Dark aerobic sulfide oxidation by anoxygenic phototrophs in the anoxic waters of Lake Cadagno

Jasmine S. Berg[1,2]*, Petra Pjevac[3], Tobias Sommer[4], Caroline R.T. Buckner[1], Miriam Philippi[1], Philipp F. Hach[1], Manuel Liebeke[5], Moritz Holtappels[6], Francesco Danza[7,8], Mauro Tonolla[7,8], Anupam Sengupta[9], , Carsten J. Schubert[4], Jana Milucka[1], Marcel M.M. Kuypers[1]

[1]Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany
[2]Institut de Minéralogie, Physique des Matériaux et Cosmochimie, Université Pierre et Marie Curie, CNRS UMR 7590, 4 Place Jussieu, 75252 Paris Cedex 05, France
[3]Division of Microbial Ecology, Department of Microbiology and Ecosystem Science, University of Vienna, 1090 Vienna, Austria
[4]Eawag, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland
[5]Department of Symbiosis, Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany
[6]Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung, Am Alten Hafen 26, 27568 Bremerhaven, Germany
[7]Laboratory of Applied Microbiology (LMA), Department for Environmental Constructions and Design (DACD), University of Applied Sciences and Arts of Southern Switzerland (SUPSI), via Mirasole 22a, 6500 Bellinzona, Switzerland
[8]Microbiology Unit, Department of Botany and Plant Biology, University of Geneva, 1211 Geneva, Switzerland
[9]Institute for Environmental Engineering, Department of Civil, Environmental and Geomatic Engineering, ETH Zurich, 8093 Zurich, Switzerland.

*corresponding author: Jasmine.Berg@mnhn.fr

ORIGINALITY-SIGNIFICANCE STATEMENT

This study reveals that sulfide oxidation within an anoxic layer of purple sulfur bacteria in the stratified water column of Lake Cadagno is largely coupled to oxygen consumption. Our findings imply that aerobic metabolism may be more prevalent in anoxic zones than previously thought. We also present a metagenome-assembled genome of *Chromatium okenii* which is the first genome sequence for the genus *Chromatium* and reveals new interesting physiological features of this environmentally relevant organism including its capacity for aerobic respiration.

SUMMARY

Anoxygenic phototrophic sulfide oxidation by purple and green sulfur bacteria plays a key role in sulfide removal from anoxic shallow sediments and stratified waters. Although some purple sulfur bacteria can also oxidize sulfide with nitrate and oxygen, little is known about the prevalence of this chemolithotrophic lifestyle in the environment. In this study, we investigated the role of *Chromatium okenii* in chemolithotrophic sulfide removal in the chemocline of Lake Cadagno. This purple sulfur bacterium appears to remain active during the night, as evidenced by its continued motility and O_2-driven carbon fixation. Our temporally resolved, high-resolution chemical profiles revealed that sulfide oxidation is largely driven by aerobic respiration in the anoxic chemocline. We postulate that the abundant and highly active *Chr. okenii* are, at least in part, responsible for this aerobic sulfide oxidation and that they bridge the spatially separated gradients of oxygen and sulfide using a novel mechanism of transport driven by the strong convection within the chemocline. The genome of *Chr. okenii* reconstructed from the Lake Cadagno metagenome confirms its capacity for microaerophilic growth and provides further insights into its metabolic capabilities. Altogether, our observations suggest that aerobic respiration may not only play an

50 underappreciated role in anoxic environments, but also that organisms typically considered strict
51 anaerobes may be involved.

52 **INTRODUCTION**

53 Anoxygenic phototrophic bacteria oxidizing sulfide and fixing CO_2 with sunlight play an important
54 role in the carbon and sulfur cycles of sulfidic, shallow sediments and stratified water columns.
55 Phototrophic sulfur bacteria, for example, are responsible for 20-85% of the total daily carbon
56 fixation in anoxic lakes (summarized in Cohen *et al.,* 1977). This primary production is so
57 important that it can control the bulk C-isotope fractionation in the water column, generating
58 isotopic signatures that are transported and preserved in sediments (Posth *et al.,* 2017). Biomass
59 from anoxygenic phototrophs feeds both grazing zooplankton in overlying oxic waters (Sorokin
60 1966) and drives sulfate reduction in anoxic waters below (Pfennig 1975). The phototrophic sulfur
61 bacteria also remove toxic sulfide from the water column enabling aerobic life at the surface while
62 recycling sulfur compounds for sulfate reducers. While their role in sulfide detoxification has long
63 been recognized in stratified lakes, there is mounting evidence that phototrophic sulfur bacteria
64 also significantly impact sulfur cycling in marine environments such as the Black Sea (Jørgensen
65 *et al.,* 1991) and the Chesapeake Bay (Findlay *et al.,* 2015).

66 Anoxygenic phototrophs generally inhabit illuminated, anoxic, reducing environments due to the
67 toxicity of oxygen to these bacteria, and to the competition with abiotic reactions involving oxygen
68 for their electron donors. Nonetheless, some anoxygenic phototrophs have evolved the capacity
69 for chemotrophic growth under microoxic conditions. Whereas the green sulfur bacteria (GSB) of
70 the *Chlorobiaceae* family are strict anaerobes, members of *Proteobacteria* collectively known as
71 the purple sulfur bacteria (PSB), can be anaerobic to microaerobic (e.g. Kampf and Pfennig, 1980;

de Witt and Van Gemerden, 1990). Both the GSB and PSB are well adapted to fluctuating environmental conditions, synthesizing and accumulating storage compounds during periods of nutrient excess. The anoxygenic phototrophs are known to store zero-valent sulfur (S^0), polyphosphate, glycogen, and in the case of the PSB alone, poly-3-hydroxyalkanoates (PHA) (Mas and Van Gemerden, 1995). The macromolecular structure and metabolism of these compounds have been intensely studied in laboratory pure cultures in order to understand conditions leading to their accumulation and breakdown. It has been suggested that glycogen may play a role in energy generation under dark conditions based on observations that cultured *Chromatium* sp. utilize glycogen to reduce stored sulfur, yielding sulfide and PHA (Van Gemerden, 1968).

Here we investigated the role of anoxygenic phototrophic bacteria in dark sulfur cycling processes in Lake Cadagno, a permanently stratified lake with high sulfate concentrations of up to 1-2 mM in the monolimnion. Microbial reduction of sulfate in the anoxic bottom waters and sediments produces large amounts of sulfide which support dense populations of GSB and PSB in the photic zone. These bacteria heavily influence the chemistry of the lake, forming a sulfide- and oxygen-free chemocline of 1-2 meters in thickness. The PSB *Chromatium okenii* is by far the most active of these bacteria, having been shown to play a disproportionately large role in inorganic carbon and ammonium assimilation despite their low abundances (<1% of total cell numbers) in the chemocline (Musat *et al.,* 2008; Posth *et al.,* 2017). In addition to their important contribution to light-driven sulfide oxidation, previous studies have shown that the anoxygenic phototrophic bacteria of Lake Cadagno remain active in the dark (Musat *et al.,* 2008; Halm *et al.,* 2009; Storelli *et al.,* 2013). However, their mechanism of energy generation in the absence of light is not yet clear. There is also evidence for dark sulfide consumption, but the electron acceptors utilized

94 remain unknown. We therefore combined high-resolution biogeochemical profiling with
95 metagenomic analyses to gain an overview of possible light-independent metabolic processes
96 impacting the sulfur biogeochemistry of Lake Cadagno. In addition to providing insights into the
97 metabolism of anoxygenic phototrophic bacteria *in situ*, we present a model to explain the
98 mechanism of dark sulfide oxidation in the chemocline of this meromictic lake.

99 **RESULTS & DISCUSSION**

100 ***Biogeochemistry of Lake Cadagno***

101 Lake Cadagno is characterized by an oxic mixolimnion and a sulfidic monolimnion spatially
102 separated from each other by a chemocline (defined by bold contour lines in Fig. 1a) free of
103 detectable oxygen (detection limit 50-100 nM) and containing very little sulfide. In August 2015,
104 oxygen disappeared just above the chemocline close to 12 m depth. The daytime increase in
105 oxygen concentrations between 11-12 m depth denotes net photosynthesis and the nighttime
106 decrease denotes net respiration (Fig. 1a). The permanent absence of oxygen in the chemocline
107 indicated that oxygen was consumed both in the day and the night.

108 Steep gradients of sulfide diffusing into the chemocline varied independently of light-dark periods
109 and the total sulfide concentration in the chemocline did not exceed 5 µM at any time point.
110 Because the lake is meromictic, these stratified conditions were also present during other
111 sampling years (see Fig. S2 for 2013 and 2014 profiles). In 2015, the 0.5-1 m wide chemocline was
112 located around 11-12 m depth, with the exact location varying over the day most likely due to the
113 action of internal waves (Egli *et al.,* 1998). In previous years, the chemocline was up to 2 m wide
114 (Fig. S2) and remained completely sulfide-free in the dark. Conservative properties such as
115 temperature and conductivity were constant throughout the chemocline in all years sampled (Fig.

S1&2) indicating mixing of this zone (Sommer *et al.,* 2017). Flat conductivity profiles revealed stronger mixing of the chemocline in 2013 and 2014 (Fig. S2) than in 2015 (Fig. S1) when the region of constant conductivity was reduced or absent.

Chr. okenii was the most significant microorganism in the chemocline in terms of biomass, accounting for ~60-80% of total microbial biovolume (Sommer *et al*. 2017), and carbon fixation (Musat *et al.,* 2008). The cell abundances of *Chr. okenii* in the Lake Cadagno chemocline were enumerated by flow cytometry during 2 daily cycles (Fig. 1b). Higher densities of *Chr. okenii* were found in 2014 ($10^6 \cdot ml^{-1}$) than in 2015 ($10^5 \cdot ml^{-1}$). *Chr. okenii* is highly motile, swimming at speeds of ~27 $\mu m \cdot s^{-1}$ and has been hypothesized to drive the convection and mixing of the chemocline (Wüest, 1994; Sommer *et al.,* 2017). *Chromatium* are known to migrate between gradients of sulfide, light, and oxygen by photo- and chemotaxis (Pfennig *et al.,* 1968). We observed that *Chr. okenii* were positioned between oxygen and sulfide gradients, regardless of changes in depth or light availability (Fig. 1a,b). Other anoxygenic phototrophs that have been consistently detected in the chemocline include the PSB *Lamprocystis, Thiocystis* and *Thiodictyon* and several GSB of the genus *Chlorobium* (Tonolla *et al.,* 1999, 2004, 2005). Together these bacteria constituted the majority of the total phototrophic cells ($10^6 \cdot ml^{-1}$) in 2015, but they are considerably smaller than *Chr. okenii*.

The oxidation of sulfide by these anoxygenic phototrophs proceeds via the formation of S^0 as an obligate intermediate (Mas and Van Gemerden, 1995). This S^0 was measured as particulate sulfur on 0.7 µm filters and may comprise S^0 stored intracellularly by PSB and S^0 adhering extracellularly to GSB. The highest concentrations of S^0 (up to 45 µM; Fig. 1c) coincided with the highest *Chr. okenii* cell numbers (Fig. 1b) in the chemocline. It is likely that this S^0 was present in the form of

both elemental S and polysulfides formed by the reaction of free sulfide with intra- and extracellular S^0, as has previously been suggested in other euxinic lakes (Overmann, 1997). Our analytical method for total S^0 did not distinguish between different forms of S^0 such as cyclooctasulfur and polysulfides. However, we could confirm the presence of polysulfides inside live *Chr. okenii* cells in environmental samples using Raman spectroscopy. The Raman spectrum of a sulfur inclusion from *Chr. okenii* exhibited two weak peaks at 152 and 218 and a prominent peak at 462 cm^{-1} (Fig. S3) which is characteristic of linear polysulfide species (Janz *et al.,* 1976). The Raman peak at ~2900 cm^{-1} corresponds to the CH_2 and CH_3 stretching vibrations (Socrates, 2004), and its co-occurrence with polysulfide peaks support the theory that the sulfur chains in these purple sulfur bacteria are terminated by organic end groups as reported previously (Prange *et al.,* 1999).

Over two diurnal cycles, the S^0 inventory (Fig. S4a), or the total amount of particulate S^0 in the chemocline, was much lower than expected from the sulfide gradients and corresponding sulfide fluxes (discussed below), suggesting that stored S^0 served only as a transient intermediate and was rapidly oxidized to sulfate. No day-night trends in S^0 accumulation were apparent in the chemocline. Nevertheless, the increase in the S^0 inventory, at several time points during the night was indicative of dark sulfide oxidation.

In culture, *Chromatium* spp. are known to store carbon compounds like glycogen and polyhydroxyalkanoates (PHAs) which have been proposed to be involved in dark sulfur metabolism (Mas and van Gemerden, 1995). We therefore quantified glycogen and PHA abundance in biomass samples from one day/night profile of the chemocline (Fig. 2). We could not detect any PHA, but the presence of glycogen during the day and night coincided with *Chr.*

160 *okenii* cell numbers (Fig. 2). This is consistent with previous reports of glycogen storage and an
161 absence of PHA in natural populations of *Chr. okenii* (Del Don *et al.,* 1994). While the highest
162 potential cellular glycogen content ($2.38 \cdot 10^{-6}$ µg/cell) was found at the top of the chemocline
163 during the day, we observed little change in the cellular glycogen content between day and night
164 (Fig. S5). Average potential cellular glycogen decreased from $5.50 \cdot 10^{-7}$ µg/cell during the day to
165 $5.33 \cdot 10^{-7}$ µg/cell during the night, which represents a 3% reduction in cellular glycogen reserves.
166 This is in contrast with a previous study of storage compounds in natural populations of *Chr. okenii*
167 in Lake Cadagno which reported 50% decrease in glycogen reserves in the dark (Del Don *et al.,*
168 1994). This apparent decrease in glycogen reported previously may be a result of undersampling,
169 as our time- and depth-resolved biogeochemical profiles revealed light-dark independent
170 variations in *Chr. okenii* cell numbers and glycogen concentrations. While it has been
171 demonstrated that *Chromatium* sp. in pure cultures obtain energy from the reduction of S^0 with
172 glycogen in the dark (Van Gemerden, 1968), we could not confirm this observation for *Chr. okenii*
173 *in situ.* From our data, we conclude that storage compounds did not play a significant role in the
174 dark respiratory metabolism of *Chr. okenii* in the Lake Cadagno chemocline.

175 Sulfate was measured as the end product of sulfide oxidation, but due to the high (1-2 mM)
176 background sulfate concentrations, the comparably small concentration changes resulting from
177 sulfide oxidation processes are non-detectable. To identify regions of sulfate production in and
178 around the chemocline, we therefore determined deviations from the sulfate-conductivity mixing
179 line drawn for each profile (see Fig. S6 for details). Strong mixing of the chemocline is expected
180 to produce a linear relationship between sulfate and conductivity, and large digressions from this
181 best-fit line indicated that sulfate was produced faster than the rate of mixing. The expected

sulfate concentration could be extrapolated based on measured conductivity, and then subtracted from the measured sulfate concentration to give excess sulfate:

$$\text{measured } [SO_4^{2-}] - \text{expected } [SO_4^{2-}] = \text{excess } [SO_4^{2-}]$$

This excess sulfate was attributed to biological sulfate production. Sulfate profiles from 2015 plotted over two diurnal cycles exhibited a peak at the top of the chemocline in the region of oxygen depletion (Fig. 1d). Interestingly, sulfate production was observed both during and at the end of the night. The overlap of excess sulfate and oxygen in 2015 profiles was the first indication that sulfide may be oxidized aerobically, without sunlight. Daytime sulfate production in 2014 related to photosynthetically active radiation (PAR) intensity (Fig. S2), suggesting that sulfide and S^0 could either have been oxidized aerobically within the chemocline using *in situ*-produced oxygen (Milucka *et al.,* 2015) or phototrophically. The comparatively broad biogenic sulfate peak in the 2014 night profile likely reflects the broader vertical distribution of the *Chr. okenii* population (Fig. S2).

The sulfate excess in the chemocline is not expected to be affected by sulfate reduction as no sulfate reduction was detected within the chemocline in 2014 or 2015. The sulfate reduction rates measured in the sulfidic zone 1 m below the chemocline were about 235 $nM \cdot d^{-1}$ and 375 $nM \cdot d^{-1}$ in 2014 and 2015, respectively.

To quantify biological sulfide consumption over time, we calculated the total sulfide flux into the chemocline (Fig. S4b). Assuming that phototrophic sulfide oxidation ceases in the dark, upwards-diffusing sulfide should accumulate in the chemocline at night. The expected sulfide accumulation was calculated based on fluxes into the layer over a 10-h night period and compared to the actual

sulfide concentration observed in the layer. From an average sulfide flux F = 0.15 µm·cm^{-2}h^{-1} (Fig. S4b), into a well-mixed layer of thickness H = 1 m over t = 10 hours, the resulting sulfide concentration $C = F*t/H$ should be about 15 µM in the chemocline. However, the sulfide measured in the layer was about 3 µM (Fig. 1a), or five times less, indicating that sulfide is consumed.

We therefore partitioned the total sulfide flux into two fractions: the flux of biologically consumed sulfide and the flux of residual sulfide in the chemocline. First, the amount of residual sulfide was calculated at each sampling time point by integrating sulfide concentrations within the mixed layer (Fig. S4c). The rate of sulfide accumulation was then calculated for each 4-h sampling interval and subtracted from the total sulfide flux to give the biologically consumed sulfide flux. The flux of sulfide consumed in the dark was in the same range as in the day (0.03 to 0.22 µmol·cm^{-2}h^{-1}) and the residual sulfide flux was very small in comparison (Fig. 3a). The observed variations did not correlate with day-night cycles and the changes of sulfide gradients could have been induced by internal waves, as mentioned above. Together, this indicates that sulfide oxidation continued in the dark and seemed to be related to the total sulfide flux (Fig. S4b) rather than the presence of sunlight. For comparison, the upwards flux of sulfide in previous years was slightly lower, or 0.011-0.024 µmol·cm^{-2}h^{-1} in 2013 and 0.032-0.072 µmol·cm^{-2}h^{-1} in 2014.

It was not possible to calculate S^0 fluxes in Lake Cadagno because S^0 is actively transported by the motile purple sulfur bacteria during chemo- and phototaxis (Pfennig *et al.,* 1968) independent of diffusive processes. The total (upwards and downwards) biogenic sulfate flux (Fig. S4d) in this region was roughly equivalent to the sulfide flux and followed a similar trend.

Overall, our high-resolution profiles revealed that sulfide in Lake Cadagno was consumed during the day and night, but only light-dependent sulfide oxidation has thus far been recognized as a major sulfide-removing process in the lake. In the absence of light, it is also possible that alternative electron acceptors such as NO_x^-, Fe^{3+}, Mn^{4+}, or O_2 play a role in sulfide oxidation. Nitrate and nitrite concentrations in the Lake Cadagno chemocline are negligible (Halm *et al.,* 2009; Milucka *et al.,* 2015). High fluxes of reduced, dissolved metals (0.027 µmol $Fe \cdot cm^{-2} \cdot d^{-1}$ and Mn 0.049 µmol $Mn \cdot cm^{-2} \cdot d^{-1}$) suggest that Fe- and Mn-oxides are rapidly reduced by microorganisms or abiotically by sulfide in the chemocline (Berg *et al.,* 2016), but re-oxidation of Fe and Mn would ultimately depend on oxygen in the dark. We therefore considered oxygen as the principal direct (or indirect) oxidant responsible for observed dark sulfide oxidation.

The oxygen flux into the chemocline varied slightly between 0.022-0.071 $\mu mol \cdot cm^{-2} h^{-1}$ over the period of 48 h (Fig. 3b). Oxygen fluxes measured in 2013 and 2014 were in the same range, or 0.013-0.048 $\mu mol \cdot cm^{-2} h^{-1}$ and 0.037-0.073 $\mu mol \cdot cm^{-2} h^{-1}$, respectively. To relate oxygen fluxes to sulfide consumption, we assumed a 2:1 stoichiometry between oxygen and sulfide for aerobic sulfide oxidation to sulfate:

(1) $2O_2 + H_2S \rightarrow 2H^+ + SO_4^{2-}$

If all oxygen was used to respire sulfide, calculated oxygen fluxes in 2013 and 2014 were in all cases sufficient to account for the sulfide oxidized in the dark. In 2015, aerobic sulfide respiration could account for up to 10-50% of sulfide oxidized during the day and 5-45% of sulfide oxidized during the night (Fig. 3c). During the day, the remainder of sulfide oxidation could be attributed to anoxygenic photosynthesis and/or aerobic sulfide oxidation fueled by *in situ* oxygen production

by photosynthetic algae. At several time points in the dark, however, we could not explain the disappearance of roughly 60-90% of upwards-diffusing sulfide. We hypothesize that the missing oxygen is supplied laterally from the turbulent transport initiated by internal wave breaking at the lake boundaries. The convection within the chemocline may be key to the transport of oxygen and sulfide to aerobic sulfide-oxidizing bacteria in the chemocline. A weakening of the mixing regime was observed in August 2015 (Sommer *et al.,* 2017) which may have signified a slowed transport of electron acceptors, thus contributing to the accumulation of sulfide in the chemocline.

Mixing and bacterial motility in Lake Cadagno

To test the importance of lateral and vertical mixing, we set up simplified laboratory incubations where water from Lake Cadagno chemocline was inoculated into agar-stabilized sulfide gradient tubes. After five weeks of incubation under permanent light conditions, dense communities of PSB developed between the gradient of upwards-diffusing sulfide and the surface colonies of photosynthetic algae (Fig. S7). Microsensor profiles revealed that sulfide was completely consumed at the base of the PSB layer in the light, but as soon as the light was turned off, the sulfide gradient diffused upwards through the agar into the zone of purple bacteria. This is in contrast to the sulfide profiles in the lake where irrespective of the day-night cycle, sulfide is consistently consumed at the bottom of the chemocline. We speculate that restricted bacterial motility in the agar and diffusion-limited conditions may have accounted for the differences observed between our cultures and *in situ* sulfide consumption as bacterial motility and mixing conditions appear necessary for continued dark sulfide oxidation in Lake Cadagno.

In fact, we could confirm that *Chr. okenii* are highly motile both in the day and the night by performing dark field video microscopy (see Movie S1 in Supplementary Materials) of environmental samples obtained during the night and monitored in a dark room to avoid any light-induced artefacts. Although the average night time swimming speed of *Chr. okenii* (9.9 µm s^{-1}; see Fig. S8) was a third of the day time swimming speed (27 µm s^{-1}; Sommer et al. 2017), it is clear that *Chr. okenii* remains motile even under dark conditions.

Metagenomic insights into the Chromatium okenii *population in Lake Cadagno*

To assess whether the genomic potential supports light-independent, aerobic sulfide oxidation by *Chr. okenii* in Lake Cadagno, we sequenced two metagenomes, one from the Lake Cadagno chemocline and one from the phototrophic, sulfide-oxidizing enrichment culture in an agar tube described above (Table S1). From a combined metagenomics assembly, we reconstructed a high quality (90% complete, <1% contaminated) metagenome-assembled genome (MAG) of a PSB highly abundant in the sulfur-oxidizing enrichment culture (Fig. S9). The recovered MAG had a low average nucleotide identity ANI (<70%) to any sequenced *Chromatiaceae* genomes (data not shown). However, it encoded an rRNA operon, including a complete 16S rRNA gene with 99% sequence identity to the 16S rRNA gene of *Chr. okenii* (Imhoff *et al.,* 1998; Tonolla *et al.*, 1999), and thus likely represents a strain of *Chr. okenii* which is the type strain of the genus *Chromatium*. At this time, *Chr. okenii* has not been successfully isolated in pure culture, nor is there any published genome available for this organism.

The key metabolic process of *Chr. okenii* in Lake Cadagno is photoautotrophic sulfur oxidation. In accordance, the *Chr. okenii* MAG contained genes encoding for a sulfide : quinone reductase (*sqr*)

and the full genomic inventory encoding for a reverse-acting dissimilatory sulfite reductase (rDSR) pathway (Fig. 4). The operon structure of the rDSR encoding genes (*dsrABEFHCMKLJOPN*) was identical to the operon structure in the well described PSB model organism *Allochromatium vinosum* (Dahl *et al.,* 2005), but no *dsrR* and *dsrS* gene were found. No genes encoding for sulfur oxidation via the SOX pathway, or homologues of sulfur globule proteins (*sgpABC*) typically found in PSB were detected in the draft genome. In line with its phototrophic metabolism, the *Chr. okenii* MAG showed the genomic potential for photosynthesis, with the genes encoding for a light harvesting complex 1 (*pufAB*) and a PSB-type photosynthetic reaction center (*pufLMC*) encoded in a single operon. Furthermore, the full genomic repertoire for a NADP-Me type C4 photosynthetic carbon assimilation cycle, and all genes (with exception of *cbbS* encoding for the small subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase) necessary for CO_2 assimilation via the Calvin-Benson-Bassham (CBB) Cycle were present (Fig. 4).

Many *Chromatiaceae* can grow chemoautotrophically, respiring oxygen under microoxic conditions (Kämpf and Pfennig, 1980). Cytochrome (Cyt) *c*-containing oxidases (e.g. Cyt *aa3*, Cyt c*bb3*) were not found in the *Chr. okenii* MAG. However, a Cyt *bd* type ubiqinol oxidase, known to function as sulfide-resistant O_2-accepting oxidase in other *Gammaproteobacteria* (Forte *et al.,* 2016), was identified (Fig. 4). Further, a plethora of genes related to heme *b* (*gltX*, *hemALBCD*, and *hemH*) and siroheme (*cysG*) synthesis, degradation (a heme oxygenase) and export (ABC-type heme exporter, *ccmABCD*), as well as hemerythrin-like metal binding proteins were encoded. Hemerythrin has been implicated in binding of oxygen for delivery to oxygen-requiring enzymes, for detoxification, or for oxygen sensing in motile, microaerobic prokaryotes (French *et al.,* 2007). The presence of these oxygen-dependent enzymes, as well as a key oxidative stress defense

309 enzyme superoxide dismutase (SOD), support the idea that *Chr. okenii* may be facultatively
310 microaerobic. A complete set of genes for flagellar biosynthesis (*fliDEGHJKLMNOPQRW*,
311 *flgABCDEFGHIK*, *flhAB*) and flagellar motor proteins (*motAB*) confer motility to this bacterium.

312 Several other genes revealed interesting metabolic capacities of *Chr. okenii.* A cytosolic
313 bidirectional [NiFe] type 3d hydrogenase and a nitrogenase were encoded in the MAG (Fig. 4),
314 implicating the potential for involvement of *Chr. okenii* in nitrogen fixation and hydrogen
315 oxidation which has previously been overlooked. Additionally, the *Chr. okenii* MAG encoded a
316 glycogen synthase and a glycogen debranching enzyme, as well as the full genomic repertoire
317 necessary for polyhydroxyalkanoate (PHA) biosynthesis. This is consistent with the detection of
318 glycogen in our biogeochemical profiles of the chemocline. Finally, it is possible that novel
319 terminal oxidases are among the hypothetical genes that could not be assigned any known
320 function.

321 **CONCLUSIONS**

322 It is intriguing that oxygen should play a major role in sulfide oxidation in the ostensibly anoxic
323 chemocline of Lake Cadagno, especially by purple sulfur bacteria generally thought to lead an
324 anaerobic lifestyle. To explain the coupling of oxygen and sulfide consumption in the oxygen- and
325 sulfide-free chemocline of Lake Cadagno, we sketched a diagram of the transport processes likely
326 driving biological activity in the chemocline (Fig. 5). As described in Sommer *et al.,* (2017), active
327 convection of the chemocline can be driven by the formation of sinking bacterial plumes.
328 Combined with turbulence induced by the breaking of internal waves at sides of the lake basin,
329 these convective currents may entrain sulfide and oxygen at the boundaries of the chemocline
330 and fuel populations of sulfide-oxidizing *Chr. okenii* there.

Sulfur-oxidizing bacteria have previously been reported to bridge distances between pools of electron donors and acceptors by intracellularly storing and transporting S^0 and NO^{3-} between redox zones (Fossing *et al.,* 1995; Jørgensen and Gallardo, 1999) and even by transferring electrons along nanowires (Pfeffer *et al.,* 2012), but the sulfide oxidation processes in Lake Cadagno represent a new mechanism of electron acceptor/donor coupling across large distances. After entrainment into the chemocline, dissolved oxygen and sulfide are consumed so rapidly that they remain below detection limits. Our metagenomic evidence shows that *Chr. okenii* possesses several high-affinity oxidases which may enable it to respire oxygen at such low, nanomolar concentrations. The physical and biological processes described here may therefore provide clues to sulfide oxidation in other anoxic environments such as the Black Sea where the mechanism of sulfide removal is not completely understood. Clearly, the biochemical limits to oxygen utilization are far below current definitions of *anoxia* and demonstrate that aerobic respiration is possible in so-called "anoxic" lacustrine (Milucka *et al.,* 2015) and marine (Garcia-Robledo *et al*., 2017) waters.

Overall, we show that in contrast to observations from laboratory cultures, *Chr. okenii* appear to have a very different metabolism in the environment where high fluxes of nutrients rather than absolute nutrient concentrations fuel microbial activity. The unexpected insights into the ecophysiology of the purple sulfur bacteria obtained here demonstrate the importance of studying these versatile bacteria *in situ* using culture-independent methods to understand their environmental function.

EXPERIMENTAL PROCEDURES

Sampling

353 The meromictic Lake Cadagno is situated in the Piora Valley in the Swiss Alps at an altitude of
354 1921 m. Data presented here were collected during field campaigns in September 2013, August
355 2014, June 2015 and August 2015. In 2013 and 2014 *in situ* measurements were performed with
356 a profiling ion analyzer (PIA; see Kirf *et al.,* 2014 for description) lowered from a platform
357 anchored at the deepest part of the lake (20.7 m). Conductivity, turbidity, depth (pressure),
358 temperature and pH were measured with a multi-parameter probe (XRX 620, RBR). Dissolved
359 oxygen was recorded online with a type PSt1 normal (detection limit 125 nM) micro-optode and
360 a type TOS7 trace (reliable detection limit 50-100 nM) micro-optode (PreSens). The oxygen
361 sensors were calibrated by parallel Winkler titrations. Water samples for chemical analyses and
362 cell counts were collected with a rosette syringe sampler equipped with twelve 60-ml syringes
363 triggered online at selected depths. Due to a technical failure of the PIA, the 6 AM profile in August
364 2014 and all subsequent profiles in 2015 were measured with a SBE 19 plus V2 CTD probe (Sea-
365 Bird Electronics, WA, USA) equipped with sensors for pressure, temperature and conductivity,
366 and with additional sensors for turbidity (WET Labs Eco), oxygen (SBE 43), pH (18-I) and two
367 fluorescence wavelengths (WET Labs ECO-AFL, FL, USA). The detection limit of the SBE 43 oxygen
368 probe was about 1 µmol/l. In parallel with *in situ* measurements, water for chemical analyses was
369 pumped to the surface through Neoprene tubing attached to the CTD and filled into 60-ml
370 syringes (rinsed 2 X with in situ water) on board. Two parallel metal plates of diameter ~15 cm
371 attached to the submersed end of the tubing served to channel water horizontally, resulting in
372 more discrete vertical profiling.

373 Water samples in syringes were aliquoted on board immediately after collection. Samples for
374 sulfate analyses were filtered (0.22 µm pore size) directly into sterile Eppendorf vials. Sulfide

samples were fixed with Zn-acetate to a final concentration of 0.1 % (w/v). Biomass was concentrated onto glass fiber filters (0.7 µm pore size) and stored at -20°C for analyses of intracellularly stored elemental sulfur and organic carbon compounds. Filtrate (0.22 µm pore size) was also collected and frozen at -20°C for metabolome analysis of dissolved compounds. Samples for fluorescence *in situ* hybridization were immediately fixed with 2% (v/v) formaldehyde. Samples for DNA analysis were collected from the chemocline in August 2014 by concentrating microbial cells on polycarbonate filters (0.22 µm pore size) on site and freezing at -20°C until further processing.

Additional water for cultivation and motility experiments was pumped directly from the chemocline into 1-L Duran bottles and sealed with butyl rubber stoppers without a headspace to maintain anoxic conditions.

Chemical Analyses

Sulfide was measured using the colorimetric method of Cline (1969). Sulfate was measured on a 761 Compact ion chromatograph (Metrohm, Filderstadt, Germany) equipped with a Metrosep A SUPP 5 column. Intracellular sulfur on filters was extracted by sonication in methanol for 15 min in an ice bath. Samples were analyzed on an Acquity H-Class UPLC system (Waters Corporation, USA) with an Acquity UPLC BEH C18 column coupled to a photodiode array (PDA) detector using UPLC-grade methanol as eluent. Data was acquired and processed using the Empower III software.

Intracellular glycogen was analyzed following the procedures of the assay kit (MAK016 Sigma Aldrich). Briefly, cells were extracted by scraping them from GFF filters and homogenizing in 200

µL extraction buffer and centrifuged two times to clear the supernatant. The supernatant was analyzed fluorometrically after incubation with enzyme mix and fluorescent peroxidase substrate. Intracellular PHA was analyzed using the protocol from Braunegg *et al*. (1978). Hydrolyzation of the polymer and conversion to a methyl-ester of the monomeric hydroxyalkanoate fraction was done in acidified alcohol solution (6% H_2SO_4 in methanol) and chloroform under heating (100°C, 2h). After addition of water and phase separation the organic phase was analyzed with GC-MS (Agilent 7890B GC connected to Agilent 5977A MSD) to detect the methylhydroxyalkanoates using the following settings: Agilent 30 m DB-5-MS column, splitless injection of 1 µl, temperature program was 50°C for 1min than heating 10°C/min until 120°C followed by 45°C/min until 320°C and hold for 5 minutes. Benzoic acid was used as internal standard in each sample and quantification was done with pure polyhydroxybutyrate standard (Sigma_Aldrich).

Sulfate reduction rates were measured by adding the radiotracer $^{35}SO_4^{2-}$ (5 MBq) to anoxic lake water in 50-ml glass syringes and incubated in the dark. A solution of unlabeled Na_2S was added to a final concentration of ~50 µmol·l^{-1} as a background sulfide pool in case of sulfide re-oxidation. At each sampling point, 10 ml of sample was dispensed into 5 ml of 20% (w/v) Zn-acetate. Reduced sulfur species (e.g. sulfur and sulfide as ZnS) were separated out via the chromium distillation method described in (Kallmeyer *et al.,* 2004) and the radioactivity per sample was determined via scintillation counting (Packard 2500 TR).

Confocal Raman spectroscopy

In glove box under 90:10 N_2-CO_2 atmosphere, a drop of fresh sample from the chemocline was mounted between two glass coverslips and sealed with electrical tape to prevent contact with

air. A polysulfide solution containing 5.06 g $Na_2S \cdot 9H_2O$ and 5.8 g elemental sulfur per 100 ml H_2O, with a final pH of 9.5 and sulfide concentration of 210 mM was used as reference. Measurements were conducted with an NTEGRA Spectra confocal spectrometer (NT-MDT, Eindhoven, Netherlands) coupled to an inverted Olympus IX71 microscope. The excitation light from a 532-nm solid-state laser was focused on the sample through an Olympus 100X (numerical aperture [NA], 1.3) oil immersion objective. Raman scattered light was collected by an electron-multiplying charge-coupled device (EMCCD) camera (Andor Technology, Belfast, Northern Ireland) cooled to -70°C. Spectra were recorded between 0 and 4,500 cm^{-1} with a spectral resolution of 0.2 cm^{-1} and analyzed with the software NT-MDT software Nova_Px 3.1.0.0.

Flux Calculations

Turbulent fluxes (J) of sulfide, sulfur, sulfate, and oxygen at the chemocline were calculated assuming steady state by applying Fick's first law: $J=-D\partial C/\partial x$. For sulfide, sulfate, and oxygen we used the turbulent diffusion coefficient (D) of 1.6×10^{-6} m^2 s^{-1} from (Wüest, 1994) corresponding to turbulence at the Lake Cadagno chemocline boundaries. For sulfur gradients within the well-mixed chemocline the coefficient D = 1.5×10^{-5} m^2 s^{-1} (Wüest, 1994) was used. The change in concentration (∂C) was computed for each species over the depths with the steepest gradients. Oxygen and sulfide fluxes were determined for the regions immediately above and below the chemocline, defined as the zone of constant conductivity.

Microbial cultivation

Anoxygenic phototrophic bacteria from the Lake Cadagno chemocline were cultivated in agar-stabilized, sulfide gradient medium in anoxic test tubes. Solid agar (1.5% w/v agar) and semi-solid agar (0.25% w/v agar) were prepared separately by autoclaving triple-washed agarose and sterile-

filtered water from the Lake Cadagno chemocline, and degassing for 1 h with mixture of 80% N_2 and 20% CO_2 during cooling to ~50°C. The solid agar was amended with a sterile Na_2S solution to a final concentration of ~4mM before pouring into degassed test tubes to form a ~2 cm bottom layer and allowed to set. The semisolid agar was amended with vitamins and trace elements as described for cultivation of purple sulfur bacteria (Eichler and Pfennig, 1988) before pouring a ~7 cm top layer, and immediately capped with a butyl rubber stopper. After cooling to ~30°C, 1 ml of fresh Cadagno chemocline water was used to inoculate the top agar via a degassed syringe. Tubes were inverted once to mix and allowed to set. Agar cultures were incubated under low, 24-h light conditions at 15°C to favor the development of anoxygenic phototrophs.

Microsensor measurements

Gradients of pH and H_2S in agar cultures were measured using microelectrodes built in-house as described previously (Jeroschewski *et al*., 1996; de Beer *et al*., 1997). Immediately before use, the pH sensor was calibrated in standard buffers and the H_2S sensor was calibrated in a dilution series of an acidified Na_2S solution. Electrodes were mounted on a micromanipulator connected to a computer and profiles were measured in 250 µm intervals from the agar surface to the base of the sulfide plug. Agar tubes were uncapped for the insertion of microsensors, and the headspace was flushed with N_2 gas before recapping immediately after each measurement. Total sulfide concentrations were calculated from pH and H_2S gradients as described in Schwedt *et al*., (2012).

Motility analysis

Water samples containing *Chr. okenii* cells were collected under anoxic conditions from the chemocline during the night, protected from artificial light with aluminum foil, and analyzed

immediately on site. Motile cells were transferred via a degassed glass syringe to a sealed rectangular millimetric chamber (dimensions 20 mm × 10 mm × 2 mm) prepared using glass slides separated by a 2-mm thick spacer, which provided an anoxic environment during motility characterization. Experiments were conducted in a dark room, and imaging was performed using the dark field microscopy mode at 25 fps, with the lowest intensity illumination. No transient response was observed right at the start of the imaging, and the swimming velocity remained steady throughout the duration of the measurements. This is in contrast to swimming behavior at higher light intensities where the swimming cells exhibited a positive phototactic response (Sommer *et al*., 2017). We could therefore rule out a light-induced effect on motility at the minimum illumination level used for our measurements. Videos of swimming cells were acquired and subsequently analyzed using the ImageJ Particle Tracker routine to obtain the coordinates of the cells (geometric centers) at each time interval. These were used to calculate the swimming speeds and extract the trajectories of individual cells.

DNA extraction, sequencing, and analysis

Environmental DNA was extracted from polycarbonate filters with the Ultra Clean MoBio PowerSoil DNA kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's protocol with the following modification: the bead beating step was reduced to 30 sec followed by incubation on ice for 30 sec, repeated 4x. The DNA was gel-purified using SYBR Green I Nucleic Acid Gel Stain (Invitrogen) and the QIAquick Gel Extraction Kit (Qiagen) according to the accompanying protocols. DNA concentration was determined fluorometrically at 260 nm, using the Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay KIT (Invitrogen) and sent to the Max Planck-Genome Centre (Cologne, Germany) for sequencing. The metagenome was sequenced

(100 bp paired end reads) by Illumina HiSeq (Illumina Inc., USA) sequencing following a TruSeq library preparation. Metagenomic reads were adapter- and quality-trimmed (phred score 15, bbduk function of the BBMap package, https://sourceforge.net/projects/bbmap/) and paired-end reads were *de novo* assembled with the uneven depth assembler IDBA-UD (Peng *et al.,* 2012).

The metagenome assembly was binned based on tetranucleotide frequencies, differential coverage, taxonomic classification, and conserved single-copy gene profiles with the Metawatt binning software (version 3.5.2; Strous *et al*., 2012). The completeness and contamination of the binned MAGs was evaluated with CheckM (Parks et al 2014). The bulk metagenome and the MAG identified as *Chr. okenii* were automatically annotated in IMG (Markowitz et al 2011), and the *Chr. okenii* MAG was manually screened for the presence of genes of interest to this study. Assembled data is available in IMG, under the IMG genome IDs 3300010965 (bulk assembly) and 2700988602 (*Chr. okenii* MAG).

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Fig 1: (a) Combined oxygen (top) and sulfide (bottom) profiles of the Lake Cadagno water column revealing the persistence of an oxygen- and sulfide- free zone over a period of 48 hours, with contour lines indicating sulfide concentrations. The bold contour lines delimiting the region with > 5 µM sulfide were used to define the chemocline in parallel profiles of *Chr. okenii* cell counts (b), particulate S^0 (c), and sulfate (d). Black dots represent sampling points for all parameters except O_2 which was measured with a microsensor mounted on a CTD probe. Shaded boxes represent dark periods between sunset at ~20:50 and sunrise at ~6:10. Time plots were interpolated from original profiles measured in August 2015 and are provided in Fig S1.

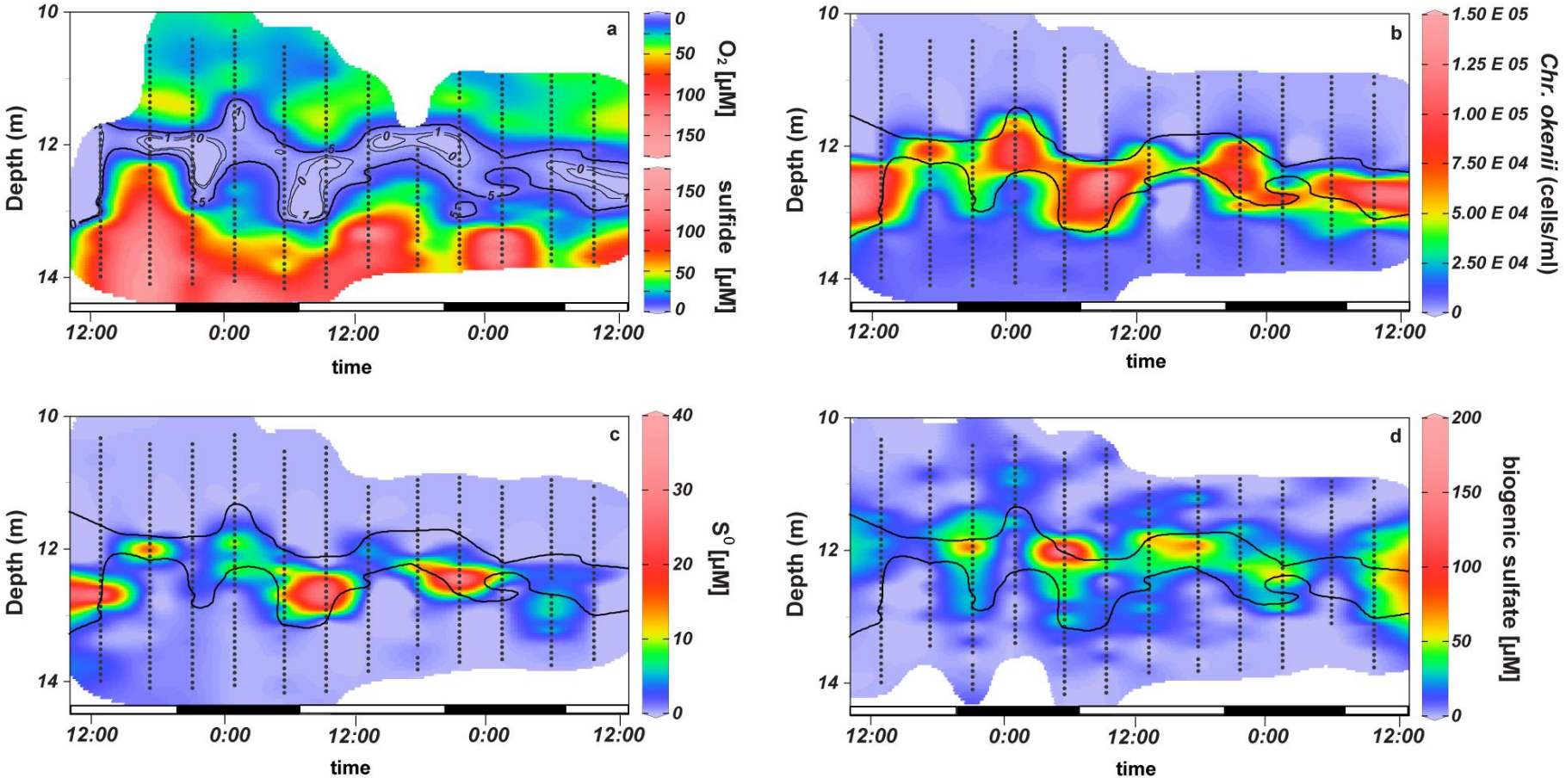

Figure 2: A day (13:00) and a night (1:30) profile through the chemocline illustrating glycogen and S^0 concentrations in relation to *Chr. okenii* cell numbers, oxygen, and sulfide gradients in the chemocline. Profiles were measured in August 2015. PHA was below detection limits and no oxygen data is available for the day profile.

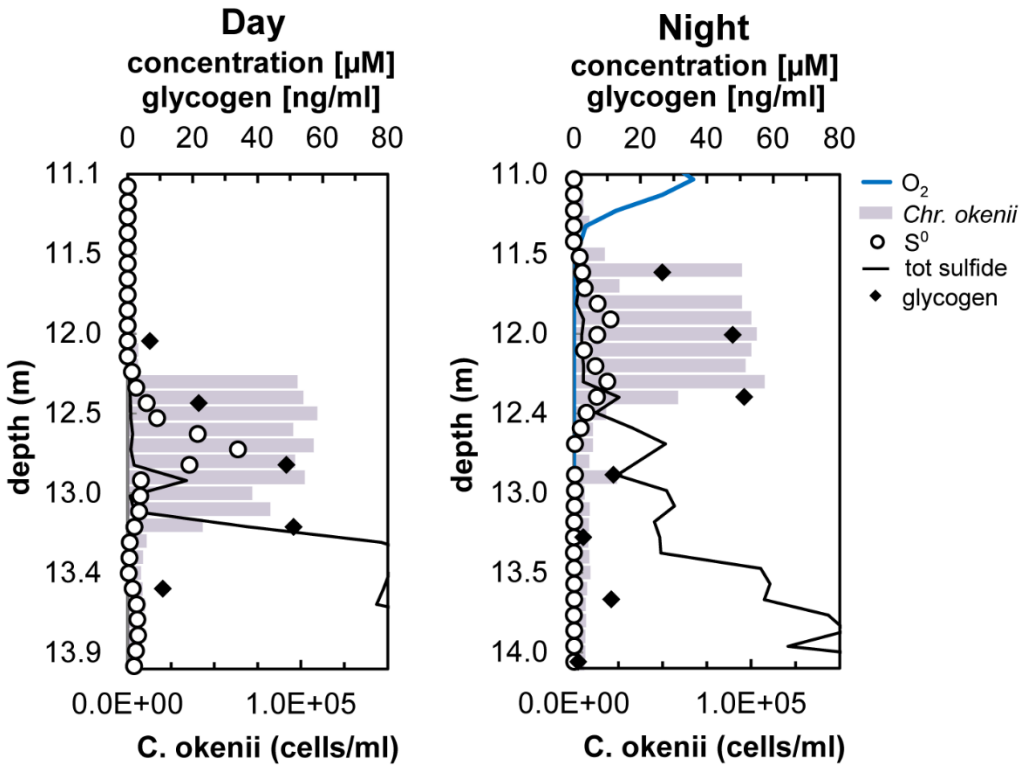

Figure 3: Sulfide and oxygen fluxes in the Lake Cadagno chemocline were calculate from profiles measured 4-h intervals over 2 day-night cycles. (a) The consumed sulfide flux (solid line) was calculated by subtracting the residual sulfide flux (dashed line) from the total sulfide flux into the mixed layer. (b) The downwards oxygen flux into the chemocline was used to estimate (c) the maximum % of sulfide aerobically respired, assuming the complete oxidation of sulfide to sulfate. Shaded regions represent dark periods.

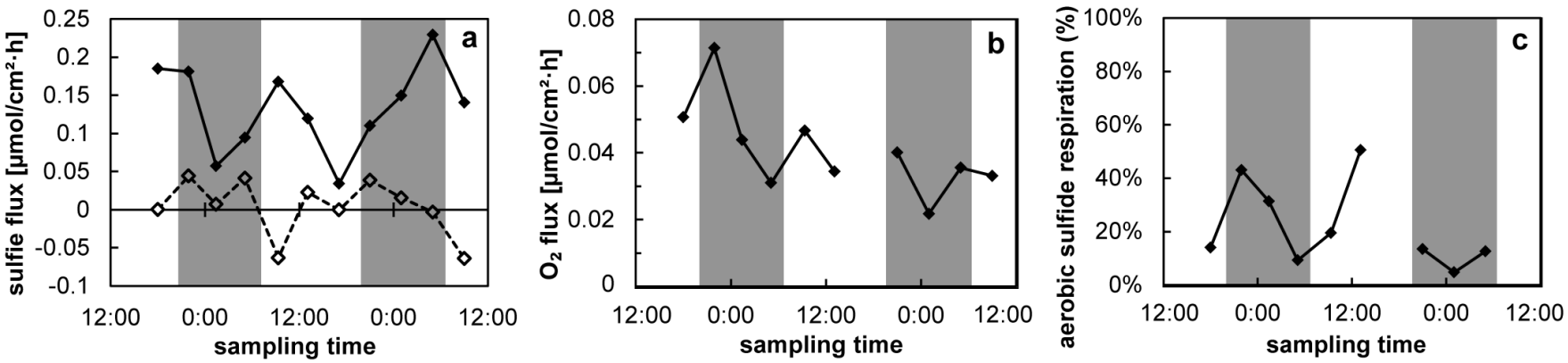

Figure 4: *Chr. okenii* cell illustration, showing the metabolic potential inferred from the metagenome-assembled genome with a particular focus on the genetic machinery implicated in photosynthesis, sulfur oxidation, aerobic metabolism, motility, glycogen and PHA storage, nitrogen fixation and transmembrane transport. The respiratory chain enzyme complexes are labeled with Roman numerals.

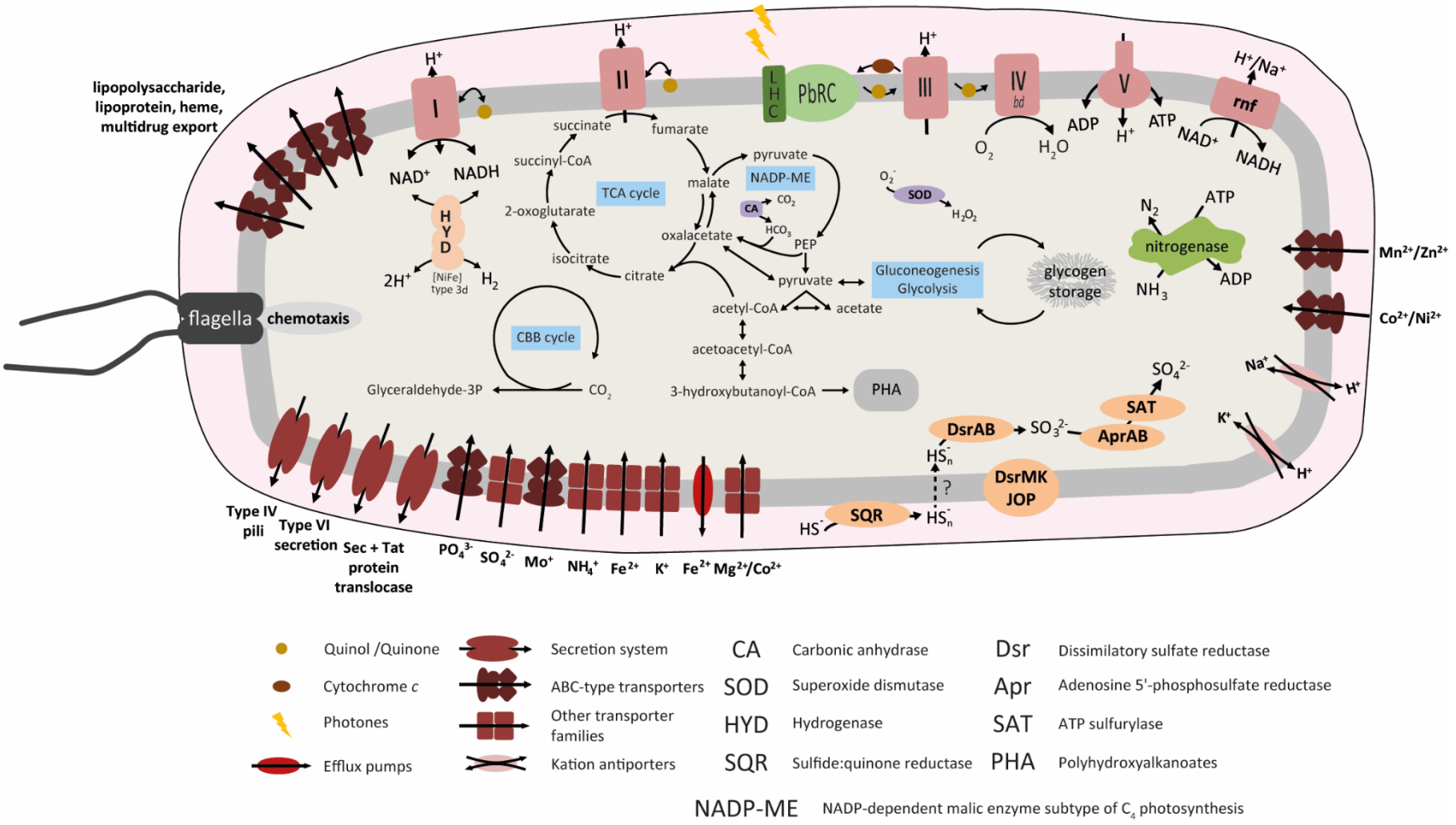

Figure 5: Schematic of phototrophic and aerobic sulfide oxidation processes in the Lake Cadagno chemocline. Convection in the chemocline may be driven by a combination of turbulence and sinking bacterial plumes, represented by the large number of descending *Chr. okenii* cells on the left. As a result, oxygen and sulfide are entrained into the chemocline and immediately consumed by purple sulfur bacteria, keeping concentrations of these compounds below detection limits. *Chr. okenii* cells, depicted with internal sulfur globules (yellow dots), are pulled in the direction of their flagellar bundle.