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Dark carbon fixation is a common process in the water column of stratified boreal lakes

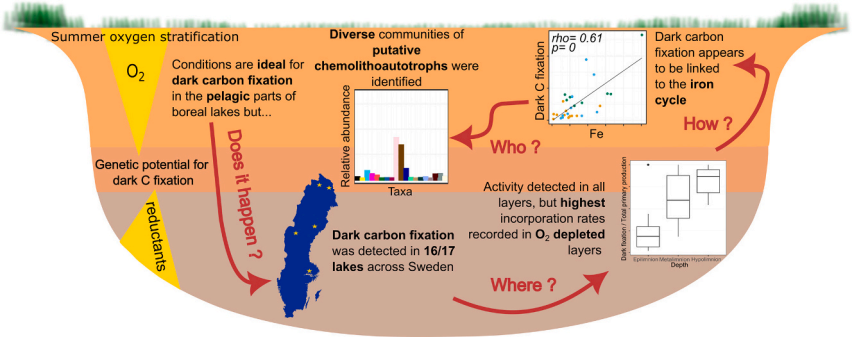
Gaëtan Martin [a,b,*], Antti J. Rissanen [c,d], Sarahi L. Garcia [e,f], Sari Peura [a,g]

[a] Department of Forest Mycology and Plant Pathology, Science for Life Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden
[b] Department of Tropical Plant and Soil Sciences, College of Tropical Agriculture and Human Resources, University of Hawai'i at Mānoa, Honolulu, United States of America
[c] Faculty of Engineering and Natural Sciences, Tampere University, Finland
[d] Natural Resources Institute Finland (Luke), Helsinki, Finland
[e] Department of Ecology, Environment and Plant Sciences, Science for Life Laboratory, Stockholm University, Stockholm, Sweden
[f] Institute for Chemistry and Biology of the Marine Environment (ICBM), School of Mathematics and Science, Carl von Ossietzky Universität Oldenburg, Oldenburg, Germany
[g] Swedish Nuclear Fuel and Waste Management Co. (SKB), Solna, Sweden

HIGHLIGHTS

- Pelagic dark carbon fixation (DF) is rarely considered in C budgets of boreal lakes.
- Using ^{14}C labelling, we show that DF is a common process in the water column of stratified boreal lakes.
- DF rates were higher in oxygen depleted layers of lakes.
- Metagenomic and environmental data suggest that DF is linked to the iron cycle.
- DF likely plays a key role in the ecology and carbon cycle of boreal stratified lakes.

GRAPHICAL ABSTRACT



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ABSTRACT

CO_2 fixation (i.e. primary production) is a key function of all ecosystems, providing the carbon and energy that fuel the entire food web. It also plays an important role in mitigating climate change as CO_2 is the most important greenhouse gas. While photosynthesis is regarded as the most important carbon fixation pathway, prokaryotes able to fix carbon in the absence of light (chemolithoautotrophs) can also be a significant source of energy in a light-limited ecosystem. Boreal lakes, notoriously colored and stratified with respect to oxygen and nutrients, present ideal conditions for this so-called dark carbon fixation by the chemolithoautotrophs. However, the prevalence of dark carbon fixation in boreal lakes remains unknown. Here, we measured dark carbon fixation in Swedish lakes from the boreal and boreo-nemoral zones, during summer stratification. We detected dark carbon fixation in 16 out of the 17 lakes studied, and concluded that dark fixation is a widespread phenomenon in boreal lakes. Moreover, the average dark primary production ranged from 18.5 % in the epilimnion to 81.4 % in the

* Corresponding author at: Department of Tropical Plant and Soil Sciences, College of Tropical Agriculture and Human Resources, University of Hawai'i at Mānoa, Honolulu, United States of America.
E-mail address: gaetan@hawaii.edu (G. Martin).

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hypolimnion of all tested lakes. Our data further suggests that chemolithoautotrophic activity is mostly driven by iron-oxidizing bacteria. The chemolithoautotrophic guild is diverse and seems to be composed of both ubiquitous bacteria, like *Gallionellaceae or Chromatiaceae*, and endemic taxa, such as *Ferrovaceae,* which appears to be favored by a low pH. These results are particularly exciting as they suggest that dark carbon fixation could partly compensate for the low photosynthetic capacity in lakes with dark-colored water.

1. Introduction

Emissions from freshwater ecosystems were often dismissed (Falkowski et al., 2000,) until the fifth assessment report of the IPCC acknowledged the importance of lakes in the global carbon cycle (Ciais et al., 2013). Even though some lakes are known to be potential net carbon sinks (Reed et al., 2018), inland waters are generally considered net emitters of CO_2 as their surface water is commonly supersaturated with CO_2 (Sobek et al., 2005; Tranvik et al., 2009; Raymond et al., 2013). Boreal lakes are of special interest as they represent 27 % of the global lake area, while boreal ecosystems cover only 14 % of the global land surface area (Verpoorter et al., 2014). Importantly, the CO_2 emissions from boreal lakes are projected to increase with climate change (Bogard and del Giorgio, 2016; Hastie et al., 2018).

CO_2 fixation is also important for the functioning of ecosystems as it provides a source of organic carbon and energy for heterotrophic organisms. The best-known and most common process of CO_2 fixation is photosynthesis, where light is used to provide the necessary energy for reducing CO_2 to organic carbon. However, it has been known since the time of Winogradsky that chemolithoautotrophs can also turn mineral carbon into biomolecules in the absence of light (Ackert, 2006) in a process called dark carbon fixation. Chemolithoautotrophic organisms (ChLithAu) are prokaryotes (i.e., bacteria and archaea) that are highly diverse in terms of phylogeny, physiology, and ecology (Enrich-Prast et al., 2009). For this reason, dark carbon fixation has been observed in a wide variety of environments, such as oceans (Markager, 1998), soil (Tolli and King, 2005; Ackert, 2006), river estuaries (Bräuer et al., 2013), marine sediments, freshwater and saline lakes (Santoro et al., 2013; Boschker et al., 2014; Wang et al., 2021), volcanic environments (King, 2007), thermal springs (Reigstad et al., 2011), subglacial ecosystems (Boyd et al., 2014), streams (Machado-Silva et al., 2023) and water columns of lakes (Camacho et al., 2001; Alfreider et al., 2017; Huang et al., 2023).

The importance of dark carbon fixation in different ecosystems varies. In the ocean, dark carbon fixation is assumed to represent only a small fraction of the total carbon fixation (in the range of 1.5 % of the oceanic photosynthesis) and might be considered secondary production as the energy source (e.g. inorganic sulfur, iron, N compounds) is often derived from organic matter (Raven, 2009; Middelburg, 2011). Dark carbon fixation nevertheless plays an important role as it supports the functioning of food webs in deep-sea ecosystems (Molari et al., 2013). In other ecosystems, like caves or Antarctic lakes deprived of light by hundreds of meters of ice, dark carbon fixation is potentially the only source of organic carbon (Chen et al., 2009; Achberger et al., 2016). In lakes, dark carbon fixation can represent an important portion of the inorganic carbon assimilation (Cloern et al., 1983; Kuuppo-Leinikki and Salonen, 1992; Camacho et al., 2001; Hadas et al., 2001; Nõges and Kangro, 2005). This proportion can be up to 50 % of the total primary production (Kuuppo-Leinikki and Salonen, 1992; Camacho et al., 2001) and the assimilation rate by chemolithoautotrophic organisms can be as high as photosynthetic assimilation in euphotic layers (Nõges and Kangro, 2005). Consequently, ChLithAu should be considered as important consumers of the most concerning greenhouse gas and key organisms in lake food webs.

In freshwater ecosystems, ChLithAu are found at the top layer of the sediment or at the chemocline in the water column where oxidized and reduced compounds are simultaneously present (Camacho et al., 2001; Santoro et al., 2013). The chemocline is located at the interface between the oxic and anoxic waters in stratified lakes with anoxic bottom layers. It is characterized by a steep and stable gradient of oxygen, nutrient concentrations, and redox potential. (Børsheim et al., 1985; Camacho et al., 2001). Most lakes develop thermal stratification during the warm season, but their hypolimnion does not necessarily become anoxic. Indeed, light penetration can allow oxygenic phototrophs to maintain oxic conditions below the thermocline (Fee, 1976; Nürnberg and Shaw, 1998). On the other hand, small colored lakes with low light penetration are more likely to possess an anoxic hypolimnion (Nürnberg and Shaw, 1998), even when very shallow. This is the result of the combination of limited oxygenic photosynthesis due to poor light penetration and high heterotrophic respiration due to high dissolved organic carbon (DOC) content. For these small lakes, the area and color also have a strong impact on the depth and the stability of the epilimnion (Fee et al., 1996), which in turn has a strong impact on the photosynthetic primary production. For example, it has been demonstrated that photosynthetic rates in the euphotic zone are higher in colored lakes than in clear lakes (Nürnberg and Shaw, 1998). However, the limited depth of the euphotic layer in the colored lakes means that despite the higher carbon fixation per unit of volume, the fixation rate per unit of lake area is lower than in clear water lakes (Nürnberg and Shaw, 1998). This combined with high respiration rates in the lower layers of colored lakes (Sobek et al., 2006) has led to a conclusion that these lakes are net heterotrophic.

Intriguingly, dark carbon fixation is rarely considered a part of the autotrophic production of the pelagic zones of boreal lakes. Following this, studies addressing the primary production in boreal lakes have not considered ChLithAu but focused on the photosynthetic plankton (Parkin and Brock, 1980; Arvola, 1983, 1986; Rohrlack et al., 2020). Although some studies provide a broader perspective on energy mobilization in boreal lakes, they have not considered the potential importance of dark carbon fixation in the water column (Andersson, 1983; Salonen et al., 1992; Carpenter et al., 1998; Nürnberg and Shaw, 1998; Ask et al., 2009; Kodama et al., 2012), even when the measured primary production seems unable to support the total carbon demand of bacteria (Kankaala et al., 2013). Further, in some studies, where ^{14}C assimilation has been used to measure primary production, the dark carbon fixation has been considered as noise and subtracted from the primary production values (Ask et al., 2012; Seekell et al., 2015). This is intriguing as previous studies have shown both the genetic potential for ChLithAu (Taipale et al., 2009; Peura et al., 2018) as well as actual measurements of dark carbon fixation in boreal lakes (Kuuppo-Leinikki and Salonen, 1992; Nõges and Kangro, 2005). Furthermore, dark carbon fixation is well documented in the sediments of boreal lakes (Santoro et al., 2013).

Due to anthropogenic effects including the combination of browning, higher temperatures, longer summers, and advance of the tree line, the duration of both thermal and chemical stratification in boreal lakes are expected to increase, leading to a shallower euphotic zone. (Fee et al., 1996; Brothers et al., 2014; de Wit et al., 2016; Jenny et al., 2016; Klaus et al., 2021; Woolway et al., 2021). All these changes are expected to increase CO_2 emissions from boreal lakes as these features favor heterotrophic respiration over photosynthesis (Bogard and del Giorgio, 2016; Hastie et al., 2018; Kuhn and Butman, 2021). But again, such predictions dismiss that these changes are also potentially beneficial to ChLithAu. However, even though the presence of a chemocline in boreal lakes suggests favorable conditions for ChLithAu (Taipale et al., 2009; Peura et al., 2018), measurements of dark carbon fixation are still scarce (Kuuppo-Leinikki and Salonen, 1992; Nõges and Kangro, 2005). So, based on i) physicochemical conditions found in boreal lakes, ii)

previous research performed by our team where we saw a prevalence of genetic potential for dark carbon fixation in boreal lakes (Peura et al., 2018), iii) as well as existing, although limited proof of dark carbon fixation in humic lakes (Kuuppo-Leinikki and Salonen, 1992; Nõges and Kangro, 2005), we hypothesized that dark carbon fixation should be a common process in boreal lakes during the summer stratification season and that dark carbon fixation should be related to ChlithO taxa. The aim of our study was therefore to test these hypotheses by i) detecting and measuring dark carbon fixation in 17 small dark water lakes located in the boreal and boreo-nemoral zones, ii) analyzing reads of metagenomes of these lakes to identify potential organisms responsible for dark carbon fixation, and iii) determining how environmental variables influence the dark carbon fixation activity and community composition of ChLithAu. To reach those aims, we performed ^{14}C assimilation experiments, sampled DNA for metagenomic sequencing, and collected environmental data from 17 small boreal lakes covering latitudes from 59.5°N to 68°N in Sweden. To the best of our knowledge, this work is the first multi-lake study on dark carbon fixation in the water columns of stratified lakes in boreal ecosystems. It offers a new insight into an often-forgotten microbial guild by combining metagenomic and environmental data with in-situ measurements of ^{14}C incorporation.

2. Methods

2.1. Sampling site selection

17 lakes with an approximate diameter below 150 m were selected using satellite pictures (Table 1). Each lake, except for Lake Plåten (PL), was sampled once. PL was sampled twice. On site, we located the deepest point of the lake and measured the O_2 concentration 50 cm above the sediment surface at that point. If the deepest point of the lake was anoxic, a full vertical water column profile for temperature, O_2, pH, conductivity, and redox potential was measured using a probe (YSI ProDSS, Xylem Analytics, Rye Brook, USA.). The incremental steps varied from 10 cm to 1 m depending on the maximum depth at the site. In every case, incremental steps were reduced to 10 cm around the chemocline. The profiles were measured from bottom to top after stabilization of the O_2 probe at 0 mg/l. Secchi depth was measured using a Secchi disk. Based on this information, three depths were selected for sampling. The first depth to sample (later referred as epilimnion) was determined as half the value of the Secchi depth and represents the conditions in the euphotic zone. The second and third sampling depths (hereafter referred to as the metalimnion and hypolimnion, respectively) were selected based on the O_2 concentration and redox profile. The metalimnion samples were selected to represent the chemocline where O_2 concentration decreased sharply. Therefore, metalimnion samples were set at the first depth from the surface where O_2 concentration was below 1 mg/l. Finally, hypolimnion samples were selected to be the depth with O_2 levels below detection limit, but as close to the chemocline as possible. For each depth, the same sampling procedure was applied. First, we slowly dropped a weighted Tygon® tube (Masterflex®, Vernon Hills, IL, United States) at the targeted depth and used a clean 60 ml syringe connected with a three-way stopcock to pump water. At each new depth the total volume of the tube was discarded at least twice before the collection of the samples. Water for all analyses was then sampled in 1 l HDEP bottles, whereas water for ^{14}C essay was collected in 25 ml syringes. All samples were kept in an ice box in the dark until the samples were processed on the shore. Preparation of ^{14}C incubations and collection of microbial cells for DNA analysis was performed simultaneously.

2.2. ^{14}C incorporation assay

Incubations to measure CO_2 incorporation were done in 25 ml serum bottles sealed with butyl rubber stoppers and aluminum crimps. Prior to sampling, all the bottles were muffled at 400 °C for 8 h to remove all traces of carbon. To avoid contamination with potential growth inhibitors, stoppers were autoclaved submerged in deionized water. After autoclaving, the stoppers were rinsed and boiled in sterile deionized water, and finally cooled in sterile deionized water. Once assembled, the bottles were flushed four times with N_2 to remove all oxygen. At each field site, nine bottles were filled with 20 ml of lake water for each depth and stored in the dark. The bottles were filled using a needle connected to a syringe with a 3-way stopcock. This allowed us to relieve the overpressure once the bottles were full. We then added to each bottle 100 µl of $NaH^{14}CO_3$ solution (activity 10 µCi/ml, DHI LAB products, Hørsholm, Denmark) using a 100 µl glass syringe (Hamilton, Reno, U.S.A). Once spiked with the ^{14}C, the bottles were put back in the dark. For each depth, the bottles were randomly assigned to three different treatments. Three bottles were put in thick and opaque polypropylene plumbing tube to measure dark carbon fixation (dark treatment). The following set of three bottles was put in a PET transparent container (light treatment). Finally, the last three bottles were spiked with 0.5 ml of 50 % glutaraldehyde (killed control treatment) and put in a container similar to the one used for the dark treatment. Weights were added to each setup to prevent buoyancy. Once incubation setups were ready for

Table 1
Sampling sites.

Lake	Location	Latitude (N)	Longitude (E)	Max depth (m)	Secchi depth (m)	Sampling date (month-year)	EPI (m)	META (m)	HYPO (m)
Ja2	Jämtland	63,3868	14,455	6	na	18-jul	2	2,8	3,5
Li1	Norrbotten (east)	67,7321	22,4244	5	0,85	19-aug	0,4	1	1,4
Li2	Norrbotten (east)	67,7314	22,4254	6,1	1,8	19-aug	0,9	1,5	2
Li3	Norrbotten (east)	67,7787	22,3575	5,1	1,2	19-aug	0,6	2	2,5
Li4	Norrbotten (east)	67,7915	22,3744	4,7	1,6	19-aug	0,8	2,15	2,5
Li5	Norrbotten (east)	67,7369	22,2619	5	0,98	19-aug	0,5	2,35	2,5
Ki1	Norrbotten (west)	67,9287	20,9612	4	1,3	18-jul	0,7	1,6	2,2
Ki2	Norrbotten (west)	67,9233	20,3691	4,2	1,4	18-jul	1	3,6	4,2
Or1	Örebro	59,4081	14,6015	10	1,05	19-jul	0,5	1,2	2
Or2	Örebro	59,4494	14,5831	7,5	1,02	19-jul	0,5	1,2	2
Or3	Örebro	59,4505	14,5826	5,8	0,6	19-jul	0,3	1	1,5
Or4	Örebro	59,4459	14,5836	9,6	1,15	19-jul	0,6	1,4	1,6
PL1[a]	Uppland	59,8629	18,5423	6	0,65	19-jun	0,35	2	2,5
PL2[a]	Uppland	59,8629	18,5423	6	0,9	19-aug	0,45	1	1,75
Up1	Uppland	59,9901	17,0958	4,1	0,55	19-jul	0,25	2	3,2
Um1	Västerbotten	64,1504	18,8	8	1,3	18-sep	1,3	3,5	4
Um2	Västerbotten	64,1231	18,7805	8,4	0,5	18-sep	0,25	3,7	5
Um3	Västerbotten	64,1216	18,7836	9	0,45	18-sep	0,25	6,5	7

General information about the sampling sites. Locations are the classes used for PERMANOVA analysis. EPI, META, and HYPO give the sampling depths in meters for the epi-, meta- and hypolimnion samples, respectively.

[a] Same lake sampled at two different time points.

all depths, they were brought back to the sampling site, where all the incubation containers were filled with water, and lowered to the depths at which the samples were collected. After 24 h, the bottles were pulled out of the water and 0.5 ml of 50 % glutaraldehyde was injected into all the dark and light treatment vials to terminate all microbial activity. Samples were then stored in the dark at 4 °C until further processing.

To measure the incorporated CO_2 (IC), three replicate samples of 6 ml were pipetted from each of the bottles into scintillation vials, acidified with concentrated phosphoric acid, and left for 48 h to evaporate unincorporated $H^{14}CO_3^-$. After that, 9 ml of scintillation liquid (Optiphase HiSafe 3, PerkinElmer, Waltham, U.S.A) was added. Simultaneously, two replicates were prepared for measurement of the total activity (TA) by pipetting 0.5 ml of each of the samples in scintillation vials with 0.5 ml of 1:7 ethanolamine-ethanol –solution and 9 ml of scintillation liquid. Activity was measured using a Hidex 300sl scintillation counter (Hidex, Turku, Finland) at the department of Ecology and Genetics (Limnology) at Uppsala University.

CO_2 raw fixation (RwF) was calculated with the following formula (Zopfi et al., 2001):

$$RwF = \frac{IC^{*}DIC x 1.06}{TA}$$

DIC is the total dissolved inorganic carbon concentration at each depth (see chemical analysis section), 1.06 is the correction factor for isotopic fractionation between ^{12}C and ^{14}C. For each depth, the total carbon fixation (TF) was calculated as the difference between the light treatment and the killed control treatment, photosynthetic carbon fixation (PF) was calculated as the difference between the mean RwF in the light treatment and the dark treatment, and dark carbon fixation (DF) as the difference between the mean RwF of the dark treatment and the killed control treatment.

$$TF = RwF^{\text{light treatment}} - RwF^{\text{killed control}}$$

$$PF = RwF^{\text{light treatment}} - RwF^{\text{dark treatment}}$$

$$DF = RwF^{\text{dark treatment}} - RwF^{\text{killed control}}$$

In all cases, *t*-tests were performed to determine whether the difference between the two treatments was significant. If the *p*-value was above 0.05, the difference between the treatments was assumed to be null.

2.3. DNA and water chemistry analysis

Two liters of water per depth were collected, pre-filtered using a 63 μm sieve (J. Engelsmann AG, Ludwigshafen, Germany) and stored in PP bottles. Once on shore, the water was pushed through a 0.22 μm Sterivex filter (Millipore) to collect cells for DNA extraction using a 60 ml syringe mounted on a caulk gun. For each sample, 1 l of water was filtered unless the filter clogged before. Filters were then wrapped in aluminum foil and flash-frozen in liquid nitrogen before being stored at −80 °C. Some of the filtrate was collected in falcon tubes and stored at −20 °C for ion chromatography, as well as DOC and dissolved nitrogen (DN) analyses. Finally, 10 ml of unfiltered water was collected in falcon tubes with 50 μl of 2 M HCl for iron measurement.

DNA extractions and library preparations were done as described in Buck et al. (2021). In short, for each sample we extracted DNA from half a filter using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). After verifying the extraction yield using a Qubit dsDNA HS kit (Thermo Fisher Scientific Inc.), the libraries were prepared for shotgun sequencing using the ThruPLEX DNA-seq Prep Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The shotgun sequencing was conducted at the Science for Life Laboratory (Uppsala University, Sweden) on the Illumina NovaSeq6000-platform. All sequences are deposited in the European Nucleotide Archive (ENA, mirrored to SRA, and accessible at the NCBI) under the project numbers PRJEB38681 (Buck et al., 2021) and PRJEB76863. The metadata of those two submissions were compiled in the Supplementary Table S1.

DOC and DN were measured by a Shimadzu TOC-L TNM-L instrument (Shimadzu, Kyoto, Japan). Ion (NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, and NH_4^+) concentrations were measured with a Metrohm IC system 883 Basic IC Plus using the 919 Autosampler Plus (Metrohm AG, Herisau, Switzerland). Anion separation was performed at 0.7 ml/min with a carbonate eluent (3.2 mM Na_2CO_3 + 1.0 mM $NaHCO_3$) in a Metrosep A Supp 5 analytical column (250 × 4.0 mm) fitted with a Metrosep A Supp 4/5 guard column. Cation separation was performed using 4.0 mM nitric acid and 1.0 mM dipicolinic acid as eluent and a flow rate of 0.9 ml/min in Metrosep C6 column (250 × 2.0 mm) with a Metrosep C6 guard column. Iron concentrations were measured using photometry (Hach DR 3900, Loveland, CO, U.S.A) according to Viollier et al. (2000). Absorbance at 420 nm (α420) was also recorded as an indicator of the water color (Kritzberg and Ekström, 2012; Weyhenmeyer et al., 2014).

For dissolved gas quantification, 5 ml samples were collected with a 10 ml syringe connected to the stopcock and immediately transferred to 20 ml glass vials filled with high-purity nitrogen gas (N_2) and 150 μL H_3PO_4 (85 %). Dissolved methane and DIC (i.e., CO_2) concentrations were measured using gas chromatography (Clarus 500, Perkin Elmer, USA, Polyimide Uncoated capillary column 5 m × 0.32 mm, with TCD and FID detector).

2.4. Bioinformatics and statistics

Quality assessment and trimming of the raw data were performed as described in Buck et al. (2021). To taxonomically classify the quality trimmed shotgun reads, we used Kaiju with default settings (Menzel et al., 2016). Classification was performed at the genus level, using the NR-UK database (downloaded on February 25, 2021, https://kaiju.binf.ku.dk/). After removal of eukaryote entries, the classified data set was rarefied using the phyloseq package (McMurdie and Holmes, 2013) in R version 4.0.2 (R Core Team, 2020). The mixOmics package (Rohart et al., 2017) was used for Partial Least Squares (PLS) regression. Mantel testing was performed using the VEGAN package (Oksanen et al., 2019). When running Mantel correlation tests, we used Bray-Curtis distance matrix for the community genus compositions and Euclidian distance for the environmental variables. All correlations and PERMANOVA analysis of dark carbon fixation rates or photosynthesis rates were calculated and plotted excluding the samples where fixation rates were not significantly different from the killed samples.

Putative ChLithAu were examined by looking for taxa for which read-based abundances correlated (Spearman) with dark carbon fixation. This approach aimed to identify interesting candidates that could be investigated in later studies and find hints regarding the main metabolism responsible for dark carbon fixation in our lakes. However, this approach is very crude and has a strong potential to detect false positives. For instance, any groups positively correlated with dark carbon fixing microorganisms in the microbial nutritional network (such as, heterotrophic microorganisms fed by dark carbon fixation) could be mistakenly identified as dark carbon fixing groups. To mitigate, but not fully eliminate, the risk of false positives, we then searched the literature for evidence for any of the correlating families to be known to harbor potential for ChLithAu. We also considered the following taxa which were previously detected in boreal lakes and assumed chemolithoautotrophic due to their genetic potential for dark fixation: *Ferrovaceae*, *Nitrospiraceae,* and *Hyphomicrobiaceae* (Taipale et al., 2011; Peura et al., 2018).

3. Results

3.1. Carbon fixation

Dark carbon fixation was detected in 16 out of the 17 study lakes (Fig. 1). This is based on sampling depths for which we had a full set of

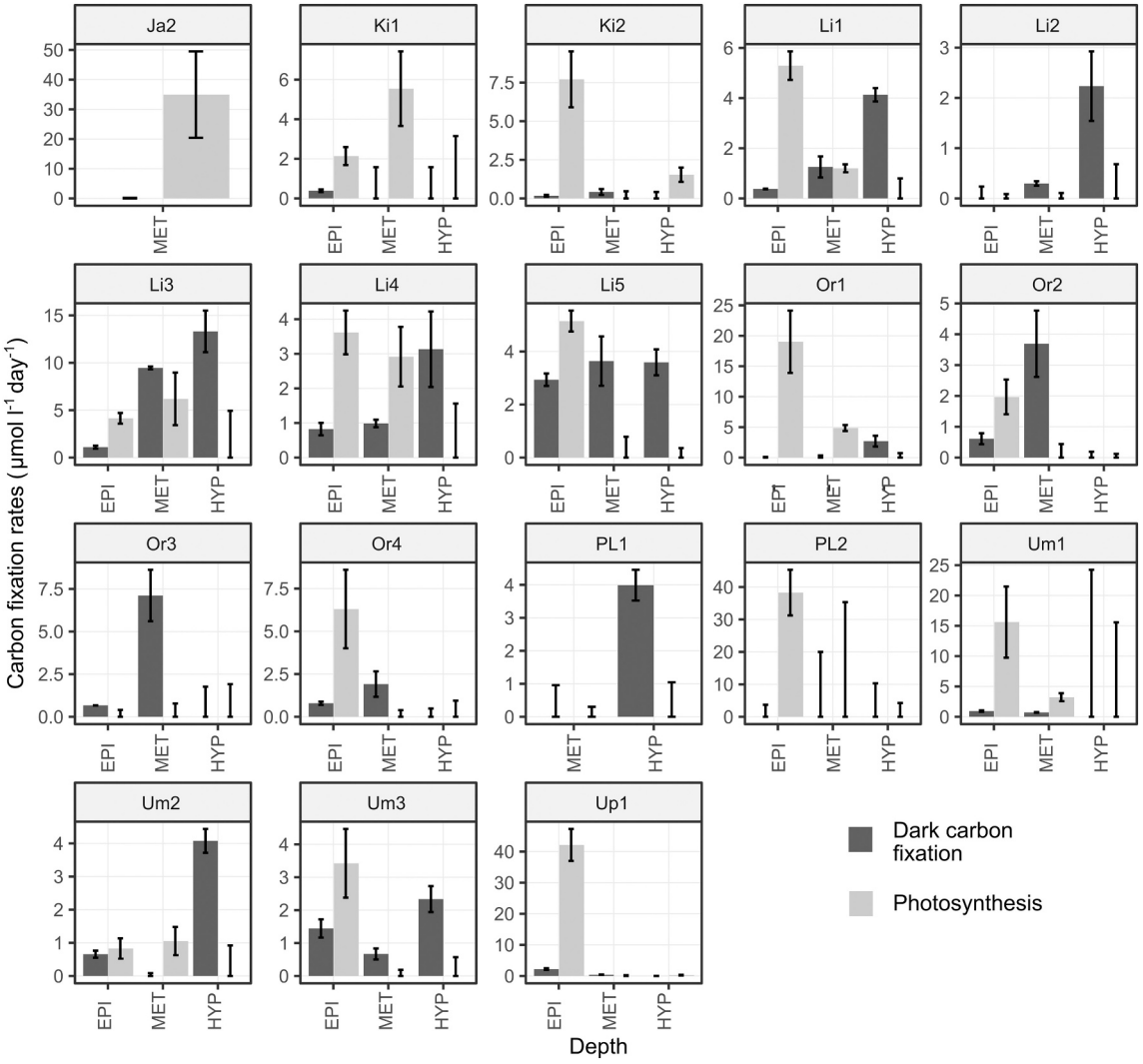

Fig. 1. Photosynthetic and dark carbon fixation rates
Mean carbon fixation rates of biological replicates of ^{14}C incubation essays. Net values are presented. For the dark carbon fixation this is the difference between the rates measured in the dark and the killed control. For photosynthesis, this is the difference between the rates measured in the light and the dark carbon fixation. If the difference between the two treatments was not significant ($p > 0.05$), the C incorporation rate was set to 0. The error bars represent the standard error. For null values only the upper bar was represented for better readability. Each vignette represents a different lake, apart from PL1 and PL2 that present values for the same lake (PL) sampled twice. EPI, MET, and HYP refer to the epilimnion, the metalimnion, and the upper hypolimnion, respectively, as described in the materials and methods. Please note the differences in y-axis scales between the lakes.

triplicate data. Daily incorporation rates of C in the dark ranged from 0.17 µM to 13.31 µM. The highest dark carbon fixation rates within a lake were mostly found in the hypolimnion (8 lakes) followed by the metalimnion (5 lakes). Photosynthesis was detected in all but two lakes, and the highest values were generally found in the epilimnion (11 lakes). Photosynthesis was detected in the hypolimnion of only two lakes. When dark fixation was detected, both the dark carbon fixation incorporation rates and the ratio of dark carbon fixation of the total primary production increased with depth (Fig. 2). The median dark carbon fixation incorporation rates ranged from 1 µM in the epilimnion to 3.6 µM in the hypolimnion ($p < 0.005$), whereas the median proportion of the primary production due to dark carbon fixation ranged from 18.5 % in the epilimnion to 81.4 % in the hypolimnion ($p < 0.0005$, Fig. 2).

Dark carbon fixation rates were moderately to strongly correlated (rho $\geq|0.5|$) with O_2, temperature, CO_2, CH_4, DOC, DN, and Fe^{total} (Fig. 3). The correlations of dark carbon fixation with O_2 and of dark carbon fixation with temperature were negative, and all the other correlations were positive. Photosynthesis was only correlated positively with O_2 and temperature (rho $\geq|0.5|$). Dark carbon fixation was strongly correlated to Fe^{total} (rho $\geq$0.8.), but only weakly to Fe^{2+} and Fe^{3+} (rho = 0.4 for both).

3.2. Microbial community composition

After rarefaction, 2.2*10^8 reads were attributed to 3516 genera grouped in 693 families. Based on PERMANOVA analysis, community structure was mostly influenced by the lake and location ($R^2 \approx 0.3$ for each) and the layer of sampling ($R^2 \approx 0.2$). We did not detect any significant difference between the communities at sites where dark carbon fixation was detected and the communities at sites lacking dark carbon fixation. Based on Mantel test, only CH_4, temperature, redox potential (ORP) and CO_2 had a correlation factor suggesting at least a weak correlation with the microbial community structure ($R^2 > 0.2$, Table 2). In contrast, there was no correlation between dark carbon fixation rates and the microbial community structure.

We found 47 out of 693 families with read-based abundances correlating (rho $\geq$0.5) with dark carbon fixation rates (Table 3). Among

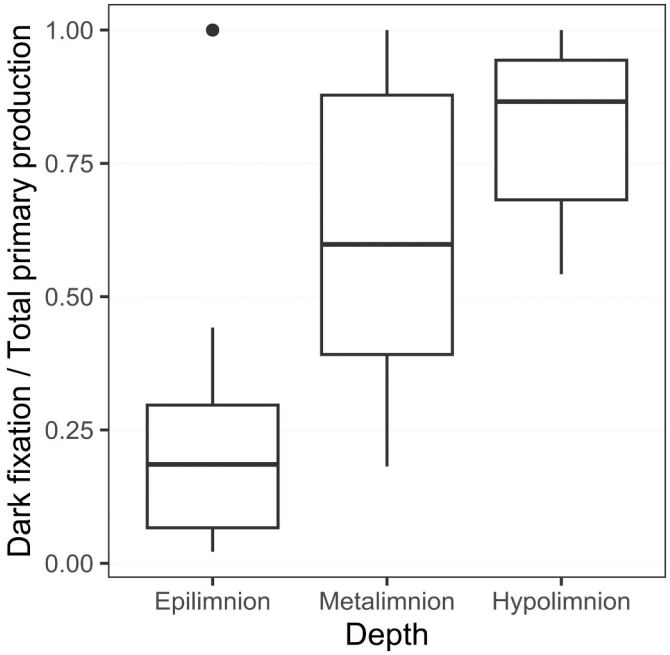

Fig. 2. Proportions of dark fixation of all primary production
For each depth, the ratio of the net dark fixation /net primary production across all lakes is presented. Only sites (i.e. depth x lake) where both dark fixation and primary production were detected are presented. If the calculated ratio was higher than one, the value was set to 1.

the families of interest, 16 families are known for harboring ChLithAu taxa. Based on median abundances in the normalized data set, the families of interest with the highest numbers of reads attributed to it were *Methylococcaceae*, *Chromatiaceae,* and *Gallionellaceae*. When maximum abundances were considered, *Methylococcaceae* was still the most abundant family of interest, followed by *Holophagaceae* and *Geobacteraceae*. *Gallionellaceae* and *Chromatiaceae* were also among the most abundant families with known ChLithAu taxa and were in 4th and 6th position, respectively (Fig. 4 and Supplementary Fig. S1).

When only the potential ChLithAu revealed by correlation with dark carbon fixation were considered, the community structure was grouped by lake, area of origin, and layer. However, unlike the total community, the grouping by layer was stronger ($R^2 \approx 0.3$) than the lake and location effect ($R^2 = 0.23$ and 0.17, respectively). When the ChLithAu families defined based on literature (i.e., *Ferrovaceae*, *Nitrospiraceae,* and *Hyphomicrobiaceae*) were added to the analysis, lake and general location accounted for approximately 60 % of the effect ($R^2 = 0.26$ and 0.30, respectively), and depth accounted for 20 %. Based on Mantel test, the distance between ChLithAu communities was mostly correlated with CH_4, CO_2, and Fe^{2+} ($R^2 > 0.2$), but no variable had a strong or medium strength correlation ($R^2 > 0.5$) with the change in the community composition. When the literature-based ChLithAu were added to the analysis, R^2 values for CO_2 and Fe^{2+} decreased. When *Ferrovaceae* was included in the ChLithAu list, there was a significant, although weak, correlation between the community composition and pH ($p < 0.005$, $R^2 = 0.18$).

The sum of the potential ChLithAu abundances correlated with the same environmental variables as dark carbon fixation (Fig. 5). One major exception was dissolved N that was not correlated at all with the sum of all ChLithAu revealed by correlations (Table S2). When *Ferrovaceae*, *Nitrospiraceae,* and *Hyphomicrobiaceae* were included, only CH_4 concentration, temperature, pH and O_2 concentration were correlated with the abundance of potential ChLithAu. Correlations between the abundances of the putative ChLithAu families with environmental variables varied a lot (Fig. 5, Table S2). The strongest covariables to *Ferrovaceae* were pH and CH_4, whereas *Gallionellaceae* correlated well with CH_4, Fe^{2+}, Fe^{total}, CO_2, temperature, and O_2 (Fig. 5, Table S2).

4. Discussion

4.1. High prevalence of dark carbon fixation in Swedish lakes

The presence of dark carbon fixation in most lakes was expected, but more surprising was the absence of it on two occasions. Although it is possible that some boreal lakes do not harbor dark carbon fixation, it seems unlikely that it would be the case for the lakes we tested. All lakes, including the ones without active dark carbon fixation, have similar conditions (oxygen stratification, strong coloration, small area), and all tested lakes harbor the same potential ChLithAu, albeit relative abundances were lower when no active dark carbon fixation was detected. Dark carbon fixation was detected in Lake Plåten (PL) during the first sampling session, but not during the second sampling later in the summer. This suggests that dark carbon fixation activity might not be constant throughout the ice-free season of boreal lakes. Substantial mixing events that can affect the metalimnion are common (Kuha et al., 2016) and could explain the apparent drop in dark carbon fixation in Lake Plåten. Furthermore, the only lake for which we do not have proof of dark carbon fixation activity (lake Ja2) was sampled relatively early in the season, less than two months after the ice-off of a major lake in the area. Consequently, it is possible that the observed stratification in the lake was fresh (Mammarella et al., 2018), and the ChLithAu community thriving in anoxic/microoxic conditions was not large enough for significant dark carbon fixation rates. This could also explain the below average dark carbon fixation rates observed in the lakes sampled at Norrbotten West (Ki1 and Ki2), as these also were sampled about two months after ice-off. Our dataset does not allow us to assess the seasonality of dark carbon fixation, but it would not be surprising to see variation in both dark carbon fixation rates and ChLithAu community composition across seasons. It has been documented that microbial community (Lew et al., 2015) as well as CO_2 concentrations (Huotari et al., 2009) vary during the ice-free season. However, regardless of the results of Lake Ja2, our data clearly shows that dark carbon fixation is a common process in stratified boreal lakes.

4.2. ChLithAu strive in cold and oxygen-limited water

The negative correlation of dark carbon fixation with oxygen concentration is in line with the fact that ChLithAu are known to favor micro-oxic environments (Camacho et al., 2001; Emerson et al., 2013). It seems therefore likely that the observed correlation between O_2 and dark carbon fixation reflects a dichotomic separation between samples from the oxic epilimnion, and hypoxic-to-anoxic metalimnion and hypolimnion. This dichotomy is visible in Fig. 3, where all samples with high O_2 and low dark carbon fixation grouped in one corner of the plot. It is also evident in the PERMANOVA results, suggesting that the community, including the ChLithAu guild, is shaped by the depth layers as previously described (Peura et al., 2012; Sakai et al., 2014; Alfreider et al., 2017; Diao et al., 2017). The impact of O_2 on the community does not necessarily mean that dark carbon fixation rates are stimulated by low O_2. This idea is reinforced by the absence of correlation between dark carbon fixation rates and ORP, suggesting that lower oxidation potential is not significantly correlated to higher dark carbon fixation rates. Low O_2 concentration is crucial for coexistence of oxidized and reduced compounds (Maisch et al., 2019), but did not seem to directly affect dark carbon fixation. The concept of an environmental variable reflecting habitat conditions rather than the preferences of ChLithAu is also relevant for temperature. Higher temperatures are expected in the upper layers that are in contact with the warm summer atmosphere and under sun exposure, while chemocline, where ChLithAu are expected to be found, depends on the absence of light, making it also likely to be colder than the surface. Thus, we cannot know whether the ChLithAu have a specific preference for cold conditions, or if it is rather the

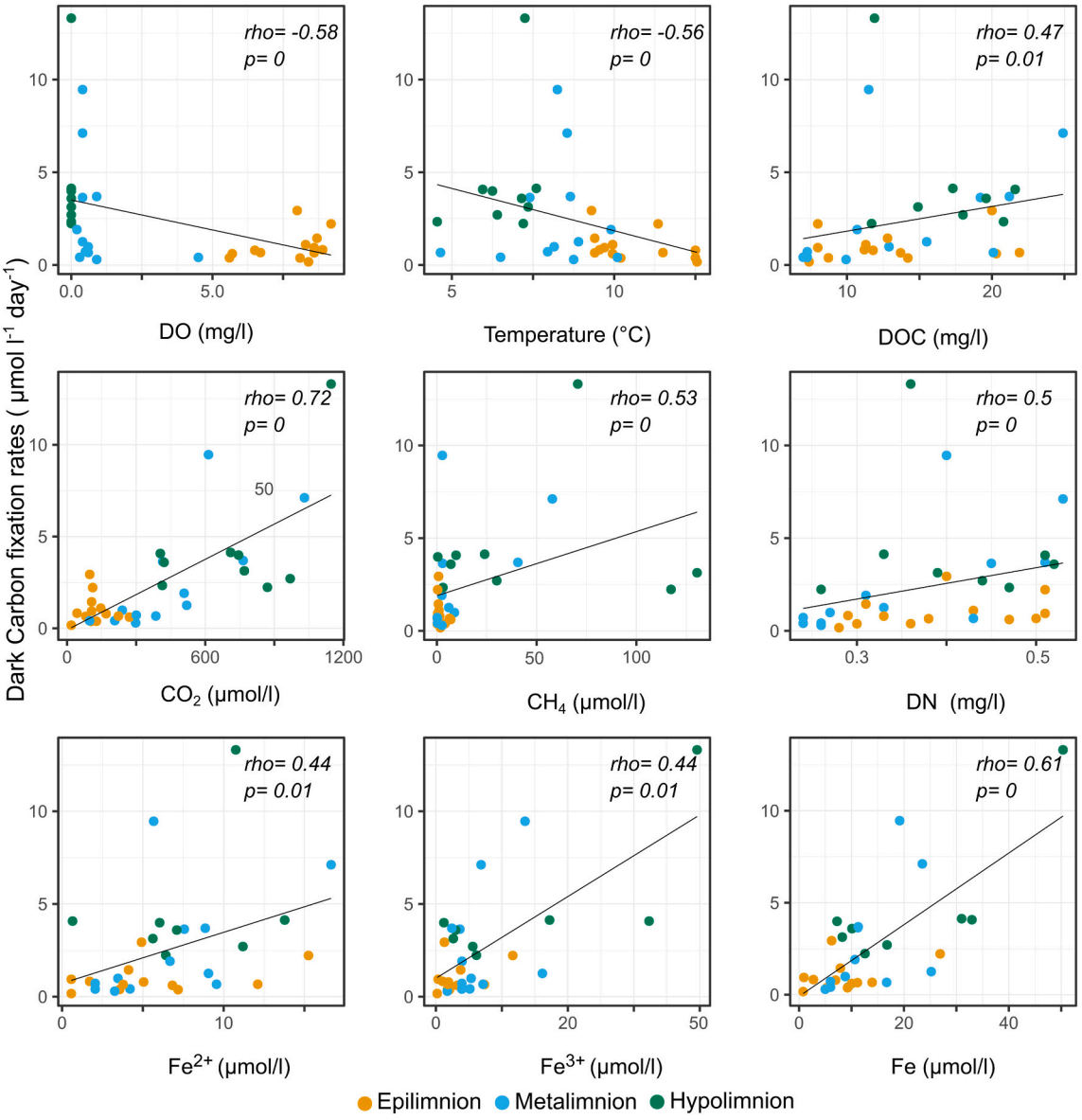

Fig. 3. The relationship between dark carbon fixation rates and selected environmental variables.
Plots presenting all significant correlations between dark carbon fixation and environmental variables (Spearman rank correlation; all variables with rho ≥0.5 are presented), as well as the relationship between dark carbon fixation and the different forms of iron (rho shown in the plots). DO = dissolved oxygen, DOC = dissolved organic carbon, DN = dissolved nitrogen. Exact values for each environmental and experimental variable are available in Supplementary Table S1.

optimum oxygen, nutrient, and redox conditions driving their location in cold water. In any case, in our study, we found ChLithAu to be active at lower temperatures.

The influence of location and depth on the microbial community is not surprising, as this is what is generally observed in multi-lake and depth studies (Sakai et al., 2014; Alfreider et al., 2017; Diao et al., 2017). What is more unexpected is that there was no measurable community change associated with dark carbon fixation. Our data suggest that there is a subset of ChLithAu that are present in most lakes and correlate well with dark carbon fixation rates. This subcommunity of potential ChLithAu seems to be influenced by the same variables as the total community. Although their relative abundances vary from lake to lake, the layer where they were sampled is more influential. On the other hand, we showed that there are some potential ChLithAu that were present in all lakes but did not correlate with dark carbon fixation. When those were considered, the lake of origin was a stronger factor of community separation than the layer. This suggests that some ChLithAu are active only in certain lakes when conditions are favorable for them. For example, in the case of *Ferrovaceae*, pH could be a key factor. *Ferrovaceae* relative abundance was not only correlated with pH, but its addition to ChLithAu community was also sufficient to turn the whole community sensitive to pH. Observations from our data suggest that *Ferrovaceae* is most abundant when pH is below 5, which is in accordance with the literature suggesting that this taxon is found in acidic environments (Johnson and Hedrich, 2013). This would also explain why *Ferrovaceae* did not come up as a potential ChLithAu in correlations with dark carbon fixation.

4.3. Correlation with dark carbon fixation helped but was not perfect at detecting potential ChLithAu

Searching for taxa which had read abundance correlated with dark carbon fixation was intended as a crude tool to detect potential ChLithAu, as creating an exhaustive list of ChLithAu taxa based on literature seemed unrealistic. Consequently, it is not surprising to see their summed abundance correlating with the same variables as dark carbon

Table 2
Mantel test results.

Variable	R^2	p-value	R^2	p-value	R^2	p-value
	Full community		ChLithO (corr)		ChLithO (all)	
O_2 (mg/l)	0,12	0,04	0,19	0	0,14	0,03
pH	0,04	0,31	0,05	0,18	0,18	0
ORP	**0,23**	**0,01**	**0,16**	**0**	**0,08**	**0,07**
SO_4 (µg/l)	0,2	0,02	-0,02	0,59	0	0,42
NO_3 (µg/l)	-0,01	0,48	-0,07	0,9	-0,03	0,66
PO_4 (µg/l)	0	0,47	-0,05	0,72	-0,06	0,74
NH_4 (µg/l)	0,02	0,33	0,05	0,21	0,07	0,17
DOC (mg/l)	0,14	0,01	0,12	0,02	0,13	0,01
DN (mg/l)	-0,05	0,84	0,05	0,23	0,04	0,22
CO_2 µM	**0,34**	**0**	**0,3**	**0**	**0,22**	**0**
CH_4 µM	**0,17**	**0,06**	**0,42**	**0**	**0,44**	**0**
Fe^{2+} µM	0,16	0,04	0,26	0	0,18	0,01
Fe^{3+} µM	0,08	0,2	0,1	0,07	-0,01	0,52
Fe µM	0,12	0,1	0,2	0	0,08	0,11
Day length	0,2	0,01	-0,06	0,84	-0,02	0,58
Temperature	**0,29**	**0**	**0,19**	**0**	**0,04**	**0,21**
α 420	0	0,46	0,02	0,32	0,03	0,26

Results of Mantel tests comparing the variance of the Bray-Curtis distances between communities and the Euclidian distances for environmental variables. Presented here are the results for the total prokaryote community (full community), the ChLithAu community based on the taxa correlating with dark carbon fixation (ChLithAu (corr)), and the ChLithAu community including taxa from literature ChLithAu (all). Variables with an R2 above 0.2 are highlighted in bold.

fixation. But interestingly, the correlations with read abundances were not as good as for dark carbon fixation rates. This could be a statistical issue as the values used are not absolute, but relative abundances (Gloor et al., 2017). Consequently, a higher or lower abundance of an unrelated taxon can have a strong impact on the relative abundances of ChLithAu reads. But even if we had absolute abundance values, the observed differences between correlation coefficients for dark carbon fixation and taxa abundance would not necessarily be surprising as the abundance and activity are not necessarily correlated (De Vrieze et al., 2016). Another issue is that the two most abundant ChLithAu families on the list (i.e., *Gallionellaceae* or *Chromatiaceae*) are among the least correlated. While *Gallionellaceae* is known as a family of iron-oxidizing ChLithAu, the family also has members with other types of metabolisms (Huang et al., 2022). Thus, it is unclear what portion of the reads mapping to this family are really related to organisms with potential for ChLithAu. Similarly, *Chromatiaceae* is a broad family harboring several genera and species with chemolithoautotrophic capacity as well as phototrophs and heterotrophs. So even if the most abundant genera of *Chromatiaceae* in both the metalimnion and hypolimnion samples are indeed potential ChLithAu, the lack of correlation is not surprising.

Furthermore, not all of the taxa correlating with dark carbon fixation were potential ChLithAu. For instance, the most abundant family with correlation to dark carbon fixation was *Methylococcaceae*. As methanotrophs, *Methylococcaceae* are expected members of an active chemocline (Rissanen et al., 2020) and also could contribute significantly to the high CO_2 concentrations observed in dark carbon fixation hotspots (Reis et al., 2022). While not in contradiction with high CO_2 concentration driven by heterotrophic respiration, it is an alternative hypothesis worth considering. The correlation of *Methylococcaceae* abundance with dark carbon fixation could also be the product of cofounding factor with iron considering that methanotrophs might use iron as an electron acceptor (He et al., 2019). Additionally, there were other examples of abundant taxa correlating with dark carbon fixation and associated with the Fe cycle, not associated with any known ChLithAu (Table 2).

Although spotted for their correlations with dark carbon fixation, none of the potential ChLithAu on the list was perfectly correlated with dark carbon fixation, and neither was their collective abundance. Besides the calculation bias mentioned previously, a key reason appears to be that some relevant potential ChLithAu were not correlating at all with dark carbon fixation. That was the case for *Ferrovaceae*, which has previously been described as a potential ChLithAu in boreal lakes (Peura et al., 2018). While not correlated with dark carbon fixation, *Ferrovaceae* was the most abundant ChLithAu taxon in several samples associated with high dark carbon fixation rates, suggesting that it might be a key player in some lakes. More critically this clearly shows that local conditions, like pH, can favor growth of lake-specific ChLithAu. This also highlights the limitation of using correlations for detecting ChLithAu. While the approach allowed us to spot potential organisms responsible for dark carbon fixation, it could also give false positives. Indeed, using correlation as a main tool we risked detecting random co-occurrence types of correlations as well as organisms that correlate with ChLithAu, because they are part of the same food network (e.g. organisms that feed on carbon produced by ChLithAu). We also have to acknowledge that comparing our list of dark carbon fixation correlating organisms with literature is not failproof as organisms taxonomically related to ChLithAu can also be phototrophs or heterotrophs and share similar environmental preferences (He et al., 2019; Tsuji et al., 2020). It is also clear that the list of correlating organisms is not exhaustive. That is, some ChLithAu may only be active under conditions specific to certain lakes or time points, as is the case with *Ferrovaceae*. It is therefore important to consider the presented list of potential ChLithAu as putative. Further studies will be necessary to confirm or reject the role of these putative ChLithAu organisms. Nevertheless, our crude approach offers a valuable insight into communities and guilds associated with dark carbon fixation. However, our data suggest that dark carbon fixation is driven by a diverse community. Part of this community seems to be ubiquitous, while another appears to be lake or time specific. Two of the most abundant potential ChLithAu were taxa associated with Fe, suggesting that Fe oxidation could play an important role in the energy mobilization of stratified boreal lakes (Schiff et al., 2017; Liu et al., 2022).

4.4. Dark carbon fixation correlates with carbon-related variables (DOC, CO_2, α420, and CH_4)

The correlation of dark carbon fixation rates with carbon-related variables (DOC, CO_2, α420, and CH_4) not only gives an insight into which conditions are favorable for dark carbon fixation, but also suggests that waters with a large ChLithAu community and active dark carbon fixation are hotspots for carbon cycling. First, it is worth noting that while epilimnion samples were generally grouped and associated with low values of CO_2 and CH_4, we do not see a clear grouping by depth as in the O_2 plot (Fig. 3). This suggests that the relationship between these C variables and dark carbon fixation is subtler than the one with O_2. The relationship between dark carbon fixation and DOC is particularly interesting as it is not confirmed by the relationship with the α420. These two variables are generally regarded as good indicators of the water color in boreal lakes. The positive correlation with DOC could be interpreted as a sign of ChLithAu preferring dark waters. This would make sense as low light could limit the competition with phototrophs (Thrane et al., 2014). However, the lack of correlation with α420, which is a direct measurement of light absorption, seems to contradict this possibility. This also suggests that the high DOC concentrations associated with high dark carbon fixation are not driven by colored compounds. As these colored compounds are generally associated with allochthonous carbon originating from soil, the discrepancy between DOC and α420 likely reflects the origin of the DOC. As it has been shown that ChLithAu can exudate significant amounts of carbon (Schnaitman and Lundgren, 1965; Borichewski, 1967; Ñancucheo and Johnson, 2010; Emerson et al., 2013), the high DOC associated with high dark carbon fixation rates may be partly produced by ChLithAu. The production and exudation of labile carbon compounds would in turn explain the higher CO_2 concentration in samples with high dark carbon fixation as it could boost heterotrophic respiration (Wickland et al., 2007). This is noteworthy because culture experiments have demonstrated that

Table 3
Bacterial families correlated with dark carbon fixation.

Family	rho	min	median	max	ChLithO	S met	N met	Fe met	Methane	References
Holophagaceae	0,67	0,04	0,11	4,33	no			yes		Coates et al., 1999
Prolixibacteraceae	0,65	0,04	0,15	1,23	no		yes			
Candidatus Brocadiaceae	0,65	0,02	0,03	0,08	yes					Kartal et al., 2008
Williamwhitmaniaceae	0,65	0	0,01	0,03	no					Su et al., 2014
Chrysiogenaceae	0,65	0	0,01	0,02	no	yes	yes			Häggblom & Bini 2014
Crenotrichaceae	0,64	0,01	0,06	0,47	no			yes	Methanotroph	Dworkin et al., 2006; Oswald et al., 2017
Desulfobacteraceae	0,63	0,08	0,11	0,31	yes	yes				Kuever, 2014c
Candidatus Magnetaquicoccaceae	0,62	0	0,01	0,43	yes	yes		yes		Koziaeva et al., 2019
Marinilabiliaceae	0,62	0,03	0,06	0,21	no					
Desulfohalobiaceae	0,61	0,01	0,01	0,03	yes	yes				Kuever, 2014c
Syntrophaceae	0,6	0,03	0,04	0,28	yes	yes				Kuever, 2014c
Melioribacteraceae	0,6	0	0	0,01	no					
Thermoanaerobacterales Family IV. Incer	0,6	0	0	0,01	no	no				
Methanomicrobiaceae	0,59	0,01	0,01	0,03	yes				Methanogen	Balch et al., 1979
Methanospirillaceae	0,59	0	0	0,01	yes				Methanogen	Balch et al., 1979
Methanosarcinaceae	0,59	0,02	0,04	0,07	yes				Methanogen	Balch et al., 1979
Paludibacteraceae	0,59	0,01	0,04	0,58	no					
Syntrophorhabdaceae	0,58	0,01	0,02	0,07	no					Kuever, 2014c
Desulfobulbaceae	0,58	0,04	0,07	0,31	yes	yes				Kuever, 2014c
Syntrophotaleaceae	0,57	0	0,01	0,02	no					
Mariprofundaceae	0,56	0,01	0,01	0,03	yes			yes		Moreira et al., 2014
Rikenellaceae	0,56	0,02	0,04	0,11	no					Graf, 2014
Marinifilaceae	0,56	0,01	0,02	0,09	no					
Methylococcaceae	0,56	0,25	0,85	9,09	no				Methanotroph	Bowman, 2014
Desulfuromonadaceae	0,55	0,03	0,05	0,22	no	yes	yes	yes		Greene, 2014
Syntrophobacteraceae	0,55	0,01	0,03	0,11	yes	yes				Kuever, 2014c
Geobacteraceae	0,55	0,11	0,16	2,18	no	yes		yes		Röling, 2014
Candidatus Methanoperedenaceae	0,55	0	0	0,01	no		yes	yes	Methanotroph	Guerrero-Cruz et al., 2018; Leu et al., 2020
Methanoregulaceae	0,54	0,01	0,02	0,11	no	yes				Oren, 2014
Bacteroidaceae	0,54	0,07	0,12	0,32	?					
Tannerellaceae	0,53	0,02	0,04	0,13	no					Sakamoto, 2014
Desulfonatronaceae	0,53	0,01	0,01	0,02	yes	yes				Kuever, 2014b
Victivallaceae	0,53	0	0	0,08	no					Plugge & Zoetendal 2014
Dysgonomonadaceae	0,52	0,02	0,05	0,14	no					Sakamoto, 2014
Desulfomicrobiaceae	0,52	0,01	0,01	0,04	yes	yes				Kuever & Galushko 2014
Hungateiclostridiaceae	0,52	0,01	0,02	0,05	no					
Sedimentisphaeraceae	0,51	0	0,01	0,04	no	yes				
Heteroscytonemataceae	0,51	0	0	0						Sendall & McGregor 2018
Methanocellaceae	0,5	0	0	0,01	yes				Methanogen	Sakai et al., 2014
Porphyromonadaceae	0,5	0,01	0,03	0,09	no					Sakamoto, 2014
Desulfarculaceae	0,5	0,01	0,01	0,03	no	yes				Kuever, 2014a
Lentimicrobiaceae	0,5	0	0,01	0,07	no					
Gallionellaceae	0,5	0,06	0,25	0,97	yes			yes		Hallbeck & Pedersen 2014
Barnesiellaceae	0,5	0	0	0,01	no					
Pontiellaceae	0,49	0,01	0,03	0,1	no					van Vliet et al., 2020
Haloplasmataceae	0,49	0	0	0						Antunes, 2014
Chromatiaceae	0,49	0,14	0,26	1,04	yes					Imhoff, 2014

List of families with read-based abundances correlating (rho>0.5) with dark carbon fixation. Correlation values (rho) were calculated using Spearman rank correlation. Abundances (min, median, and max) are in %. The ChLithAu column indicates which family harbors known chemolithoautotrophs, including methanogens. X_met indicates if some taxa of the family are known to use the compound X. Families are ordered based on the strength of their correlation (i.e., rho) with dark carbon fixation. "met" in the titles of the columns stands for metabolism.

heterotrophs can enhance ChLithAu activity (Liu et al., 2011; Vyrides et al., 2015; Vardanyan et al., 2017). A similar point can be made for dissolved N (DN), as it also correlated with dark carbon fixation, unlike none of the N mineral forms. This suggests that the DN driving the correlation could be organic, possibly produced and exudated by ChLithAu or other microorganisms taking advantage of dark carbon fixation-derived labile DOC. This fresh DOC and DN could also be produced by phototrophic organisms, particularly certain anoxygenic phototrophs (e.g. the genus *Chlorobium*), that are known to thrive in similar environments as ChLithAu (Camacho et al., 2001). However, this seems unlikely as no correlation was found between DOC and photosynthesis rates. It is not clear why photosynthesis is not correlated with DOC. It could be that photosynthetic organisms are better at keeping their C, but a potential correlation could be hindered by factors that are not as important for ChLithAu. Thus, it seems likely that photosynthesis rates are more stochastic and weather-sensitive. For instance, variations in day length and cloud cover are more likely to impact photosynthesis than dark carbon fixation, which can potentially be active continuously if the chemocline is not perturbed. Finally, the correlation with CH_4 should be analyzed with caution as most samples had a very low CH_4 concentration. While being statistically significant, the correlation appeared to be influenced by only a few samples. However, these samples suggest that pelagic methanogens might be responsible for a part of the dark carbon fixation. This would fit in a growing amount of literature showing that methanogenesis is not restrained to the sediment (Iversen et al., 1987; Karr et al., 2006; Bogard et al., 2014; Biderre-Petit et al., 2019). Overall, our data suggest that dark carbon fixation could play an important role in energy mobilization and nutrient cycling of the boreal lakes. This is particularly relevant as several studies have pointed out that phototrophy and bacterial production could not fully support the carbon

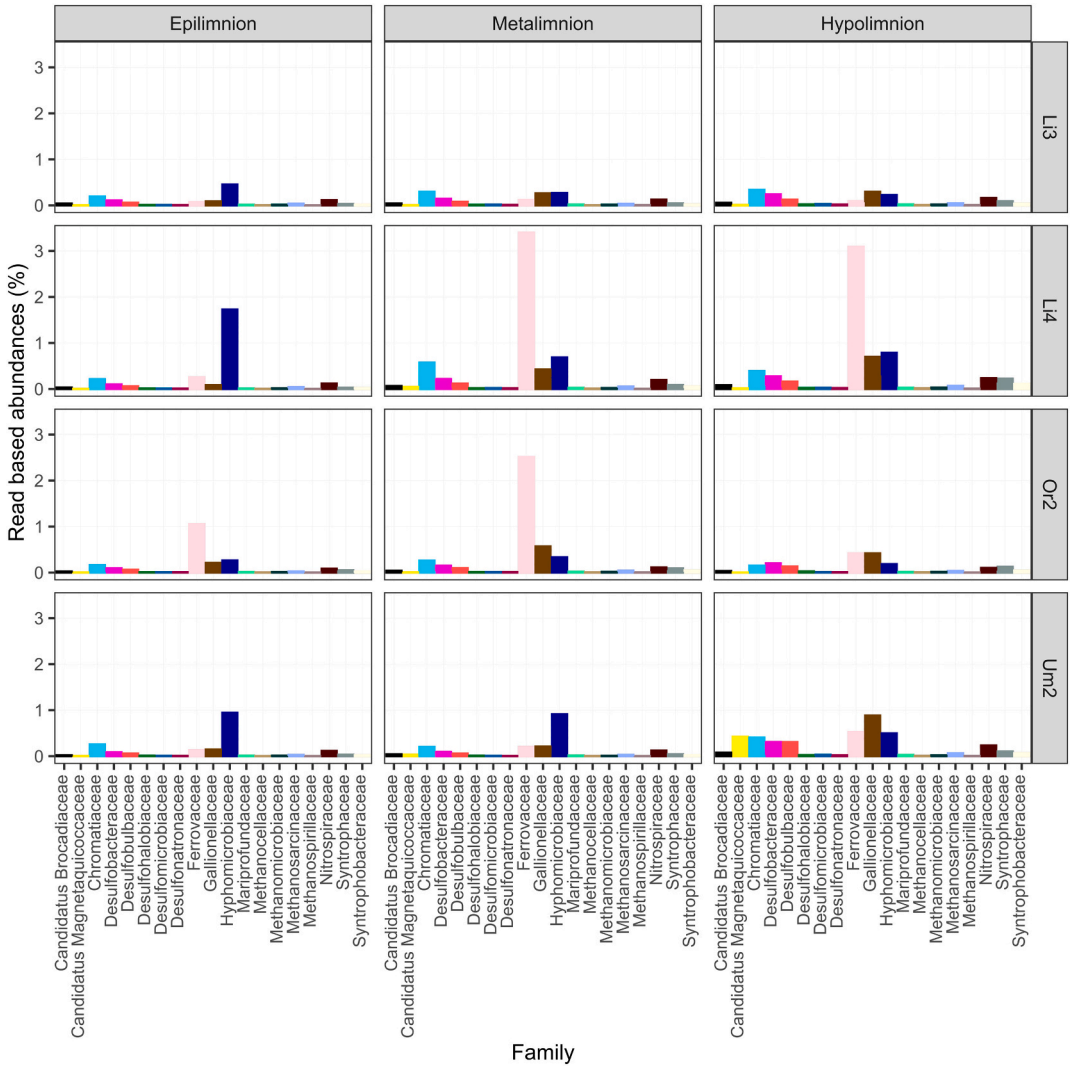

Fig. 4. Relative abundances of putative ChLithAu families in selected lakes
Relative abundances based on reads attributed to each taxon in some of the lakes with high dark carbon fixation rates. Values are in % of the total normalized prokaryotic read counts for each layer. The lakes that are not shown here can be seen in Supplementary Fig. S1.

demand of bacteria (Salonen et al., 1992; Kankaala et al., 2013). Thus, the data point towards the dark carbon fixation being the missing link complementing allochthonous C as a carbon source for the food web. Not only is dark carbon fixation common in boreal lakes, but it also seems to represent an important part of the total primary production (Fig. 2). It is hard to estimate the actual thickness of the most active dark fixation depth and it seems unlikely that the values measured are relevant for the whole hypolimnion. Similarly, the actual thickness of the metalimnion is not straightforward to estimate. However, several studies have shown that metalimnic carbon incorporation in stratified lakes, while highly variable, can represent up to 87 % of the total daily gross primary production of a lake (Camacho and Vicente, 1998; Casamayor et al., 2012; Giling et al., 2017), and that dark carbon fixation alone can represent >50 % of the total primary production of a lake (Camacho et al., 2001). In this study by Camacho et al. (2001) the ratio of dark fixation to photosynthesis at the chemocline was in line with our measurements. However, unlike the ratio in the epilimnia of boreal lakes we studied, they observed no or little contribution of dark fixation in the euphotic epilimnion. This overall suggests that dark carbon fixation may be an important, even if only temporary, contributor to primary production specifically in stratified boreal lakes. However, although our data demonstrate that dark carbon fixation is prevalent in boreal lakes across Sweden, further studies are needed to properly estimate the contribution of dark carbon fixation to the carbon budget of boreal lakes.

4.5. Iron is the most likely source of energy and electrons for dark carbon fixation

Peat and bog lakes are generally associated with low N content, and boreal lakes appear to display a low S concentration during summer stratification (Schiff et al., 2017). On the other hand, Fe is an important factor in brownification and also plays an important role in the biogeochemistry of boreal lakes (Pokrovsky et al., 2012; Weyhenmeyer et al., 2014; Xiao and Riise, 2021). Among the elements that are potential sources of electrons for dark carbon fixation (i.e., N, S, and Fe) the only strong and significant correlation was with Fe, supporting our hypothesis that dark carbon fixation in boreal lakes is associated with iron cycling. The correlation between all the measured iron species (Fe^{2+}, Fe^{3+} and, total Fe) suggests a quick and balanced cycling of the element. If reduction or oxidation were to dominate, we would expect an anticorrelation between Fe^{2+} and Fe^{3+}, as the production of one would deplete the stock of the other. The fact that both forms of iron correlated with dark carbon fixation suggests that higher Fe concentrations were driving higher dark carbon fixation rates. Surprisingly high concentrations of reduced iron (Fe^{2+}) were measured in the oxic epilimnion. This

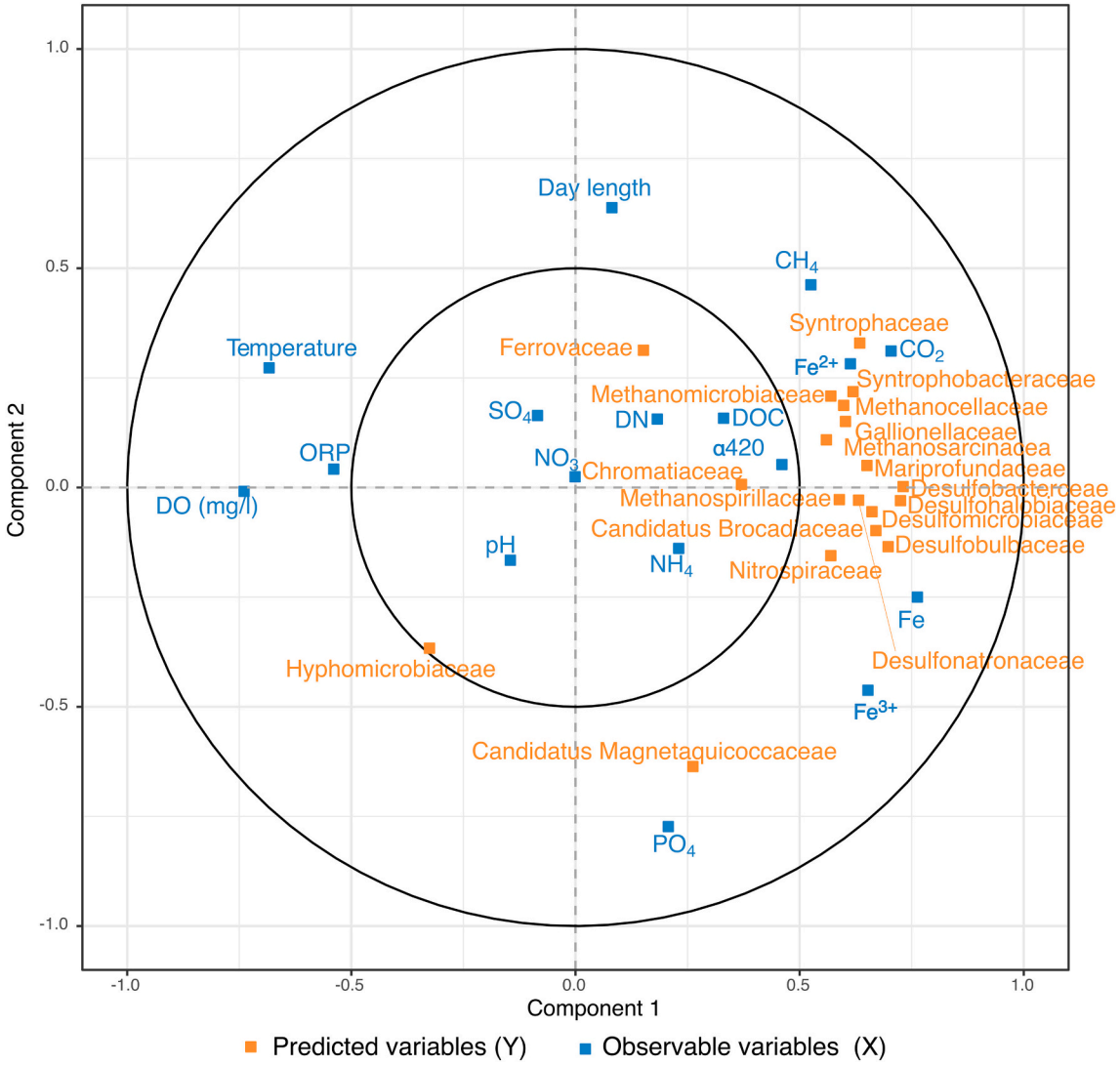

Fig. 5. Correlation plot of the Partial Least Squares (PLS) regression analysis evaluating the relationship between environmental variables and the abundance of potential ChLithAu families Component axes represent latent variables (i.e., a linear combination of the original observed variables). Positions of the observed variables (X) indicate how much each X variable explains the component axis. Positions of the predicted variables (Y) indicate how much of each Y variable variance is carried by the latent variable. The distance to the axis indicates the strength of the correlation between the variables (X or Y) and the latent variables (component 1 or 2). More detailed values for the correlation between the abundances of the families and environmental variables can be found in Supplementary Table S2. DO = dissolved oxygen, ORP = redox potential, DOC = dissolved organic carbon, DN = dissolved nitrogen, and α420 = the light absorbance at 420 ηm.

could be the product of photoreduction (Mcknight et al., 1988) and would also explain why lower concentrations of Fe^{3+} were observed in the epilimnion than in the hypolimnion (Fig. 3). If Fe^{2+} is the main substrate for dark carbon fixation in boreal lakes, photoreduction of Fe could sustain the dark carbon fixation that we detected in the oxic and euphotic epilimnion of several boreal lakes, whereas previous studies in an alpine lake lacking similar Fe metabolism could not detect dark carbon fixation in the epilimnion (Camacho et al., 2001). It could also explain why the correlation of dark carbon fixation with redox potential was weak as photoreduction could allow the coexistence of ferrous iron and oxygen in highly oxidative conditions. Alternatively, the dark carbon fixation could be fueled by the high levels of DOM in boreal lakes, as a recent study suggests that DOM could play a role in both extracellular electron transfer (Olmsted et al., 2023), and reductive pathways (Ross et al., 2011; Rowe et al., 2021). However, there is no proof that this happens in natural conditions as the DOM-induced reduction of CO_2 observed in Ross et al. (2011) and Rowe et al. (2021) depended on an electrode as an electron source in controlled laboratory conditions.

Iron and DOM are important factors in lake brownification (Pokrovsky et al., 2012; Weyhenmeyer et al., 2014; Xiao and Riise, 2021). Brownification is one of the main causes of the expected increase in the GHG emissions of boreal lakes as it is expected to limit light availability for photosynthesis (Kritzberg et al., 2020), while increasing the duration of hypoxia and anoxia in lakes (Couture et al., 2015). The relationship between iron and dark carbon fixation that we observed therefore suggests that Fe-fueled dark carbon fixation could play a role in mitigating future GHG emissions from boreal lakes. Indeed, brownification could lead to conditions favorable to dark carbon fixation (i.e. more iron, anoxic hypolimnion). However, further research will be needed to understand the relationship between dark carbon fixation, iron, and brownification in boreal lakes. While our work did not investigate brownification, it does suggest a strong link between dark carbon fixation and variables associated with brownification, such as, iron, DOM, and very low levels of oxygen. To confirm this hypothesis future work will need to investigate the link between brownification and dark carbon fixation and to test the temporal dynamics of dark carbon fixation and iron cycling throughout the seasonal dynamics of mixing and stratification typical of boreal lakes.

5. Conclusions

This study demonstrated that dark carbon fixation is widely present in stratified boreal lakes and is responsible for an important share of the total primary production of these lakes, particularly in the deeper darker water. Our study also suggests that dark carbon fixation is driven by a diverse set of microbes, some of which are somewhat ubiquitous, such as *Gallionellaceae or Chromatiaceae*, and some of which seem to be linked to more specific lake conditions, such as *Ferrovaceae*. While our work shows the potential ubiquity of dark carbon fixation in boreal lakes, it lacks a time component. Our work suggests that dark carbon fixation rate may be lower in the early ice-free season before a stable stratification is formed or when the stratification is disrupted, but further research will be needed to confirm this hypothesis. Similarly, nothing is known about the situation during winter when the ice cover limits the light. Those questions are critical as the potentially ubiquitous presence of ChLithAu in small boreal lakes could be of major importance for understanding energy mobilization and mitigation of GHG emissions from these lakes. Furthermore, with lake browning on the rise, a better understanding of iron-based dark carbon fixation seems particularly relevant, as in some lakes primary production rates were highest in light-limited layers that are dominated by dark carbon fixation. ChLithAu communities and dark carbon fixation in boreal lakes could, hence, help compensate for decreasing photosynthesis as DOC and Fe concentrations increase. Finally, our work also highlights the importance of considering other forms of primary production than photosynthesis and offers a new path to explain the discrepancies in the carbon budgets of boreal lakes.

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CRediT authorship contribution statement

Gaëtan Martin: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antti J. Rissanen:** Writing – review & editing, Supervision. **Sarahi L. Garcia:** Writing – review & editing, Supervision. **Sari Peura:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All sequences are deposited in the European Nucleotide Archive (ENA, mirrored to SRA, and accessible at the NCBI) under the project numbers PRJEB38681 (Buck et al. 2021) and PRJEB76863.

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