Interactions between microorganisms and oxic-anoxic transitions

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Chapter 6 | Synthesis and Outlook

Synthesis and Outlook
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This thesis described the diversity, dynamics, and community assembly of microorganisms in a seasonally stratified lake. Within the project, we have studied the spatio-temporal dynamics of bacterial communities and the interactions between microorganisms and oxic-anoxic transitions (Chapter 2). Combining microbial community dynamics, mathematical models, and biogeochemical processes, oxic-anoxic regime shifts and hysteresis were investigated in Chapter 3. Sulfur bacteria proved to play vital roles during oxic-anoxic regime shifts, therefore the diversity and dynamics of sulfate-reducing bacteria (SRB), green sulfur bacteria (GSB), purple sulfur bacteria (PSB) and colorless sulfur bacteria (CSB) have been surveyed (Chapter 4). Nitrogen compounds are of paramount importance to lake ecosystems, hence the diversity, abundance and dynamics of bacteria and archaea involved in the nitrogen cycle were investigated and the results were described in Chapter 5.

Results in this thesis emphasize that oxic-anoxic transitions have substantial influences on the diversity, dynamics and functional roles of microbial communities, and in particular on microorganisms involved in sulfur and nitrogen cycling. Furthermore, interactions between cyanobacteria, SRB, GSB, PSB and biogeochemical processes may induce hysteresis during oxic-anoxic regime shifts. This thesis has provided novel information on the seasonal variation of microbial communities involved in the microbial sulfur and nitrogen cycle of seasonally stratified lakes. The thesis has also led to several new questions on the functioning of these microbial communities that will be of interest for future research.

Links between the microbial sulfur and nitrogen cycle

The results in this thesis show that the microbial communities involved in the sulfur cycle (Chapter 4) and nitrogen cycle (Chapter 5) both displayed striking seasonal patterns, which were strongly influenced by oxic-anoxic transitions in the lake (Figure 6.1). Most of the microorganisms involved in the oxidation-reduction reactions of the sulfur and nitrogen cycle were present in the deeper parts of the lake, i.e., in the metalimnion, hypolimnion or sediment of Lake Vechten. Ammonium oxidizers and anammox bacteria were mainly present in the sediment. Similar
microbial distributions have also been observed in other lakes (Hastings et al., 1998; Bollmann et al., 2014).

In spring, at the onset of lake stratification, oxygen was depleted in the hypolimnion and the microbial community shifted towards alternative electron acceptors for the degradation of organic matter. At first, denitrifying bacteria became abundant in the hypolimnion, prior to the appearance of SRB (Figure 6.1), which is likely because nitrate reduction yields more energy than sulfate reduction. Once nitrate was depleted and the hypolimnion became sulfidic in summer, the abundance of nitrogen bacteria (i.e. denitrifying bacteria and nitrogen-fixing bacteria) decreased sharply and diverse sulfur bacteria (SRB, PSB, GSB) bloomed in the metalimnion and hypolimnion of Lake Vechten. During fall turnover, CSB (mainly Arcobacter) dominated bacterial communities in the bottom water, which can oxidize the accumulated sulfide using oxygen and sometimes also nitrate (Gevertz et al., 2000). Furthermore, ammonium-oxidizing bacteria and archaea increased in abundance in the sediment during late fall, and the ammonium that had accumulated in the bottom water was oxidized to nitrate. At first, these oxygen-consuming processes maintained a low dissolved oxygen concentration, creating hypoxia throughout the water column. This was followed by a period with relatively high dissolved oxygen, nitrate and sulfate concentrations throughout the water column during the winter months.

We note that several SRB (e.g. Desulfobulbus propionicus) can use nitrate instead of sulfate as alternative terminal electron acceptors (Marietou 2016), and some bacteria that are commonly regarded as SRB (e.g. Sulfurospirillum) can also oxidize sulfide with nitrate (Eisenmann et al., 2000). Therefore, several of the sulfur bacteria can also become active as denitrifying bacteria. Conversely, nitrogen bacteria such as Nitrospinae and Nitrospirae were discovered to have a potential contribution in sulfur oxidation (Anantharaman et al., 2018). Thus, it can be seen that the microbial sulfur and nitrogen cycle are closely intertwined and can interact through species that play a role in both cycles. It would be interesting to further explore interrelationships between the microbial sulfur and nitrogen cycle in future studies.
Figure 6.1. Conceptual model of the seasonal succession of (A) oxygen, nitrogen (stippled areas) and sulfur compounds; (B) nitrogen bacteria, archaea, and sulfur bacteria in the water column and sediment of Lake Vechten. AOA, ammonium-oxidizing archaea; AOB, ammonium-oxidizing bacteria; Anammox, anaerobic ammonium-oxidizing bacteria; NFB, nitrogen-fixing bacteria; nirK DNB, denitrifying bacteria with the nirK gene; nirS DNB, denitrifying bacteria with the nirS gene; CSB, colorless sulfur bacteria; PSB, purple sulfur bacteria; GSB, green sulfur bacteria; SRB, sulfate-reducing bacteria.
Activity and diversity of microorganisms in the sulfur and nitrogen cycle

In our study, we described the relative abundances of sulfur bacteria by 16S rRNA gene amplicon sequencing (Chapter 4), and quantified the abundances of various functional genes (e.g. *amoA* and *nifH* genes) involved in the nitrogen cycle by qPCR (Chapter 5). However, relative abundances of different functional groups do not necessarily reflect their activity in the sulfur and nitrogen cycle. For instance, it has been reported that SRB were low in abundance in peat soil, but were the main drivers of sulfate reduction by enhancing their ribosome content (Hausmann *et al.*, 2016). In a study of microbial communities in grassland soil, it was discovered that AOA were more abundant than AOB, whereas AOB dominated the nitrification process (Sterngren *et al.*, 2015). Therefore, quantifying the expression of functional genes and measuring the activities of specific microbial groups (e.g. Mosier and Francis, 2010; Turk *et al.*, 2011) can provide more comprehensive insights into the contributions of different microorganisms to the sulfur and nitrogen cycle.

Members of *Acidobacteria* were recently identified as SRB (Hausmann *et al.*, 2018). Another recent study discovered that 13 bacterial and archaeal phyla which had not previously been recognized as SRB actually have the genetic capacity for sulfate/sulfite reduction (Anantharaman *et al.*, 2018). Eight of these newly identified SRB are candidate phyla without isolated representatives. Thus, it seems that the diversity of sulfur bacteria has been underestimated for a long time despite their vital ecological roles.

Previous studies showed that nitrogen-fixing bacteria and denitrifying bacteria were phylogenetically diverse (Zumft, 1997; Raymond *et al.*, 2004). In this thesis, the diversity of nitrogen-fixing bacteria and denitrifying bacteria was detected by cloning of the corresponding functional genes (*nifH*, *nirS* and *nirK*) (Chapter 5). However, the number of sequenced clones was limited and therefore the bacterial diversity may not be fully covered. Furthermore, several archaeal groups can also perform nitrogen fixation and denitrification (Zumft, 1997; Raymond *et al.*, 2004), but were not investigated in this thesis. Metagenomics and metatranscriptomics techniques now can detect the genetic capacities and expressions of organisms directly, which will provide more comprehensive information on the diversity and functional roles of microorganisms involved in nitrogen fixation and denitrification.
Other processes in the microbial nitrogen cycle

Some functional groups that might play important roles in the nitrogen cycle were not included in this research. For instance, we did not investigate the newly discovered comammox bacteria, which have high affinity for ammonia and high growth yield compared to canonical nitrifiers (Kits et al., 2017), and appear to be widely distributed in freshwater environments (van Kessel et al., 2015; Gonzalez-Martinez et al., 2016; Pinto et al., 2016). Therefore, comammox bacteria may play significant roles in the nitrification process of freshwater lakes and their ecological functions should be explored in the future.

Furthermore, bacteria performing dissimilatory nitrate reduction to ammonium (DNRA) can compete with denitrifying bacteria for nitrate as electron acceptor (Kraft et al., 2014; van den Berg et al., 2015). DNRA has been observed in estuarine, marine and freshwater lake sediments (Brunet and GarciaGil, 1996; Giblin et al., 2013), and it can be stimulated in sulfidic environments (Jones et al., 2017). The hypolimnion of Lake Vechten becomes sulfidic during the stratification period, and hence the role of DNRA and the DNRA/denitrification ratio should be evaluated in future studies.

The carbon cycle and other biogeochemical cycles

As this thesis has emphasized the sulfur and nitrogen cycle, information on the carbon and other elemental cycles (e.g., the iron cycle) in Lake Vechten is still elusive. Yet, these biogeochemical cycles are also essential to lake ecosystems.

For instance, methane (CH$_4$) produced in the carbon cycle is a potent greenhouse gas which has substantial influence on global warming (IPCC, 2013). Freshwater environments contribute a large part of the global methane emission (Bastviken et al., 2011; Aben et al., 2017), and the involvement of microbial communities has been studied extensively (Costello and Lidstrom, 1999; He et al., 2015; Samad and Bertilsson, 2017). Aerobic and anaerobic oxidation of methane by methanotrophic bacteria and archaea are critical processes in controlling the flux of methane from lakes to the atmosphere (Hanson and Hanson, 1996; Haroon et al., 2013; Yang et al., 2016). Nitrate/nitrite-dependent anaerobic methane oxidation was discovered to be a significant methane sink in Lake Constance (Deutzmann et al., 2014) and also in three freshwater wetlands in China (Hu et al., 2014). Previous
studies in Lake Vechten revealed commensalistic relations between SRB and methanogens, although SRB produced sulfide which can limit vertical distributions of methanogens (Cappenberg, 1974; Cappenberg, 1975). Since oxic-anoxic transitions can determine the distribution and dynamics of SRB (Chapter 4), it may also influence the distribution and dynamics of methanogens. The dynamics of nitrate were also strongly affected by oxic-anoxic transitions in Lake Vechten (Chapter 5), which consequently can influence nitrate/nitrite-dependent anaerobic methane oxidation process. Bacterial 16S rRNA gene amplicon sequencing showed that *Methylobacter* bloomed after fall turnover (Chapter 2, Figure 2.5C). Since *Methylobacter* oxidizes methane and consumes oxygen, it is very likely that *Methylobacter* plays an important role in the carbon cycle during oxic-anoxic transitions.

It has been reported that GSB and PSB had great contributions to the primary production of Lake Vechten (Steenbergen, 1982) and other lakes (Takahashi and Ichimura, 1968; Storelli *et al.*, 2013). Sulfur-oxidizing *Arcobacter* could dominate carbon fixation in the chemocline of freshwater lakes (Noguerola *et al.*, 2015). Chemoautotrophic *Nitrospinae* can oxidize nitrite into nitrate, and fix 15-45% of the inorganic carbon in the dark ocean (Pachiadaki *et al.*, 2017). Methanogens were reported to be the major nitrogen fixers in a freshwater wetland (Bae *et al*. 2018). Furthermore, anaerobic oxidation of methane can be coupled to the reduction of iron, manganese, sulfate and nitrate (Boetius *et al.*, 2000; Beal *et al.*, 2009; Haroon *et al.*, 2013). The iron cycle is closely coupled with the sulfur cycle as sulfide can precipitate with iron at the water-sediment interface (Riera *et al.*, 1988). In the sediment of Lake Vechten, iron-oxidizing *Geobacter* had a relatively high abundance (Chapter 2), and may have played an important role in both the carbon and iron cycle and possibly the sulfur cycle. Thus, it can be seen that the biogeochemical cycles of sulfur, nitrogen, carbon and other elements are highly interrelated and further research on these interactions should be conducted.

**Mechanisms of oxic-anoxic regime shifts**

Although oxic-anoxic transitions are common phenomena in many lakes, coastal waters and open oceans (Diaz and Rosenberg, 2008; Jenny *et al.*, 2016; Breitburg *et al.*, 2018), the detailed mechanisms underlying oxic-anoxic regime shifts are still
largely unknown. Our results indicate that hysteresis loops and tipping points are common features of oxic-anoxic regime shifts, causing rapid drops in oxygen levels that are not easily reversed (Chapter 3). Moreover, our results also show that microorganisms play a major role in these oxic-anoxic regime shifts, as they mediate many of the oxidation-reduction reactions in aquatic ecosystems.

It is well known that several factors can influence oxic-anoxic regime shifts in seasonally stratified lakes. As the main force of thermal stratification, temperature has a major effect on oxic-anoxic transitions and microbial succession in temperate lakes (Lindstrom et al., 2005; Kara et al., 2013; Yu et al., 2014). For instance, increasing temperature will decrease oxygen solubility and enhanced thermal stratification will suppress the mixing of $\text{O}_2$-rich surface water into deeper layers (Deutsch et al., 2011). Hence, global warming is likely to extend the duration and expansion of hypoxia in seasonally stratified lakes (Livingstone, 2003; North et al., 2014), which may delay the transition to oxic conditions during fall turnover (Chapter 2). Eutrophication, which is caused by elevated nutrient discharge, can also expand hypoxia and anoxia in lakes and coastal waters (Jenny et al., 2016, Breitburg et al., 2018). However, the models of oxic-anoxic transitions that have been developed so far, including our own work (Chapter 3), are still a major simplification of reality. For instance, realistic quantification of many of the microbially-mediated oxidation-reduction reactions taking place during oxic-anoxic transitions is a major challenge, and accurate predictions of the tipping points in lakes and coastal waters are therefore not yet feasible. Further elucidation and quantification of the underlying mechanisms of oxic-anoxic regime shifts and hysteresis loops will help us to better understand and predict how increasing temperature and eutrophication will affect the spread of hypoxia and anoxia. In particular, comparative studies on lakes and coastal waters with different thermal and eutrophic conditions may improve quantification of the key microbiological and biogeochemical processes involved in oxic-anoxic transitions.

**Meta-omics analysis of microbial communities**

Isolating microorganisms from environments is pivotal to explore their ecophysiology and functional roles in ecosystems (Gevertz et al., 2000; Könneke et al., 2005). In this project, we tried to isolate AOA and AOB from Lake Vechten to investigate their
ecophysiology. Although we enriched AOA and AOB in the laboratory for more than 1 year, we did not succeed in isolating representatives of these microorganisms. It has been reported before that isolating microorganisms from environments, especially from freshwater lakes, is particularly difficult (Rappé and Giovannoni, 2003; Newton et al., 2011). Over the last decade, technical improvements in nucleic acid sequencing and mass spectrometry have advanced the study of non-culturable microorganisms through the development of metagenomics, metatranscriptomic, metaproteomic, and metabolomic analysis. Applications of meta-omics techniques have largely expanded our understanding of microbial diversity, for instance, the recent discoveries of comammox bacteria (Daims et al., 2015; van Kessel et al., 2015) and the Candidate Phyla Radiation (Brown et al., 2015).

Metagenomics can provide detailed insights into the diversity and functional potential of microbial communities in lakes, especially for microbial groups involved in the carbon, nitrogen and sulfur cycles (Llorens-Mares et al., 2015). Metagenome-assembled genomes of Verrucomicrobia revealed that they are potential (poly)saccharide degraders in freshwater lakes (He et al., 2017). Metatranscriptomics analysis of microbial communities in Mono Lake identified transcriptionally active microorganisms (Thioalkalivibrio) involved in the sulfur and arsenic cycle (Edwardson and Hollibaugh, 2017).

To obtain more comprehensive information on microbial communities in Lake Vechten, we also employed metagenomics and metatranscriptomics approaches in our study. Samples from the water column (at 1 m intervals) and sediment were collected on 1 September, 2015. Profiles of temperature and dissolved oxygen (DO) show that the water column of Lake Vechten was clearly stratified into an oxic epilimnion and anoxic hypolimnion (Figure 6.2A,B). The hypolimnion was characterized by high concentrations of ammonium and dissolved organic carbon (DOC) (Figure 6.2C,D), indicating active degradation of organic material. Sulfate was present in the epilimnion whereas sulfide accumulated in the hypolimnion (Figure 6.2E,F), indicative of sulfate reduction in the hypolimnion. Overall, environmental conditions were similar as in our observations of Lake Vechten in previous years (Chapters 2-5).

Bacterial 16S rRNA gene amplicon sequencing data showed large differences in bacterial community composition between water layers and also the sediment during the stratification period (Chapter 2). Therefore, samples from the epilimnion
(1 m depth), metalimnion (5 m depth), hypolimnion (10 m depth) and sediment of Lake Vechten were selected for metagenomics and metatranscriptomics analysis.

Figure 6.2 Profiles of environmental parameters in Lake Vechten on September 1, 2015. (A) Temperature, (B) Dissolved oxygen (DO), (C) ammonium, (D) dissolved organic carbon (DOC), (E) sulfate, (F) sulfide.
Table 6.1 Overview of metagenomics data and analysis (sampled on September 1, 2015).

<table>
<thead>
<tr>
<th></th>
<th>Epilimnion</th>
<th>Metalimnion</th>
<th>Hypolimnion</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling depth</td>
<td>1 m</td>
<td>5 m</td>
<td>10 m</td>
<td>sediment</td>
</tr>
<tr>
<td>Total Sequences</td>
<td>466,569,664</td>
<td>468,966,450</td>
<td>467,711,418</td>
<td>419,937,370</td>
</tr>
<tr>
<td>Sequence length (bp)</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>%GC</td>
<td>46.5</td>
<td>46</td>
<td>47</td>
<td>52</td>
</tr>
<tr>
<td>Quality trimming</td>
<td>CLC Genomics Workbench</td>
<td>CLC Genomics Workbench</td>
<td>CLC Genomics Workbench</td>
<td>CLC Genomics Workbench</td>
</tr>
<tr>
<td>Assembly</td>
<td>Megahit¹</td>
<td>Megahit</td>
<td>Megahit</td>
<td>Megahit</td>
</tr>
<tr>
<td>Annotation</td>
<td>Creep &amp; KEGG²</td>
<td>Creep &amp; KEGG</td>
<td>Creep &amp; KEGG</td>
<td>Creep &amp; KEGG</td>
</tr>
<tr>
<td>Binning</td>
<td>MetaBat³</td>
<td>MetaBat</td>
<td>MetaBat</td>
<td>MetaBat</td>
</tr>
<tr>
<td>Bin quality check</td>
<td>CheckM⁴</td>
<td>CheckM</td>
<td>CheckM</td>
<td>CheckM</td>
</tr>
<tr>
<td>No. of bins</td>
<td>357</td>
<td>453</td>
<td>676</td>
<td>312</td>
</tr>
<tr>
<td>No. of good bins</td>
<td>22</td>
<td>46</td>
<td>69</td>
<td>22</td>
</tr>
</tbody>
</table>

¹ Li et al., 2014.
² Kanehisa et al., 2016.
³ Kang et al., 2015.
⁴ Parks et al., 2015.
⁵ Bins with >65% completeness, <15% contaminations, and a size of >1Mb.

DNA and RNA were extracted from the 4 selected samples and sequenced by commercial companies. An overview of the metagenomics data and analysis is presented in Table 6.1. Hundreds of bins, which are draft genomes of microorganisms, were retrieved from the data for all 4 samples. After quality control, we obtained 22 good bins from 1 m depth, 46 good bins from 5 m depth, 69 good bins from 10 m depth, and 22 good bins from the sediment. Currently, we are analyzing these draft genomes to explore the diversity and genetic capacities of microorganisms in Lake Vechten.
Table 6.2 Overview of metatranscriptomics data analyzed by MG-RAST (sampled on September 1, 2015).

<table>
<thead>
<tr>
<th>Sampled depth</th>
<th>Epilimnion</th>
<th>Metalimnion</th>
<th>Hypolimnion</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sequences</td>
<td>5,109,483</td>
<td>5,759,111</td>
<td>7,796,301</td>
<td>4,190,446</td>
</tr>
<tr>
<td>Sequence length (bp)</td>
<td>98 ± 33</td>
<td>92 ± 29</td>
<td>93 ± 29</td>
<td>97 ± 32</td>
</tr>
<tr>
<td>%GC</td>
<td>51 ± 10</td>
<td>51 ± 11</td>
<td>53 ± 10</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>Predicted protein features</td>
<td>1,314,302</td>
<td>1,608,954</td>
<td>3,377,685</td>
<td>1,020,676</td>
</tr>
<tr>
<td>Predicted rRNA features</td>
<td>194,685</td>
<td>249,310</td>
<td>203,687</td>
<td>290,276</td>
</tr>
<tr>
<td>Identified protein features</td>
<td>306,167</td>
<td>306,634</td>
<td>590,990</td>
<td>103,269</td>
</tr>
<tr>
<td>Identified rRNA features</td>
<td>87,127</td>
<td>89,228</td>
<td>52,313</td>
<td>72,240</td>
</tr>
<tr>
<td>Identified functional categories</td>
<td>254,740</td>
<td>250,766</td>
<td>462,394</td>
<td>76,720</td>
</tr>
</tbody>
</table>

Metatranscriptomics data was analyzed by MG-RAST (Meyer et al., 2008), and an overview of the results is present in Table 6.2. Preliminary analysis of the metatranscriptomics data shows that the microbial communities in Lake Vechten were quite diverse (Figure 6.3). Overall, microbial communities from the epilimnion and metalimnion were similar, but quite different from the communities in the hypolimnion and sediment.
Figure 6.3 Taxonomic hit distribution of sequence reads from the metatranscriptomes of (A) epilimnion; (B) metalimnion; (C) hypolimnion, and (D) sediment. The metatranscriptomes were sampled on September 1, 2015. Only taxa with a relative presence of >1% in at least one of the four samples are shown. The results were obtained with MG-RAST (Meyer et al., 2008) after removal of the eukaryotic reads.

More specifically, *Gammaproteobacteria* and to a lesser extent also *Bacteroidetes* were active throughout the water column and in the sediment. *Betaproteobacteria* and *Actinobacteria* were active in the water column, but not in the sediment. Conversely, *Euryarchaeota* and *Firmicutes* were mainly active in the anaerobic hypolimnion and sediment. Furthermore, *Chlorobi* and *Chloroflexi* were prevalent in the hypolimnion, and *Chloroflexi* were active in the metalimnion as well.
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Compared with bacterial 16S rRNA gene amplicon sequencing data, results from metagenomics and metatranscriptomics approaches are more reliable since they can avoid the amplification bias in PCR. Furthermore, the information obtained from metagenomics and metatranscriptomics is more comprehensive than bacterial 16S rRNA amplicon sequencing. In particular, metatranscriptomics data showed high activity of *Euryarchaeota* in the hypolimnion and sediment of Lake Vechten (Figure 6.3), whereas this phylum had not been detected in our previous studies in this lake (Chapters 2-5). Also, according to the metatranscriptome *Chloroflexi* were very active in the metalimnion and hypolimnion, even though their relative abundance was <0.5% according to our previous 16S rRNA gene analysis (Chapter 2). Draft genomes of microorganisms retrieved from metagenomics data will provide detailed information both on the diversity and genetic capacities of these microorganisms, and metatranscriptomes can further verify the expression of their functional genes. We believe this information will greatly enhance our understanding of activity and interactions of microorganisms in lakes.

**Concluding remarks**

In this thesis, we have investigated the diversity and seasonal dynamics of bacteria and archaea in Lake Vechten by state-of-the-art sequencing techniques. In particular, microorganisms involved in the sulfur and nitrogen cycling were investigated in great detail. It was evident that dynamic changes in microbial community composition were strongly influenced by oxic-anoxic transitions. In turn, the biogeochemical oxidation-reduction processes mediated by the microbial community (e.g., SRB, GSB, PSB, cyanobacteria, *Arcobacter* and *Methylobacter*) appeared to affect the oxic-anoxic transitions, thus generating feedbacks between biogeochemical conditions and microbial community composition. Information from this thesis is conducive to understand how environmental changes (e.g., eutrophication, global warming) influence microbial communities in aquatic environments experiencing oxic-anoxic transitions. Efforts still need to be made to get more comprehensive views on other important biogeochemical cycles (e.g. the carbon cycle), and especially on the activities and interactions of microorganisms involved in these processes. Furthermore, we emphasize that the work presented here is only a first step towards a deeper mechanistic understanding of the interactions between microorganisms.
and oxic-anoxic transitions, and quantitative prediction of the tipping points during oxic-anoxic transitions is still beyond reach. Comparative studies of seasonally stratified lakes with different thermal and eutrophic conditions may further expand our understanding of the dynamics and assembly of microbial communities, and their role in nutrient cycling and oxic-anoxic regime shifts in lake ecosystems. Such an improved understanding will help us to predict and perhaps even mitigate the spread of hypoxia and anoxia in many aquatic ecosystems across the globe.