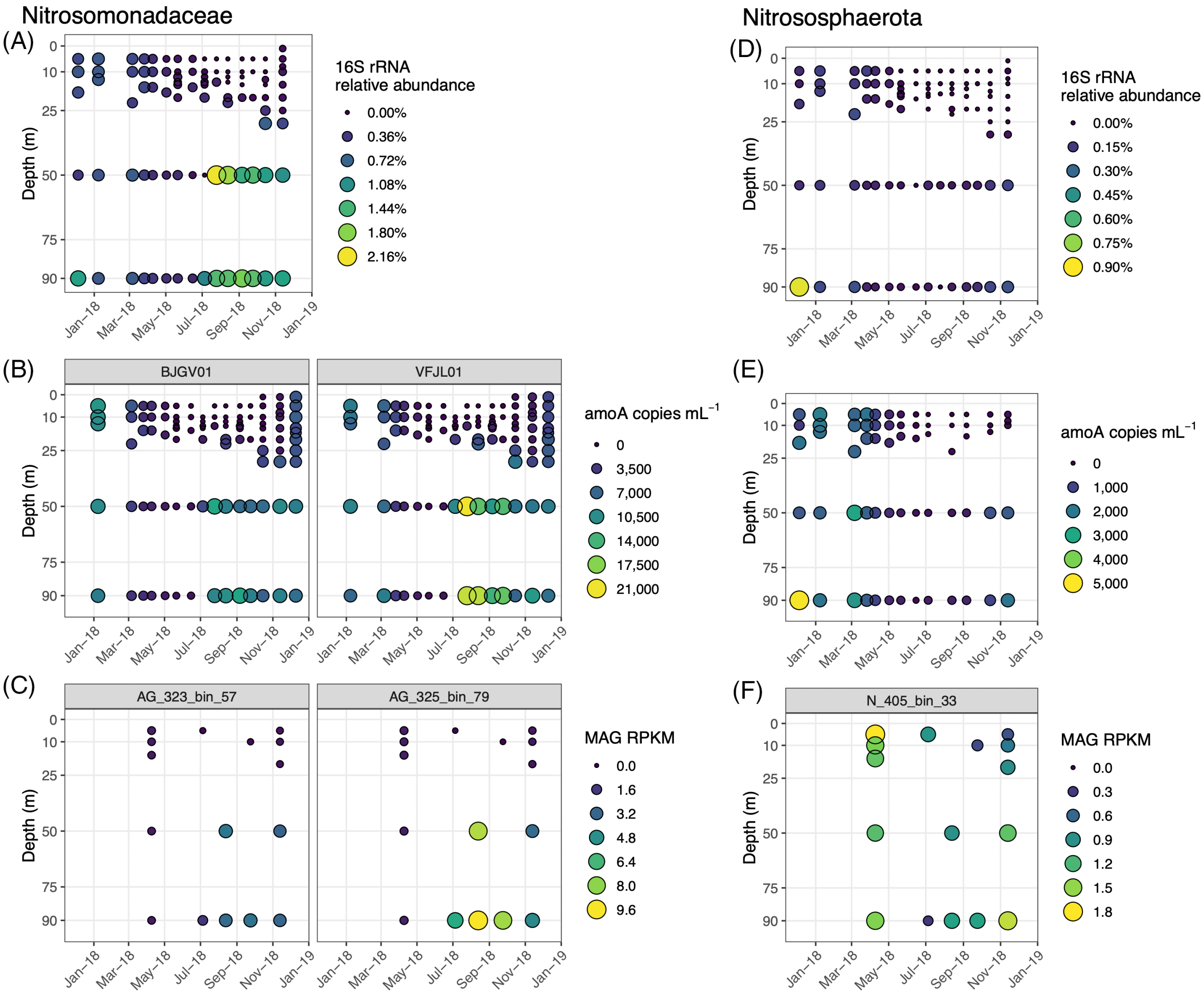


FIGURE 2 Ammonia-oxidizing organisms show seasonal succession in Flathead Lake. Amplicon 16S rRNA gene relative abundances (A, D), droplet digital PCR abundances of the ammonia monooxygenase subunit A (*amoA*) gene (B, E), and read recruitment against representative metagenome-assembled genomes (C, F) of members of the Nitrosomonadaceae (A–C) and Nitrososphaerota (D–F). Genomes in C reflect members of the BJGV01 and VFJL01 clades, respectively. Note that the scales are different between plots. MAG, metagenome-assembled genome; RPKM, reads per kilobase million reads.

likely due to assembly issues of nearby genes or multiple closely related gene copies. Metagenomic read recruitment against representative Flathead Lake genomes revealed the same temporal and depth-dependent patterns as that obtained using 16S rRNA gene amplicon sequencing (Figure 2). Flathead Lake MAGs of all three ammonia-oxidizing organisms were similar to those reported from other oligotrophic lakes, especially those within the Great Lakes, sharing >99.5% ANI. Comparative genomics revealed that the Flathead BJGV01 Nitrosomonadaceae have a number of genes that are missing in the more-abundant VFJL01 clade, including those involved in (1) the use of alternative nitrogen sources, such as *ureABCDEFG* and *eutMN* for urea and ethanolamine, respectively, and (2) light harvesting and light damage, including a proteorhodopsin for ATP generation and DNA photolyase for repairing UV-induced DNA damage (Figure A5). Proteorhodopsin operon comparisons showed this gene cluster is similar to that present in genomes from the Great Lakes (Figure A6).

We next used ddPCR to amplify *amoA* genes and quantitatively estimate the abundances of nitrifier populations in the lake. Ammonia monooxygenase gene sequences obtained from the metagenomes related to the Nitrosomonadaceae clustered into two distinct clades, consistent with MAG analysis (Figure A1). In Flathead Lake, the *amoA* gene representing clade VFJL01 (ecotype NspGL2b) within the Nitrosomonadaceae was the most abundant, with peak abundances exceeding 21,000 copies mL^{-1}, while *amoA* gene abundances of BJGV01 (NspGL1) were nearly 2-fold lower (∼12,800 copies mL^{-1}; Figure 2). Although some AOB have multiple copies of the *amoA* gene

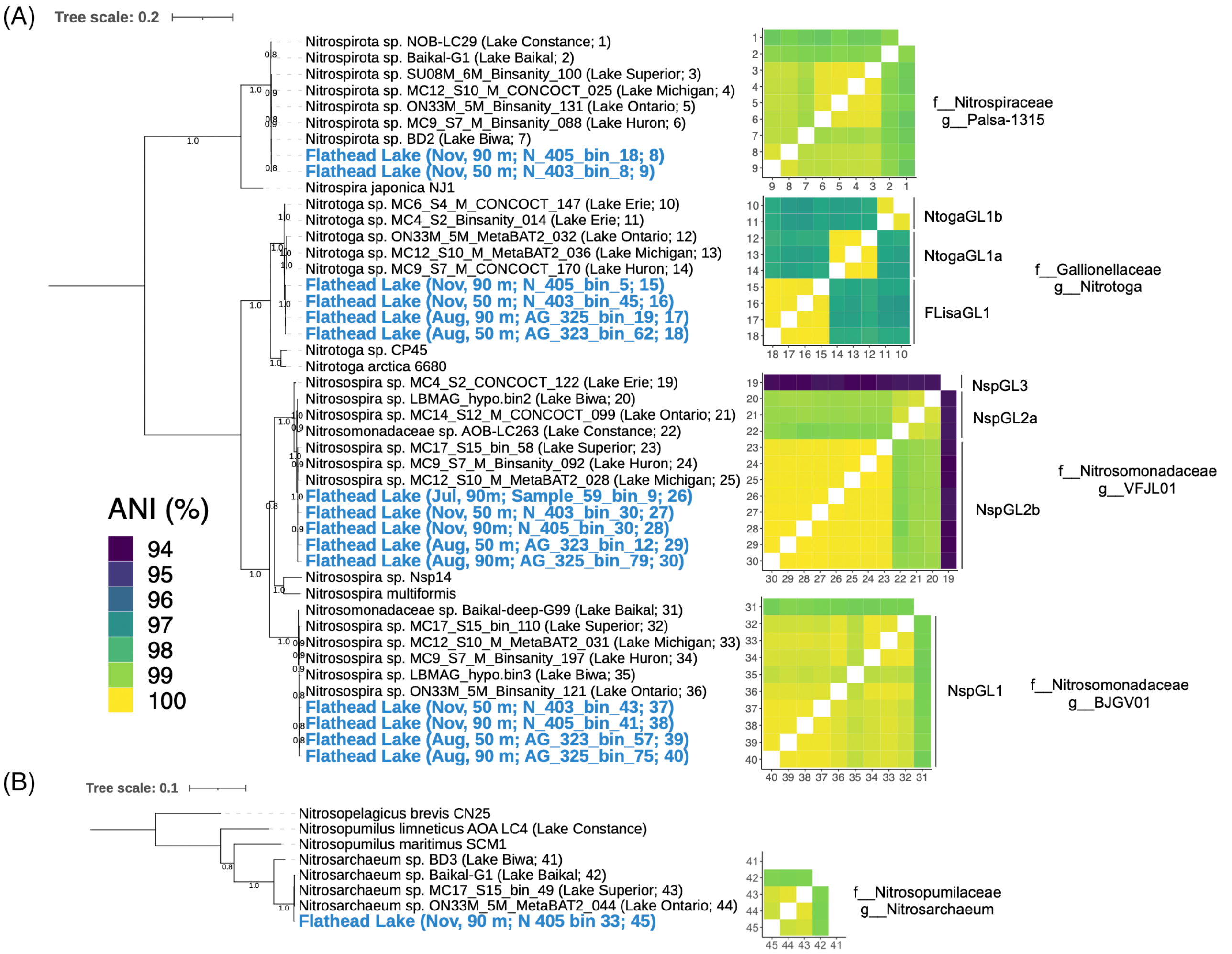


FIGURE 3 Flathead Lake nitrifiers appear near-identical to those found in other lakes, sharing more than 99% ANI. Whole genome marker gene phylogenetic trees and average nucleotide identity (ANI) comparisons of bacterial (A) and archaeal (B) nitrifiers, including ammonia oxidizers (Nitrosomonadaceae, Nitrosopumilaceae) and nitrite oxidizers (Nitrospiraceae, Gallionellaceae). ANI comparison numbers correspond to numbers in the phylogenetic tree labels. ANI colours are the same in both A and B. White (empty) boxes reflect same-genome comparisons or ANI lower than 94%. Putative subspecies ecotype delineations (Nsp##, FL##) and taxonomic names at the family (f) and genus (g) levels are listed next to their respective clades.

(Klotz, 1998; Norton et al., 1996), assuming a single gene copy per cell and using total cell abundances previously reported (Evans et al., 2024), maximum abundances of each clade represent 3.4%, and 2.9% of the total cellular community, with a maximum total combined abundance of ~6.0% in September at 90 m. Ammonium monooxygenase genes deriving from the archaeal Nitrososphaerota were the least abundant of the targeted groups (peak of ~5000 copies mL^{-1}; 1% of all cells with maxima December–March). Overall, the *amoA* gene distributions showed similar seasonal and depth-specific patterns as that obtained based on 16S rRNA gene amplicon sequencing, with the Nitrosomonadaceae most abundant in the hypolimnion during the summer, while Nitrososphaerota peaked in the fall and winter.

We also investigated the dynamics of organisms responsible for the second step of nitrification, nitrite oxidation. Based on 16S rRNA gene amplicon sequencing and genome binning, two nitrite-oxidizing organisms were identified that were related to members of the phylum Nitrospirota and the genus *Nitrotoga* (family Gallionellaceae; Figure 3; Table S1). Amplicon 16S rRNA gene sequencing and metagenomic read recruitment revealed similar seasonal and depth patterns for these lineages as observed for the ammonia oxidizers (Figures 4 and 5). Both groups demonstrated peak relative abundances in the hypolimnion, with the Gallionellaceae most abundant throughout the summer (relative abundances up to ~1.4%), and Nitrospirota elevated in the fall. Analysis of 16S rRNA amplicons indicated that both the phylum Nitrospirota and the family Gallionellaceae were dominated by only one ASV each (Figure A4). The Flathead Lake Nitrospirota (two representative MAGs) shared >99% ANI with Great Lakes MAGs (Figure 3). In contrast, the Flathead Lake

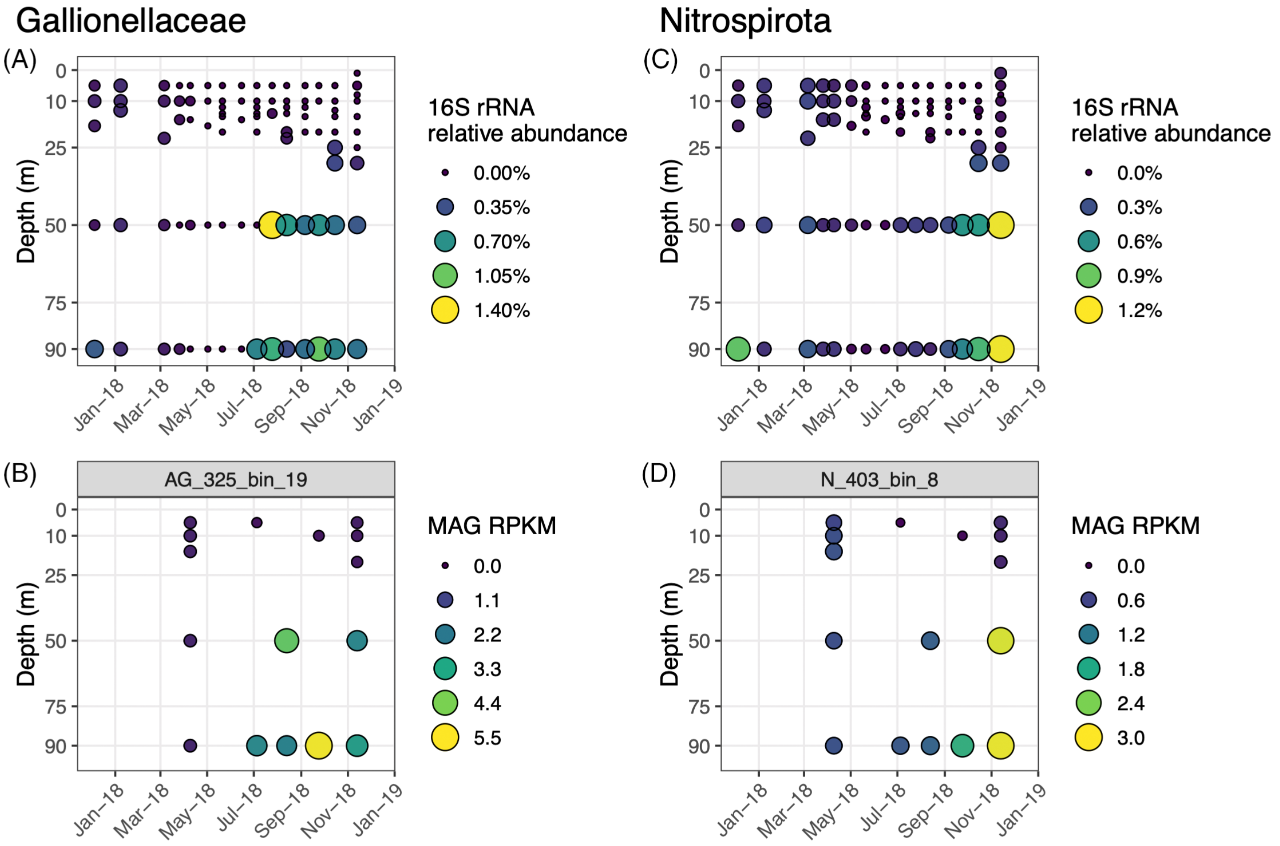


FIGURE 4 Nitrite-oxidizing organisms show seasonal succession at depth in Flathead Lake. Amplicon 16S rRNA gene relative abundances (A, C) and read recruitment against representative metagenome-assembled genomes (B, D) of members of the family Gallionellaceae (genus *Nitrotoga*; A, B) and the phylum Nitrospirota (C, D). Note that the scales are different between plots. MAG, metagenome-assembled genome; RPKM, reads per kilobase million reads.

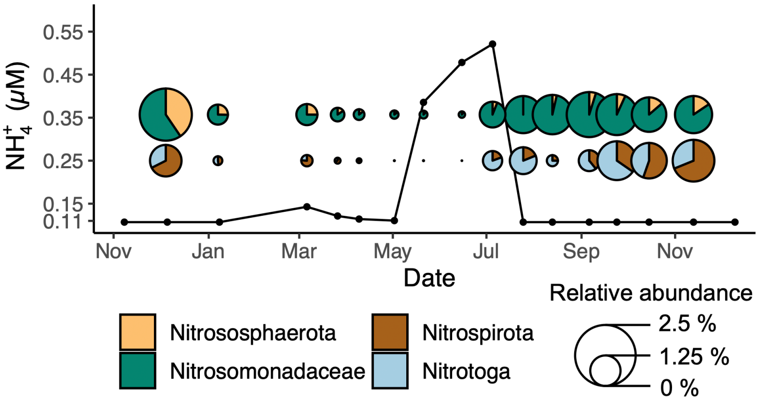


FIGURE 5 Ammonium (NH_4^+) and nitrifier dynamics at 90 m depth in Flathead Lake during the year 2018. The line graph shows NH_4^+ over time. Circle sizes reflect relative abundances of ammonia oxidizers (top) or nitrite oxidizers (bottom) based on 16S rRNA gene amplicon community sequencing at 90 m depth throughout 2018. The colours within each pie chart reflect the relative taxonomic composition of ammonia or nitrite oxidizers.

Nitrotoga (four representative MAGs) were more distinct, sharing ∼97.8% ANI to other genomes, consistent with a new subspecies ecotype. While Flathead Lake is a great lake, it is not a Great Lake, and therefore we designate this ecotype as *FLisaGL1*. Comparative genomics revealed the *phr* gene encoding a DNA photolyase was present in both the Nitrospirota and *Nitrotoga* genomes (Figure A5). While members of the Nitrospirota can be capable of complete ammonia oxidation (commamox; Daims et al., 2015; van Kessel et al., 2015) in some rivers and lakes (e.g., Harringer & Alfreider, 2021; Liu et al., 2020; Lu et al., 2020), we did not identify *amoABC* or *hao* genes related to the Nitrospirota in the metagenomes or in our MAGs. This suggests the Nitrospirota detected here in Flathead Lake are likely not capable of commamox.

DISCUSSION

In this study, we show that NH_4^+, NO_x^-, and the organisms catalysing nitrification in Flathead Lake vary seasonally with strong depth-dependent patterns through the water column. Maximum NH_4^+ concentrations in Flathead Lake ($\sim$0.7 μM) are low but similar to those reported in other oligotrophic lakes where nitrification has been studied, including Lake Superior (mean $\sim$ 0.2 μM, maximum $\sim$ 1.1 μM; Kumar et al., 2007; Small et al., 2013) and Lake Constance (maximum $\sim$1 μM; Herber et al., 2019; Klotz et al., 2022). In contrast, maximum NO_x^- concentrations in Flathead Lake (7 μM) appear substantially lower than other lakes, where concentrations exceed 20 μM and can reach 70 μM (e.g., Finlay et al., 2007; Herber et al., 2019; Klotz et al., 2022; Sterner et al., 2007). The observed seasonal oscillations of NH_4^+ and NO_x^- provide insight into the timing between key aspects of N cycling. The near complete drawdown of both NH_4^+ and NO_x^- between August and October in the epilimnion of Flathead Lake suggests microbial growth could become N limited during periods of the summer and fall (Dodds et al., 1989, 1991). Interestingly, peak NO_x^- concentrations in the hypolimnion appeared offset in time relative to the drawdown of NH_4^+; specifically, NH_4^+ appeared largely consumed throughout the water column by August, while NO_x^- concentrations in the hypolimnion peaked in October or November. Furthermore, the seasonal maximum NO_x^- concentrations in the hypolimnion were greater than concentrations of NH_4^+. We suspect these patterns provide insight into the time scales of nitrogen assimilation into organic nitrogen (as biomass), recycling to NH_4^+, and eventual oxidation by nitrifiers to NO_2^- and NO_3^-. Specifically, these findings may point to the rapid coupling between the production and consumption of NH_4^+ such that it may be produced throughout the summer and fall but that it is consumed faster than it can accumulate. Such dynamics would be “invisible” when tracking pools of these substrates (as performed here) but not when tracking rates. The overall strong seasonality and low concentrations of N species in Flathead Lake are consistent with the lakes oligotrophic character, short residence time, and undeveloped surrounding watershed.

Using three distinct but complementary techniques, our findings indicate nitrifying microorganisms in Flathead Lake demonstrate unique seasonal dynamics. We identified three ammonia oxidizers—two members of the Nitrosomonadaceae and one archaeal *Nitrosarchaeum* species—and two nitrite oxidizers—members of the genus *Nitrotoga* and phylum Nitrospirota—in Flathead Lake. These organisms showed distinct seasonal cycles consistent with linked dynamics among the two groups; the AOB Nitrosomonadaceae and NOB *Nitrotoga* are present in the summer when NH_4^+ concentrations are highest, while AOA *Nitrosarchaeum* and NOB Nitrospirota appear in the fall and winter. Previous studies describe apparent seasonality among nitrifying organisms in lakes, including changes in diversity (e.g., Auguet et al., 2011; Fujimoto et al., 2016; Hampel et al., 2018; Okazaki & Nakano, 2016; Vissers et al., 2013a, 2013b) and the importance of nitrification in the winter, especially under ice (e.g., Cavaliere & Baulch, 2019; Massé et al., 2019; Powers et al., 2017a, 2017b). In the hypolimnion of Flathead Lake, seasonal changes can include differences in oxygen content ($\sim$9.5–12 mg L^{-1}), temperature ($\sim$2–6°C), and pH ($\sim$7–8), all of which may contribute to shifts in community composition. We hypothesize that changes in nitrifier distributions in part reflect differences in the affinity for N between these two seasonal groups. Previous studies point to AOB generally having a lower affinity for ammonia, preferring higher concentrations, compared to AOA which tend to have higher substrate affinities and are often more abundant when ammonia concentrations are low (French et al., 2012, 2021; Martens-Habbena et al., 2009). The lower end of AOB ammonia affinities is $\sim$0.1–1 μM, consistent with the highest concentrations seen in Flathead Lake in the summer and fall, while AOA substrate affinities are typically 2–3 orders of magnitude lower (Jung et al., 2022). Similar observations have been made between NOB *Nitrotoga* and Nitrospirota species, which show preferences for higher and lower concentrations of nitrite, respectively (e.g., Boddicker & Mosier, 2018; Keuter et al., 2022; Kinnunen et al., 2017; Kitzinger et al., 2018; Nowka et al., 2015; Wegen et al., 2019). Given that ammonia-oxidizing and nitrite-oxidizing organisms can show tightly coupled metabolic cross-feeding (e.g., Daims et al., 2016; Graham et al., 2007; Juretschko et al., 1998; Knapp & Graham, 2007; Koch et al., 2015; Maixner et al., 2006), our work provides evidence that species-specific temporal and spatial coupling between nitrifiers may be present in lakes.

Our findings suggest that highly-similar, low-diversity nitrifier populations may exist across North American lakes. Quasi-clonal, low-diversity nitrifying *Nitrosopumilus* archaeal populations are present throughout Europe, suggesting low rates of diversification despite geographic isolation and differences in lake age and ecology (Ngugi et al., 2023). Flathead Lake genomes, including members of the *Nitrosarchaeum*, Nitrosomonadaceae, and Nitrospirota, share >99.5% ANI with ecotypes from the oligotrophic Great Lakes, well beyond the typical 95% species threshold (Kim et al., 2014; Olm et al., 2020). Indeed, a putatively horizontally transferred rhodopsin gene in North American Nitrosomonadaceae BJGV01 genomes (Podowski et al., 2022), including MAGs from Flathead Lake, is absent in Eurasian genomes despite sharing 99% ANI. This gene transfer event therefore likely occurred before the geographic isolation of Flathead Lake and Great Lakes species but after their divergence from those in Europe and Asia. These populations may have been seeded with similar subglacial nitrifiers following the retreat of North American ice sheets ~20 kya. We note exceptions to these shared populations exist. One notable example is Lake Erie, which has distinct nitrifiers likely due to significantly higher NH_4^+ and nutrient content than the other Great Lakes (e.g., Hampel et al., 2019; Podowski et al., 2022). Another exception appears to be the *Nitrotoga*, as Flathead Lake genomes share only ~98% ANI to other published genomes and therefore likely represent a distinct ecotype. Further, the presence of multiple Nitrosomonadaceae species within the same lakes suggests discrete niches for similar organisms. While both ammonia and nitrite oxidizers peaked in abundance in the dark waters of the hypolimnion during the summer, consistent with previously reported distributions of these organisms (e.g., Auguet et al., 2012; Herber et al., 2019; Mincer et al., 2007; Ngugi et al., 2023), the apparent ecotype-specific distributions of genes associated with light exposure or urea use suggest this may be a potential avenue for niche speciation (Podowski et al., 2022; Qin et al., 2024). While we did not observe clear depth or temporal differences in the distributions of the two Nitrosomonadaceae clades in the summer, BJGV01 is equally or more abundant than VFJL01 during the winter when nitrifier distributions are homogenous at all depths. Rhodopsins and photolyases for fixing light-induced DNA damage may therefore provide BJGV01 a competitive advantage when the lake is fully mixed and these organisms are exposed to light, as has been suggested for deep-adapted Chloroflexi (Denef et al., 2016). Therefore, while nitrifiers appear highly similar in many lakes and reflect continental-scale geographic isolation, different groups may be undergoing speciation at different rates, driven perhaps by differences in N concentrations and seasonal dynamics.

One observation from our study that appears contrary to reports from other relatively deep, temperate lakes is that AOA represented a relatively small proportion of the Flathead Lake prokaryotic community. While our findings are consistent with abundances reported in the Great Lakes, in various lakes across Europe AOA can reach >30% of communities (e.g., Cabello-Yeves et al., 2020; Callieri et al., 2016; Klotz et al., 2022; Ngugi et al., 2023), similar to proportions of AOA reported at depth in the open ocean (Karner et al., 2001). While nitrifiers can show patchy dynamics (Paver et al., 2020), our evidence suggests that the 2018 community was likely representative of other years, with N concentrations and temperature showing reproducible patterns across our time series. If NH_4^+ concentrations and affinities underlie the temporal patterns in AOB and AOA in Flathead Lake, we might have expected higher abundances of the presumably oligotrophic AOA given the relatively low concentrations of NH_4^+. However, some eutrophic lakes can show high abundances of AOA (Hampel et al., 2020), and not all AOA have high affinities for ammonia (Jung et al., 2022). Furthermore, it was recently reported that some ammonia oxidizers prefer urea to ammonia (Qin et al., 2024), suggesting changes in N substrate type may control nitrifier distributions. Therefore, while we hypothesize that ammonia concentrations may be in part responsible for seasonal dynamics, future work will be needed to understand the variables that control the distributions and abundances of these organisms in freshwater ecosystems.

AUTHOR CONTRIBUTIONS

Logan M. Peoples: Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; formal analysis. **Miranda H. Seixas:** Methodology; writing – review and editing; formal analysis; investigation. **Kate A. Evans:** Investigation; writing – review and editing; methodology; formal analysis. **Evan M. Bilbrey:** Investigation; methodology; writing – review and editing; formal analysis. **John R. Ranieri:** Investigation; writing – review and editing; methodology; formal analysis. **Tyler H. Tappenbeck:** Investigation; methodology; writing – review and editing; formal analysis. **John E. Dore:** Conceptualization; writing – review and editing; funding acquisition; investigation; methodology. **Adam Baumann:** Conceptualization; investigation; writing – review and editing; methodology; formal analysis. **Matthew J. Church:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; methodology; supervision.

ACKNOWLEDGEMENTS

This work was performed within the territory of the Bitterroot Salish, Upper Pend d'Oreille, and Kootenai tribes, within the Flathead Reservation of the Confederated Salish and Kootenai Tribes. Permits for sampling of Flathead Lake were issued by the Confederated Salish and Kootenai Tribes and we are grateful for their partnership. Thanks to Jim Craft, Sydni Racki, Tom Bansak, James Elser, Emma Wear, and all other individuals who have contributed to the Flathead Monitoring Program. FMP receives support from the state of Montana and philanthropic donations. This work was funded in part by the National Science Foundation grants DEB-1951002 and 1950963.

CONFLICT OF INTEREST STATEMENT

The authors disclose that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All sequence data is publicly available at NCBI under BioProject accession numbers PRJNA948362: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA948362 and PRJNA973100: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA973100. Temperature, nutrient concentrations, oxygen, and light measurements are available through the Flathead Lake Biological Station FMP website: https://flbs.umt.edu/newflbs/research/flbs-public-data.

ORCID

Logan M. Peoples https://orcid.org/0000-0002-0163-2769

REFERENCES

Akima, H., Gebhardt, A., Petzold, T. & Maechler, M. (2016) Package 'akima', v0.6-2.3.

Alfreider, A., Grimus, V., Luger, M., Ekblad, A., Salcher, M.M. & Summerer, M. (2018) Autotrophic carbon fixation strategies used by nitrifying prokaryotes in freshwater lakes. *FEMS Microbiology Ecology*, 94, fiy163. Available from: https://doi.org/10.1093/femsec/fiy163

Appling, A.P., Leon, M.C. & McDowell, W.H. (2015) Reducing bias and quantifying uncertainty in watershed flux estimates: the R package loadflex. *Ecosphere*, 6, 1–25. Available from: https://doi.org/10.1890/ES14-00517.1

Arkin, A.P., Cottingham, R.W., Henry, C.S., Harris, N.L., Stevens, R.L., Maslov, S. et al. (2018) KBase: the United States Department of Energy Systems Biology Knowledgebase. *Nature Biotechnology*, 36, 566–569. Available from: https://doi.org/10.1038/nbt.4163

Auguet, J.C., Nomokonova, N., Camarero, L. & Casamayor, E.O. (2011) Seasonal changes of freshwater ammonia-oxidizing archaeal assemblages and nitrogen species in oligotrophic alpine lakes. *Applied and Environmental Microbiology*, 77, 1937–1945. Available from: https://doi.org/10.1128/AEM.01213-10

Auguet, J.C., Triadó-Margarit, X., Nomokonova, N., Camarero, L. & Casamayor, E.O. (2012) Vertical segregation and phylogenetic characterization of ammonia-oxidizing archaea in a deep oligotrophic lake. *The ISME Journal*, 6, 1786–1797. Available from: https://doi.org/10.1038/ismej.2012.33

Baird, R.B., Eaton, A.D., Rice, E.W. (Eds.). (2017) 4500-NH3 nitrogen (ammonia). In: *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association: Washington (DC), USA. Available from: https://doi.org/10.2105/SMWW.2882.087

Baird, R.B., Eaton, A.D., Rice, E.W. (Eds.). (2017) 4500-NO3-nitrogen (nitrate). In: *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association: Washington (DC), USA. Available from: https://doi.org/10.2105/SMWW.2882.089

Beman, J.M., Popp, B.N. & Francis, C.A. (2008) Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. *The ISME Journal*, 2, 429–441. Available from: https://doi.org/10.1038/ismej.2007.118

Boddicker, A.M. & Mosier, A.C. (2018) Genomic profiling of four cultivated Candidatus *Nitrotoga* spp. predicts broad metabolic potential and environmental distribution. *The ISME Journal*, 12, 2864–2882. Available from: https://doi.org/10.1038/s41396-018-0240-8

Bolger, A.M., Lohse, M. & Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30, 2114–2120. Available from: https://doi.org/10.1093/bioinformatics/btu170

Bollmann, A., Bullerjahn, G.S. & McKay, R.M. (2014) Abundance and diversity of ammonia-oxidizing archaea and bacteria in sediments of trophic end members of the Laurentian Great Lakes, Erie and Superior. *PLoS ONE*, 9, e97068. Available from: https://doi.org/10.1371/journal.pone.0097068

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A. et al. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 37, 852–857. Available from: https://doi.org/10.1038/s41587-019-0209-9

Bouskill, N.J., Eveillard, D., Chien, D., Jayakumar, A. & Ward, B.B. (2011) Environmental factors determining ammonia-oxidizing organism distribution and diversity in marine environments. *Environmental Microbiology*, 14, 714–729. Available from: https://doi.org/10.1111/j.1462-2920.2011.02623.x

Bouskill, N.J., Tang, J., Riley, W.J. & Brodie, E.L. (2012) Trait-based representation of biological nitrification: model development, testing, and predicted community composition. *Frontiers in Microbiology*, 3, 364. Available from: https://doi.org/10.3389/fmicb.2012.00364

Cabello-Yeves, P.J., Zemskaya, T.I., Rosselli, R., Coutinho, F.H., Zakharenko, A.S., Blinov, V.V. et al. (2018) Genomes of novel microbial lineages assembled from the sub-ice waters of Lake Baikal. *Applied and Environmental Microbiology*, 84, e02132-17. Available from: https://doi.org/10.1128/AEM.02132-17

Cabello-Yeves, P.J., Zemskaya, T.I., Zakharenko, A.S., Sakirko, M.V., Ivanov, V.G., Ghai, R. et al. (2020) Microbiome of the deep Lake Baikal, a unique oxic bathypelagic habitat. *Limnology and Oceanography*, 65, 1471–1488. Available from: https://doi.org/10.1002/lno.11401

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. & Holmes, S.P. (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods*, 13, 581–583. Available from: https://doi.org/10.1038/nmeth.3869

Callieri, C., Hernández-Avilés, S., Salcher, M.M., Fontaneto, D. & Bertoni, R. (2016) Distribution patterns and environmental correlates of Thaumarchaeota abundance in six deep subalpine lakes. *Aquatic Sciences*, 78, 215–225. Available from: https://doi.org/10.1007/s00027-015-0418-3

Cavaliere, E. & Baulch, H.M. (2019) Winter nitrification in ice-covered lakes. *PLoS One*, 14, e0224864. Available from: https://doi.org/10.1371/journal.pone.0224864

Chaumeil, P.A., Mussig, A.J., Hugenholtz, P. & Parks, D.H. (2019) GTDB-Tk: a toolkit to classify genomes with the genome taxonomy database. *Bioinformatics*, 36, 1925–1927. Available from: https://doi.org/10.1093/bioinformatics/btz848

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M. et al. (2015) Complete nitrification by *Nitrospira* bacteria. *Nature*, 528, 504–509. Available from: https://doi.org/10.1038/nature16461

Daims, H., Lücker, S. & Wagner, M. (2016) A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends in Microbiology*, 24, P699–P712. Available from: https://doi.org/10.1016/j.tim.2016.05.004

Denef, V.J., Mueller, M.S., Chiang, E., Liebig, J.R. & Vanderploeg, H.A. (2016) Chloroflexi CL500-11 populations that predominate deep-lake hypolimnion bacterioplankton rely on nitrogen-rich dissolved organic matter metabolism and C_1 compound oxidation. *Applied and Environmental Microbiology*, 82, 1423–1432. Available from: https://doi.org/10.1128/AEM.03014-15

Dodds, W.K., Johnson, K.R. & Priscu, J.C. (1989) Simultaneous nitrogen and phosphorus deficiency in natural phytoplankton assemblages: theory, empirical evidence, and implications for lake management. *Lake and Reservoir Management*, 5, 21–26. Available from: https://doi.org/10.1080/07438148909354677

Dodds, W.K., Priscu, J.C. & Ellis, B.K. (1991) Seasonal uptake and regeneration of inorganic nitrogen and phosphorus in a large oligotrophic lake: size-fractionation and antibiotic treatment. *Journal of Plankton Research*, 13, 1339–1358. Available from: https://doi.org/10.1093/plankt/13.6.1339

Dworkin, M. & Gutnick, D. (2012) Sergei Winogradsky: a founder of modern microbiology and the first microbial ecologist. *FEMS Microbiology Reviews*, 36, 364–379. Available from: https://doi.org/10.1111/j.1574-6976.2011.00299.x

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792–1797. Available from: https://doi.org/10.1093/nar/gkh340

Ellis, B.K., Stanford, J.A., Goodman, D., Stafford, C.P., Gustafson, D.L., Beauchamp, D.A. et al. (2011) Long-term effects of a trophic cascade in a large lake ecosystem. *Proceedings of the National Academy of Sciences*, 108, 1070–1075. Available from: https://doi.org/10.1073/pnas.1013006108

Elser, J.J., Devlin, S.P., Baumann, A., Church, M.J., Dore, J.E., Hall, R.O., Jr. et al. (2022) Sustained stoichiometric imbalance and its ecological consequences in a large oligotrophic lake. *Proceedings of the National Academy of Sciences*, 119, e2202268119. Available from: https://doi.org/10.1073/pnas.2202268119

Evans, J.T. & Denef, V.J. (2020) To dereplicate or not to dereplicate? *mSphere*, 5(3), e00971-19. Available from: https://doi.org/10.1128/msphere.00971-19

Evans, K.A., Peoples, L.M., Ranieri, J.R., Wear, E.K. & Church, M.J. (2024) Mixing-driven changes in distributions and abundances of planktonic microorganisms in a large, oligotrophic lake. *Limnology and Oceanography*. Available from: https://doi.org/10.1002/lno.12509

Fernàndez-Guerra, A. & Casamayor, E.O. (2012) Habitat-associated phylogenetic community patterns of microbial ammonia oxidizers. *PLoS One*, 7, e47330. Available from: https://doi.org/10.1371/journal.pone.0047330

Finlay, J.C., Sterner, R.W. & Kumar, S. (2007) Isotopic evidence for in-lake production of accumulating nitrate in Lake Superior. *Ecological Applications*, 17, 2323–2332. Available from: https://doi.org/10.1890/07-0245.1

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E. & Oakley, B.B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences*, 102, 14683–14688. Available from: https://doi.org/10.1073/pnas.0506625102

French, E., Kozlowski, J.A. & Bollmann, A. (2021) Competition between ammonia-oxidizing archaea and bacteria from freshwater environments. *Applied and Environmental Microbiology*, 87, e01038-21. Available from: https://doi.org/10.1128/AEM.01038-21

French, E., Kozlowski, J.A., Mukherjee, M., Bullerjahn, G. & Bollmann, A. (2012) Ecophysiological characterization of ammonia-oxidizing archaea and bacteria from freshwater. *Applied and Environmental Microbiology*, 78, 5773–5780. Available from: https://doi.org/10.1128/AEM.00432-12

Fujimoto, M., Cavaletto, J., Liebig, J.R., McCarthy, A., Vanderploeg, H.A. & Denef, V.J. (2016) Spatiotemporal distribution of bacterioplankton functional groups along a freshwater estuary to pelagic gradient in Lake Michigan. *Journal of Great Lakes Research*, 42, 1036–1048. Available from: https://doi.org/10.1016/j.jglr.2016.07.029

Gilchrist, C.L.M. & Chooi, Y.H. (2021) Clinker & clustermap.js: automatic generation of gene cluster comparison figures. *Bioinformatics*, 37, 2473–2475. Available from: https://doi.org/10.1093/bioinformatics/btab007

Graham, D.W., Knapp, C.W., Van Vleck, E.S., Bloor, K., Lane, T.B. & Graham, C.E. (2007) Experimental demonstration of chaotic instability in biological nitrification. *The ISME Journal*, 1, 385–393. Available from: https://doi.org/10.1038/ismej.2007.45

Hampel, J.J., McCarthy, M.J., Aalto, S.L. & Newell, S.E. (2020) Hurricane disturbance stimulated and altered ammonia oxidizer community structure in Lake Okeechobee and St. Lucie Estuary (Florida). *Frontires in Microbiology*, 11, 1541. Available from: https://doi.org/10.3389/fmicb.2020.01541

Hampel, J.J., McCarthy, M.J., Gardner, W.S., Zhang, L., Xu, H., Zhu, G. et al. (2018) Nitrification and ammonium dynamics in Taihu Lake, China: seasonal competition for ammonium between nitrifiers and cyanobacteria. *Biogeosciences*, 15, 733–748. Available from: https://doi.org/10.5194/bg-15-733-2018

Hampel, J.J., McCarthy, M.J., Neudeck, M., Bullerjahn, G.S., McKay, R.M.L. & Newell, S.E. (2019) Ammonium recycling supports toxic Planktothrix blooms in Sandusky Bay, Lake Erie: evidence from stable isotope and metatranscriptome data. *Harmful Algae*, 81, 42–52. Available from: https://doi.org/10.1016/j.hal.2018.11.011

Harringer, M. & Alfreider, A. (2021) Primer evaluation and development of a droplet digital PCR protocol targeting *amoA* genes for the quantification of Comammox in lakes. *Scientific Reports*, 11, 2982. Available from: https://doi.org/10.1038/s41598-021-82613-6

Hatzenpichler, R. (2012) Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. *Applied and Environmental Microbiology*, 78, 7501–7510. Available from: https://doi.org/10.1128/AEM.01960-12

Hayden, C.J. & Beman, J.M. (2014) High abundances of potentially active ammonia-oxidizing bacteria and archaea in oligotrophic, high-altitude lakes of the Sierra Nevada, California, USA. *PLoS One*, 9, e111560. Available from: https://doi.org/10.1371/journal.pone.0111560

Herber, J., Klotz, F., Frommeyer, B., Weis, S., Straile, D., Kolar, A. et al. (2019) A single Thaumarchaeon drives nitrification in deep oligotrophic Lake Constance. *Environmental Microbiology*, 22, 212–228. Available from: https://doi.org/10.1111/1462-2920.14840

Herrmann, M., Saunders, A.M. & Schramm, A. (2009) Effect of lake trophic status and rooted macrophytes on community composition and abundance of ammonia-oxidizing prokaryotes in freshwater sediments. *Applied and Environmental Microbiology*, 75, 3127–3136. Available from: https://doi.org/10.1128/AEM.02806-08

Hink, L., Gubry-Rangin, C., Nicol, G.W. & Prosser, J.I. (2018) The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. *The ISME Journal*, 12, 1084–1093. Available from: https://doi.org/10.1038/s41396-017-0025-5

Horak, R.E.A., Qin, W., Bertagnolli, A.D., Nelson, A., Heal, K.R., Han, H. et al. (2018) Relative impacts of light, temperature, and reactive oxygen on thaumarchaeal ammonia oxidation in the North Pacific Ocean. *Limnology and Oceanography*, 63, 741–757. Available from: https://doi.org/10.1002/lno.10665

Hyatt, D., Chen, G.L., LoCascio, P.F., Land, M.L., Larimer, F.W. & Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*, 11, 119. Available from: https://doi.org/10.1186/1471-2105-11-119

Jung, M.Y., Sedlacek, C.J., Kits, K.D., Mueller, A.J., Rhee, S.K., Hink, L. et al. (2022) Ammonia-oxidizing archaea possess a wide range of cellular ammonia affinities. *The ISME Journal*, 16, 272–283. Available from: https://doi.org/10.1038/s41396-021-01064-z

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Röser, A., Koops, H.P. et al. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Applied and Environmental Microbiology*, 64, 3042–3051. Available from: https://doi.org/10.1128/AEM.64.8.3042-3051.1998

Kanehisa, M., Sato, Y. & Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *Journal of Molecular Biology*, 428, 726–731. Available from: https://doi.org/10.1016/j.jmb.2015.11.006

Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H. et al. (2019) MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ*, 7, e7359. Available from: https://doi.org/10.7717/peerj.7359

Karner, M.B., DeLong, E.F. & Karl, D.M. (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature*, 409, 507–510. Available from: https://doi.org/10.1038/35054051

Keuter, S., Koch, H., Sass, K., Wegen, S., Lee, N., Lücker, S. et al. (2022) Some like it cold: the cellular organization and physiological limits of cold-tolerant nitrite-oxidizing *Nitrotoga*. *Environmental Microbiology*, 24, 2059–2077. Available from: https://doi.org/10.1111/1462-2920.15958

Kim, M., Oh, H.S., Park, S.C. & Chun, J. (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology*, 64, 346–351. Available from: https://doi.org/10.1099/ijs.0.059774-0

Kinnunen, M., Gülay, A., Albrechtsen, H.J., Dechesne, A. & Smets, B.F. (2017) *Nitrotoga* is selected over *Nitrospira* in newly assembled biofilm communities from a tap water source community at increased nitrite loading. *Environmental Microbiology*, 19, 2785–2793. Available from: https://doi.org/10.1111/1462-2920.13792

Kitzinger, K., Koch, H., Lücker, S., Sedlacek, C.J., Herbold, C., Schwarz, J. et al. (2018) Characterization of the first "Candidatus *Nitrotoga*" isolate reveals metabolic versatility and separate evolution of widespread nitrite-oxidizing bacteria. *mBio*, 9, e01186-18. Available from: https://doi.org/10.1128/mbio.01186-18

Klotz, M.G. (1998) Multiple copies of ammonia monooxygenase (amo) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. *FEMS Microbiology Letters*, 168, 303–311. Available from: https://doi.org/10.1111/j.1574-6968.1998.tb13288.x

Klotz, F., Kitzinger, K., Ngugi, D.K., Büsing, P., Littmann, S., Kuypers, M.M.M. et al. (2022) Quantification of archaea-driven freshwater nitrification from single cell to ecosystem levels. *The ISME Journal*, 16, 1647–1656. Available from: https://doi.org/10.1038/s41396-022-01216-9

Knapp, C.W. & Graham, D.W. (2007) Nitrite-oxidizing bacteria guild ecology associated with nitrification failure in a continuous-flow reactor. *FEMS Microbiology Ecology*, 62, 195–201. Available from: https://doi.org/10.1111/j.1574-6941.2007.00380.x

Koch, H., Lücker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E. et al. (2015) Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proceedings of the National Academy of Sciences*, 112, 11371–11376. Available from: https://doi.org/10.1073/pnas.1506533112

Könneke, M., Bernhard, A.E., de la Torre, J., Walker, C.B., Waterbury, J.B. & Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*, 437, 543–546. Available from: https://doi.org/10.1038/nature03911

Kowalchuk, G.A. & Stephen, J.R. (2001) Ammonia-oxidizing bacteria: A model for molecular microbial ecology. *Annual Review of Microbiology*, 55, 485–529. Available from: https://doi.org/10.1146/annurev.micro.55.1.485

Kumar, S., Sterner, R.W., Finlay, J.C. & Brovold, S. (2007) Spatial and temporal variation of ammonium in Lake Superior. *Journal of Great Lakes Research*, 33, 581–591. Available from: https://doi.org/10.3394/0380-1330(2007)33[581:SATVOA]2.0.CO;2

Langmead, B. & Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nature Methods*, 9, 357–359. Available from: https://doi.org/10.1038/nmeth.1923

Letunic, I. & Bork, P. (2021) Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Research*, 49, W293–W296. Available from: https://doi.org/10.1093/nar/gkab301

Lantz, M.A., Boddicker, A.M., Kain, M.P., Berg, O.M.C., Wham, C.D., & Mosier, A.C. (2021). Physiology of the nitrite-oxidizing bacterium Candidatus Nitrotoga sp. CP45 enriched from a Colorado river. *Frontiers in Microbiology*, 12, 709371. https://doi.org/10.3389/fmicb.2021.709371

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N. et al. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics*, 25, 2078–2079. Available from: https://doi.org/10.1093/bioinformatics/btp352

Li, D., Liu, C.M., Luo, R., Sadakane, K. & Lam, T.W. (2015) MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*, 31, 1674–1676. Available from: https://doi.org/10.1093/bioinformatics/btv033

Liu, S., Wang, H., Chen, L., Wang, J., Zheng, M., Liu, S. et al. (2020) Commamox *Nitrospira* within the Yangtze River continuum: community, biogeography, and ecological drivers. *The ISME Journal*, 14, 2488–2504. Available from: https://doi.org/10.1038/s41396-020-0701-8

Lu, S., Sun, Y., Lu, B., Zheng, D. & Xu, S. (2020) Change of abundance and correlation of *Nitrospira inopinata*-like commamox and populations in nitrogen cycle during different seasons. *Chemosphere*, 241, 125098. Available from: https://doi.org/10.1016/j.chemosphere.2019.125098

Maixner, F., Noguera, D.R., Anneser, B., Stoecker, K., Wegl, G., Wagner, M. et al. (2006) Nitrite concentration influences the population structure of *Nitrospira*-like bacteria. *Environmental Microbiology*, 8, 1487–1495. Available from: https://doi.org/10.1111/j.1462-2920.2006.01033.x

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R. & Stahl, D.A. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. *Nature*, 461, 976–979. Available from: https://doi.org/10.1038/nature08465

Massé, S., Botrel, M., Walsh, D.A. & Maranger, R. (2019) Annual nitrification dynamics in a seasonally ice-covered lake. *PLoS One*, 14, e0213748. Available from: https://doi.org/10.1371/journal.pone.0213748

McMurdie, P.J. & Holmes, S. (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, 8, e61217. Available from: https://doi.org/10.1371/journal.pone.0061217

Merbt, S.N., Stahl, D.A., Casamayor, E.O., Marti, E., Nicol, G.W. & Prosser, J.I. (2012) Differential photoinhibition of bacterial and archaeal ammonia oxidation. *FEMS Microbiology Letters*, 327, 41–46. Available from: https://doi.org/10.1111/j.1574-6968.2011.02457.x

Mincer, T.J., Church, M.J., Taylor, L.T., Preston, C., Karl, D.M. & DeLong, E.F. (2007) Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific subtropical gyre. *Environmental Microbiology*, 9, 1162–1175. Available from: https://doi.org/10.1111/j.1462-2920.2007.01239.x

Mosier, A.C. & Francis, C.A. (2008) Relative abundance and diversity of ammonia-oxidizing archaea and bacteria in the San Francisco Bay estuary. *Environmental Microbiology*, 10, 3002–3016. Available from: https://doi.org/10.1111/j.1462-2920.2008.01764.x

Mukherjee, M., Ray, A., Post, A.F., McKay, R.M. & Bullerjahn, G.S. (2016) Identification, enumeration and diversity of nitrifying planktonic archaea and bacteria in trophic end members of the Laurentian Great Lakes. *Journal of Great Lakes Research*, 42, 39–49. Available from: https://doi.org/10.1016/j.jglr.2015.11.007

Ngugi, D.K., Salcher, M.M., Andrei, A.S., Ghai, R., Klotz, F., Chiriac, M.C. et al. (2023) Postglacial adaptations enabled colonization and quasi-clonal dispersal of ammonia-oxidizing archaea in modern European large lakes. *Science Advances*, 9, eadc9392. Available from: https://doi.org/10.1126/sciadv.adc9392

Nicol, G.W., Leininger, S., Schleper, C. & Prosser, J.I. (2008) The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environmental Microbiology*, 10, 2966–2978. Available from: https://doi.org/10.1111/j.1462-2920.2008.01701.x

Norton, J.M., Low, J.M. & Klotz, M.G. (1996) The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. *FEMS Microbiology Letters*, 139, 181–188. Available from: https://doi.org/10.1111/j.1574-6968.1996.tb08200.x

Nowka, B., Daims, H. & Spieck, E. (2015) Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. *Applied and Environmental Microbiology*, 91, 745–753. Available from: https://doi.org/10.1128/AEM.02734-14

Okazaki, Y. & Nakano, S.I. (2016) Vertical partitioning of freshwater bacterioplankton community in a deep mesotrophic lake with a fully oxygenated hypolimnion (Lake Biwa, Japan). *Environmental Microbiology Reports*, 8, 780–788. Available from: https://doi.org/10.1111/1758-2229.12439

Olm, M.R., Crits-Cristoph, A., Diamond, S., Lavy, A., Carnevali, P.B.M. & Banfield, J.F. (2020) Consistent metagenome-derived metrics verify and delineate bacterial species boundaries. *mSystems*, 5, e00731-19. Available from: https://doi.org/10.1128/mSystems.00731-19

Olson, R.J. (1981) Differential photoinhibition of marine nitrifying bacteria: possible mechanism for the formation of the primary nitrite maximum. *Journal of Marine Research*, 39(2), 1541. Available from: https://elischolar.library.yale.edu/journal_of_marine_research/1541

Parada, A.E., Needham, D.M. & Fuhrman, J.A. (2016) Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, 18, 1403–1414. Available from: https://doi.org/10.1111/1462-2920.13023

Parks, D.H., Imelfort, M., Skennerton, C., Hugenholtz, P. & Tyson, G.W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research*, 25, 1043–1055. Available from: https://doi.org/10.1101/gr.186072.114

Paver, S.F., Newton, R.J. & Coleman, M.L. (2020) Microbial communities of the Laurentian Great Lakes reflect connectivity and local biogeochemistry. *Environmental Microbiology*, 22, 433–446. Available from: https://doi.org/10.1111/1462-2920.14862

Peoples, L.M., Dore, J.E., Bilbrey, E.M., Vick-Majors, T.J., Ranieri, J.R., Evans, K.A. et al. (2023) Oxic methane production from methylphosphonate in a large oligotrophic lake: limitation by substrate and organic carbon supply. *Applied and Environmental Microbiology*, 89, e01097-23. Available from: https://doi.org/10.1128/aem.01097-23

Podowski, J.C., Paver, S.F., Newton, R.J. & Coleman, M.L. (2022) Genome streamlining, proteorhodopsin, and organic nitrogen metabolism in freshwater nitrifiers. *mBio*, 13, e02379-21. Available from: https://doi.org/10.1128/mbio.02379-21

Powers, S.M., Baulch, H.M., Hampton, S.E., Labou, S.G., Lottig, N.R. & Stanley, E.H. (2017a) Nitrification contributes to winter oxygen depletion in seasonally frozen forested lakes. *Biogeochemistry Letters*, 136, 119–129. Available from: https://doi.org/10.1007/s10533-017-0382-1

Powers, S.M., Labou, S.G., Baulch, H.M., Hunt, R.J., Lottig, N.R., Hampton, S.E. et al. (2017b) Ice duration drives winter nitrate accumulation in north temperate lakes. *Limnology and Oceanography*, 2, 177–186. Available from: https://doi.org/10.1002/lol2.10048

Price, M.N., Dehal, P.S. & Arkin, A.P. (2010) FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One*, 5, e9490. Available from: https://doi.org/10.1371/journal.pone.0009490

Prosser, J.I. (1990) Autotrophic nitrification in bacteria. *Advances in Microbial Physiology*, 30, 125–181. Available from: https://doi.org/10.1016/S0065-2911(08)60112-5

Prosser, J.I. & Nicol, G.W. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology*, 20, P523–531. https://doi.org/10.1016/j.tim.2012.08.001

Qin, W., Wei, S.P., Zheng, Y., Choi, E., Li, X., Johnston, J. et al. (2024) Ammonia-oxidizing bacteria and archaea exhibit different nitrogen source preferences. *Nature Microbiology*, 9, 524–536. Available from: https://doi.org/10.1038/s41564-023-01593-7

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P. et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 41, D590–D596. Available from: https://doi.org/10.1093/nar/gks1219

R Core Team. (2020) *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Available from: https://www.R-project.org/

Rotthauwe, J.H., Witzel, K.P. & Liesack, W. (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*, 63, 4704–4712. Available from: https://doi.org/10.1128/aem.63.12.4704-4712.1997

Runkel, R.L., Crawford, C.G. & Cohn, T.A. (2004) *Load estimator (LOADEST): a FORTRAN program for estimating constituent loads in streams and rivers*. USA Techniques and Methods 4-A5: USGS Publications Warehouse. Available from: https://doi.org/10.3133/tm4A5

Small, G.E., Bullerjahn, G.S., Sterner, R.W., Beall, B.F.N., Brovold, S., Finlay, J.C. et al. (2013) Rates and controls of nitrification in a large oligotrophic lake. *Limnology and Oceanography*, 58, 276–286. Available from: https://doi.org/10.4319/lo.2013.58.1.0276

Sterner, R.W., Anagnostou, E., Brovold, S., Bullerjahn, G.S., Finlay, J.C., Kumar, S. et al. (2007) Increasing stoichiometric imbalance in North America's largest lake: nitrification in Lake Superior. *Geophysical Research Letters*, 34, L10406. Available from: https://doi.org/10.1029/2006GL028861

Strickland, J.D.H. & Parsons, T.R. (1968) Determination of reactive nitrate. A practical handbook of seawater analysis. *Fisheries Research Board of Canada, Bulletin*, 167, 71–75.

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B. et al. (2015) Complete nitrification by a single microorganism. *Nature*, 528, 555–559. Available from: https://doi.org/10.1038/nature16459

Vissers, E.W., Anselmetti, F.S., Bodelier, P.L.E., Muyzer, G., Schleper, C., Tourna, M. et al. (2013a) Temporal and spatial coexistence of archaeal and bacterial amoA genes and gene transcripts in Lake Lucerne. *Archaea*, 2013, 289478. Available from: https://doi.org/10.1155/2013/289478

Vissers, E.W., Blaga, C.I., Bodelier, P.L.E., Muyzer, G., Schleper, C., Damsté, J.S.S. et al. (2013b) Seasonal and vertical distribution of putative ammonia-oxidizing thaumarchaeotal communities in an oligotrophic lake. *FEMS Microbiology Ecology*, 83, 515–526. Available from: https://doi.org/10.1111/1574-6941.12013

Wegen, S., Nowka, B. & Spieck, E. (2019) Low temperature and neutral pH define "*Candidatus* Nitrotoga sp." as a competitive nitrite oxidizer in coculture with *Nitrospira defluvii*. *Applied and Environmental Microbiology*, 85, e02569-18. Available from: https://doi.org/10.1128/AEM.02569-18

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. & Madden, T.L. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, 13, 1–11. Available from: https://doi.org/10.1186/1471-2105-13-134

Yoon, S., Ha, S., Lim, J., Kwon, S. & Chun, J. (2017) A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie Van Leeuwenhoek*, 110, 1281–1286. Available from: https://doi.org/10.1007/s10482-017-0844-4

Yu, G. & Xu, S. (2023) Package "scatterpie". https://cran.r-project.org/package=scatterpie

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Peoples, L.M., Seixas, M.H., Evans, K.A., Bilbrey, E.M., Ranieri, J.R., Tappenbeck, T.H. et al. (2024) Out of sight, but not out of season: Nitrifier distributions and population dynamics in a large oligotrophic lake. *Environmental Microbiology*, 26(3), e16616. Available from: https://doi.org/10.1111/1462-2920.16616

APPENDIX A

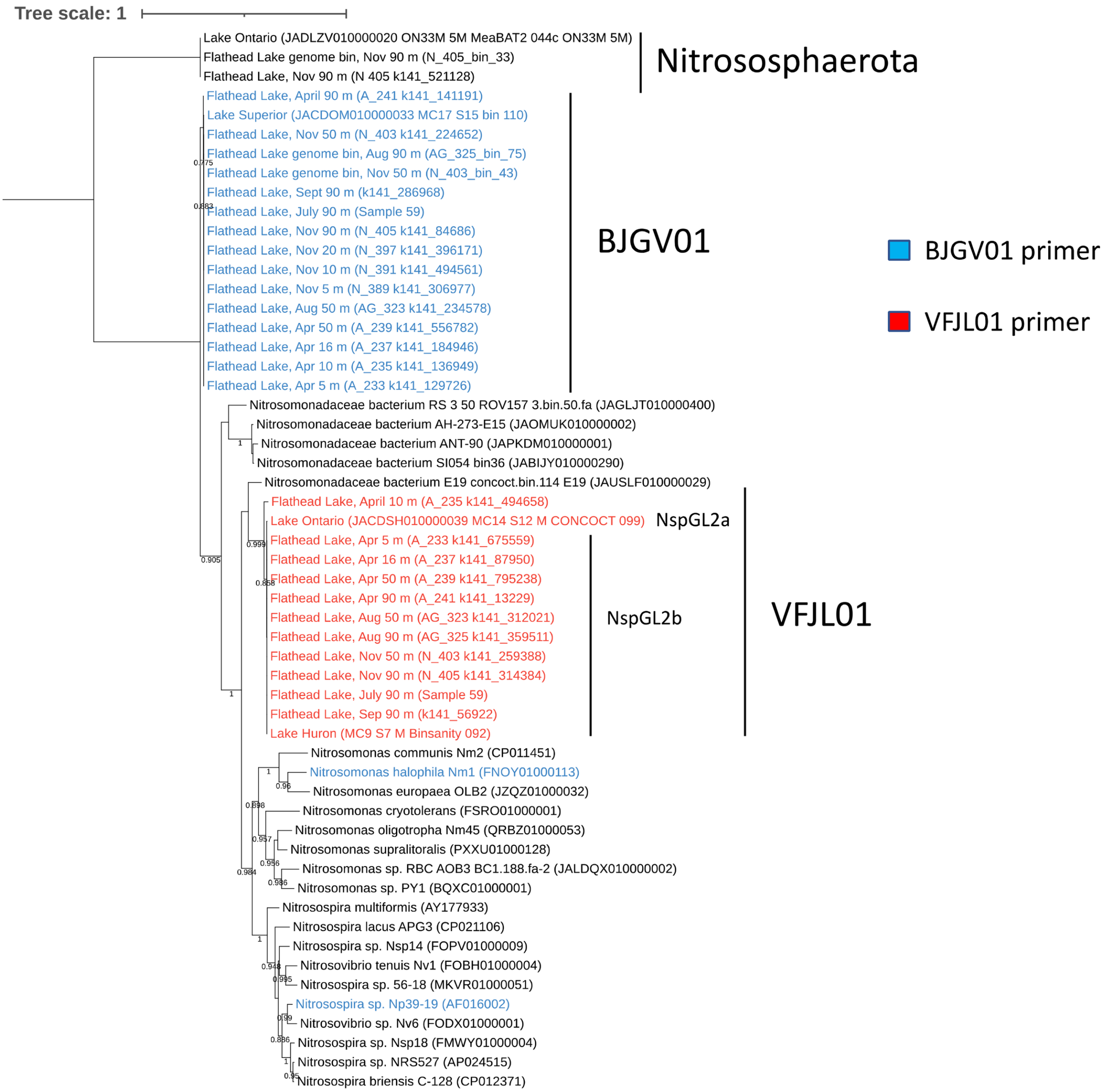


FIGURE A1 Ammonia monooxygenase subunit A (*amoA*) gene diversity within Flathead Lake and select comparison sequences. There are three abundant ammonia-oxidizing organisms in Flathead Lake: two Nitrosomonadaceae bacteria that belong to the BJGV01 and VFJL01 clades and one archaeon related to the Nitrososphaerota (Thaumarchaeota). Sequences that have identical matches to the BJGV01 (blue) and VFJL01 (red) primers used in this study are shown in colour.

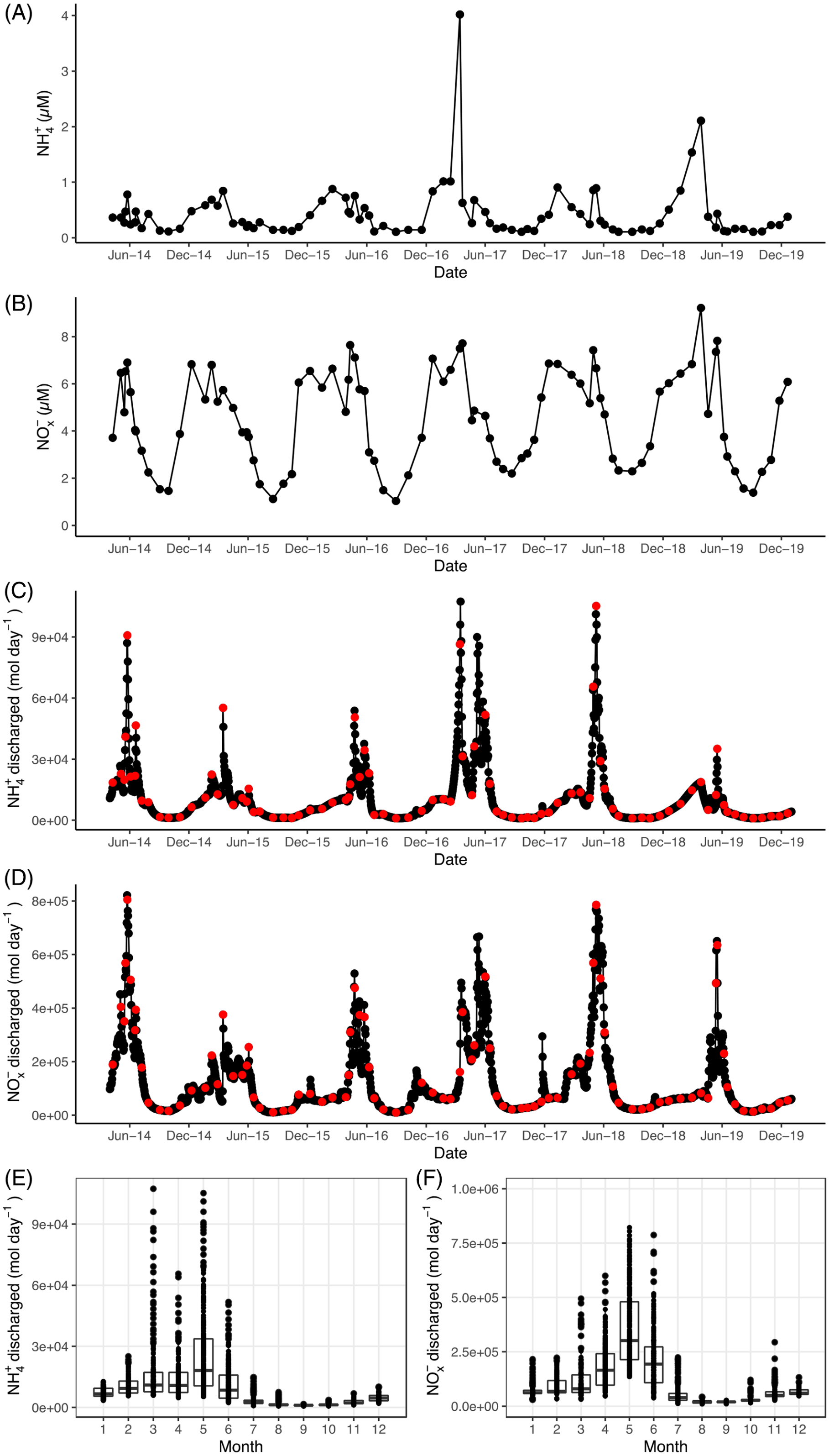


FIGURE A2 Measured riverine concentrations and flux of NH_4^+ and NO_x^- (nitrate + nitrite) entering Flathead Lake from the Flathead River. (A, B) Measured concentrations of NH_4^+ (A) and NO_x^- (B) in the Flathead River. (C, D) Daily discharge of NH_4^+ (C) and NO_x^- (D) from the Flathead River into Flathead Lake. Points in black were modelled using loadflex (Appling et al., 2015) while those in red were calculated using known concentrations in A and B and average discharge on the day of collection. (E, F) Modelled daily discharge binned by month.

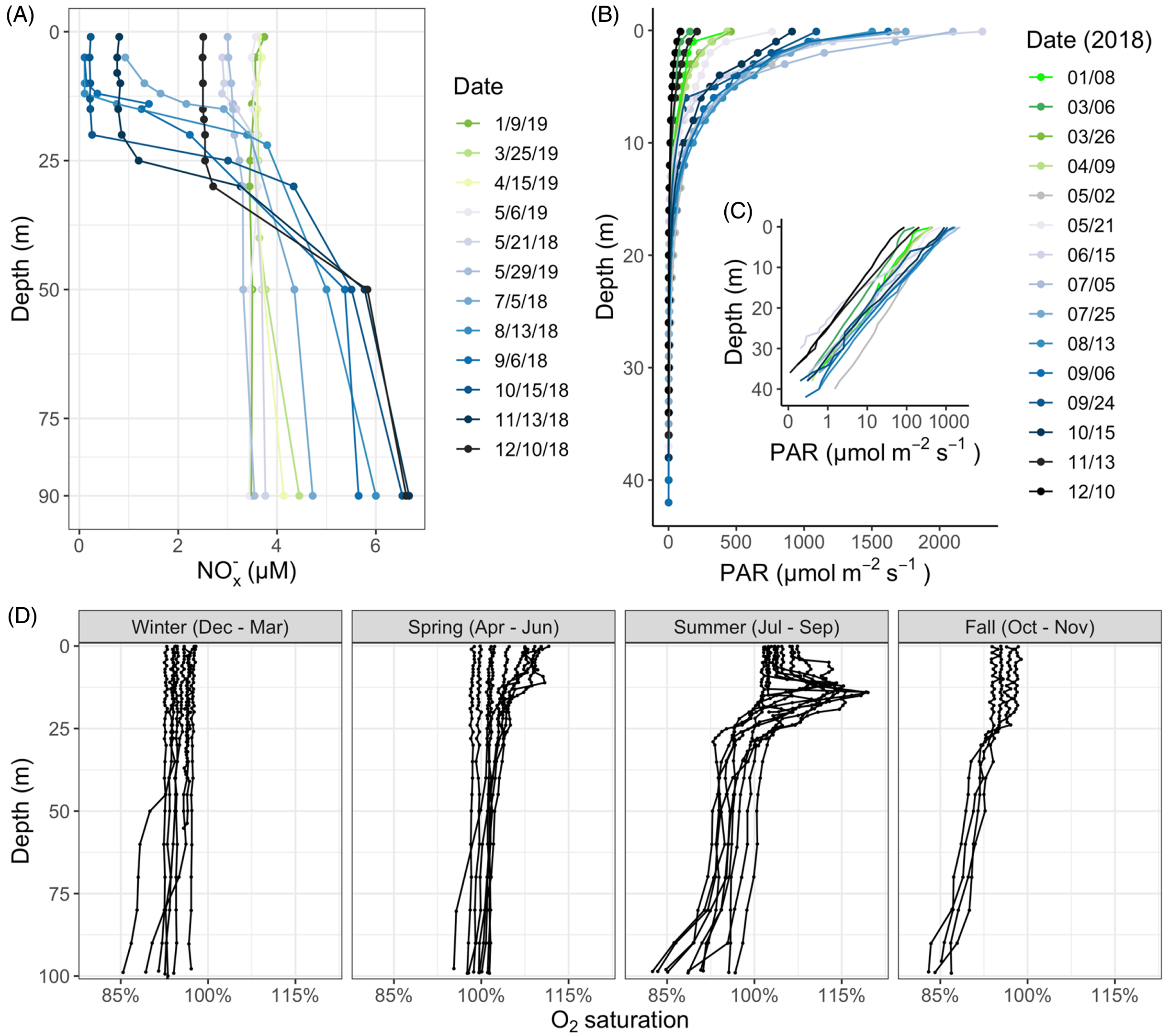


FIGURE A3 (A) NO_x^- (nitrate + nitrite) concentrations, (B,C) photosynthetically active radiation (PAR), and (D) O_2 saturation (2018–2019) in Flathead Lake.

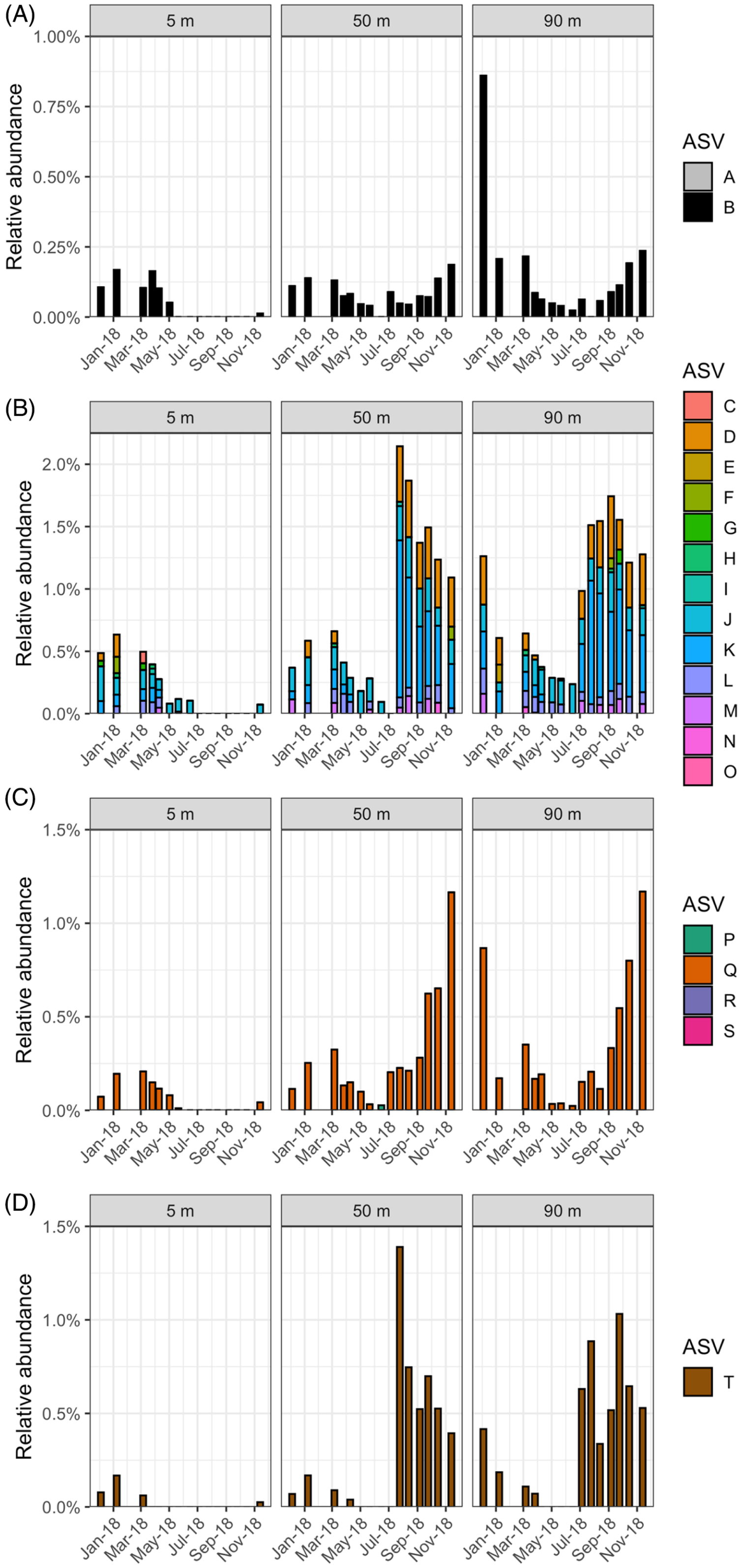
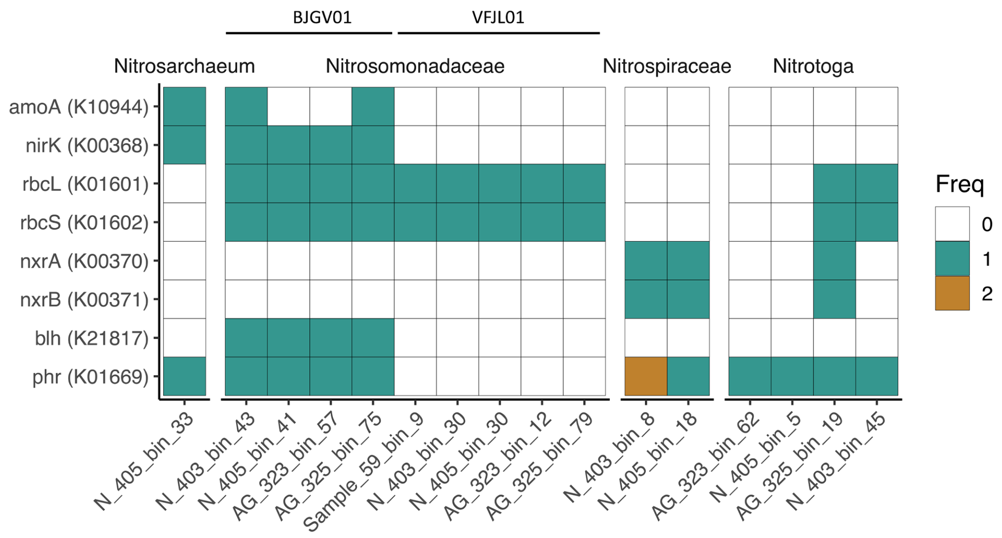


FIGURE A4 Abundances of amplified sequence variants (ASVs) within the phylum Nitrososphaerota (A; named Crenarchaeota in this version of SILVA), the family Nitrosomonadaceae (B), the phylum Nitrospirota (C), and the family Gallionellaceae (D) based on 16S rRNA gene amplicon sequencing. Note that the axes are different. Because the ASVs represent sub-species level classifications with no taxonomy, they are represented by a letter.



	N_405_bin_33	N_403_bin_43	N_405_bin_41	AG_323_bin_57	AG_325_bin_75	Sample_59_bin_9	N_403_bin_30	N_405_bin_30	AG_323_bin_12	AG_325_bin_79	N_403_bin_8	N_405_bin_18	AG_323_bin_62	N_405_bin_5	AG_325_bin_19	N_403_bin_45
amoA (K10944)	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
nirK (K00368)	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
rbcL (K01601)	0	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1
rbcS (K01602)	0	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1
nxrA (K00370)	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0
nxrB (K00371)	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0
blh (K21817)	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
phr (K01669)	1	1	1	1	1	0	0	0	0	0	2	1	1	1	1	1

FIGURE A5 Distribution of specific genes of interest within Flathead Lake nitrifier metagenome-assembled genomes. Colours reflect the frequency of that gene within the genome. K numbers are KEGG identifiers. Further genome information can be found in Table S1. *amoA*, ammonia monooxygenase subunit A; *blh*, beta-carotene 15,15′-dioxygenase; *nirK*, nitrite reductase; *nxrA*, nitrite oxidoreductase alpha subunit; *nxrB*, nitrite oxidoreductase beta subunit; *phr*, deoxyribodipyrimidine photolyase; *rbcL*, ribulose-bisphosphate carboxylase large chain; *rbcS*, ribulose-bisphosphate carboxylase small chain.

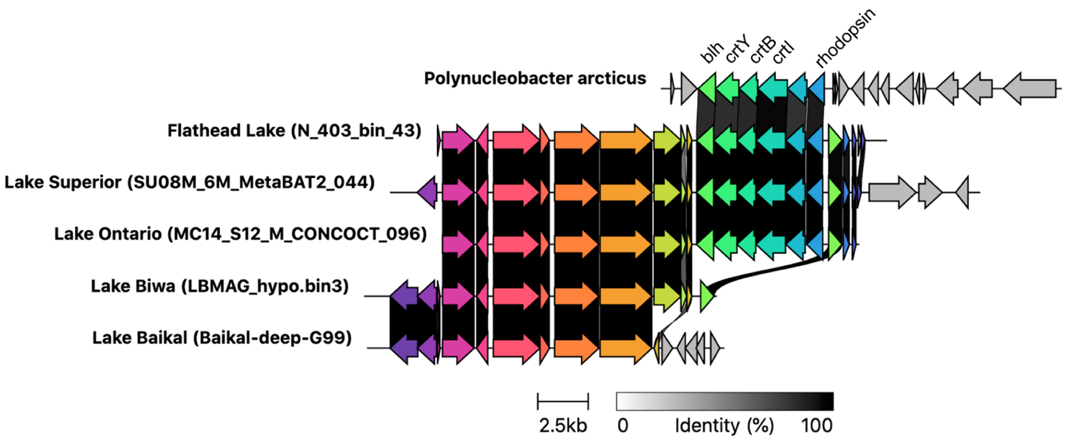


FIGURE A6 Rhodopsin gene operon within a Nitrosomonadaceae clade BJGV01 Flathead Lake MAG compared against other closely related genomes.