

Archaea and Bacteria in deep lake hypolimnion: *in situ* dark inorganic carbon uptake

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ABSTRACT

The interest for microorganisms inhabiting the hypolimnion and for their role in biogeochemical cycles of lakes is considerable, but knowledge is far from complete. The presence of chemolithoautotrophic Bacteria and mesophilic Archaea (e.g., Thaumarchaeota) assimilating inorganic carbon in the deep hypolimnion of lakes has been ascertained. We measured, for the first time at 350 m in Lake Maggiore (Northern Italy), the prokaryotic *in situ* dark [¹⁴C]HCO₃ incorporation with a new custom-made apparatus, which takes samples and adds tracers *in situ*. Thereby stress factors affecting prokaryotes during sample recovery from the depth were avoided. We tested the new instrument at different depths and conditions, performing parallel conventional on board incubations. We found that dark [¹⁴C]HCO₃ incorporations had lower standard deviation in *in situ* incubations with respect to the on board ones, but their means were not statistically different. At 350 m we estimated an uptake of 187.7±15 μg C m⁻³ d⁻¹, which is in line with the published uptake rates in aquatic systems. By inhibiting the bacterial metabolism, we found that Archaea were responsible for 28% of the total CO₂ uptake. At the same depth, Thaumarchaeota, on average, constituted 11% of total DAPI counts. Dark [¹⁴C]HCO₃ incorporation integrated along the aphotic water column was 65.8±5.2 mg C m⁻² d⁻¹ which corresponds to 87% of picophytoplanktonic autotrophic fixation in the euphotic layer. This study provides the first evidence of Bacteria and Archaea dark CO₂ fixation in the deep hypolimnion of a subalpine lake and indicates a potentially significant prokaryotic CO₂ sink.

Key words: Archaea and Bacteria, Thaumarchaeota, dark inorganic carbon uptake, Lake Maggiore, hypolimnion.

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INTRODUCTION

The hypolimnion of deep lakes is a significant freshwater volume, which has received attention in the early 2000s as a sensitive indicator for climate warming (Ambrosetti and Barbanti, 1999; Dokulil *et al.*, 2006). Since most studies have centered on epilimnetic microbes the knowledge on prokaryotes inhabiting the dark hypolimnion is comparatively scarce (Newton *et al.*, 2001; Okazaki *et al.*, 2013; Salcher *et al.*, 2011). In particular, the ecological function of hypolimnetic prokaryotes in the carbon cycle are so far understudied.

The presence of mesophilic Thaumarchaeota (formerly: Crenarchaeota) (Brochier-Armanet *et al.*, 2008) has been ascertained in the deep oxygenated hypolimnion of Crater Lake (589 m) (Urbach *et al.*, 2001), of Lake Maggiore (372 m) (Callieri *et al.*, 2009) and in the moderately deep (73 m) high altitude Lake Redon (Auguet *et al.*, 2012). Thaumarchaeota have also been found in the epilimnion and upper hypolimnion (~50 m) of Laurentian Great Lakes in North America, African Lake Victoria, and Lakes Ladoga and Onega in Northeastern Eurasia (Keough *et al.*, 2003), and in a hypertrophic saline lake (La Cono *et al.*, 2013). Moreover, Thaumarchaeota are widespread among marine systems, e.g., in the Pacific

Ocean (Karner *et al.*, 2001), in the Atlantic Ocean (Teira *et al.*, 2006; Schattnerhofer *et al.*, 2009), and in the Mediterranean Sea (Yakimov *et al.*, 2011).

In the 16S rRNA-based tree Thaumarchaeota clearly separate from Euryarchaeota and comprise, among others, Marine Group I (MGI.1a), which are commonly found in oceanic and freshwater plankton (formerly 1.1a Crenarchaeota) (Pester *et al.*, 2011). Members of the MGI.1a clade were abundant in deeper water layers of a high-altitude lake during stratification whereas the clade SAG-MGC-1, closely related to MGI.1a, was retrieved in high densities in the neuston and in surface water layers during winter (Auguet *et al.*, 2012).

The discovery of putative genes for the ammonia-monooxygenase alpha-subunit (*amoA*) exclusive for Thaumarchaeota (Venter *et al.*, 2004) gave a hint to their ecological importance, by emphasizing their role in the first step of nitrification and their possible competition with nitrifying Bacteria. Even though *amoA* gene frequency hints to the ecological function of ammonia-oxidizing prokaryotes, it does not demonstrate their metabolic activity and gives only very indirect evidence of their role in carbon cycling. A more direct estimate of prokaryotic activity can be achieved by measurements of

tracer incorporation, such as dark $[^{14}\text{C}]\text{HCO}_3$ assimilation. Inorganic carbon assimilation can be performed by chemoautotrophs affiliated with Bacteria and Archaea, albeit using different pathways. Chemolithoautotrophic Bacteria can incorporate CO_2 in the dark through a variety of carboxylation reactions to fulfill diverse metabolic requirements such as anaplerotic demands or synthesis of fatty acids, nucleotides and amino acids (Casamayor *et al.*, 2012). Thaumarchaeota can use the hydroxypropionate–hydroxybutyrate carbon assimilation pathway and reduced nitrogen compounds as energy source, fueling the autotrophic metabolism (Berg *et al.*, 2007).

To date, all dark CO_2 fixation measurements in hypolimnia of deep lakes or in oceans have been done by taking samples and incubating on deck or in incubation chambers (Herndl *et al.*, 2005; Yakimov *et al.*, 2011). This procedure hampers an accurate assessment of the activity of the deep layers, since changes in pressure, temperature, and light exposure may cause stress on prokaryotic communities. Therefore we invested in planning and constructing a device for *in situ* incubations, and measured dark $[^{14}\text{C}]\text{HCO}_3$ incorporation. On board measurements were performed in parallel and used in order to test the instrument in different conditions. Our apparatus was designed for *in situ* incubation and therefore avoids the decompression of the samples from great depths as it automatically takes water-sample, adds the tracers and stays at the chosen depth during the whole incubation time. With this apparatus we assessed the putative archaeal *in situ* dark $[^{14}\text{C}]\text{HCO}_3$ incorporation in the deep oxygenated hypolimnion of a subalpine lake by inhibiting the bacterial metabolism and calculating their contribution to the total CO_2 uptake. At the same depth we counted Thaumarchaeota 1.1a (from now on referred to as Thaumarchaeota) and Bacteria using CARD-FISH and estimated their contribution to prokaryote abundance. For the study, we have selected Lake Maggiore because Thaumarchaeota presence along its water column has already been studied (Callieri *et al.*, 2009) and because the lake is regularly monitored since 1980 (Bertoni *et al.*, 2010).

METHODS

Site description

Lake Maggiore is a large, deep, subalpine lake (212 km^2 , Z_{max} 370 m) located in Northern Italy, and belongs to the Long-Term Ecological Research (LTER) network as Southern Alpine Lake. Long-term physical, chemical and biological data are available (Salmaso *et al.*, 2012). The lake recovered from a eutrophic state, which culminated in the late 1970s, and is now oligo-mesotrophic with total phosphorus around $10 \mu\text{g L}^{-1}$ (Bertoni *et al.*, 2004; Salmaso *et al.*, 2006). Lake Maggiore is holo-oligomictic and the complete turnover only takes place in winters with strong

wind and low air temperatures. Nevertheless the hypolimnion is always oxygenated due to river and littoral water intrusions into deep layers (Ambrosetti *et al.*, 2003). During this study the oxygen profiles showed oxic condition of the water column with concentrations ranging from 8.6 to $12 \text{mg O}_2 \text{L}^{-1}$. The temperature profiles indicated persistence of the stratification from June to October.

In situ and on board incubations

In situ incubations were performed with a new custom-made device (Fig. 1) suspended at the selected depth. The new device was tested by making parallel *in situ* and

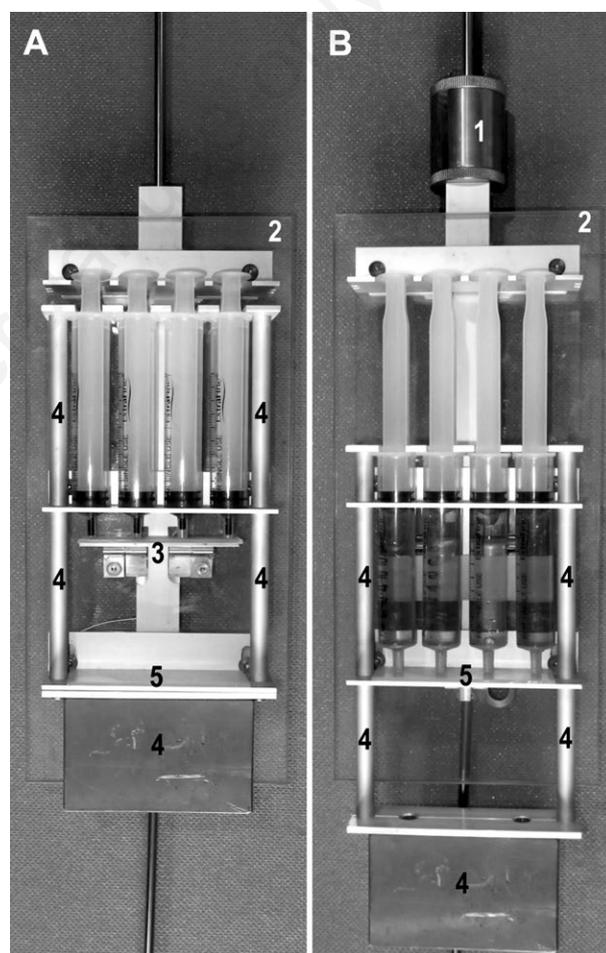


Fig. 1. Custom-made instrument for *in situ* incubation in deep waters (see *Methods* for a detailed description). A) Image of the instrument before sampling with syringes loaded with tracers. B) Image of instrument after sampling and during incubation time, with syringes filled with sample. 1, Messenger; 2, acrylic frame; 3, pivoting frame closing the tips of syringes until the messenger is released; 4, sliding cursor pulling down the bodies of the syringes; 5, fixed frame covered by a silicon layer closing the tip of the syringes after sampling and during the incubation.

on board measurements of $[^{14}\text{C}]\text{HCO}_3$ uptake at different stations, months and hypolimnetic depths in Lake Maggiore (Tab. 1), in the year 2010. The two sampling stations were Ghiffa (45°56'N, 8°38'E) at the deepest point (maximum depth 370 m), and Maggia (46°08'N, 8°46'E) located close to the homonymous river outflow, a source of potential allochthonous input. These different stations, months and depths provided various conditions for testing the instrument. In the following year (2011) total dark $[^{14}\text{C}]\text{HCO}_3$ uptake was measured only *in situ*, at Ghiffa station, at 350 m, to separate the Archaea activity from the total, using appropriate treatments as described in details below.

The device, operated by a messenger, allowed *in situ* sample collection, tracer addition, and incubation in deep waters because it can be maintained at the selected depth. The instrument is composed of an acrylic rectangular frame provided with a syringe holder to accommodate 4 plastic disposable syringes (3 replicates and 1 control) of 50 mL volume. The syringes were prepared in the laboratory by introducing the tracers and the metabolic inhibitors in the replicates and the fixative in the control. After adjusting the plunger of each syringe keeping their content on the edge of the tip, the syringes were placed in the syringe holder and fixed to the frame. The bodies of the syringes were engaged in a sliding cursor and kept in place by a pivoting frame, thus closing the tips of syringes until the beginning of the incubation. The pivoting frame was kept in a fixed position by a trigger mechanism; when the trigger was operated through the release of a messenger, the pivoting frame freed the ends of the syringes. Incubations started when the sliding cursor pulled down by gravity filling the syringes with water samples and adding the tracer to it. The run of the syringe body stopped at a fixed frame covered by a silicon layer. The device was left at the sampling depth suspended to a buoy and recovered after 6 h of incubation.

The samples for on board incubations were taken with a Niskin bottle; tracers and fixative were added immediately after sampling. Incubations were performed in triplicate 50 mL Falcon® tubes kept in thermo boxes pre-filled

with water collected from the same depths and kept at *in situ* temperature in the dark.

Measurements of dark $[^{14}\text{C}]\text{HCO}_3$ uptake

We used labeled $[^{14}\text{C}]\text{HCO}_3$ with a specific activity of 52 mCi mmol⁻¹ (PerkinElmer, Life Sciences) according to the protocol of Steeman-Nielsen (1951). Triplicate samples of 50 mL were amended with 500 µL $[^{14}\text{C}]\text{HCO}_3$ (10 µCi), to have a final activity of 0.2 µCi mL⁻¹. Controls were fixed with 2.5 mL of filtered formaldehyde (2% [v/v], final concentration). To avoid $[^{14}\text{C}]\text{HCO}_3$ losses, after addition of the fixative, appropriate aliquots of NaHCO₃ at pH 9.5 were added to the syringes for the *in situ* incubations.

To measure the total and non-bacterial *in situ* dark $[^{14}\text{C}]\text{HCO}_3$ uptake we used two custom-made devices suspended at 350 m. One was utilized to measure the total dark uptake and the second to assess the non-bacterial dark $[^{14}\text{C}]\text{HCO}_3$ uptake by inhibiting bacterial activity with metabolic inhibitors. The first device was loaded with 500 µL of $[^{14}\text{C}]\text{HCO}_3$ to triplicate syringes, as described above. The three syringes of the second device were all amended with 500 µL $[^{14}\text{C}]\text{HCO}_3$, 120 µL sodium azide (SA) 10 mM (25 µM L⁻¹), and 400 µL allylthiourea (ATU) 10 mM (86 µM L⁻¹). At these concentrations, ATU inhibits the bacterial monooxygenase and SA is a selective bacteriostatic agent that inhibits ammonia and nitrite oxidation of Gram-negative bacteria (Ginestet *et al.*, 1998; Santoro and Casciotti, 2011); they both have been successfully used in similar experiments (Yakimov *et al.*, 2011). In the control pH adjustment necessary to avoid CO₂ loss was performed with NaHCO₃ as described above.

The total activity of $[^{14}\text{C}]$ added was measured in the laboratory for each sample and control to obtain accurate $[^{14}\text{C}]\text{HCO}_3$ concentrations. Briefly, 6 mL of scintillation liquid (Instangel, Beckman) were added to 1 mL sample and immediately counted in a Beckman LS6000 scintillation counter. The remaining sample was then filtered over 0.22 µm polycarbonate filters (Poretics, 25 mm diameter) in Swinnex® Millipore filter holders and with dis-

Tab. 1. *In situ* dark $[^{14}\text{C}]\text{HCO}_3$ uptake performed with the new instrument tested vs on board incubation. The measurements were done in Lake Maggiore at two stations (Maggia and Ghiffa), at different depths and months.

Station	Month	Depth m	Dark $[^{14}\text{C}]\text{HCO}_3$ uptake ($\mu\text{g C m}^{-3}\text{h}^{-1}$)			
			<i>In situ</i>		On board	
			Mean	±sd	Mean	±sd
Maggia	July	100	8.8	0.6	25.2	12.9
Maggia	July	200	9.4	1.5	5.2	1.8
Ghiffa	October	300	7.4	na	16.3	4.0
Ghiffa	August	350	8.3	2.2	2.9	0.7

sd, standard deviation; na, not available.

posable plastic syringes. Filters were acidified with 200 μL 1N HCl, kept under the hood for 60 min to eliminate the inorganic fraction as $[^{14}\text{C}]$ labeled CO_2 . Then, 5 mL scintillation liquid (Filter Count, Beckman) were added to the filters and samples were counted in the scintillation counter for maximum 10 min. The counts per minute (cpm) were automatically corrected to obtain *disintegration per minutes* (dpm) using the $[^{14}\text{C}]$ reference standard with a quenching around zero. The efficiency of the conversion was on average 95% and the luminescence near 0%. Inorganic carbon dark uptake was calculated based on the dpm of the total activity, the dpm of the filters and the dissolved inorganic carbon (DIC) of each sample following a previously described protocol (Callieri and Stockner, 2002) using a Shimadzu 5000A apparatus. Statistical analyses to estimate the difference between *in situ* and on board incubation were performed with GraphPad Statistics, Prism package, 2007.

Fluorescence *in situ* hybridization catalyzed reporter deposition (CARD-FISH) and DAPI counting

Parallel to the *in situ* dark $[^{14}\text{C}]\text{HCO}_3$ uptake measurements performed in 2011, samples from 350 m were analysed for prokaryote abundance (DAPI counting) and community composition (CARD-FISH). Samples were fixed with 0.2 μm filtered paraformaldehyde (2% [w/v], final concentration) according to Pernthaler *et al.* (2004) and Teira *et al.* (2004). Aliquots of 6-12 mL were filtered onto 0.2 μm polycarbonate filters (Poretics, 47 mm filter diameter), rinsed twice with deionized water, embedded in low melting point agarose, air dried and stored at -20°C until further processing. Filters were cut in sections and selected sections were treated for cell permeabilization with lysozyme or proteinase K (for Bacteria and Thaumarchaeota probes, respectively) according to the protocol by Teira *et al.* (2004). The following oligonucleotide probes were used: EUB I-III, for all Bacteria (Daims *et al.*, 1999; Amann and Fuchs, 2008), Cren537, for Thaumarchaeota MGI.1a (Teira *et al.*, 2004; Herndl *et al.*, 2005) and Non338 as negative control for Bacteria (Thermo-Hybrid, Germany). Filter sections were hybridized with HRP- (horseradish-peroxidase) labeled probes and Alexa488-labeled tyramide was used for signal amplification. Filters were counterstained with DAPI, embedded in Citifluor (Citifluor Ltd., London, UK), and inspected under an epifluorescence microscope (Zeiss Axioplan) equipped with filter sets for DAPI (BP365, FT395, LP420) and for Alexa488 (BP450-490, FT510, LP520). Filter sections were analyzed by fully automated high-throughput microscopy (Zeder and Pernthaler, 2009) by capturing 3 pictures from the same field corresponding to total DAPI-stained cells, cells stained with the specific probe, and auto-fluorescence of cyanobacteria and debris. All images were analyzed with the image analysis free-

ware ACMEtool (<http://www.technobiology.ch>), and interfering autofluorescent cyanobacteria or debris were individually subtracted from images of hybridized cells. At least 10 high quality images or >1000 DAPI stained cells were analyzed per sample.

For total prokaryotic cell counting, after staining with 4',6-diamino-2-phenylindole (DAPI, final concentration 1 $\mu\text{g mL}^{-1}$) replicate samples were filtered onto 0.2 μm pore-size polycarbonate membranes (Poretics, 25 mm diameter), and counted by epifluorescence microscope (Zeiss Axioplan equipped with an HBO 100 W lamp, a Neofluar 100 x objective 1.25 x additional magnification and filter sets for UV: BP365, FT 395, LP420). A minimum of 400 DAPI-stained cells per sample were counted on at least 10 fields.

RESULTS

In situ dark $[^{14}\text{C}]\text{HCO}_3$ uptake: testing the instrument

We tested the functioning of a new device for *in situ* incubations at different stations (Ghiffa and Maggia) in Lake Maggiore at different depths and months. In order to compare the results obtained with the new device, classical on board incubations were performed concomitantly. Falcon[®] tubes for on board and syringes for *in situ* incubations were of the same volume and syringe tips large enough to sample particles or aggregates present in the water. We are thus confident that the two sample-types were comparable.

Dark $[^{14}\text{C}]\text{HCO}_3$ uptake measured *in situ* ranged from 6.6 to 10.7 $\mu\text{g C m}^{-3} \text{ h}^{-1}$ (mean coefficient of variation, $\text{CV}=0.16$), whereas on board measurements ranged from 2.2 to 38 $\mu\text{g C m}^{-3} \text{ h}^{-1}$ ($\text{CV}=0.89$) with slightly higher mean (Fig. 2). The on board uptake measurements showed high spatial and temporal variability compared to the *in*

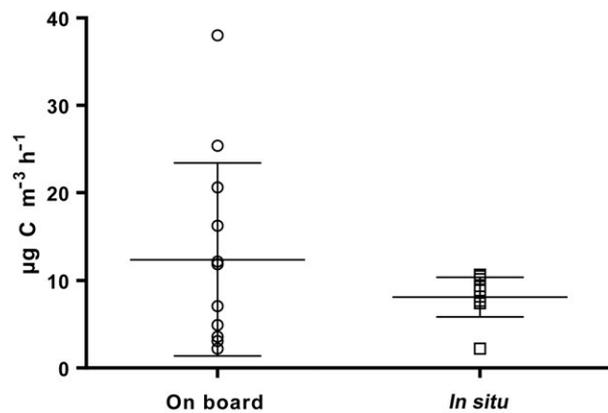


Fig. 2. Mean, standard deviation and single values of the dark $[^{14}\text{C}]\text{HCO}_3$ uptake ($\mu\text{g C m}^{-3} \text{ h}^{-1}$) obtained with on board and *in situ* incubations.

situ incubations nevertheless, analyzed altogether the data of the two treatments were not significantly different (unpaired *t*-test, $df=22$, $t=1.308$, $P=0.204$). The unpaired *t*-test performed per single depth showed significant differences at 200 and 300 m ($P=0.034$ and 0.024 respectively) and no significant differences at 100 and 350 m ($P=0.093$ and 0.200 respectively).

***In situ* dark [¹⁴C]HCO₃ uptake: Archaea vs Bacteria**

Dark [¹⁴C]HCO₃ uptake of Archaea was estimated by inhibiting the bacterial community *in situ* (Tab. 2). Measurements were performed with two custom-made devices at 350 m depth (Ghiffa), in August and October 2011. The non-bacterial daily uptake rates resulted in almost identical values in the two dates with an average of $52.7 \pm 0.4 \mu\text{g C m}^{-3} \text{ d}^{-1}$; this represents 28% of total dark uptake. Dark [¹⁴C]HCO₃ uptake values of Bacteria were higher both in August and October with an average value of $135.0 \pm 15.6 \mu\text{g C m}^{-3} \text{ d}^{-1}$ (Tab. 2).

To estimate the bulk impact of dark [¹⁴C]HCO₃ uptake in the hypolimnion of Lake Maggiore we considered the aphotic water column from 20 m to 370 m depth. In this zone the mean total prokaryotic dark [¹⁴C]HCO₃ uptake was $65.8 \pm 5.2 \text{ mg C m}^{-2} \text{ d}^{-1}$, with a non-bacterial uptake of $18.4 \pm 0.1 \text{ mg C m}^{-2} \text{ d}^{-1}$ (Tab. 2).

Thaumarchaeota and Bacteria in the deep hypolimnion

Thaumarchaeota (probe Cren537) at 350 m constituted 10.2% of the total DAPI counts in August and 12% in October, resulting in cell numbers of 43 and 51×10^3 cells mL^{-1} , respectively. Bacteria (probe EUB I-III) were 73.6% and 85.2%, or 313 and 365×10^3 cells mL^{-1} , in August and October, respectively.

DISCUSSION

The prokaryotic community inhabiting the hypolimnion is a crucial component of lake functioning but knowledge in respect to its activity is scarce. The aim of this study was to elucidate the role of Archaea in the autotrophic carbon fixation in a deep hypolimnion. In particular, we measured the dark [¹⁴C]HCO₃ uptake of the prokaryotic community inhabiting the deep hypolimnion (350 m) of the large subalpine Lake Maggiore by direct *in situ* incubation performed with a custom-made device (Fig. 1). We were able to measure low activities of prokaryotes adapted to the dark, cold, and pressure conditions present at 350 m, by avoiding sampling stresses. We made a comparison between *in situ* and on board incubations testing the device in different conditions (2 stations, 3 months, 4 depths). Interestingly, values of dark [¹⁴C]HCO₃ uptake measured *in situ* were similar in all the conditions ranging from 6.6 – $10.7 \mu\text{g C m}^{-3} \text{ h}^{-1}$, while

measurements on board showed high variability (Fig. 2). Considering that the hypolimnion is a stable environment not prone to high fluctuations, constant values of *in situ* measurements seemed to reflect the dark CO₂ fixation better. Altogether the data sets of the on board and *in situ* incubations were not significantly different from each other. Taken separately per depth they were significantly different at 200 and 300 m and not different at 100 and 350 m. Therefore, at present, our data do not support a difference between on board and *in situ* incubations. Actually the dark [¹⁴C]HCO₃ uptake technique and the protocol used were the same for the two incubation procedures. Nevertheless, the lower variation obtained render the *in situ* measurements more precise. In addition, the presented data are the first available on dark [¹⁴C]HCO₃ directly measured *in situ* at great depth.

The use of metabolic inhibitors allowed to measure total and non-bacterial dark CO₂ assimilation *in situ*. As inhibitors of Bacteria we used allylthiourea that, at concentration of $86 \mu\text{M}$, inhibits bacterial ammonium monooxygenase, by chelating the Cu-active site of the Amo protein (Ginestet *et al.*, 1998; Santoro and Casciotti, 2011); we also used sodium azide which is a bacteriostatic agent and an inhibitor of ammonia and nitrite oxidation (Mallmann *et al.*, 1941; Ginestet *et al.*, 1998). These metabolic inhibitors caused no significant decline in autotrophic activity of Archaea in the bathypelagic Mediterranean Sea (Yakimov *et al.*, 2011) and in a hypertrophic saline lake (La Cono *et al.*, 2013). Conversely, laboratory experiments (Santoro and Casciotti, 2011) evidenced that allylthiourea inhibited the rates of ammonia oxidation by a 58% at $86 \mu\text{M}$ concentration after 40 days of incubation, whereas no effect on ammonia-oxidizing Archaea growth was observed within the first 48 hours of incubations (Santoro and Casciotti, 2011). Accordingly, the incubation period conducted in the present study should not have inhibited archaeal growth nor radiolabelled bicarbonate uptake. This consideration in the light of the demonstrated increase of Thaumarchaeota abundances with depth in Lake Maggiore (Callieri *et al.*, 2009) indicate that the

Tab. 2. Bacteria and Archaea mean dark [¹⁴C]HCO₃ uptake at 350 m in Lake Maggiore, in August and October. Daily uptake was also calculated *per surface* ($\text{mg C m}^{-2} \text{ d}^{-1}$), considering the aphotic water column from 20 to 370 m.

Month	Domain	$\mu\text{g C m}^{-3} \text{ d}^{-1}$		$\text{mg C m}^{-2} \text{ d}^{-1}$	
		Mean	$\pm\text{sd}$	Mean	$\pm\text{sd}$
August	Bacteria	146	49.6	51.1	17.4
	Archaea	52.4	13.9	18.3	10.2
October	Bacteria	124	18.0	43.6	6.5
	Archaea	53.0	18.4	18.5	6.5

sd, standard deviation.

non-bacterial CO₂ uptake may be assigned to Archaea, likely belonging to Thaumarchaeota.

The estimate of [¹⁴C]HCO₃ uptake in the deep hypolimnion of Lake Maggiore was 187.7±15 μg C m⁻³ d⁻¹, substantially higher than reported for a hypertrophic saline lake (6-51 μg C m⁻³ d⁻¹) (La Cono *et al.*, 2013). Moreover, our rates were higher than in the Mediterranean deep-sea (50-60 μg C m⁻³ d⁻¹ at 2000 m) (Yakimov *et al.*, 2011) and in line with Levantine Intermediate Water (230 μg C m⁻³ d⁻¹) (Yakimov *et al.*, 2011). Though the deep hypolimnion of Lake Maggiore is undoubtedly shallower than the marine bathypelagic zone, a comparison of the two zones is meaningful as they are both dark, cold, oxygenated, and oligotrophic diluted water masses with bottom sediment boundaries. Moreover, the relevance of dark CO₂ uptake in freshwater deep hypolimnetic zones opens a new perspective on the role of prokaryotes as a sink for inorganic carbon. Our results indicate a predominant role of Bacteria with respect to Archaea in the dark CO₂ fixation: the former being responsible for 72% of total dark CO₂ uptake and the latter for the remaining 28%.

Although there is evidence of autotrophic ammonia oxidation of Archaea (Herndl *et al.*, 2005; Ingalls *et al.*, 2006) their heterotrophic or mixotrophic growth cannot be excluded (Ouverney and Fuhrman, 2000; Teira *et al.*, 2006) and it has even been proposed as the prevalent metabolism in Archaea (Agogué *et al.*, 2008). With the method used in our work we cannot specify which metabolic pathways were used by prokaryotes in the deep hypolimnion. We are aware of the fact that prokaryotes can also use bicarbonate for anaplerotic reactions to replenish TCA cycle intermediates (Romanenko, 1964), or for the synthesis of amino acids and biosynthesis of fatty acids and that therefore our dark CO₂ fixation measurements may also include these activities.

We measured the relative importance of Bacteria and Thaumarchaeota at 350 m in Lake Maggiore using CARD-FISH. To detect and enumerate Bacteria we used the mix EUBI-III which includes two additional probes complementary to Eub338: Eub338 II and III that target members of Verrucomicrobia and Planctomycetales (Daims *et al.*, 1999). To identify Thaumarchaeota we avoided to use the general probe Arch915 which unspecifically binds to some bacteria (Pernthaler *et al.*, 2002) and picocyanobacteria (Callieri, *unpublished results*), thus, would greatly overestimate the number of Archaea. We instead used the probe Cren537 which specifically targets the Thaumarchaeota MGI.1a clade (95.1% coverage, 0.2% outgroup hits) (Teira *et al.*, 2004, 2006; Callieri *et al.*, 2009). The finding that in a deep alpine lake up to 90% of the 16S rRNA gene sequences of Thaumarchaeota belonged to MGI.1a and SAGMGC-1, the former being prevalent in the deep layers (Auguet *et al.*, 2012) supports our choice of the probe targeting

Thaumarchaeota. Moreover, MGI.1a sequences have been found to dominate the archaeal assemblage of the oxygenated layer of a saline lake (La Cono *et al.*, 2013). Nevertheless we cannot exclude that we were not able to detect all Archaea, as also Euryarchaeota play a role in freshwater habitats. However, these microbes seem to be more important in lakes with a higher trophic status (Auguet and Casamayor, 2013) or in anoxic water layers of a saline lake (La Cono *et al.*, 2013). In general, CARD-FISH hybridization rates were in the range of other lake profiles (Salcher *et al.*, 2011), suggesting that no substantial part of the community was missed.

To gain better insight into the relevance of dark CO₂ fixation for lake ecosystem functioning, we compared the photosynthetic CO₂ fixation in the photic zone of Lake Maggiore (Callieri and Piscia, 2002) with the dark CO₂ fixation in the aphotic zone. The average primary production, measured in a time series study in 2002 by performing 15 measurements at 5 different depths in the euphotic zone of Lake Maggiore, was 483 mg C m⁻² d⁻¹ (E. Caravati, *unpublished data*), while the average dark CO₂ fixation in the aphotic zone was 66 mg C m⁻² d⁻¹ (Table 2). Despite the different extension of the euphotic (0-20 m) and the aphotic (20-370 m) zone, the dark fixation corresponds to 14% of the total photosynthetic fixation. This comparison is imbalanced, as it also includes eukaryotic phototrophic activity (*i.e.*, algae). If only prokaryotic activities are considered, the ratio photosynthetic:dark CO₂ fixation is 1.2, and the percentage of dark CO₂ fixation corresponds to 87% of the pico-phytoplankton production (average: 76 mg C m⁻² d⁻¹; E. Caravati, *unpublished data*). This value is only slightly higher than a 1:1 photosynthetic:dark CO₂ fixation ratio reported from the Mediterranean Sea (Yakimov *et al.*, 2011), although the extension of the aphotic zone is larger in the Mediterranean Sea than in Lake Maggiore (200-3480 m vs 20-370 m). Furthermore, the photic layer in the Mediterranean Sea is ultra-oligotrophic, thus, phototrophic CO₂ fixation rates are much lower than in Lake Maggiore.

CONCLUSIONS

Our study provides the first direct estimate of the *in situ* dark CO₂ fixation conducted by prokaryotes in the deep hypolimnion of an oligotrophic lake. Our results point out that freshwater deep hypolimnia, although often disregarded, are places of important microbial metabolisms of the carbon and nitrogen cycle, and dark CO₂ fixation rates are even comparable with the photosynthetic fixation occurring in photic zones.

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