Environmental controls of benthic nitrogen cycling in Lake Lugano South Basin, Switzerland – Pathways, rates, isotopic signatures and microbial communities

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“A person who never made a mistake never tried anything new. “

Albert Einstein

Summary

Nitrogen (N) is a key constituent of biomolecules required by all living organisms so understanding of its fluxes and in situ environmental availability is of crucial importance. In aquatic ecosystems, N availability regulates primary production and, to an extent, mass and energy transfer between trophic levels. Naturally, the vast reservoir of atmospheric N_2 is only available to highly specialized diazotrophic microbes, but development and broad implementation of the Haber–Bosch process produces 450 million tons of nitrogen fertilizer per year from the N_2 in the atmosphere, thereby changing the global N cycle entirely. Excessive loadings of reactive N (i.e., NO_3^-, NH_4^+) from fertilizer overuse have dramatically disturbed Earth ecosystems and inland waters in particular. One of the immediate consequences of P and N pollution in lakes and in the ocean is excessive biomass production, which supplies large amounts of highly labile organic matter to the sediments. Subsequent microbial degradation of this surplus organic matter quickly reduced the oxygen (O_2) availability leading to proliferation of hypoxia and anoxia in bottom waters.

Anaerobic respiration by microorganisms can remove significant quantities of reactive N from the system, specifically in the sediments where rates of microbial N processing are highest. Anaerobic denitrification and ammonium oxidation (anammox), which convert fixed N to N_2, are globally the most important sinks for reactive N. In contrast, anaerobic dissimilatory nitrate reduction to ammonium (DNRA) hampers the removal of nitrogen and instead leads to recycling of nutrients (e.g. NH_4^+) in the water column. The relative partitioning between N-removal and its recycling plays a critical role in modulating eutrophication and is highly relevant for regulating the N budget of lakes and the ocean. However, our current understanding of the nitrogen cycling in lacustrine sediments, and in particular the exact biogeochemical controls on the relative partitioning between these N-transformation processes remains limited. Recent discovery of new N-transforming microorganisms with previously unknown metabolisms and unexpected links with other biogeochemical cycling (e.g. iron, manganese, sulfur and methane) implies that

despite, decades of research, aquatic nitrogen cycling still hides mysteries. Lake Lugano (South Basin) is a monomictic eutrophic system, and thus an excellent model system to disentangle the relative importance of numerous microbial redox-driven transformations and identify their environmental controls. The main goal of my PhD project was to quantify the different benthic N-transformation processes and understand the potential environmental controls on their relative contribution to N reduction, and associated NO_3^- isotopic signatures. In addition, we investigated the microbial community's structure and its seasonal dynamics at the surface sediments.

The results highlight the overall importance of the biogeochemical controls O_2, sediment reactivity, Fe^{2+} and H_2S on the partitioning between N-loss and N-recycling, as well as on N isotopic signatures associated with NO_3^- reduction. Denitrification was the main anaerobic N-transformation processes in the sediments. The relative contribution of DNRA to total NO_3^- reduction varied from 31 to 52% depending on the season. In contrast, anammox contributed only about 1%. We demonstrated the major importance of oxygenation in controlling the fate of N in incubation experiments using natural sediments subject to fluctuating oxygen concentrations. Denitrification was favored over DNRA at relatively low oxygen concentrations (≤ 1 µM O_2). In contrast N-processing via DNRA was prevalent at higher O_2 levels. The O_2 penetration depth was found to control the NO_3^- isotopic signatures in overlying water, through effects on nitrification (aerobic oxidation of NH_4^+ to NO_3^-) in particular. Finally, we showed that the availability of Fe^{2+} and H_2S regulated the balance between denitrification and DNRA. Generally, at low Fe^{2+} (≤ 250 µM) and free H_2S (≤ 80 µM) levels, denitrification was favored over DNRA, while DNRA was stimulated when Fe^{2+} concentration exceeded 700 µM. In contrast, Mn^{2+} did not play an important role in regulating the fate of benthic N.

Among bacterial functional groups, only sulfate-reducing, sulfur-oxidizing and methanotrophic bacteria were affected by seasonally changing redox conditions at the sediment-water interface. The annual water-column turnover and subsequent oxygenation of bottom waters likely decreases Fe^{2+} and H_2S availability in surface sediments, which may enhance NO_3^- removal to N_2 at the

oxic-anoxic interface layer. On the other hand, DNRA is less O_2 sensitive than denitrification, and water-column mixing may enhance NH_4^+ release from the sediments. Therefore, the experimental results suggest the importance of fluctuating environmental conditions in regulating the partitioning between N-loss and N-recycling in freshwater sediments, however, it remains difficult to predict how these environmental changes act together to possibly shift the balance between the different N-cycling processes and to regulate the overall fixed N-elimination rate in lake sediments.

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Chapter 1

Introduction

The modern nitrogen cycle

Nitrogen (N) is a key constituent of many important biomolecules (i.e., amino acids, nucleic acids) and essential to all living organism. The microbial N cycle has thus been intensively investigated over the last centuries from the molecular to the environmental level. The classical loop "nitrogen fixation-nitrification-denitrification" was already stated by the end of the 19th century (Payne, 1986; Smil, 2011), whereas the importance of other processes such as dissimilatory nitrate reduction to ammonium (DNRA) or anaerobic ammonium oxidation (anammox) was reported much later (reviewed in Burgin and Hamilton 2007). The improvement of experimental isotope-based and molecular techniques helped to expand our knowledge on the global N cycle in environments.

N is naturally introduced into the biosphere from fixation of atmospheric N_2 by diazotrophs. This natural fixation pathway is reported to introduce substantial amounts of N into terrestrial and aquatic ecosystems (Fowler et al., 2013). Subsequently, N_2 is transformed into organic N that is mostly recycled into inorganic compounds through ammonification (Figure 1.1). Microbes can then perform diverse N redox reactions to gain energy for growth, and thereby become important drivers of the fate of nitrogen in environments. At the same time as our understanding of N transformation pathways has tremendously improved in the last century, recent human activities have dramatically altered the global N cycle.

The nitrogen cycle is one of the most anthropogenically impacted biogeochemical cycle (Fowler et al., 2013). The population explosion during the 20th century required the use of synthetic N fertilizers to enhance food production (Figure 1.2). At that time, about half of the human population depended on these fertilizers for their food (Galloway et al., 2004; Fowler et al., 2013). Therefore, the creation of the Haber-Bosch process ($N_2 + 3H_2 \rightarrow 2NH_3$) helped to sustain life on Earth, but intensive use of the process led to a dramatic increase of ammonia synthesis from 3.7 Mt N in 1950 to 100 Mt in 2010, with about 75% of the total NH_3 produced used as fertilizer (Smil 2011). Excessive

loading of this fixed N in groundwater and rivers in developed areas of the world has caused the eutrophication of lakes and coastal marine waters.

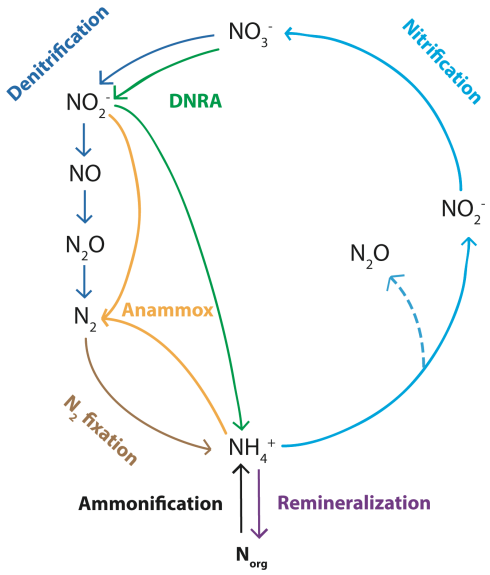

Figure 1.1: Microbial nitrogen transformations in aquatic systems.

This surplus of N supported algal blooms; their subsequent microbial degradation using oxygen as electron acceptor resulted in the development of oxygen-depleted zones in bottom waters. These events have had many consequences for the environment, including emissions of the greenhouse gas nitrous oxide (N_2O), global acidification, alteration of other biogeochemical cycles (Gruber and Galloway, 2008), and reduced habitat for higher trophic levels, like fish. Our goals in understanding the N cycle have thus shifted from how to sustain life to how intensive human activities have damaged ecosystems. Given expected trends in population growth, models predict that anthropogenically derived riverine N loadings will be 1.2-fold higher in 2050 than in the early 1990s, when they had already increased by 1.6-fold since 1860 (Galloway et al., 2004).

To tackle this situation, it is crucial to get a complete understanding of the interplay among microbial N processes to better constrain the fate of this excess "new" N in environments. Some microbially-mediated processes (e.g.

denitrification, anammox), which are discussed in the next section, can help to mitigate these excessive N-loadings by producing N_2. In contrast, other processes (e.g. nitrification, DNRA) maintain the internal eutrophication of the system through production of bioavailable forms of N for microbes. This thesis will focus on how introduced fixed N is microbially transformed in lacustrine environments with a particular focus on understanding the environmental controls (O_2, Fe^{2+}, Mn^{2+}, H_2S) on these processes and associated microbial communities.

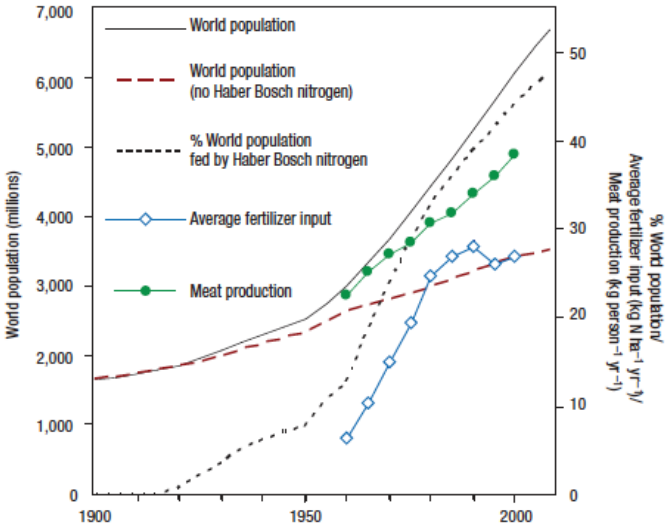

Figure 1.2: Fertilizer use and world human population trends in the 20th century (from Erisman et al., 2008)

Microbial N transformation pathways

Nitrogen is introduced to aquatic systems via biological N_2 fixation by prokaryotes or by allochthonous inputs from natural or human activities. Only prokaryotes containing the enzyme nitrogenase are able to fix atmospheric N_2 and convert it to ammonium through ammonification. This section will provide an overview of the most important aerobic and anaerobic fixed N transformation pathways (Figure 1.1), though the rest of thesis will mainly focus on anaerobic N processes.

Nitrification

Nitrification plays an essential role in the global N cycle, particularly by mitigating NH_4^+ production from organic matter decomposition and providing the required electron acceptor for denitrification. Nitrification consists of stepwise reactions from ammonium to nitrate via hydroxylamine, NO and nitrite. It is performed primarily by aerobic ammonia oxidizers and nitrite oxidizers.

$$NH_4^+ \rightarrow (NH_2OH) \rightarrow (NO) \rightarrow NO_2^- \rightarrow NO_3^-$$

Classically, the reaction was considered to be only performed by chemolithoautotrophs, though it was recently reported that some chemoorganotrophic bacteria are also capable of "heterotrophic nitrification" and may play an important role in the environment (Stein, 2011). The first step consists of ammonium oxidation to hydroxylamine (NH_2OH) catalyzed by the membrane-associated enzyme ammonium monooxygenase. The subsequent oxidation of hydroxylamine by the periplasmic enzyme hydroxylamine oxidoreductase (HAO) was long thought to yield nitrite directly. Only recently, Caranto and Lancaster (2017) confirmed that HAO oxidizes hydroxylamine to NO and not NO_2^-, which makes NO an obligate intermediate in nitrification. This set of reactions is carried out by ammonia oxidizing bacteria of the genera *Nitrosomonas* and *Nitrosospira* (Kowalchuk and Stephen, 2001). Nitrite oxidation is performed by the membrane-bound enzyme nitrite oxidase found in bacteria of the genera *Nitrobacter*, *Nitrococcus*, *Nitrospir*a, and *Nitrosospira* (Daims et al., 2016), producing nitrate that can be used to fuel denitrification. Recently, bacteria capable of carrying out the complete nitrification pathway (Comammox) were discovered and are widely distributed (Daims et al., 2015). Furthermore, it has been found that many heterotrophic nitrifiers are also capable of aerobic denitrification (Robertson and Kuenen, 1990). Therefore, nitrite and nitrate produced from nitrification are simultaneously reduced (e.g.

coupling with denitrification and/or DNRA) and do not accumulate in environments, which often led to underestimates of the contribution of nitrification in the past (Stein, 2011). Further discussion on nitrification is, however, beyond the scope of this thesis.

Denitrification

Denitrification was reported in the 19th century when the first denitrifying bacteria strain was isolated (Payne, 1986). Denitrification is the major sink for fixed N, converting NO_3^- to N_2 with NO_2^-, NO and N_2O as intermediates.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The respiratory nitrate reduction to N_2 is a stepwise reaction carried out by four different enzymes (Zumft, 1997). Nitrate reductases can either be membrane-bound (Nar) or periplasmic (Nap). Nitrite reductases (Nir) are located in the cell periplasm and the two enzymes (NirS and NirK) are not found together in the same strain. Nitric oxide (NO) reduction to nitrous oxide (N_2O) is catalyzed by the membrane-bound NO reductase (Nor). The last step of denitrification (N_2O to N_2) is performed by the periplasmic N_2O reductase (Nos). Most microorganisms are capable of complete denitrification, though some denitrifiers are lacking one or more of the reductases leading to intermediates accumulation (Zumft 1997). However, in this thesis, we define denitrification as the complete reduction of NO_3^- to N_2.

The "canonical" respiratory denitrification process is the dominant pathway for fixed N elimination in aquatic systems using nitrate as terminal electron acceptor to oxidize organic matter (organotrophic denitrification). More recently, microbes capable of nitrate reduction using inorganic compounds such as Fe^{2+} and H_2S (chemolithotrophic denitrification) have been discovered in pure culture (Straub et al., 1996; Dannenberg et al., 1992) as well

as in a wide range of environments (discussed below). The denitrification pathway has been investigated in pure culture experiments using microbial strains of *Pseudomonas* and *Paraccocus* species amongst others (Knowles, 1982; Ferguson, 1994; Zumft, 1997). Denitrification in the environment is, however, very widespread among bacterial communities, and the phylogenetic affiliations of denitrifiers are extremely broad. Most of denitrifiers are prokaryotes, and most of these are found within the Proteobacteria (α, β, γ, δ, ε) whereas only very few denitrifying archaea have been identified so far (e.g. *Pyrobaculum aerophilum*, *Haloarcula denitrificans;* Canfield et al., 2005).

Dissimilatory Nitrate Reduction to Ammonium (DNRA)

The existence of microbes capable of dissimilatory nitrate reduction to ammonium (DNRA), or nitrate ammonification, was highlighted in the early 20th in pure culture experiments (Woods, 1938). However, in the past decades, DNRA had received very little attention, particularly regarding its contribution to the N cycle in environments (Bonin, 1996). In contrast to denitrification, DNRA retains a bioavailable form of N within aquatic systems by reducing nitrate to ammonium with nitrite as the intermediate.

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$$

The initial NO_3^- reduction step of DNRA and denitrification is performed by the same nitrate reductase enzymes (Nar and Nap; Mohan and Cole 2007). The distinction between the two processes thus arises at the nitrite reduction step, which involves the cytoplasmic enzyme Nir in denitrification and the periplasmic enzyme Nrf in DNRA. In contrast to denitrification, nitrate ammonifiers have not yet been shown to possess the periplasmic NO_3^--reductase together with the cytoplasmic NO_2^--reductase, and vice-versa. The cytoplasmic pathway is restricted to very few facultative anaerobes in presence of high NO_3^-

concentration, implying that microorganisms have mostly retained Nap and Nrf enzymes in NO_3^--poor environments (Mohan and Cole, 2007).

Nitrate ammonification can be performed through fermentative (Cole and Brown, 1980) and respiratory pathways (Burgin and Hamilton, 2007). Fermentative bacteria primarily gain energy by using NO_2^- as electron sink from the oxidation of organic compounds, with additional ATP generated by substrate level phosphorylation. In respiratory DNRA, microbes gain energy by electron transfer from inorganic (H_2, Fe^{2+}, H_2S) and organic (formate, acetate) electron donors to the nitrite reductase, and ATP is generated by electron transfer across the membrane and oxidative phosphorylation. With these two possible mechanisms, the DNRA microbial community can have a strong advantage over denitrifiers in many environments, an aspect that is discussed further in a subsequent section. DNRA is known to be carried out by heterotrophic and chemoautotrophic bacteria from diverse phylogenetically lineages (Mohan et al., 2004), most of which are affiliated with the Proteobacteria, but also including Gram positive bacteria, Bacteroidetes and Planctomycetes (Tiedje, 1988; Bu et al., 2017). Recently, eukaryotes such as diatoms have been discovered to also perform DNRA (Kamp et al., 2011), however their exact metabolism remains to be investigated.

Anammox

Five decades ago, researchers observed that ammonium did not accumulate in marine oxygen minimum zones (OMZ) where denitrification occurred, and they hypothesized that another biological process oxidizing NH_4^+ anaerobically must thus occur (Richards, 1965). Based on thermodynamic calculations, Broda (1977) found that NH_4^+ oxidation with nitrite is as favorable as aerobic nitrification, and so speculated that some chemolithoautotrophic bacteria were able to perform the following reaction:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

However, microbial anaerobic ammonium oxidation (anammox) was only discovered in wastewater bioreactors much more recently (van de Graaf et al., 1995; Mulder et al., 1995). As anammox bacteria are very slow-growing and difficult to cultivate (Strous et al., 2006), it has been difficult to elucidate their metabolic pathway (Kartal et al., 2011). It was initially speculated that the electron acceptor NO_2^- was reduced to hydroxylamine (NH_2OH) during the first reaction step (Jetten et al., 2001). However, it is now well understood that NO is formed from NO_2^- reduction and simultaneously condenses with the electron donor NH_4^+ to produce N_2 via hydrazine (N_2H_4) as intermediate (Kartal et al., 2011). Based on molecular analysis, various enzymes were proposed to catalyze this stepwise reaction; 1) either a cd1 NirS nitrite reductase or a novel enzyme to catalyze NO_2^- reduction to NO, 2) a hydrazine synthase (hzsA) to conduct the reaction between NH_4^+ and NO to produce N_2H_4, and 3) a hydrazine oxidoreductase (hzo) to oxidize N_2H_4 to N_2 (Harhangi et al., 2012; Hu et al., 2019).

Anammox microbes are anaerobic chemolithoautrophs. So far only five genera capable of anammox have been identified, all affiliated within the Planctomycete lineage, and more precisely within the *Brocadiales* order: *Candidatus* Brocadia*, Candidatus* Kuenenia*, Candidatus* Anammoxoglobus*, Candidatus* Jettenia, *Candidatus* Scalindua;(van Niftrik and Jetten, 2012).

The fate of nitrogen in sediments

Sediments play a major role in the N budget in aquatic systems (Middelburg et al., 1996; Codispoti et al., 2001). Sediments can be a source of nitrogen through organic matter remineralization, nitrification, and DNRA; and an N-sink via denitrification and anammox. However, in contrast to marine sediments, little is known about the overall N-transformation rates in freshwater sediments, and N-budgets are thus difficult to assess. Denitrification and DNRA co-occur under similar conditions in absence or near absence of O_2, but nitrate concentration at the depth of nitrate reduction may be an important driver of the fate of nitrogen (discussed below). Until recently, however, the

significance of DNRA in environmental settings was little considered compared to denitrification (Bonin, 1996), and denitrification was thus often reported as the major process. The detection of DNRA in many different environments has challenged this prior assumption. In some settings the contribution of DNRA to N-reduction has been found to be as important as denitrification (e.g. An and Gardner 2002; Gardner et al. 2006; Brunet and Garcia-Gil 1996) or even, in more reduced sediments with high H_2S levels, to be the main NO_3^--reduction process (Dong et al. 2011; discussed below). The fate of nitrogen can have critical implications for the environment (Gruber and Galloway, 2008), so particular emphasis has been placed on understanding how sedimentary biogeochemistry controls benthic N-reduction pathways. But to date, experimental investigations generally used high substrate levels that are not representative of in situ conditions and/or they did not systematically measure all potential N-products (e.g. N_2, NH_4^+; see Table 2 in Chapter 4). Therefore, despite increased research, our understanding of the environmental factors controlling the denitrification versus DNRA partitioning in natural ecosystems still remains limited.

Environmental controls on the partitioning between NO_3^--reducing processes

Both denitrification and DNRA have been recognized in a wide range of aquatic environments and their respective contribution seemed to be strongly regulated by biogeochemical conditions (Burgin and Hamilton 2007). The carbon to nitrate ratio of available substrates has been suggested as one of the most important factors regulating the relative contribution of denitrification versus DNRA (Strohm et al., 2007). Generally, denitrifiers were stimulated under high NO_3^- availability and carbon source limitation (Strohm et al., 2007), whereas DNRA was favored under nitrate-limiting conditions when there was excess of electron donors because of the capacity of nitrate ammonifiers to accept eight electrons per molecule of nitrate rather than the five required to produce N_2 (Nizzoli et al., 2010; Chutivisut et al., 2014; van den Berg et al., 2015).

Seasonal cycles of water column mixing and stagnation can modulate the penetration of oxygen into the sediments, which may significantly affect redox gradients in surface lake sediments (Figure 1.3). Microbes preferentially consume O_2 since it yields more energy than other electron acceptors (e.g. NO_3^-, MnOx, FeOx, SO_4^{2-} and CO_2) for their growth. When oxygen is depleted, such as during water-column stratification, anaerobic processes are enhanced and reduced compounds accumulate. These compounds can be used by NO_3^--reducers as alternative electron donors (Figure 1.3; discussed below), which may in turn strongly alter the fate of nitrogen because of the extremely different paths taken by denitrifiers and nitrate ammonifiers.

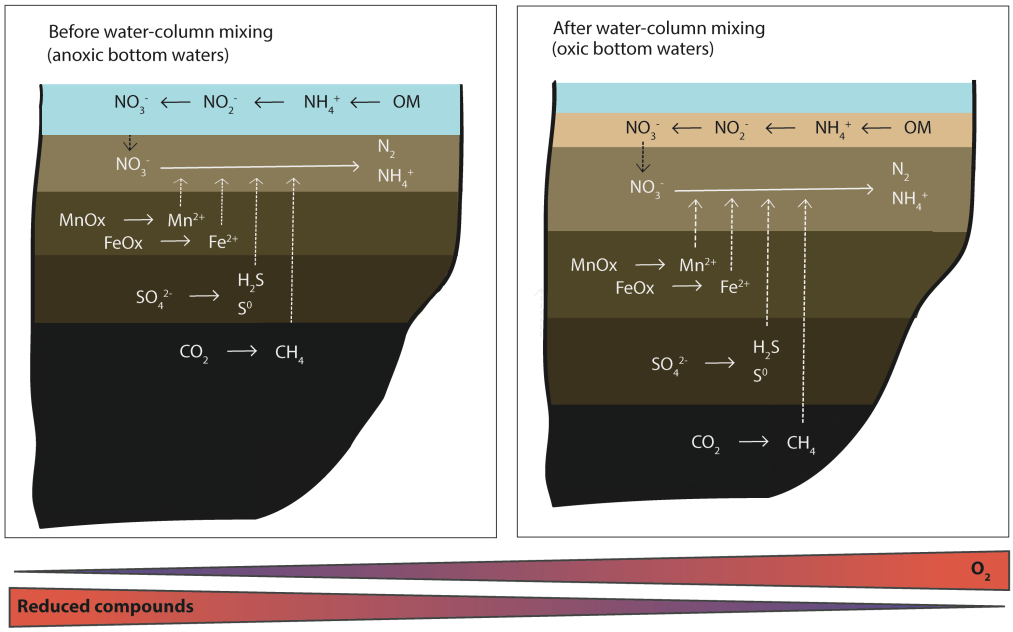

Figure 1.3: Schematic representation of potential interaction between nitrate reduction and other biogeochemical cycles in sediments over a seasonal cycling of water-column mixing. Text in black and white corresponds to aerobic and anaerobic reactions, respectively. The color gradients below the figure represent the schematic concentrations of reduced compounds (Fe^{2+}, Mn^{2+}, H_2S, CH_4) and O_2 at each season.

Oxygen has been shown to be an important driver of microbial processes in marine water column and sediments (Beman and Carolan, 2013; Broman et al., 2017). Similarly, oxygen control on denitrification and anammox rates has been thouroughly investigated in the ocean water column (e.g. Jensen et al. 2008; Kalvelage et al. 2011; Babbin et al. 2014; Dalsgaard et al. 2014). But

investigations into the O_2 inhibition and tolerance of benthic N-reduction remain rather rare and have been limited to sandy marine sediments (Rao et al., 2007; Gao et al., 2010; Hietanen et al., 2012; Marchant et al., 2017). Similarly, a systematic investigation of the exact O_2 control on DNRA does, to our knowledge, not exist.

Ferrous iron may also play an important role in regulating the partitioning between benthic denitrification and DNRA. Microbial chemolithotrophic denitrification and DNRA with Fe^{2+} have been recognized in pure and enrichment cultures of species including *Thiobacillus* sp., *Acidovorax* sp., and *Geobacter* sp. (Straub et al., 1996; Weber et al., 2006; Melton et al., 2014). However, the exact functional metabolism of the reaction is still not well constrained. Recently, some studies focused on investigating the significance of the process under environmentally relevant conditions in estuarine and freshwater sediments (Roberts et al., 2014; Robertson et al., 2016; Robertson and Thamdrup, 2017). Yet despite intensified research (reviewed in detail in Chapter 4), the importance of Fe^{2+}-dependent nitrate reduction in natural environments remains unclear, and the exact role of Fe^{2+} in controlling the balance between denitrification and DNRA is thus not well understood. In contrast, although suggested as a significant process in sediments (Aller, 1990; Schulz et al., 1994; Luther et al., 1997), the importance of Mn^{2+} oxidation coupled to NO_3^- reduction in environments has not yet been demonstrated experimentally.

Denitrification and DNRA using sulfide as electron donor are another alternative to heterotrophic denitrification and DNRA in sediments. The processes have been reported in pure and enrichment culture (Dannenberg et al., 1992; Campos et al., 2008; Kraft et al., 2014) and diverse environments (e.g. Brunet and Garcia-Gil 1996; Jensen et al. 2009; Bowles et al. 2012; Chapter 4), implying microbes affiliated within the genera *Thiobacillus*, *Sulfurimonas* and *Sulfuricella*, among others (Beller et al. 2006; Sievert et al. 2008; Watanabe et al. 2014; Chapter 2). In environmental settings, high H_2S concentration generally favored DNRA over denitrification whereas denitrification was mostly enhanced

under low H_2S levels (Brunet and Garcia-Gil, 1996; Christensen et al., 2000; Senga et al., 2006; Burgin et al., 2012). The coupling between N and S cycles may play an important role in detoxifying aquatic systems by removing the toxic compound H_2S and the contaminant NO_3^-, but the relevance of the process in natural freshwater sediments is still unclear.

Methane may also be a suitable electron donor for N reduction. Recently, new genera (e.g. *Candidatus* Methanoperedens and *Candidatus* Methanomirabilis) capable of performing this reaction have been identified in an enrichment culture obtained from anoxic freshwater sediment rich in nitrate (Raghoebarsing et al., 2006), but the importance of this process in the environment is still not well constrained.

By investigating the biogeochemical control (e.g. O_2, Fe^{2+}, Mn^{2+}, H_2S) on benthic N-reduction processes under environmentally relevant conditions, this thesis will thus bring new insights into the exact regulatory factors driving the fate of nitrogen in lake sediments.

How to trace N transformation pathways?

There is a multitude of approaches that can help to quantify microbial N transformation processes in the environment, including chemical profile analysis, molecular microbiological techniques, tracer experiments (e.g. isotope pairing technique) and the use of natural abundance stable isotope ratios of N and O. For many years, the use of experimental isotope-based methods proved very challenging due to the complexity of the aquatic N cycle and the potential influence of the co-occurrence of multiple processes (e.g. denitrification, anammox, DNRA). With the improvement of these techniques, we are now able to better constrain the relative contribution of N transformation pathways in environments and, in turn, obtain more accurate estimation of global N-budgets.

Isotope pairing technique (IPT)

The nitrogen isotope pairing technique (IPT) was developed by Nielsen and colleagues (1992) to quantify denitrification rates in sediments. The method is based on the random coupling between the two stable isotopes of N (^{14}N and ^{15}N) to produce ^{15}N-N_2 from the addition of labeled $^{15}NO_3^-$ to the system. The IPT allows thus the tracing of ^{15}N-N_2 production despite of high atmospheric (^{14}N-N_2) background. The formation of unlabeled ($^{14}N^{14}N$), single-labeled ($^{15}N^{14}N$), and double-labeled pairs ($^{15}N^{15}N$) is measured by mass spectrometry (Figure 1.4).

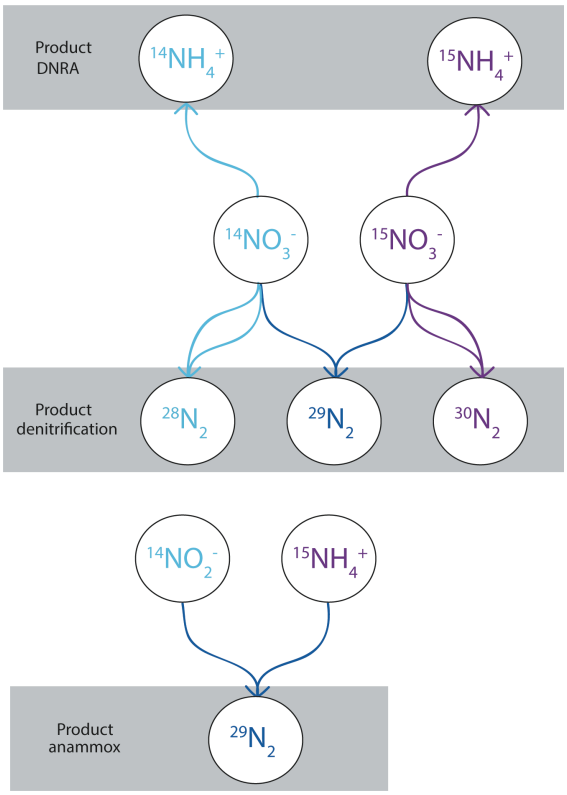

Figure 1.4: Overview of potential isotope pairing coupling during N-transformation processes (adapted from Holtappels et al., 2011)

The IPT can also be used to quantify anammox and DNRA in environments (e.g. sediments, water-column), using different substrates or experimental protocols (Risgaard-Petersen et al. 1995; Thamdrup and Dalsgaard 2002; Holtappels et al.

2011; see methodology sections of chapter 3 and 4). It is important to accurately quantify each N-transformation process under a wide variety of environmental conditions as environmental factors and/or the co-occurrence of multiple N-processes (e.g. denitrification, anammox and DNRA) may pose issues to the use of the IPT and, consequently, lead to misinterpretation of the results (reviewed in Robertson et al. 2019). We used the IPT to quantify benthic N-reduction rates in Chapter 3 and 4.

Natural abundance of stable N and O isotopes

Nitrogen has two stable isotopes: ^{14}N and ^{15}N, and oxygen has three: ^{16}O, ^{17}O, ^{18}O. Isotopic compositions are expressed in terms of "delta" (δ) values which quantifies the per mil (‰) deviation relative to a standard:

$$\delta_{sample} = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000$$

where $R = {}^{15}N/{}^{14}N$ or ${}^{18}O/{}^{16}O$, respectively. Delta values for both N and O are standardized to atmospheric N_2 (^{15}N abundance of 0.003677) and Standard Mean Ocean Water (SMOW; ^{18}O abundance of 0.0020052), respectively.

Biological kinetic isotope fractionation is based on the fact that different isotopes have similar chemical properties but, because of their distinct masses, different reaction rates. Typically, in microbially-mediated reactions, the isotopic fractionation is mass-dependent, where molecules containing lighter isotopes (e.g ^{14}N) react more quickly than those with heavier isotopes. Consequently, the residual substrate (e.g. NO_3^-) pool becomes enriched with the heavier isotope. Specific microbial pathways can be associated with differential isotope effect (more details in Chapter 5). The isotope effect ε quantifies the relative magnitude of isotopic enrichment in the reactant pool:

$$^{15}\varepsilon = \left(\frac{^{14}k}{^{15}k} - 1\right) \times 1000$$

where ^{14}k and ^{15}k correspond to the reaction rates of ^{14}N and ^{15}N, respectively. The isotopic fractionation can thus provide important insight into rates of N transformation processes. However, it is crucial to have detailed knowledge about individual reactions and what drives isotope fractionation in environmental settings to accurately quantify global N budgets. It is thought that NO_3^- fractionation during denitrification is mainly enzymatically-driven (Granger et al., 2008). However, environmental parameters also have significant mitigating effects on the expression of N-isotopic signatures. For instance, the net isotope effect of denitrification at the sediment-water interface was found to be lower than expected in marine and freshwater environments (Brandes and Devol, 1997; Lehmann et al., 2004b; Lehmann et al., 2007). Modelling results suggested that NO_3^- diffusion-limitation and/or sedimentary nitrification (e.g. Lehmann et al., 2007) may be the main parameters responsible for this discrepancy, but experimental evidence for these results does not yet exist, and the exact environmental controls on NO_3^- isotopic signature remain unclear (Chapter 5).

Molecular techniques

The development of molecular techniques has tremendously improved our understanding of microbial processes in the environment. We are now able to identify and quantify microbial communities through the next-generation sequencing (NGS), though still with some limitations. Phylogenetic 16S rRNA analysis does not, for instance, allow the identification of exact functional metabolism and additional molecular analyses are often necessary (e.g. qPCR). Nevertheless, it allows large amounts of sequencing to be performed in a single assay and thus replicate analyses of whole microbial communities in a large number of samples (Caporaso et al., 2012; Knight et al., 2012). The method is based on four steps: library preparation, cluster generation, sequencing and

data analysis (Figure 1.5). In this thesis, we took advantage of the NGS to investigate the benthic microbial community's structure in the studied lake (Chapter 2).

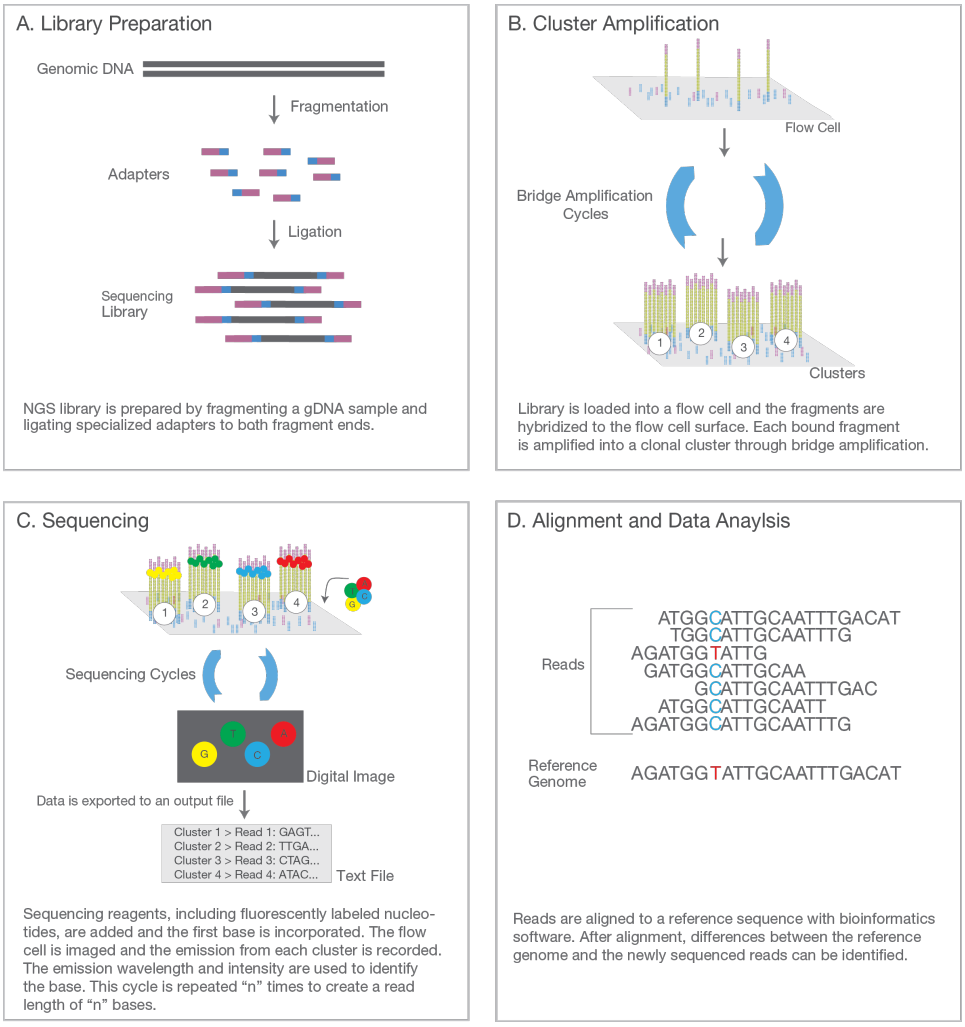

Figure 1.5: Principle of the NGS method (from Illumina, Inc; www.illumina.com/technology/next-generation-sequencing.html)

Rationale and PhD project objectives

Despite years of very intensive research, the nitrogen cycle is continuously called into question as deeper knowledge is constantly brought on to N-transformation processes themselves, their controlling geochemical factors, and their associated microbial communities. With this study we aimed to better constrain the biogeochemical controls (O_2, Fe^{2+}, Mn^{2+}, H_2S) on the

benthic microbial processes responsible for fixed-N reduction and associated isotopic signatures by extending the existing knowledge obtained from bacterial culture experiments, substrate-rich experiments and/or modeled-based observation to more environmentally relevant conditions. In addition, we identified the main actors catalyzing the principal biogeochemical processes occurring in the sediments of Lake Lugano (south basin) and assessed their seasonal (fall, winter, spring) variability in the surface sediments.

Lake Lugano has suffered from eutrophication since the start of industrialization and increased population density in its catchment area in the beginning of the 20th century (Barbieri and Mosello, 1992). The southern basin of the lake is a monomictic system that is annually mixed (fall/winter) leading to seasonal change in redox conditions, microbial community structure, and processes rates (Lazzaretti et al. 1992; Chapter 2 and 4). Based on earlier mass balance calculations, a large part of the external fixed N loading is being eliminated within the lake (Lehmann et al., 2004a), and prolonged anoxia of bottom waters before the annual water-column turnover resulted in the accumulation of large amounts of reduced chemical species (i.e., ammonium, methane, sulfide, reduced Fe and Mn) in sediments (Lazzaretti et al. 1992; Chapter 2). In a previous project on Lake Lugano, Wenk et al. (2013) observed the occurrence of S-dependent denitrification in the water-column of the meromictic northern basin. The significance of chemolithotrophic processes, including S-dependent denitrification, and also Fe^{2+} and Mn^{2+}-dependent nitrate reduction, in the sediments of the southern basin of the lake remains, however, unknown. In particular, the exact environmental controls on the relative partitioning between denitrification and DNRA are not yet constrained in Lake Lugano (south basin) sediments.

We were thus interested in the following questions:

- How does the seasonal water-column turnover affect the sediment (e.g. porewater, particulate phase) chemistry and the microbial community's structure at two different sites of the southern basin of the lake; Figino and Melide?

- What is the relative contribution of denitrification, anammox, and DNRA to benthic N-transformation at these sites?
- How, and to what extent, do changes in environmental conditions (O_2, Fe^{2+}, Mn^{2+}, H_2S) influence the partitioning between N-removal and N-recycling?
- What environmental parameters (e.g. sediment reactivity and associated O_2 penetration depth, nitrification) are responsible for the under-estimation of the isotope effect associated with benthic NO_3^- reduction at the sediment-water interface?

Chapters and publication outline

This thesis includes four manuscripts, two of which have been published in peer-reviewed journals and one of them is in preparation for submission within the coming months. My contribution to the publications is detailed below.

1. Spatial and temporal variability of microbial communities in ferruginous sediments of a eutrophic lake

Adeline N.Y. Cojean, Moritz F. Lehmann, Guangyi Su, Jakob Zopfi

In preparation for: *Frontiers in Microbiology*

This study will be presented in Chapter 2. The determination of most chemical compounds in the water column and sediments was performed by the SUPSI institute and myself, respectively, and CH_4 concentration was measured by Guangyi Su. I extracted DNA samples and reprocessed the sequencing data with the help of Jakob Zopfi. I wrote the manuscript with input from all co-authors.

2. Direct O_2 control on the partitioning between denitrification and dissimilatory nitrate reduction to ammonium in lake sediments

Adeline N.Y. Cojean, Jakob Zopfi, Alan Gerster, Claudia Frey, Fabio Lepori, Moritz F. Lehmann

Published as: *Biogeosciences* (2019), **16**, 4705-4718

This study will be presented in Chapter 3. I designed the experimental set-up and performed all rate measurements together with Alan Gerster in the context of his Bachelor thesis. Fabio Lepori provided concentration profiles of chemical compounds in water column. I wrote the manuscript with input from all co-authors.

3. Controls of H_2S, Fe^{2+}, and Mn^{2+} on microbial NO_3^--reducing processes in sediments of an eutrophic lake

Adeline N.Y. Cojean, Moritz F. Lehmann, Elizabeth K. Robertson, Bo Thamdrup, Jakob Zopfi

Published as: *Frontiers in Microbiology* (2020), **11**(1158), 1-17

This study will be presented in Chapter 4. Elizabeth Robertson taught me the method for ^{15}N-labelled incubation experiments in the laboratory of Bo Thamdrup at the University of Southern Denmark. All experimental work and process rate measurements were performed by myself. I wrote the manuscript with input from all co-authors.

4. Environmental control on the nitrogen isotope effect of sedimentary nitrate reduction at the sediment-water interface

Adeline N.Y. Cojean, Jakob Zopfi, Fiona Galliker, Thomas Kuhn, Anna-Neva Visser, Moritz F. Lehmann

This study will be presented in Chapter 5. I designed and conducted all experimental work with the help of Moritz Lehmann and Jakob Zopfi. In the context of her Bachelor thesis, Fiona Galliker also performed some incubation experiments and rate measurements. Thomas Kuhn and Anna-Neva Visser reprocessed the isotope data. I wrote the manuscript with input from all co-authors.

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Chapter 2

Spatial and seasonal variability of microbial communities in ferruginous sediments of a eutrophic lake

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Abstract

Lake sediments are sites of organic matter accumulation, degradation and nutrient recycling. In anoxic sediments such as in the eutrophic south basin of Lake Lugano, these biogeochemical processes are essentially carried out by prokaryotic microorganisms. Despite of investigations on biogeochemical processes in the sediments of the lake, little is known about the main microbial actors catalyzing these different reactions. With the improvement of molecular techniques, we are now able to better assess microbial community structures and potentially deduce the role of certain community members in specific biogeochemical reactions. Both study sites presented here share high geochemical similarity and exhibited very similar microbial community structures. Generally, a greater α-diversity was observed in surface sediments than in deeper layers. Yet, the 16S rRNA phylogenetic analyses revealed the presence of microbes potentially involve in N, Fe, S and C biogeochemical processes. Furthermore, despite of changing redox conditions at the surface sediments due to water-column turnover, the microbial community structure was relatively stable at different season and both sites. The putative sulfate-reducers, sulfide-oxidizers and methanotrophs were the most dynamic communities while the microbial community structure of other functional groups remained similar at all seasons. By stimulating H_2S-oxidizers and methanotrophs in particular, the water-column may play a significant role in the lake detoxification by removing the toxic gas H_2S and the potent greenhouse gas CH_4.

Introduction

Lake sediments are hot spots of microbial life and play an important role in organic matter decomposition and nutrient recycling (Forsberg, 1989). Benthic microbial communities are vertically stratified in the sediment column accordingly to specific redox zonation. Oxygen is generally rapidly consumed within the very first millimeter of the sediments as its use as terminal electron

acceptor yields more energy to microbes during respiration (Glud, 2008). When O_2 is depleted, anaerobically respiring microorganisms subsequently use NO_3^-, Mn(IV), Fe(III), SO_4^{2-}, and ultimately CO_2 to mineralize organic matter. However, identifying links between microbial processes and microbial community structure is very challenging. Investigations on marine and freshwater sediments have identified key taxa involved in these reactions but they also highlighted the variability of microbial communities among studied sites (e.g. Broman et al., 2017; Reyes et al., 2017). Yet, many microbes remain phylogenetically very widespread, or even unidentified, which makes our understanding of the microbial community structure in sediments largely incomplete.

Like many lakes in Switzerland, Lake Lugano is a eutrophic system (Lepori et al., 2018), in which intense microbial activities have led to prolonged anoxia of bottom waters, resulting in the accumulation of large amounts of reduced chemical species (i.e., NH_4^+, Mn(II), Fe(II), H_2S, CH_4) in sediments (Lazzaretti et al., 1992). During these last decades, studies have investigated the different biogeochemical cyles (e.g. P, N, C) in the lake sediments through porewater concentration determination (Lazzaretti et al., 1992), process rate measurements (Wenk et al., 2014; Cojean, in prep.), and isotope-based methods (Lehmann et al., 2004; Bechtel and Schubert, 2009). However, despite having gained an increased knowledge of the biogeochemical processes in Lake Lugano sediments, investigations on the microbial community's structure do not exist, and the main microbial actors catalyzing these processes remain unknown.

In general, very little is known about the depth- and time-related variations of microbial community structures in sediment and how they respond to the biogeochemical conditions in their habitat. The main hurdle has been the limitations of techniques for quantifying microbial community structure, both in sequencing depth and limited resolution of fingerprinting (Werner et al. 2011). This situation has changed dramatically with the advent of the next-generation sequencing (NGS) technologies, which allow the massive parallel sequencing of

a large number of samples in a very short period of time and at comparatively low costs. Thanks to the parallel development of adapted bioinformatics and statistical tools we are, for the first time, in the position to perform replicate analyses of whole communities of microorganisms and to follow them or individual taxa in a large number of samples with time (Caporaso et al., 2012; Knight et al., 2012; Nemergut et al., 2013). Yet such analyses are expensive and, in general, only one core per site has been investigated, implying low-resolution power of the molecular methods. As a consequence, little is known about the main microbial actors catalyzing the biogeochemical cycles.

The goals of this study were thus to i) identify the main microbial actors catalyzing the principal biogeochemical processes in iron rich (ferruginous) sediments of a eutrophic lake basin, ii) to compare the sedimentary microbial community structures in two spatially separated sampling locations that share high geochemical similarity, iii) to assess the vertical and seasonal (fall, winter, spring) variability of microbial communities in replicate sediment cores, and finally, iv) to attempt a link between microbial community structure and prior biogeochemical process rate measurements.

Materials and methods

Study area and sample collection

Lake Lugano is located at the Swiss/Italian border and is separated by a natural dam into a permanently stratified northern basin and a eutrophic monomictic southern basin (Barbieri and Polli, 1992; Lepori et al., 2018). Sediment cores were collected using a small gravity corer (inner diameter 6.2 cm) at two different stations in the south basin of Lake Lugano, Station Figino (8°53'37"E, 45°57'31"N, 94 m depth) and Station Melide (8°57'29"E, 45°56'22"N, 85 m depth). Samples for seasonal dynamics of sediment geochemistry were taken in February and May 2015, November 2016, June and

October 2017, and March 2018. In November 2016, June 2017 and March 2018, samples were also collected for DNA extraction and microbial community analysis. Water column O_2 concentration and temperature were determined with a CTD (Idronaut Ocean Seven 316Plus). Concentrations of chemical compounds (NO_3^-, NO_2^-, NH_4^+) were measured by the SUPSI institute as part of the CIPAIS monitoring program (IST-SUPSI, 2018).

Sediment and porewater characterization

Directly after return to the laboratory, sediment cores were sectioned for porewater extraction and particulate phase characterization. From 0 to 6 cm depth, the sediment was sectioned into 1 cm thick slices and immediately placed into 50 mL Falcon™ tubes and centrifuged at 4700 rpm, 10 min. In the meanwhile, deeper layers (6 to 20 cm depth) were sectioned every 2 cm and subsequently centrifuged. The supernatant was filtered (0.2 µm) and samples (1 mL) for H_2S determination were stabilized with 20 µL aqueous zinc acetate solution (20 % w/v) and stored at 6 °C until measurement. Samples for Fe^{2+}, Mn^{2+} were fixed with hydrochloric acid (40 µL 1M HCl). Additionally, particulate Fe (Fe^{II}, Fe^{III}) and total Mn were extracted by adding 1 g of sediment into 50 mL Falcon tubes that were pre-filled with 30 mL HCl (1M). Tubes were placed on a shaker for 24 h and then centrifuged at 4700 rpm for 10 min. Supernatants were filtered and frozen for later measurements. The remaining filtered porewater was frozen until analysis of the other compounds (NO_3^-, NO_2^-, NH_4^+, SO_4^{2-}). Additional sediment from each depth was frozen in 15 mL Falcon for later determination of porosity, organic matter content, and grain-size. For porosity measurement, 4 mL of wet sediment were placed in an aluminum tray and weighed precisely. After 12 hours at 70 °C, the dried sediments were weighed again. The porosity was determined as: ((((ww-dw)/ww))*100)*d/100, where ww and dw correspond to wet and dry weight, respectively, and d the density of 4 mL wet sediment. For determination of the organic matter content about 12 mg of dried sediment was placed into a silver capsule and 1-2 drops of Milli-Q water were added. The samples were exposed to fumic HCl in a desiccator for 24

h to remove inorganic carbon. Samples were dried (48 h, 50 °C) and analyzed using an integrated and ^{13}C and ^{15}N analyzer (Sercon, Integra 2). Total inorganic carbon content was calculated from the difference between the carbon content of HCl-treated samples (organic carbon) and untreated samples (total carbon). Grain-size measurement was performed by weighing 1g of sample that was preliminarily treated with H_2O_2 (30%) to remove organic matter, and measurement through the laser diffraction technique using a Malvern Mastersizer 2000.

Concentration profiles of dissolved O_2 were measured using commercial microsensors with a tip diameter of 100 µm (Unisense). The water layer (~10-15 cm) above the sediment surface was continuously aerated by a gentle gas flow through a glass micropipette and to ensure the establishment of a stable diffusive boundary layer (DBL). Typically, triplicate profiles were measured in each of the duplicate cores.

Analytical methods

Dissolved sulfide in the porewater was quantified by the colorimetric methylene blue method (Cline, 1969). Concentration of Fe^{2+} in porewaters and particulate phases (Fe^{II} and Fe^{III}) was determined using the Ferrozine assay (Stookey, 1970). Determination of Fe^{III} concentration was calculated from the difference between total Fe^{II} (hydroxylamine addition, Fe^{III} + Fe^{II}) and Fe^{II} (no hydroxylamine addition; Jensen and Thamdrup 1993). Manganese concentration in the acidic sediment extracts (including dissolved and particulate phases) was determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES; Agilent Technologies 5100). Nitrite concentrations were determined colorimetrically using sulfanilamide and Griess-reagent, according to Hansen and Koroleff (1999). Total NO_x (i.e., NO_3^- + NO_2^-) concentration was measured using a NOx-analyzer (Antek Model 745) after reduction to nitric oxide (NO) in an acidic V^{3+} solution, and detection of NO by chemiluminescence (Braman and Hendrix, 1989). Nitrate concentration was

calculated from the difference between [NO_x] and [NO_2^-]. Ammonium was measured by suppressor ion chromatography (940 Professional IC Vario, Metrohm) and conductivity detection with a column comprised of silica gel with carboxyl groups. Major anions, including chloride and sulfate, were quantified by suppressed anion chromatography (Metrohm) and conductivity detection.

DNA extraction

Sediment cores for molecular analyses were collected in November 2016, June 2017, and March 2018 when the bottom water oxygen concentrations (2 m above the sediment surface) were about 0 µM, 30 µM, and 140 µM respectively. After returning to the laboratory, duplicate sediment cores from each sampling site were sectioned every 2 cm down to 20 cm depth. Aliquots of homogenized sediment from each layer were transferred into 2 mL Eppendorf tubes and immediately frozen at -80 °C for later DNA extraction. DNA was extracted from about 0.4 g w.w. of sediment using the Fast DNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's protocol, including an initial bead beating step of 30 seconds and 6500 rpm (Precellys® 24, Bertin Technologies). The concentration of DNA in the purified extracts was determined fluorometrically using Qubit assay (Invitrogen).

PCR amplification, Illumina sequencing, and data analysis

Extracted DNA samples were sent to the Quantitative Genomics Facility Basel (https://www.biozentrum.unibas.ch), Switzerland, for amplicon library production and high throughput sequencing. A two-step PCR approach was applied for library preparation as described in detail in Su et al. (submitted). Briefly, a first PCR of 25 cycles was performed using universal primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3') targeting the V4 and V5 regions of the 16S rRNA gene (Parada et al., 2016). Sample indices and Illumina adaptors were added in a second PCR of eight cycles. Purified indexed amplicons were finally pooled at equimolar

concentration into one library and sequenced on an Illumina MiSeq platform using reagent kit V3 and the 2×300 bp paired-end protocol. After sequencing, quality of the raw reads was checked using FastQC (v 0.11.8; Andrews et al., 2010). FLASH was used to merge forward and reverse reads into amplicons of about 374 bp length, allowing a minimum overlap of 15 nucleotides and a mismatch density of 0.25 (Magoc and Salzberg, 2011). Primer sites were trimmed with cutadapt (Martin, 2011), and thereafter quality filtering (min Q20, no Ns allowed) was carried out using PRINSEQ (Schmieder and Edwards, 2011). Classical OTU (operational taxonomic unit) clustering with a 97% cutoff was performed using the UPARSE-OTU algorithm in USEARCH v10.0.240 (Edgar, 2010). Taxonomic assignment was done using SINTAX v10.0.240_i86linux64 (Edgar, 2016) and the SILVA 16S rRNA reference database v128 (Quast et al., 2013). Subsequent analyses were carried out in the R environment (v. 3.5.1; R Core Team, 2018) using the packages phyloseq (McMurdie and Holmes, 2013), DEseq2 and vegan (Love et al. 2014; Oksanen et al., 2017). Chloroplast and mitochondria sequences were removed from the dataset before any further analysis was done.

Statistical analysis

In total a number of 64 samples have been sequenced, including 2x16 samples from Station Figino and 2x16 from Station Melide. In November 2016, a depth profile consisting of 10 depths (0-2 cm, 2-4 cm,…18-20 cm) has been sampled at both stations and from duplicate cores. Additional samples were collected at the same locations in June 2017 and in March 2018, where only selected depths (0-2 cm, 2-4 cm, 4-6 cm, 10-12 cm, 18-20 cm) were examined to assess the variability of repeated sampling, and potentially, to detect temporal variations in community structures. For the basic characterization of the microbial community structures different α-diversity measures (Observed OTU richness, Chao1, Shannon, and inverse Simpson) were determined, based on rarefied sequence data to compensate for the different sequencing depths. To determine the degree of dissimilarity in the microbial community composition

among different samples (beta-diversity), Bray-Curtis as well as weighed Unifrac (which considers both abundance and phylogenetic relatedness of taxa; Lozupone et al., 2011) distances were calculated on relative abundances of OTU's. Principal Coordinates Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS) ordinations of microbial community structures were calculated using the ordinate function implemented in the vegan package (Oksanen et al., 2017) in the R environment (v.5.3.1; R Core Team, 2018). A statistical test was performed in order to check whether changes in the microbial community composition among different sampling dates and sediment depths were significant. Briefly, prior analysis sample count data were transformed to achieve a negative binomial distribution (McMurdie and Holmes, 2014) using the *DEseq2* package (Love et al. 2014). It is crucial to transform the data prior calculation of the dissimilarity distance matrix. We performed analyses of variances for multivariate data (PERMANOVA; 999 permutations) based on the weighed Unifrac distance of the sample using the *adonis* function in the *vegan* package in R (Oksanen et al., 2017; R Core Team, 2018). Additionally, in order to check whether statistical differences were observed for each functional group (e.g. potential sulfate-reducing bacteria, sulfide-oxidizers, iron-oxidizers, etc.), potential genera for each corresponding group were subset from the overall microbiome prior the statistical analysis described above.

Results

Porewater and solid-phase chemistry

The sediments at both sampling sites were rich in organic matter (C_{org} ~8% d.w.), very fine grained (< 125 µm grain-size) and quite liquid in the top 2-4 cm. Oxygen penetration depth in sediments was ~ 3.7 ± 0.6 and 2 ± 0.5 mm at Figino and Melide, respectively. At both stations, the chemical gradients were relatively similar across seasons and thus no clear seasonal trends were observed within the surficial sediments (Figure 2.1). Generally, the sediments were characterized by high concentrations of dissolved Fe^{2+}, Mn^{2+}, and CH_4 in

the porewater. Dissolved Fe^{2+} concentrations at Figino ranged from 180 to 790 µmol L^{-1} at the sediment surface, depending on the season, and reached up to ~1200 µmol L^{-1} at about 19 cm depth (Figure 2.1). At Melide, concentrations were only slightly lower and rose from ~20-100 µmol L^{-1} to 600 µmol L^{-1}. Similarly, particulate Fe^{II} contents were consistently higher at Figino than Melide in the surficial sediments (~24-98 and 11-43 µmol g^{-1}, respectively) and increased with depth to ~ 162 µmol g^{-1}. Yet, higher particulate Fe^{III} and total Mn levels were observed at Figino, and measured contents were similar for both compounds (~4-32 and 1-12 µmol g^{-1} at Figino and Melide, respectively). In contrast, porewater Mn^{2+} concentrations were relatively equivalent at the two stations across all seasons (from ~120-480 to 550 µmol L^{-1} in surface and deep sediments, respectively).

At both stations, sediments efficiently removed NO_3^-, exhibiting a strong decrease of NO_3^- concentration from bottom waters to surface sediments (from ~26-70 to ~0 µmol L^{-1}, respectively; Figure 2.1). Similarly, nitrite concentrations in sediments remained below detection limit. Ammonium profiles exhibited an important accumulation up to ~1750 µmol L^{-1} in deeper sediment layers, suggesting high organic matter remineralization rates. Similar to the other chemical compounds, the seasonal change in NH_4^+ concentration at the surface sediments was more pronounced at Figino than Melide.

Sulfate concentration rapidly decreased with depth (Figure 2.1). In contrast to chemical gradients described above, sulfate concentrations in surficial sediments were generally higher at Melide than Figino for all sampling dates. At both stations, free H_2S was not detected in porewaters. Measurements of dissolved free sulfide (H_2S) concentration in porewaters were strongly affected by the presence of ferrous iron and partial removal of H_2S as FeS. Yet, CH_4 concentration was important at both stations, particularly at Figino where concentrations exceeded 20 mM in May 2015 in deepest layers (Figure 2.1).

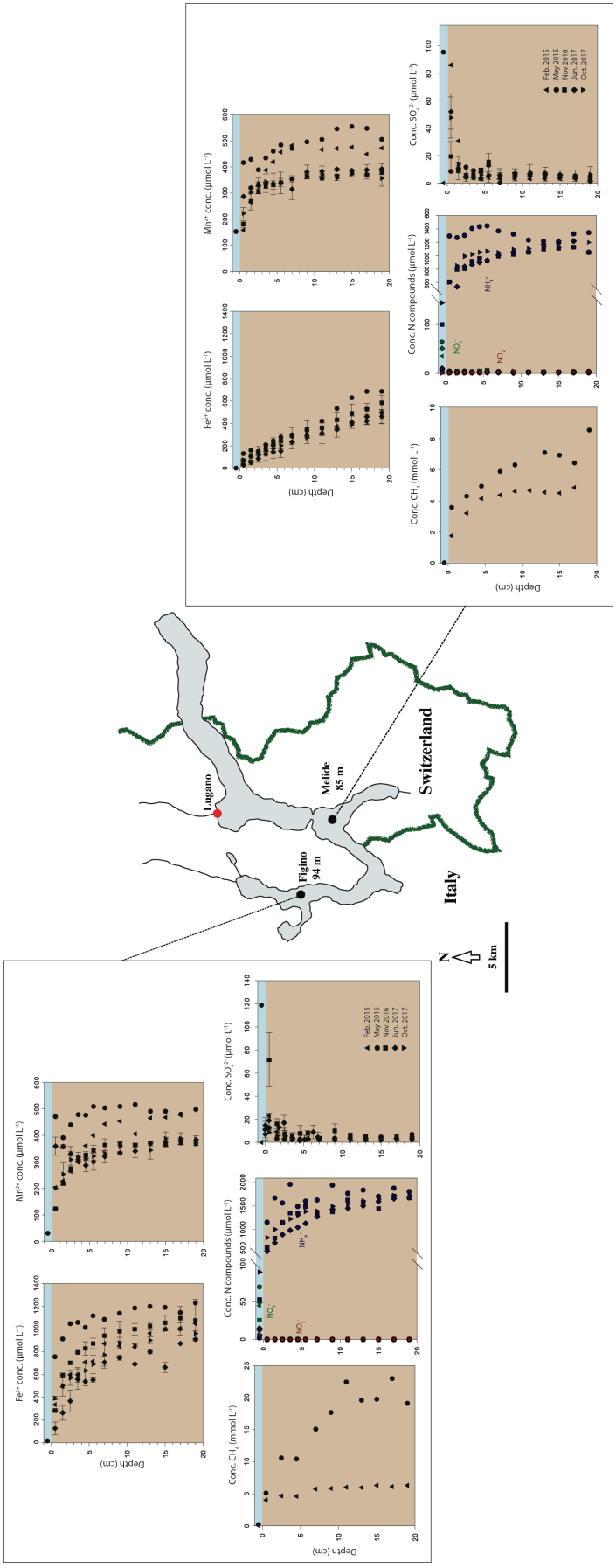

Figure 2.1: Map of Lake Lugano and vertical porewaters profiles at variable seasons at Figino and Melide.

Comparison of the microbial diversity between sites

At each sampling date, DNA was extracted from duplicate cores. A total of 26337 OTUs were obtained in all the 64 samples combined. Chloroplast (n = 164) and mitochondria (n = 183) sequences were removed from the dataset before any further analysis was done. The number of OTUs was further reduced to 18659 by eliminating all taxa with < 3 occurrences in all samples combined. Table 2.1 summarizes the sequencing results, including read per sample, singletons, observed OTU richness as well as other alpha diversity measures (Chao1, Shannon, and inverse Simpson). The box plots in Figure 2.2 present the alpha diversity for all combined samples of Figino and Melide, respectively.

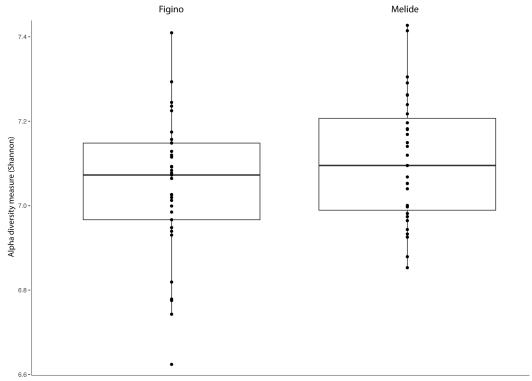

Figure 2.2: Variation of α-diversity (Shannon index) of microbial communities at the two sites

The distance weighed UniFrac was chosen over Bray-Curtis as it better explained the variability between samples taken into account the relative abundance and phylogenetic relatedness between taxa. The variation in microbial community structure was mainly driven by the first axis (46.1 and 33.9% using weighed UniFrac and Bray-Curtis, respectively) while the second axis had a minor contribution (10.3 and 13.2% using weighed UniFrac and Bray-Curtis, respectively). Similarly, Non-Metrical Dimensional Scaling (NMDS) was chosen over PCoA being more suitable in this case. Indeed, NMDS defines a two-dimension space that allowed the best spatial representation of sample dissimilarity based on weighed UniFrac similarity indices. The NMDS ordination

of 16S rRNA gene sequence OTU distributions revealed a consistent picture of the changes in the microbial community structure with depth and a good reproducibility of samples from replicate cores for most of the cases (Figure 2.3). Some surface sediment samples from Melide, however, group rather with samples from 3-5 cm depth (Figure 2.3).

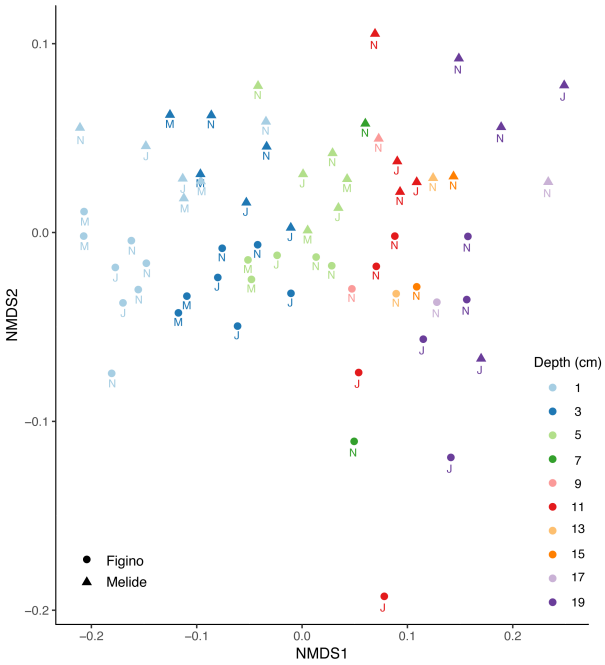

Figure 2.3: NMDS ordination plot of weighted Unifrac distances based on 16S rRNA gene sequences, representing the sedimentary microbial community structures at the two sampling sites in the south basin of Lake Lugano.

This could indicate that the surface layer of this core had been lost during sampling or that the uppermost sediment layers got mixed (e.g. by escaping methane bubbles, core handling) as a consequence of their soupy nature. Despite of a lower reproducibility at Melide, it seems that the microbial community structure is essentially driven by depth, as adjacent sediment depths were most similar to each other at both sites (Figure 2.3). This representation of the data shows that the microbial communities at the two different sampling locations in the south basin of Lake Lugano are similar but sufficiently different so that they form separate clusters. Comparing the α-diversity (Shannon) of all Melide samples combined with the Figino samples, there is only a minor, yet statistically

not significant, difference between the two sampling locations (Figure 2.2). The variability of the observed OTU richness as well as calculated α-diversity measures along the sediment profile are shown in Figure 2.4. With the notable exception of the uppermost sediment sample at both stations, there seems to be a tendency towards decreasing values for these parameters with sediment depth. Maximum observed OTU richness was observed at 3-5 cm at both stations (5692.8 ± 572.6 OTUs at Figino, 5903.2 ± 1012.7 OTUs at Melide).

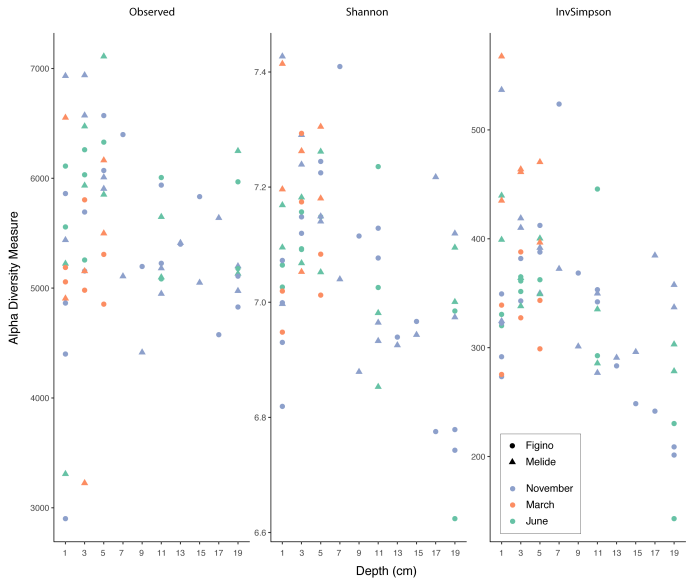

Figure 2.4: Seasonal and depth distribution of microbial α-diversity at Figino and Melide. The figure summarizes the Observed OTUs, Shannon and the inverse Simpson (InvSimspon) diversity indexes.

Figino									Melide								
Month	Depth	Replicate	Read per sample	Singleton	Observed	Chao1	Shannon	InvSimpson	Month	Depth	Replicate	Read per sample	Singleton	Observed	Chao1	Shannon	InvSimpson
November	1	R1	29860	2068	4400	7315.8	6.9	291.7	**November**	1	R1	49004	2264	5438	8098.1	7.0	324.4
		R2	34427	2159	4864	7664.0	7.1	349.4			R2	53835	2858	6932	10289.4	7.4	536.7
	3	R1	47441	2328	5693	8407.1	7.1	381.9		3	R1	68362	2378	6573	9110.0	7.2	410.1
		R2	37925	2174	5156	7783.4	7.1	342.9			R2	67635	2572	6939	9605.4	7.3	418.9
	5	R1	54442	2147	6071	8169.1	7.2	412.3		5	R1	57315	2237	5905	8393.5	7.1	350.0
		R2	79137	1842	6571	8063.6	7.2	387.8			R2	59852	2167	6009	8263.4	7.1	391.8
	7	R1	52210	2349	6398	8804.4	7.4	523.7		7	R1	42720	2072	5108	7638.1	7.0	372.4
	9	R1	44451	1864	5197	7060.0	7.1	368.5		9	R1	33267	1898	4415	6768.3	6.9	301.2
	11	R1	54012	2195	5938	8175.8	7.1	353.2		11	R1	43931	1895	4949	6940.7	7.0	349.8
		R2	41196	2030	5226	7316.8	7.1	342.1			R2	50518	1905	5181	7221.0	6.9	276.9
	13	R1	58556	1812	5400	7096.8	6.9	283.3		13	R1	54447	2022	5412	7514.1	6.9	290.8
	15	R1	67134	1831	5833	7547.8	7.0	248.6		15	R1	48099	1854	5050	6897.0	6.9	296.1
	17	R1	46065	1658	4576	6360.0	6.8	241.7		17	R1	41937	2237	5639	7991.7	7.2	384.7
	19	R1	48897	1718	4828	6468.6	6.7	201.4		19	R1	46892	1848	4975	6886.1	7.0	337.1
		R2	57175	1694	5109	6667.7	6.8	208.9			R2	42536	1905	5200	7067.7	7.1	357.7
March	1	R1	43812	2108	5057	7735.9	7.0	339.1	**March**	1	R1	48396	2851	6552	10272.4	7.4	567.5
		R2	46684	2130	5188	7785.2	6.9	275.4			R2	28990	2343	4906	8130.0	7.2	435.1
	3	R1	34755	2062	4981	7406.7	7.2	327.4		3	R1	14110	1709	3225	5893.2	7.1	463.8
		R2	44355	2307	5804	8406.7	7.3	387.9			R2	30259	2431	5155	8390.1	7.3	461.3
	5	R1	50235	1919	5307	7290.1	7.1	343.4		5	R1	44892	2510	6164	8921.3	7.3	470.4
		R2	38670	1959	4854	7050.9	7.0	298.9			R2	40097	2352	5499	8280.5	7.2	396.6
June	1	R1	63986	2262	6111	8509.9	7.1	330.6	**June**	1	R1	13430	1801	3308	6276.7	7.1	439.7
		R2	51742	2233	5557	8194.1	7.0	320.2			R2	35946	2345	5223	8307.6	7.2	399.1
	3	R1	44244	2044	5256	7547.9	7.1	365.1		3	R1	64507	2461	6473	9320.6	7.2	364.1
		R2	81914	1844	6260	7834.8	7.1	351.6			R2	59029	2226	5934	8297.0	7.1	338.2
	5	R1	59745	2192	6032	8345.4	7.2	361.2		5	R1	75574	2489	7110	9621.2	7.3	400.3
		R2	66293	2325	6329	8794.0	7.1	362.4			R2	61273	2033	5852	7845.8	7.1	349.1
	11	R1	36698	2022	5084	7288.1	7.2	445.7		11	R1	68253	1801	5649	7384.4	7.0	335.3
		R2	66326	2073	6007	7997.4	7.0	292.6			R2	62686	1628	5099	6598.9	6.9	285.6
	19	R1	58773	2102	5968	7959.1	7.0	230.3		19	R1	67050	1299	5132	6123.8	7.0	303.1
		R2	69084	1600	5178	6636.6	6.6	143.0			R2	60748	2279	6249	8643.6	7.1	278.4

Table 2.1: Sequencing and diversity information of all replicate samples at Figino and Melide.

Bacteria dominated the microbial community by far and accounted for about 85% of the reads in all samples combined. The contribution of Archaea, however, increased with depth from 3 to 8% and from 8 to 17% at Figino and Melide, respectively. Similar to the geochemical profiles, both stations displayed very similar taxonomic results. The microbial community was dominated by Proteobacteria, Firmicutes, Euryarchaeota, Cyanobacteria, Chloroflexi, Bacteroidetes, and Aminicenantes (data not shown).

Vertical distribution of different putative microbial metabolismic pathways

The characterization of the microbial community structure along the sediment core profile was performed during anoxia of bottom waters (November 2016). The diversity of 16S rRNA gene OTUs affiliated with all putative microbial functional groups (e.g. nitrate-reducers, ammonium-oxidizers, iron-reducers, iron-oxidizers, sulfate-reducers, sulfide-oxidizers, methanogens and methanotrophs) decreased with depth (Figure 2.5). Nevertheless, we observed a slight increase in relative abundances of most microbial functional groups at ~ 11 and 19 cm depth, which may be due to greater C_{org} levels that were comparable to those of surface sediments at these depths. While the bacterial composition was very similar at the two sites, the total microbial relative abundance within surficial sediments (2 cm depth) was generally about 2-fold greater at Figino than Melide.

Potential nitrate reducers are relatively widespread among bacterial communities as many taxa are able to reduce NO_3^-. Since their taxonomic affiliation remains uncertain for most of them, we focused on the most well-known and abundant potential genera associated with NO_3^- reduction. At both sites, many OTUs were identified as *Decholoromonas* (≤1.9%) as well as other OTUs assigned to *Pseudomonas*, *Propionivibrio*, *Nocardioides*, and *Bradyrhizobium* in surface sediments in particular (Figure 2.5). The taxonomic assignment of representative sequences of potential aerobic N-oxidizers related mainly to *Nitrospira* and *Nitrobacter* (≤0.8 and 0.6% relative abundance,

respectively). Minor relative abundances of OTUs assigned to *Candidatus* Nitrososphaera, *Candidatus* Nitrosopumilus and *Candidatus* Nitrosoarchaeum were also observed at all sediment depths.

In contrast to other microbial functional groups, the microbial community structure of potential iron reducers shifted with depth at both stations. With a relative abundance of about 1% to 2.3%, *Albidiferax* was the most abundant Fe^{III}-reducer taxon in surface sediments, while the relative abundance of OTUs was mostly affiliated with *Bacillus* from 5 to 20 cm depth (~ 0.2 to 1.4%; Figure 2.5). Yet, at both sites, OTUs listed as potential Fe^{II}-oxidizers were mostly assigned to *Letptothrix* and, at Figino in particular, few OTUs affiliated with *Acidovorax*, *Gallionella* and *Sideroxydans* were also observed.

Sulfate-reducing bacteria are phylogenetically well constrained, hence this metabolic trait can be predicted with more certainty than others (e.g. nitrate-reducers) from the 16S rRNA sequence information. Among all microbial functional groups examined, they exhibited the greatest diversity, in terms of the number of detected genera. Their total relative abundance decreased from ~ 5.5 and 2.5% in surface sediments of Figino and Melide, respectively, to ~ 1% in deeper layers of both sites (Figure 2.5). The representative sequences were mainly affiliated with *Desulfomonile, Sva0081*, H16, *Desulfatirhabdium* and *Desulfatiglans*. At the surface, additional genera were also present such as *Desulfatirhabdium* as well as other minor OTUs (e.g. *Desulfonema*, *G55, Desulfobulbus)*. In contrast, the total relative abundance of taxa affiliated with potential sulfide-oxidizers was low (≤1% total relative abundance) and the diversity remained more limited. The taxa *Thiobacillus*, *Sulfuritalea* and *Sulfurimonas* dominated the S-oxidizing microbial community at both sites and all depths (Figure 2.5). OTUs affiliated with *Sulfurimonas* were also particularly abundant in Figino surface sediments.

Figure 2.5 Relative abundance of OTUs affiliated with taxa of different potential functional groups associated with nitrogen, sulfur, and iron cycling.

Methanogens were also one of the dominant functional groups in the south basin sediments of Lake Lugano (~0.1 to 2.5% relative abundance). At all sediment depths of both sites, the methanogens community was mainly represented by the *Methanosaeta* (0.2-1.6% relative abundance), and OTUs assigned to *Methanobacterium* were also abundant in the top sediments of

Figino (~ 0.9% relative abundance). In contrast, methanotrophs were the least abundant community with a maximum of total relative abundance ≤ 0.8%. The most abundant OTUs were affiliated with *Methylobacter*, *Desulfobulbus,* and *Methlylocystis*. To a lesser extent, the community was also represented by *Methylocaldum*, as well as the two anaerobic methanotrophs *Candidatus* Methanoperedens and *Candidatus* Methylomirabilis (Figure 2.5).

Temporal variability of bacterial functional groups

The temporal variability of the different functional groups presented above was investigated at three different seasons: fall (November 2016; 0 µM O_2 bottom water concentration), spring (June 2017; 30 µM O_2), and winter (March 2018; 140 µM O_2). For this analysis we considered only the samples from 0-5 cm depth, as no rapid temporal changes are to be expected in deeper layers of unbioturbated anoxic sediments. A first visual examination of the combined data presented in Figure 2.6 reveals a striking similarity between sites. Though, statistically, it appears that the seasonal water-column turnover exerted a stronger influence on the microbial communities at Figino where potential sulfate reducers, sulfide oxidizers and methanotrophs exhibited significant changes between sampling dates. Conversely, at Melide, H_2S-oxidizers were the only impacted guild (Adonis, $p<0.05$; Figure 2.6). Bacterial diversities of other functional groups (e.g. potential NO_3^--reducers, NH_4^+-oxidizers, Fe^{III}-reducers, and Fe^{II}-oxidizers) were, in contrast, relatively stable over seasons at the two sites (Adonis; $p>0.05$).

Although the sulfate-reducers community (e.g. *Desulfatiferula*, *Desulfitobacterium*, *Desulfococcus*, *Desulfonema*, *Desulfobulbus,* in particular*)* was significantly altered with season at Figino, no clear seasonal trend was observed regarding their specific relative abundance. Furthermore, at Figino,

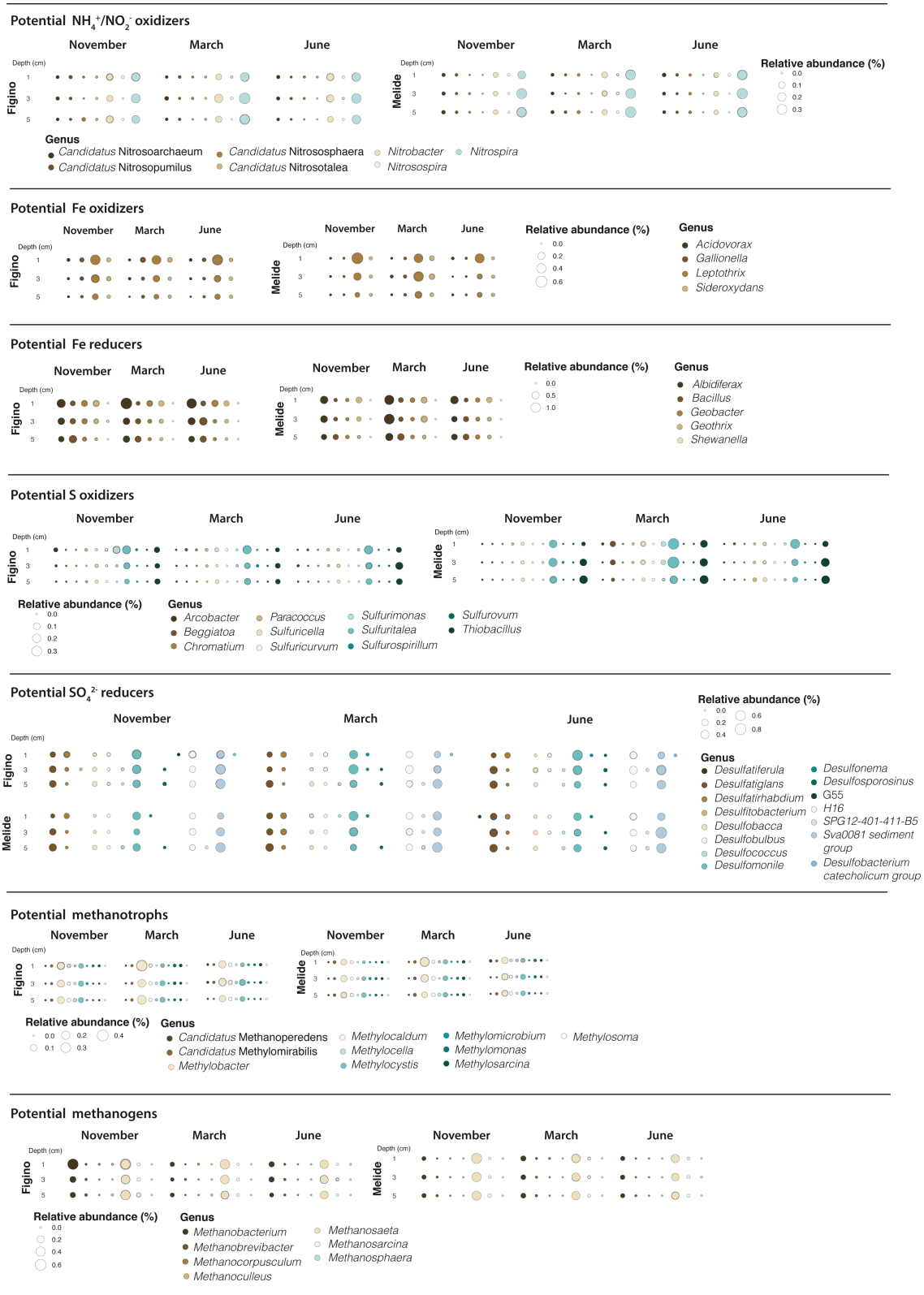

Figure 2.6 Temporal variations of relative abundances in the uppermost 6 cm sediment of microbial taxa potentially involved in the cycling of N, Fe, S, and C. Samples were collected at both stations in the south basin of Lake Lugano in November 2016, March 2018, and June 2017.

the stratification of the water column (November) strongly stimulated the relative abundance of *Sulfurimonas* and *Arcobacter* genera while, at Melide *Beggiatoa-, Sulfuricella-* and *Sulfurimonas*-related OTUs were favored after the water-column mixing (March). Though, *Sulfuritalea* and *Thiobacillus* were the dominant sulfur-related taxa at the two stations at all seasons. At both sites, the methanotrophs community was *Methylobacter*-dominated at both sites and all seasons, and the water-column turnover seemed to affect the relative abundance of both *Methylobacter* and *Methylocystis* in particular (Figure 2.6).

Discussion

Geochemical and microbial vertical gradients

High concentrations of Fe^{2+}, Mn^{2+} and CH_4 indicated intensive Fe-, Mn- and CO_2-reduction in Lake Lugano (south basin) sediments. Similarly, intensive microbial nitrate and sulfate reduction was evidenced by the sharp decrease in their concentrations from bottom waters to the surface sediments. Though, in contrast to previous work at the same locations (Lazzaretti et al., 1992), no clear seasonal gradients were observed for the porewater chemistry at the sediment-water interface. The sampling resolution-depth was similar between the two studies (e.g. 1-1.5 cm), however, their *in situ* sampling strategy was likely more precise than the pore water extraction method used in the work presented here. Moreover, the porewater peepers of Lazzaretti et al. (1992) have been placed precisely with the aid of a submarine, within a narrow range. The sediment cores in our study have been retrieved from onboard a small research boat, thus encompassing likely more spatial heterogeneity. Despite all the variability in the data, the sediments of the two stations in the south basin of Lake Lugano share the same geochemical characteristics. The sediments at both locations are particularly rich in organic matter, and the porewater chemistry characterizes these sediments as ferruginous (Fe^{2+}-rich, SO_4^{2-}-poor and H_2S-free; Vuillemin et al., 2016). The potential electron acceptors for anaerobic respiration NO_3^- and

SO_4^{2-} were depleted within the very first 3 centimeters. Manganese and iron oxides were detected at all depth, however, they are usually less easily reducible by bacteria. Therefore, methanogenesis remains the dominant pathway of organic matter degradation in the Lake Lugano sediments, as also witnessed by very high CH_4 concentrations and large CH_4 bubbles in the sediment cores.

The microbial communities assembly at the two stations is not identical but very similar (Figure 2.3), and essentially structured with depth. Microbial communities were more diverse (α-diversity) and taxonomically more dynamic in surface sediments (0-5 cm) than in deeper layers. The diversity is strongly driven by the availability of electron acceptors that can be used by microbes for growth (Canfield et al., 2005). The limited supply of electron acceptors to deeper sediment layers results in longer generation times for microorganisms, which thus decrease their diversity (Røy et al., 2012; Jørgensen and Marshall, 2016). When solute transport is only driven by diffusion such as in Lake Lugano sediments, the selection (e.g. more competitive) becomes the predominant means of microbial community assembly (Nemergut et al., 2013; Walsh et al., 2016). While it is likely that the microbial community structure is correlated to specific geochemical zonation, we did not observe any clear distribution of microbial functional groups (i.e., NO_3^--reducers, Fe^{III}-reducers) with depth. This may be due in part to the low sampling resolution used (e.g. 2 cm depth), which overlooked any gradients, most microbial activities occuring within the very first few millimeters/centimeters of the sediments. Nevertheless, the predominance of certain putative microbial functional groups is a good indicator of the different biogeochemical reactions occurring in the sediments. Despite of a recent work by Vuillemin et al. (2018), to our knowledge, investigations on the vertical distribution of benthic microbial communities in lakes do not exist, and the present study provide important insights about the depth-related dynamics of microbial communities and associated putative metabolisms.

Putative metabolisms involved in N, Fe, S, and C cycles

The phylogenetic analysis performed in the present study highlight the presence of important microbial actors that are potentially involved in the principal biogeochemical processes occurring in the sediments of Lake Lugano South Basin. The anaerobic ammonium-oxidizing guild was only represented by *Candidatus* Brocadia with a very low relative abundance (<0.01%), which correlates well with prior negligible anammox rates measured in the same sediments. Assigning specific metabolisms to nitrate reducers is, however, more delicate as microbes capable of denitrification also hold a multitude of functionalities (i.e., aerobic respiration, fermentation) and particular care is thus required. Nevertheless, we were able to putatively identify some well-known nitrate reducers such as *Pseudomonas* and *Dechloromonas* (Bedzyk et al., 1999; Horn et al., 2005) that were abundant at both sites and all depths. In marine sediments inhabited by the filamentous *Thioploca* and *Beggiatoa* species, prior work has shown the significant NH_4^+ production from the reaction between H_2S and NO_3^- (Otte et al., 1999; Sayama et al., 2005). It has also been shown that freshwater *Thioploca* behaved similarly than marine species and can accumulate large amount of nitrate (Kojima et al., 2007). However, to our knowledge, clear evidence of their implication in nitrate reduction is still lacking.

The ferruginous nature of the sediment shows the importance of the Fe cycle in the south basin of Lake Lugano sediments. The relative abundance of putative Fe^{III}-reducers was quite high at the two stations. In surface sediments, the taxa *Albidiferax, Geothrix, and Geobacter* dominated the Fe^{III}-reducing community, while *Bacillus* was the main guild in deeper layers. Thanks to its fermentative metabolism (Nakano et al., 1997), *Bacillus* can survive under electron acceptor limitation and may thus outcompetes many other species. Furthermore, most Fe^{III}-reducing microbial groups are versatile, and it is thus likely that other species such as putative SO_4^{2-}-reducers (e.g. *Desulfobacter;* Reyes et al., 2016) may have also catalyzed the process. In addition to Fe^{III}-reducers, some putative Fe^{II}-oxidizers have been identified. For instance,

Letpothrix was particularly abundant at the two stations and dominated the overall Fe^{II}-oxidizing community at all depths. The taxa *Gallionella* and *Acidovorax* have also been detected at the surface sediments (0-2 cm) of Figino. *Gallionella* is a microaerophilic taxon, however, its presence in anoxic sediments during water-column stratification suggests that it used alternative electron acceptors such as NO_3^-. Yet, both *Gallionella* and *Acidovorax* were only present at Figino where NO_3^- is never depleted in bottom waters, in contrast to Melide. These two taxa have the potential to reduce NO_3^- with Fe^{2+} as electron donor (Miot et al., 2009; He et al., 2016), and their presence strengthens prior biogeochemical results that highlighted the significance of Fe^{2+}-dependent nitrate reduction in the same sediments (Cojean et al., in prep.).

The sulphur cycle in lakes has long been neglected (Holmer and Storkholm, 2001). Indeed, lake sediments generally contain low sulfate concentration and therefore researchers have assumed that the S cycling must have been insignificant compared to other biogeochemical processes. The sediments of Lake Lugano (south basin) host high relative abundance of potential SO_4^{2-}-reducers (e.g. *Desulfomonile, Sva0081*, H16, *Desulfatirhabdium, Desulfatiglans*), which even exhibited the highest relative abundance among all putative microbial functional groups investigated. Interestingly, we also observed the presence of sulfate-reducing (*Desulfobulbus)* and sulfur-oxidizing (*Beggiatoa*, *Thioploca*) cable bacteria. Until recently (Risgaard-Petersen et al., 2015), cable bacteria had only been reported in marine sediments. These microbes are able to perform long-distance electron transfer and thus obtain the electron acceptors (O_2, NO_3^-, SO_4^{2-}) they need from widely separated locations (e.g. from sediment-water interface to deeper sediment layers; Bjerg et al., 2018). Therefore, the presence of such microbes may allow the occurrence of intensive sulfur cycling in sediments limited in sulfate. Yet, similarly to Fe^{II}-oxidizers, we identified putative NO_3^--reducing S-oxidizers, such as *Thiobacillus*, *Sulfurimonas, Sulfuricella* (Beller et al. 2006; Sievert et al. 2008; Watanabe et al. 2014), which supports our prior biogeochemical results (Cojean et al., in prep.).

In Lake Lugano, high concentration of methane is likely biologically produced by methanogens such as *Methanosaeta* and *Methanobacterium* (Garcia et al., 2000), which were particularly abundant in the surficial sediments of Figino. Based on the concentration profiles of SO_4^{2-} and CH_4, anaerobic methane oxidation using SO_4^{2-} may be occurring. Indeed, 16S rRNA gene sequences of some anaerobic methanotrophs have been detected. The archaeon *Candidatus* Methanoperedens, for instance has recently been shown to couple anaerobic methane oxidation with sulfate reduction with the aid of *Desulfobubus* sp. as syntrophic partner (Su et al., in review). However, in presence of suitable electron acceptors such as Fe^{III} and NO_3^-, *Candidatus* Methanoperedens can also oxidize CH_4 alone. In addition *Candidatus* Methylomirabilis, a NO_3^-/NO_2^--reducing bacterial anaerobic methanotroph has also been detected at both sites.

Temporal variability of microbial communities

Microbes are key drivers of nutrients cycling in aquatic ecosystems, and they are, generally, particularly responsive to changes of the environmental conditions. In Lake Lugano sediments, however, the microbial communities were relatively stable at different sampling time and both locations. Though, it is possible that, as mentioned above, the sampling resolution may have not been sufficient to capture minor variability in the community structures in the very first few millimeters subject to the seasonal changes.

Deepened statistical analysis helped to reveal some of the responsive populations in the microbial community. Potential sulfate-reducers, sulfide-oxidizers, and methanotrophs were the most dynamic communities, while the other putative microbial functional groups (e.g. NO_3^--reducers, NH_4^+/NO_2^- oxidizers, Fe^{III}-reducers, Fe^{II}-oxidizers, and methanogens,) seemed well adapted to seasonal biogeochemical changes and displayed a similar microbial community structure across seasons. In particular, sulfide-oxidizers exhibited the highest seasonal variability, though with differential responses between stations. At Figino, the relative abundance of OTUs assigned to *Sulfurimonas* was

highest during anoxia of bottom waters, while the abundance of *Beggiatoa*-, *Sulfuritalea*-, and *Sulfuricella*-like OTUs increased during oxygenation at Melide. Important changes in S-oxidizing communities due to O_2-changing conditions were also observed in marine water column and sediments (Beman and Carolan, 2013; Broman et al., 2017) where, similar to our results at Figino, the abundance of *Sulfurimonas*-like OTUs in surface sediments significantly increased during anoxia of bottom waters. Nitrate concentration at Figino generally never decreases below ~ 20 µM during anoxia of bottom waters (November), which may have thus stimulated nitrate-reduction coupled to H_2S-oxidation by *Sulfurimonas* (Sievert et al., 2008), which is in contrast to Melide where NO_3^- is depleted during winter. In comparison with Samad and Bertilsson (2017), we also observed a greater abundance of the *Methylobacter*-like aerobic methanotroph during oxygenation of bottom waters. The relative abundance of the putative sulfate-reducing *Desulfobulbus,* which can be a syntrophic partner during aneraobic CH_4 oxidation, also seemed to increase after the water-column turnover. However, additional statistical and experimental work is required to ascertain the specific anaerobic metabolism of methanotrophs. Furthermore, some putative microbial functional groups responded differently to biogeochemical changes than observed in other environments where, for instance, NH_4^+-oxidizers were generally strongly impacted with changing redox conditions (i.e., O_2; Duff et al. 2017), whereas the sulfate-reducing community remained relatively stable across seasons (Fukui and Takii, 1990; Mori et al., 2018). Hence, the microbial response to biogeochemical changes seems to vary from one environment to another, and the exact environmental controlling factors are still not fully constrained.

Conclusion

With this study, we present the first overview of spatial and temporal variations of microbial community structures and associated metabolisms in freshwater lake sediments. The microbial community structure was relatively similar between the two studied sites sharing similar geochemical conditions.

Yet, the presented phylogenetic data complement the vast amount of (biogeo-)chemical information established for the two sampling sites, Figino and Melide, in Lake Lugano. The results support previous studies on chemolithotrophic NO_3^- reduction, in which representative taxa could be identified that are known to anaerobically oxidize Fe^{2+} and H_2S utilizing nitrate as terminal electron acceptor. The water-column turnover in fall had a significant influence on the microbial community structure in surface sediments at one of the sampling sites, where bottom water oxygenation occurred. In particular, the relative abundance of sulfate-reducing, sulfide-oxidizing, and methanotrophs were affected. The water-column mixing leads to the oxidation of S-compounds and CH_4 by stimulating sulfur-oxidizing bacteria (e.g. *Sulfuritalea, Sulfuricella*) and methanotrophs (e.g. *Methylobacter*), respectively. Hence, the water-column may play a significant role in the lake detoxification by removing the toxic gas H_2S and the potent greenhouse gas CH_4.

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Chapter 3

Direct O_2 control on the partitioning between denitrification and dissimilatory nitrate reduction to ammonium in lake sediment

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Abstract

Lacustrine sediments are important sites of fixed nitrogen (N) elimination through the reduction of nitrate to N_2 by denitrifying bacteria, and are thus critical for the mitigation of anthropogenic loading of fixed N in lakes. In contrast, dissimilatory nitrate reduction to ammonium (DNRA) retains bioavailable N within the system, promoting internal eutrophication. Both processes are thought to occur under oxygen-depleted conditions, but the exact O_2 thresholds particularly of DNRA inhibition are uncertain. In O_2-manipulation laboratory experiments with dilute sediment slurries and $^{15}NO_3^-$ additions at low- to sub-micromolar O_2 levels, we investigated how, and to what extent, oxygen controls the balance between DNRA and denitrification in lake sediments. In all O_2-amended treatments, oxygen significantly inhibited both denitrification and DNRA compared to anoxic controls, but even at relatively high O_2 concentrations (≥ 70 µmol L^{-1}), nitrate reduction by both denitrification and DNRA was observed, suggesting a relatively high O_2 tolerance. Nevertheless, differential O_2 control and inhibition effects were observed for denitrification versus DNRA in the sediment slurries. Below 1 µmol L^{-1} O_2, denitrification was favored over DNRA, while DNRA was systematically more important than denitrification at higher O_2 levels. Our results thus demonstrate that O_2 is an important regulator of the partitioning between N-loss and N-recycling in sediments. In natural environments, where O_2 concentrations change in near bottom waters on an annual scale (e.g., overturning lakes with seasonal anoxia), a marked seasonality with regards to internal N eutrophication versus efficient benthic fixed N elimination can be expected.

Introduction

Over the last decades, intensive human activities have dramatically affected the nitrogen (N) cycle in aquatic systems through elevated inputs of reactive (biologically available) N. In some lakes, external N loading can lead to excessive algal blooms in the upper water column, and the subsequent

decomposition of the sinking algal biomass is often associated with O_2 depletion in the deeper water column, and possibly, seasonal or permanent anoxia (e.g. Blees et al., 2014; Lehmann et al., 2004, 2015). Depending on the O_2-concentrations in the water column, and the reactivity of the sediment organic matter, the oxygen penetration depth within lacustrine sediments can vary (e.g. Lehmann et al., 2009), and so will the transition zone that separates aerobic from anaerobic biogeochemical reactions. Under oxygen-depleted conditions, both in the water column and in sediments, anaerobic N-transformation processes such as denitrification, anammox and/or dissimilatory nitrate reduction to ammonium (DNRA) become important. While denitrification and anammox can mitigate excessive N loading (eutrophication) by converting reactive nitrogen (NO_3^-, NO_2^-, NH_4^+) to N_2, which subsequently returns to the atmosphere, DNRA retains a bioavailable form of nitrogen within the ecosystem, fostering internal eutrophication of lakes (Tiedje, 1988).

The biogeochemical conditions that regulate the partitioning between these different N-transforming processes (and others) in benthic environments remain uncertain, but links to other biogeochemical cycles are likely an important factor. It is commonly accepted, that when organic matter and nitrate concentrations are relatively high, nitrate is mostly reduced to N_2 by denitrifying bacteria (Gruber, 2008; Seitzinger et al., 2006; Seitzinger, 1988). In contrast, DNRA seems to be favoured in sediments with an excess of electron donors (total organic carbon (TOC), H_2S, Fe^{2+}) relative to nitrate (Brunet and Garcia-Gil, 1996; Roberts et al., 2014). In organic matter-rich lake sediments, the contribution of anammox to the total fixed-N transformation fluxes across the sediment-water interface is most likely minor relative to both denitrification and DNRA, since anammox seems to occur primarily in sediments with low organic matter content (Babbin et al., 2014; Thamdrup and Dalsgaard, 2002).

Changes in the redox zonation may have profound impact on the benthic N cycle (Otte et al., 1996). Seasonal cycles of water column mixing and stagnation can modulate the penetration of redox boundaries into the sediments,

potentially changing the redox environments of, for example, nitrifiers, denitrifiers, anammox, and DNRA bacteria. Oxygen can hence be considered a key regulator of benthic N exchange (Glud, 2008; Tiedje, 1988), and its effects are multifold. On one side, increasing O_2 concentrations may expand the oxic/suboxic zone where nitrification can supply NO_3^- and NO_2^- for denitrification and anammox, enhancing the overall fixed-N loss (Lehmann et al. 2015). On the other hand, O_2 can inhibit nitrate/nitrite reduction. When surface sediments are oxygenated, the facultative anaerobic microbes will preferably use oxygen, as the heterotrophic respiration with O_2 yields more energy to cells for growth than with other oxidants (i.e., NO_3^-, NO_2^-; Froelich et al., 1979; Payne et al., 2009; Thauer et al., 1977). Moreover, under oxygenated conditions, the synthesis and/or the activity of the key enzymes involved in nitrate/nitrite reduction may be suppressed (Körner and Zumft 1989, Baumann et al. 1996, Dalsgaard et al., 2014). Existing reports on O_2 tolerance and inhibition of denitrification and anammox in environments differ quite significantly. Inhibition may occur already at very low (nanomolar) O_2 concentrations (Dalsgaard et al., 2014), but experimental studies also revealed that relatively high O_2 levels may be required (up to 16% saturation levels) to induce a 50% inhibition of anammox (Jensen et al., 2008; Kalvelage et al., 2011). The apparent persistence of denitrification at relatively high O_2 concentration levels led to a revision of the classical paradigm regarding the absolute O_2 inhibition of nitrate reduction in nature (Tiedje et al., 1988), with important implications regarding the total volume of hypoxic zones in the ocean or in lakes that hosts microbial N_2 production (Paulmier and Ruiz-Pino, 2009).

While oxygen inhibition/tolerance of denitrification and anammox has been studied previously in the ocean water column (Jensen et al. 2008, Kalvelage et al. 2011, Babbin et al. 2014, Dalsgaard et al. 2014), investigations into the O_2 control on benthic N-reduction are rather rare, and limited to sandy and low organic matter marine sediments (Gao et al., 2010; Jäntti and Hietanen, 2012; Rao et al., 2007). Despite intensified research, the exact O_2 thresholds with regards to the direct inhibition of benthic N reduction are still poorly

constrained. This is particularly true for DNRA. Recent work has highlighted the significance of DNRA even in the presence of relatively high O_2 concentrations (i.e., at hypoxic levels (i.e., 10-62 µmol L^{-1}), or concentrations even greater than 62 µmol L^{-1}) in estuarine sediments (Roberts et al., 2012, 2014) and marine sediments (Jäntti and Hietanen, 2012), but a systematic investigation of how DNRA is impacted at low micromolar O_2 levels in aquatic sediments (and how in turn the balance between denitrification and DNRA is affected), does to our knowledge not exist.

In this study, we provide first experimental evidence for direct O_2 control on the fate of reactive N in lacustrine sediments with high organic matter content. Through slurry incubation experiments with sediment from a eutrophic lake in Switzerland (Lake Lugano), ^{15}N-labelled substrates and manipulated O_2 concentrations, we investigated the functional response of benthic N-reducing processes to changing O_2 levels. We demonstrate that denitrification and DNRA are differentially sensitive towards O_2, which has important implications for fixed N removal in environments that undergo short- and longer-term O_2 changes, such as seasonally stratified (anoxic) lakes or other aquatic environments with expanding volumes of hypoxia/anoxia.

Sampling site, materials and methods

Sampling location

Sediment sampling took place in the south basin of Lake Lugano, a natural alpine lake situated at the border between Switzerland and Italy. Between April and January, the water column of the basin is stratified, with bottom-water suboxia/anoxia starting in late spring/early summer (e.g., Lehmann et al. 2004; Lehmann et al. 2015). During winter (February/March) the lake turns over and bottom waters are oxygenated until the water column re-stratifies in spring, and bottom-water O_2 concentrations decrease again (Fig. 3.1). Water column O_2 and

N-compound (NO_3^-, NO_2^-, NH_4^+) concentrations were measured as part of a long-term monitoring campaign promoted by the International commission for the protection of Italian-Swiss waters (CIPAIS; Commissione Internazionale per la Protezione delle Acque Italiano-Svizzere) and run by SUPSI (University of Applied Sciences and Arts of Southern Switzerland) on behalf of the Admnistration of Canton Ticino. Sediment cores were taken at two locations, Figino (8°53'37"E, 45°57'31"N, 94 m depth) and Melide (8°57'29"E, 45°56'22"N, 85 m depth) in October 2017, using a small gravity corer (inner diameter 6.2 cm). Figure 1 displays representative seasonal trends in the deep south basin. During oxygenation of the bottom waters, nitrate concentrations in the water 2 m above the sediments reach about 75 µmol $L^{-1,}$ and even during water column anoxia, near-sediment nitrate concentrations rarely dropped below 15 and 5 µmol L^{-1} at Figino and Melide, respectively (Fig. 3.1; SUPSI data), so that the sediments are constantly exposed to nitrate-containing bottom waters. Ammonium concentrations in bottom water were relatively high (~ 30-140 µmol L^{-1}) during anoxia and close to the detection limit during months when the water column was mixed.

Porewater sampling

Porewater oxygen microprofiles were generated using an O_2 microsensor (Unisense) with a tip diameter of 100 µm in duplicate cores from both sites. The overlying water was gently stirred (without disturbing the sediment-water interface) and aerated to determine the O_2 penetration depth under oxygenated conditions at room temperature (~20 °C). Porewater samples for the analysis of dissolved inorganic nitrogen concentrations were obtained by sectioning of a separate set of cores from the same sites at 1 cm-interval under normal atmosphere and at room temperature (~20 °C), and centrifuging of the samples (4700 rpm, 10 min).

N-transformation incubation experiments

In a first step, incubations to measure potential denitrification and DNRA rates under control (i.e., anoxic) conditions were performed. At each site, fresh surface sediments (upper 2 cm) from duplicate sediment cores were homogenized to prepare dilute sediment slurries. Aliquots of 1 g sediment and 70 mL anoxic artificial lake water (NO_3^-, NO_2^-, NH_4^+-free; Smith et al., 2002) were transferred into 120 mL serum bottles. The use of dissolved-NO_x-free artificial water is important to avoid any potential underestimation of N-transformation process rates due to $^{28}N_2$ production from ambient NO_3^- or NO_2^-- present in bottom waters. Serum bottles were sealed and crimped using blue butyl rubber stoppers and aluminum caps. The sediment slurries (generally in triplicates, Table 1) were He-flushed for 10 min to lower the atmospheric N_2 and O_2 backgrounds, and placed overnight on a shaker (80 rpm) at 8 °C in the dark to remove any residual O_2. It needs to be noted that this He-flushing step, although crucial in our experimental set-up, may have interfered with in situ conditions by altering microbe-particle interactions through disruption of larger aggregates in the sediments or by slightly changing the pH in the sediment slurries. Labeled ^{15}N substrate (i.e., $Na^{15}NO_3^-$, 99% ^{15}N, Cambridge Isotopes Laboratories, 120 ± 2 µmol L^{-1} final conc.) was added in order to quantify potential rates of denitrification and DNRA. During the incubation period (ca. 10 hours), anoxic sediment slurries were kept in an incubator on an orbital shaker (80 rpm; 8 °C). Preliminary tests were performed in order to assess the minimal incubation time required to obtain clear and robust ^{15}N-N_2 production trends, and during which it was feasible to maintain a more or less constant O_2 concentration in parallel slurry experiments. For subsampling of gas and liquid samples, the incubation vials were transferred to an anaerobic chamber with N_2-atmosphere. There, 2-mL gas samples were taken at four successive time points (T_0, T_1, T_2, T_3) for N_2 isotope measurement, in exchange with 2 mL He (T_0) or anoxic Milli-Q water (T_1 to T_3) in order to compensate for any pressure decrease inside the vials. Gas samples were then transferred into 3 mL exetainers (Labco), prefilled with anoxic water, and stored upside down until isotope analysis.

Liquid samples (6 mL) were taken at T_0 and T_3 for the quantification of DNRA rates through N-NH_4^+ isotope analysis (see below) and for the assessment of nutrient (NO_3^-, NO_2^-, NH_4^+) concentrations.

O_2 manipulation experiments

For the O_2 manipulation experiments, serum bottles were equipped with TRACE Oxygen Sensor Spots (TROXSP5, detection limit = 6 nmol L^{-1} O_2, Pyroscience, Germany), allowing non-invasive, contactless monitoring of dissolved O_2 concentrations in the dilute slurry. The sensor spots were fixed at the inner side of the glass wall with silicone glue and the sensor signal was read out from outside using a Piccolo2 fiber-optic oxygen meter (PyroScience). Different volumes of pure O_2 (99,995%) were injected into the headspace of pre-conditioned and $^{15}NO_3^-$-amended slurries using a glass syringe (Hamilton). For each treatment, the gas volume required to reach the targeted O_2 equilibrium concentration (0.8, 1.2, 2, …, 78.6 µmol L^{-1}) was calculated based on the headspace versus slurry volumes, salinity, and temperature (Garcia and Gordon, 1992). Measured O_2 concentrations in slurries after injection of the respective O_2 gas volumes were always close to the ones calculated (the first measurement was performed 30 minutes after injection to ensure gas equilibration between the gas and the liquid phase). Oxygen concentrations in the slurry incubations were monitored with the fiber-optic oxygen meter every 30 minutes and, in case of a decline in dissolved O_2 due to microbial consumption, O_2 was added in order to return to the initial target O_2 concentrations (Supp. Table SI.1). In addition to continuous agitation on the shaking table, the dilute slurries were vigorously shaken by hand every 30 minutes to avoid the formation of anoxic microniches, which may act to increase rates of anaerobic N-transformation processes (Kalvelage et al., 2011).

Hydrochemical analyses

Nitrite concentrations were determined colorimetrically using sulfanilamide and Griess-reagent, according to Hansen and Koroleff (1999). Total NO_x (i.e., NO_3^- + NO_2^-) concentrations were measured using a NO_x-analyser (Antek Model 745, detection limit = 0.02 µmol L^{-1}), by reduction to nitric oxide (NO) in an acidic V^{3+} solution, and quantification of the generated NO by chemiluminescence detection (Braman and Hendrix, 1989). Nitrate concentrations were then calculated from the difference between NO_x and NO_2^- concentrations. Ammonium was measured by suppression-ion chromatography with conductivity detection (940 Professional IC Vario, Metrohm, Switzerland).

^{15}N-based rate measurements

For the determination of denitrification rates, gas samples from the ^{15}N-isotope enrichment experiments were analysed for $^{15}N/^{14}N$ isotope ratios in the N_2 using a Delta V Advantage isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific) with a ConFlo IV interface (Thermo Fisher Scientific). Denitrification (and negligible anammox) rates were calculated based on the quantification of ^{15}N in the N_2 gas in excess of the natural abundance, i.e. by calculating the linear regression of $^{30}N_2$ concentrations (and to a minor extent $^{29}N_2$) over incubation time (Nielsen, 1992; Thamdrup and Dalsgaard, 2002; Supp. Fig. SI.1). DNRA rates were quantified using the isotope-pairing method described by Risgaard-Petersen et al. (1995). Briefly, 2 mL liquid samples were transferred into 6 mL exetainers (Labco) and closed with plastic screw septum caps. The headspace was flushed with He for 2 min to reduce the $^{28}N_2$ background, and 25 µL mL^{-1} of alkaline (16 mol NaOH) hypobromite iodine solution (3.3 mol L^{-1}) were added through the septum to convert NH_4^+ to N_2 (Robertson et al., 2016). Exetainers were stored upside down and placed on a shaker (100 rpm) for 24 h at room temperature. The produced N_2 was then analysed by IRMS as described above. DNRA rates were determined based on the $^{15}NH_4^+$ production with time, as calculated from the total ^{15}N-N_2 in the

hypobromite-treated samples (i.e., calculated from the excess $^{29}N_2$ and $^{30}N_2$ signals). The recovery of ^{15}N-label from identically treated standards was >95%.

Statistics

Results are presented as the mean and standard error of n replicate experiments (Table 1). Correlation analyses were performed in Microsoft Excel software, and significant differences between results were verified using a Student's *t*-test ($P < 0.05$).

Results

Porewater hydrochemistry

The O_2 microsensor profiles revealed that the O_2 penetration at the two sites under aerated conditions ranged between 2.4 mm (Melide) and 3.7 mm (Figino, Fig. 3.2). The relatively low oxygen penetration depth is consistent with a high organic carbon content (~8%, data not shown). According to the observed O_2 concentration gradients at the two stations, the potential O_2 flux into sediments was greater at Melide suggesting a higher reactivity of the sedimentary organic matter.

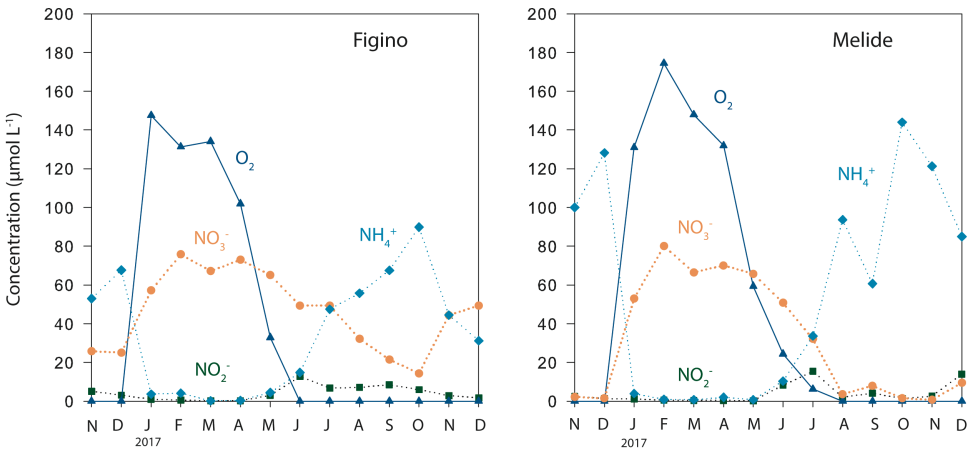

Figure 3.1: Concentrations of dissolved O_2 and reactive nitrogen in the bottom waters (2 m above the sediments) of the Lake Lugano South Basin in 2017.

In contrast to the microsensor profiling, the sectioning-centrifuging technique was not sufficient to resolve the exact porewater nitrate concentration gradient, yet the observed nitrate concentration data across the sediment-water interface (Fig. 3.2) clearly indicate that the sediments at both sites represent a sink for water-column nitrate, and that nitrate is consumed to completion already within the top centimeter of the sediments. In contrast, ammonium concentrations just below the sediment-water interface at Figino and Melide increased steeply from 830 and 600 µmol L^{-1} NH_4^+ to 1.7 and 1.2 mmol L^{-1}, respectively.

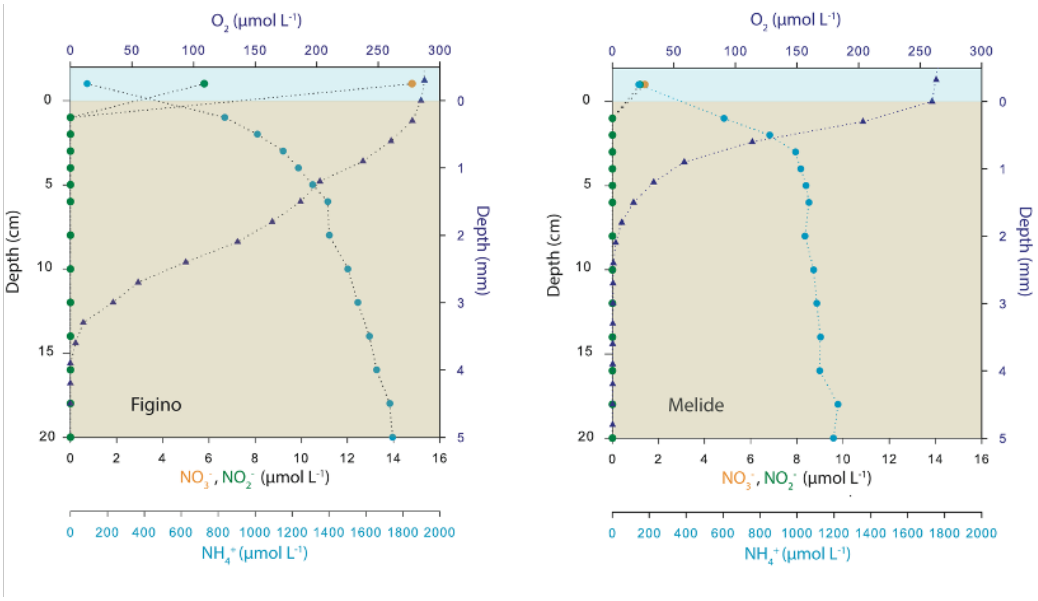

Figure 3.2*:* Ex situ sediment porewater profiles (O_2 and dissolved inorganic nitrogen) at the two sampling stations of the Lake Lugano South Basin in sediment cores collected in October 2017. Symbols correspond to the mean value of O_2 and dissolved N species concentrations measured in triplicate and duplicate cores, respectively. Oxygen concentration profiles (note different depth units) were determined in aerated cores, and thus are representative of the O_2 penetration during aerated conditions in the water column, as seen between January and April (see Fig. 3.1).

N-transformations in control experiments

Potential rates of denitrification and DNRA under true anoxic conditions were quantified at both sampling sites in October 2017. Anammox rates were measured in a previous study at different times of the year, and its contribution to the total fixed-N removal was always less than 1%, thus negligible with respect to other processes (Cojean et al., 2020). Indeed, in all experiments,

denitrification and DNRA were the main benthic N-transformation processes with an essentially equal contribution to the total nitrate reduction (≈0.1 µmol N g^{-1} wet sed. d^{-1}; Table 1 caption). We ensured that measured DNRA rates were not underestimated due to $^{15}NH_4^+$ loss through adsorption on mineral surfaces. Previous results (Cojean et al., 2020) demonstrate that adsorption of ambient or tracer ammonium does not occur at detectable levels in the dilute sediment slurries. Ammonium consumption by nitrifiers in presence of O_2, however, might slightly affect quantification of DNRA rates. Indeed, oxic slurry incubation experiments (≥ 73 µmol L^{-1} O_2) revealed that at least at high O_2 concentrations net NO_3^- production occurs (≤ 1 µmol N g^{-1} wet sed. d^{-1}).

Impact of O_2 on NO_3^- reduction in sediments

The O_2 sensitivity of denitrification and DNRA and inhibition kinetics were investigated through slurry incubation experiments under O_2-controlled conditions. At both sites, potential denitrification and DNRA rates consistently decreased with increasing O_2 concentration (Fig. 3.3). While the general pattern was systematic for both processes (i.e., an exponential attenuation of both denitrification and DNRA rates with increasing O_2), the response of denitrifiers versus nitrate ammonifiers towards manipulated O_2 differed across sites and treatments. We compared O_2-addition experiments to the anoxic controls to estimate the inhibition of nitrate reduction by O_2. At the lowest O_2 concentration (~ 1 µmol L^{-1} O_2), denitrification was less inhibited than DNRA at Figino (29 ± 20 % and 51 ± 7 % inhibition, respectively) while the suppression was almost equivalent at Melide (43 ± 8 % and 37 ± 9 % inhibition of denitrification and DNRA respectively, Table 1). At O_2 concentrations around 2 ± 0.2 µmol L^{-1}, both denitrification and DNRA rates were more than 50% inhibited compared to the anoxic control (Table 3.1, Fig. 3.3). At O_2 concentration ≥ 2 µmol L^{-1}, DNRA rates were generally higher than those of denitrification (with one exception, i.e., 16 µmol L^{-1} O_2 at Figino; Fig. 3.3), indicating that denitrification was more sensitive than DNRA to elevated O_2 levels. Oxygen concentrations higher than 73 µmol L^{-1} resulted in almost complete inhibition of denitrification at both sites (96 ± 1 %

and 93 ± 2 % at Figino and Melide, respectively, Table 3.1). Oxygen inhibition thresholds for DNRA were even higher, as DNRA rates were significantly less impaired compared to denitrification at these elevated O_2 levels (79 ± 5 % and 75 ± 4 % inhibition compared to the anoxic controls at Figino and Melide, respectively; Table 3.1). A correlation analysis between the relative contribution of DNRA to the total NO_3^- reduction (%) and the increase of O_2 concentration displayed a positive correlation coefficient of 0.57 and 0.91 for Figino and Melide, respectively (Supp. Fig. SI.2). Hence, the relative contribution of the two processes to total nitrate reduction was significantly affected by changing O_2 concentrations. At anoxic and submicromolar levels of O_2 (≤ 1 ± 0.2 µmol L^{-1} O_2), denitrification rates were higher than those of DNRA, while at higher O_2 concentration the ratio between denitrification and DNRA was shifted in favour of the nitrate ammonifiers (Fig. 3.4).

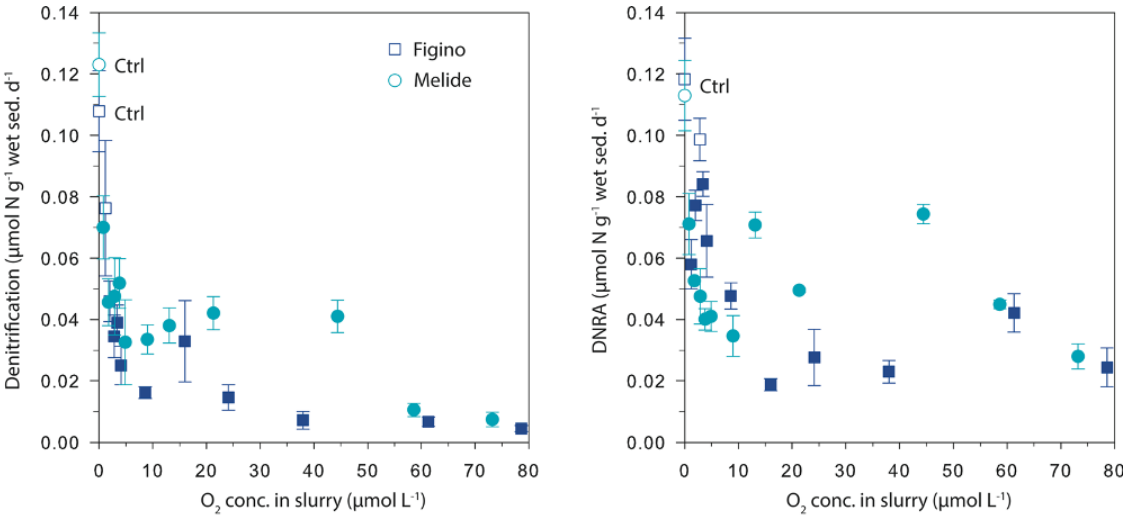

Figure 3.3: Denitrification and DNRA rates as a function of dissolved O_2 concentration in dilute sediment slurry from Figino and Melide. Statistically significant differences between denitrification/DNRA rates measured in the different O_2 treatments versus those in the respective control (Ctrl) experiments are shown by the filled symbols ($P < 0.05$, Supp. Table SI.2). Error bars represent the standard error of n replicate experiments and measurements (Table 3.1).

Consistent with the observed decline in denitrification and DNRA rates based on the ^{15}N-N_2 and $^{15}NH_4^+$ measurements in the ^{15}N-label incubations, nitrate consumption in slurries decreased with increasing O_2 concentration at both stations (Table 1). Similarly, maximum ammonium accumulation was

observed in the anoxic controls, whereas at higher O_2 levels ammonium underwent net consumption, indicating the concomitant decrease of DNRA and the increasing importance of nitrification under more oxic conditions, particularly at Melide. In incubations where nitrate concentrations decreased, the ratio of $(NO_3^-)_{consumed}$: $(N\text{-}N_2 + {}^{15}NH_4^+)_{produced}$ was always significantly higher (>5:1) than expected (1:1). This observation is consistent with previous work in the Lake Lugano South Basin (Wenk et al., 2014). Here, whole-core flow-through incubations also revealed that NO_3^- fluxes into the sediments significantly exceeded benthic N_2 production, an imbalance, which could neither be explained by nitrate reduction to ammonium nor incomplete reduction to N_2O. As it is not the scope of this study, we will not discuss this puzzling discrepancy further, but we speculate that excess NO_3^- consumption may be linked to bacterial and algal uptake (Bowles et al., 2012).

Biotic immobilization of NO_3^- in marine sediments has been attributed previously to the intracellular storage of nitrate by filamentous bacteria (Prokopenko et al., 2013; Zopfi et al., 2001) and/or diatoms (Kamp et al., 2011), but we do not know yet whether such nitrate sinks are important also in Lake Lugano sediments.

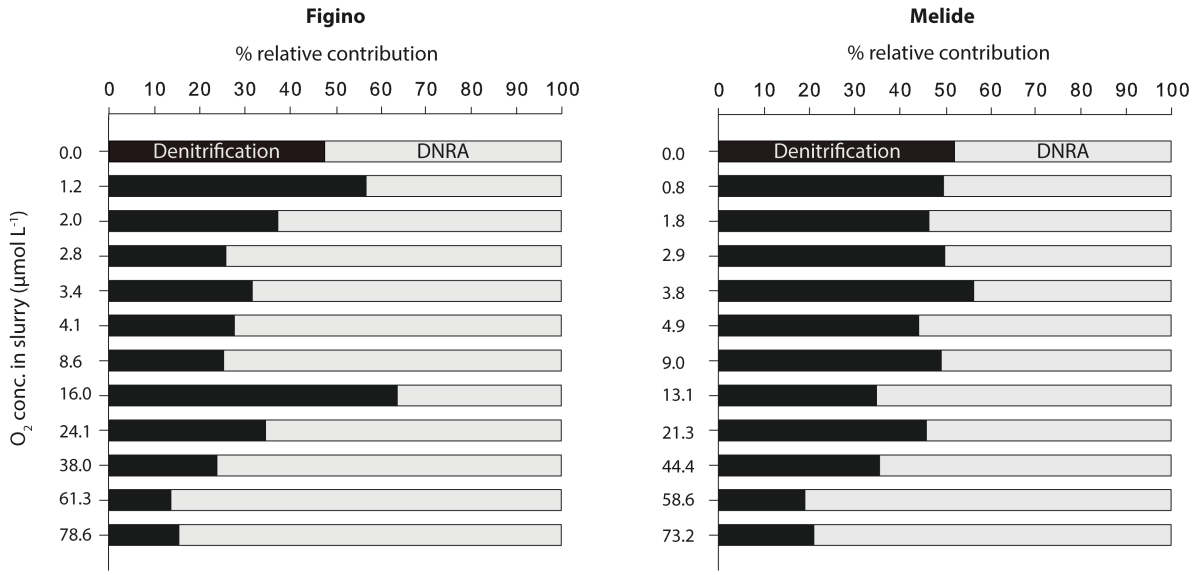

Figure 3.4: Relative contribution (%) of denitrification and DNRA to total nitrate reduction under variable O_2 conditions.

Table 3.1: Overview of N transformation rates in O_2-controlled slurry incubation experiments. Negative and positive values correspond to net NO_3^- or NH_4^+ consumption and production rates over incubation time, respectively. Standard errors are indicated in bracket for *n* replicates. Average denitrification and DNRA rates (µmol N g^{-1} wet sed. d^{-1}) in anoxic control experiments were: 0.11 ± 0.01 and 0.12 ± 0.04, respectively, at Figino; 0.12 ± 0.01 and 0.11 ± 0.01, respectively, at Melide.

Sampling site	O_2 conc. in slurry	*n*	Inhibition compared to control (%)		NO_3^-	NH_4^+
	µmol L^{-1}		Denitrification	DNRA	*µmol N g^{-1} wet sed. d^{-1}*	
Figino	0 (control)	12			-1.4 (0.1)	0.7 (0.03)
	1.2	3	29	51	-0.8 (0.2)	0.8 (0.4)
	2	3	57	35	-0.4 (0.1)	0.8 (0.2)
	2.8	3	68	17	-0.4 (0.3)	0.7 (0.1)
	3.4	2	64	29	-0.6 (0.2)	0.6 (0.1)
	4.1	3	77	45	-0.9 (1.3)	0.5 (0.2)
	8.6	3	85	60	-1.1 (0.3)	0.4 (0.0)
	16	4	70	84	-0.2 (0.5)	0.1 (0.2)
	24.1	3	86	77	-0.2 (0.6)	0.2 (0.1)
	38	3	93	39	0.2 (2.1)	0.0 (0.2)
	61.3	3	94	64	-0.3 (0.3)	-0.2 (0.1)
	78.6	6	96	79	1.1 (1.4)	-0.1 (0.0)
Melide	0 (control)	9			-1.0 (0.4)	0.2 (0.0)
	0.8	2	43	37	-0.7 (0.2)	-0.1 (0.1)
	1.8	2	63	53	-0.6 (0.1)	0.2 (0.2)
	2.9	3	61	58	-0.5 (0.3)	-0.1 (0.2)
	3.8	4	58	65	-0.2 (0.1)	-0.1 (0.3)
	4.9	3	74	64	-0.3 (0.2)	-0.1 (0.3)
	9	7	73	69	0.0 (0.1)	-0.1 (0.2)
	13.1	2	69	37	-0.6 (0.1)	-0.1 (0.0)
	21.3	2	66	56	-0.4 (0.1)	-0.1 (0.1)
	44.4	2	67	34	-0.3 (0.2)	-0.1 (0.2)
	58.6	3	91	60	-0.1 (0.1)	-0.1 (0.2)
	73.2	4	93	75	0.2 (0.2)	-0.4 (0.1)

Discussion

Anaerobic N-cycling in the South Basin of Lake Lugano

Benthic denitrification and DNRA were the predominant anaerobic N-transformation processes at the two studied stations. Interestingly, the contribution of DNRA was systematically higher than observed in flow-through whole-core incubations performed with sediment from the same basin. Wenk et al. (2014) reported a maximum DNRA contribution to NO_3^- reduction of not more than 12%, but also argued that their DNRA rate measurements must be considered conservative, because they did not account for the production of $^{14}NH_4^+$ from ambient natural-abundance nitrate. The reason for such a discrepancy is unclear, but there seems to be a tendency for slurry incubations to yield higher DNRA rates compared to denitrification (Kaspar, 1983), implying biasing methodological effects. The observed discrepancies may also be related to natural sediment heterogeneity and/or seasonal/interannual fluctuations in benthic N transformation rates. As for the latter, in 2016, the annual water overturn and bottom-water ventilation was exceptionally suspended and sediments remained anoxic for more than a year. In contrast, in 2017, the water column mixed in January and surface sediments were oxygenated throughout June. Our O_2 manipulation experiments revealed that redox conditions have a marked impact on the partitioning between the two nitrate reduction pathways, and consistent with the slurry incubation data, the extended O_2 exposure of microbes at the sediment-water interface in 2017 compared to the preceding year may have favoured nitrate ammonifiers over denitrifiers. Independent of any possible spatio-temporal variability, in this study, DNRA rates were equal, or even higher, than denitrification. Such a partitioning of the two nitrate reducing processes is not implausible and was similarly observed in a wide range of environments, particularly in more reduced sediments with high organic matter content and comparatively low nitrate levels (Brunet and Garcia-Gil, 1996; Dong et al., 2011; Papaspyrou et al., 2014). More generally, substrate-availability changes induced by O_2 fluctuations may be important drivers of the

partitioning between denitrification and DNRA (Cojean et al., 2020), and environmental conditions that favour DNRA over denitrification may be quite common. However, to our knowledge, experimental evidence for the direct O_2 control on the balance between these two nitrate-reducing processes is still lacking.

O_2 inhibition thresholds of benthic nitrate reduction

Our study shows that submicromolar O_2 levels significantly lowered both, denitrification and DNRA rates. Denitrification and DNRA were inhibited by about 30-50% at 1 µmol L^{-1} O_2, while in previous studies that investigated O_2 effects on fixed-N elimination in the water column, denitrification was almost completely suppressed at this O_2 level already. For example, by conducting incubation experiments using samples from oxygen minimum zones in the Eastern Tropical Pacific, a 50% inhibition of denitrification was noticed already at 0.2 µmol L^{-1} O_2, and complete suppression at 1.5-3 µmol L^{-1} O_2 (Dalsgaard et al., 2014, Babbin et al., 2014). Similarly, incubation experiments with samples from a Danish fjord exhibited full inhibition of denitrification at 8-15 µmol L^{-1} O_2 (Jensen et al., 2009). In marine sediments, in contrast, denitrification was occurring even at O_2 concentrations greater than 60 µmol L^{-1} (Gao et al., 2010, Rao et al., 2007). This is in agreement with our results showing that at higher O_2 levels (≥73 µmol L^{-1}) denitrification was still active although at very low rates compared to the anoxic control (≥ 93% inhibition). Similarly, DNRA was still occurring, and was less impaired by the elevated O_2 concentration compared to denitrification (≥ 75% inhibition relative to the anoxic control). An increase of DNRA relative to denitrification rates under oxic conditions (> 100 µmol L^{-1} O_2) was also observed in estuarine sediments, though N-removal remained predominant (Roberts et al., 2012, 2014). In brackish sediments in the Gulf of Finland in the Baltic Sea, at elevated O_2 concentrations (from 50 to 110 µmol L^{-1} in bottom waters), benthic DNRA rates were generally higher than denitrification rates (Jäntti and Hietanen, 2012), further supporting our findings. Yet, in contrast to our study, their observations suggest a higher O_2

sensitivity (i.e., greater inhibition) of DNRA compared to denitrification in sediments with higher bottom water O_2 concentrations (> 110 µmol L^{-1}). Given the paucity and discrepancy of existing data in this context, it is premature to conclude that DNRA microbes are generally less or more oxygen-tolerant than denitrifiers. A direct comparison of DNRA O_2 inhibition thresholds in this study and in the study of Jäntti and Hietanen (2012) is difficult because of the differing methodological approaches. There, nitrate reduction rates were determined in whole-core incubations, without manipulating (and measuring) the O_2 concentrations at the sediment depth where nitrate is actually reduced. And although the O_2 penetration depth and porewater O_2 concentrations will respond to a certain degree to the O_2 content in the bottom water, deducing the actual O_2 concentrations for the active nitrate reduction zone within the sediment from O_2 concentrations in the overlying water is problematic. Here, we tested the oxygen sensitivity of a microbial community in suspension, directly exposed to defined O_2 conditions. These incubation data indicate that DNRA is less inhibited than denitrification at O_2 concentrations ≥ 73 µmol L^{-1} and, at the same time, imply that anoxia per se is not a strict requirement for DNRA, as previous ecosystem-scale work has also suggested (Burgin and Hamilton, 2007). Our results also are consistent with observations made in soil microcosms showing that DNRA is less sensitive to increasing O_2 partial pressures than denitrification within the range of 0-2% O_2 v/v (Fazzolari et al., 1998; Morley and Baggs, 2010).

The observed O_2 inhibition thresholds for nitrate reduction are significantly higher than reported from most incubation studies with water column samples (Dalsgaard et al., 2014, Babbin et al., 2014, Jensen et al., 2008). Elevated O_2 tolerance in prior studies was often attributed to the formation of anoxic microniches that may foster anaerobic N-reduction (Kalvelage et al., 2011). It is unlikely that such microniches formed during our incubation experiments since slurries were heavily diluted (1 g sediment in 70 mL water) and vigorously shaken by hand every 30 min, in addition to the continuous agitation on a shaking table during the incubation. Also, experiments were

replicated 2-3 times for some O_2-amended treatments, and measured rates were very similar between replicates. If anoxic microniches had formed, we would have expected that their formation is more variable, resulting in a lower reproducibility of the determined rates.

The existence of aerobic denitrifiers (e.g. microbes that reduce NO_3^-/NO_2^- to N_2 in presence of O_2) in soils and sediments has been confirmed through isolation of bacterial strains (e.g. Robertson et al., 1995), and it was suggested that they contribute to the total fixed N loss in marine sediments (Carter et al., 1995; Patureau et al., 2000; Zehr and Ward, 2002). Recent studies of permeable marine sediments (Gao et al., 2010) and soils (Bateman and Baggs, 2005; Morley et al., 2008) also observed significant N_2 production in the presence of O_2 and attributed it to aerobic denitrification.

DNRA favoured under less reducing conditions

It is generally assumed that strongly reducing conditions favour DNRA over denitrification, yet in our study, particularly at elevated O_2 concentrations, DNRA rates were higher than those of denitrification. That DNRA often seems to be more important under true anoxic conditions may therefore not be linked directly to the absence of O_2 and differential O_2 inhibition levels of the two nitrate-reducing processes. Indirect mechanisms are likely to be important. For instance, H_2S accumulation, which often accompanies prolonged anoxia, can inhibit denitrification and simultaneously enhance DNRA (An and Gardner, 2002; Rysgaard et al., 1996). Another indirect, redox-dependent factor may be the availability of nitrate. Higher DNRA rates were observed under more NO_3^--limiting conditions induced by prolonged anoxia, probably because nitrate ammonifiers are able to gain more energy per NO_3^- reduced than denitrifiers (Dong et al., 2011). As nitrate concentrations are generally much lower under oxygen-free conditions, it appears plausible that anoxia-associated nitrate and nitrite depletion is conducive to higher DNRA/denitrification rates. While these examples seem to support that DNRA is favoured under true anoxic conditions,

results of other studies are more consistent with our observation of higher DNRA than denitrification rates at elevated O_2 concentrations. For example, in estuarine sediments, DNRA was stimulated relative to denitrification under more oxidizing conditions (Roberts et al., 2014, 2012). The authors argued that DNRA is enhanced by increasing Fe^{2+} availability at the oxic-anoxic sediment layer during more oxygenated conditions. These studies highlight the importance of redox conditions in regulating the balance between dentrification and DNRA, however, to what extent O_2 directly controls the partitioning between the two nitrate-reducing processes at the enzyme levels remains, to our knowledge, still unknown. Apparent contradictions with regards to how changing O_2 levels may impact nitrate reduction may simply be due to the counteracting and variable influence of direct versus indirect effects of the variable O_2 concentrations.

We cannot fully exclude that through O_2 manipulation in this study, we partly affected nitrate-reduction indirectly through its control of H_2S or Fe^{2+}. Yet, we set up the experiments in a way that indirect effects should be minimized (e.g., no free sulfide measured in any of the incubations, same organic matter content, same excess NO_3^- concentrations), and this study can thus be considered an investigation into the direct O_2 effect on the partitioning between N-loss by denitrification and N-recycling by DNRA in aquatic sediments. The fact that in our experiments we can essentially exclude the effects of redox-related parameter changes (i.e., H_2S, NO_3^-, and Fe^{2+}) leads us to the conclusion that in the studied sediments from Lake Lugano, O_2 likely controls the balance between denitrification and DNRA at the organism-level, and that denitrification is in fact more sensitive towards increasing O_2 concentrations than DNRA.

Direct O_2 control on benthic NO_3^- reduction

It has been previously reported that O_2 can either suppress the synthesis of enzymes involved (Baumann et al., 1996) or the enzyme activity itself (Dalsgaard et al., 2014). The observed DIN concentration trends (i.e. decreasing

nitrate consumption) with increasing O_2 concentrations suggest that the overall activity is modulated mainly at the nitrate reduction step. Without conclusive information on enzyme activities in hand, we can only speculate at this point about any real difference in O_2-dependent response of the enzymes involved in denitrification versus DNRA. The differential response of denitrifiers and nitrate ammonifiers may, however, suggest a distinct O_2 sensitivity of the nitrate reductase enzymes involved. Denitrifiers and nitrate ammonifiers utilize the same nitrate reductase enzymes (Nar, Nap), and while a differential O_2 sensitivity of the same type of enzyme is difficult to explain, it is certainly possible for different enzymes. Indeed, the membrane-bound (Nar) and the periplasmic (Nap) nitrate reductases have distinct affinities towards NO_3^- and O_2 tolerance (Mohan and Cole, 2007). Periplasmic nitrate reduction is almost exclusively found in the Proteobacteria and many of the organisms possess both Nar and Nap systems, whose production is regulated in response to ambient NO_3^- and O_2 concentrations (Simon and Klotz, 2013). When NO_3^- is scarce, Nap provides a high-affinity (for NO_3^-) but low-activity pathway that does not require NO_3^- transport into the cell cytoplasm (Mohan and Cole, 2007). In presence of oxygen, nitrate transport across the cell membrane is repressed, preventing nitrate reduction by the membrane-bound enzyme Nar with its cytoplasm-facing active site (Moir and Wood, 2001). In contrast, nitrate reduction in the periplasm is less O_2 sensitive, so that microbes possessing and relying on Nap are likely to have an ecological advantage in environments that are subject to O_2 fluctuation (Carter et al., 1995). In nature, nitrate reduction by denitrifiers is assumed to be catalysed primarily by Nar (Richardson et al., 2007), while most nitrate ammonifiers seem to use Nap (Mohan and Cole, 2007).

Clearly, more fundamental research is required in environmentally relevant non-model microorganisms or mixed communities, to understand better the combined effects of O_2 on the nitrogen-transforming metabolic pathways and their regulation. Additional O_2 inhibitory effects at one of the down-cascade enzyme levels (Nir, Nrf, Nor, Nos) are likely to exhibit variable O_2 sensitivities (Baumann et al., 1996, 1997; Körner H. and Zumft, 1989; Poock et

al., 2002). While we are aware that the treatment above is speculative, we argue that our observations of higher DNRA/denitrification ratios at higher O_2 provides at least putative evidence that microorganisms performing DNRA using Nap may be more O_2-tolerant than denitrifiers using Nar, a hypothesis that requires further testing.

Implication for N-elimination versus N-recycling in lakes with fluctuating O_2 conditions

The redox-sensitive partitioning of nitrate elimination (through N_2 production by denitrification) versus fixed-N recycling (by nitrate ammonification) has likely important ecosystem-scale consequences. The annual water column turnover of holomictic lake basins such as the south basin of Lake Lugano plays an important role in regulating the contribution of N-removal and N-recycling in the water column (Lehmann et al. 2004; Wenk et al, 2014). To which extent O_2 fluctuations affect N transformation reactions within the sediments remains uncertain. Winter water column turnover ventilates the bottom waters and re-oxygenates surface sediments that were anoxic for several months. Hence, at least in the top millimeters of the sediment column, we can expect changes in the benthic N cycling. Based on our incubation experiments, the O_2 inhibition threshold was lower for denitrification than for DNRA, possibly reflecting differential adaption of the in situ microbial community of denitrifiers and nitrate ammonifiers to fluctuating O_2 conditions of bottom waters. Indeed, many nitrate ammonifiers possess both nitrate reductase enzymes (Nap and Nar) and can switch between the two respiratory systems providing them with an ecological advantage over denitrifiers when substrates become limiting (i.e., with regards to the primary reductant used in energy metabolism; Mohan and Cole, 2007). During oxygenated bottom-water conditions, within the benthic redox transition zone, nitrate-reducing microbes at the sediment-water interface will be exposed to elevated O_2 concentrations, similar to the ones tested here. Our experimental data imply that then, at least in the uppermost sediments, DNRA is favoured over denitrification. We may even expect an O_2-

regulated zonation of DNRA and denitrification. As a consequence, when denitrification-driven nitrate-reduction is pushed down, it is possible that NO_3^- will be partially consumed through DNRA before it gets to the "denitrification layer", as nitrate ammonifiers are less O_2 sensitive than denitrifiers. In contrast, denitrification is likely to be a more important nitrate-reducing process compared to DNRA during water column stratification (suboxia/anoxia of bottom waters), when the sediments are fully anoxic.

In the discussion thus far, we implicitly assume that the main control O_2 exerts on the absolute and relative rates of denitrification and DNRA is due to its inhibitory effects at the organism-level, yet the effect of O_2 on the coupling of nitrification and nitrate reduction by either denitrification or DNRA remained unaddressed. Oxygen fluctuations in the natural environment will affect nitrate regeneration by nitrification, and hence determine how much nitrate is available for microbial reduction. It has been shown previously that through oxygenation events (e.g., the increase in bottom water O_2 concentrations during episodic mixing/ventilation), the overall benthic N elimination in lakes may be enhanced through coupled nitrification-denitrification, at least transiently (Hietanen and Lukkari, 2007; Lehmann et al., 2015). So, while the direct effect of elevated O_2 would be to hamper fixed N elimination by denitrification at the organism-level, the oxygenation of previously ammonium-laden but nitrate free (pore-) waters would help to better exploit the benthic nitrate-reduction potential by increasing the nitrate availability for nitrate-reducing microbes within the sediments, so that the overall nitrate reduction may be stimulated (Lehmann et al. 2015). Yet, as shown in the present study, oxygenation of the water column and the upper surface sediments may also act to shift the balance between denitrification and DNRA towards DNRA, thus promoting N-recycling rather than fixed-N elimination through denitrification. Total nitrification rates were not measured in this study, but nitrate concentration changes in sediment slurries suggest that at elevated O_2 levels there is at least some production of nitrate. There is no obvious reason to assume that O_2-stimulation of the coupling of nitrification and denitrification on the one hand, and of nitrification and DNRA

on the other would per se be different. Yet, as demonstrated here, DNRA appears to be less O_2 sensitive compared to denitrification. It is thus reasonable to expect a higher coupling of nitrification with DNRA than with denitrification during oxygenated bottom-water conditions. Indeed, there is putative evidence for such an indirect link between O_2 and elevated coupled nitrification-DNRA. In a recent study with estuarine sediments, stronger stimulation of DNRA compared to denitrification was observed during oxygenation of bottom waters, in parts attributed to the coupling to nitrification (Roberts et al., 2012). Additional experimental work is required to better understand the role of nitrification in regulating the balance between benthic denitrification and DNRA during oxygenation of bottom waters.

It is important to understand that in the natural environment, O_2 will not be the only regulator of the balance between denitrification and DNRA. As previously mentioned, the partitioning of the two nitrate-reducing processes can also be modulated by the substrate (e.g., NO_3^-, NO_2^-, TOC, H_2S, Fe^{2+}) availability. The latter may be redox controlled or not. Such regulation may be linked to the differential substrate affinity of the two processes when competing for the same electron acceptor (e.g., nitrate/nitrite) providing selective pressure that can drive communities either towards denitrification or DNRA (Kraft et al. 2014), or simply due to differing substrate requirements in the case of chemolithotrophic versus organotrophic nitrate reduction.

For example, nitrate concentrations in the water column of the lake sampled in this study (Lake Lugano) varied significantly over the year, with very low NO_3^- concentrations during the stagnation period (during anoxia; Fig. 1). As a consequence, it is reasonable to assume that the relative partitioning between denitrification and DNRA in a natural environment is affected by the fluctuating nitrate concentrations (e.g., Tiedje et al., 1988, Dong et al., 2011). Similarly, Fe^{2+} levels in near-bottom waters and sediment porewaters in Lake Lugano are greater during the anoxia/stratification period (Lazzaretti et al., 1992). At least in environments where chemolithotrophic processes contribute to the overall

nitrate reduction, such redox-dependent Fe^{2+} concentration changes (or changes of other electron donors such as HS^-) may affect the balance between DNRA and denitrification (e.g., Robertson et al. 2015). Hence, in addition to the direct regulating effects of O_2 on the partitioning between denitrification and DNRA, which we have demonstrated here experimentally, O_2 can act as indirect regulator of fixed N elimination versus regeneration. The ultimate ecosystem-scale DNRA/denitrification ratio in environments that are subject to O_2 fluctuating conditions is difficult to predict, because direct and indirect O_2 regulation may act concomitantly and in opposite ways.

Conclusion

The presented results broaden the range of O_2 inhibition thresholds of benthic denitrification at micromolar O_2 levels, demonstrating that benthic denitrification may resist full inhibition up to almost 80 µM O_2. Similarly, sedimentary DNRA does not necessarily require true anoxia, and was even less sensitive than denitrification to higher O_2 levels. Our data suggest that the balance between DNRA and denitrification is modulated by O_2 at the nitrate-reducing enzyme level. However, more in-depth investigations on the exact role of oxygen in regulating other denitrification and/or nitrate-ammonification enzymes in microbial pure culture experiments are needed. The differential tolerance of denitrifiers versus nitrate ammonifiers towards O_2 has important implications for natural environments with fluctuating O_2 conditions. Based on our results, one might argue that DNRA may be more important during phases of bottom-water oxygenation, while anoxic conditions during the stratification period may favour full denitrification to dinitrogen. Whether and when fixed nitrogen is preserved in a lake or eliminated by denitrification is, however, difficult to predict, as this will depend also on multiple indirect effects of changing O_2 levels. For example, nitrification and the redox-dependent modulation of substrates that may be relevant for denitrification or DNRA (such as nitrite, the substrate at the branching point between the two processes, and/or sulfide as potential inhibitor of denitrification and stimulator of

chemolithotrophic DNRA) will play an important role both with regards to the overall nitrate reduction rate, as well as the balance between different nitrate reducing processes. Internal eutrophication from N in high-productivity lakes is generally less of a concern than from P. Nevertheless, it needs to be considered that oxygenation may reduce the overall fixed N-elimination capacity of the bottom sediments by impairing denitrification more than DNRA, partially counteracting the generally positive effects of hypolimnetic ventilation in the context of benthic nutrient retention/elimination, and with implications on the nutrient status in the water column.

Author contribution

JZ and MFL initiated the project. ANYC performed all sample collection and conducted the experimental work with help from AG. FL provided the water column chemistry profiles. ANYC, JZ and MFL performed data analysis and interpretation. ANYC and MFL prepared the manuscript with input from all co-authors.

Competing interest

The authors declare that they have no conflict of interest.

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Supplementary information

Table SI.1: Average O_2 concentrations in replicate slurries (see Table 1) at different time points during the incubation period (~ 10 h). The first time point (T1) was always at 30 minutes after the beginning of the incubation while the other time points (T2, T3, …, T9) were different for each treatment (i.e. more frequent O_2 measurements at low O_2 levels).

	Average O_2 concentration during incubation (µmol L^{-1})	O_2 concentration in slurry at different time points (µmol L^{-1})								
		T1	T2	T3	T4	T5	T6	T7	T8	T9
Figino	1.2	1.4	1.2	1.4	1.2	1.2	1.1	1.0	1.1	1.2
	2.0	1.7	2.2	2.0	2.2	1.9	2.0	1.7	2.0	2.1
	2.8	2.2	2.6	2.9	3.1	2.9	2.9	2.9	2.8	3.0
	3.4	3.0	3.2	3.9	3.2	3.6	3.7			
	4.1	3.5	3.5	3.9	4.3	4.1	4.2	4.9		
	8.6	7.5	7.8	8.8	9.1	8.1	8.9	10.1		
	16.0	17.6	17.1	15.0	16.7	16.5	13.6	15.5	15.8	
	24.1	26.3	25.8	22.9	24.5	21.0	24.2			
	38.0	39.3	35.2	39.5	36.8	38.3				
	61.3	66.8	62.1	57.2	56.9	63.4				
	78.6	81.0	84.8	73.1	81.6	73.6	77.4			
Melide	0.8	0.8	0.8	0.6	0.8	0.8	0.7	1.0	1.1	
	1.8	1.2	2.0	1.7	2.0	1.8	1.7	1.9	2.1	
	2.9	2.7	3.0	2.9	3.1	2.8	3.0			
	3.8	3.2	3.5	3.3	3.8	3.7	4.1	3.5	4.3	4.4
	4.9	4.8	4.8	5.1	4.7	4.7	4.9	5.2		
	9.0	8.3	8.2	8.8	8.7	9.5	9.4	10.3	11.1	11.1
	13.1	11.3	11.3	13.3	12.7	13.8	13.8	15.1		
	21.3	20.6	19.0	21.4	21.4	24.2				
	44.4	40.3	36.4	47.8	46.7	50.6				
	58.6	44.6	53.3	56.3	52.0	57.8	66.6	68.9	69.0	
	73.2	39.3	70.8	73.8	69.7	76.8	81.2	86.4	87.3	

Table SI.2: Results of the t-test analysis of the difference between denitrification and DNRA rates in O_2 treatments and those in control experiments. *P* values with significant differences ($P < 0.05$) are highlighted in bold character.

	O_2 concentration in slurry ($\mu mol\ L^{-1}$)	Denitrification	DNRA
Figino	1.2	0.3529	**0.0023**
	2.0	**0.0011**	**0.0127**
	2.8	**0.0003**	0.2137
	3.4	**0.0004**	**0.0305**
	4.1	**0.0001**	**0.0182**
	8.6	**< 0.0001**	**0.0003**
	16.0	**0.0056**	**< 0.0001**
	24.1	**< 0.0001**	**0.0093**
	38.0	**< 0.0001**	**< 0.0001**
	61.3	**< 0.0001**	**0.0002**
	78.6	**< 0.0001**	**< 0.0001**
Melide	0.8	**0.0099**	**0.0256**
	1.8	**0.0002**	**0.0008**
	2.9	**0.0055**	**0.0019**
	3.8	**0.0001**	**0.0002**
	4.9	**0.0045**	**0.0002**
	9.0	**< 0.0001**	**< 0.0001**
	13.1	**< 0.0001**	**0.0459**
	21.3	**< 0.0001**	**0.0005**
	44.4	**< 0.0001**	**0.0433**
	58.6	**< 0.0001**	**0.0062**
	73.2	**< 0.0001**	**< 0.0001**

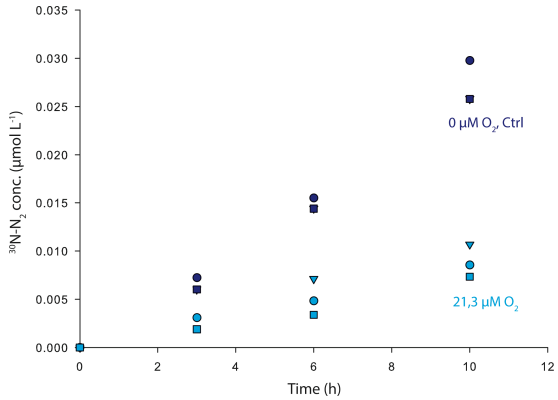

Figure SI.1: Exemplary times-series of $^{30}N_2$ concentration data during incubation experiments in two different O_2 treatments (triplicate) at Melide.

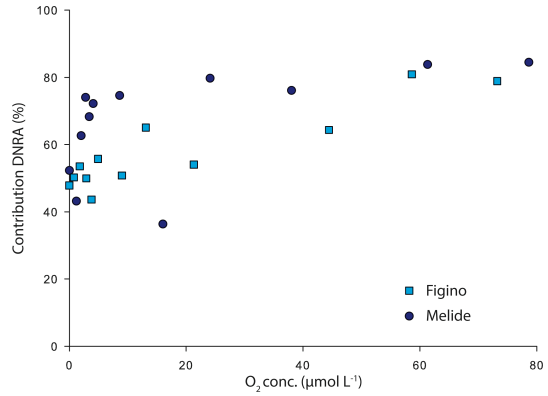

Figure SI.2: Relative contribution of DNRA (%) to total NO_3^- reduction at variable O_2 concentrations at Figino and Melide.

Chapter 4

Controls of H_2S, Fe^{2+}, Mn^{2+} on NO_3^--reducing processes in sediments of an eutrophic lake

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Abstract

Understanding the biogeochemical controls on the partitioning between nitrogen (N) removal through denitrification and anaerobic ammonium oxidation (anammox), and N recycling via dissimilatory nitrate (NO_3^-) reduction to ammonium (DNRA) is crucial for constraining lacustrine N budgets. Besides organic carbon, inorganic compounds may serve as electron donors for NO_3^- reduction, yet the significance of lithotrophic NO_3^- reduction in the environment is still poorly understood. Conducting incubation experiments with additions of ^{15}N-labeled compounds and reduced inorganic substrates (H_2S, Fe^{2+}, Mn^{2+}), we assessed the role of alternative electron donors in regulating the partitioning between the different NO_3^--reducing processes in ferruginous surface sediments of Lake Lugano, Switzerland. In sediment slurry incubations without added inorganic substrates, denitrification and DNRA were the dominant NO_3^--reducing pathways, with DNRA contributing between 31% and 46% to the total NO_3^- reduction. The contribution of anammox was less than 1%. Denitrification rates were stimulated by low to moderate additions of ferrous iron (Fe^{2+} ≤ 258 μM) but almost completely suppressed at higher levels (≥ 1300 μM). Conversely, DNRA was stimulated only at higher Fe^{2+} concentrations. Dissolved sulfide (H_2S, i.e. sum of H_2S, HS^- and S^{2-}) concentrations up to ~ 80 μM, strongly stimulated denitrification, but did not affect DNRA significantly. At higher H_2S levels (≥ 125 μM), both processes were inhibited. We were unable to find clear evidence for Mn^{2+}-supported lithotrophic NO_3^- reduction. However, at high concentrations (~ 500 μM), Mn^{2+} additions inhibited NO_3^- reduction, while it did not affect the balance between the two NO_3^- reduction pathways. Our results provide experimental evidence for chemolithotrophic denitrification or DNRA with Fe^{2+} and H_2S in the Lake Lugano sediments, and demonstrate that all tested potential electron donors, despite the beneficial effect at low concentrations of some of them, can inhibit NO_3^- reduction at high concentration levels. Our findings thus imply that the concentration of inorganic electron donors in lake sediments can act as an important regulator of both benthic denitrification and DNRA rates, and

suggest that they can exert an important control on the relative partitioning between microbial N-removal and N-retention in lakes.

Introduction

The water quality of lakes in Switzerland has greatly improved over the last few decades due to the ban of phosphates in laundry detergents, improved wastewater management and modern treatment technologies (Jakob et al. 2002; Zobrist and Reichert 2006; Zobrist et al, 2018). Phosphate concentrations have largely returned to pre-eutrophication levels, yet reactive nitrogen levels in Swiss lakes are still relatively high, likely due to continued inputs from agriculture (Zobrist and Reichert, 2006). Lake sediments are hot spots of N transformations and play an important role in the remediation of excess reactive N inputs through nitrate (NO_3^-)-reducing processes (e.g. Wenk et al., 2014). Efficient NO_3^- elimination in lakes is mainly due to denitrification, the microbially-mediated dissimilatory reduction of nitrate to gaseous N_2 using organic or inorganic substrates (organotrophic vs. chemolithotrophic denitrification). The anaerobic oxidation of ammonium (anammox) with NO_x^- to N_2 can also play a role in N removal in lake sediments (Schubert et al., 2006; Wenk et al., 2013, Crowe et al., 2017). Conversely, dissimilatory reduction of nitrate to ammonium (DNRA), results in the retention of reactive N in the environment. The coupling and the partitioning of the different NO_3^--transforming metabolisms will thus determine the ultimate fate of reactive N.

Denitrification and DNRA compete for the same substrates and electron acceptors (NO_2^-, NO_3^-). In field and laboratory incubations, the ratio of organic carbon (OC) to NO_3^- availability has been shown to control the relative importance of the different N-transforming processes (Kraft et al., 2014; Hardison et al., 2015, Palacin-Lizarbe et al., 2019). For instance, when the OC/NO_3^- ratio is low, NO_3^- is commonly reduced via denitrification. On the other hand, when the OC/NO_3^- ratio is high, DNRA is often the dominant N-reduction pathway (Nizzoli et al., 2010; Yoon et al., 2015, van den Berg et al., 2015 and

2016; Chutivisut et al, 2018).

Traditionally, NO_3^- reduction via denitrification and DNRA has been considered as purely organotrophic processes (Tiedje, 1988). Geochemical evidence (Froelich et al, 1979) and the discovery of nitrate-reducing microorganisms using Fe^{2+} as substrate (e.g. Straub et al., 1996; Hafenbradl et al., 1996) stimulated research on the microbiology and biogeochemistry of this novel mode of N-transformation. Culturing studies, as well as sediment incubation experiments provided putative evidence that this process is performed by diverse microbes in a variety of aquatic environments (Table 1). However, bacterial culture experiments with high substrate concentrations (e.g. 2.5-4 mM NO_3^-, 10 mM Fe^{2+}; Weber et al., 2006b) are not representative for natural conditions. On the other hand, experimental studies with environmentally relevant substrate concentrations did not always investigate the end products of NO_3^- reduction (i.e. N_2 vs. NH_4^+ production; Chakraborty et al., 2013; Laufer et al., 2016). Recent work has shown an increasing contribution of DNRA relative to denitrification with increasing environmental Fe^{2+} concentration (Roberts et al., 2014; Robertson et al., 2016, Robertson and Thamdrup, 2017). Yet, the environmental relevance of Fe^{2+}-dependent nitrate transformations is still poorly understood, as is the effect of Fe^{2+} on the partitioning between N-removal by denitrification and N-retention in the case of DNRA. Similarly, knowledge on the potential role of Mn^{2+}, which has also been suggested as potential electron donor for chemolitotrophic denitrification (Aller 1990; Luther et al., 1997), is completely lacking in lacustrine sediments.

In contrast, the importance of H_2S as a direct substrate for both denitrification and DNRA is well established (Table 4.1). In organic-rich lake sediments, sulfate is efficiently reduced to sulfide (Holmer and Storkholm, 2001), which reacts with iron species to sulfur intermediates or FeS (Zopfi et al., 2004). Some nitrate reducers can use both dissolved and particulate forms of reduced sulfur for their metabolism (e.g. Dannenberg et al., 1992; Kamp et al., 2006; Yan et al., 2018). In natural environments, NO_3^- reduction coupled to sulfide oxidation has been studied in water columns (e.g. Jensen et al., 2009;

Wenk et al., 2013; Table 4.1) and sediments (Brunet and Garcia-Gill, 1996; Senga et al., 2006). However, studies on the end products of sulfide-dependent NO_3^- reduction pathways (i.e., N_2 versus NH_4^+) under environmentally relevant substrate concentrations are still scarce.

In the present study we aimed to assess the role of different potential inorganic electron donors (Fe^{2+}, H_2S, Mn^{2+}) in regulating the overall rates and the partitioning between N-removing (denitrification, anammox) and N-recycling (DNRA) processes in the ferruginous sediments of the eutrophic southern basin of Lake Lugano (Switzerland). Previous studies in this seasonally anoxic basin demonstrated that an important portion of the external NO_3^- load is removed through sedimentary denitrification (Wenk et al., 2014). By conducting incubation experiments with ^{15}N-labelled substrates and additions of different inorganic electron donors, we determined whether, and to what extent, lithotrophic nitrate reduction is significant under environmentally relevant substrate concentrations.

Materials and methods

Sampling site

The south alpine Lake Lugano is located on the Swiss/Italian border and is divided by a shallow sill into a permanently stratified northern basin and a eutrophic, monomictic southern basin (Barbieri and Polli, 1992). Using a gravity corer, we collected sediments at two locations in the southern basin: Figino (8°53'37"E, 45°57'31"N, 94 m depth) and Melide (8°57'29"E, 45°56'22"N, 85 m depth; Supplementary Figure 1). Sampling campaigns took place in 2015 (December), 2016 (March and September) and 2017 (March and June). Depth profiles of water column temperature, conductivity, and oxygen concentrations were obtained by a winch-operated CTD (Idronaut Ocean Seven 316Plus). Bottom water concentration data for NO_3^-, NH_4^+, total dissolved Fe, Mn and S

were determined within the frame of a long-term monitoring campaign promoted by the International Commission for the Protection of Italian-Swiss Waters (CIPAIS; Commissione Internazionale per la Protezione delle Acque Italiano-Svizzere) on behalf of the Administration of the Canton of Ticino, and were provided by F. Lepori (SUPSI; University of Applied Sciences and Arts of Southern Switzerland).

Table 4.1: Overview of prior experimental studies that have investigated Fe(II) and H_2S oxidation coupled to nitrate reduction

Electron donor	Experimental set-up	Electron donor conc. (mM)	Main N-product	Studied ecosystem	Reference
Fe^{2+}	Pure culture	3.5	N_2O (N_2 n/d)	Freshwater lake [a]	Chen et al., 2018
		10	N_2	Swine waste lagoon [a]	Chaudhuri et al., 2001
		10	n/d	Lake sediments [a]	Muehe et al., 2009
		10	n/d	Anaerobic baffled reactor [a]	Mattes et al., 2013
		10	n/d	ATCC strain 25259 [a]	Beller et al., 2013
	Enrichment culture	0.25	n/d	Stream sediments [a]	Chakraborty et al., 2013
		4	N_2O, N_2	Freshwater/marine/brackish sediments [a]	Benz et al., 1998
		4	n/d	Lake sediments [a]	Hauck et al., 2001
		5-6	NH_4^+	River sediments [a]	Coby et al., 2011
		6.6-10	N_2	Town ditches/brackish water lagoon [a]	Straub et al., 1996
		7-8	NH_4^+	Wetland sediments [a]	Weber et al., 2006a
		10	n/d	Swine waste lagoon [a]	Lack et al., 2002
	Incubation	0.08-0.15	N_2,NH_4^+	Lake water column	Michiels et al., 2017
		0.49-0.65	NH_4^+	Estuarine sediments	Robertson et al., 2016
		0.1-5	NH_4^+	Lake sediments	Robertson and Thamdrup, 2017
		2	N_2	Activated sludge	Nielsen and Nielsen, 1998
		5	NH_4^+	Estuarine sediments	Roberts et al., 2014
H_2S	Pure culture	0.001	N_2O	ATCC strain 23642	Sublette and Sylvester, 1987
		0.02	n/d	Lake water [a]	Kojima and Fukui, 2011
		0.04-0.07	NH_4^+	Freshwater sulfate reducing bacteria	Dannenberg et al., 1992
		0.05-0.135	NH_4^+	Purified *Thioplocca* filaments	Otte et al., 1999
		0.3	N_2O/N_2	Nest of a deep-sea hydrothermal vent polychaete [a]	Takai et al., 2006
		5	NH_4^+	n/d	Eisenmann et al., 1995
	Enrichment culture	1	N_2	Marine tidal sediments [a]	Kraft et al., 2014
		1.5-3	N_2 (NH_4^+ n/d)	USAB reactor [a]	Campos et al., 2008
		1.5-3	N_2,NH_4^+	Freshwater sludge reactor [a]	Chutivisut et al., 2014
		4-8	n/d	Freshwater stream mud [a]	Kamp et al., 2006
	Incubation	0.002-0.05	N_2 (NH_4^+ n/d)	Fjord water column	Jensen et al., 2009
		0.01	N_2	Lake water column	Wenk et al., 2013
		0.05	N_2O (N_2 n/d)	Marine water column	Brettar and Rheinheimer, 1991
		0.1-0.8	n/d	Marine sediments	Schulz et al., 1999
		0-5	N_2O (N_2 n/d)	Lake sediments	Senga et al., 2006
		0.1-5	N_2	Marine sediments	Bowles et al., 2012
		1	NH_4^+	Lake sediments	Brunet and Garcia-Gil, 1996
		1	n/d	Fluidized bed reactor	Cytryn et al., 2005

[a] Origin of the inoculum for pure culture experiments

Sediment depth profiles

Upon return to the laboratory, duplicate sediment cores from each site were sectioned into 1-cm slices in the uppermost 6 cm of the core, and 2-cm slices from 6 to 20 cm depth. Samples were taken for porosity, TOC, particulate Fe^{II} / Fe^{III}, total Mn, and dissolved pore water constituents (Fe^{2+}, Mn^{2+}, H_2S, SO_4^{2-}, NH_4^+, NO_3^-, and NO_2^-). For porewater collection, sediment sections were rapidly transferred to 50 mL Falcon tubes and centrifuged at 4700 rpm for 10 min. The supernatant was immediately filtered (0.2 µm), and samples (1 mL) for Fe^{2+} and Mn^{2+} were fixed with hydrochloric acid (40 µL 1 M HCl). Samples for H_2S determination were stabilized with 20 µL aqueous zinc acetate solution (20% w/v) and stored at 6 °C until analysis. The remaining filtered porewater was frozen until analysis of all other compounds. Oxygen microprofiles were measured in the laboratory (21 °C) using an amperometric microsensor with a tip diameter of 100 µm (Unisense).

Sediment slurry incubations

Seasonal variation of the potential benthic N-transformation processes was investigated through sediment slurry incubation experiments performed at different times of the year (December 2015, March and September 2016). For the preparation of the slurries, fresh surface sediment (upper 2 cm) was homogenized, and aliquots of 1 g sediment were transferred into 120 mL serum bottles and complemented with 80 mL of anoxic (He purged for 45 min) artificial lake water (Smith et al., 2002; Supplementary Table 1). The artificial lake water was free of NO_3^-, NO_2^-, and NH_4^+, reducing the production of N_2 from NO_x present in natural bottom waters. Serum bottles were sealed with blue chlorobutyl-rubber stoppers and crimped before purging with He for 10 min to remove O_2 and to lower the N_2 background for further isotopic measurements. Slurries were pre-incubated overnight on a shaker table (80 rpm) at 8 °C in the dark to remove any traces of O_2, which may still have been present after the initial purging. Labeled ^{15}N (e.g. $Na^{15}NO_3^-$, $^{15}NH_4Cl$; ^{15}N 99%; Cambridge Isotopes

Laboratories; final conc. ~ 120 µM and 71 µM, respectively; Supplementary Table 2) and ^{14}N-substrates (e.g. $^{14}NO_2^-$; final conc. ~ 34 µM) were added to identify and quantify potential rates of denitrification, DNRA, and anammox (Nielsen et al., 1992, Thamdrup and Dalsgaard, 2002). During substrate addition and sampling, the slurries were transferred to an anaerobic chamber with N_2 atmosphere, and were then returned to the shaker table held at 8 °C. Gas samples for ^{15}N-N_2 isotope analysis were taken from the headspace at each time point (4 in total) using a 5 mL gas-tight glass syringe (Hamilton). Two milliliters of the gas were transferred to 3 mL Exetainers (Labco) pre-filled with anoxic Milli-Q water. In exchange for the extracted gas sample 2 mL of anoxic Milli-Q water were added to the incubation vials to maintain constant pressure inside. Exetainers with the gas samples were stored upside down at room temperature until isotopic measurement of N_2. At $T_{initial}$ and T_{end}, 6 mL liquid samples were collected, and immediately filtered for nutrient determination and quantification of DNRA rates. The incubation time for each treatment was determined based on preliminary tests and lasted 3 to 4 days. Liquid samples were kept frozen or acidified with sulfamic acid (40 mM final concentration; Klueglein and Kappler, 2013) until further analysis of dissolved nutrients and metals, respectively. Preliminary tests, during which samples were taken at four time points, consistently showed linear $^{15}NH_4^+$ production over time. We also tested for the adsorption of NH_4^+ to sediment material following an adapted procedure of Behrendt et al. (2013). Briefly, sediment slurries were prepared as described above and known quantities of NH_4Cl were added, corresponding to final concentrations 5, 10, 25 and 50 µM. The percentage of adsorption was calculated from the difference between the target concentration and the actually determined concentration in the supernatant minus the NH_4^+ concentration in control slurries without NH_4^+ addition. All preparations were done in triplicates.

Incubation experiments with different electron donors

To assess the influence of various inorganic electron donors on the mode and regulation of benthic N-transformation, we performed incubation experiments with microbial biomass from fresh sediment. Sediments used were collected in March and June 2017 for Fe^{2+}, H_2S, and Mn^{2+} addition experiments. Through stirring and centrifugation, microbial biomass was separated from the sediment matrix in order to minimize the effect of the sedimentary organic matter, solid-phase iron sulfides and metal-oxyhydroxides. Briefly, the first two centimeters of duplicate sediment cores were sectioned and transferred into a 2-L Erlenmeyer flask with anoxic (He purged) artificial lake water (Supplementary Table 1), which was immediately closed using a thick grey rubber stopper. After intense shaking and stirring, the slurry was transferred into 50 mL Falcon tubes and centrifuged at 300 rpm for 3 min to separate solid-phase particles from detached microbial biomass. The supernatants were pooled, and aliquots of 70 mL were transferred into 120 mL serum bottles (in triplicate) that were then sealed and crimped. The liquid phases were purged with He (10 min) and pre-incubated on a shaker table (80 rpm) in the dark at 8 °C. Different volumes of anoxically prepared solutions of $FeCl_2 \cdot 4H_2O$ (100 mM), $Na_2S \cdot 9H_2O$ (50 mM) and $MnCl_2 \cdot 4H_2O$ (50 mM) were added (Supplementary Table 2). Then the pH was adjusted with anoxic HCl or NaOH (1 M) to the pH of control incubations without added substrate, and kept constant (± 0.2 units) for the duration of the experiment. Substrate additions and subsampling of gas or liquid phase was done in an anaerobic chamber, as described above. Samples (1 mL) for Fe^{2+} and Mn^{2+} determination were fixed with 40 mM sulfamic acid (Klueglein and Kappler, 2013). 1-mL aliquots for H_2S determination were preserved with 20 µL zinc acetate (20% w/v). The experiment was started with the addition of $Na^{15}NO_3^-$ (~ 116 ± 11 µM) and lasted 5 to 7 days (Supplementary Table 2). Incubations took place in the dark, under gentle agitation at 8 °C. Given the stark deviation from in situ conditions (i.e., the separation of microbial biomass from sediment and modification of the natural solute concentrations), these incubations were not intended to assess absolute benthic N

transformation rates that are representative for the natural conditions. Instead, these incubations allowed us to investigate any differential stimulation/inhibition by the tested electron donors within a well-controlled experimental set-up. As a consequence, results below are presented as percent (%) "stimulation" or "inhibition" of a given NO_3^--reducing processes relative to the corresponding controls without additions. Student's t-tests ($P < 0.05$, Excel) were applied to determine significant differences between measured rates.

^{15}N-based rate measurements

Denitrification and anammox rates were calculated using ^{15}N isotope-pairing technique (Nielsen, 1992), through monitoring the production of $^{14}N^{15}N$ or $^{15}N^{15}N$ (Nielsen, 1992; Thamdrup and Dalsgaard, 2002) using a Delta V Advantage isotope-ratio mass spectrometer (IRMS; Thermo Fisher Scientific) coupled to a gas chromatograph for gas purification. Rates of denitrification and anammox were calculated from the total accumulation of single ($^{14}N^{15}N$) and double-labeled ^{15}N-N_2 ($^{15}N^{15}N$) over time as determined by linear regression analysis of excess $^{14}N^{15}N$ /$^{14}N^{14}N$ and $^{15}N^{15}N$ /$^{14}N^{14}N$ trends (Supplementary Figure 2). In incubations with $^{15}NH_4^+$ and $^{14}NO_2^-$,

$$N_{2\ anammox} = {}^{14}N^{15}N \times F_{NH4+}$$

where F_{NH4+} is the fraction of ^{15}N in NH_4^+ (Thamdrup and Dalsgaard, 2002). As anammox was insignificant, the following equation was used to quantify denitrification in incubations with $^{15}NO_3^-$,

$$N_{2\ denitrification} = {}^{14}N^{15}N + 2 \times {}^{15}N^{15}N$$

DNRA rates were quantified by oxidation of NH_4^+ to N_2 using alkaline hypobromite (Risgaard-Petersen et al., 1995), as in Robertson et al. (2016). The produced N_2 was then analyzed by GC-IRMS as described above. In incubations with $^{15}NO_3^-$, DNRA rates were determined by linear regression of the

concentration of $^{15}NH_4^+$ versus time,

$$NH_4^+{}_{DNRA} = {}^{14}N^{15}N + 2 \times {}^{15}N^{15}N$$

$^{15}NH_4^+$ standards were prepared in parallel with the samples in order to test the efficiency of the hypobromite oxidation step (recovery typically > 95%).

Chemical analyses

Nitrite concentrations were determined colorimetrically according to (Hansen and Koroleff 1999). Concentrations of NO_x^- (i.e., NO_3^- + NO_2^-) were measured by chemiluminescence detection using a NOx-analyzer (Antek Model 745; Braman and Hendrix, 1989). Nitrate concentrations were then calculated from the difference between NO_x^- and NO_2^-. Ammonium in porewater and incubation experiments was measured generally by suppression-ion chromatography with conductivity detection (940 Professional IC Vario, Metrohm, Switzerland). In treatments with Mn^{2+} additions, NH_4^+ was measured spectrophotometrically, due to interferences during the ion-chromatographic separation. Photometric quantification of NH_4^+ was done using the indophenol method (Krom, 1980).

Dissolved Fe^{2+} in porewater and incubation samples was measured with ferrozine (Stookey, 1970). Solid-phase reactive Fe^{II} and Fe^{III} was quantified by extraction in 0.5 M HCl for 1 h according to Jensen and Thamdrup (1993) and subsequent analysis with ferrozine. Manganese concentrations in the acidic sediment extracts (including Mn^{II} and Mn^{IV}) and in Mn^{2+}-amended incubations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent Technologies 5100).

Dissolved sulfide (H_2S, i.e. sum of H_2S, HS^- and S^{2-}) in the porewater was quantified by the colorimetric methylene blue method (Cline, 1969). Concentrations in slurries were affected by the presence of Fe^{2+} and, hence, the

partial removal of the added H_2S from solution through precipitation as FeS. Therefore, both the added quantity and the measured concentration in the incubations are reported. Porewater chloride and sulfate (SO_4^{2-}) concentrations were quantified by suppressed anion chromatography with conductivity detection (940 Professional IC Vario, Metrohm).

Results

Geochemical setting of sampling sites

Fine organic-rich (~ 8% OC dry mass) sediments were found at both sampling stations in the southern basin of Lake Lugano. High microbial activity within the sediments was reflected by the shallow O_2 penetration depths of 3.7 ± 0.6 and 2 ± 0.5 mm at Figino and Melide, respectively (n = 4, data not shown), as determined by microsensor measurements in the laboratory with oxygenated (~ 220-235 µM O_2) lake water covering the sediment surface. Active bioturbation by higher organisms was not evident, consistent with seasonal bottom-water anoxia for more than 7 months (e.g., Blees et al. 2014). Typically, deep-hypolimnion oxygenation occurs once a year during winter mixing. In 2016, however, the bottom water at Figino remained anoxic throughout the year (Figure 4.1), whereas at Melide at least low oxygen concentrations were measured throughout most of the annual cycle. The bottom-water concentrations of the different nitrogen compounds varied seasonally as a function of water column stratification conditions. Ammonium concentrations 2 m above the sediments increased during the thermal stratification period at both stations, reaching 68 µM and 128 µM at Figino and Melide, respectively. With the onset of water-column mixing in February, the downwelling of oxygenated water led to the almost complete oxidation of ammonium and the concomitant production of NO_2^- and NO_3^-. The sum of produced NO_2^- and NO_3^- did not match the loss of NH_4^+, suggesting that most NO_x^- was further metabolized by respiratory processes in the water column and/or the surface

sediments. The bottom water concentrations of NO_3^- varied between 25-65 µM and 1-67 µM at Figino and Melide, respectively, depending on the season (Figure 4.1). Nitrite concentrations ranged between 0.05 µM and 14 µM at both stations.

Porewater concentration data of dissolved N compounds (NH_4^+, NO_2^-, and NO_3^-), as well as dissolved and particulate phases of Mn and Fe are shown in Figure 4.2. At both stations, NH_4^+ concentrations near the sediment-water interface were ~ 600 µM and increased with depth indicating the active mineralization of organic matter. In the surface sediments, NO_3^- and NO_2^- concentrations were below detection limit (Figure 4.2), despite the presence of NO_x in the bottom waters at the time of sampling (Figure 4.1), indicating that the sediments represent an efficient sink for these compounds. The porewaters at both stations were further characterized by high concentrations of Mn^{2+} and Fe^{2+}. Dissolved Mn^{2+} concentrations were almost identical at both stations (~ 350-400 µM, Figure 4.2) and did not show any strong variation with depth. Concentrations of Fe^{2+} were much higher at Figino, where they increased from ~ 150 µM at the sediment-water interface to ~ 900 µM at 20 cm depth (Figure 4.2). Dissolved Fe^{2+} and Mn^{2+} concentrations in the surface sediment layers (0-2 cm) varied throughout the year at both stations, though no clear temporal trends were observed (Cojean, 2019). Particulate Fe^{II}, Fe^{III}, and total Mn concentrations were relatively constant with depth, and similar between sites (Figure 4.2). Free H_2S was not detected in the porewater at either of the two stations (detection limit 1 µM).

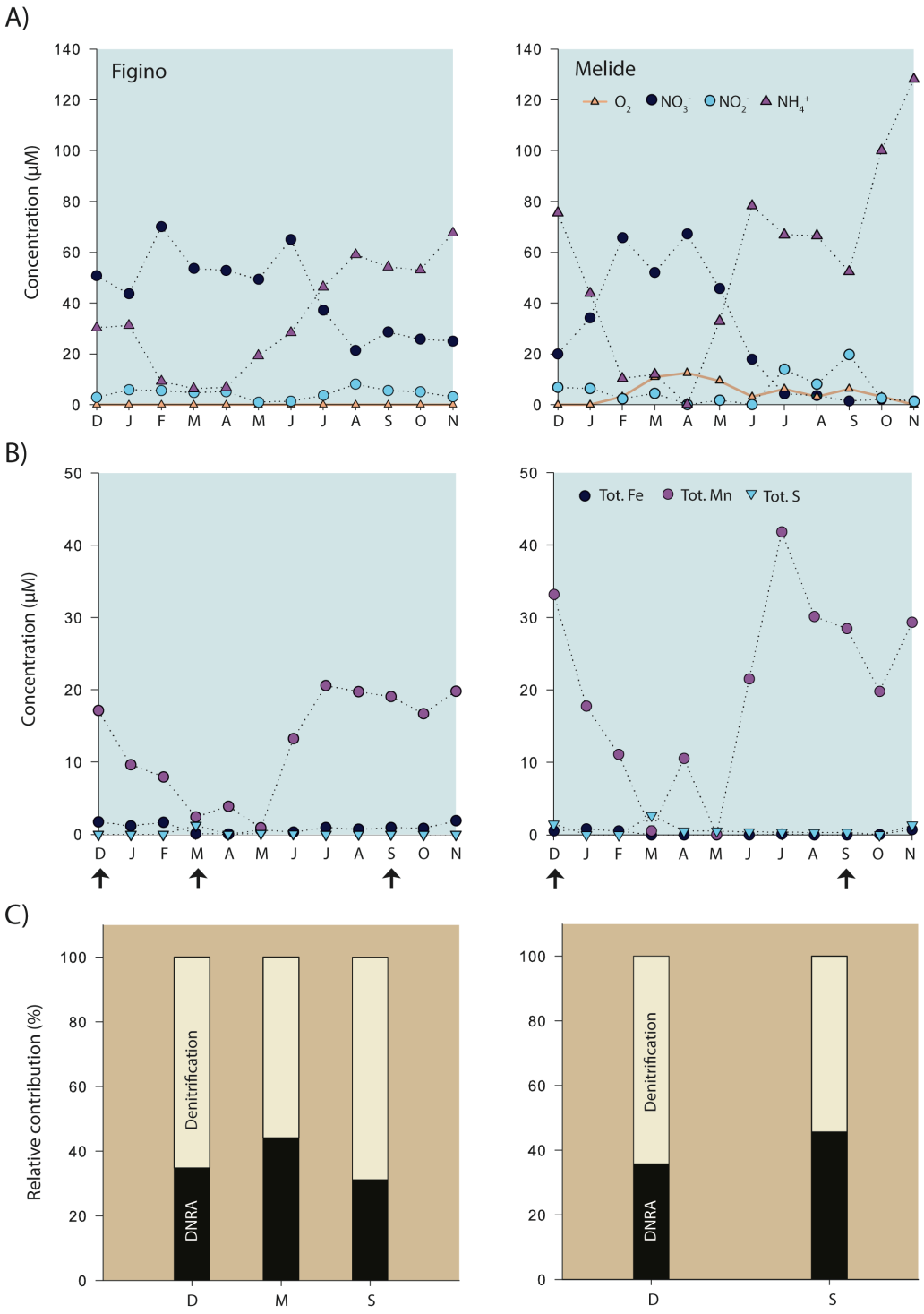

Figure 4.1: (**A**) Seasonal variation of bottom-water (2 m above sediment surface) concentrations of dissolved oxygen and nitrogen compounds, and (**B**) total dissolved Fe, Mn and S at the two study locations in the south basin of Lake Lugano (Supplementary Figure 1). No oxygen was detected at Figino during that year. (**C**) Relative contribution of DNRA and denitrification (%) to the total benthic NO_3^- reduction, as determined by sediment slurry incubation experiments at selected time points (arrows). Capital letters at x-axes correspond to the first letter of each month from December 2015 to November 2016.

Benthic N-transformations

We determined potential rates of benthic denitrification, DNRA, and anammox in anoxic slurry incubation experiments with fresh surface-sediment material, collected at different times of the year (Figure 4.1). At each site, similar results were obtained for the different sampling months. In $^{15}NO_3^-$ amended slurries, production rates of ^{15}N-N_2 via denitrification were consistently higher than $^{15}NH_4^+$ production through dissimilatory nitrate reduction to ammonium (DNRA). Adsorption of generated $^{15}NH_4^+$ to sediment particles could lead to underestimated DNRA rates. Initial adsorption tests showed, however, that NH_4^+ did not bind significantly to the mineral phases in our dilute slurries. Hence, the measured DNRA rates can be considered as not being affected significantly by adsorption artefacts. The contribution of DNRA relative to total nitrate reduction varied seasonally between 31% and 46% (Figure 4.1). The ratio of denitrification to DNRA (DEN:DNRA) varied accordingly between 1.2 and 2.2. Despite this relatively consistent partitioning between denitrification and DNRA at each station, the absolute potential rates differed markedly across sites. For all sampling dates, except for December 2015, denitrification was higher at Figino than Melide, with measured maximum rates of 85 and 53 nmol N g^{-1} wet sediment d^{-1}, respectively. In contrast, DNRA rates were slightly higher at Melide than at Figino, with maximum rates of 44 and 38 nmol N g^{-1} wet sediment d^{-1}, respectively. In slurries amended with $^{15}NH_4^+$ and natural abundance NO_2^-, N_2 production rates were close to 0.01 nmol N g^{-1} wet sediment day^{-1} at both sites, indicating that the contribution of anammox to the total N-removal was < 1% (data not shown). Anammox was therefore not further investigated in this study.

Incubations with microbial biomass only

Incubations with microbial biomass that had been separated from most of the organic and inorganic sedimentary solids were established as a baseline for the treatments amended with inorganic electron donors (Fe^{2+}, H_2S and Mn^{2+}). These experiments were designed to investigate any differential stimulation/inhibition by the tested electron donors under controlled conditions. The removal from sedimentary solids had a major influence on the potential rates and the relative contribution of DNRA to NO_3^- reduction, which decreased from about 31-46% in the regular slurry incubations to < 12%. Given the significant deviation from in situ conditions, this is not too surprising. In fact, our observation provides putative evidence for nitrate-reduction/partitioning controls other than those that were specifically tested here. More specifically,

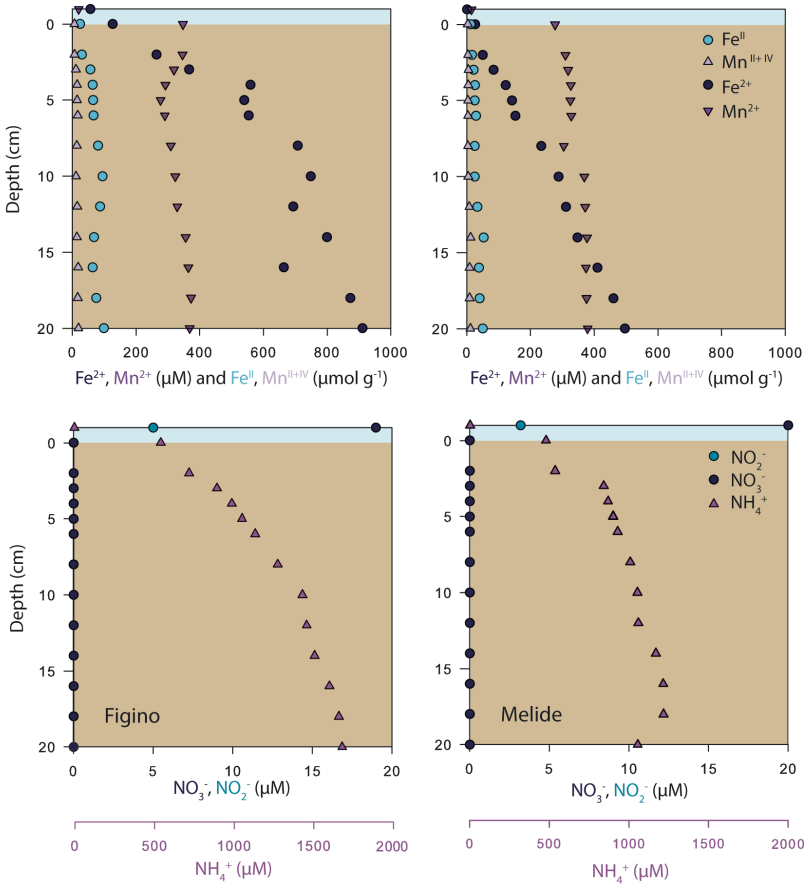

Figure 4.2: Vertical profiles of porewater solutes (Fe^{2+}, Mn^{2+}, NO_3^-, NO_2^-, NH_4^+) and particulate phases (Fe^{II} and total Mn) in sediments sampled in June 2017 at Figino (left) and Melide (right). The bottom water samples were collected 2 m above the surface sediments.

during the separation step, total particulate organic carbon (POC) was reduced from ~ 300 to 55 mg L^{-1} (Figino) and ~ 180 to 25 mg L^{-1} (Melide), while dissolved organic carbon (DOC) concentrations increased from about 510 to 900 mg L^{-1} and 640 to 1400 mg L^{-1} for Figino and Melide, respectively. Thus, our findings hint at the importance of organic matter as regulatory factor determining the ratio between denitrification and DNRA (as shown earlier, Nizzoli et al., 2010; Chutivisut et al, 2018), or alternatively suggest that DNRA bacteria are to a larger extent particle-associated. The goal of the present study, however, was to explore the role of inorganic electron donors as potential substrates for anaerobic NO_3^--reduction processes, and their impact on the repartition between denitrification and nitrate ammonification.

Incubations with Fe^{2+} additions

In order to investigate the effect of Fe^{2+} on NO_3^- reduction at environmentally relevant concentrations, we conducted incubation experiments with additions of $^{15}NO_3^-$ and various concentrations of dissolved Fe^{2+} (Table 4.2). While we aimed to lower the ambient Fe^{2+} concentration by separating the microbial biomass from most of the Fe-containing sediment solids and dilution of sediment porewater with Fe-free artificial lake water, total Fe^{2+} background concentrations in the controls were still about 201 µM and 113 µM at Figino and Melide, respectively, suggesting desorption of Fe^{2+} or dissolution of Fe(II) phases during the separation of microbial biomass. These background Fe^{2+} levels represent 20% of the average ambient Fe^{2+} porewater concentration.

In all incubation experiments, we observed systematic trends across treatments and between the two sites. At both stations, the lower Fe^{2+} additions (≤ 258 µM) significantly enhanced N_2 production, with maximum stimulation relative to the controls of 75% and 250%, at Figino and Melide, respectively (Figure 4.3). With increasing Fe^{2+} concentrations (≥ 406 µM), however, N_2 production decreased to ~ 0.01 µmol N L^{-1} d^{-1}, indicating almost complete

inhibition of denitrification at the highest Fe^{2+} concentration (Figure 4.3; Table 4.2). In contrast, DNRA was partly inhibited by Fe^{2+} at the low and intermediate concentrations, while the highest Fe^{2+} levels (≥ 2433 and 1303 µM at Figino and Melide, respectively), enhanced DNRA 408% (Figino) and 88% (Melide) relative to the corresponding controls (Figure 4.3). Consequently, the relative contribution of DNRA to the total NO_3^- reduction compared to denitrification (i.e., the DEN:DNRA ratio) varied according to the final Fe^{2+} concentration in the incubations. In the controls, DNRA contributed not more than ~ 8% to the total nitrate reduction rate. At low Fe^{2+} concentrations, when denitrification was Fe-stimulated the most, the importance of DNRA relative to denitrification decreased (Table 4.2). In contrast, at Fe^{2+} concentrations higher than 661 µM (Figino) and 406 µM (Melide), the DNRA contribution was ≥ 20% at both sites (with one exception at 662 µM Fe^{2+} at Melide; Table 4.2), clearly indicating the differential stimulation/inhibition of DNRA versus denitrification by Fe^{2+}.

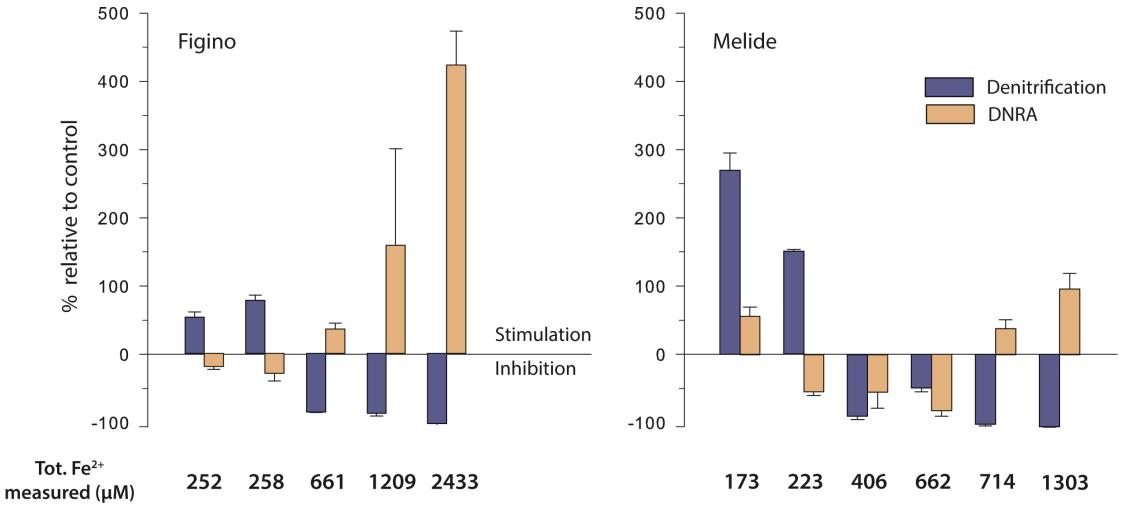

Figure 4.3: Effect of dissolved ferrous iron on denitrification and DNRA rates in anoxic sediment-biomass incubation experiments amended with $^{15}NO_3^-$ and different Fe^{2+} concentrations. Stimulation (positive values)/inhibition (negative values) is expressed relative to the respective controls without Fe^{2+} additions for each set of experiments (see supplementary Table 2). Error bars show standard errors (n = 3).

Consistent with the ^{15}N-tracer results, nitrate consumption increased relative to controls when low concentrations of Fe^{2+} were added, particularly at Figino (Table 4.2). With increasing Fe^{2+} concentrations (i.e., 661 µM and higher),

NO_3^- reduction was significantly reduced (Table 4.2). The nitrate concentration measurements revealed that the net amount of $^{15}NO_3^-$ removed did not match up with the determined products (NO_2^-, ^{15}N-N_2 and/or $^{15}NH_4^+$). In most experiments, nitrate consumption was ~ 1.3 times higher than the sum of measured ^{15}N-labeled products,-which may be due to the assimilation or storage uptake by microorganisms and algal cells. The total recovery of dissolved Fe^{2+} added at the beginning of the incubation (after 2-5 days of pre-incubation for pH stabilization) was complete in most treatments (70-100%). But in all experiments, added Fe^{2+} was mostly found in the particulate phase, with only 2% to 6% remaining in solution. This suggests that Fe^{2+} may have sorbed to surfaces, like remaining sedimentary solids.

Table 4.2: Summary of denitrification, DNRA, NO_3^- consumption rates (all µmol N L^{-1} d^{-1}), and the contribution of DNRA to total nitrate reduction (%) in $^{15}NO_3^-$ addition incubation experiments with different Fe^{2+} amendments. The percentage of recovery was calculated prior to the NO_3^- addition. Positive and negative values correspond to production and consumption, respectively. Standard errors are indicated in parentheses.

	Total $[Fe^{2+}]$ measured	% recovery $[Fe^{2+}]$ added	Denitrification	DNRA	NO_3^-	DNRA contrib.
	µM	*%*	*µmol N L^{-1} d^{-1}*			*%*
Figino	201		5.30 (0.65)[a]	0.32 (0.06)[a]	-8.44 (1.00)[a]	5.7
	252	100	8.18 (0.42)*	0.33 (0.02)	-11.07 (0.12)*	3.9
	258	63	9.47 (0.39)*	0.29 (0.04)	-12.75 (0.27)*	3.0
	661	97	0.79 (0.03)*	0.20 (0.01)	-3.03 (0.18)*	20.2
	1209	100	0.72 (0.17)*	0.37 (0.2)	-1.33 (0.74)*	33.9
	2433	100	0.01 (0.01)*	0.74 (0.07)*	-1.00 (0.22)*	98.7
Melide	113		1.12 (0.12)[a]	0.10 (0.01)[a]	-5.29 (1.00)[a]	8.3
	173	100	4.45 (0.29)*	0.19 (0.04)	-5.97 (0.58)	4.1
	223	100	3.05 (0.03)*	0.13 (0.05)	-3.94 (0.13)	4.1
	406	100	0.22 (0.06)*	0.08 (0.03)	-0.92 (0.18)*	26.7
	662	100	0.70 (0.06)*	0.04 (0.01)*	-1.42 (0.10)*	5.4
	714	56	0.06 (0.02)*	0.12 (0.01)	-1.20 (0.35)*	66.7
	1303	53	0.03 (0.00)*	0.17 (0.02)*	-1.33 (0.41)*	85

[a] Average of two different sets of control experiments (n = 6). Percentages of stimulation and inhibition of denitrification and DNRA (Figure 4.3) have been calculated from the respective control for each experiment.

* Significantly different ($p < 0.05$) from the respective controls without Fe^{2+} addition for each set of experiments (see Supplementary Table 2)

Incubations with H_2S additions

Similar to the treatments with Fe^{2+}, H_2S was added at different concentrations to the incubations amended with $^{15}NO_3^-$. In all treatments, denitrification was a more important NO_3^--reducing pathway than DNRA. In control experiments without H_2S addition, ^{15}N-N_2 production was 8 and 12 times higher than of $^{15}NH_4^+$ at Figino and Melide, respectively (Table 4.3). Upon addition of H_2S, the largest fraction of sulfide was removed from solution by reacting with Fe or Mn. Both, the targeted H_2S ("added") concentration as well as the actual concentrations of dissolved H_2S ("measured") are therefore presented in Table 4.3. Values reported below represent the targeted concentrations of H_2S, unless stated otherwise.

Denitrification was significantly enhanced when sulfide was added in the range of 50-500 µM H_2S (corresponding to ≤ 80 µM of measured dissolved H_2S; Table 4.3). Within this concentration range, denitrification was stimulated in proportion to increasing H_2S at Melide, while it remained relatively constant at Figino. At 100 and 500 µM H_2S, the stimulation was highest and reached 414% (Figino) and 959% (Melide), respectively, relative to unamended control incubations (Figure 4.4). The addition of H_2S with a targeted concentration of 1 mM, resulted in measured dissolved H_2S concentrations ≥ 125 µM, and caused a 96% and 80% decline in the denitrification rates at Figino and Melide, respectively (Figure 4.4). The response of the nitrate ammonifiers to the different H_2S concentrations was less pronounced and, in most cases, statistically not significant (Table 4.3). At Figino, $^{15}NH_4^+$ production decreased slightly with increasing H_2S concentration, while at Melide, DNRA was stimulated by 60% and 30% with 100 µM and 500 µM H_2S added, respectively (Figure 4.4). At both sites, DNRA seemed also to be inhibited at the highest H_2S levels, although to a lesser extent than denitrification (83% and 47% DNRA decrease at Figino and Melide, respectively).

In slurries exposed to ≤ 500 µM H_2S added, the relative contribution of DNRA to the total nitrate reduction decreased to a minimum of ~ 2% (Figino) and ~ 1% (Melide), respectively (compared to ~ 11% and 8% in the controls, respectively). At the highest measured H_2S concentration, the DNRA contribution increased to about 36% (Figino) and 16% (Melide), suggesting that the balance between denitrification and DNRA is regulated by the environmental H_2S concentration, as was also the case with Fe^{2+}. These experiments were repeated with sediment material collected in March 2017 and displayed very similar patterns (data not shown).

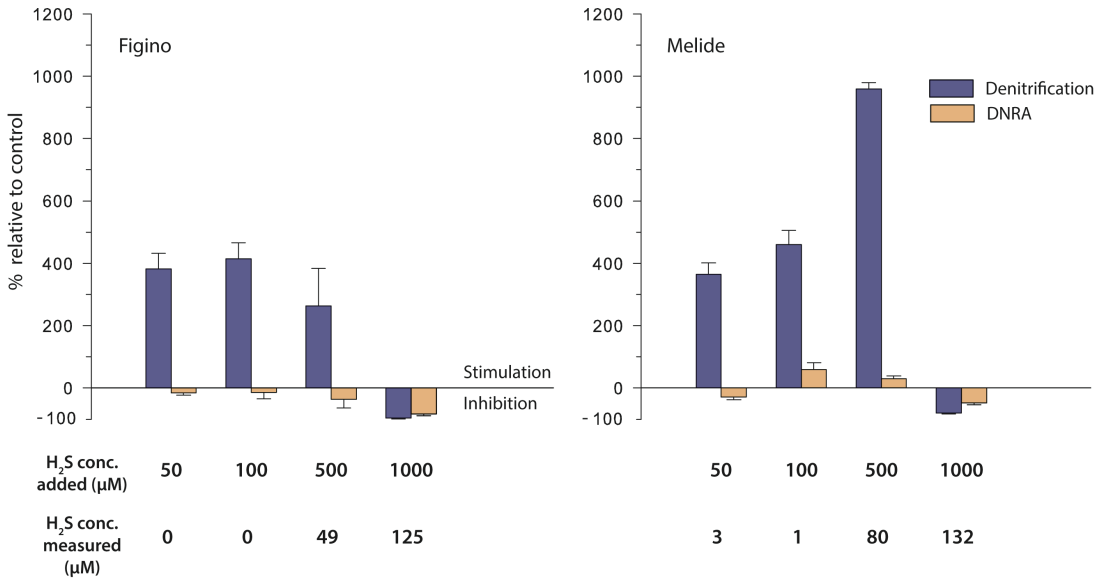

Figure 4.4: Effect of sulfide on denitrification and DNRA in anoxic sediment biomass incubation experiments amended with $^{15}NO_3^-$ and different H_2S additions. Stimulation (positive values)/inhibition (negative values) is expressed relative to controls without additions. Error bars represent standard errors (n = 3).

In line with the ^{15}N tracer results, NO_3^- consumption increased with increasing H_2S availability (up to 500 µM H_2S added; Table 4.3). As for the other amendment experiments with Fe^{2+}, NO_3^- consumption was not balanced by the formation of NO_2^- and $^{15}N\text{-}N_2/^{15}NH_4^+$ production. In control incubations, SO_4^{2-} accumulated at a rate of 1.9 ± 1.8 µmol L^{-1} d^{-1}. The production increased with the amount of added H_2S and reached a maximum rate of 5.9 ± 0.5 µmol L^{-1} d^{-1} in the experiment where 500 µM of H_2S was added. The rate dropped to 0.3 ± 1.5 µmol

Table 4.3: Transformation rates of denitrification, DNRA, NO_3^- consumption rates (all µmol N L^{-1} d^{-1}), and DNRA contribution to total nitrate reduction (%) from experiments supplemented with $^{15}NO_3^-$ and H_2S. Positive and negative values correspond to production and consumption respectively, with standard errors given in parenthesis (SE). Concentration of H_2S and percentage recovery were calculated at $T_{initial}$.

	[H_2S] added	[H_2S] measured	Recovery [H_2S] added	Denitrification	DNRA	NO_3^-	DNRA contrib.
	µM		*%*	*µmol N L^{-1} d^{-1}*			*%*
Figino	0	0		1.90 (0.02)	0.24 (0.02)	-5.05 (0.24)	11.2
	50	0	0	9.24 (0.95)*	0.20 (0.02)	-18.34 (1.6)*	2.3
	100	0	0	9.84 (1.00)*	0.21 (0.05)	-23.98 (0.44)*	2.0
	500	49	10	6.95 (2.31)	0.15 (0.07)	-17.48 (7.23)	2.1
	1000	125	13	0.07 (0.07)*	0.04 (0.01)*	-0.22 (0.75)*	36.4
Melide	0	0.6		1.53 (0.40)	0.13 (0.04)	-5.69 (0.12)	7.8
	50	3	5	7.47 (0.62)*	0.09 (0.01)	-12.25 (3.03)	1.2
	100	1	0	9.35 (0.75)*	0.21 (0.03)	-20.37 (1.57)*	2.2
	500	80	16	17.67 (0.33)*	0.17 (0.01)	-20.74 (3.45)*	1
	1000	132	13	0.32 (0.04)	0.06 (0.01)	-1.63 (0.15)*	15.8

* Significantly different from the corresponding control without H_2S addition ($p < 0.05$)

L^{-1} d^{-1} at the highest H_2S concentration tested (Table 4.3).

Incubations with Mn^{2+} additions

As for the Fe^{2+} addition experiments, we aimed to lower the ambient Mn^{2+} concentration in the microbial-biomass separation step (see above), but dissolved Mn^{2+} background concentrations remained substantial, at ~30 µM and ~36 µM for Figino and Melide incubations, respectively. Both in the controls and in all incubations amended with Mn^{2+}, production of ^{15}N-N_2 was consistently greater than of $^{15}NH_4^+$. At the Figino station, denitrification and DNRA rates in unamended controls equaled 0.97 ± 0.14 and 0.13 ± 0.03 µmol L^{-1} d^{-1}, respectively, and at Melide 1.24 ± 0.09 and 0.06 ± 0.02 µmol L^{-1} d^{-1}, respectively. At Figino, at moderate Mn^{2+} addition (95 µM), denitrification was stimulated by about 40% compared to the corresponding controls (Figure 4.5). At higher Mn^{2+} concentrations, denitrification activity decreased with increasing Mn^{2+} concentration. At Melide, no stimulation of denitrification was observed. Our

results revealed a proportional reduction of $^{15}N\text{-}N_2$ formation with increasing Mn^{2+}, corresponding to ~ 80% inhibition at the highest Mn^{2+} concentration tested. At both stations, DNRA rates also decreased with rising Mn^{2+} levels.

Transformation rates based on NO_3^- and Mn^{2+} concentration changes were very similar between sites and among treatments. In control incubations, nitrate consumption rates equaled 4.9 ± 0.5 µmol L^{-1} d^{-1} at both stations, and decreased to 2.4 ± 0.4 µmol L^{-1} d^{-1} in incubations supplemented with the highest Mn^{2+} concentration (891 µM and 931 µM at Figino and Melide, respectively). On the other hand, Mn^{2+} removal rates increased with increasing Mn^{2+} from 0 ± 0.2 µmol L^{-1} d^{-1} in the controls to 3.0 ± 1.6 µmol L^{-1} d^{-1} at highest Mn^{2+} levels. The lack of any significant stimulation of either DNRA or denitrification in most treatments supplemented with Mn^{2+} suggests that Mn^{2+}, while being removed from the liquid phase (e.g. through adsorption onto particles), did not play a major role as electron donor for NO_3^- reduction in Lake Lugano sediments.

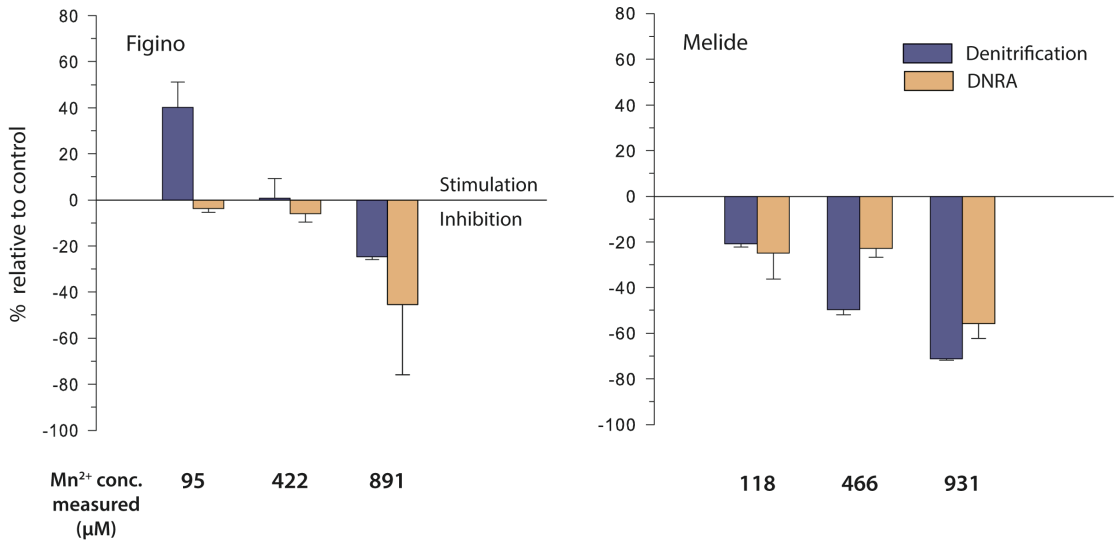

Figure 4.5: Effect of dissolved manganese on denitrification and DNRA in anoxic sediment biomass incubation experiments amended with $^{15}NO_3^-$ and different Mn^{2+}concentrations. Stimulation (positive values)/inhibition (negative values) is expressed relative to controls without additions. Error bars show standard errors (n = 3).

Discussion

Denitrification versus DNRA in Lake Lugano sediments

During the sampling campaigns in this study, denitrification was the main N-reduction process in slurries of Lake Lugano (South Basin) sediments. At both stations, the DNRA contribution to the total NO_3^- reduction showed moderate fluctuations between the different seasons, ranging between 31% to 46% but no clear seasonal trend was observed despite of the seasonal variations in bottom water oxygenation and NO_3^- contents. The contribution of DNRA to NO_3^- reduction was significantly higher than observed previously in flow-through whole-core incubations performed with sediments from the same basin (Wenk et al. 2014). Wenk et al. reported a maximum DNRA contribution to NO_3^- reduction of ~ 12%, but also suggested that these measurements should be considered conservative, because they neither accounted for the production of $^{14}NH_4^+$ from ambient background NO_3^- nor for product NH_4^+ potentially retained in the intact sediment cores. The use of intact sediment cores provides a more accurate representation of in situ N-cycling conditions by maintaining the biogeochemical zonation of the sediment relative to slurry methods (Hansen et al 2000, Robertson et al. 2019). However, relative potential process rates and their specific regulating factors can better be resolved through controlled manipulation experiments (Robertson et al., 2019), such as those performed here (discussed below).

Similar partitioning of the two dissimilative NO_3^--reducing processes, with a relatively large contribution of DNRA has been observed in a wide range of environments, particularly in reduced sediments with high organic matter content and comparatively low nitrate levels (Burgin and Hamilton, 2007, Dong et al., 2011). The contribution of DNRA to total NO_3^- reduction can be particularly important in both estuarine sediments (~ 5 to 91%; An and Gardner, 2002; Megonigal et al., 2004; Gardner et al., 2006; Burgin and Hamilton, 2007; Dong et al., 2009; Giblin et al., 2013; Roberts et al., 2014; Plummer et al., 2015; Kessler

et al., 2018) and freshwater lake sediments (~ 3 to 50%; Nizzoli et al., 2010, Robertson and Thamdrup, 2017; Wenk et al., 2014). Yet, the natural variability in these studied environments is quite high. The OC/NO_3^- ratio is often considered an important regulating factor of the relative contribution between the different benthic NO_3^--reducing processes. DNRA can be stimulated at high OC and low (limiting) NO_3^- levels, while denitrification is generally dominant when NO_3^- is not limiting (van den Berg et al., 2016; Kraft et al., 2014). Differences with respect to the occurrence of DNRA versus denitrification across different ecosystems, however, may also be attributed to variable H_2S and Fe^{2+} availability, with DNRA presumably being favored over denitrification at high levels of these inorganic substrates.

Biogeochemical control of Fe^{2+}on denitrification and DNRA

In order to investigate the effect of inorganic substrates on NO_3^- reduction rates and on the partitioning between denitrification and DNRA we performed incubation experiments with sediment biomass, from which most of the sediment solids have been removed. For the investigation of Fe^{2+}, H_2S and Mn^{2+}, which may adsorb to solid phases or react with particle-associated compounds, the exclusion of particulates is helpful in following the consumption of electron donors over time. While the transformation rates in such a system cannot be directly compared to the volume-based biogeochemical rate measurements with natural sediment, these incubations provide insights as to the metabolic potential for stimulation of denitrification and DNRA by alternative electron donors within a carefully controlled system.

The sediments in the southern basin of Lake Lugano are rich in dissolved Fe^{2+} and Mn^{2+} and show seasonal variation in the concentrations of these solutes in the sediment surface layer (Figure 2; Lazzaretti et al, 1992). The apparent relation to fluctuations in bottom water oxygenation and the presence of NO_3^- suggests a potential coupling between NO_3^- reduction and Fe^{2+} oxidation. Our results revealed a complex control from Fe^{2+} on the balance between

denitrification and DNRA with relative stimulation of the former and latter at low and high concentrations, respectively. The relative stimulation of DNRA at high Fe^{2+} concentration (≥ 2433 and 1303 µM Fe^{2+} at Figino and Melide, respectively), agrees qualitatively with observations by Robertson et al. (2016) and Robertson and Thamdrup (2017), who reported a stimulation of DNRA and Fe^{2+} oxidation rates in estuarine and lake sediments with Fe^{2+} addition ranging between 165 and 5000 µM. However, they did not observe a relative stimulation of denitrification at the lower range of Fe^{2+} concentrations tested, which appears to contrast with the present study, where denitrification was favored over DNRA at the lower Fe^{2+} concentrations (≤ 258 µM) at both stations. Our observations are in line, however, with a pure-culture study by Chakraborty et al. (2011) who also observed enhanced rates of denitrification and increased growth yields in presence of environmentally relevant substrate concentrations (≤ 250 µM Fe^{2+}, 20 µM acetate, 100 µM NO_3^-) in mixotrophic *Acidovorax* sp., while at higher Fe^{2+} levels growth ceased as cells became encrusted with Fe^{III} oxides. Indeed, *Acidovorax* sp and *Siderooxydans* sp., another abundant and putative Fe^{2+}-oxidizing NO_3^--reducing bacterium, have been detected in the top sediment layers at Figino and Melide using 16S rRNA gene amplicon sequencing (Cojean, 2019). To date, microbial Fe^{2+}-dependent NO_3^- reduction is mainly attributed to microbes that sustain their growth energy from the mixotrophic use of Fe^{2+} together with an organic co-substrate (Straub et al., 1996; Melton et al., 2014). The coupling between Fe^{2+} oxidation and denitrification has also been highlighted in a variety of natural environments including lake waters (Michiels et al., 2017), marine and estuarine sediments (Laufer et al., 2016; Robertson et al., 2016), soils (Ratering and Schnell, 2001), and activated sludge from waste-water treatment plants (Nielsen and Nielsen, 1998). However, the majority of these previous studies used millimolar substrate concentrations in order to investigate the influence of Fe^{2+} on NO_3^- reduction (Table 4.1).

In contrast to results from previous studies, it appears that in our experiments, nitrate ammonifiers did not preferentially use Fe^{2+} as electron donor. At low Fe^{2+} levels, there was no indication for any Fe^{2+}-induced

stimulation of DNRA. In this regard, we argue that the increase in the relative contribution of DNRA to total nitrate reduction at higher Fe^{2+} levels, may not have been a direct result of an increase in the rate of DNRA coupled to Fe^{2+} oxidation. Rather, we speculate that nitrate ammonifiers were less sensitive than denitrifiers to high Fe^{2+} levels, and that very high concentrations of Fe^{2+} may have indirectly favored DNRA bacteria by suppression of organotrophic denitrification and a resulting increased availability of organic substrates.

In previous studies investigating the coupling between Fe^{2+} oxidation and NO_3^- reduction, inhibition of denitrification under Fe-rich conditions has been attributed to cell encrustation from Fe^{III}-oxide formation around the cell membrane and inside the periplasm (Kappler et al., 2005, Muehe et al., 2009, Klueglein et al., 2014, Nordhoff et al., 2017), which affects bacterial metabolism by limiting substrate uptake, and may even lead to cell damage. However, cell encrustation was, so far, only observed using millimolar Fe^{2+} concentrations whereas our data displayed a significant inhibition of denitrification at around 400 µM Fe^{2+} already, pointing to a potential direct Fe^{2+} toxicity effect on the metabolism. Iron toxicity under anoxic conditions has previously been attributed to inhibition of the F-ATPase (Dunning et al, 1998) and replacement of active-site metal cofactors (Crichton, 2009). But so far, this toxicity effect has only been examined in cultures of anoxygenic phototrophs and streptococci (Dunning et al., 1998; Poulain and Newman, 2009). Our results show that Fe^{2+} inhibition on denitrification in natural habitats may occur at lower Fe^{2+} concentration than previously thought based on studies of pure or enrichment culture experiments.

Stimulation of denitrification by H_2S

In lake sediments, H_2S is continuously produced by mineralization of sulfur-containing biomass and respiratory SO_4^{2-} reduction. In ferruginous surface sediments like in the south basin of Lake Lugano, rapid reaction of H_2S with dissolved Fe^{2+} or Fe-oxides leaves the porewater free of dissolved H_2S

(Lazzaretti et al., 1992). Despite this, independent molecular analyses using 16S rRNA gene sequencing (Cojean, 2019) revealed a variety of sulfur-oxidizing bacteria, among them several abundant taxa with a metabolic potential for anaerobic respiration with nitrate or nitrite (e.g. *Sulfuritalea* sp., *Sulfurimonas* sp., *Sulfurovum* sp. *Thiobacillus* sp.). Moreover, among the three inorganic substrates tested in this study, sulfide additions showed the strongest stimulation on NO_3^- consumption and denitrification.

Stimulation of denitrification by H_2S has been observed in pure and enrichment cultures (Senga et al., 2006; Campos et al., 2008; Table 1), stratified water columns (Brettar and Rheinheimer, 1991; Burgin et al., 2012; Wenk et al., 2013), sediments (Brunet and Garcia-Gill, 1996, Hayakama et al., 2013, Deng et al., 2015), but also in engineered systems such as anaerobic digesters (Sher et al., 2008). Often, the degree of H_2S-induced stimulation was directly related to the H_2S concentration. In natural systems, denitrification was enhanced primarily at lower H_2S concentration (< 100 µM; e.g. Senga et al., 2006; Burgin et al., 2012; Bowles et al., 2012), while it was strongly inhibited at higher sulfide levels (discussed below). At first sight, denitrification in our study may appear less sensitive towards high H_2S additions. Considering, however, that most of the added H_2S (> 84%) was removed from solution by reaction with Fe or Mn, we find that our results are in good agreement with these reports. It is likely that increased free H_2S concentrations ≥ 125 µM (Table 4) in our treatments with highest H_2S addition inhibited microbial NO_3^- reduction. Uncharged sulfide (H_2S) can easily diffuse across cell membranes and it is therefore recognized as the most toxic form of the compound (Barton et al, 2014), exerting a stronger inhibitory effect on bacteria than FeS, S^0 or $S_2O_3^{2-}$. At elevated concentrations (> 100 µM), free H_2S can act as growth-inhibitor by e.g. denaturing proteins through disruption of disulfide cross-links between polypeptide chains, or inactivating the redox centers of metalloenzymes, and it can, ultimately lead to cell death (Khan et al., 1990; Wu et al., 2015). In contrast, FeS has been considered as a non-toxic repository of suitable electrons for sulfur bacteria, and inhibition of denitrification by FeS was only observed at > 10 mmol S L^{-1} (Garcia-

Gil and Golterman, 1993). To date, knowledge on specific effects of H_2S on NO_3^--reducing microbial groups is scarce. A study on marine microbial communities reports a lag of bacterial growth of potential denitrifiers (*Vibrio* sp., *Marinobacter* sp., *Pseudomonas stutzeri*) with increasing H_2S concentration (Mirzoyan and Schreier, 2014). Based on pure culture studies reporting on increased concentrations of N-intermediates (NO_2^-, NO, N_2O) in the presence of H_2S, it has been proposed that H_2S can have an inhibitory effect on the respective N-compound reducing enzymes (Sørensen et al., 1980; Aelion and Warttinger, 2009). Aside from such conclusions based on the accumulation of N-intermediates, however, knowledge is lacking regarding the mechanisms of inhibition by H_2S at the enzyme level.

As opposed to denitrification, DNRA is often enhanced under highly sulfidic conditions (Brunet and Garcia-Gil, 1996; Gardner et al., 2006; Lu et al., 2013; Plummer et al., 2015) and may even dominate reductive NO_3^- transformation, leading to the retention of reactive N in the ecosystem (An and Gardner, 2002; Dong et al, 2011; Murphy et al., 2020). Some pure cultures of H_2S-oxidizing DNRA bacteria can grow at millimolar concentrations of free H_2S (e.g. Eisenmann et al, 1995), showing that physiological mechanisms exist that allow them to cope with such high levels of this toxic compound. This contrasts with our observations where DNRA activity was somewhat suppressed already in the presence of low H_2S concentrations, and, just as denitrification, inhibited at free H_2S concentrations > 125 µM. Unlike previous studies in other environments (e.g. Brunet and Garcia Gil, 1996; Otte et al., 1999; Sayama et al., 2005) we do not find that H_2S is the preferred substrate for DNRA bacteria in the ferruginous sediments in the south basin of Lake Lugano where the autochthonous microbial communities are not exposed to significant concentrations of free dissolved H_2S. We speculate therefore that the selective pressure was not sufficient to enable communities of sulfide-tolerant DNRA bacteria to become enriched. As a consequence, the microbial communities are not well adapted, and thus sensitive, to high H_2S concentrations. It seems that low free H_2S concentrations, along with FeS and S^0 as the most important reduced sulfur species fueling NO_3^-

reduction, exert a beneficial remediation effect by increasing the contribution of N-removal (denitrification) relative to N-retention (DNRA).

Inhibitory effects of high Mn^{2+} concentration on nitrate reduction

Based on thermodynamic considerations (Luther et al., 1997) and porewater concentration profiles (Aller et al., 1990; Schulz et al., 1994), Mn^{2+}-driven nitrate reduction has been suggested to occur in natural systems, particularly in manganese-rich sediments, but solid experimental proof is still lacking (Madison et al., 2013). The main goal of this part of our work was to assess, whether nitrate-dependent Mn^{2+} oxidation exists in Mn^{2+}-rich freshwater sediments. Excluding the Figino Station, where denitrification seemed to be stimulated in the presence of 100 µM Mn^{2+}, our results show the rates of both denitrification and DNRA decreased with increasing Mn^{2+} concentrations. No comparable stimulation was observed in sediments from Melide. Based on these results, we conclude that Mn^{2+} does not play a biogeochemically relevant role as reductant for NO_3^- in the sediments under investigation. In line with our data, Schippers et al. (2005) did not find enhanced manganese oxidation with sensitive ^{54}Mn radiotracer experiments, in the anoxic water column of the Black Sea when NO_3^-, NO_2^-, or N_2O was present. On the other hand, a stimulation of denitrification and DNRA with Mn^{2+} addition was observed in the anoxic water column of the Baltic Sea. It remained unclear, however, whether this stimulation was due to an indirect effect of Mn^{2+}, e.g. by the removal of an inhibitory substance (Bonaglia et al., 2016).

In most of our experiments, Mn^{2+} clearly exerted an inhibitory effect on both denitrification and DNRA. The reason for this observation is not clear, but could reflect a general toxicity of high Mn^{2+} concentrations on the activity of microbes. Mn toxicity has been reported for mammals, plants, and prokaryotes (Hohle and O'Brian, 2014), where Mn over-accumulation is often correlated with deficiencies in other transition-metals of physiological relevance. This can then lead to mismetallation of regulatory transcription factors and key enzymes,

affecting growth, sensitivity towards reactive oxygen species, and virulence (Zeinert et al., 2018). For instance, Mn can bind to the ferric uptake regulator (Fur) in the Gamma-Proteobacterium *E. coli.* As a consequence, iron import systems are repressed, intracellular Fe levels drop, and, for example, heme synthesis is impeded (Martin et al, 2015). Mn also interferes competitively with secondary Fe import (Martin et al., 2015), and high extracellular Mn^{2+} levels can also competitively inhibit magnesium (Mg^{2+}) uptake (Silver and Clark, 1971). Manganese toxicity has been investigated in detail for the soil Alpha-Proteobacterium *Bradyrhizobium japonicum* (Hohle and O'Brian, 2014). It was demonstrated that Mn^{2+} can enter the cells through open Mg^{2+} transporters under low Mg conditions, and exert a toxic effect by displacing Mg in proteins or other macromolecules (Hohle and O'Brian, 2014). Replacing Mg^{2+} with Mn^{2+} as a cofactor in enzymes affects their activity and possibly lead to the disregulation of metabolic pathways (Hohle and O'Brian, 2014).

Whether the mechanisms of Mn^{2+} toxicity established in model organisms also apply to complex environmental communities is not known yet. In our experiments, where we added up to 900 µM $MnCl_2$ (in presence of only 0.34 mM Mg^{2+}; Supplementary Table 1) and observed a decreasing N-transformation activity with increasing Mn^{2+} levels, Mn^{2+} toxicity by interference with Mg^{2+} homoeostasis is plausible. Yet much more research with environmentally relevant microorganisms is required to better understand the effects of increased transition metal concentrations such as Mn and Fe on environmental microbes and the biogeochemical processes they catalyze.

Conclusion

Benthic NO_3^- reduction in the South Basin of Lake Lugano is mainly driven by denitrification and DNRA, whereas anammox is negligible. Our results show that Fe^{2+} and H_2S are important controlling factors for the partitioning between denitrification and DNRA, and that the effect is concentration-dependent. Nitrate was mostly reduced through denitrification at lower levels of dissolved

H_2S (< 80 µM) and Fe^{2+} (< 258 µM).The relative contribution of DNRA to the overall benthic N reduction increased under highly ferruginous conditions (~ 1000 µM Fe^{2+}), possibly as a consequence of reduced substrate competition with denitrifiers, which were almost completely inhibited. The role of Mn^{2+} as electron donor for NO_3^- reduction was negligible, as was its influence on the partitioning between denitrification and nitrate ammonification. All three inorganic substrates, however, had strong inhibitory effects at concentrations significantly higher than the prevailing environmental concentrations. Our study implies that the fate of N may be linked to changes in the availability of inorganic substrates within surface sediments.

During periods of bottom water anoxia in eutrophic lakes, reduced inorganic species diffuse out of the sediment into the bottom waters. In the south basin of Lake Lugano, and potentially other iron-rich eutrophic lakes, the zone of Fe^{2+} driven NO_3^- reduction will extend far into the water column. The prevailing conditions of low Fe^{2+} concentrations and nearly absent free H_2S should be favorable for denitrification rather than DNRA. Periods of extended anoxia under enhanced stratified conditions, however, may lead to the accumulation of significant amounts of Fe^{2+}, Mn^{2+}, or even H_2S (Lazzaretti et al. 1992; Lehmann et al, 2015), and the conditions for denitrification and DNRA in near-bottom lake waters or surface sediments may thereby develop from a stimulating into an inhibitory mode, with important implications for the ultimate fate of reactive N in a lake.

We clearly demonstrated the differential role Fe^{2+}, H_2S and Mn^{2+} can play in regulating the partitioning between denitrification and DNRA. Predictions, however, on how these potential inorganic electron donors act together to possibly shift the balance between the two N-cycling processes and to regulate the overall fixed N-elimination rate in lake sediments remains a challenge.

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Author contribution

JZ and MFL initiated the project. ANYC performed all sample collection and conducted the experimental work. ANYC, MFL and JZ performed data analysis and interpretation. ANYC and JZ prepared the manuscript with input from all co-authors.

Conflict of interest

The authors declare no conflict of interest.

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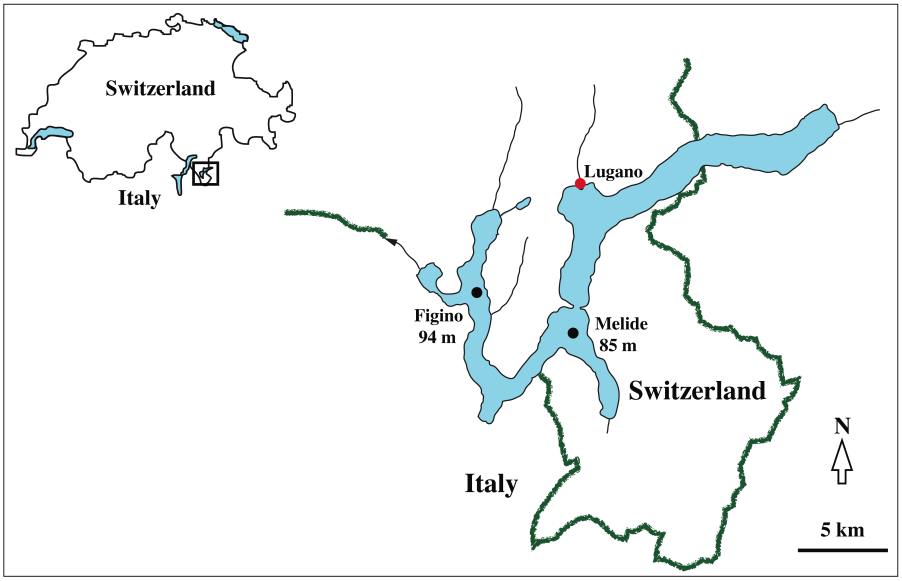
Supplementary information

Supplementary Table 1. Composition of artificial lake water used for incubation experiments. Synthetic lake water was prepared according to Smith et al., (2002) to emulate the major ion composition of Lake Lugano water, devoid of dissolved nitrogen compounds.

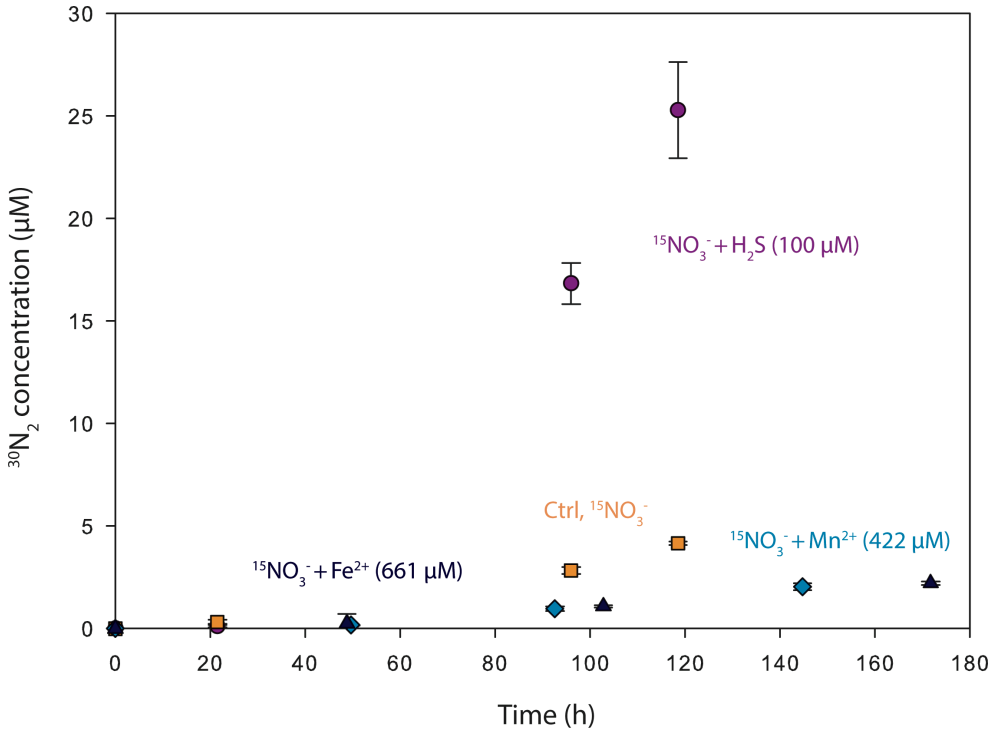
Compound	Targeted species	Species conc. (μM)
$MgCl_2 \cdot 6H_20$	Mg^{2+}	344
$CaCl_2 \cdot 2H_20$	Cl^-	226
$CaCO_3$	Ca^{2+}	905
Na_2SO_4	SO_4^{2-}	102
$KHCO_3$	K^+	50
$NaHCO_3$	Na^+	290

Supplementary Table 2. Overview of the different experiments performed in this study.

Site	Experimental set-up	Treatment	Set of experiments	^{15}N substrate (µM)	Inorganic electron donors added (µM)	Inorganic electron donors measured (µM)	Incubation time (day)
Both sites	Slurry incubation	$^{15}NO_3^-$		120			3-4
		$^{15}NH_4^+ + {}^{14}NO_2^-$		71			3-4
Figino	Incubation with microbial biomass	$^{15}NO_3^- + Fe^{2+}$	1st	115	0	227	6
				113	26	252	6
				115	32	258	6
			2nd	110	0	175	7
				110	486	661	7
				113	1034	1209	7
				109	2258	2433	7
		$^{15}NO_3^- + H_2S$		114	0	0	5
				125	50	0	5
				123	100	0	5
				130	500	49	5
				126	1000	125	5
		$^{15}NO_3^- + Mn^{2+}$		136	0	30	6
				130	65	95	6
				130	392	422	6
				124	861	891	6
Melide	Incubation with microbial biomass	$^{15}NO_3^- + Fe^{2+}$	1st	95	0	119	
				89	55	173	7
				98	104	223	7
				99	287	406	7
				98	543	662	7
			2nd	126	0	107	6
				118	595	714	6
				119	1196	1303	6
		$^{15}NO_3^- + H_2S$		116	0	0	5
				123	50	3	5
				119	100	1	5
				120	500	80	5
				122	1000	132	5
		$^{15}NO_3^- + Mn^{2+}$		122	0	36	6
				118	82	118	6
				116	430	466	6
				113	895	931	6



Supplementary Figure 1. Map of Lake Lugano and the two sampling sites Figino and Melide.



Supplementary Figure 2. Exemplary times-series data of $^{30}N_2$ concentrations during denitrification incubation experiments with microbial biomass and additions of Fe^{2+}, H_2S and Mn^{2+} at Figino. Error bars correspond to standard error of triplicate experiments.

Chapter 5

Environmental control on the nitrogen isotope effect of sedimentary nitrate reduction at the sediment-water interface

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Abstract

In many ocean regions, as a limiting nutrient, bioavailable nitrogen (N) controls marine primary productivity and thus the ocean's capacity to fix and sequester atmospheric CO_2 in its interior. In lakes, N from both natural and anthropogenic sources is an important driver of eutrophication. Therefore, it is crucial to understand the sources and sinks of fixed N in both in the ocean and in lakes. Denitrification, the microbial reduction of nitrate to dinitrogen (N_2), and other modes of suboxic N_2 production (e.g., the anaerobic oxidation of ammonium, or anammox), are the most important sinks of fixed N in aquatic environments, however, particularly with regards to the N cycle in the ocean, there is a persistent debate regarding the overall size of sinks and sources. Isotope ratios of nitrogenous species (e.g., $^{15}N/^{14}N$) can provide important constraints natural N turnover processes and thus on the N cycle itself. However, in order to use stable isotope measurements to trace fluxes of N in aquatic systems, it is imperative to understand the isotope effects associated with these fluxes. While denitrification at the organism-level is known to be associated with a marked N isotope fractionation, the expression of the N isotope effect of benthic (i.e., sedimentary) denitrification in the overlying water is only poorly constrained, and likely varies with the environmental conditions. Here, we conducted whole-core incubations using sediments of variable reactivity and O_2 penetration regimes, in order to investigate experimentally the factors that regulate diffusion-limitation within sediments, nitrate regeneration, as well as their impact the net N isotope effects of benthic nitrate elimination. Our data confirm that the N isotope effect of denitrification in highly reactive sediments is generally strongly suppressed at the scale of sediment-water exchange. In less reactive sediments, where O_2 penetration is significantly deeper, coupled nitrification-denitrification is stimulated, and can even lead to an apparent inverse benthic nitrate isotope effect. Ultimately, across a relatively wide range of reactivity regimes, the cohesive denitrifying lake sediments tested were never a strong source of ^{15}N-depleted nitrogen validating the generally low N isotope effects in global or regional N isotope budgets. Understanding the exact

biogeochemical control exerted on the N isotope effect at the sediment-water interface will help to better parameterize ocean and freshwater biogeochemical models, which in turn will improve global N-budget estimates.

Introduction

Nitrogen (N) is an important element that regulates primary productivity in surface waters, both of the ocean and in lakes. In aquatic environments, fixed N (NO_3^-, NO_2^-, NH_4^+) is introduced into the water column via N_2 fixation and/or directly through loading from contaminated groundwater and adjacent rivers. Denitrification, the stepwise reduction of nitrate (NO_3^-) to N_2, is the most important sink of fixed N in aquatic systems. Nitrification and dissimilatory nitrate reduction to ammonium (DNRA) recycle nitrogen from NH_4^+ into NO_3^- and from NO_3^- into NH_4^+, respectively. The balance between the main sinks and sources of N determines its availability in the ocean or lakes. There is a persistent debate regarding the overall size of global sinks and sources of bioavailable (or fixed) N in aquatic systems, and to what extent input and output terms are balanced (Codispoti 1995, 2007; Brandes and Devol 2002). Understanding the balance of N budgets is important because bioavailable N (such as nitrate) is a limiting nutrient throughout vast areas of the surface ocean, and thus controls the ocean's capacity to fix and sequester atmospheric CO_2 (e.g. primary production) in its interior via the biological pump (Falkowski 1997). On the other hand, excessive N loadings can cause irremediable damage, in particular for coastal marine and freshwater (e.g. lakes, rivers) ecosystems (Gruber and Galloway 2008).

Over the last three decades, natural-abundance isotope-based approaches using ^{15}N and ^{18}O measurements have been constantly improved in order to constrain different N-transformation pathways in the environment (e.g., Sigman et al. 2001; Casciotti et al. 2002). Particular efforts have been made to understand the effects of different N cycling processes on the N and O isotopic composition of NO_3^- (Casciotti 2016). For instance, NO_3^- reduction at the

organism-level is associated with a significant dual nitrate (e.g. $\delta^{15}N$, $\delta^{18}O$) isotope effect that is characteristic for this microbial process. Consequently, its occurrence leads to a marked increase in the $\delta^{15}N$ (and $\delta^{18}O$) of the residual nitrate and a decrease in $\delta^{15}N$-N_2 in suboxic regions of the ocean (e.g. Brandes et al., 1998; Cline and Kapplan, 1975; Sigman et al., 2003), lakes (Lehmann et al. 2003; Wenk et. al. 2014), and within sediment porewaters (Lehmann et al. 2007). Yet, the dual analysis of N and O nitrate isotope can provide more information about the denitrifying metabolism (Nar versus Nap; Granger et al., 2008) and help to distinguish the co-occurrence of different N-turnover processes in the environment (Casciotti 2016).

The mean $\delta^{15}N$ of nitrate in aquatic systems is a function of the relative rates of fixed N inputs (N_2 fixation, riverine inputs) and sinks (pelagic and benthic denitrification; Galbraith et al. 2013; Casciotti 2016), as well as the associated N isotope effects. Assuming an N isotope budget in steady state, the mean nitrate isotopic $\delta^{15}N$ of water column and sedimentary denitrification must equal the average $\delta^{15}N$ of global fixed N inputs (~0‰; Brandes and Devol 2002). Based on the canonical endmember isotope effects traditionally assumed (i.e. ε_{N2_Fix} ~-1‰, ε_{WC_Denit} ~25‰, $\varepsilon_{WC\_Sed\text{-}Denit}$ ~25‰) and a mean ocean nitrate $\delta^{15}N$ of ~5‰, a global ratio of benthic versus water-column denitrification of up to 4:1 has been proposed (Brandes and Devol, 2002). Accepting the range of 60 to 90 Tg N yr^{-1} for water-column denitrification (Gruber 2004), a balanced N isotope budget would in turn necessitate approximately 240 to 360 TgN/yr of benthic denitrification (Brandes and Devol 2002; Codispoti 2007), and would suggest a global N budget far out of balance, with excessive of N loss relative to the sources. While the isotopic fingerprinting of fixed N sources is well established (Casciotti, 2016), canonical estimates on the global N isotope effect (ε) associated with denitrification, particularly in sediments, may need some reconsideration.

Several studies have focused on quantifying the isotope effect of denitrification in the marine and lake water column testifying to a relatively

robust N isotope effect on the order of 25 ± 5‰ (e.g. Brandes et al. 1998; Voss et al. 2001; Lehmann et al. 2003; Sigman et al. 2003; Casciotti et al. 2013). In contrast, studies on the N isotope effect for denitrification in sediments are rather rare. As in water columns, a large organism-level isotope effect (ε_{cell} ~ 11-35‰) for denitrification has been observed within sediment porewaters (Lehmann et al. 2007; Alkhatib et al. 2012; Dähnke and Thamdrup 2013, 2016; Kessler et al. 2014). However, sedimentary denitrification has traditionally been assumed to display essentially no marked N-isotope effect at the sediment-water interface (i.e., $\varepsilon_{app} \sim 0$ ‰; Brandes and Devol 1997; Lehmann et al. 2004, 2007; Alkhatib et al. 2012; Kessler et al. 2014). The suppression of the N isotope effect at the point of sediment-water solute exchange has commonly been attributed to the complete consumption of nitrate within the sedimentary denitrification zone; in both reactive and less reactive sediments, the supply of nitrate by diffusion is thought to be limiting (Brandes and Devol 1997; Lehmann et al. 2007). But in recent years, it has become clear that the apparent N isotope effect associated with sedimentary denitrification is both more variable and higher than previously assumed. For example, an isotopic effect of about 8‰ was found in sediments of the Mauritanian oxygen minimum zone (Dale et al. 2014). Therefore, a large uncertainty still persists regarding the regulation and factors that influence the nitrate isotopic fractionation associated with sedimentary N-loss that is perceived in overlying waters. In a modeling study, Lehmann et al. (2007) have argued that depending on the organic matter reactivity, the redox conditions, the nitrate penetration depth and the occurrence of nitrification, the benthic N isotope effect of denitrification can vary quite significantly. However, despite of model-based observations, supporting experimental data in this regard is still lacking.

This study aims at experimentally investigating the controls on how the biological fractionation of denitrification (i.e., at the cellular-level) is expressed at the ecosystem level, thereby controlling N-isotopic budgets. We focus particularly on understanding the control of organic matter reactivity and associated O_2 penetration depth on N and O isotope effects of benthic

denitrification at the sediment-water interface. We hypothesize that a greater O_2 penetration depth in less reactive sediments will limit nitrate transport to the zone of denitrification, and in turn decrease the associated net N isotope effect. The present work also verifies the isotopic effects that N-transformation processes other than denitrification (e.g., nitrification) can have on the isotopic composition of NO_3^- exchanged at the sediment water interface.

Materials and methods

Sediment core preparation

Model sediments with a range of reactivities (high, intermediate and low organic matter content) were used to investigate how oxygen penetration into sediments influences the isotopic signature associated with sedimentary nitrate reduction, as perceived in overlying water. The model sediments consisted of various mixtures of natural sediments from a Swiss lake (including a natural consortium of microorganisms) and an inert matrix of ultra clean quartz sand (0.3-0.9 mm grain-size, Büchi). Aliquots of natural sediments and quartz sand were proportionally weighed and mixed to reach 100%, 20%, 5% and 1% "reactivity" (4.1, 0.2, 0.02 and 0.01% total C_{org} dry mass, respectively). Sediments were transferred to plastic tubes (20 cm long, 5.3 cm diameter) that were sealed on the bottom using large grey rubber stoppers and 3M-tape to avoid any water leakage (Figure 1). Artificial lake water (NO_3^-, NO_2^-, and NH_4^+-free; Cojean et al., submitted) was gently added on top of the sediments using a 50 mL syringe connected to a silicon tubing in order to avoid any sediment resuspension. Cores were then covered with aluminum foil and submerged into a refrigerated bath (10°C) in the dark. Flushing tubes connected to aquarium pumps were placed in each core at about 4 cm above the sediment surface to ensure full oxygenation of overlying waters.

Oxygen penetration depth

Prior the beginning of the incubation, porewater oxygen microprofiles were generated using a microsensor with an O_2 microelectrode with a tip diameter of 100 µm (Unisense). The overlying water was gently stirred, without disturbing the sediment water interface, and aerated to determine the O_2 penetration depth under oxygenated conditions. Triplicate measurements were done in all sediment cores.

Whole core incubation and sampling

Overlying water was constantly and gently stirred using a homemade electric stirrer separately positioned in each core (see Figure 5.1), in order to avoid stagnating waters that may turn anoxic during the incubations. Nitrate ($\delta^{15}N$ =14.15‰ and $\delta^{18}O$= 25.6‰; final conc. ~ 25 µM) was added to overlying waters, and incubations started (e.g. first sample collection) about 30 minutes after substrate addition. Water samples were collected at distinct time intervals (8 to 10 time points), and the overall incubation time was adapted to the different treatments. Samples for N-NO_x (NO_3^- + NO_2^-) isotope and concentration determination were filtered (0.2 µm) and directly stored frozen in 15mL Falcon tubes until further processing. For NO_3^- samples, a preliminary NO_2^- removal step was necessary prior to freezing, since interferences of NO_2^- with the NO_3^- isotope analyses are expected when using the denitrifier method. Moreover, NO_2^- is rather instable during storage (e.g. O exchange water, reoxidation to NO_3^- ; Granger and Sigman 2009). Hence, immediately after sampling, an aliquot of 10µL sulfamic acid (8% w/v) was added to each sample to react with ambient NO_2^-. After 15 min of shaking, 8 µL NaOH (4M) was added to each sample to bring back the pH to 7 (Granger and Sigman 2009). Samples were stored at -20°C until further analysis. Triplicate experiments were performed for each treatment, except for experiments using 0.2 and 0.01% C_{org}, which were performed in one core only.

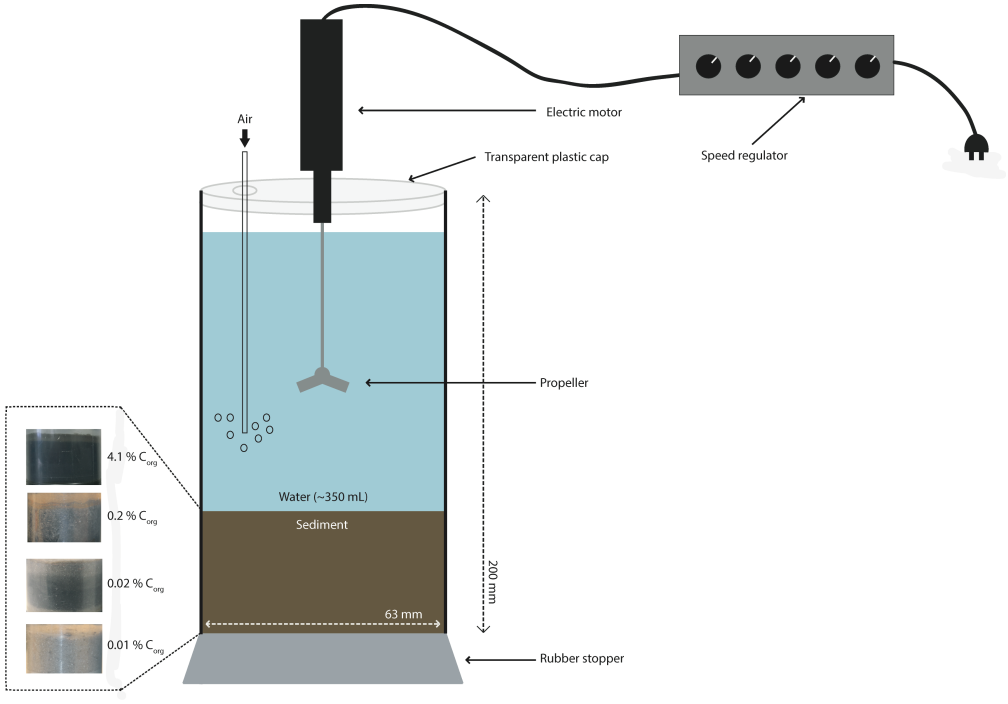


Figure 5.1: Scheme of the whole-core incubation system used in the study.

The effect of nitrification on the net NO_3^- isotope effects of benthic-water column exchange associated with nitrate reduction was investigated in a separate set of triplicate cores containing the same sediment for each respective treatment. However, in these experiments, an inhibitor of nitrification (nitrapyrin, 1 mM; Lehtovirta-Morley et al. 2013) was directly mixed with sediments during sediment core preparation (see above). Samples for nitrate and NO_x isotopic composition and concentration analysis were taken as described above.

Dissolved inorganic nitrogen concentrations

Nitrite and ammonium concentrations were determined spectrophotometrically (Krom 1980; Hansen and Koroleff 1999). Total NO_x (i.e., NO_3^- + NO_2^-) concentration was measured using a NOx-analyzer (Antek Model 745) after reduction to nitric oxide (NO) in an acidic V^{3+} solution, and detection of NO by chemiluminescence (Braman and Hendrix 1989). Nitrate concentration was calculated from the difference between [NO_x] and [NO_2^-]. Nitrate

transformation rates were determined by linear regression of the concentration of NO_3^- versus time.

N and O isotope analysis

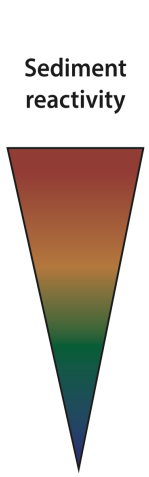
Natural abundance NO_3^- ($\delta^{15}N$ and $\delta^{18}O$) and NO_x ($\delta^{15}N$) isotope ratios were determined using the denitrifier method modified from Sigman et al. (2001). Briefly, aqueous nitrate from our samples was reduced to nitrous oxide (N_2O) through microbial respiration of denitrifiers (*Pseudomonas aureofaciens*) lacking the N_2O reductase enzyme. The isotopic composition of produced N_2O was then analyzed using an isotope ratio mass spectrometer (Delta V IRMS Thermo Fisher Scientific). Oxygen isotope exchange with H_2O was generally less than 5% and corrected for by standard bracketing. Ratios of N and O isotope were reported as δ values (‰; $\delta = (R_{sample}/R_{standard} - 1)*1000‰$, where $R = {}^{15}N/{}^{14}N$ or ${}^{18}O/{}^{16}O$) relative to air N_2 and Vienna Standard Mean Ocean Water (VSMOW), respectively. Internal ($\delta^{15}N$ =14.15‰ and $\delta^{18}O$= 25.6‰) and international KNO_3 reference materials with reported $\delta^{15}N$ and $\delta^{18}O$ values of 4.7‰ and 25.6‰ (IAEA-N3) and -1.8‰ and -27.9‰ (USGS34), respectively, were used for isotope value calibration. Isotope effect values (ε) were calculated through linear regression in a plot of $\delta^{15}N$ or ${}^{18}O$ versus the natural logarithm of the residual nitrate (or NO_x) for the different time points (Mariotti et al., 1981).

Results

O_2 penetration depth and nitrate transformation rates

Nitrate reduction rates varied as function of sediment reactivity and associated O_2 penetration depth. The oxygen penetration depth was a few

millimeters in highly reactive sediments and increased up to 3.5 cm depth in C_{org}-poor sediments (Figure 5.2).



Sediment reactivity	O_2 penetration depth (mm)	$^{15}\varepsilon_{NO3-}$	Nitrification $^{15}\varepsilon_{NO3-}$	$^{18}\varepsilon_{NO3-}$	Nitrification $^{18}\varepsilon_{NO3-}$
	~2.4	0.4 (0.2)	1.0. (0.7)	3.4 (0.9)	3.2 (0.3)
	~ 4.5	-5.3	0.5	-8.3	1.9
	~24	-6.7 (0.7)	3.2 (0.7)	-8.8 (0.2)	4.3 (0.4)
	~34.5	18.5	1.4	24.25	0.3

Figure 5.2: Table summarizing O_2 penetration depth and N and O isotope effects, with and without nitrification, in sediments of variable reactivity. Note that the apparent positive isotope effects observed in the less reactive sediments are due to the simultaneous depletion in ^{15}N- NO_3^- and production of NO_3^-. and must not considered net isotope effects of benthic nitrate elimination. Numbers in parentheses correspond to standard errors.

Similarly, NO_3^- reduction rates declined from ~ -0.2 µmol L^{-1} h^{-1} in sediments containing the highest C_{org} content (4.1% dry mass) to ~ -0.05 µmol L^{-1} h^{-1} in those hosting intermediate C_{org} levels (0.2 and 0.02% dry mass). In incubations with the lowest C_{org} levels (0.01% dry mass), we observed even net NO_3^- production (+0.02 µmol L^{-1} h^{-1}). In order to quantify the importance of nitrification, a separate set of sediments was supplemented with nitrapyrin (nitrification inhibitor). In these sediment cores, nitrate was completely consumed over the incubation time in all cores, and NO_3^-reduction rates were generally about 1.5-fold higher than in sediments hosting active nitrifying bacteria (Figure 5.3). Ammonium transformation rates were also determined in all treatments (with and without nitrification) but did not present any clear trends that would indicate us the differential occurrence of nitrification.

Nitrate N and O isotope effects in different treatments

The N and O isotopic composition and concentrations of the "initial" nitrate was slightly different among treatments, which may be due to the production of ^{15}N-depleted nitrate from ambient NH_4^+ (e.g. nitrification) during pre-incubation time. In the most reactive sediments, we observed an inverse correlation between [NO_3^-] and both $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$, whereas sediments of intermediate reactivity exhibited a continuous decrease in $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ isotope values concomitantly with the net nitrate drawdown over time, suggesting a production of ^{15}N-depleted NO_3^- (Figure 5.3). In experiments with the least reactive sediments, however, we observed continuous production of NO_3^- (i.e., increasing nitrate concentrations with time) concomitant with depletion of ^{15}N and ^{18}O isotopes in the accumulating nitrate pool. In contrast, when nitrification was inhibited, isotope values consistently increased over time, particularly in core-incubation experiments with sediments containing only 0.02% C_{org} (Figure 5.3).

The general trend suggests that sediment of lower reactivity displayed more reduced $^{15}\varepsilon_{NO3}$ and $^{18}\varepsilon_{NO3}$ isotope effects compared to more reactive sediments. The most reactive sediments displayed similar results with and without nitrification inhibition, showing an overall $^{15}\varepsilon_{NO3}$ and $^{18}\varepsilon_{NO3}$ isotope effect of about 0.4-1‰ and 3.3‰, respectively (Figure 5.2 and 5.3). In contrast, in less reactive sediments, different patterns were observed depending on the active suppression of nitrification. In particular, sediments of intermediate reactivity displayed an apparent inverse isotope effect (i.e. a decrease in δ values as nitrate concentration decreased) when nitrification was not shut off, while a normal isotope effect (i.e., enrichment of ^{15}N and ^{18}O with decreasing nitrate concentrations) was observed without nitrification ($^{15}\varepsilon \sim 3.2$ and $^{18}\varepsilon \sim 4.3$‰). Similarly, in the least reactive sediments, the production of ^{15}N-depleted NO_3^- generated a "positive anomaly" of the NO_3^- isotope effect in Rayleigh space (e.g. decrease in N and O delta values but positive $^{15}\varepsilon_{NO3}$, Figure 5.2 and 5.3).

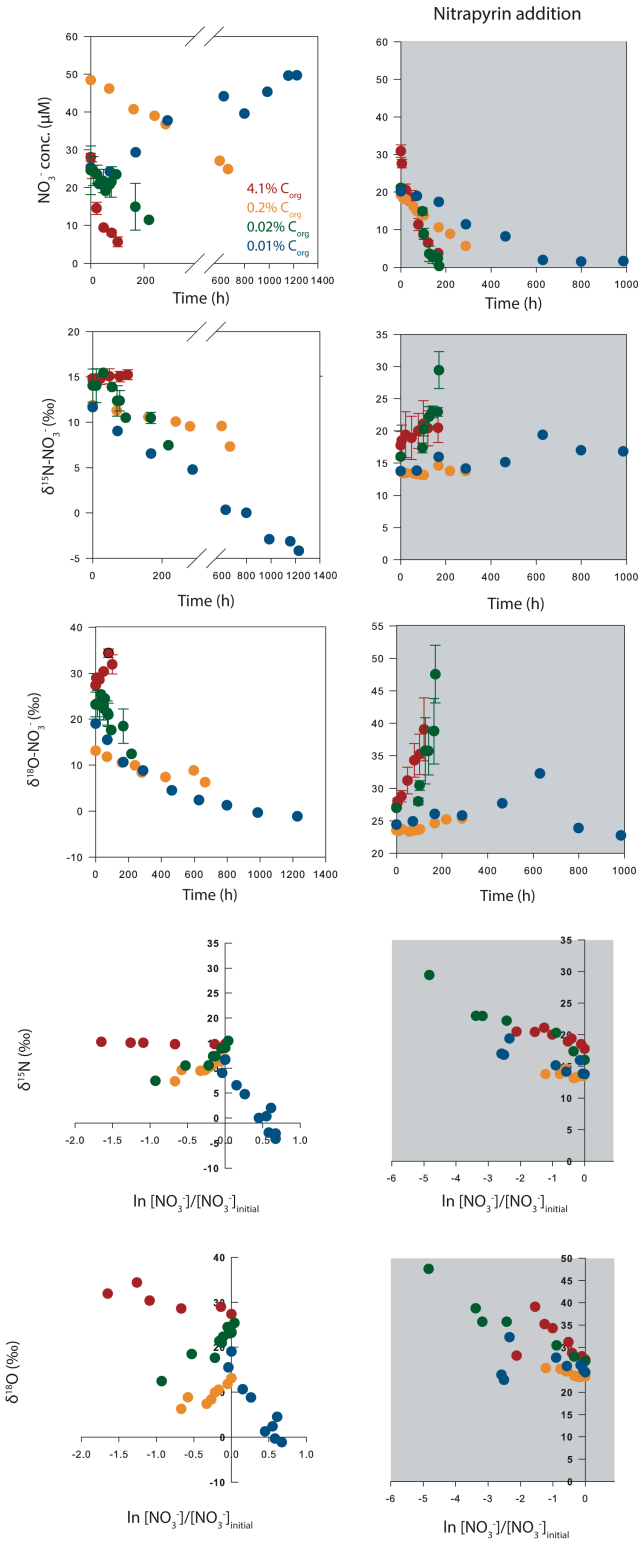


Figure 5.3: Nitrate concentration and isotopic composition changes over time, and $\delta^{15}N_{NO3}$ and $^{18}O_{NO3}$ versus $\ln[NO3\text{-}]_t/[NO3\text{-}]_i$ in sediments of variable reactivity (% C_{org} dry mass), with and without nitrification inhibition (nitrapyrin addition ; grey background). Error bars represent atandard error (n=3). Note different scales on y-axis.

Similarly, "regular" $^{15}\varepsilon_{NO3}$ and $^{18}\varepsilon_{NO3}$ enrichment factors (~1.4‰ and 0.3‰, respectively) were observed when nitrification was suppressed (Figure 5.2). Yet, a decoupling of nitrogen and oxygen isotope fractionation in nitrate was consistently observed in most incubations, which was mainly due to a stronger fractionation of $\delta^{18}O_{NO3}$ than $\delta^{15}N_{NO3}$ (^{18}O:^{15}N > 1; Figure 5.4).

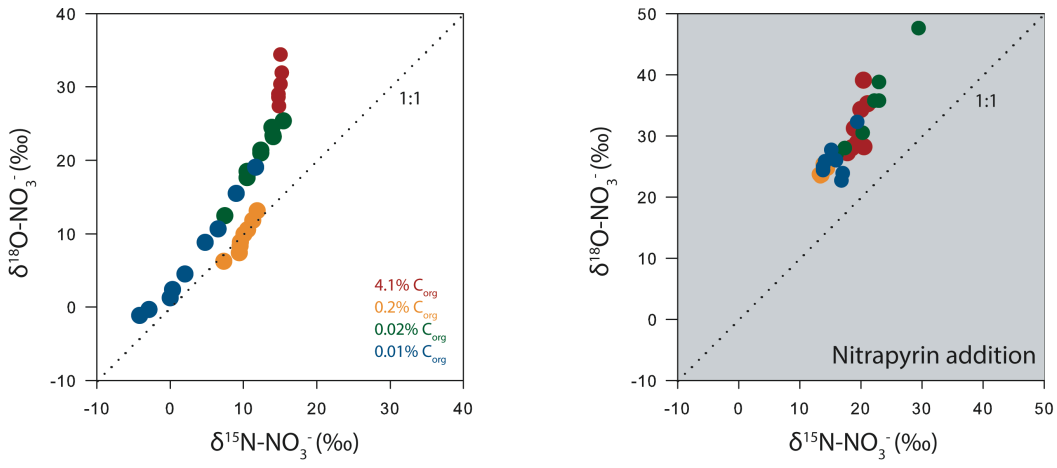


Figure 5.4: Relationship between the N and O isotopic composition of NO_3^- in overlying waters of whole-core incubations with artificial sediments of variable reactivity with and without nitrification (nitrapyrin addition ; grey background).

Influence of NO_x production

A general common feature of all incubations was that NO_3^- and NO_x (NO_3^- + NO_2^-) reduction trends were relatively similar to each other and, with one exception, no NO_2^- accumulation over time was observed. Only in the most reactive sediments a significant production of NO_2^- was observed (up to ~20 µM), but only when nitrification proceeded (Figure 5.5).

Oxygen isotope effects were not measured in NO_x samples because nitrite oxygen isotopes tend to equilibrate with water quite fast (Casciotti et al., 2002), and it is therefore difficult to separate the different processes. In the most reactive sediments, when nitrification was not suppressed, the depletion in ^{15}N isotopes was more pronounced in the NO_x pool than in the NO_3^- pool (Figure 5).

However, since the NO_x concentration fluctuated over time, it was impossible to calculate robust isotope effects $^{15}\varepsilon_{NOX}$ for these experiments (Figure 5.5). In sediments of intermediate reactivity, both $^{15}\varepsilon_{NO3}$ and $^{15}\varepsilon_{NOX}$ were relatively similar to each other, with and without nitrification (~ -5.3‰ and 1.1‰, respectively).

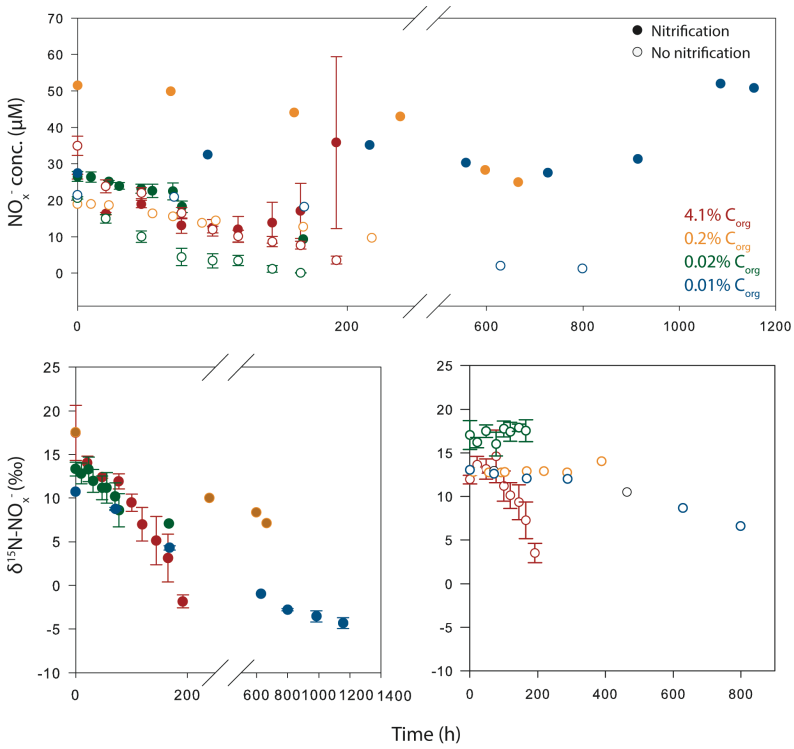


Figure 5.5: Concentration and N isotopic signature of NO_x in sediments of variable reactivity, with and without nitrification. Error bars represent standard error of triplicate incubations.

Discussion

The isotope effect associated with sedimentary nitrate reduction consistently remained low, and likely negligible compared to the organism-scale isotope effect, at the sediment-water interface in all environments investigated in this study. Nitrate isotope effects measured in porewaters (ε_{cell}) generally ranged between 11-35 ‰ (Lehmann et al., 2007; Alkhatib et al., 2012; Kessler et al., 2014), while no-marked isotope effect was perceived in overlying waters (ε_{app} < 3‰). In previous studies, it has been postulated that there are at least

three important controls on ε_{app}: 1) organism-scale isotope effect of denitrification (ε_{cell}), 2) substrate diffusion-limitation to the denitrification zone, and 3) occurrence of nitrification that can fuel ^{15}N-depleted nitrate to overlying waters and thus mask any porewater nitrate ^{15}N enrichment (Brandes and Devol 1997; Lehmann et al., 2004; Lehmann et al., 2007). However, experimental evidence to verify the relative importance of the respective mechanisms is still lacking. As ε_{app} is used to estimate model-derived N global budgets, it is crucial to better constrain the main regulatory factors of its under-expression in aquatic systems. To simplify the discussion, we attributed the term denitrification to the overall nitrate reduction, including potential DNRA, as we will discuss further below. Indeed, the N isotopic composition of the possible end-products N_2, and NH_4^+ was not quantified, and separating denitrification and DNRA was not possible. Both microbial processes use the same nitrate-reductase enzymes (Nar, Nap; Mohan and Cole. 2007), suggesting that their associated NO_3^- isotope fractionation is similar. Yet, in terms of the overall N isotope effect of nitrate elimination, it will be important to distinguish between the two nitrate-reducing processes, as only complete denitrification (or anammox) to N_2 will represent an external N-cycle process, and thus prescribe how much the $\delta^{15}N$ of a reactive N pool (e.g. NO_3^-, NO_2^-, NH_4^+) will be elevated (or not) above the average $\delta^{15}N$ of global fixed N inputs (~0‰).

Substrate diffusion-limitation

In a first step, any potential effects from concomitant nitrate regeneration were suppressed to look at the quasi-isolated effect of denitrification. We thus inhibited nitrification by addition of nitrapyrin directly to the sediment. The O_2 penetration depth in sediments is regulated by the supply of reactive organic matter (here expressed as the C_{org} content) serving as electron donor for aerobic heterotrophic respiration, and the O_2 concentration in bottom waters (Glud 2008). As oxygen is thermodynamically a better electron acceptor than NO_3^-, it is assumed that deeper O_2 penetration will force nitrate reduction deeper into the sediments, leading to less steep concentration gradients and in turn to

slower diffusive nitrate fluxes. The resulting substrate limitation leads to a quantitatively consumption of nitrate, and in turn to a barely expressed discrimination between the heavier and lighter nitrate isotopologues (i.e., under-expression of ε_{app}). Indeed, even in little reactive sediments with low denitrification rates, deep O_2 penetration into the sediments will likely limits the NO_3^- supply and cause complete consumption of NO_3^- within the denitrification zone. As a consequence, the fractional gross efflux of a ^{15}N-enriched porewater NO_3^- pool to overlying waters is negligible. Similarly, in more reactive sediments with low O_2 penetration, high denitrification rates result in a negligible nitrate efflux out of sediments. Hence, in both types of sediment (reactive versus less reactive), NO_3^- concentration at the denitrification zone act as a major control of the expression of NO_3^- isotope effect at the scale of sediment-water solute exchange. Although, the influence of substrate-limitation on NO_3^- isotope effect is theoretically well constrained, experimental evidence regarding the exact controls (e.g. exact O_2 penetration depth) was lacking.

In agreement with prior studies on marine and freshwater sediments (Brandes and Devol 1997; Sebilo et al. 2003; Lehmann et al. 2004, 2007; Alkhatib et al. 2012; Kessler et al. 2014), the measured ε_{app} displayed a large under-expression in both highly and less reactive sediments (0.5-4.3‰). As mentioned above, one would have expected that the most and least reactive sediments display no-marked isotope effect, whereas ε_{app} would have been higher in sediments of intermediate to high reactivity. However, our results did not show any clear trends that would suggest a clear systematic influence of sediment reactivity on the apparent isotope effect; ε_{app} was relatively similar for all types of sediment. In contrast, previous work showed that ε_{app} was generally more under-estimated in well-oxygenated sediments hosting less reactive organic matter, while it was greater in highly reactive sediments with low O_2 penetration depth (Alkhatib et al., 2012; Kessler et al., 2014). Nevertheless, the apparent isotope effect ε_{app} remained relatively small (<5‰) at both higher and lower O_2 penetration depths, suggesting additional biogeochemical controls. While variability in sediment reactivity did not exert a significant direct control on ε_{app}

in the present study, it is recognized as a main regulator of the ε_{app} expression in bottom waters, by controlling denitrification rates, O_2 penetration into sediments, and NO_3^- concentration at the denitrification site.

In particular, Lehmann et al. (2007) suggested that the nitrate concentration at the denitrification site is a major parameter regulating the magnitude of ε_{cell} and, in turn, the degree of ε_{app} expression in bottom waters. As a matter of fact, if ε_{cell} is low due to nitrate supply limitation at the denitrification site, its expression in bottom waters will also be reduced. Similarly, in pure cultures of denitrifying bacteria, reduced ε_{cell} of nitrate reduction was attributed to nitrate-uptake limitation when NO_3^- concentration was minimal, as NO_3^- supply becomes insufficient to maintain saturated enzymatic activity (Granger et al. 2008; Kritee et al. 2012). In other words, limitation in NO_3^- concentration induced slow reduction rates and, as a consequence, low nitrate uptake. In this case, all nitrate introduced into the cell was reduced and no efflux of ^{15}N-bearing nitrate out of the cell could occur. In contrast, NO_3^- uptake was enhanced if previously intracellular accumulated NO_3^- was reduced at high rates (when NO_3^- was not limiting) and thus led to an efflux of ^{15}N-bearing nitrate. Similarly, it seems that the redox state (i.e., reduced versus oxidized) of the carbon source is also an important regulator of the N isotope effect of denitrification. Depletion in readily consumable and thermodynamically favorable (i.e. glucose versus acetate versus toluene/benzoate) carbon sources caused a decrease in ε_{cell} (Kritee et al. 2012; Wunderlich et al. 2012). Although the carbon content of the sediments varied strongly among experiments (from 0.01 to 4.1 % dry mass C_{org}), ε_{app} remained very similar. These results suggest that the carbon source had a relatively small influence on the N isotope effect in the present study, or at least that nitrate diffusion-limitation at the zone of nitrate reduction was not primarily controlled by the reactivity of the sediments.

The decoupling between N and O isotope ratio changes observed in all types of sediments suggests the co-occurrence of other N-transformation processes. We noticed that the average of the apparent isotope effect across all

treatments was greater for O (~2.4‰) than for N (~1.5 ‰), which differs from the 1:1 relationship of N and O isotope fractionations that is typically observed during nitrate reduction for pure cultures of denitrifying bacteria (Granger et al., 2008). This "anomaly", or deviation, from the expected 1:1 ^{18}O to ^{15}N ratio has already been observed in various natural environments, and has been attributed to simultaneous NO_3^- reduction and NO_2^- oxidation (Sigman et al. 2005; Casciotti and McIlvin 2007; Dale et al. 2014). In this case, nitrate is consumed with the 1:1 $\delta^{18}O$ to $\delta^{15}N$ relationship expected from denitrification, while the reoxidation of transient NO_2^- to NO_3^- returns NO_3^- with a similar $\delta^{15}N$. In contrast, the $\delta^{18}O$ of NO_3^- produced is expected to be higher than that of the nitrate consumed, as ^{16}O is preferentially removed during reduction of NO_3^- to NO_2^- (Casciotti et al. 2002). This combination leads to a greater overall $\delta^{18}O$ than $\delta^{15}N$ nitrate when nitrite is re-oxidized. However, the nitrification as a potential control on the nitrate N:O isotope signature and explanation for the deviation from a 1:1 ratio can be excluded at least for those sediment incubations in which nitrification was inhibited (as for the other experiments, the overall influence of nitrification on N and O isotope effects will be discussed in the next section). Also, our results imply that other processes within the sediments must affect nitrate N and O isotope fractionation in differential ways. Recently, Grabb et al. (2017) showed that chemodenitrification, the chemical NO_2^- reduction with ferrous iron, displayed a greater fractionation of $\delta^{18}O$ relative to $\delta^{15}N$ ($^{18}\varepsilon:^{15}\varepsilon \sim 2$) in presence of aqueous Fe^{2+}. The natural sediments used to perform the experiments (Lake Lugano, Switzerland) host high levels of Fe^{2+} in the sediment porewaters (Lazzaretti et al. 1992; Cojean et al., in prep.), suggesting that chemodenitrification could be responsible for the decoupling between N and O isotopes. However, before evaluating the contribution of chemodenitrification and its role in decoupling N and O isotopic signatures in net denitrifying environments further, more work is needed to quantify the nitrate isotope effect associated with this process in environmental settings.

Nitrification

The potential influence of nitrification on ε_{app} was addressed by comparing N isotope effects from incubations both with and without nitrification. Furthermore, NO_2^- is also produced as intermediate during nitrification, and the measurement of N isotope ratios of NO_x (NO_3^- + NO_2^-) helped to better assess the overall influence of nitrification on $\varepsilon_{app.}$ Indeed, the nitrite produced through nitrification is important to consider because it could be at some point reoxidized to nitrate and, in turn, affect the net nitrate isotope fractionation (Sigman et al., 2005; Casciotti and McIlvin, 2007). It is thus important to consider the overall $^{15}\varepsilon_{NOx}$, which takes the overall nitrate transformation and not just $^{15}\varepsilon_{NO3}$ into account. Direct measurements of N and O nitrite isotopes were not performed, so we do not have precise data regarding NO_2^- isotope effects of nitrification. Yet, while we cannot precisely quantify $^{15}\varepsilon_{NO2}$ of nitrification, we can assume that the difference between NO_3^- and NO_x isotope effects stems from the presence of NO_2^-. Except in the most reactive sediments, nitrite did not accumulate and $^{15}\varepsilon_{NOx}$ was similar to $^{15}\varepsilon_{NO3}$ in all experiments. Hence it seems that NO_2^- production did not play a major role in regulating the expression of ε_{app}, particularly in less reactive sediments, and we therefore only consider $^{15}\varepsilon_{NO3}$ for the rest of the discussion.

The occurrence of nitrification seems to be the main cause for the underexpression of ε_{app} in experiments where no nitrapyrin was added. Suppression of the denitrification N isotope effect occurs in particular through ^{15}N-depleted nitrate efflux (Lehmann et al. 2004). The most reactive sediments exhibited the lowest nitrate production/regeneration rates and the weakest contribution to the measured "community N isotope effect", as ε_{app} was < 1‰ and thus very similar to what we observed in the experiments without nitrification. These results are in line with previous studies on marine sediments (Brandes and Devol, 1997; Lehmann et al., 2004; Lehmann et al., 2007), where nitrification proceeded, but the consumption of ambient and nitrification-derived nitrate was immediate, and complete at the sediment depth of

denitrification, and no NO_3^- efflux occurred. In contrast, in the less reactive sediments, coupling between nitrification-denitrification was likely more significant. Here, the deeper O_2 penetration in sediments enhanced nitrification, and the gross efflux of ^{15}N-depleted NO_3^- to overlying waters in the context of net nitrate consumption via denitrification led to an inverse isotope effect ($\varepsilon_{app} \sim$ -5.3 to -11.6‰). But when nitrification was inhibited, ^{15}N enrichment in the residual nitrate pool was clearly observed, with a $^{15}\varepsilon_{app}$ of 0.5-3.2‰. A similar apparent "inverse N isotope effect" due to nitrification in a net denitrifying environment, was observed in the estuarine water column of Elkhorn Slough, California (Wankel et al. 2009). This and our study demonstrate that sedimentary nitrogen cycling with fractional ammonium oxidation can, despite the net nitrate consumption and ^{15}N discrimination due to denitrification, result in a substantial ^{15}N-depleted nitrate efflux to overlying waters and continuous nitrate removal. Yet in the least reactive sediments, the O_2 penetration depth was at the bottom of the sediment core ($\sim$ 4 cm depth) resulting in almost full inhibition of denitrification. Here, nitrification was the main benthic N-process and the continuous production of ^{15}N-depleted nitrate led to an "anomalous" positive ε_{app}. Denitrification was inhibited in the whole sediment column and the results obtained from this experiment are thus likely not representative of natural conditions as in natural systems, NO_3^- would be, at some point, transported to the site where denitrification can proceed (e.g., deeper anoxic sediments, suboxic/anoxic bottom waters).

As observed in this study, the efflux of ^{15}N-depleted nitrate from partial nitrification will offset any ^{15}N enrichment in the reactive water-column N pool from denitrification, and thus generate a lower ε_{app}. In contrast, previous work also attributed higher ε_{app} to the occurrence of nitrification. As previously mentioned, partial nitrification will also lead to ^{15}N-enriched ammonium efflux out of the sediments that may be subsequently transformed to NO_3^-. In this case, high ammonium efflux would lead to higher $\delta^{15}N_{NH4}$, and subsequently $\delta^{15}N_{NO3}$, in overlying waters, which in turn will substantially increase the apparent N isotope effect (Granger et al., 2011). Hence, Lehmann et al. (2007) suggested that

ε_{sed} (akin to ε_{app} but also including ammonium fluxes) is more representative of the NO_3^- isotope effects associated with sedimentary denitrification. In the present study, however, we were not able to measure ammonium isotopes, and NH_4^+ concentration did not show any clear trend that would indicate us the differential occurrence of nitrification in the different treatments. Similar incubation work focusing on NH_4^+ isotope dynamics is therefore required to further our insight about the overall importance and effects of nitrification on ε_{app} in a wide range of sediment reactivity regimes.

Alternate N-transformation processes

The dissimilatory nitrate reduction to ammonium (DNRA) contributes significantly (34 to 50%, Cojean et al., submitted) to the total nitrate reduction in the sediments/sedimentary inoccula we used to prepare incubations. While its contribution is likely to decrease with lowered C_{org} content to nitrate ratios (Strohm et al. 2007; Nizzoli et al. 2010), it is plausible that in the most reactive sediments, DNRA will significantly contribute to the overall NO_3^- reduction N and O isotope effects. The first irreversible-step of both denitrification and DNRA involves the same enzymes, Nar and Nap (Mohan et al. 2006), and it is thus possible that they display similar NO_3^- isotope fractionation during nitrate reduction, but more work is required to assess the exact organism-scale NO_3^- isotope effect of DNRA. However, DNRA is a source of ^{15}N-depleted ammonium (McCready et al. 1983), which, at least in the natural water column at some point, can be re-oxidized to NO_3^- and, as a consequence, decrease $^{15}\varepsilon_{sed}$. Further investigations on N and O isotope fractionation during DNRA would help to better constrain the degree of its contribution on the apparent NO_3^- isotope effects.

Chemolithotrophic denitrification is an alternative process that reduces nitrate using inorganic electron donors such as Fe^{2+} or H_2S. In a different study focusing on potential alternative electron donors for NO_3^- reduction, we observed a substantial coupling between Fe^{2+} and H_2S oxidation with nitrate reduction occurring in the natural sediments used in the present work (Cojean

et al., submitted). However, the N and O isotope fractionation of sulfur- and iron-oxidizing nitrate reducers (Frey et al. 2014; Wenk et al. 2014; A.N Visser, in prep.) generally displayed a O:N ratio ≤ 1. Similarly, abiotic nitrite reduction with ferrous iron led to a smaller $^{18}\varepsilon$:$^{15}\varepsilon$ ratio except, as mentioned above, in presence of dissolved Fe^{2+} (Buchwald et al. 2016; Grabb et al. 2017). Hence, it seems that these reactions do not explain the "anomalous" $^{18}\varepsilon$:$^{15}\varepsilon$ (>1) observed in the present study.

Conclusions and implication for N-isotope budgets

We present one of the first experimental datasets on the biogeochemically-driven under-expression of NO_3^- isotope effect at the sediment-water interface. Our data demonstrate the importance of sediment reactivity, O_2 penetration depth, and nitrification in regulating benthic N and O isotope fractionation and the corresponding isotope effects at the scale of sediment-water nitrate exchange. In reactive sediments, the under-expression of ε_{app} was induced by high denitrification rates that led to the complete consumption of nitrate. In sediments of intermediate reactivity, deeper O_2 penetration enhanced the nitrification and coupled nitrification-denitrification, which resulted in an apparent inverse community N isotope effect (ε_{app}<0‰). Hence, all together these data confirm previous model-based conclusions by Lehmann et al. (2007), as well as they provide insight into the NO_3^- isotopic signature of coupled nitrification-denitrification.

Both in the ocean and in lakes, understanding the biogeochemical control on N isotope effects will allow us to better parameterize global isotope models, and thus better estimate N budgets, which remain, to date, inconsistent among studies (e.g., see Codispoti 1995, 2007; Brandes and Devol 2002). The accurate estimation of global N budgets is difficult as models extrapolate N fluxes measured at a limited number of sites in the ocean and often neglect the interplay between the different N-transformation processes that may alter N isotopic signatures. Our study broadens the range of N and O isotope effect

values at the sediment-water interface for various types of sediments (reactive versus less reactive, cohesive versus permeable) and shows the importance of coupled processes (e.g. nitrification-denitrification) in the expression of NO_3^- isotope effect at the sediment-water interface.

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Chapter 6

Conclusions and outlook

This dissertation contributes to a more comprehensive understanding of the N cycle in the sediments of the southern basin of Lake Lugano. More broadly, it brings new insights into the environmental controls that regulate NO_3^- isotopic signatures at the sediment water interface, sedimentary microbial communities, and the fate of nitrogen in aquatic ecosystems (Figure 6.1).

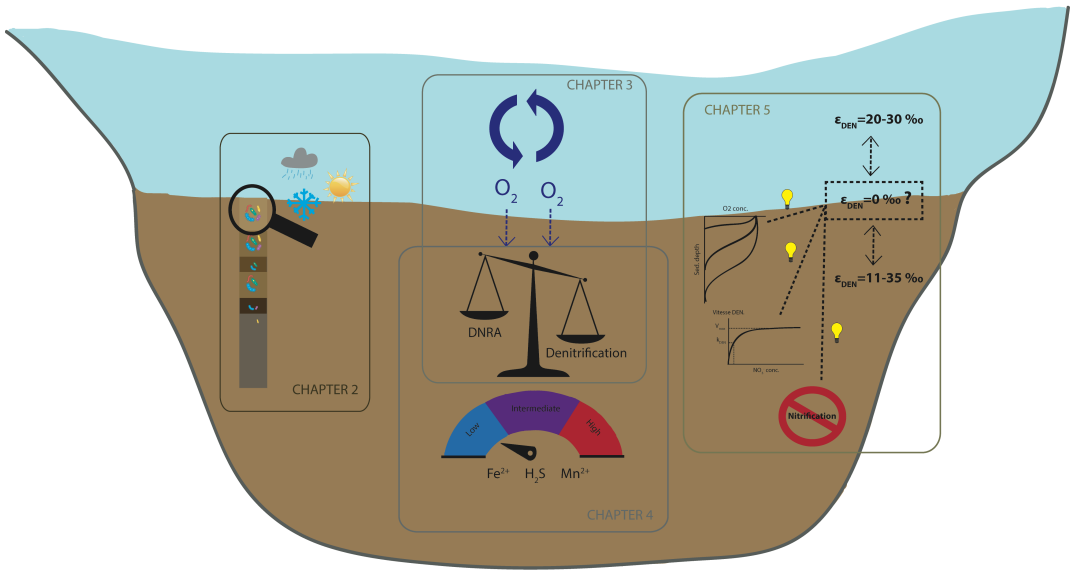

Figure 6.1: Schematic overview of the work performed in each chapter of this thesis.

By applying an interdisciplinary approach that includes both biogeochemical (e.g. isotope-based) and molecular (e.g. next generation sequencing) methods, we performed a systematic comparison of two different sites in the south basin of the lake, Figino and Melide. Both sites exhibited similar porewater chemistry, microbial community's structure and benthic N-process rates:

- Porewaters were characterized by high concentrations of Fe^{2+}, Mn^{2+}, and CH_4.
- The microbial diversity was greater in surface sediments, which are constantly supplied with electron acceptors than in deeper layers.

- At Figino, in particular, the seasonal water-column turnover significantly altered the community composition with regards to sulfate-reducers, sulfide-oxidizers and methanotrophs, while at both sites the N- and Fe-metabolizing microbial communities were noticeably more stable across seasons.

- Denitrification and DNRA were the main benthic N transformation processes, while anammox contribution to N-reduction was negligible compared to other processes (less than 1%).

- No clear seasonal trends were observed regarding the partitioning between denitrification and DNRA in 2016, when the water column was exceptionally stable (e.g. no mixing). In 2017, however, the annual water column mixing event occurred and after mixing, in October, the DNRA contribution to N-reduction relative to denitrification was greater than in the previous year

Molecular analysis of the sediments revealed the presence of genera that may be involved in the N cycle. While some microbial metabolisms (e.g. sulfate-reducers; nitrifiers) are well constrained, it is more difficult to draw solid conclusion about nitrate reducers, as this trait is extremely widespread phylogenetically in environmental microbes. Nevertheless, we were able to assign some putative taxa to NO_3^- reduction in Lake Lugano (south basin) sediments. These results, in combination with porewater concentration profiles and N-process rate measurements, suggested a potential coupling between the N cycle and other biogeochemical cycles (discussed below and in Chapter 4).

Water column turnover induced O_2 to the surface sediments that had remained suboxic/anoxic since the previous mixing. According to seasonal measurements of benthic N-process rates, it is likely that episodic O_2 supply may affect NO_3^- reducers at the surface sediments, however, our phylogenetic data showed the stability of the community over seasonal cycles. Limitations in the

molecular approach as well as the high depth resolution of sampling (i.e. 2 cm depth) may have resulted in overlooking seasonal variations in nitrate reducers. Therefore, we also performed additional incubation experiments with continuous O_2 monitoring in order to better constrain the influence of O_2 in regulating the partitioning between denitrification and DNRA (discussed below and in Chapter 3).

Environmental controls of the fate of N – summary and implications

Our results highlight the importance of O_2, Fe^{2+}, and H_2S in regulating the partitioning between denitrification and DNRA in sediments. In contrast, Mn^{2+} played a minor role in partitioning processes, however, it exerts a similar inhibitory effect on processes rates (Figure 6.2). In addition to organotrophic nitrate reduction, chemolithotrophic processes involving Fe^{2+} and H_2S may play a significant role in Lake Lugano (south basin) sediments.

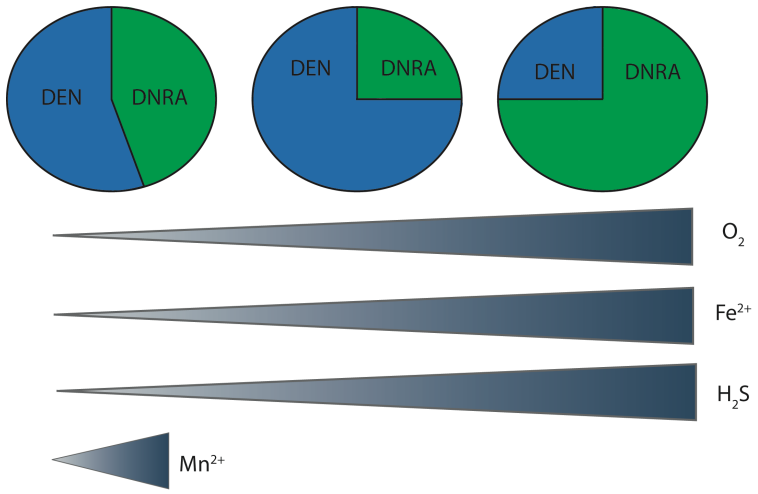

Figure 6.2: Scheme summarizing the results obtained from incubation work investigating the environmental controls on the partitioning between benthic denitrifcation (DEN) and DNRA in the southern basin of Lake Lugano.

Oxygen

We demonstrated the important regulatory control of O_2 on the rates and relative partitioning of benthic denitrification and DNRA (Chapter 3; Figure 6.2). At lower O_2 concentration (≤ 1 µM), denitrification was favored over DNRA, whereas denitrification was more O_2 sensitive than DNRA at higher O_2 levels (≥ 2 µM). Before our study, investigations on the systematic O_2 control on DNRA in the micromolar range did not exist, and our results are thus of particular importance as they provide a better understanding of the N cycle in environments subject to O_2-fluctuating conditions where denitrifiers and nitrate ammonifiers co-exist.

In lake sediments, intensive organic matter remineralization occurs and O_2 penetration is generally restricted to the very first few millimeters. Therefore, the fate of N is barely, if at all, controlled by oxygen in such sediments. But in less reactive sediments or water columns, the oxic/hypoxic layer is more extended, and higher O_2 levels may play a role in favoring internal N recycling over N-removal. The contribution of DNRA in these environments hosting low organic carbon levels is generally assumed to be minor compared to denitrification or anammox, but, until recently, DNRA had received little attention compared to the other processes and a re-assessment of the occurrence of the process may help to improve global N-budgets. While this thesis brings new insights, the exact O_2 control in environmental settings is far from fully constrained and more work is required to better estimate the partitioning between denitrification and DNRA in ecosystems subject to O_2 fluctuations (see outlook).

Artificial hypolimnetic oxygenation has been suggested as an efficient way to detoxify eutrophic lakes (Beutel and Horne, 1999; De Brabandere et al., 2015). Aeration of bottom waters may stimulate phosphorous and ammonia removal through binding to iron to iron oxyhydroxides and the coupled nitrification-denitrification process, respectively (Beutel and Horne, 1999). The

application of the process in lakes seemed beneficial as it significantly reduced ammonium levels (reviewed in Beutel, 2006). In contrast, our study showed that N-regeneration was favored over N-removal at higher O_2 levels. One important factor explaining the difference between our study and prior work may be the oxygenation time. It seems that long oxygenation time (> 12 months against only 10 h in our study) decreased the contribution of DNRA to total N-reduction compared to denitrification, which was in part due to less reducing conditions in sediments that favored denitrification (de Brabandere et al., 2015). Although artificial oxygenation seems beneficial for the restoration of lakes (Beutel, 2006), the exact influence of O_2 on the partitioning between denitrification and DNRA is still not well constrained, and it would be important to better understand the mechanism before to use the method intensively for lake restoration.

Ferrous iron

Studies investigating Fe^{2+}-dependent NO_3^--reduction in natural conditions are scarce. This thesis provides new insight into the role of Fe^{2+} in regulating the partitioning between NO_3^--reducing pathways under environmentally relevant conditions (Chapter 4). Denitrification was significantly stimulated at low Fe^{2+} levels (≤ 280 µM) whereas it was strongly inhibited at higher Fe^{2+} concentrations (> 400 µM). DNRA was less sensitive to Fe^{2+} than denitrification, and its contribution to N-reduction increased with increasing Fe^{2+} levels. In this study, we were, for the first time, able to show a Fe^{2+} concentration-dependence for the fate of N under natural conditions (Figure 6.2). However, more work is required to 1) assess if Fe^{2+} exerts a direct effect on DNRA (see outlook), and 2) better constrain the exact stimulating or inhibiting mechanism of Fe^{2+} on both denitrification and DNRA.

Sulfide

Sulfide played a major role in regulating the fate of N in incubation experiments conducted in this thesis. Denitrification was strongly enhanced at low sulfide levels (<80 µM). Most free sulfide added was not recovered, likely due to binding with Fe(II) or Fe(III). We suggested thus that FeS, not H_2S, served as electron donor for denitrification. Surprisingly, H_2S did not stimulate DNRA, which contradicts most prior studies (Chapter 4 and references therein). The contribution of DNRA to N-reduction increased with higher H_2S levels (Figure 6.2), though this was mainly due to less competition with denitrifiers, as denitrification was strongly inhibited at the highest sulfide levels (≥ 125 µM). This lack of stimulation may be explained by the fact that nitrate ammonifiers in Lake Lugano (south basin) sediments are only adapted to low H_2S (or high FeS), since most of the sulfide binds to Fe, and so were not familiar with free H_2S. Therefore excessive H_2S concentration (≥ 125 µM) may have stressed nitrate ammonifiers and at lower H_2S levels they were likely outcompeted by denitrifiers. While it is commonly assumed that reducing conditions favor DNRA over denitrification, our results showed the opposite and underscore the importance of this chemolithotrophic fixed N elimination pathway in aquatic ecosystems.

The water-column turnover event increased the relative abundance of many putative sulfide-oxidizers such as *Beggiatoa*, *Sulfurimonas*, *Sulfuritalea*, and *Sulfuricella*. This was particularly notable at Melide, where in contrast to Figino, NO_3^- is limited during water-column stratification. Some of these genera were also identified as potential denitrifiers (e.g. *Sulfurimonas*, *Sulfuricella*; Sievert et al., 2008; Watanabe et al., 2014). Hence, in holomictic lakes or other reduced environments subject to seasonal bottom water mixing (i.e.; rice paddy soils), the supply of electron acceptors (e.g., O_2, NO_3^-) to surface sediments may enhance the detoxification of the system by removing the toxic gas H_2S and the contaminant NO_3^-.

Reduced manganese

Among all substrates tested (e.g. O_2, Fe^{2+}, H_2S), Mn^{2+} displayed the lowest impact on the relative partitioning between denitrification and DNRA. Based on thermodynamic considerations and porewater concentration profiles, coupling between nitrate reduction and manganese oxidation has been suggested to occur in sediments (Aller, 1990; Schulz et al., 1994; Luther et al., 1997). However, we observed no overall stimulation of either denitrification or DNRA. On the contrary, both processes seemed inhibited by increasing Mn^{2+} concentration, suggesting a possible toxicity effect on the activity of the microbes involved. To our knowledge, we provide the first environmental investigation into Mn^{2+}-dependent nitrate reduction, and the exact mechanism of Mn^{2+} toxicity on nitrate reducers is thus still unknown.

Environmental controls of NO_3^- isotope effect at the sediment water interface

The under-estimation of NO_3^- isotope effect of nitrate reduction at the sediment-water interface (ε_{app}) has been reported in many studies (reviewed in Chapter 5). Relying on modeled-based results, multiple environmental drivers (e.g. sediment reactivity, nitrate-diffusion limitation, nitrification) of NO_3^- isotope effect have been suggested, however, experimental evidence for or against these suggestions did not so far not exist. With this thesis, we provide the first experimental investigation of this question and demonstrate the significance of sediment reactivity and nitrification in regulating ε_{app}. In reactive sediments, the under-expression of ε_{app} was mainly driven by high denitrification rates, whereas in less reactive sediments hosting greater O_2 penetration, nitrification was the main factor. Isotope-based models are a very powerful tool to quantify overall N budgets in aquatic ecosystems. Imbalance between global N budget estimations still persists (e.g. Codispoti, 2007), which may be due to the use of inaccurate NO_3^- isotope effect values. Our pilot study helps to better understand what are the main environmental factors driving N and O isotopic

signatures at the sediment water-interface, however further work is required such as e.g. investigation of additional potential biogeochemical controls, estimation of N and O isotopic signatures of other processes (e.g. DNRA, abiotic NO_3^- reduction with Fe^{2+}).

Outlook

This study investigated the different anaerobic N-transformation processes in Lake Lugano (south basin) sediments and elucidated the environmental controls on their relative partitioning. However, this thesis also opens a multitude of new questions:

***Oxygen control on denitrification and DNRA at the enzyme-level in the environment*:** We have shown the importance of O_2 in regulating benthic denitrification and DNRA partitioning. However, additional experiments are required to constrain the exact control of O_2 on N turnover processes at *in situ* conditions. One possibility would be to extract DNA from the very first few millimeters of surface sediments and investigate the expression of denitrifying (*nap*, *nar*, *nir*) and nitrate-ammonifying (*nap*, *nrfA*) genes during a complete seasonal cycle in Lake Lugano (south basin). Additionally, a comparison with other environments would be interesting to assess whether the O_2 mechanism on benthic N reduction is typical of the lake or if this is a general feature.

***DNRA stimulation at high Fe^{2+}* levels:** In this thesis, we suggested that Fe^{2+} did not directly stimulate DNRA, but rather that its contribution to N-reduction increased due to less competition with denitrifiers for NO_3^- uptake. To investigate this hypothesis, I suggest monitoring the *nrfA* gene expression over time during incubation experiments with variable Fe^{2+} concentration similar to those conducted in this thesis. If *nrfA* expression is similar in incubations with higher Fe^{2+} levels than in a control incubation without Fe^{2+} addition, it is likely that Fe^{2+} does not directly stimulate DNRA. Abiotic controls would also be necessary in order to check whether NH_4^+ is chemically or biologically produced.

N and O isotopic signature of nitrate-ammonifiers*:* Nitrate-ammonifiers are very difficult to cultivate, and investigations on NO_3^- isotope effect of DNRA do not exist. It has been shown that each denitrifying enzymes (Nar and Nap) have a specific NO_3^- isotopic signature (Granger et al., 2008). Both denitrification and DNRA involve the same nitrate-reducing enzymes, however most nitrate-ammonifiers identified so far possessed Nap, and it is thus possible that the nitrate isotope effect of DNRA is different from that of denitrification. As shown in this study, DNRA can be an important process in some environments, and investigating its isotopic signature in pure culture experiments would help to better assess NO_3^- isotope effect of overall nitrate reduction in ecosystems where denitrification and DNRA co-exist.

N and O isotopic signature of nitrate reduction in DNRA- versus denitrification-dominated systems*:* As mentioned above, nitrate-ammonifiers are difficult to cultivate and it may take some time until the investigation of N and O isotopic signatures in pure culture will be possible. It has been shown that the relative contribution between fermentative DNRA, respiratory DNRA and denitrification strongly depends on the carbon source and the ratio carbon to nitrate (van den Berg et al., 2017). Hence, another possibility to investigate NO_3^- isotope effect of DNRA versus denitrification would be to conduct chemostat experiments enriched with nitrate-ammonifiers and denitrifiers and amended with different carbon sources (e.g. fermentable versus non-fermentable) and various concentrations of C and NO_3^-.

Bentho-pelagic transport of microbial communities via bubble shuttle: It has been suggested that methanotrophs may be transported to overlying water through bubble-mediated transport in a natural marine seep vent (Schmale et al., 2015). The south basin of Lake Lugano is a hot spot for CH_4 production, and intensive bubbling may lead to important microbial transport to bottom water through bubble shuttle, which may have important implications for the lake. For instance, the transport of nitrate-reducers to bottom waters that are not NO_3^--limiting may have significantly influence N budgets. Therefore, I suggest to

investigate bubble-mediated transport of the overall microbial community using an *in situ* bubble catcher (Schmale et al., 2015). The microbes transported into the bubble catcher would be analyzed through 16S rRNA sequencing.

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Cojean A.N.Y., Lehmann M.F., Robertson E.K., Thamdrup B., Zopfi J. (2020) Fe^{2+}, H_2S, and Mn^{2+} controls on benthic NO_3^--reducing processes in surface sediments of an eutrophic lake. *Frontiers in Microbiology*, **11**(1158), 1-17

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Cojean A.N.Y., Lehmann M.F., Su G., Zopfi J. (in prep.) Seasonal dynamics of microbial communities in ferruginous lacustrine sediments.

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Adeline N.Y. Cojean, Maciej Bartosiewicz, Moritz F. Lehmann, Katrina Kremer and Stefanie B. Wirth. *Different lacustrine pockmark systems in Lake Thun, Switzerland, and their potential influence on the hydrological and biogeochemical budget of the lake.* Swiss Geoscience Meeting (SGM) 2020. [poster]

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Cojean A., Zopfi J., Gerster A., Lepori F., Lehmann M.F. *Oxygen control on nitrate reduction processes in lacustrine sediment: a reassessment of O_2 inhibition thresholds*. European Geosciences Union Meeting (EGU). EGU2018-16476 [oral]

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Cojean A., Zopfi J., Robertson E., Thamdrup B., Lehmann M.F. *Fe^{2+} and H_2S as electron donors during benthic N transformations in anoxic lake sediments*. Goldschmidt 2017. gold2017:abs:2017002217 [oral]

Cojean A., Zopfi J., Robertson E., Thamdrup B., Lehmann M.F. *Fe^{2+}, Mn^{2+} and H_2S as electron donors for benthic N-processes in lacustrine sediments.* European Geosciences Union Meeting (EGU). EGU2017-8777 [poster]

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