RESEARCH ARTICLE

Anoxygenic photo- and chemo-synthesis of phototrophic sulfur bacteria from an alpine meromictic lake

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One sentence summary: The ecological role of three different species of anoxygenic phototrophic sulfur bacteria from the chemocline of an alpine meromictic lake was investigated through a combination of field and laboratory experiments.

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ABSTRACT

Meromictic lakes are interesting ecosystems to study anaerobic microorganisms due their permanent stratification allowing the formation of a stable anoxic environment. The crenogenic meromictic Lake Cadagno harbors an important community of anoxygenic phototrophic sulfur bacteria responsible for almost half of its total productivity. Besides their ability to fix CO₂ through photosynthesis, these microorganisms also showed high rates of dark carbon fixation via chemosynthesis. Here, we grew in pure cultures three populations of anoxygenic phototrophic sulfur bacteria previously isolated from the lake, totaling 72.8% of the total microbial community and exhibiting different phenotypes: (1) the motile, large-celled purple sulfur bacterium (PSB) Chromatium okenii, (2) the small-celled PSB Thiodictyon syntrophicum and (3) the green sulfur bacterium (GSB) Chlorobium phaeobacteroides. We measured their ability to fix CO₂ through photosynthesis, these microorganisms also showed high rates of dark carbon fixation via chemosynthesis. Here, we grew in pure cultures three populations of anoxygenic phototrophic sulfur bacteria previously isolated from the lake, accounting for 72.8% of the total microbial community and exhibiting different phenotypes: (1) the motile, large-celled purple sulfur bacterium (PSB) Chromatium okenii, (2) the small-celled PSB Thiodictyon syntrophicum and (3) the green sulfur bacterium (GSB) Chlorobium phaeobacteroides. We measured their ability to fix CO₂ through photo- and chemo-synthesis, both in situ in the lake and in laboratory under different incubation conditions. We also evaluated the efficiency and velocity of H₂S photo-oxidation, an important reaction in the anoxygenic photosynthesis process. Our results confirm that phototrophic sulfur bacteria strongly fix CO₂ in the presence of light and that oxygen increases chemosynthesis at night, in laboratory conditions. Moreover, substantial differences were displayed between the three selected populations in terms of activity and abundance.
INTRODUCTION

Primary production is the conversion of energy and inorganic carbon into organic matter by autotrophic organisms. The most familiar form of primary production is photosynthesis, where carbon dioxide is converted into organic substances drawing energy from sunlight. Not all primary production, however, results from photosynthesis; under anaerobic conditions, some microorganisms are capable of chemosynthetic growth (Yang et al. 2011; Smith 2012). Unlike photosynthesis, microbial production by chemosynthesis converts inorganic carbon into organic matter using chemical energy from the oxidation of reduced compounds. Among such microorganisms are anoxygenic phototrophic sulfur bacteria which possess both photo- and chemo-synthetic abilities (Overmann 2008). Anoxygenic phototrophic sulfur bacteria play an important role in anoxic environments where they are the main primary producers of light and sulfide are present, including sediments (Preisler et al. 2007), hot springs (Hanada 2003) and freshwater lakes (Sorokin, Kuenen and Muzyer 2011; Watanabe et al. 2013).

Meromictic lakes, characterized by a permanent stratification of the water column which allows the formation of a stable anoxic compartment, represent a suitable environment to study the growth of anoxygenic phototrophic sulfur bacteria (Zadereev, Gulati and Camacho 2017). Alpine meromictic Lake Cadagno, located in the Ticino canton of Switzerland, has been intensely investigated over the last decades (Tonolla et al. 2017). Its chemocline, typically lying at about 12 m depth, separates theoxic, salt-poor surface water of low density from the anoxic, salt-rich bottom water of high density. The opposite gradients of sulfide and light irradiance present in the chemocline support the growth of a dense community of purple sulfur bacteria (PSB) of the genera Chromatium, Lamprocystis, Thioctys and Thiodictyon and green sulfur bacteria (GSB) of the genus Chlorobium (Tonolla, Demarta and Peduzzi 1999; Tonolla et al. 2003; Tonolla, Peduzzi and Hahn 2005; Rajasekar et al. 2012; Danza et al. 2018). Moreover, Lake Cadagno is considered an open living archive system to study microorganisms that have been key for the development of early life on Earth (Crowe et al. 2008; Canfield, Farquhar and Zerkle 2010; Dahl et al. 2010; Wirth et al. 2013).

The anoxygenic phototrophic sulfur bacteria community of Lake Cadagno plays an important ecological role with an high rate of $^{14}$CO$_2$ fixation, as demonstrated by Camacho et al. (2001) who observed that, although representing only about 10% of the volume of the lake, up to 40% of the total $^{14}$CO$_2$ photosynthesis occurs in the chemocline. Further studies, using nanoscale secondary ion mass spectrometry (NanoSIMS; Musat et al. 2008; Zimmermann et al. 2015) and again $^{14}$C isotope quantitative assimilation (Storelli et al. 2013; Poth et al. 2017; Luedin et al. 2019), highlighted the different efficacy of individual bacterial populations. Indeed, among PSB, large-celled Chromatium okenii resulted the most effective photo-assimilator, while the dark chemo-assimilation (~30%) was mostly carried out by small-celled PSB Thiodictyon syntrophicum and Lamprocystis purpurea. In all studies both GSB populations, although the most abundant numerically, resulted in a low assimilation of inorganic carbon. Recently, PSB C. okenii (Berg et al. 2019) and PSB T. syntrophicum (Luedin et al. 2019) have been shown to mediate micro-aerobic metabolism in the dark. High levels of dark inorganic carbon assimilation have also been observed in other stratified lakes (Hadas, Pinkas and Erez 2001; Casamayor 2010; Casamayor et al. 2012; Morana et al. 2016; Pjevac et al. 2019), suggesting that alternative metabolic pathways, such as chemosynthesis, are involved in the primary production of these environments.

Ecologically similar but phylogenetically distant, PSB and GSB represent a good example of convergent evolution, having evolved in the same natural environment exhibiting different evolutionary strategies (Stayton 2015). A major difference is the set of pigments necessary to harvest light, GSB possess chlorosomes structures conferring an higher affinity to low light intensity compared to PSB (Bryant and Frigaard 2006). A second important difference lies in the accumulation of sulfur globules (S$^0$) inside and outside the cell for PSB and GSB, respectively (Frigaard and Dahl 2008). Sulfur globules can further be oxidized to sulfate when hydrogen sulfide becomes scarce in the environment. Recently, Danza et al. (2017) investigated the response to hydrogen sulfide oxidation of the anoxygenic photosynthetic sulfur bacteria in the chemocline of Lake Cadagno confirming the link between light-dependent H$_2$S uptake rates and important changes in the intracellular complexity. Consequently, sulfide oxidation to elemental sulfur results to be light-intensity dependent (Hanson et al. 2013). Normally, microbial communities in sediments and anoxic zones organize according to the redox tower, this perspective suggests that chemical potential can be used to predict the activity of microbial communities and their biogeochemical impact in redox models. These are models that take into account the most important reduction-oxidation (redox) reactions involved in the metabolisms of microbial populations within ecosystem models, providing additional information on bacterial assimilatory and respiratory fluxes (Zakem, Polz and Follows 2020). Moreover, different evolutionary strategies can be found even within the same group. For example, large-celled PSB C. okenii possesses flagella, unlike small-celled PSB which rely on gas vacuoles for movement, conferring a rapid reaction time with a resulting better capacity to adapt to changes of environmental conditions. Once a minimum number of cells is reached, C. okenii’s phototactic but aerophilic movement (Pfenning 1962) produces a local remixing of water, triggering a process called bioconvection (Sommer et al. 2017; Sepúlveda Steiner, Bouffard and Wüst 2019). This phenomenon is strongly linked to the number of C. okenii cells and to the presence of oxygen, which, by stopping the cells, increases the density of the water that precipitates downwards. The presence of a similar number of Cryptomonas spp, a highly motile algae, in the upper part of the chemocline of Lake Cadagno, is also to be taken into account, although apparently it does not produce the same process of bioconvection (Rodrigo et al. 1999; Camacho et al. 2001; Camacho, Vicente and Miracle 2001). However, the ecological significance of this phenomenon for the population of C. okenii, and for the phototrophic sulfur bacteria community in general, remains still unknown.

The aim of this study is to investigate the primary production by comparing photo- and chemo-synthetic activity of three phenotypically different anoxygenic phototrophic sulfur bacteria species living in the chemocline of meromictic Lake Cadagno. We selected three strains such as the large-celled, motile by
flagella PSB C. okenii strain LaCa, the small-celled PSB T. syne-  
trophicum strain Cad16Δ2 and the GSB Chlorobium phaeobacteroides  
strain 1VII D7, all previously isolated from Lake Cadagno and  
grown in pure cultures in the laboratory. First, we tested the CO₂  
photo- and chemo-assimilation in situ using dialysis bags and  
radioactive ¹⁴CO₂ and compared the single population activity  
with the microbial community present in the chemocline. Then,  
in the laboratory, we investigated the capacity to photo-oxidize  
hydrogen sulfide at different light intensities and substrate con-  
centrations, and we evaluated the role of low concentrations of  
oxogen in chemotrophic carbon fixation.

**MATERIALS AND METHODS**

**Study site and sampling**

Lake Cadagno is a crenogenic meromictic lake located in the  
Piora Valley at 1921 m above sea level, in the southern Swiss  
Alps (46°33′N, 8°43′E and depth approximately 21 m). In addition  
to surface water tributaries, there are sublacustrine springs that  
feed the lake with high-density water flowing through gypsum-  
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Phototrophic sulfur bacteria growth conditions

Phototrophic sulfur bacteria were grown in Pfennig’s medium I  
(Eichler and Pfennig 1988), containing per liter: 0.25 g of KH₂PO₄,  
0.34 g of NH₄Cl, 0.5 g of MgSO₄ × 7H₂O, 0.25 g of CaCl₂ × 2H₂O,  
0.34 g of KCI, 1.5 g of NaHCO₃, 0.02 mg of vitamin B₁₂ and 1.0 mL  
of trace elements solution SL10. The medium was prepared in a  
2.0 L bottle using a flushing gas composition of 80% N₂ and  
20% CO₂ according to (Widdel and Bak 1992) and was reduced  
by the addition of a Na₂S × 9H₂O solution a to a concentration  
of 1.0 mM S²⁻ and adjusted to a pH of approximately 7.1. All  
cultures were incubated at room temperature (22–23°C) and  
subjected to a light/dark photoperiod of 16/8 h with a light  
intensity of 136.4 μmol/m²/s PAR measured with a portable  
LI-180 Spectrometer (LI-COR Biosciences, Lincoln, NE) with  
incandescent 100 W bulbs emitting the entire visible spectrum  
(Figure S1, Supporting Information).

**Flow cytometry**

Phototrophic sulfur bacteria in pure cultures were enumerated  
by flow cytometry (FCM) measuring chlorophyll-like autofluo-  
rescence particle events (Tonolla et al. 2017; Danza et al. 2018). A BD  
Accuri C6 cytometer (Becton Dickinson, San José, CA) equipped  
with two lasers (488 and 680 nm), two scatter detectors and  
four fluorescence detectors (laser 488 nm: FL1 = 533/30, FL2 =  
585/40, FL3 = 670; laser 640 nm: FL4 = 675/25) was used for  
samples analysis. A total of two principal parameters were used  
for event characterization: forward scatter (FSC), which allows  
for the discrimination of cells by size, and 90° sideward scat-  
ter (SSC), which is proportional to the internal complexity of the  
cells. A threshold of 10 000 on FSC-H was applied to exclude most  
of the unwanted abiotic particles. Furthermore, to discriminate  
cells emitting autofluorescence due to chlorophyll and bacteri-  
oclorophyll, a FL3-A > 1100 threshold was applied on FL3 (red  
fluorescence). A volume of 50 μL was analyzed in triplicate for  
each sample at a 66 μL/min flow rate, and dilutions in Milli-Q  
water were performed for those samples with more than 10 000  
events per second.

Green sulfur (GSB) and purple sulfur bacteria (PSB) coloniz-  
ging the chemocline of Lake Cadagno were distinguished based  
on morphological characteristics (Danza et al. 2017). Large-celled  
PSB C. okenni LaCa, small-celled PSB T. syntrophicum Cad16Δ2  
and very small GSB C. phaeobacteroides 1VII D7 Lake Cadagno were  
well distinguished in a SSC vs FSC dot plot and gating on the  
respective populations permitted their quantification.

**Definition of the cellular biovolume**

Cell volumes were calculated as in Tonolla et al. (2003). Biovol-  
umes of bacterial cells were analyzed on images captured with a  
Zeiss Axioscam 305 color camera connected to a Zeiss Axio  
Scope A1 epifluorescence microscope (Zeiss, Oberkochen, Ger-  
many) using the ZEN 2.6 (blue edition) imaging software (Zeiss,  
Oberkochen, Germany). For each species, between 30 and 40  
cells in the exponential phase were taken into account to deter-  
mine the mean size.

**Measure of inorganic carbon assimilation using ¹⁴C**

The ¹⁴C-radiosotope uptake experiment was carried out as pre- 
viously described (Storrelli et al. 2013).  

Pure bacterial cultures enriched up to 10⁶ cells/mL (late expo-  
nential phase) were sealed in 50-cm-long dialysis bags (inflated  
diameter of 62.8 mm; Karl Roth GmbH Co. KG, Karlsruhe, Ger-  
many), that allowed for the free diffusion of molecules smaller  
than 20 kDa while preventing contamination of in situ incubated  
cultures by environmental bacteria. The final number of dialysis  
bags attached and incubated in the chemocline of Lake Cadagno  
was a sum of triplicate for each microorganism tested multi-  
plied by two for both testing conditions of light and dark, for  
a total of 18. Moreover, the dialysis bags supporting frame was  
equipped with HOBO UA-002–64 Pendant passive data loggers  
(Onset Computer Corporation, MA) measuring relative light (Lux;  
180–1200 nm) and temperature at 60 min intervals. A total of two  
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Light-dependent sulfide oxidation

PSB C. eikenii LaCa, T. syntrophicum Cad16T and GSB C. phaeobacteroides 1VII D7 isolated from Lake Cadagno chemocline were incubated in a single microrespiratory chamber system equipped with a sulfide gas sensor (SULF; range 0–300 μM, detection limit 0.3 μM; Unisense A/S, Aarhus, Denmark). The system was kept under continuous magnetic stirring at 200 rpm and at a constant temperature of 24 °C. A total of four different H2S concentrations of 5, 10, 100 and 300 μM were tested by a sulfide pulse added before starting the measurement to achieve the desired initial concentration. The sensor detects the partial pressure of H2S gas, which is only one component of the total sulfide equilibrium system. Therefore, according to the following equation (Jeroschewski, Steuckart and Kuhl 1996):

\[ [S^2^-] = [H_2S] \left( 1 + \frac{K_1}{[H_3O^+]} \right) \]

where \( K_1 \) is a reaction rate constant and \([H_3O^+]\) is a proxy for the pH of the sample solution, the H2S concentrations correspond to \( S^2^- \) concentrations of 0.02, 0.04, 0.40 and 1.20 μM.

A light/dark photoperiod (5 min/5 min) was applied using 100 W incandescent bulbs emitting the entire spectrum for a total of 90 min. The sample in the microrespiratory chamber was incubated under ten following increasing light intensities (0, 1, 2, 3, 5, 10, 20, 30, 40, 50 μmol/m²/s PAR). Light intensity was measured using a LI-193SA spherical quantum sensor (LI-COR Biosciences, Lincoln, NE) next to the incubation chamber.

Replicates incubated in the dark and a batch of medium incubated in the presence of light were used as negative controls with a concentration of 0.4 mM S²⁻.

The rate of sulfide metabolism for phototropic sulfur bacteria is assumed to be dependent on both light and sulfide concentration according to the following equation:

\[ \text{rate} = k[H_2S] [\text{light}] \]

Assuming a constant initial sulfide concentration, the rate equation then becomes:

\[ \text{rate} = k[\text{light}] \]

The sulfide oxidation rate related to the respective light intensity was calculated through the integration of values at the start and the end of the light phase period. All different concentrations of sulfide were measured in triplicates.

The data were fitted to a Hill equation (Hill 1910) representing sulfide oxidation as a function of light intensity:

\[ V = \frac{V_{\text{max}}[\text{light}]^n}{(K)^n + [\text{light}]} \]

Where \( V_{\text{max}} \) is the maximum rate of sulfide oxidation, \( n \) is the Hill coefficient which typically indicates substrate binding cooperativity in enzyme kinetics, or more generally a non-linear dose-response, \( K \) is a light half-saturation constant and \([\text{light}]\) is the light radiation intensity. The equation was used to perform non-linear curve fitting to the experimental data using Levenberg–Marquardt algorithm.

In the fitting procedure, the baseline level of sulfide oxidation in the dark was added to the Hill equation and not varied throughout the fitting procedure.

Nucleic-acid content determination

The relative total nucleic acid content for each sample was estimated through staining with SYBR Gold (Molecular Probes, Eugene, OR) with a final concentration of 1:10 000. The stained samples were incubated for 13 min at 37 °C in the dark, and then 50 μL were measured using the flow cytometer (Becton Dickinson, San José, CA). Median fluorescence signal FL1-H (>1100) of gated populations allowed detecting changes in signal intensity due to the variation of nucleic acid content.
Table 1. FISH and FCM (± standard deviation) quantification of PSB C. okenii, PSB T. syntrophicum and GSB C. phaeobacteroides in the chemoclone of Lake Cadagno and in dialysis bags (22 August 2019).

| Time (h) | Depth (m) | FCM | FISH | FISH | FCM |
|----------|-----------|-----|------|------|-----|
| 10:00    | 12.29     | 1.07 ± 0.35 | 1.49 ± 0.34 | 12.6 ± 3.0 | 3.69 ± 0.42 |
| 22:00    | 12.65     | 0.48 ± 0.05 | 2.23 ± 0.04 | 10.9 ± 6.4 | 4.76 ± 0.22 |

Dialysis bags

| PSB C. okenii (10⁶ cells mL⁻¹) | PSB T. syntrophicum (10⁶ cells mL⁻¹) | GSB C. phaeobacteroides (10⁶ cells mL⁻¹) |
|-------------------------------|-------------------------------------|---------------------------------------|
| FCM                           | FCM                                 | FCM                                   |

Before 6-week incubation

| FCM | FCM | FCM |
|-----|-----|-----|
| 2.16 ± 0.02 | 11.0 ± 0.02 | 34.4 ± 0.17 |

After 6-week incubation

| Time (h) | Depth (m) | FCM | FCM | FCM |
|----------|-----------|-----|-----|-----|
| 10:00    | 11.78     | 3.48 ± 0.11 | 13.6 ± 1.1 | 5.64 ± 0.73 |
| 22:00    | 2.67 ± 0.05 | 11.1 ± 1.2 | 6.15 ± 0.74 |

Measure of intracellular ATP

The mean activity of microbial cells in pure cultures has been determined by quantifying the ATP present using the BacTiter-Glo Microbial Cell Viability Assay (Promega Corporation, Dübendorf, CH) and a BioOrbit 1253 luminometer (BioOrbit OY, Turku, Finland). The BacTiter-Glo reagent was prepared according to the manufacturer’s instructions. The kit protocol recommends incubating an equal volume of reagent and sample (100 μL:100 μL) at room temperature for 5 min before recording the luminescence, expressed in Relative Light Units (RLU). The RLU values were converted to ATP concentrations using a calibration curve, measured with separate preparations of the ATP reagent.

Fluorescent in situ hybridization (FISH)

The total cell number of PSB T. syntrophicum and GSB C. phaeobacteroides in the bacterial layer of the Lake Cadagno chemoclone were identified and quantified using FISH with species-specific Cy3-labeled oligonucleotides, 5′-GCCGATATGATTCCAGCGGCAAGGCG and 5′-CCGATATGATTCCAGCGGCAAGGCG, respectively. A total of 20 mL of lake water were filtered onto 0.2 μm polycarbonate filters, fixed for 30 min with a 4% paraformaldehyde solution and washed twice in PBS. Bacteria were then resuspended in 600 μL of PBS/EtOH 1:1. Both samples were observed in 2 and 5 μL aliquots of paraformaldehyde-fixed water samples (n = 3) spotted onto gelatin-coated slides (0.1% gelatin, 0.01% KCr(SO₄)₂; Glöckner et al. 1996). Hybridizations were performed as described by Zarda et al. (1997) with concomitant DAPI staining for total bacterial community quantification. The slides were treated with Citifluor AF1 (Citifluor Ltd., London, UK) and examined by epifluorescence microscopy using filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP and D460/50 for DAPI) and F41 (AHF Analysentechnik; HQ535/50, Q565LP and HQ610/75 for Cy3). The microorganisms were counted at 1000× magnification in 40 fields of 0.01 mm².

RESULTS

Physical and chemical analysis of Lake Cadagno water column

Measurements of the physicochemical parameters of Lake Cadagno water column were carried out on the 22nd August 2019. The profiles showed in Fig. 1 were taken at the beginning of both 4 h ¹⁴CO₂ incubation periods in Lake Cadagno, at 10:00 h (Fig. 1A and B) and at 22:00 h (Fig. 1C and D). The CTD analysis of the water column put in evidence the three distinct zones in the meromictic Lake Cadagno, the mixolimnion, well highlighted by profiles of oxygen (a) and (c) extending the aerobic water layer from the surface until approx. 12 m depth, where oxygen disappears (0.31 mg/L). In the mixolimnion profiles of oxygen, temperature and conductivity are homogenized down to approximately 6 m depth as a result of wind and convectively-driven surface mixing. Below the homogeneous zone, the temperature decreased from 14.6 °C to its lowest value of 4.4 °C, while conductivity increased from 0.108 to 0.205 mS/cm at 12 m of depth (Fig. 1, graph A and C). The values of temperature and conductivity did not change between day and night. The second zone is called chemoclone (from approximately 12 to 14 m depth) and coincides with the beginning of
Figure 1. Vertical distribution of physicochemical profiles of the 22nd of August 2019. Physicochemical profiles of meromictic Lake Cadagno measured at 10:00 h (A and B) and at 22:00 h (C and D). Measurements were taken from a platform (5 m long and 3 m wide) installed in the deepest part of the lake, after a CTD equilibration period of 5 min at 0.5 m depth. Top graphs (a) and (c) show profiles of temperature (grey), conductivity (red) sulphide (green) and oxygen concentration (blue), and bottom graphs (b) and (d) show profiles of turbidity (purple), phycocyanin (cyan) and light (yellow). Moving average was calculated on a factor of 10 elements for Turbidity and BGAPC data set of graphs b and d. Profiles of physical parameters, such as temperature, conductivity, oxygen, hydrogen sulphide and light, are similar to past observation. However, biological parameters such as turbidity and BGAPC can change if measured at different period of the year.

the anaerobic layer, confirmed by the increase of the hydrogen sulphide concentration until a maximal value of 1.4 mg/L at 18 m of depth (Fig. 1A and C, green lines). Here the profile of conductivity remains constant in correspondence of the turbidity peak. In the chemocline the turbidity profile strongly increases to a maximal value up to 16.0 FTU (Fig. 1B and d, purple lines). During the day, the turbidity layer absorbed the light completely, and light did not penetrate below 14 m depth (Fig. 1Ba and D, yellow lines). The profile of light shows an uncommon behavior, with a steep reduction in the first 2 m, until approximately 4 m depth (Fig. 1B, yellow line). The measurement on the Lake Cadagno are normally carried out by the way of a platform (5 m long and 3 m wide) in the middle of the lake, where the depth is maximal (21 m depth). The CTD is lowered from the centre of the platform, to reduce oscillations as much as possible, producing this artificial profile derived from the shadow of the platform. A couple of meters before the complete disappearance of oxygen, the measure of BGAPC (Blue-Green Algae Phycocyanin) reached the maximum value of approximately 50 ppb (Fig. 1B and D, cyan lines; values multiplied by 5). This peak of phycocyanin pigments highlights the presence of cyanobacterial-like microorganism close to the anaerobic water. The lower layer of the lake, called monimolimnion, from 14 m to the bottom, is dark, anoxic and with high-density water due to the low temperature and presence of dissolved salts.

The anoxicogenic phototrophic sulfur bacteria community

The turbidity peaks showed in Fig. 1 (B and D, purple lines) between 12 and 14 m depth were analyzed using flow cytometry (FCM) and fluorescent in situ hybridization (FISH) in order to identify our population of interest within the entire microbial community.

FCM analysis, using a red autofluorescence signal (laser 488, emission 670 nm), confirmed a high concentration of photosynthetic microorganisms in correspondence to the turbidity peaks (Fig. 1B and D). The top of the bacterial layer changed slightly during the day, at 10:00 h it was at 12.29 m and 22:00 h at 12.65 m of depth, while the number of photosynthetic cells at the top of the turbidity peak did not vary much with $6.34 \times 10^5$ and $6.60 \times 10^5$ cells/mL in the morning and in evening, respectively.
Big-celled C. okenii were directly identified and counted by FCM on SSC/FSC dot plots after positive FL3 fluorescence discrimination, revealing some difference in number between day and night, of $1.07 \times 10^5$ and $4.88 \times 10^4$ cells/mL, respectively. The remaining of the phototrophic community in the bacterial layer was composed mainly of PSB and GSB, as further confirmed by FISH analyses. GSB C. phaeobacteroides was the most abundant among the three strains, with day and night numbers of $1.26 \times 10^6$ and $1.09 \times 10^6$ cells/mL while PSB T. syntrophicum had a cell number of $1.49 \times 10^6$ and $2.23 \times 10^6$ cells/mL day and night, respectively (Table 1). All together this three species account for the 72.8% of the anoxygenic phototrophic sulfur bacteria community of Lake Cadagno, the remaining 27.2% being represented by other species not considered in this study, because phenotypically similar to small celled PSB T. syntrophicum (e.g. Lamprocystis roseopersicina and purpurea; and Thiocystis chemocinalis and cadagonensis) and to GSB C. phaeobacteroides (e.g. C. chlatriforme) (Decristophoris et al. 2009).

Day and night $^{14}$CO$_2$ fixation in Lake Cadagno

After 4 h of incubation, the total fixed $^{14}$CO$_2$ in the upper part of the bacterial layer (positive control) was $2672.9 \pm 689.0$ $^{14}$C nM/h in the presence of light and 264.3 $\pm 102.1$ $^{14}$C nM/h in the dark. These values were transformed using the total number of phototrophic cells previously determined by FCM, with the result of a cell fixation rate of $210.7 \pm 27.2$ and $17.8 \pm 4.1$ $^{14}$C amol (atto-moles)/cell/h, during day and night, respectively.

The uptake of inorganic carbon was also measured using pure cultures of the three different populations, the PSB C. okenii strain LaCa and T. syntrophicum strain Cad16$^T$, and the GSB C. phaeobacteroides strain 1VII D7, incubated directly in the chemocline of Lake Cadagno in 50-cm-long dialysis bags (11.78–12.28 m depth). In the presence of light during the day (between 10:00 and 14:00 h) the most active $^{14}$CO$_2$ assimilator microorganism was the small-celled T. syntrophicum Cad16$^T$ with 95.2 $\pm 11.6$ $^{14}$C amol/cell/h (Fig. 2B purple bar, grey box). Big-celled C. okenii LaCa, assimilated around one half of T. syntrophicum with 53.6 $\pm 11.6$ $^{14}$C amol/cell/h, while the least effective was the GSB C. phaeobacteroides 1VII D7 with 36.1 $\pm 21.9$ $^{14}$C amol/cell/h (Fig. 2B violet and green bars, white box). During the night without any light irradiation (between 22:00 and 02:00 h), the situation of $^{14}$CO$_2$ fixation for C. okenii LaCa and C. phaeobacteroides 1VII D7 was similar to that observed during the day in presence of light, with 42.0 $\pm 5.6$ and 32.9 $\pm 5.6$ $^{14}$C amol/cell/h (Fig. 2B violet and green bars, grey box). Instead, the assimilation rate of T. syntrophicum Cad16$^T$ in the night was five times lower, 19.3 $\pm 4.0$ $^{14}$C amol/cell/h, compared to the day (Fig. 2B purple bar, grey box).

From the beginning of the pre-incubation period (from the 11th of July to 22nd of August), then during (the 22nd and 23rd of August) and after the the experiment days (until the 27th of September) the temperature and light were recorded by HOBO loggers attached on the structure holding the dialysis bags (top, middle and bottom). Temperature increased over the summer, varying from 4.5 up to 6 $^\circ$C at 11.8 m of depth. The high peaks of temperature correspond to the time the dialysis bags were collected, for the $^{14}$C experiment (Figure S3, Supporting Information). Light intensity ranged from 0 during the night to higher values during the day of around 7.6, 3.8 and 2.4 $\mu$mol/m$^2$/s PAR on the top, in the middle and at the bottom of the incubation structure, respectively.

This experiment highlighted the importance of photosynthesis in the $^{14}$C fixation process for the bacterial chemocline community of Lake Cadagno with a yield 10 times higher than at night. As for pure cultures, only PSB T. syntrophicum showed similar behavior, while C. okenii and C. phaeobacteroides did not seem to show a big difference between day and night.

Role of oxygen in the $^{14}$CO$_2$ fixation

Inorganic carbon fixation was further analyzed in the laboratory evaluating two environmental conditions such as low temperature (around 4 $^\circ$C) and the presence of microaerophily (around 5% of O$_2$). The oxygen was added in order to verify the possibility of PSB chemotrophic microrespiration suggested in recent studies (Berg et al. 2019; Luedin et al. 2019). The low temperature measurement, similar to the natural environment, has been selected to verify the possibility of artifacts related to the higher temperatures used in growing PSB and GSB in the laboratory. In the 5% O$_2$ incubation, no sulfide was present in the culture bottles whereas in the other incubation conditions (RT and 4 $^\circ$C) sulfide concentration was similar to that observed in the chemocline of the lake. In Fig. 3, both environmental conditions are compared to the standard growth conditions (see materials and methods) in presence of light and in the dark. In general, for all situations tested, the fixation of $^{14}$CO$_2$ was higher compared to the values showed in the previous experiment where fixation was analyzed in the chemocline of Lake Cadagno (Fig. 2), except for the two dark fixation rates without oxygen of C. okenii LaCa.

C. okenii LaCa cultures showed almost no fixation in the dark, except for low activity in the presence of 5% O$_2$, where the amount of CO$_2$ assimilated settled at 220.6 $\pm 61.2$ $^{14}$C amol/cell/h. Interesting, the activity almost doubled for samples incubated at low temperatures (4 $^\circ$C) compared to room temperature, 2017.6 $\pm 565.2$ and 890.4 $\pm 323.7$ $^{14}$C amol/cell/h, respectively. Similarly to the dialysis bags experiment (Fig. 2), T. syntrophicum Cad16$^T$ showed the highest values of CO$_2$ fixation also in laboratory conditions, both in the presence of light and in the dark. Its rates of 3715.9 $\pm 640.2$ and 2538.4 $\pm 283.1$ $^{14}$C amol/cell/h under light exposure at room and low-temperature conditions, respectively, were the most significant amount of CO$_2$ fixed recorded in the experiment. As for C. okenii, in the presence of 5% oxygen, photo-assimilation strongly decreased to a value of 504.5 $\pm 215.2$ $^{14}$C amol/cell/h. T. syntrophicum strain Cad16$^T$ is known to be able to fix CO$_2$ also in the absence of light (Peduzzi et al. 2012; Storelli et al. 2013, 2014), explaining its strong dark activity. GSB C. phaeobacteroides 1VII D7 showed a similar activity around 700 $^{14}$C amol/cell/h in the presence of light but without oxygen, where activity is strongly reduced. In the dark, the fixation rate was low, around 200 $^{14}$C amol/cell/h, also in presence of oxygen, confirming GSB’s strictly anaerobic behavior (Frigaard and Dahl 2008; Haas et al. 2018).

Therefore, as already observed in the field experiment, photosynthesis was more efficient in terms of $^{14}$CO$_2$ fixation in the laboratory experiment as well, with both PSB being more performing than C. phaeobacteroides. The presence of oxygen strongly reduced photosynthesis, but had no effect for chemosynthesis, except for an increase in activity for C. okenii.

Photo-oxidation of sulfide at different light intensities

Following the $^{14}$CO$_2$ assimilation assessment, the light-dependent H$_2$S oxidation rates of both dialysis bags and laboratory cultures was also tested. Growth rates of the laboratory grown three phototrophic sulfur bacteria species analyzed have been determined by FCM (Figure S4, Supporting Information). Bacterial cells were grown to their late exponential phase, at a concentration of approx. $4.0 \times 10^5$ cells/mL for C. okenii LaCa and approx. $1.0 \times 10^7$ cells/mL for both T. syntrophicum.
Figure 2. Day and night $^{14}$CO$_2$ fixation in the Lake Cadagno using dialysis bags. (A) Total $^{14}$CO$_2$ assimilated [nM/h] by the bacterial layer water sampled at 12.29 m during the day (white bar) and 12.65 m in the night (grey bar) from the chemocline of Lake Cadagno. The standard error of four replicates is showed on top of every bar. (B) $^{14}$C-assimilation rates for all three microorganisms incubated in the dialysis bags, C. okenii LaCa, T. syntrophicum Cad16T and C. phaeobacteroides 1VII D7. Error bars represent standard errors.

Figure 3. $^{14}$CO$_2$ assimilation under laboratory conditions. Inorganic carbon fixation [$^{14}$C amol/cell/h] by pure cultures of PSB C. okenii LaCa, PSB T. syntrophicum Cad16T and GSB C. phaeobacteroides 1VII D7 isolated from Lake Cadagno in the presence of light and in the dark (grey areas). RT (room temperature) was 22 $^\circ$C. Fixation of $^{14}$CO$_2$ was quantified during in situ autotrophic cultures. Standard errors are shown as error bars.

Under both conditions, in all the three strains, the increase in light intensity irradiation (from 0 to 50 $\mu$E m$^{-2}$/s) resulted in an increase in the sulfide oxidation rates, confirming the positive effect of light irradiance on the photosynthetic process (Figs 4 and 5). A saturation effect on the $\text{S}^-\text{S}$ consumption is also visible. For this reason, the photo-oxidation activity is measured for different increasing sulfide concentrations. The kinetic parameters considered for the strains are summarized in Table 2.

Dialysis bags cultures

Cultures of C. okenii LaCa reached their maximal sulfide oxidation rate of $2.29 \times 10^{-8}$ $\mu$mol/cell/h with a starting $\text{S}^-\text{S}$ concentration of 0.02 mM, with a corresponding light intensity constant K of 4.41 $\mu$E/m$^2$/s (Fig. 4A and Table 2). The lowest sulfide oxidation...
Table 2. Comparison of kinetic parameters, $V_{\text{max}}$ (μmol/cell/h) and $K$ (μE/m/s), derived from enzyme activity model.

| S²⁻ (mM)   | C. okenii LaCa | T. syntrophicum Cad16T | C. phaeobacteroides 1VII D7 |
|------------|----------------|------------------------|-----------------------------|
|            | $V_{\text{max}}$ | $K$                    | $V_{\text{max}}$ | $K$       | $V_{\text{max}}$ | $K$ |
| Dialysis bags cultures | | | | | | |
| 0.02       | $2.29 \times 10^{-8}$ | 4.41           | $1.57 \times 10^{-8}$ | 235.05 | $2.66 \times 10^{-10}$ | 3.54 |
| 0.04       | $5.42 \times 10^{-9}$ | 10.64         | $1.21 \times 10^{-8}$ | 19.31 | $9.20 \times 10^{-10}$ | 90.62 |
| 0.4        | $7.19 \times 10^{-9}$ | 9.27          | $3.23 \times 10^{-9}$ | 14.52 | $1.51 \times 10^{-8}$ | 598.64 |
| Laboratory cultures | | | | | | |
| 0.04       | $2.53 \times 10^{-9}$ | 3.40          | $3.06 \times 10^{-9}$ | 4.89 | $1.75 \times 10^{-9}$ | 3.15 |
| 0.4        | $3.42 \times 10^{-8}$ | 11.07         | $3.04 \times 10^{-9}$ | 6.33 | $2.57 \times 10^{-9}$ | 8.92 |
| 1.2        | $9.72 \times 10^{-8}$ | 32.01         | $7.15 \times 10^{-9}$ | 11.14 | $8.00 \times 10^{-9}$ | 5.38 |

Figure 4. Light-dependent sulfide oxidation in dialysis bags cultures. Sulfide oxidation rate [μmol/cell/h] vs light [μE/m²/s] for dialysis bags cultures of (A) C. okenii LaCa, (B) T. syntrophicum Cad16T and (C) C. phaeobacteroides 1VII D7 at different light intensities. Error bars represent standard deviation ($N = 3$). If no error bars are shown, SD was smaller than the symbols used.

Figure 5. Light-dependent sulfide oxidation in laboratory cultures. Sulfide oxidation rate [μmol/cell/h] vs light [μE/m²/s] for laboratory cultures of (A) C. okenii LaCa, (B) T. syntrophicum Cad16T and (C) C. phaeobacteroides 1VII D7 at different light intensities. No oxidation was observed at the concentration of 0.02 mM S²⁻. Error bars represent standard deviation ($N = 3$). If no error bars are shown, SD was smaller than the symbols used.

The rate of C. okenii was of $5.42 \times 10^{-9}$ μmol/cell/h under the concentration of 0.04 mM S²⁻, with a corresponding K value of 10.64 μE/m²/s (Fig. 4A and Table 2). T. syntrophicum Cad16T showed similar values with both 0.02 and 0.04 mM S²⁻, with an oxidation rate of $1.57 \times 10^{-8}$ μmol/cell/h with K of 235.05 μE/m²/s and $1.21 \times 10^{-8}$ μmol/cell/h with K of 19.31 μE/m²/s, respectively. Differently to C. okenii, T. syntrophicum had its minimal sulfide uptake of $3.23 \times 10^{-9}$ μmol/cell/h under the 0.4 mM concentration, with a K of 14.52 μE/m²/s. C. phaeobacteroides 1VII D7 showed the lowest sulfide oxidation rates under every concentration, with a maximal oxidation rate of $1.51 \times 10^{-8}$ μmol/cell/h and a K value of 598.64 μE/m²/s under the concentration of 0.4 mM S²⁻ (Fig. 4C and Table 2). In all cases, a saturation effect produced by the light intensity increase has been observed. The highest sulfide concentration of 1.2 mM was not analysed for dialysis bags cultures because too high compared to that encountered in the natural environment.

Laboratory cultures
In C. okenii LaCa cultures, the increase in light intensity irradiation resulted in marked changes in the sulfide oxidation rates, with little variation among analytical replicates. Maximal sulfide oxidation rates of $9.72 \times 10^{-9}$ μmol/cell/h was reached with a starting S²⁻ concentration of 1.2 mM, with a corresponding light intensity constant K of 32.01 μE/m²/s (Fig. 5A and Table 2). The lowest sulfide oxidation rate of C. okenii was of $2.53 \times 10^{-9}$ μmol/cell/h under the initial concentration of 0.04 mM S²⁻, with a corresponding K value of 3.40 μE/m²/s (Fig. 5A and Table 2).
**Light-dependent intracellular variations**

As a consequence to the exposure to different light intensities and sulfide concentrations, PSB C. okenii LaCa and T. syntrophicum Cad16T showed significant dynamic cellular complexity variations during the photosynthetic activity. These changes correlated with variations in SSC signal intensity (Fig. 6), due to the formation of sulfur globules (Danza et al. 2017). For C. okenii laboratory cultures, with initial concentrations of 0.04 and 0.4 mM S\(^{2-}\), sulfide consumption correlated with an increase of SSC (Δ approx. 7.1 × 10\(^4\) and 5.0 × 10\(^5\), respectively) between the start and the end of the experiment. The 1.2 mM S\(^{2-}\) concentration corresponded to the maximum increase in SSC (Δ approx. 6.0 × 10\(^6\)). In dialysis bags cultures of C. okenii, an increase in the concentration of sulfide (0.02-0.4 mM S\(^{2-}\)) corresponded to a progressively lower Δ of approx. 5.3 × 10\(^3\), 1.7 × 10\(^4\) and 1.3 × 10\(^5\), respectively (Fig. 6E-G). On the contrary, the FCM forward scatter (FSC) signal did not vary significantly during photo-oxidation of sulfide, indicating no change in the cell size (Figure S6, Supporting Information). Small-celled PSB T. syntrophicum Cad16T showed similar behavior in both laboratory and dialysis bags cultures, although with a less pronounced increase in the SSC signal over the measurements. The maximum increases in SSC signal (Δ approx. 4.4 × 10\(^5\) and 8.3 × 10\(^5\)) were observed with an initial S\(^{2-}\) concentration of 1.2 mM and 0.4 mM, for laboratory and dialysis bags cultures, respectively (Fig. 6D and G).

C. phaeobacteroides 1VII D7 laboratory and dialysis bags cultures showed no effect on both SSC or FSC (Fig. 6 and Figure S6, Supporting Information).

Sulfur globules are not the only compounds supposed to alter the cellular complexity and, as a consequence, the SSC parameter. SYBR Gold staining to detect changes in nucleic-acid content of the three strains did not show any particular variation between the start and the end of the experiment (Figure S7, Supporting Information), indicating no changes in the concentration of double- or single-stranded DNA or RNA following the exposure to S\(^{2-}\) and light.

Together with nucleic acids, the formation of glycogen was estimated by way of the measurement of the cellular ATP concentration. PSB C. okenii showed a slight increase in the ATP production after the exposure to light under all the S\(^{2-}\) concentrations tested (Figure S8a and d, Supporting Information). For PSB T. syntrophicum a substantial increase in ATP was observed under the 1.2 mM S\(^{2-}\), with small differences among the other S\(^{2-}\) initial concentrations and incubation conditions, although with a significantly lower ATP concentrations compared to the other strains (Figure S8b and e, Supporting Information). Similarly to PSB, we observed an increase in the ATP concentration for GSB C. phaeobacteroides in both laboratory and dialysis bags cultures for all the hydrogen sulfide concentrations tested (Figure S8c and f, Supporting Information).

As already shown in the past (Danza et al. 2017), the oxidation of sulfide correlates with the production of intracellular sulfur globules of PSB, resulting in an increase of the SSC parameter. At low concentrations of H\(_2\)S only cultures from dialysis bags showed a substantial increase in SSC, further confirming their adaptation to lower concentrations of sulfide.

**DISCUSSION**

**Physicochemical and biological properties of Lake Cadagno**

In meromictic Lake Cadagno, CTD profiles observed on the 22nd of August 2019 were in line with past measurements of the same period (Storelli et al. 2013; Danza et al. 2017; Luedin et al. 2019). The profiles of sulfide concentration during day and night are almost similar (Fig. 1A and C; green graphs), however, in presence of light the beginning of sulfide and the disappearance of oxygen (Fig. 1A and C; blue graphs) had a distance of about 2 m, in correspondence with the turbidity peak, which highlights phototropic activity with consequent oxidation of HS\(^-\) (Fig. 1B and D; purple graphs). This turbidity peak coincides with the maximal density of anoxicogenic phototrophic sulfur bacteria community, as confirmed by FCM and FISH analyses (Table 1). This bacterial layer changed in depth between the two observations at 10:00 h and 22:00 h lowering its depth of 36 cm, from 12.29 m to 12.65 m, respectively. This short-term difference is likely caused by gravity-based oscillations within the lake water column due to internal waves, also called internal seiches, typical of stratified water bodies where differences in temperature and/or salinity determine density variations with depth (Imboden and Wüst 1995; Bouffard and Boegman 2012; Bouffard and Wüst 2019).

Within the bacterial layer correlating with the turbiuity peak, we observed an almost uniform trend in the temperature and salinity profiles below the oxic-anoxic interface (Fig. 1A and C; grey and red graphs). This uniformity has been considered an indication of mixing, in this case bioconvection, as it has been demonstrated to be caused by the movement of PSB C. okenii (Sommer et al. 2017). In fact, C. okenii is capable of swimming using a flagellum in search of the optimal conditions of light (phototaxis), and sulfide (positive gradient) and oxygen (negative gradient) concentration (Pfennig 1962; Egli et al. 2004; Sommer et al. 2017).

**CO\(_2\) assimilation in the chemocline of Lake Cadagno**

In this study, measurement of in situ 14C assimilation from dialysis bags further confirmed a strong total inorganic carbon fixation rate in the chemocline of Lake Cadagno, especially during the day, which showed a 10-fold higher values in the presence of light (Fig. 2A). The CO\(_2\) assimilation rate recorded in the dark was of the same order of magnitude as that found recently by Luedin et al. (2019), but much lower compared to earlier studies using dialysis bags (Storelli et al. 2013) and punctual in situ analysis (Camacho et al. 2001). In both studies, measurements...
were taken in September, when C. okenii population normally declines, making way for the development of small-celled PSB populations (Danza et al. 2018), known to be able to fix CO\(_2\) in the dark (Storelli et al. 2013). In our case the dominance of C. okenii was also confirmed by the presence of bioconvection, as shown by temperature and conductivity profiles (Fig. 1A and C; blue and green graphs), normally absent in September. Moreover, the dark fixation measurement was carried out during the day in parallel to the phototrophic fixation by the aid of opaque bottles, so ongoing phototrophic activity might have influenced the results increasing the dark fixation values.

In the presence of light, our values were in between higher values recorded by Storelli et al. (2013) and Camacho et al. (2001) and lower values observed by Musat et al. (2008) and Luedin et al. (2019). In 2019 we were confronted with a particular weather situation with a very cold Spring that caused ice-cover on Lake
Cadagno to thaw only at the beginning of June (usually at the end of April). This late Winter condition and late Summer stratification negatively influenced the development of chemocline microorganisms highlighted during the season by much lower turbidity in the bacterial layer than normal (15–20 instead of 35–40 FTU). The lack of a complete development of the bacterial community can partially explain the total lower $^{14}$CO$_2$ assimilation observed during our experiment. This may also be due to the seasonal variations observed in the phototrophic sulfur bacteria community composition (Danza et al. 2018). PSB C. okeni is present in high concentrations in mid-Summer (July), when bioconvection is more intense (Sommer et al. 2017), while other PSB and GSB are less abundant. The decrease in C. okeni population observed towards the end of Summer (late September), when bioconvection ceases, correlates with an increase in small-celled PSB and GSB (Danza et al. 2018).

Among the phototrophic sulfur bacteria strains selected for this study, PSB T. syntrophicum Cad16T had the highest CO$_2$ fixation activity in the presence of light, almost double compared to the value of PSB C. okeni LaCa and GSB C. phaeobacteroides 1VII D7 (Fig. 2B). This observation confirms data from previous studies (Storelli et al. 2013, 2014; Luedin et al. 2019) about the inorganic carbon fixation effectiveness of T. syntrophicum Cad16T during daytime. The higher inorganic $^{14}$C-uptake rates we registered during the day compared to those observed at night suggests that PSB can actively carry out photosynthesis even at the low light intensities of the chemocline (between approx. 0.8 and 6.7 $\mu$mol/m$^2$/s PAR), as described by Fischer et al. (1996).

Despite being the most abundant strain but the smallest in cell size, GSB C. phaeobacteroides 1VII D7, was the least efficient CO$_2$-fixing strain in the light. Following this observation, CO$_2$ assimilation rates were normalized to the average cell volume of 62 ± 14 $\mu$m$^3$ calculated for C. okeni and 4.2 ± 1.3 $\mu$m$^3$ and 0.8 ± 0.3 $\mu$m$^3$ obtained for T. syntrophicum and C. phaeobacteroides, respectively. This resulted in C. phaeobacteroides becoming far more efficient than both PSB strains (data not shown). However, rates per cell volume do not reflect the true ecological relevance of each strain in the chemocline because surface to volume ratio is higher for small cells, therefore overestimating C. phaeobacteroides and T. syntrophicum’s contribution at the expense of large-celled C. okeni. For this reason, number of cells rather than volume was considered in this study.

The average C. phaeobacteroides carbon uptake rate of 36.1 ± 21.9 $^{14}$C mol/cell/h during daytime is comparable to that previously observed in Lake Cadagno for the other GSB Chlorobium clathratiforme strain Cad4DE living in Lake Cadagno (Musat et al. 2008; Storelli et al. 2013). This further suggests that GSB are generally less effective in assimilating CO$_2$ than PSB in the presence of light. On the other hand, was surprising the fact that T. syntrophicum had the lowest CO$_2$ assimilation rate in the absence of light (Storelli et al. 2013), but in this study the dark fixation phase was performed during the night (between 22:00 h and 02:00 h) and not during the day with opaque bottles. It is likely that the effectiveness of PSB in fixing CO$_2$ differs during the all day (24 h) not only according to the presence or absence of light, as already suggested by a 24 h experiment under laboratory condition (Storelli et al. 2013), and for other microorganisms (Hancé et al. 2015; Sandrini et al. 2016; Alcamañ-Arias et al. 2018). Therefore, the choice of a 4h-time span during the day for the $^{14}$CO$_2$ fixation analysis is critical for reproducibility, and comparison with other studies, of the presented results.

Dark activity of C. okeni in Lake Cadagno has previously been described by Berg et al. (2019), who observed a significant amount of C-fixation in the absence of light. It is known that some PSB species possess the capacity of microaerobic respiration (Cama-cho, Vicente and Miracle 2000; Casamayor, García-Cantizano and Pedrós-Alí 2008; Peduzzi et al. 2011; Luedin et al. 2019), and therefore it may well be possible that C. okeni showed chemoautotrophic growth on oxygen, in the absence of light. This is corroborated by our results from the laboratory experiment, where we observed an increased in C. okeni’s dark fixation in the presence of oxygen. This consideration is also supported by the fact that, on the day of the test (at 10:00 h), the chemocline resulted partly oxygenated with around 0.57 mg O$_2$/L (36 $\mu$M) in presence of light and 0.95 mg O$_2$/L (59 $\mu$M) in the dark at the depth where the dialysis bags were positioned. This small amount of oxygen probably diffused from the oxic upper layer produced during the day by photosynthetic microplankton, such as cryptomonads and other cyanobacteria-like microorganisms (Fig. 1B and D, cyan graphs), which are known to be present at the oxic-anoxic boundary (Camacho et al. 2001).

The CO$_2$ fixation of C. pheao bacterioides 1VII D7 is similar both in the day and at night, confirming earlier observations of members of the Chlorobium genus being able to assimilate inorganic carbon in the absence of light (Habicht et al. 2011). Since chemoautotrophic growth of GS B has not been reported so far, this dark activity might be due to the fermentation of organic compounds, such as glycolgen, stored in the cell and not to the presence of oxygen (Casamayor, García-Cantizano and Pedrós-Alí 2008; Habicht et al. 2011; Casamayor et al. 2012). In fact, it has been proposed that glycolgen can be consumed in the dark as an energy source via a mixed acid fermentation where CO$_2$ is required to convert phosphoenol pyruvate to oxaloacetate, which is then transformed to succinate using the reverse tricarboxylic acid cycle ($r$TCA; Sirevåg 1995; Overmann 2006; Habicht et al. 2011).

CO$_2$ assimilation under controlled laboratory conditions

The $^{14}$CO$_2$ assimilation rate of pure cultures was also analyzed under laboratory conditions, testing the low temperature and low concentration of oxygen typical of the chemocline of Lake Cadagno. Overall, the CO$_2$ uptake rates were higher compared to the values obtained in the in situ experiment, where fixation was analyzed in the chemocline of the lake (Fig. 2). This was due to the nearly 40-fold higher light intensity used in the laboratory (136.4 $\mu$mol/m$^2$/s PAR) compared to that of the chemocline (approximately 3.75 $\mu$mol/m$^2$/s PAR on average), in fact in situ and in vitro maximum $^{14}$CO$_2$ fixation rates differ by approximately the same factor.

T. syntrophicum Cad16T showed the highest values of CO$_2$ fixation also under the different laboratory conditions, both in the presence of light and in the dark (Fig. 3, pink bars). Chemo-assimilation of inorganic carbon in the presence of low oxygen concentration was similar to both other conditions, suggesting the ability to survive under microaerophilic conditions, although the chemosynthesis process is not only related to the presence of oxygen (Peduzzi et al. 2012; Luedin et al. 2019). Past proteomics analysis highlighted the presence of overexpressed enzymes part of the beta-oxidation of fatty acid and polyhydroxybutyrate (PHB) granules that should produce reducing power in the form of NAD(P)H, then potentially used in the CO$_2$ fixing process ($r$TCA or/and dicarboxylate/4-hydroxybutyrate cycle or others) during the dark period (Storelli et al. 2014).

During the day, C. okeni was also a strong CO$_2$ photoassimilator, particularly at low temperatures, while in the dark the presence of oxygen strongly enhance the chemo-assimilation (Fig. 3, red bars in the grey box). Our observations,
both in vitro and in situ, show that, in the presence of low levels of oxygen, dark CO₂ fixation activity of *C. okenii* LaCa is present, thereby confirming that these bacteria can take energy in the dark from aerobic respiration but with much lower efficiency, in accordance with recent results (Berg et al. 2019). The fact that *C. okenii* and other members of the family Chromatiaceae are capable of aerobic respiration (Kampf and Pfennig 1980) is also suggested by the presence of several oxygen-dependent enzymes such as cytochrome bd oxidase, superoxide dismutase and hemerythrin-like proteins (French, Bell and Ward 2008; Forte et al. 2016; Berg et al. 2019). Other PSB were also found to oxidize thiosulfate and sulfide under aerobic chemolithoautotrophic conditions, using RubiscO and the Calvin cycle to assimilate CO₂ (Kondratieva et al. 1976).

So far, there has not been much evidence of metabolic activity of GSB under aerobic conditions, even if Berg et al. (2019) observed that two species of GSB from Lake Cadagno possess genes encoding for terminal oxidases. This finding is corroborated by previous observations (Eisen et al. 2002; Frigaard et al. 2003; Ng et al. 2010) that GSB *Chlorobium tepidum* is equipped with several proteins against oxidative damage, suggesting that these bacteria can sustain aerobic conditions and that such enzymes play a role in protecting the cell when exposed to O₂. GSB are considered strict anaerobes, thus sensitive to even low oxygen concentrations, and therefore can be expected to have limited capabilities for oxidative stress protection. Especially in presence of light, our results showed a clear reduction in the photosynthesis of *C. phaeobacteroides* 1VII D7 when exposed to 5% of oxygen (Fig. 3) with 3-fold less ¹⁴C O₂ fixed compared to both other anaerobic conditions. Even in the dark, the presence of oxygen coincides with a lower chemo-oxidation value, different from that observed for PSB.

**Response to sulfide oxidation of PSB and GSB**

In aquatic environments, productivity of anaerobic phototrophic sulfur bacteria normally correlates well to the amount of light reaching the anaerobic layers (Parkin and Brock 1980). A similar relationship can be determined between the amount of CO₂ assimilated through anoxygenic photosynthesis by these organisms and the amount of light reaching the bacterial layer (Montesinos and van Gemerden 1986). Therefore, light is likely the main factor controlling the photosynthesis of anoxygenic phototrophic sulfur bacteria. Following these considerations, we investigated the rates of sulfide photo-oxidation of PSB *C. okenii* LaCa and *T. syntrophicum* Cad16⁷ and GSB *C. phaeobacteroides* 1VII D7.

The three strains had a similar response in the oxidation rates to variations in light intensity, albeit with considerable differences among species. All three strains, in fact, showed an increase in the sulfide uptake rate in direct proportion with the initial concentration of sulfide (Table 2). In general, however, the response to increasing light intensities is analogous to that recorded for GSB in the Chesapeake Bay (Luther, Ferdelman and Tsamakis 1988; Hanson et al. 2013; Findlay et al. 2015), where observations over the years confirmed light-dependent anaerobic sulfide uptake as a component of the sulfur cycle.

Overall, laboratory cultures of *C. okenii* LaCa showed higher *Vₘₐₓ* values indicating a faster response to the presence of S²⁻ compared to both *C. phaeobacteroides* 1VII D7 and *T. syntrophicum* Cad16⁷, that anyway had lower Kₗ values, indicating their high sensitivity to light at low radiance (Table 2). GSB, in particular, can grow at the lowest light intensities of all known phototrophs (Manske et al. 2005; Haas et al. 2018). The strains used in this experiment were grown under laboratory conditions and exposed to light intensities much higher than those normally encountered in the chemocline of Lake Cadagno. Therefore, the higher light intensity irradiance necessary to obtain the saturation with laboratory grown cultures, compared to what observed in a previous work using fresh isolates (Danza 2018), might be due to a higher production of photoprotective pigments caused by the exposure to strong artificial light (Šlouf et al. 2012). Indeed, both dialysis bags PSB cultures showed, on average, a higher response to lower sulfide concentrations (*Vₘₐₓ*) compared to laboratory cultures, particularly showing photo-oxidation activity even at 0.02 mM S²⁻ (Fig. 4A and B). At the same sulfide concentrations, GSB *C. phaeobacteroides* had oxidation rates comparable to those observed in the laboratory cultures (Fig. 4C). The better response showed by dialysis bags cultures to lower sulfide concentrations is probably due to the 6-weeks incubation period in the chemocline of the lake during which the cultures acclimatized to the conditions of the natural environment, where they are exposed to a lower light irradiance but, at the same time, are more capable of using it. As shown above, the intensity of light used in the growth of the cultures is very important to regulate the affinity, especially of PSB, to small amounts of sulfide. As for the quality of light, we did not observed substantial differences in the growth rates using 3 different light spectra (data not shown).

As for CO₂ assimilation, cell volume normalization of sulfide oxidation rates resulted in substantial differences, with large-celled *C. okenii* becoming by far the least efficient under any S²⁻ concentration (Figure S9, Supporting Information), suggesting that, for the purpose of this work, photosynthetic activity too should not be investigated in terms of cell volume, which tends to overestimate the real sulfide and light absorption of small-celled bacteria.

Of note, fitting the kinetic data of sulfide oxidation (Fig. 4) revealed a deviation from linearity in the dose/response curve (sulfide oxidation/light irradiance) of the three bacterial strains. All species displayed a kinetic curve with a Hill coefficient between 1.5 and 2.0 (data not shown). Hill coefficient was historically employed to quantify binding cooperativity to enzymes or transport proteins with multiple substrate binding sites (Hill 1910), and its usage further extended to quantify non-linear dose/response in different contexts (Ferrell and Machleder 1998). A typical example of binding cooperativity is the case of hemoglobin, which contains four oxygen binding sites, and a measured Hill coefficient of 2.8. This indicates a positive effect exerted by the binding of a oxygen molecule onto the next oxygen molecule binding (i.e. the nth oxygen bound to hemoglobin, facilitates the binding of the (n-1)th oxygen molecule). Similarly, in this present study, the deviation from the linear range observed in Fig. 4, indicates a homotypic form of cooperativity, in which the sulfide oxidation rate is enhanced at intermediate light irradiance in a switch-like manner. Further experiments are needed to determine if this effect is due to cooperative binding of sulfide molecules to the enzymes that metabolize them, due to enhanced import mechanisms on the cell surface after a dosage threshold, or at a more macroscopic level, at which cells might deploy a concerted response after a sulfide concentration is reached (e.g. by quorum sensing).

**Light-dependent intracellular variations**

PSB *C. okenii* LaCa and *T. syntrophicum* Cad16⁷ displayed a significant alteration in the intracellular complexity in response to photo-oxidation of sulfide, as clearly shown by the SSC signal (Fig. 6). This increase in intracellular complexity is directly related to the formation of sulfur globules (S⁹) after the
oxidation of sulfide. In the laboratory cultures, the increase in SSC signal observed after the incubation, and the relative maximal SSC values, positively correlated with the initial sulfide concentration (Fig. 6A–D), whereas in dialysis bags cultures a major increase in the internal complexity was observed at lower S²⁻ concentrations, particularly for C. okenii (Fig. 6E). The absence of sulfide oxidation and SSC variation recorded under dark incubation corroborates this observation (Figure S5b and c, Supporting Information). These findings are in line with those from Danza et al. (2017) and other studies (Casamayor et al. 2007; Frigaard and Dahl 2008) correlating sulfide oxidation with the intracellular accumulation of sulfur globules, as a result of their photosynthetic activity (Mas and Van Gemerden 1995). Moreover, the higher SSC values observed at low sulfide concentrations in dialysis bags seem to suggest that, under natural conditions, PSB reach the optimum at low sulfide concentrations. This consideration is corroborated by the fact that in the lake the lowest sulfide concentrations are found in the upper part of the bacterial layer, where light penetration is higher.

Anoxygenic phototrophic sulfur bacteria are also known for storing other inclusion bodies, such as glycogen, that might influence the SSC signal (Mas and Van Gemerden 1995). Moreover, it has been observed that accumulation of storage carbohydrates is usually coupled with the production of elemental sulfur during anoxygenic photosynthesis (Mas and Van Gemerden 1995; Casamayor et al. 2007). In PSB and GSB, the glycogen synthetic pathway involves ATP and α-D-glucose-1-phosphate as substrates of the enzyme glucose-1-phosphate adenyltransferase in the biosynthesis of ADPglucose, which is then transferred to an α-glucan primer to form the α-(1, 4) linkages in the polymer in a reaction catalyzed by glycogen synthase (Sirévág 1995; Wilson et al. 2010; Machtet et al. 2012). The observed increase in ATP concentration in all the strains, particularly under the 0.4 and 1.2 mM S²⁻ concentrations, could then result from the achievement of conditions where electron transport initiated by light reaction triggers the production of the ATP and the reducing power needed for the synthesis and storage of glycogen. In fact, the synthesis of glycogen has been found to be maximal in presence of a high energy state of the cell (Preiss and Sivak 1999; Ballicora, Iglesias and Preiss 2004). The accumulation of glycogen, together with polyhydroxybutyrate, is supposed to be involved in the process of dark CO₂ assimilation, particularly in T. syntrophicum Cad16⁷ (Storelli et al. 2014). Moreover, the little increases in SSC values of C. pheobacteroides after the exposure to light (Fig. 6) were probably the consequence of variations in glycogen and other inclusion bodies detected by SSC, rather than a result of internal storage of S²⁻, since it has been widely demonstrated that GSB secrete sulfur globules outside the cell (Frigaard et al. 2003; Frigaard and Dahl 2008; Holkenbrink et al. 2011).

Overall, the metabolic activity during the light-dependent sulfide oxidation did not affect the cell size (FSC signal, Figure S6, Supporting Information). Our data demonstrate that photosynthetic activity in phototrophic sulfur bacteria determined intracellular physiological alterations by the accumulation of inclusion bodies with no effects on cell size.

CONCLUSIONS

In this paper, we highlight the ecological importance as primary producers of the anoxygenic phototrophic sulfur bacteria community living in the chemocline of Lake Cadagno. The ¹⁴CO₂ assimilation rates of PSB C. okenii strain LaCa, PSB T. syntrophicum strain Cad16⁷ and GSB C. pheobacteroides strain 1VII D7, corresponding to the 72.8% of the total community in Lake Cadagno, indicated that they are much more active in the presence of light via photosynthesis than in the dark via chemotrophy, both in situ and in vitro.

In both experiments, in the presence of light, small-celled PSB T. syntrophicum Cad16⁷ proved to be the most effective in CO₂ photo-assimilation, followed by the other PSB C. okenii LaCa. As in previous studies, GSB C. pheobacteroides 1VII D7 showed a low photo-assimilation activity also because of its very small size compared to PSB. Moreover, for all strains, the presence of oxygen strongly reduced the photosynthetic activity, while in absence of light, especially for PSB C. okenii LaCa, the presence of a small amount of O₂ strongly increased the fixation of CO₂. Interestingly, the condition of microaerophily (around 5% of O₂) did not have the same amplifying effect for the other PSB T. syntrophicum Cad16⁷, which suggests the presence of another different mechanism of chemotrophy. In general, dark CO₂ fixation was substantially lower compared to photosynthesis for all three species.

Photosynthetic CO₂ fixation is directly correlated to the efficacy of pigments in collecting photons of light and to the capability of oxidizing reduced compounds, such as H₂S. Here, we compared the ability of our three anoxygenic phototrophic sulfur bacteria strains to oxidize different concentrations of sulfide with increasing light intensities. As observed in the ¹⁴CO₂ assimilation experiment, both PSB species were more efficient than C. pheobacteroides 1VII D7 in terms of photosynthetic activity. Big-celled PSB C. okenii LaCa was able oxidize the highest amount of sulfide probably due to its large cell size. In fact, once the results were normalized to cellular biovolume, the small GSB C. pheobacteroides 1VII D7 became the most efficient. After the 6-weeks incubation in Lake Cadagno chemocline inside dialysis bags, the cultures showed an higher affinity to lower sulfide concentration compared to laboratory cultures.

From an ecological point of view, at the level of individual species, T. syntrophicum Cad16⁷ seems to be the most effective in fixing CO₂, especially in the presence of light, even if it is the least present in chemocline. Instead, the most abundant but also the smallest, C. pheobacteroides 1VII D7, is the least active in terms of CO₂ fixation, besides being the most sensitive to oxygen. The largest and most efficient in the photo-oxidation of H₂S in vitro, C. okenii LaCa, in our study does not seem to be so strong in fixing CO₂, as shown in the past. In the natural environment, however, it can produce a turbulence process known as bioconvection, which could represent a competitive advantage in the quest for the optimal environmental conditions and affect the effectiveness of other microorganisms.

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**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSEC online.

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**Conflicts of Interest.** None declared.

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