

## ORIGINAL ARTICLE

# Structure and function of natural sulphide-oxidizing microbial mats under dynamic input of light and chemical energy

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**We studied the interaction between phototrophic and chemolithoautotrophic sulphide-oxidizing microorganisms in natural microbial mats forming in sulphidic streams. The structure of these mats varied between two end-members: one characterized by a layer dominated by large sulphur-oxidizing bacteria (SOB; mostly *Beggiatoa*-like) on top of a cyanobacterial layer (B/C mats) and the other with an inverted structure (C/B mats). C/B mats formed where the availability of oxygen from the water column was limited (<5 μM). Aerobic chemolithotrophic activity of the SOB depended entirely on oxygen produced locally by cyanobacteria during high light conditions. In contrast, B/C mats formed at locations where oxygen in the water column was comparatively abundant (>45 μM) and continuously present. Here SOB were independent of the photosynthetic activity of cyanobacteria and outcompeted the cyanobacteria in the uppermost layer of the mat where energy sources for both functional groups were concentrated. Outcompetition of photosynthetic microbes in the presence of light was facilitated by the decoupling of aerobic chemolithotrophy and oxygenic phototrophy. Remarkably, the B/C mats conserved much less energy than the C/B mats, although similar amounts of light and chemical energy were available. Thus ecosystems do not necessarily develop towards optimal energy usage. Our data suggest that, when two independent sources of energy are available, the structure and activity of microbial communities is primarily determined by the continuous rather than the intermittent energy source, even if the time-integrated energy flux of the intermittent energy source is greater.**

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## Introduction

Life is driven by essentially two types of energy: chemical energy, in the form of chemical disequilibria, and physical energy carried by light of suitable wavelengths. On geological timescales, changes in energy fluxes and the development of new strategies of life are closely coupled. One of the most important changes in energy availability was the evolution and proliferation of oxygenic photosynthesis. The onset of this process introduced the thermodynamically most favourable electron-acceptor oxygen to the wider reduced environment. Oxygenic photosynthesis therefore provided previously unexplored thermodynamic disequilibria to other microorganisms thereby allowing for the evolution and diversification of aerobic

metabolisms (Castresana and Saraste, 1995; Dismukes *et al.*, 2001; Catling *et al.*, 2005).

Insights into the revolutionary milestones associated with the evolution and proliferation of oxygenic photosynthesis can be gained by studying modern model ecosystems in environments whose geochemical/energetic characteristics exhibit spatial gradients that are analogous to the temporal transition from a reduced to an oxidized state of the Earth's surface. The thin microbial mats forming at the bottom of light-exposed cold sulphidic springs at Frasassi, Italy, (Galdenzi *et al.*, 2008) are such an analogue system as they are distributed across a gradient from reduced to oxidized conditions in the overlying water column. These contemporary phototrophic microbial mats are of particular interest as they represent analogues to ancient cyanobacterial mats (for example, stromatolites) that are thought to have been extensive in shallow waters throughout the Proterozoic and possibly already in the Archean (Ward *et al.*, 1992; Grotzinger and Knoll, 1999; Allwood *et al.*, 2009; Seckbach and Oren, 2010; Schopf, 2012).

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In this study, we used microsensors to quantify energy fluxes available for and conserved by the dominant processes in the mats—photosynthesis (P) and aerobic sulphide oxidation (SO)—with the aim to understand how the interaction between these processes under a fluctuating input of chemical and light energy determines the structure and function of the mats. We hypothesized that under the different conditions the mats develop towards a system that conserves the available energy optimally. We discuss the important role of energy dynamics on microbial mat structure and possible implications of our results in the context of Earth's oxygenation.

## Materials and methods

### *Study site*

This study was performed in streams and pools where the sulphidic waters from the Frasassi Cave system in the Frasassi Gorge, Italy, emerge and mix with waters of the Sentino river (Galdenzi *et al.*, 2008). The study sites included the outflows of two perennial springs, the Fissure Spring and the Main Spring (43°24'4"N, 12°57'56"E).

### *Water chemistry*

Sampling for bulk water chemistry analysis was carried out in May and September 2009 and in September 2012. Samples were taken during night and around midday within a few cm above the microbial mat patches and were preserved immediately in gas-tight vials containing a mixture of 100 µl of 20% ZnCl<sub>2</sub> and 50 µl of saturated HgCl<sub>2</sub> solution. The vials were filled with water samples until there was no head space and kept at room temperature until quantification in Bremen. Dissolved inorganic carbon and ammonium were determined using flow injection analysis (Hall and Aller, 1992). The sum of nitrate and nitrite was quantified according to Braman and Hendrix (1989) using an NO<sub>x</sub> analyser equipped with a chemiluminescence detector (Model CLD 66, Eco Physics, Dürnten, Switzerland). pH and concentrations of dissolved O<sub>2</sub> and total sulphide ( $S_{\text{tot}} = [S^{2-}] + [HS^-] + [H_2S]$ ) were determined with microsensors. Temperature at the mat surface was measured with a PT1000 mini-sensor (Umwelt-sensortechnik, Geschwenda, Germany).

### *Microscopy*

The dominant members of the mat community were identified by microscopy. Imaging by bright field, phase contrast and fluorescence microscopy was carried out using an Axiophot epifluorescence microscope (Zeiss, Jena, Germany). Photopigments were identified on a single-cell level by hyperspectral imaging (Polerecky *et al.*, 2009).

### *Microsensors*

O<sub>2</sub>, pH and H<sub>2</sub>S microsensors with a tip diameter of 10–80 µm and response time of <1 s were built, calibrated and used as described previously (Revsbech, 1989; Jeroschewski *et al.*, 1996; de Beer *et al.*, 1997). Calibration of the H<sub>2</sub>S microsensors was performed in acidified spring water (pH < 2) to which NaS<sub>2</sub> was added stepwise. The total sulphide concentrations,  $S_{\text{tot}}$ , in the calibration solutions were determined according to Cline (1969). Calculation of  $S_{\text{tot}}$  from the local H<sub>2</sub>S concentrations and pH values measured with microsensors was carried out according to Millero (1986), using the pK<sub>1</sub> value of 7.14–7.17 depending on temperature (Jeroschewski *et al.*, 1996; Wieland and Köhl, 2000).

### *In situ microsensor and light measurements over a diel cycle*

*In situ* measurements of O<sub>2</sub>, pH and H<sub>2</sub>S in the mats were carried out using previously described microsensor setups (Weber *et al.*, 2007, www.microsen-wiki.net). Parallel O<sub>2</sub>, pH and H<sub>2</sub>S profiles were measured under naturally variable light conditions by measuring continuously over a complete diel cycle on a cloudless day.

In parallel to microsensor measurements, downwelling irradiance of the photosynthetically available radiation was recorded using a calibrated light logger (Odyssey Dataflow Systems, Christchurch, New Zealand) or a calibrated scalar irradiance microprobe (Lassen *et al.*, 1992) placed next to the microsensors. The irradiance microprobe was additionally used to quantify reflectance of the mats, as previously described (Al-Najjar *et al.*, 2010).

### *Microsensor measurements under controlled light conditions*

Measurements under controlled light conditions were performed both *in situ* and *ex situ*. *In situ*, we first measured steady-state profiles of O<sub>2</sub>, H<sub>2</sub>S and pH in the dark and at an incident irradiance of 650 µmol photons m<sup>-2</sup> s<sup>-1</sup> generated by a halogen lamp (KL1500, Schott, Müllheim, Germany) corresponding to the natural illumination around midday. Subsequently, volumetric rates of gross oxygenic P were determined using the O<sub>2</sub> microsensor-based light–dark shift method of Revsbech and Jørgensen (1983). Finally, the same light–dark shift approach was applied using H<sub>2</sub>S and pH microsensors instead of a O<sub>2</sub> microsensor, which allowed quantification of volumetric rates of gross anoxygenic P in terms of consumed  $S_{\text{tot}}$  (Klatt *et al.*, 2015). For this measurement, the light was switched on and off over a few minute intervals while the signals were recorded in 0.3-s intervals. All of these measurements were carried out in the same spot of the mat.

To confirm that the cyanobacterial population in the mats was able to perform simultaneous oxygenic and anoxygenic P, similar measurements were

performed *ex situ*. The mat sample was placed in a temperature-controlled (15 °C) flow chamber, and O<sub>2</sub>, H<sub>2</sub>S and pH were measured in parallel in the same spot of the mat under variable illumination from the halogen lamp. To assess the potential role of obligate anoxygenic phototrophs that can use light in the near infrared part of the spectrum and H<sub>2</sub>S as the electron donor for anoxygenic P, similar measurements were additionally performed using near infrared light-emitting diodes (maximal emission at  $\lambda_{\text{max}} = 740$  and 810 nm; H2A1 series, Roithner-Lasertechnik, Vienna, Austria). The measurements with and without the additional near infrared light were carried out before and after the addition of 5  $\mu\text{M}$  of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; Aldrich, Seelze, Germany), a specific inhibitor of oxygenic P.

#### Calculation of fluxes and daily energy budgets

Diffusive fluxes of O<sub>2</sub> and S<sub>tot</sub> were calculated from the measured concentration gradients multiplied by the corresponding diffusion coefficients and factors correcting for the azimuthal angle at which the profiles were measured (Berg *et al.*, 1998; Polerecky *et al.*, 2007). Diffusion coefficients, *D*, were corrected for temperature, salinity and, in case of S<sub>tot</sub>, for the composition of the total sulphide pool as previously described (Sherwood *et al.*, 1991; Wieland and Kühl, 2000). Specifically, diffusion coefficients were  $1.78 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  for O<sub>2</sub> and  $1.35 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  for S<sub>tot</sub>, varying by  $\leq 3\%$  depending on the temperature in each particular measurement.

Fluxes of incident light energy were estimated from the measured downwelling irradiance, assuming that the average energy content of photons of photosynthetically available radiation is 217.5 kJ (mol photons)<sup>-1</sup> (Al-Najjar *et al.*, 2010). The fraction of the incident light energy absorbed by the cyanobacterial population in the mats was estimated from the measured reflectance of the mats (see Supplementary Information 1).

Fluxes of energy conserved by oxygenic and anoxygenic P as well as by aerobic SO were estimated by multiplying the estimated CO<sub>2</sub> fluxes with the molar energy required for CO<sub>2</sub> fixation by the respective process. For oxygenic and anoxygenic P, the CO<sub>2</sub> flux was estimated from the measured fluxes of O<sub>2</sub> produced and S<sub>tot</sub> consumed in the photosynthetically active zone assuming the stoichiometry of O<sub>2</sub>/CO<sub>2</sub> = 1 and S<sub>tot</sub>/CO<sub>2</sub> = 2, respectively. For CO<sub>2</sub> fixation coupled to aerobic SO, we employed the approach of Klatt and Polerecky (2015), which allows the prediction of the complete stoichiometry of autotrophic SO based on the measured S<sub>tot</sub>/O<sub>2</sub> flux ratios. Prerequisite for these calculations is the relationship between the S<sup>0</sup>/SO<sub>4</sub><sup>2-</sup> production ratio and the energy conservation efficiency, that is, the ratio between the energy demand for CO<sub>2</sub> reduction and the energy available from O<sub>2</sub> reduction. To derive this relationship, we made two

main assumptions: (i) When SO is in a steady state, sulphide is oxidized completely to SO<sub>4</sub><sup>2-</sup> (Jørgensen *et al.*, 2010); (ii) When the S<sub>tot</sub>/O<sub>2</sub> consumption ratio in the SO layer varies strongly over a diel cycle, the highest measured S<sub>tot</sub>/O<sub>2</sub> consumption ratio occurs when sulphide is oxidized incompletely to S<sup>0</sup>, whereas the lowest measured S<sub>tot</sub>/O<sub>2</sub> consumption ratio corresponds to complete sulphide oxidation to SO<sub>4</sub><sup>2-</sup>. These assumptions enabled us to determine the energy conservation efficiency as a function of the S<sup>0</sup>/SO<sub>4</sub><sup>2-</sup> production ratio, based on which we estimated the fluxes of CO<sub>2</sub>, energy gained and energy conserved by SO from the measured fluxes of S<sub>tot</sub> and O<sub>2</sub> at different times during the diel cycle. Details of the CO<sub>2</sub> and energy flux calculations are given in Supplementary Information 2.

Daily budgets were calculated by integrating the instantaneous fluxes over the 24-h period for all relevant reactants and energy-harvesting processes involved.

## Results

#### Mat structure vs chemical composition of the spring water

The light-exposed microbial mats from the Frasassi sulphidic springs are dominated by two functional groups: cyanobacteria, identified by the presence of the characteristic cyanobacterial pigments chlorophyll *a* and phycocyanin in the cells revealed by hyperspectral microscopy (Supplementary Figure S1), and large *Beggiatoa*-like sulphur-oxidizing bacteria (SOB; filamentous, containing sulphur inclusions, motile through gliding) (Figures 1a and b). Inferring from microscopy, hyperspectral imaging and reflectance measurements of the mats (Supplementary Figure S2), the abundance of phototrophs other than cyanobacteria was not significant in the studied mat layers.

A close inspection revealed that the structure of the mats fell essentially between two end-members: mats with a distinct cyanobacterial layer on top of a distinct SOB layer (Figure 1c), and mats with SOB on top of cyanobacteria (Figure 1d). These two mat types are hereafter referred to as C/B and B/C mats, respectively.

In the spring outflow streams, the distribution of these two mat types was patchy and varied on a centimetre to decimetre scale. Water chemistry and light measurements revealed that the physico-chemical parameters directly above the mats also changed at this scale. This was mainly due to mixing of the spring water with the freshwater from the Sentino river under highly spatially variable flow conditions, which lead, for instance, to the formation of stagnant anoxic pools next to an aerated water column. The formation of a certain mat type did not correlate with the daily light dose, with temperature, pH, nitrate, ammonium, dissolved inorganic carbon, H<sub>2</sub>S or S<sub>tot</sub> concentrations in the water column





**Figure 1** Microscopic images of the dominant cyanobacterial (a) and SOB (b) morphotypes in sulphide-oxidizing microbial mats from the light-exposed sulphidic streams in Frasassi, Italy, and photographs of end-member mat structures (c: C/B mat; d: B/C mat) (scale bars: a–b: 10  $\mu\text{m}$ , c: 20 cm, d: 5 cm).

during day and night nor with the maximum  $\text{H}_2\text{S}$  and  $S_{\text{tot}}$  concentrations measured inside the mat (Table 1 and Supplementary Table S1). However, the locations harbouring the two end-member mat types clearly differed with respect to the dissolved  $\text{O}_2$  concentration in the water column above the mats, with the C/B and B/C mats exclusively found in areas where the  $\text{O}_2$  concentration during the night was  $<5 \mu\text{M}$  and  $>45 \mu\text{M}$ , respectively (Table 1). Changes in the water column  $\text{O}_2$  concentration during the day, which occurred owing to the high rates of oxygenic P in the mats when the overlying water column was stagnant, had no effect on the formation of a certain mat type (compare data for mats C/B-1 and C/B-2 in Supplementary Figure S3 and Supplementary Table S1).

#### *Photosynthetic activity of the cyanobacterial community*

Laboratory microsensor measurements revealed light-induced production of  $\text{O}_2$  and consumption of  $\text{H}_2\text{S}$  in the cyanobacterial layer of C/B (Figure 2) and B/C mats (data not shown). Although  $\text{O}_2$  production was due to oxygenic P, removal of  $\text{H}_2\text{S}$  could be caused by three processes: chemical or biologically mediated oxidation with the photosynthetically produced  $\text{O}_2$ , a shift in the equilibrium of sulphide speciation ( $\text{H}_2\text{S}$ ,  $\text{HS}^-$  and  $\text{S}^{2-}$ ) induced by a pH increase associated with the uptake of dissolved inorganic carbon by oxygenic P, or by anoxygenic P that uses  $\text{H}_2\text{S}$  as the electron donor. First,  $\text{H}_2\text{S}$  oxidation with  $\text{O}_2$  could be excluded because the  $\text{H}_2\text{S}$  dynamics changed abruptly upon light–dark transitions and were independent of  $\text{O}_2$  concentrations. Additionally, the depth-integrated rate of gross oxygenic P matched the net rate derived from steady-state diffusive profiles (see below), showing that there is negligible oxygen reduction activity (such as owing to the reaction with  $\text{H}_2\text{S}$ ) in the photosynthetically active layer. Second, assuming that total sulphide concentrations,  $S_{\text{tot}}$ , stayed constant during the light–dark transitions and pH varied as measured (by about 0.005 pH units; Figure 2), the calculated

variation in  $\text{H}_2\text{S}$  would be about 1–2 orders of magnitude lower than measured (data not shown). Therefore, we exclude also the second possibility and conclude that the measured light-induced variation in  $\text{H}_2\text{S}$  was a direct result of anoxygenic P in the cyanobacterial layer of the mat.

This conclusion was confirmed by *ex situ* measurements in a C/B mat treated with DCMU, a specific inhibitor of oxygenic P but not of anoxygenic P. Specifically,  $\text{H}_2\text{S}$  concentrations in the DCMU-treated mat decreased and increased upon the addition and removal of light, respectively, while the effect on pH was marginal (data not shown) and  $\text{O}_2$  was below detection limit (Figure 3a). For the incident irradiance of  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , the volumetric rates of anoxygenic P derived from light–dark shift microsensor measurements ranged between 1 and  $4 \text{ mmol } S_{\text{tot}} \text{ m}^{-3} \text{ s}^{-1}$  (Figure 3b), and their depth-integrated value ( $1.53 \mu\text{mol } S_{\text{tot}} \text{ m}^{-2} \text{ s}^{-1}$ ) closely matched the flux of  $S_{\text{tot}}$  removed by the cyanobacterial anoxygenic P derived from the steady-state microprofiles ( $1.55 \mu\text{mol } S_{\text{tot}} \text{ m}^{-2} \text{ s}^{-1}$ ). This implies that anoxygenic P was the only significant sink of sulphide in the cyanobacterial layer.

The observed light-induced variability of  $S_{\text{tot}}$  could also be due to the activity of obligate anoxygenic phototrophs. The activity of such phototrophs is, however, expected to be affected by the local oxygen concentration. For example, filamentous anoxygenic phototrophs (also known as green non-sulphur bacteria) can switch from sulphide-driven anoxygenic P to photoorganoheterotrophy or aerobic respiration when oxygen and cyanobacterial exudates are available in the light (Van der Meer *et al.*, 2005; Polerecky *et al.*, 2007). Additionally, anaerobic anoxygenic phototrophs, such as green and purple sulphur bacteria, are expected to be poisoned by high oxygen concentrations (Van Gemerden and Mas, 1995). In our measurements, however, the net and gross rates of anoxygenic P before the addition of DCMU to the mat sample, that is, in the presence of oxygenic P, were almost equal to those measured after inhibition of oxygenic P by

**Table 1** Physico-chemical parameters in the overlying water column and in the studied mats

Mat type	Site	W <sup>f</sup>		C <sup>c</sup>		SOB <sup>f</sup>		O <sub>2</sub> (μM) <sup>b</sup>	DIC (mM) <sup>c,d</sup>	pH <sup>f</sup>	NH <sub>4</sub> <sup>+</sup> (μM) <sup>c,d</sup>	NO <sub>x</sub> (μM) <sup>c,d</sup>	PAR <sup>g</sup> (μmol photons m <sup>-2</sup> s <sup>-1</sup> )	T (°C)
		H <sub>2</sub> S (S <sub>tot</sub> ) (μM) <sup>b</sup>	C <sup>c</sup>	W <sup>f</sup>	SOB <sup>f</sup>									
C/B-1	Main Spring	73 (582)	345 (2755)	510 (3339)	0	4.2 (±0.18)	8.0	77 (±3.3)	34.4 (±1.9)	133	14			
C/B-2	Main Spring	23 (151)	36 (287)	36 (353)	5	3.9 (±0.6)	8.0	56 (±4.7)	60 (±1.8)	93	14			
C/B-3	Fissure Spring	401 (628)	415 (642)	435 (681)	5	8.9	6.9	150	<1	78	14.5			
B/C-1	Main Spring	89 (278)	125 (390)	95 (175)	45	4.4 (±1.5)	7.5	206 (±10.2)	3.6 (±0.07)	124	13			
B/C-2	Main Spring	50 (92)	81 (149)	73 (88)	65	4.7 (±0.11)	7.3	—	—	163	13			
B/C-3	Fissure Spring	16 (248)	61	34	80	—	8.3	<1	50	—	15			

Abbreviations: DIC, dissolved inorganic carbon; PAR, photosynthetically available radiation; SOB, sulphur-oxidizing bacteria.

<sup>a</sup>Total sulfide concentration, S<sub>tot</sub>, was calculated from the measured H<sub>2</sub>S concentration and pH.

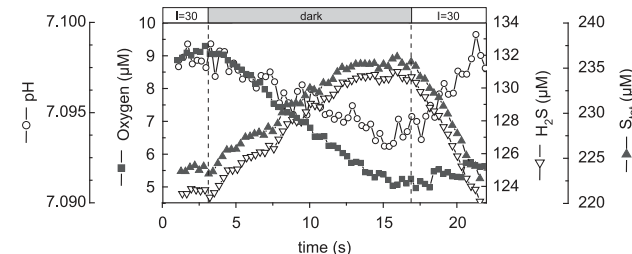
<sup>b</sup>Values are derived from microsensor measurements performed during night. The values obtained during the day are shown in Supplementary Table S2.

<sup>c</sup>Values were determined from water samples taken during night. The values determined from samples taken during the day are shown in Supplementary Table S2.

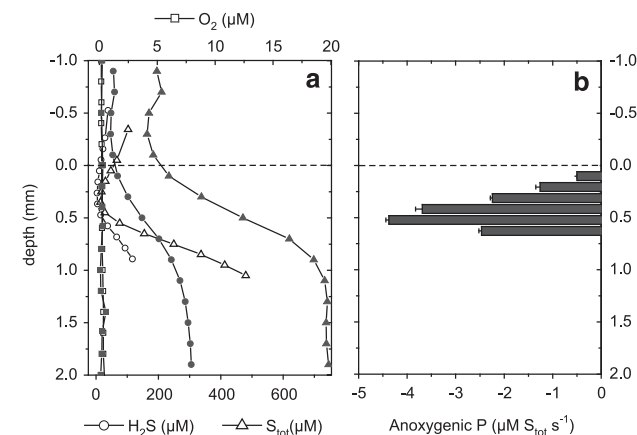
<sup>d</sup>Values in parentheses refer to s.d. (n=3–6).

<sup>e</sup>Downwelling irradiance of the PAR, given as the average photon flux during 15 h of light.

<sup>f</sup>Concentrations in the water column (W) inside the cyanobacterial layer (C) and the layer dominated by large SOB, as derived from microsensor measurements.



**Figure 2** Light-induced dynamics of O<sub>2</sub>, H<sub>2</sub>S, S<sub>tot</sub> and pH inside the cyanobacterial layer of a freshly collected C/B mat. Total sulphide concentrations, S<sub>tot</sub>, were calculated from the measured H<sub>2</sub>S concentrations and pH. The incident irradiance was 30 μmol photons m<sup>-2</sup> s<sup>-1</sup>. Distance between sensor tips was about 5 mm.



**Figure 3** *Ex situ* microsensor measurements in a C/B mat after the addition of DCMU. **(a)** Steady-state depth profiles of O<sub>2</sub> and H<sub>2</sub>S and S<sub>tot</sub> measured in the light (open symbols) and in the dark (filled symbols). **(b)** Depth profile of volumetric rates of anoxygenic photosynthesis, as derived from the light–dark shift method adapted for S<sub>tot</sub>. For both panels, total sulphide concentrations, S<sub>tot</sub>, were calculated from the measured H<sub>2</sub>S concentrations and pH, and the incident irradiance during the light measurements was 100 μmol photons m<sup>-2</sup> s<sup>-1</sup>.

DCMU (data not shown), suggesting that obligate anoxygenic phototrophs were not significantly contributing to the light-driven sulphide consumption in the cyanobacterial layer. This was confirmed by the observation that exposure to near infrared light, which specifically targets bacteriochlorophylls of obligate anoxygenic phototrophs, did not induce sulphide consumption (Supplementary Figure S4).

Together these data show that the cyanobacterial community in the studied mats is able to perform oxygenic and sulphide-driven anoxygenic P simultaneously and that cyanobacterial anoxygenic P is fully responsible for the measured light-induced H<sub>2</sub>S variation in the cyanobacterial layer of the mats.

### Activity of C/B mats

Continuous *in situ* micro-profiling in C/B mats under naturally variable illumination gave consistent results with those obtained in the laboratory. In the dark, sulphide consumption rates were minute or

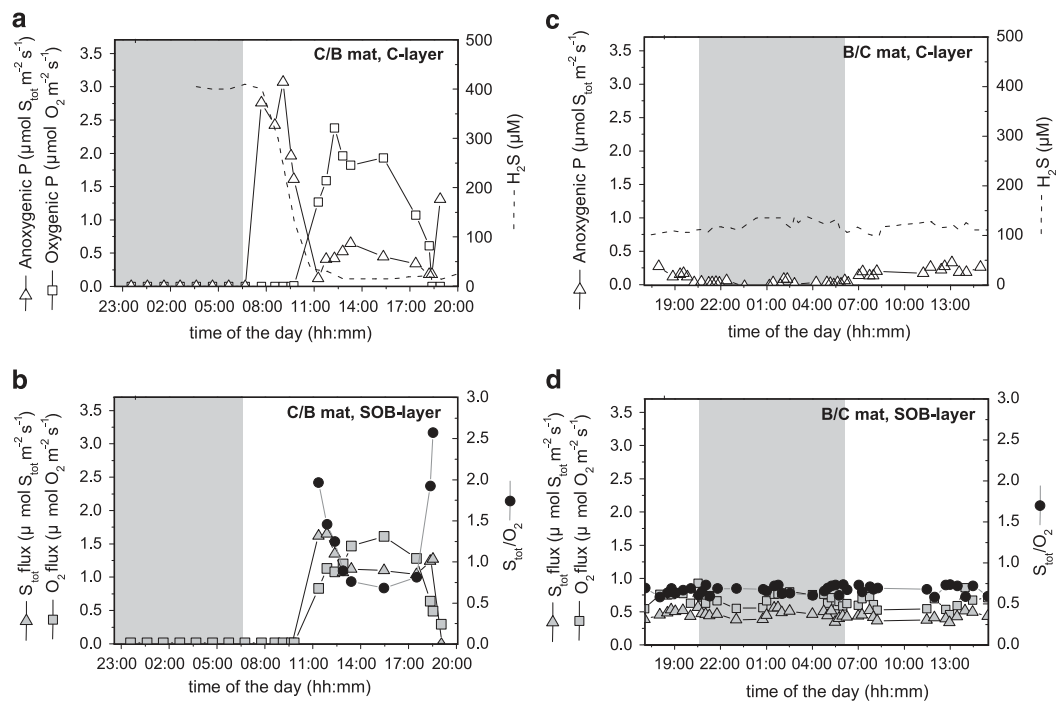
below detection limit, as implied by essentially flat  $S_{\text{tot}}$  profiles across the mats during the night, and  $O_2$  was not detected (Supplementary Figure S3). At sunrise, while still at low light, the rate of sulphide removal owing to anoxygenic P in the cyanobacterial layer sharply increased, resulting in a gradual decrease in  $H_2S$  concentrations (Figure 4a). Around midday, when the incident irradiance exceeded about  $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , sulphide concentrations within the cyanobacterial layer decreased and the photosynthetic production of oxygen sharply increased (Figure 4a). These dynamics occurred essentially in reverse order at sunset.

*In situ* measurements under controlled light conditions revealed additional insights into the activity of C/B mats. At high incident irradiance ( $650 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), the cyanobacterial community performed both oxygenic and anoxygenic P simultaneously, as shown by the light–dark shift measurements (Figure 5b). Aerobic respiration in the photosynthetically active zone was negligible, as the net diffusive flux of oxygen from the zone was very similar to the gross rates of oxygenic P depth-integrated over the zone ( $2.43$  and  $2.47 \mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively). Although oxygenic P was detectable at depths  $0$ – $0.6$  mm, anoxygenic P was detectable only at depths  $0$ – $0.4$  mm, that is, in zones of the cyanobacterial layer where both  $H_2S$  and light were abundant. The overlap between the zone of anoxygenic photosynthetic activity and the decrease in  $S_{\text{tot}}$  from the overlying water indicated that the sulphide

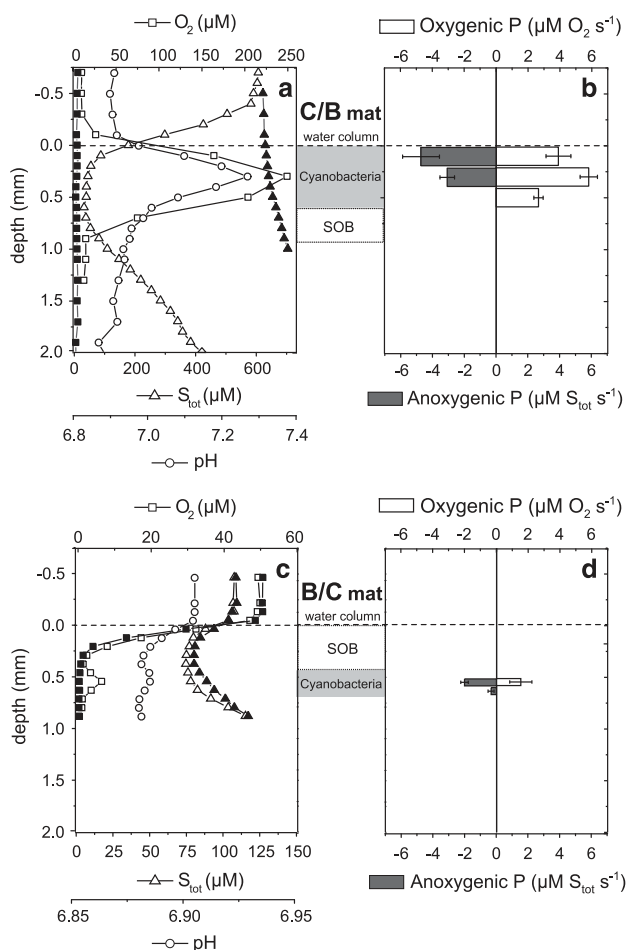
used by the cyanobacteria originated exclusively from the overlying water. This was supported by the close match between the depth-integrated rates of gross anoxygenic P ( $1.56 \mu\text{mol } S_{\text{tot}} \text{ m}^{-2} \text{ s}^{-1}$ ) and the downward  $S_{\text{tot}}$  flux from the water column ( $1.55 \mu\text{mol } S_{\text{tot}} \text{ m}^{-2} \text{ s}^{-1}$ ) (Figures 5a and b). When oxygenic P was, however, not active (that is, in the morning and later in the afternoon), the flux of  $S_{\text{tot}}$  consumed by anoxygenic P had significant contributions from both the downward and upward components of the diffusive  $S_{\text{tot}}$  flux.

Sulphide consumption in the SOB layer was detectable only after the onset of oxygenic P by the cyanobacteria (Figures 4b and 5a). This observation is consistent with the absence of an external supply of an electron acceptor, that is,  $O_2$ , from the water column and additionally indicates that anaerobic sulphide oxidation with  $NO_3^-$  as the electron acceptor was not important. Once  $O_2$  became available, sulphide consumed by the SOB originated exclusively from below, while the sulphide entering the mat from above was consumed by the cyanobacteria (see above).

Analysis of the profiles in Figure 5a revealed that the photosynthetically produced  $O_2$  (downward flux  $0.85 \mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$ ) and the sulphide supplied from below (upward flux  $0.48 \mu\text{mol } S_{\text{tot}} \text{ m}^{-2} \text{ s}^{-1}$ ) were consumed at a  $S_{\text{tot}}/O_2$  flux ratio of about  $0.6$ . However, a detailed analysis of the complete sets of profiles measured in the C/B mat over a diel cycle (Supplementary Figure S3) revealed that the  $S_{\text{tot}}/O_2$



**Figure 4** Activity of the cyanobacterial (a and c) and SOB (b and d) populations in the C/B (a and b) and B/C (c and d) mats exposed to natural light fluctuations over a 24-h period. The fluxes were derived from *in situ* microsensor profiles of  $O_2$  and  $H_2S$  concentration (Supplementary Figures S5 and S6) and pH. The corresponding average  $H_2S$  concentration in the cyanobacterial layer, and the  $S_{\text{tot}}/O_2$  flux ratio in the SOB layer, are also shown. Open and shaded areas correspond to light and dark, respectively.



**Figure 5** Microsensor measurements in the C/B and B/C mats under artificially controlled light conditions. Measurements in the C/B (a and b) and B/C mats (c and d) were performed *in situ* and *ex situ*, respectively. Left panels show steady-state depth profiles of  $O_2$ ,  $S_{tot}$  and pH in the light (open symbols) and in the dark (closed symbols). Right panels show the corresponding depth profiles of volumetric rates of oxygenic and anoxygenic photosynthesis, as derived from light–dark shift microsensor measurements of  $O_2$  and  $S_{tot}$ , respectively. For all panels, total sulphide concentrations,  $S_{tot}$ , were calculated from the measured  $H_2S$  concentrations (not shown) and pH. The incident irradiance during the light measurements in the C/B and B/C mats was 650 and 710  $\mu mol\ photons\ m^{-2}\ s^{-1}$ , respectively. For orientation, approximate structures of the mats are also shown.

consumption ratio changed substantially over the course of the day, reaching about 2 after  $O_2$  became available, gradually decreasing to about 0.7 before increasing back to about 2.6 just before  $O_2$  depletion towards the night (Figure 4b). Similar patterns in the diurnal variations of the  $S_{tot}/O_2$  consumption ratio between 0.7 and 2.6 were also observed in a replicate C/B mat (Supplementary Figures S5A and B).

The observed diurnal variation in the  $S_{tot}/O_2$  consumption ratio in the SOB zone can be explained by assuming that the SOB activity varied between incomplete oxidation of sulphide to  $S^0$  and complete oxidation of sulphide to  $SO_4^{2-}$  when the  $S_{tot}/O_2$  ratio reached the maximum and minimum value, respectively. This assumption made it possible to estimate

the energy conservation efficiency of the SOB community. Specifically, using the maximum value of  $S_{tot}/O_2=2.6$  (oxidation to  $S^0$ ) and additionally taking into account the measured local concentrations of the substrates and products involved, we found the efficiency values of 16.6% and 16.5% for the two replicate C/B mats. Conversely, the minimum value of  $S_{tot}/O_2=0.7$  (complete oxidation to  $SO_4^{2-}$ ) measured in the respective mats gave the efficiency values of 17.2% and 17.1% (calculation details given in Supplementary Information 2.2). These calculations suggest that the energy conservation efficiency of the SOB community in the studied mats was around 16.9% and did not significantly depend on the  $S^0/SO_4^{2-}$  production ratio.

The  $S_{tot}/O_2$  consumption ratio in both replicate C/B mats was significantly negatively correlated with the downward  $O_2$  flux from the cyanobacterial layer ( $R^2=0.91$ ,  $P<0.0001$ ), as well as with the local concentration of  $O_2$  ( $R^2=0.91$ ,  $P<0.0001$ ) and  $H_2S$  ( $R^2=0.66$ ,  $P=0.008$ ), but not correlated with the upward  $S_{tot}$  flux ( $R^2=0.27$ ,  $P=0.15$ ; Supplementary Figure S6). In one of the C/B mats, the availability of sulphide from below was low and thus the minimum  $S_{tot}/O_2$  consumption ratio of 0.7, which indicates complete oxidation of sulphide to sulphate (see above), was reached early during the day (Supplementary Figure S5B). When this ratio was reached, we observed a pronounced downward shift of the zone where sulphide was aerobically consumed (Supplementary Figure S5C). This downward shift did not affect the correlation between the  $S_{tot}/O_2$  consumption ratio and the  $O_2$  flux or the local  $O_2$  and  $H_2S$  concentrations.

Taken together, our data suggest that in the layer underneath the photic zone aerobic sulphide oxidation by SOB was the only significant sink of oxygen in the layer while aerobic heterotrophic activity was negligible. Therefore, we conclude that the SOB population in the C/B mats most likely varied its activity between incomplete and complete aerobic sulphide oxidation to  $S^0$  and  $SO_4^{2-}$ , respectively (that is, at a variable  $S^0/SO_4^{2-}$  production ratio), whereby this variability was regulated by the availability of  $O_2$  produced locally by the overlying cyanobacteria and by the local  $H_2S$  concentration but not by the flux of sulphide from below.

#### Activity of B/C mats

Despite the fact that B/C mats were exposed to similarly high incident light intensities as the C/B mats (Table 1), sulphide removal in these mats owing to anoxygenic P was not very pronounced (Figure 4c). Also oxygenic P was very low or even below detection limit in the B/C mats (Figure 4c; Supplementary Figure S7). This was confirmed by *ex situ* measurements under controlled light conditions. Specifically, the depth-integrated rates of gross anoxygenic and oxygenic P were about one order of magnitude lower than in C/B mats at similar irradiances and remained low even when the applied



irradiance exceeded the maximal values measured *in situ* (Figure 5d; Supplementary Figure S7). Additionally, the rate of O<sub>2</sub> consumption in the cyanobacterial layer (such as by aerobic respiration), calculated as the difference between the gross and net rates of O<sub>2</sub> produced in the layer (Figures 5c and d), was not significant in the B/C mats. Also, the depth-integrated rates of gross anoxygenic P (0.20 μmol S<sub>tot</sub> m<sup>-2</sup> s<sup>-1</sup>) matched closely the net S<sub>tot</sub> flux (0.19 μmol S<sub>tot</sub> m<sup>-2</sup> s<sup>-1</sup>) consumed in the cyanobacterial layer, indicating that anoxygenic P was the only significant sink of sulphide in this layer. The source of this photosynthetically removed sulphide was exclusively from below.

Sulphur and oxygen cycling in B/C mats was dominated by the light-independent aerobic chemolithotrophic SO in the top SOB layer (Figures 4d and 5c). In the dark, sulphide for aerobic SO originated from both the water column and the underlying sediment. During high light conditions, the downward sulphide flux from the water column was the main source, while the upward sulphide flux was mostly consumed in the cyanobacterial layer (see above; Figure 5c). Additionally, during the maximum incident irradiance, consumption of O<sub>2</sub> produced locally by the underlying cyanobacterial layer only contributed <15% to the overall O<sub>2</sub> consumption in the SOB layer. Thus SO in the top layer relied mostly on O<sub>2</sub> supplied externally from the overlying water column.

Over the course of the day, the S<sub>tot</sub>/O<sub>2</sub> consumption ratio remained constant at about 0.67 (Figure 4d). Thus aerobic SO by the SOB community in the B/C mats was in steady state and therefore most likely proceeded completely to sulphate (Nelson *et al.*, 1986; Jørgensen *et al.*, 2010). Using this stoichiometry, we estimated the energy conservation efficiency of the SOB community in the two replicate B/C mats to be 16.4% and 17.1% (calculation details given in Supplementary Information 2.2), which is very similar to the value estimated for the SOB community in the C/B mats (see above).

#### *Light absorption by the cyanobacteria*

C/B and B/C mats back-reflected 4.5% and 81% of the incident irradiance, respectively. This suggests that the estimated fractions of the incident flux of light energy absorbed by the cyanobacterial populations were 95.5% and 19%, respectively (see Supplementary Information 1). However, these values likely define the upper limits of the light energy utilized by cyanobacteria in the mats, as light absorption could occur also owing to abiotic components in the mats (Al-Najjar *et al.*, 2012).

#### *Carbon and energy budgets*

Assuming that photosynthesis by cyanobacteria and aerobic sulphide oxidation by SOB were the only processes responsible for oxygen and sulphide cycling, we used the measured fluxes of light, oxygen

and sulphide together with the estimated energy conservation efficiency of the SOB populations to estimate daily carbon and energy budgets in the two studied mat types (Table 2). The C/B mats conserved about 2.42% of the incident light energy directly by P (split into 1.83% by oxygenic and 0.59% by anoxygenic P). This conservation efficiency was increased to about 2.54% when additionally considering that the photosynthetically produced oxygen is used for SOB-driven carbon fixation coupled to aerobic chemosynthetic SO. When expressed in terms of fixed carbon, the exploitation of the thermodynamic disequilibrium between O<sub>2</sub> and sulphide, which was internally generated by oxygenic P, thus increased the total primary productivity in the C/B mats by 15% (from 67.5 to 79.5 mmol C m<sup>-2</sup> d<sup>-1</sup>).

In contrast, the B/C mats conserved only about 0.12% of the incident light energy by P (mainly by anoxygenic P), although the daily flux of light energy available for both mat types was similar. Instead, energy conservation in the B/C mats was dominated by chemosynthesis (about 69% of the total energy conserved), although the daily flux of light energy available to the system was about 67-fold larger than the flux of chemical energy (in the form of oxygen and sulphide diffusing from the water column, utilized by aerobic SO). Thus, because of the small contribution of P, the overall energy conversion efficiency in the B/C mats was only 0.37%, that is, sevenfold lower than in the C/B mats. This grossly inefficient utilization of the available light energy in the B/C mats is also reflected in the estimated overall primary productivity, which was about threefold lower than in the C/B mats (23.4 vs 79.5 mmol C m<sup>-2</sup> d<sup>-1</sup>; Table 2). Analysis of the replicate measurements led to similar conclusions although with slightly different numerical values (Supplementary Table S2).

## Discussion

The dominant functional groups in the Frasassi spring mats, photosynthetic cyanobacteria and aerobic chemolithoautotrophic SOB are directly coupled as both oxygen and sulphide are involved in their energy-generation pathways. The two functional groups, however, depend on different energy sources, that is, on light and chemical energy, respectively. Our results show that activity in the mats is driven by both energy sources. However, depending on the direction and temporal dynamics of the energy supply, the mats stratified in essentially two distinct structures, C/B mats and B/C mats, characterized by substantially different activity patterns and, most strikingly, utilization efficiencies of the externally available energy.

#### *C/B mats*

In C/B mats, cyanobacteria inhabiting the photic layer switched between anoxygenic and oxygenic



**Table 2** Daily budgets of compounds and energy utilized by photosynthesis (P, anoxygenic and oxygenic) and by aerobic sulphide oxidation (SO) in the two end-member mat types

	C/B mat			B/C mat		
	P (anoxyP; oxyP)	SO	Total (P; SO)	P (anoxyP; oxyP)	SO	Total (P; SO)
O <sub>2</sub> flux <sup>a</sup> (mmol O <sub>2</sub> m <sup>-2</sup> d <sup>-1</sup> )	43.93	-34.11	9.82	0.0	-56.30	-56.30
S <sub>tot</sub> flux <sup>a</sup> (mmol S <sub>tot</sub> m <sup>-2</sup> d <sup>-1</sup> )	-47.12	-32.28	-79.34	-8.63	-37.67	-46.30
CO <sub>2</sub> flux <sup>a</sup> (mmol CO <sub>2</sub> m <sup>-2</sup> d <sup>-1</sup> )	-67.49 (34.9%; 65.1%)	-12.04	-79.53 (84.9%; 15.1%)	-4.32 (100%; 0%)	-19.06	-23.38 (18.5%; 81.5%)
Energy flux available <sup>b</sup> (kJ m <sup>-2</sup> d <sup>-1</sup> )	1572.53	13.22 <sup>c</sup>	1585.75 (99.2%; 0.8%)	1455.74	21.68	1477.34 (98.5%; 1.5%)
Energy flux conserved (kJ m <sup>-2</sup> d <sup>-1</sup> )	38.10 (24.4%; 75.6%)	2.24	40.34 (94.5%; 5.5%)	1.70 (100%; 0%)	3.71	5.41 (31.43%; 68.6%)
Conservation efficiency <sup>d</sup>	2.42% (0.59%; 1.83%)	16.9%	<b>2.54%</b> (2.40%; 0.14%)	0.12% (0.12%; 0)	17.1%	<b>0.37%</b> (0.12%; 0.25%)

<sup>a</sup>Positive and negative values are used when the compound is produced and consumed, respectively.

<sup>b</sup>Energy gained by aerobic SO was assumed to represent also the energy available for this process.

<sup>c</sup>Represents energy generated internally within the ecosystem as a consequence of oxygenic P.

<sup>d</sup>Calculated as energy conserved divided by energy available. The total energy conservation efficiency of the mats is highlighted in bold.

P over a diurnal cycle, with microzones performing oxygenic and anoxygenic P simultaneously (Figures 4 and 5). As C/B mats formed in areas characterized by a very low O<sub>2</sub> concentration (<5 μM) in the overlying water (Table 1), oxygenic P was the exclusive provider of electron acceptor for aerobic SO.

Because of the fluctuating availability of light, the supply of oxygen to the SOB population residing under the cyanobacterial population was also fluctuating. A possible adaptation of SOB to live under such conditions is to rapidly adjust their SO stoichiometry, that is, to vary the S<sup>0</sup>/SO<sub>4</sub><sup>2-</sup> product ratio, depending on the availability of O<sub>2</sub> and S<sub>tot</sub>, whereby the range of S<sub>tot</sub>/O<sub>2</sub> consumption ratios that can be covered by the SOB through the variation between the complete and incomplete oxidation of sulphide to sulphate and zero-valent sulphur, respectively, is determined by the corresponding energy-conservation efficiency (Klatt and Polerecky, 2015). Our data suggest that this strategy was adopted by the SOB in the C/B mats. Specifically, we estimated that the dominant SOB performed aerobic SO with an energy conservation efficiency of ~16.9%, which allowed them to adjust their S<sub>tot</sub>/O<sub>2</sub> consumption ratio to the range of S<sub>tot</sub>/O<sub>2</sub> flux ratios imposed by the environment (between ~0.7 and ~2.6) and thus harvest the available fluxes of sulphide and oxygen optimally.

Interestingly, when the S<sub>tot</sub>/O<sub>2</sub> consumption ratio reached the minimum value of 0.7, that is, when sulphide oxidation proceeded entirely to sulphate, a further increase in the rate of oxygen supply by oxygenic P led to a downward migration of the SOB. We suggest that this is because the SOB could not adjust the S<sub>tot</sub>/O<sub>2</sub> consumption ratio below the minimum value determined by their energy conservation efficiency (Klatt and Polerecky, 2015), leaving the adjustment of their position as the only option to maintain optimal utilization of the available substrates in the dynamically changing gradients of sulphide and O<sub>2</sub>. There are two plausible hypotheses concerning the exact trigger for migration: (i) the biomass-dependent maximum rate of O<sub>2</sub> consumption was reached, or (ii) the rate of O<sub>2</sub> consumption by the SOB became limited by the supply of H<sub>2</sub>S. In both cases, any further increase in oxygenic P would lead to an increase in the local O<sub>2</sub> concentration triggering downward migration owing to a phobic response to O<sub>2</sub> (Møller *et al.*, 1985).

Intriguingly, the S<sub>tot</sub>/O<sub>2</sub> consumption ratio was only determined by the O<sub>2</sub> flux and the local H<sub>2</sub>S concentration but not by the S<sub>tot</sub> flux (Supplementary Figure S6). This is consistent with the expectation that SOB should adjust their S<sup>0</sup>/SO<sub>4</sub><sup>2-</sup> production ratio so as to maximize utilization of the available O<sub>2</sub>. This is because the carbon yield per oxygen is expected to be almost constant irrespective of the S<sup>0</sup>/SO<sub>4</sub><sup>2-</sup> production ratio, that is, the O<sub>2</sub> reduction rate directly translates into growth, as suggested by the fact that the energy-conversion efficiencies estimated for the incomplete and complete SO in

the SOB inhabiting the C/B mats were almost equal (Klatt and Polerecky, 2015).

Together, the flexible O<sub>2</sub>-dependent adjustment of the S<sup>0</sup>/SO<sub>4</sub><sup>2-</sup> production ratio seems to allow the SOB to efficiently exploit the chemical energy in the system and to additionally build up storage of intracellular S<sup>0</sup> that might serve as an electron acceptor for anaerobic respiration during night when the O<sub>2</sub> is not available (Schwedt *et al.*, 2011). The dominant SOB therefore appear to be highly adapted to co-exist with oxygenic phototrophs.

Overall, under the oxygen-limited conditions in C/B mats, both photosynthetic and aerobic chemosynthetic activity are regulated by light energy supplied to the system. Despite the dependence of both anoxygenic P and chemolithotrophy on H<sub>2</sub>S as an electron donor, its light-dependent depletion was not disadvantageous for the SOB because the chemolithotrophic and phototrophic layers did not compete for the same sulphide pool. Specifically, chemolithotrophic activity was exclusively supplied by the sulphide flux from below while the photosynthetically oxidized sulphide originated from the overlying water column. Aerobic SO by SOB, or for that matter any aerobic activity, was directly coupled to both oxygenic and anoxygenic P, the latter required to locally deplete the reductant (H<sub>2</sub>S) so as to enable the former. Therefore, both anoxygenic and oxygenic photosynthetic activities were beneficial for the SOB, as they together allowed for the life-enabling production of oxygen required for the SOB to thrive under the particular oxygen-limiting conditions where C/B mats developed.

#### *B/C mats*

In B/C mats, SOB formed a layer on top of the cyanobacteria, where they could access a continuous supply of energy (H<sub>2</sub>S and O<sub>2</sub>) from the water column throughout the entire diel cycle (Figure 4d). In contrast, the energy source (light) for the cyanobacteria in the B/C mats was discontinuous. We hypothesize that the continuity of the energy supply was the main advantage that the SOB had over cyanobacteria in the B/C mats. Specifically, in the absence of light during the night the cyanobacteria were not triggered to assert themselves in the uppermost position of the mat, that is, closest to the energy sources for both functional groups during the day. This has been taken advantage of by the SOB, whose chemolithotrophic activity in this position could continue uninterrupted. During the day the position of the cyanobacteria remained in the lower part of the mat, where the light availability was significantly reduced (at least fivefold) owing to intense back-scattering in the overlying layer of SOB (Supplementary Information 1). Therefore, the cyanobacteria were not able to harvest the light optimally. It remains unclear how exactly the very distinct layering was maintained during the day and why the cyanobacteria were not able to invade the

SOB layer. An important factor was probably faster motility of the SOB that made them more successful competitors for the 'prime spot' on top of the mats.

#### *Importance of energy flux direction and dynamics for mat structure and function*

When oxygen provided externally from the water column was limiting, light availability regulated the activity and spatial organization of the dominant functional groups, leading to the formation of C/B mats. In these mats, primary productivity was exclusively driven by light. Namely, light energy was directly utilized by anoxygenic and oxygenic P and indirectly facilitated productivity of the SOB that exploited the chemical energy in the form of the thermodynamic disequilibrium driven by photosynthetically produced O<sub>2</sub>. Consequently, processes stratified predictably according to the direction and magnitude of the energy source, and the dominant functional groups, photosynthetic cyanobacteria and aerobic SOB, beneficially interacted. This led to an efficient utilization of the available external energy.

B/C mats, on the other hand, were exposed to two energy sources, both of which had the same direction, that is, they were externally supplied from the water column, and were therefore most abundant at the upper surface of the mat. One of these sources, namely, chemical energy in the form of oxygen and sulphide, was continuous, while the other, light, was diurnally fluctuating. As the functional groups competed for the space closest to their energy sources, specific adaptation mechanisms (for example, motility) and phenotypic features (for example, light-scattering S<sup>0</sup> globules) gained in importance. Intriguingly, the organisms specialized in utilizing chemical energy, SOB, outcompeted all photosynthetic microbes from the position closest to the light, even though the availability of light energy per day was orders of magnitude higher than that of chemical energy. This means that the continuously available chemical energy was used preferentially, while the additional potential for gaining oxygen via internal recycling of the other available external energy source (light) was neglected or even suppressed. As a consequence, the competition for a favourable position in the mat led to a comparatively inefficient use of the bulk external energy.

It is also likely that nitrogen retention, storage and removal in the two mat types strongly differ owing to the differences in the O<sub>2</sub> concentrations in the water column overlying the C/B and B/C mats and the differences in the activity of the SOB and cyanobacteria in the mats. To understand possible effects of this on the mat stratification, more detailed measurements of bioavailable nitrogen in the mats would be required, which could, however, not be done during this study owing to technical limitations. Nevertheless, the lack of correlation between the mat type and the water column concentrations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> (Table 1) suggests that nitrogen cycling is an

unlikely factor that determines the formation of a certain mat type.

### Implications

The scenario in C/B mats resembles what is thought to have been the arena for the evolutionary transition from anoxygenic to oxygenic P in the ancient phototrophic microbial mats in isolated shallow water environments. The environment where these critical steps in evolution occurred was chemically reduced (for example, Tice and Lowe, 2004; Sessions *et al.*, 2009; Lyons *et al.*, 2014). Anoxygenic P using the available reductants, for example, H<sub>2</sub>S, is expected to be an important process in such environments. However, H<sub>2</sub>S can become locally depleted, which provides a selective advantage to cyanobacteria that are able to switch from anoxygenic to oxygenic P (Cohen *et al.*, 1986; Jørgensen *et al.*, 1986; Klatt *et al.*, 2015) and that are therefore never limited by electron donors. In C/B mats, this versatility leads to the introduction of aerobic hotspots in the otherwise reduced environment.

The C/B mats also demonstrate the revolutionary consequences of creating such aerobic hotspots. Oxygen as the most favourable electron acceptor offers a myriad of thermodynamic strategies for other microorganisms, and oxygenic P has therefore set the basis for the co-evolution of aerobic organisms. Initially, as the proliferation of oxygenic P and aerobic metabolisms took place in otherwise anoxic and reduced environments, these two processes were spatially and temporarily closely coupled. This is illustrated in C/B mats, where aerobic SO was completely dependent on the internal conversion of light energy into chemical energy driven by the photosynthetic production of oxygen.

Despite the substantial consumption of oxygen by aerobic chemolithotrophy in the layer underneath the photic zone, C/B mats were net sources of oxygen to the water column. It is assumed that, in ancient, more reduced oceans, excess reductants have initially scavenged the photosynthetically produced oxygen, and it was only upon depletion of these sinks that oxygen could persistently accumulate in the atmosphere and upper water column of the oceans during the Great Oxidation Event. However, the Great Oxidation Event was possibly predated by transient accumulations of oxygen in the atmosphere (the 'whiffs of oxygen') (for example, Anbar *et al.*, 2007; Lyons *et al.*, 2014), and it is not unlikely that, despite reductant availability, oxygen had also persistently accumulated in the shallow water column above microbial mats, similarly to the scenario in the Frasassi sulphidic springs. Independent of the exact timing, water column oxygenation freed aerobic organisms from consortia and microenvironments where O<sub>2</sub> was provided exclusively through the close proximity to oxygenic phototrophs (that is, oxygen oases; for example, Buick, 2008). A paradoxical consequence of this is strikingly demonstrated in the

Frasassi B/C mats. Although these mats were exposed to light and contained significant populations of oxygenic phototrophs, they became a net sink for oxygen once it accumulated in the water column. This is because they were mainly driven by an aerobic, light-independent process (SO). This demonstrates that an aerobic metabolism, which had, by necessity, evolved dependent on light, could even outcompete the oxygenic phototrophs that formerly served as the exclusive provider of oxygen for its energy metabolism.

The oxygenation of the Earth's atmosphere represents an event where oxygen has evolved from a fluctuating internal source of chemical energy into a continuous external source. We suggest that aerobic chemosynthesis might have become competitive against photosynthesis, thus tempering photosynthesis-driven primary productivity and providing a negative feedback on the proliferation of oxygenic phototrophic organisms. As demonstrated by the Frasassi mats, the effect of this negative feedback could have been as radical as a turn from a hugely productive ecosystem that acts as a net O<sub>2</sub> source (C/B mat) into a considerably less productive ecosystem that acts as a net O<sub>2</sub> sink (B/C mat). Further research is required to identify possible biosignatures of this competitive interaction between phototrophs and aerobic chemolithotrophs that could be searched for in geological records to explore whether the negative-feedback hypothesis could be generalized beyond the highly localized scale of this study.

### Conflict of Interest

The authors declare no conflict of interest.

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