

## RESEARCH ARTICLE

# Nitrate-dependent anaerobic methane oxidation and chemolithotrophic denitrification in a temperate eutrophic lake

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**One sentence summary:** Biogeochemical processes measurements highlight the occurrence of complex interactions between carbon, nitrogen and sulfur cycles in the water column of a permanently stratified European lake.

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## ABSTRACT

While the emissions of methane (CH<sub>4</sub>) by natural systems have been widely investigated, CH<sub>4</sub> aquatic sinks are still poorly constrained. Here, we investigated the CH<sub>4</sub> cycle and its interactions with nitrogen (N), iron (Fe) and manganese (Mn) cycles in the oxic-anoxic interface and deep anoxic waters of a small, meromictic and eutrophic lake, during two summertime sampling campaigns. Anaerobic CH<sub>4</sub> oxidation (AOM) was measured from the temporal decrease of CH<sub>4</sub> concentrations, with the addition of three potential electron acceptors (NO<sub>3</sub><sup>-</sup>, iron oxides (Fe(OH)<sub>3</sub>) and manganese oxides (MnO<sub>2</sub>)). Experiments with the addition of either <sup>15</sup>N-labeled nitrate (<sup>15</sup>N-NO<sub>3</sub><sup>-</sup>) or <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> combined with sulfide (H<sub>2</sub>S), to measure denitrification, chemolithotrophic denitrification and anaerobic ammonium oxidation (anammox) rates, were also performed. Measurements showed AOM rates up to 3.8 μmol CH<sub>4</sub> L<sup>-1</sup> d<sup>-1</sup> that strongly increased with the addition of NO<sub>3</sub><sup>-</sup> and moderately increased with the addition of Fe(OH)<sub>3</sub>. No stimulation was observed with MnO<sub>2</sub> added. Potential denitrification and anammox rates up to 63 and 0.27 μmol N<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>, respectively, were measured when only <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> was added. When H<sub>2</sub>S was added, both denitrification and anammox rates increased. Altogether, these results suggest that prokaryote communities in the redoxcline are able to efficiently use the most available substrates.

**Keywords:** anaerobic methane oxidation; nitrate; iron; manganese; denitrification; anammox

## INTRODUCTION

The Paris Agreement ratified in 2016 states that the global average temperature must be kept below 1.5°C compared with the pre-industrial period to limit the consequences of global warming. With this perspective, methane (CH<sub>4</sub>) plays a key role, since its global warming potential is 28 times higher on a 100-year time frame than carbon dioxide (CO<sub>2</sub>) but has a much shorter residence time in the atmosphere than CO<sub>2</sub>

(IPCC 2014). The atmospheric CH<sub>4</sub> concentrations have dramatically increased compared with the pre-industrial era because huge amounts of CH<sub>4</sub> are annually released into the atmosphere (~8 GtCO<sub>2eq</sub> yr<sup>-1</sup>), 50–65% being from anthropogenic sources (mainly agriculture, waste, fossil fuel production and use) (IPCC 2014), and the remaining 35–50% being produced by natural systems, among which lakes contribute ~10% (Saunio *et al.* 2016, 2020).

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While a notable effort has been made to quantify global sources of CH<sub>4</sub> from natural environments, aquatic CH<sub>4</sub> sinks have been less investigated. In aquatic systems, CH<sub>4</sub> is mainly produced in anoxic sediments by methanogenic archaea, and is then emitted via diffusion or ebullition in the water column. A large part of the CH<sub>4</sub> produced is biologically oxidized by aerobic or anaerobic methanotrophs before reaching the atmosphere (Borrel et al. 2011). Anaerobic methane oxidation (AOM) can be coupled to different electron acceptors: sulfate (SO<sub>4</sub><sup>2-</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), nitrite (NO<sub>2</sub><sup>-</sup>), iron (Fe) oxides or manganese (Mn) oxides (Borrel et al. 2011). More recently, it has also been suggested that AOM could occur with organic compounds (Valenzuela et al. 2017; Bai et al. 2019). It was initially thought that AOM was exclusively performed by a consortium of archaea (ANME-type) and SO<sub>4</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, Fe or Mn reducers (Knittel and Boetius 2009; Cabrol et al. 2020), but it was recently demonstrated that bacteria from the phylum NC10 were also capable to perform AOM coupled to NO<sub>2</sub><sup>-</sup> reduction without any partner (Ettwig et al. 2010; Oswald et al. 2017; Graf et al. 2018). Besides, some studies also suggested that AOM could be performed by aerobic methanotrophs, in micro-oxic conditions (Blees et al. 2014; Oswald et al. 2016b), or also that some Archaea were capable of decoupling AOM and SO<sub>4</sub><sup>2-</sup> reduction (Scheller et al. 2016). Sulfate-dependent AOM is mainly encountered in marine environments (Reeburgh 2007) due to higher SO<sub>4</sub><sup>2-</sup> concentrations in seawater, but has also been shown in freshwaters (Eller, Känel and Krüger 2005; Schubert et al. 2011; Roland et al. 2017, 2018b). In freshwaters, the other potential electron acceptors can be found in higher concentrations, and are thus assumed to contribute more significantly to AOM (e.g. Borrel et al. 2011; Crowe et al. 2011; Sturm et al. 2016). Humic substances have also been found to fuel anaerobic methane oxidation in humic-rich freshwater ecosystems, such as wetlands (Valenzuela et al. 2020).

The occurrence of AOM coupled to the reduction of Fe or Mn oxides is thermodynamically favorable, and has been envisaged in lakes (Crowe et al. 2011; Sivan et al. 2011; á Norði et al. 2013; Sturm et al. 2019) and in marine sediments (Beal, House and Orphan 2009; Cui et al. 2015; Egger et al. 2015), but its natural significance and its mode of action remain poorly understood due to the complexity of natural environments in which multiple electron acceptors are present. A certain discrepancy appears in the literature, AOM sometimes being stimulated by the addition of minerals, and sometimes not (Oswald et al. 2016b; Bar-Or et al. 2017; Rissanen et al. 2017). NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup>-dependent AOM (DAMO) is more documented (e.g. Raghoebarsing et al. 2006; Ettwig et al. 2009, 2010; Deutzmann and Schink 2011; Kits, Klotz and Stein 2015; Oswald et al. 2017), but direct in-situ measurements of the process have seldom been reported in the literature. DAMO is thermodynamically more favorable than SO<sub>4</sub><sup>2-</sup>-dependent AOM, and can thus potentially play a key role in the reduction of CH<sub>4</sub> emissions from freshwaters (Raghoebarsing et al. 2006). However, it may also compete with heterotrophic denitrification (NO<sub>3</sub><sup>-</sup> reduction with organic matter as electron donor, performed by a variety of prokaryotes, including *Bacillus*, *Paracoccus* and *Pseudomonas*; Bernhard 2010) and anaerobic ammonium oxidation (anammox; NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> reduction coupled to ammonium oxidation, performed by bacteria belonging to the phylum Planctomycetes; Bernhard 2010) for substrates, two thermodynamically more favorable processes, commonly encountered in anoxic environments. The balance between the different biotic processes for access to the substrates plays a key role in the occurrence of AOM.

We previously showed that AOM occurred in a temperate, eutrophic and meromictic stone pit lake (Lake Dendre, Belgium;

Roland et al. 2017). Lake Dendre is considered as meromictic, the water column remaining anoxic below 20-m depth throughout the year. The epilimnion shows strong seasonal variability, with waters anoxic from 10-m depth in summer, while it is entirely mixed (up to 20-m depth) in winter. The lake is characterized by high SO<sub>4</sub><sup>2-</sup> concentrations all along the vertical profile (up to ~1500 µmol L<sup>-1</sup>), high CH<sub>4</sub> and sulfide (H<sub>2</sub>S) concentrations in anoxic waters (up to ~1000 and ~100 µmol L<sup>-1</sup>, respectively), and by an underwater spring located at 17-m depth (in the anoxic compartment), delivering substantial quantities of NO<sub>3</sub><sup>-</sup> (up to ~80 µmol L<sup>-1</sup>) due to generalized fertilizer contamination of groundwater in Belgium (SPW-DGO3 2021; Roland et al. 2017). In our previous study (Roland et al. 2017), we suggested that AOM was mainly coupled to SO<sub>4</sub><sup>2-</sup> reduction, given the overall high SO<sub>4</sub><sup>2-</sup> concentration throughout the entire water column. However, we also showed that NO<sub>3</sub><sup>-</sup>, Fe and Mn concentrations in the water column of Lake Dendre were not negligible, and a concomitance between maximum AOM peaks and maximum NO<sub>3</sub><sup>-</sup> concentration peaks was observed (Roland et al. 2017). We hypothesize that the underwater spring could provide the N substrate to sustain elevated denitrification and NO<sub>3</sub><sup>-</sup>-dependent AOM rates in anoxic waters. During this study, we thus investigated alternative pathways of AOM, by conducting incubations with addition of the different potential electron acceptors NO<sub>3</sub><sup>-</sup>, Fe oxides and Mn oxides, as well as measurements of denitrification, to check for the occurrence of DAMO and potentially competitive relationships.

## MATERIALS AND METHODS

### Physico-chemical parameters and sampling

Sampling in the Dendre stone pit lake (50.6157°N, 3.7949°E; Wallonia, Belgium) was carried out during the summers of 2017 and 2018. Depth profiles of dissolved oxygen (O<sub>2</sub>) concentrations, temperature, pH and specific conductivity were obtained with a YSI Exo multiparameter probe (YSI, Yellow Spring, Ohio, USA). The conductivity, pH and oxygen sensors were calibrated the day before each sampling using the protocols and standards recommended by the manufacturer (YSI). Sampling of water for the different measurements was performed with a Niskin bottle (General Oceanics, Miami, Florida, USA) through a silicon tube connected to the outlet. All the samples were left to overflow the vial volume three times before sealing.

### CH<sub>4</sub>, CO<sub>2</sub> and N<sub>2</sub>O concentration profiles

Duplicate samples for N<sub>2</sub>O and CH<sub>4</sub> concentration analyses were collected in 60-mL glass serum bottles (Derco, Ittre, Belgium), which were immediately sealed with butyl stoppers (Wheaton, Milville, New Jersey, USA) and aluminium caps (Wheaton, Milville, New Jersey, USA). In 2017, water was sampled in oxic waters at 1-, 4- and 7-m depths, at the oxic-anoxic interface at 9-, 10- and 11-m depths and in the anoxic waters at 13-, 15-, 17- and 20-m depths. In 2018, sampling for determination of the vertical profiles of dissolved gases was carried out at 1-, 7-, 10-, 13-, 15- and 17-m depths.

CH<sub>4</sub> and N<sub>2</sub>O concentrations were determined via the headspace equilibration technique (20-mL N<sub>2</sub> headspace in 60-mL serum bottles) and measured by gas chromatography (GC) with electron capture detection (ECD) for N<sub>2</sub>O and with flame ionization detection (FID) for CH<sub>4</sub> (Weiss 1981). The SRI 8610C GC-ECD-FID (SRI, Torrance, California, USA) was calibrated with certified CH<sub>4</sub>: CO<sub>2</sub>: N<sub>2</sub>O: N<sub>2</sub> mixtures (Air Liquide, Liège, Belgium)

of 1, 10, 30 and 509 ppm CH<sub>4</sub> and of 0.2, 2.0 and 6.0 ppm N<sub>2</sub>O. Concentrations were computed using the solubility coefficients of Yamamoto, Alcauskas and Crozier (1976) and Weiss and Price (1980), for CH<sub>4</sub> and N<sub>2</sub>O, respectively. The precision of measurements was ±3.9% and ±3.2% for CH<sub>4</sub> and N<sub>2</sub>O, respectively.

Triplicate samples for determination of the partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) were collected in 60-mL plastic syringes directly from the Niskin, at the same depths as the CH<sub>4</sub> and N<sub>2</sub>O vertical profiles in 2017, and at 1-, 5-, 7-, 10-, 13-, 15-, 17- and 19-m depths in 2018. The pCO<sub>2</sub> was measured with an infra-red gas analyzer (Licor Li-840, Lincoln, Nebraska, USA) after headspace equilibration in the syringe (Abril et al. 2015; Borges et al. 2015). The Li-840 was calibrated with N<sub>2</sub> and certified CO<sub>2</sub>: N<sub>2</sub> mixtures (Air Liquide, Liège, Belgium) of 388, 813, 3788 and 8300 ppm CO<sub>2</sub>. The precision of measurements was ±4.1%.

### Dissolved inorganic nitrogen species and major element concentrations

Water from the water column was collected for the determination of nitrogen nutrients (NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) and major element concentrations along the vertical profiles (the same depths as the dissolved gases' vertical profiles). NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> concentrations were determined using the sulfanilamide colorimetric method, and NH<sub>4</sub><sup>+</sup> with the dichloroisocyanurate-salicylate-nitroprussiate colorimetric method (Westwood 1981; APHA 1998). They were determined colorimetrically using a 5-cm optical path, with a Genesys 10 vis spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The detection limits of the methods were 0.15, 0.03 and 0.3 μmol L<sup>-1</sup> for NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, respectively.

Samples for determination of major elements (Fe and Mn) were taken at the same depths as the nutrients and dissolved gases, were stored in 20-mL scintillation vials and preserved with 50 μL of HNO<sub>3</sub> (65%, Suprapur grade, Sigma-Aldrich, Saint-Louis, Missouri, USA). Dissolved Mn, Fe and S concentrations were measured with inductively coupled plasma MS (ICP-MS; Agilent 7700x, Santa Clara, California, USA) calibrated with the following standards: SRM1640a from National Institute of Standards and Technology (Gaithersburg, Maryland, USA), TM27.3 (lot 0412) and TMRain-04 (lot 0913) from Environment Canada (Québec, Canada) and SPS-SW2 Batch 130 from Spectrapure Standard (Spectrapure, Tempe, Arizona, USA). The limits of quantification were 46.97, 0.002 and 0.01 μmol L<sup>-1</sup> for S, Mn and Fe, respectively. Due to a problem with sample preservation in 2018, major elements are only available for the field campaign of 2017.

### CH<sub>4</sub> oxidation measurements (sampling of 2018)

Depths for CH<sub>4</sub> oxidation measurement were chosen based upon previous field campaigns (Roland et al. 2017), at the oxic-anoxic interface (10-m depth) and below (13- and 17-m depths) in the water layer under the influence of the external source bringing NO<sub>3</sub><sup>-</sup> into the anoxic waters and penetrating the lake at a depth of 17 m.

Samples for determination of the CH<sub>4</sub> oxidation rates were collected in 60-mL glass serum bottles and immediately sealed with butyl stoppers and aluminum caps. Two samples per depth were immediately poisoned with 200 μL of a saturated HgCl<sub>2</sub> solution (VWR, Leuven, Belgium) (T0) to subsequently determine the initial CH<sub>4</sub> concentrations at the beginning of the incubation, and four unmanipulated bottles (control treatment) were

incubated in the dark and at constant temperature (close to in-situ temperature, so 20°C on the surface, 10°C in the redox zone and 5°C below a depth of 10 m). The biological activity in the incubation bottles was stopped at different time intervals (~20, 45, 75 and 90 h) by the addition of 200 μL of a saturated HgCl<sub>2</sub> solution. They were then stored in the dark until analysis in the laboratory following the method described above for CH<sub>4</sub> concentration determination.

Sixteen supplementary samples were incubated under the same conditions but were amended by the addition of different electron acceptors after a pre-incubation period of 12 h, to remove oxygen potentially inadvertently introduced during sampling. Four of them received 100 μL of a solution of nitrate KNO<sub>3</sub> (12 g L<sup>-1</sup>, final concentration of 200 μmol L<sup>-1</sup>), four received 100 μL of the same solution of nitrate plus 100 μL of a solution of H<sub>2</sub>S (60 g L<sup>-1</sup>, final concentration of 100 μmol L<sup>-1</sup>), four received 100 μL of a solution of Fe<sub>2</sub>O<sub>3</sub> (4.8 g L<sup>-1</sup>, final concentration of 50 μmol L<sup>-1</sup>) and four received 100 μL of a solution of MnO<sub>2</sub> (2.6 g L<sup>-1</sup>, final concentration of 50 μmol L<sup>-1</sup>). The concentrations of the different solutions were chosen to be in excess compared with the natural concentrations. The different electron acceptor solutions (except those with H<sub>2</sub>S) were stored in 30-ml sealed glass serum bottles and flushed with helium for 10 min to evacuate atmospheric oxygen that had potentially been inadvertently introduced. To avoid any trace oxygen contamination during the injection of the electron acceptor solution, all bottles remained closed and solutions were taken with a syringe and a needle through the septum and injected into the different samples, also through the septum.

The biological activity in the incubation bottles was stopped after the same time interval and following the same method as in the control treatment described above. They were then stored in the dark until analysis in the laboratory following the method described above for CH<sub>4</sub> concentration determination.

CH<sub>4</sub> oxidation rates were estimated as the slope of the linear regression of CH<sub>4</sub> concentration (μmol L<sup>-1</sup>) versus time during the incubation (per day). CH<sub>4</sub> oxidation was considered significant only if the slope of the linear regression was significantly lower than 0 (95% confidence interval). Oxidation rates in the experiments with the addition of different potential electron acceptors were considered as moderately stimulated when the slope in the treatment was higher than 1 standard deviation of the slope of the control (confidence interval of 68%, 1 σ), or strongly stimulated when it was higher than 2 standard deviations (confidence interval of 95%, 2 σ). Statistical testing was performed using Graphpad Prism 7 Software.

### CH<sub>4</sub> emissions calculations

CH<sub>4</sub> emissions to the atmosphere were calculated as described by Roland et al. (2017). Briefly, the CH<sub>4</sub> concentration gradient across the air-water interface was computed from the CH<sub>4</sub> concentration at a depth of 1 m, and the gas transfer velocity was computed from wind speed according to the Cole and Caraco (1998) relationship. A positive emission value corresponds to a net gas transfer from the water to the atmosphere.

### Anammox and denitrification rates measurements

#### Sampling of 2017

Denitrification measurements were performed at six depths (9, 10, 11, 13, 15 and 17 m), located at the oxic-anoxic interface and below the water layer under the influence of the external source



that brings  $\text{NO}_3^-$  into the anoxic waters, and where  $\text{H}_2\text{S}$  concentrations are high.

For each depth, two 250-mL glass serum bottles were filled directly from the tubing of the Niskin bottle taking care to avoid air bubbles and were immediately closed without headspace. These bottles were pre-incubated for 12 h before further manipulation, for the same reasons as described above. After the pre-incubation period, the bottles were spiked with tracer solutions following two different treatments: (i) addition of a solution of  $\text{K}^{15}\text{NO}_3^-$  (Eurisotop, Saint-Aubin, France) (final concentration of  $100 \mu\text{mol L}^{-1}$ ), and (ii) addition of the same solution of  $\text{K}^{15}\text{NO}_3^-$  and with a solution of dissolved  $\text{H}_2\text{S}$  (Sigma-Aldrich, Saint-Louis, Missouri, USA) (final concentration of  $100 \mu\text{mol L}^{-1}$ ) in order to test the effect of  $\text{H}_2\text{S}$  addition on the denitrification process. Given the fact that all the tracer and  $\text{H}_2\text{S}$  molecules were added in excess, we considered that measured rates should be viewed as potential rates.

After the tracer addition, the water samples contained in each of the 250-mL serum bottles were gently transferred into 12-mL Exetainer glass vials (Labco, Lampeter, UK) with a syringe and silicone tubing. Six Exetainer vials per 250-mL bottle were overfilled and closed without headspace. One Exetainer vial was immediately stopped (T0 sample) by injection of  $500 \mu\text{L}$  of 20% zinc acetate (ZnAc; VWR, Leuven, Belgium); the others were placed in the dark at a temperature close to the *in situ* temperature and were stopped after 6, 12, 18, 24 and 48 h of incubation. Exetainer vials for denitrification and anammox rates measurement were stored in the dark until quantification of the  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  with an elemental analyzer-isotope mass spectrometer (EA-IRMS, EA1112 coupled to deltaV advantage; Thermo Fisher Scientific, Waltham, Massachusetts, USA) after creating a 2-mL helium headspace. Denitrification and anammox rates were calculated according to Equations 1 and 2 (Thamdrup and Dalsgaard 2002; Thamdrup et al. 2006):

$$\text{N}_2 \text{ denitrification} = {}^{15}\text{N}^{15}\text{N}_{\text{excess}} * (\text{F}_{\text{NO}_3})^{-2} \quad (1)$$

$$\text{N}_2 \text{ anammox} = (\text{F}_{\text{NO}_3})^{-1} * \left( {}^{14}\text{N}^{15}\text{N}_{\text{excess}} + 2 * \left( 1 - (\text{F}_{\text{NO}_3})^{-1} \right) * {}^{15}\text{N}^{15}\text{N}_{\text{excess}} \right) \quad (2)$$

where  $\text{N}_2$  denitrification and  $\text{N}_2$  anammox are the production of  $\text{N}_2$  by denitrification and anammox, respectively,  ${}^{15}\text{N}^{15}\text{N}_{\text{excess}}$  and  ${}^{14}\text{N}^{15}\text{N}_{\text{excess}}$  are the production of excess  ${}^{15}\text{N}^{15}\text{N}$  and  ${}^{14}\text{N}^{15}\text{N}$ , respectively, and  $\text{F}_{\text{NO}_3}$  is the fraction of  ${}^{15}\text{NO}_3^-$  in the  $\text{NO}_3^-$  pool ( ${}^{15}\text{NO}_3^- / ({}^{15}\text{NO}_3^- + \text{NO}_3^-)$ ).  ${}^{15}\text{N}^{15}\text{N}$  and  ${}^{14}\text{N}^{15}\text{N}$  excess is the excess relative to masses of 30 and 29, respectively, in the T0 samples. The limit of detection for denitrification and anammox measurements with the above mentioned IRMS setup was estimated based on triplicate injection of a selection of samples and was  $10.4 \text{ nmol L}^{-1} \text{ d}^{-1}$  for denitrification and  $6.1 \text{ nmol L}^{-1} \text{ d}^{-1}$  for anammox.

Natural denitrification rates can be deduced from potential rates using the following equation (Thamdrup et al. 2006):

$$\begin{aligned} & \text{Natural N}_2 \text{ denitrification} \\ & = \text{Potential N}_2 \text{ denitrification} * (1 - \text{F}_{\text{NO}_3}) \quad (3) \end{aligned}$$

where natural  $\text{N}_2$  denitrification is the natural production of  $\text{N}_2$  by denitrification, potential  $\text{N}_2$  denitrification is the potential denitrification rate (as described in Equation 1) and  $\text{F}_{\text{NO}_3}$  is the fraction of  ${}^{15}\text{NO}_3^-$  in the  $\text{NO}_3^-$  pool.

### Sampling of 2018

Denitrification measurements were performed at four depths (13, 15, 17 and 19 m) with the same general procedure as

described above, but water samples were incubated following the following treatments: (i) 250-mL serum bottles were spiked with a solution of  $\text{K}^{15}\text{NO}_3^-$  (final concentration of  $200 \mu\text{mol L}^{-1}$ ), or (ii) with a solution of  ${}^{15}\text{NH}_4\text{Cl}$  (Eurisotop, Saint-Aubin, France) (final concentration of  $200 \mu\text{mol L}^{-1}$ ) and a solution of  $\text{K}^{14}\text{NO}_3^-$  (final concentration of  $200 \mu\text{mol L}^{-1}$ ) to specifically target the anammox process. After the tracer addition, Exetainer vials were filled, poisoned and processed in the laboratory as described above. Anammox rates were calculated as follows:

$$\text{N}_2 \text{ anammox } {}^{15}\text{N}^{14}\text{N} = {}^{15}\text{N}^{14}\text{N}_{\text{excess}} * (\text{F}_{\text{NH}_4})^{-1} \quad (4)$$

where  $\text{N}_2 \text{ anammox } {}^{15}\text{N}^{14}\text{N}$  is the production of  $\text{N}_2$  by anammox in the treatment with  ${}^{15}\text{NH}_4^+$  added,  ${}^{15}\text{N}^{14}\text{N}_{\text{excess}}$  is the production of excess  ${}^{15}\text{N}^{14}\text{N}$  and  $\text{F}_{\text{NH}_4}$  is the fraction of  ${}^{15}\text{NH}_4^+$  in the  $\text{NH}_4^+$  pool ( ${}^{15}\text{NH}_4^+ / ({}^{15}\text{NH}_4^+ + \text{NH}_4^+)$ ).

## RESULTS

### Chemical composition and seasonal variability of the water column

Differences occurred in the physico-chemical vertical profiles from 2017 to 2018 (summer 2018 was characterized by very high air temperatures and a severe drought across Europe) (Fig. 1). An offset in the thermocline, chemocline and oxycline positions could be observed, with deeper clines in 2018.  $\text{O}_2$  concentrations at the surface were typically around  $11 \text{ mg L}^{-1}$  during both field campaigns, and the water column was anoxic ( $\text{O}_2$  below detection limit) from a 10-m depth in 2017 and from a 12-m depth in 2018. A peak of higher  $\text{O}_2$  concentrations (up to  $14 \text{ mg L}^{-1}$ ) was observed in 2018 at the bottom of the epilimnion and photic zone (8-m depth) and related to higher chlorophyll-*a* content (Fig. S1).

A wide  $\text{NO}_3^-$  and  $\text{NO}_2^-$  accumulation zone spreading almost through the entire oxycline and the upper part of the anoxic compartment was observed in 2017 (Fig. 2), with concentrations peaking right at the oxic-anoxic transition for  $\text{NO}_3^-$  ( $33 \mu\text{mol L}^{-1}$  at 10 m) and slightly below for  $\text{NO}_2^-$  ( $20 \mu\text{mol L}^{-1}$  at 11 m). This accumulation zone extended in the anoxic compartment down to 15 m.  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations were much lower in 2018, with a maximum of  $9 \mu\text{mol L}^{-1}$  and less than  $0.5 \mu\text{mol L}^{-1}$  for  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , respectively. In contrast to 2017, no distinct accumulation zone could be observed.  $\text{NH}_4^+$  shows an opposite pattern, with an increase in concentration with depth in the anoxic compartment, particularly abrupt at the bottom of the  $\text{NO}_3^-$  and  $\text{NO}_2^-$  accumulation zone in 2017.  $\text{N}_2\text{O}$  maximum concentration peaks of 29 and  $55 \text{ nmol L}^{-1}$  (in 2017 and 2018, respectively) were observed in oxic waters, while peaks of 17 and  $8 \text{ nmol L}^{-1}$  (in 2017 and 2018, respectively) were also observed in anoxic waters.

Total Fe concentrations were low throughout the water column, with maximum concentrations of  $0.8 \mu\text{mol L}^{-1}$  observed in oxic waters. Total Mn concentrations were higher, and sharply increased in anoxic waters, up to  $\sim 15 \mu\text{mol L}^{-1}$ . The water column was very rich in S, with concentrations of up to  $\sim 650 \mu\text{mol L}^{-1}$ .

$\text{CH}_4$  and  $\text{pCO}_2$  vertical profiles showed similar patterns during both sampling campaigns, with a first increase of  $\text{pCO}_2$  with depth starting in the thermocline, below the photic zone ( $\sim 5 \text{ m}$ ), and a second in the conductivity gradient between 15 and 20 m.  $\text{CH}_4$  concentrations gradually increased with depth in the anoxic compartment, below the oxic-anoxic transition. The lake was a net source of  $\text{CH}_4$  during both sampling campaigns, with emis-

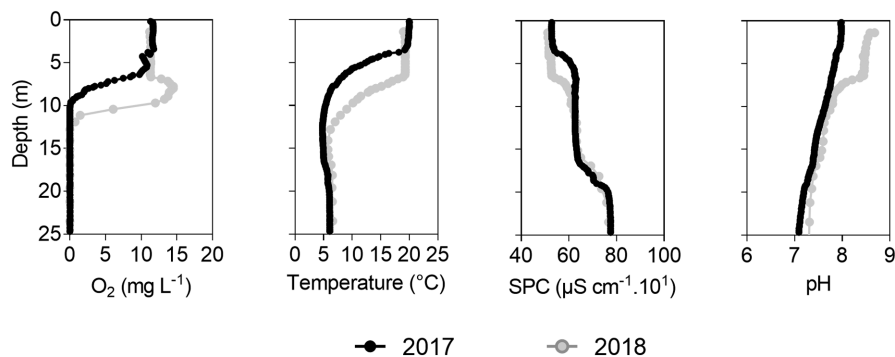


Figure 1. Physico-chemical parameters (oxygen concentrations ( $\text{mg L}^{-1}$ ), temperature ( $^{\circ}\text{C}$ ), specific conductivity (SPC,  $\mu\text{S cm}^{-1}\cdot 10^1$ ) and pH) in Lake Dendre in 2017 (black) and 2018 (gray).

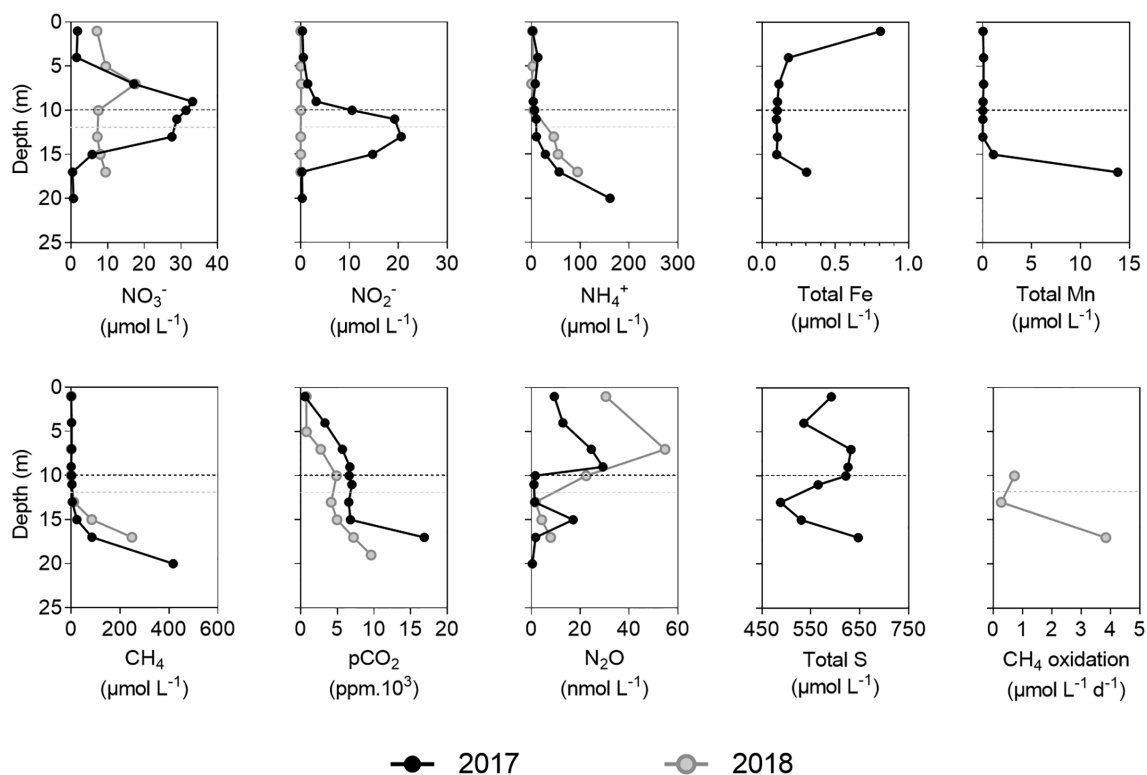


Figure 2. Vertical profiles of nitrogenous nutrients ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ), major elements (total Fe, Mn, S) and dissolved gases ( $\text{CH}_4$ ,  $\text{pCO}_2$ ,  $\text{N}_2\text{O}$ ) concentrations during both sampling campaigns, and  $\text{CH}_4$  oxidation, in 2018. Horizontal dashed lines represent the respective oxic-anoxic interfaces for each sampling campaign. Black: 2017; gray: 2018.

sion rates estimated at  $0.64$  and  $0.55 \text{ mmol m}^{-2} \text{ d}^{-1}$  in 2017 and 2018, respectively.

### $\text{CH}_4$ oxidation

The maximum of  $\text{CH}_4$  oxidation (MOX) measured in control (unmanipulated) samples was located well below the oxic-anoxic transition zone (located at  $12 \text{ m}$ ), with a maximum rate of  $3.84 \text{ } \mu\text{mol CH}_4 \text{ L}^{-1} \text{ d}^{-1}$  measured at a depth of  $17 \text{ m}$  (Figs 2 and S2). The experiment with addition of potential electron acceptors showed that MOX was moderately stimulated by the addition of  $\text{NO}_3^-$  and Fe oxides at  $10$  and  $13 \text{ m}$  (except Fe), and was strongly stimulated at  $17 \text{ m}$  (Fig. 3, Supplemental Table 1). By contrast, Mn addition did not significantly affect the  $\text{CH}_4$  oxidation rates, which were even lower than in the control samples at depths of  $13$  and  $17 \text{ m}$ .

### Denitrification and anammox

Potential denitrification rates were modest in the upper part of the hypolimnion but abruptly increased at depths below  $15 \text{ m}$ , with maximum rates measured at  $17 \text{ m}$  during both sampling cruises (up to  $17$  and  $63 \text{ } \mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$  in 2017 and 2018, respectively) (Figs 4, S2 and S4), slightly below the lower limit of the  $\text{NO}_3^-$  and  $\text{NO}_2^-$  accumulation zone (Fig. 2). Significant potential anammox rates (up to  $0.27 \text{ } \mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ ) were only detected in 2017, with two maximum peaks at depths of  $11$  and  $15 \text{ m}$ , but did not follow any clear vertical pattern.

In 2017, denitrification was significantly stimulated by the addition of  $\text{H}_2\text{S}$  (up to 1470 times increase) at every depth (Fig. 5), with the exception of  $17\text{-m}$  depth, where ambient  $\text{H}_2\text{S}$  concentrations were already high.  $\text{H}_2\text{S}$  also enhanced anammox between  $9$  and  $11 \text{ m}$  (up to 152 times increase).

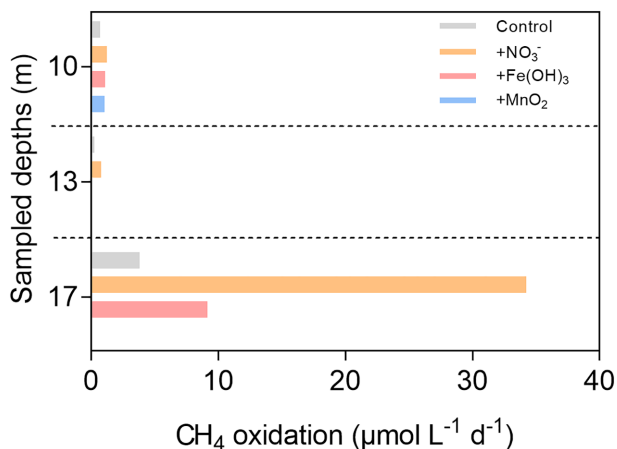


Figure 3. Methane oxidation rates in the control incubations (gray), and in incubations with the different potential electron acceptors added (light orange:  $\text{NO}_3^-$ ; red: Fe oxides; blue: Mn oxides), during the sampling campaign of 2018.

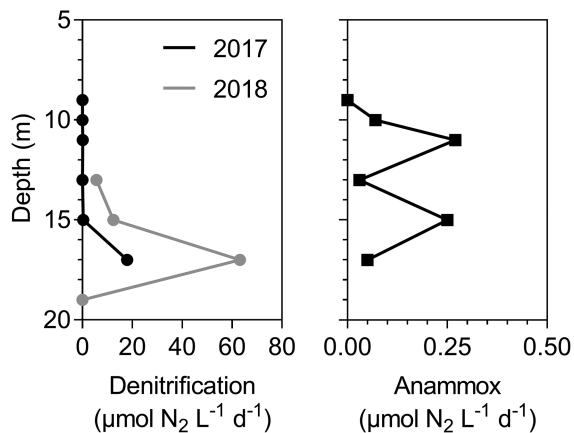


Figure 4. Vertical profiles of denitrification (circles) and anammox (squares) rates ( $\mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ ) in 2017 (black) and 2018 (gray). Note that anammox was not observed in 2018.

Maximum natural denitrification rates observed were estimated at  $2.9 \mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ .

## DISCUSSION

### An active N cycle

An active N cycle was observed in the water column, with substantial denitrification (in 2017 and 2018) and anammox (in 2017 only) rates observed in anoxic waters. Furthermore, the vertical profiles of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , with a decrease of  $\text{NH}_4^+$  at the oxic-anoxic interface and an accumulation of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in the oxycline (below the photic zone) and in the upper part of anoxic compartment, provide indirect evidence for the occurrence of active nitrification in the water column of Lake Dendre at the oxic-anoxic interface. This is supported by the observation of  $\text{N}_2\text{O}$  concentration maxima in the oxycline, which could be linked to nitrifier activity (e.g. Barnard, Leadley and Hungate 2005; Kirchman et al. 2008; Ji et al. 2015; Battaglia and Joos 2018).

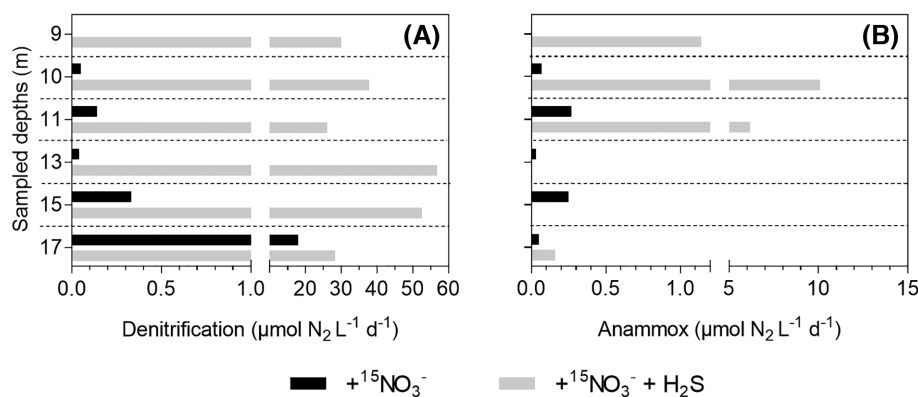
While heterotrophic denitrification is usually assumed to be ubiquitous in anoxic waters and sediments of freshwater ecosystems, observations of alternative denitrification pathways using inorganic electron donors such as  $\text{H}_2\text{S}$  (chemoautotrophic denitrification) have been mostly restricted to marine

sediments, or in the oxygen minimum zone of several marine basins such as the Baltic Sea (Labrenz et al. 2005), Black Sea (Brettar and Rheinheimer 1991) or the coastal upwelling off Chile (Galán et al. 2014). This could be related to the fact that freshwater systems usually harbor lower sulfate (and then sulfide) but higher organic matter concentrations in comparison with marine systems (Capone and Kiene 1988). Notable exceptions are sulfide-rich, deep meromictic lakes such as Lake Kivu (Roland et al. 2018b) or Lake Lugano (Wenk et al. 2013), where co-occurrence of anammox and sulfide-dependent denitrifiers was reported. Similar to Lake Cadagno, our results showed that potential denitrification was strongly stimulated by  $\text{H}_2\text{S}$  addition at every depth from the oxic-anoxic interface down to 17 m, where ambient  $\text{H}_2\text{S}$  concentrations usually increase (Roland et al. 2017), suggesting electron donor limitation of sulfide-dependent denitrification in Lake Dendre. Potential denitrification was not only detected in the nitrogenous compound accumulation zone, but also deeper, down to 17 m. This pattern might be explained by lateral intrusion of  $\text{NO}_3^-$ -rich groundwater (Borges et al. 2018) fed by a sub lacustrine spring located at a depth of  $\sim 17$  m. Anammox activity was detected in 2017 and was also stimulated by  $\text{H}_2\text{S}$  addition, although to a much lesser extent than denitrification. Reports on the effects of  $\text{H}_2\text{S}$  on anammox activity are contradictory, with some showing that they can be inhibited in the presence of  $\text{H}_2\text{S}$  (Dalsgaard et al. 2003; Jensen et al. 2008, 2009), and others showing that they could actually even be stimulated by external  $\text{H}_2\text{S}$  supply (Wenk et al. 2013; Roland et al. 2018a; Qin et al. 2019). Our observations are similar to those of Wenk et al. (2013), who suggested that in Lake Lugano anammox might rely on the  $\text{NO}_2^-$  produced as an intermediate of sulfide-dependent denitrification and benefit from a locally detoxified environment (removal of  $\text{H}_2\text{S}$  by sulfide-dependent denitrifiers) if organized in aggregate with sulfide-dependent denitrifiers.

While potential anammox rates measured in Lake Dendre during this study were similar to other sites in the literature (Table 1), higher potential denitrification rates were observed. Denitrification being dependent on the trophic status (Bai et al. 2019), the high denitrification rates observed can be linked to the eutrophic status of Lake Dendre and the continuous supply of  $\text{NO}_3^-$ -rich waters to the anoxic compartment by a sub lacustrine spring. The maximum potential sulfide-dependent denitrification measured in Lake Dendre ( $57 \mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ ) was also much higher than in Lake Lugano ( $0.2 \mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ ), and might be related to the overall higher electron donors availability ( $\text{H}_2\text{S}$ ) found in Lake Dendre (up to 100 and  $12 \mu\text{mol L}^{-1}$  in Lake Dendre and Lugano, respectively; Wenk et al. 2013; Roland et al. 2017b).

### $\text{CH}_4$ biogeochemistry in anoxic waters

The  $\text{CH}_4$  oxidation rates measured in the control treatment (without any amendment, so occurring with natural substrates, such as  $\text{SO}_4^{2-}$  present at very high concentrations; Roland et al. 2017b) were significantly lower than the maximum oxidation rate of  $15 \mu\text{mol L}^{-1} \text{ d}^{-1}$  reported in Lake Dendre during our previous research (Roland et al. 2017), suggesting a strong seasonal and inter-annual variability. Regarding anaerobic oxidation rates reported in the literature, a strong spatial variability is observed between study sites (Table 1), and rates are strongly influenced by natural  $\text{CH}_4$  concentrations of the environment (Fig. 6), except for Lake Matano and Lake Pavin, which presented higher oxidation rates for moderate  $\text{CH}_4$  concentrations and lower oxidation rates for higher  $\text{CH}_4$  concentrations, respectively (Lopes et al. 2011; Sturm et al. 2019). In Lake Dendre, the pattern is less clear,



**Figure 5.** (A) Denitrification rates ( $\mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ ) and (B) anammox rates ( $\mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ ), measured in incubations with  $^{15}\text{N-NO}_3^-$  (black) and  $^{15}\text{N-NO}_3^- + \text{H}_2\text{S}$  (gray), during the campaign of 2017.

**Table 1.** Denitrification, anammox and anaerobic methane oxidation (AOM) rates ( $\mu\text{mol N}_2 \text{ L}^{-1} \text{ d}^{-1}$  and  $\mu\text{mol CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ , respectively) in the water column of different aquatic environments (see Supplemental Table 2 for the hydro-morphological characteristics of each environment).

	Maximum rates ( $\mu\text{mol L}^{-1} \text{ d}^{-1}$ )	Source
<b>Potential denitrification</b>		
Lake Dendre	63.2	This study
Baltic Sea	1.2	Dalsgaard, De Brabandere and Hall Per (2013)
Lake Kivu (Africa)	4.2	Roland et al. (2018a)
Lake Tanganyika (Africa)	1.2	Schubert et al. (2006)
Lake Erie (North America)	0.4	Lu et al. (2018)
<b>Potential anammox</b>		
Lake Dendre	0.3	This study
Lake Kivu (Africa)	0.2	Roland et al. (2018a)
Lake Tanganyika (Africa)	0.2	Schubert et al. (2006)
Golfo Dulce	0.5	Dalsgaard et al. (2003)
Benguela upwelling system	0.2	Kuypers et al. (2005)
Lake Erie (North America)	0.9	Lu et al. (2018)
<b>AOM</b>		
Lake Dendre	3.8	This study
Lake Dendre	15	Roland et al. (2017)
Lake Kivu (Africa)	16	Roland et al. (2018b)
Lake Pavin (France)	0.4	Lopes et al. (2011)
Lake Marn (Sweden)	2.2	Bastviken, Ejlertsson and Tranvik (2002)
Lake Illersjoen (Sweden)	3	Bastviken, Ejlertsson and Tranvik (2002)
Lake Big Soda (USA)	0.01	Iversen, Oremland and Klug (1987)
Lake Matano (Indonesia)	181	Sturm et al. (2019)
Lake Tanganyika (Africa)	1.8	Rudd, Hamilton and Campbell (1974)
Lake Paul (USA)	5.6	Bastviken et al. (2008)
Lake Peter (USA)	6.3	Bastviken et al. (2008)
Lake Hummingbird (USA)	2.4	Bastviken et al. (2008)

suggesting that CH<sub>4</sub> concentrations are not the main and/or only factor determining the magnitude of CH<sub>4</sub> oxidation.

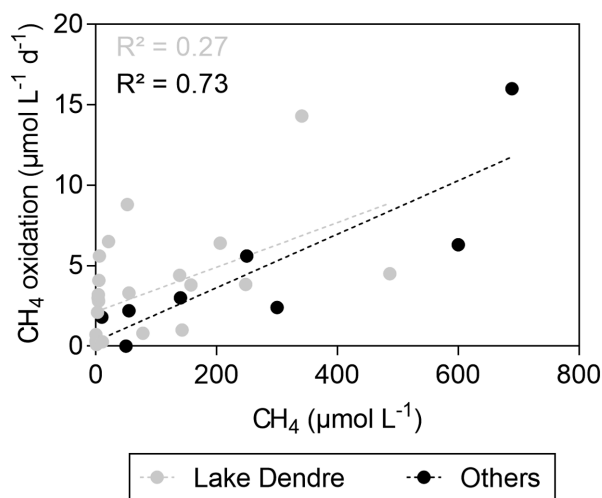
Experiments with the addition of three potential electron acceptors for AOM were carried out in 2018 to investigate deeper the electron acceptors involved in CH<sub>4</sub> oxidation in the anoxic compartment of Lake Dendre, besides sulfate, which is available at high concentrations (>500  $\mu\text{mol L}^{-1}$ ) throughout the water column (Roland et al. 2017). These experiments revealed that NO<sub>3</sub><sup>-</sup> addition significantly stimulated CH<sub>4</sub> oxidation at every depth from the oxic-anoxic interface down to 17 m.

Furthermore, according to the stoichiometry of Equation 5 (see below, Jensen et al. 2009), measurement of CH<sub>4</sub> oxidation rates in control treatment (maximum of 3.8 CH<sub>4</sub>  $\mu\text{mol L}^{-1} \text{ d}^{-1}$ ) could fully sustain the natural denitrification rates measured (maximum of 2.9 N<sub>2</sub>  $\mu\text{mol L}^{-1} \text{ d}^{-1}$ ).



Jointly, these results could suggest a coupling between CH<sub>4</sub> oxidation and denitrification, which is thermodynamically favorable and has been widely reported in laboratory





**Figure 6.** Methane oxidation rates ( $\mu\text{mol L}^{-1} \text{d}^{-1}$ ) according to  $\text{CH}_4$  concentrations ( $\mu\text{mol L}^{-1}$ ), in Lake Dendre (gray) and in different lakes in the literature (black; see Table 1). The  $\text{CH}_4$  oxidation rates in Lake Dendre are those observed during this study and during our previous study (Roland et al. 2017b).

experiments (e.g. Raghoebarsing et al. 2006; Ettwig et al. 2009). The in-situ occurrence of denitrification-dependent  $\text{CH}_4$  oxidation in lakes has been mainly reported in sediments (Deutzmann and Schink 2011; á Deutzmann et al. 2014; Kojima et al. 2012; Liu et al. 2015; Norði and Thamdrup 2014), but to the best of our knowledge, only once in a lake water column (van Grinsven et al. 2020). In Lake Dendre, we hypothesize that the underwater spring supply the  $\text{NO}_3^-$  used to oxidize anaerobically a substantial part of the upward flux of  $\text{CH}_4$ . While  $\text{SO}_4^{2-}$  availability is sufficient throughout the entire water column and for the whole year (at least  $500 \mu\text{mol L}^{-1}$ ; Roland et al. 2017) to always sustain AOM, we speculate that the importance of  $\text{NO}_3^-$ -dependent AOM varies seasonally in the function of the underwater spring discharge.

$\text{CH}_4$  oxidation rates also significantly increased with the addition of Fe oxides at every depth except 13 m (and may be due to competition with natural  $\text{NO}_3^-$  concentrations, which were high at this depth,  $\text{NO}_3^-$  being thermodynamically more favorable than Fe oxides for AOM; Borrel et al. 2011). By contrast, Mn addition did not significantly enhance  $\text{CH}_4$  oxidation rates. The  $\text{CH}_4$  oxidation coupled to Fe oxides in natural environments is still being investigated; it has been notably suggested to occur in Lake Matano (Indonesia; Crowe et al. 2011), Lake Orn (Denmark; á Norði et al. 2013), Lake Zug (Switzerland; Oswald et al. 2016), Lake La Cruz (Spain; Oswald et al. 2016a) or Siberian lakes (Cabrol et al. 2020). It could be of greater importance than originally expected (based on natural Fe oxide concentrations), because it has been estimated that Fe could be oxidized and reduced by 100 to 300 times before burial (Beal, House and Orphan 2009). Some studies also suggest that  $\text{CH}_4$  oxidation stimulation by Fe oxides could be due to rapid oxidation of  $\text{H}_2\text{S}$  by Fe oxides, and then use of  $\text{SO}_4^{2-}$  formed for AOM (Segarra et al. 2015; Su et al. 2020). In Lake Dendre,  $\text{SO}_4^{2-}$  are large and not limiting (Roland et al. 2017), and thus it is quite unlikely that a relatively small supplemental supply of  $\text{SO}_4^{2-}$  increases the AOM rates. Nevertheless, iron is an important nutrient for many prokaryotes and we cannot exclude that a fraction of the Fe oxides added at the start of the incubations, if reduced to  $\text{Fe}^{2+}$ , might have stimulated AOM in alleviating a nutrient limitation. For instance, in culture experiments (Sub, Shuhei and Tanja 2012) or in marine ecosystems (Sivan

et al. 2014), iron has been found to stimulate  $\text{SO}_4^{2-}$  reducers, and consequently  $\text{SO}_4^{2-}$ -driven AOM.

## CONCLUSION

The results of this experiment, during which the addition of several electron acceptors was found to positively affect  $\text{CH}_4$  oxidation, highlight that methanotrophy in the Lake Dendre water column seems to be diversified and would occur with different available potential electron acceptors. In this case, the availability of the different potential electron acceptors and the thermodynamics of the reactions would determine the magnitude and vertical segregation of the different processes, as suggested by the significant enhancement of  $\text{CH}_4$  oxidation concomitant with the addition of electron acceptors ( $\text{NO}_3^-$ , Fe oxides). Furthermore, our results showed that, in addition to a linkage with the C cycle, the N cycle could also be linked to the sulfur S cycle, through chemolithotrophic denitrification using  $\text{H}_2\text{S}$  as the electron donor. This process could be particularly relevant in Lake Dendre, which is characterized by sulfate-rich waters throughout the water column and deep waters rich in  $\text{H}_2\text{S}$  (Roland et al. 2017).

Altogether, these results suggest that the microbial community involved in  $\text{CH}_4$  oxidation was not specialized in the use of electron acceptors but was instead versatile, with an ability to use different substrates. We suggest the occurrence of several linkages between N, S and C cycles, notably between denitrification, anammox and sulfide oxidation, and  $\text{CH}_4$  oxidation and denitrification. These results suggest that some processes are probably underestimated and underinvestigated in the literature, because the natural conditions are considered non-optimal for their occurrence.

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## SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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**Conflict of interest.** None declared.

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