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Metabolic Responses of a Phototrophic Co-Culture Enriched from a Freshwater Sediment on Changing Substrate Availability and its Relevance for Biogeochemical Iron Cycling

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ABSTRACT

Metabolic flexibility controls microbial survival and shapes ecological structures. Although the utilization of a variety of substrates has been studied for individual metabolically versatile microorganisms, only little is known about the response of microbial communities toward substrate fluctuation. Here, we exposed a phototrophic co-culture (*Rhodopseudomonas* sp. and *Chlorobium* sp.) that was isolated from a littoral freshwater sediment to a variety of substrates (acetate, Fe(II), or H_2) and followed the abundance of individual microbial partners based on their characteristic light absorption pattern. *Chlorobium* sp. dominated the culture when Fe(II) was supplied, whereas *Rhodopseudomonas* sp. decreased below detection. *Rhodopseudomonas* sp. dominated when the co-culture was transferred onto acetate (*Chlorobium* sp. below detection). When grown on H_2, the dominance pattern depended on pre-culture conditions. Pre-cultures on Fe(II) or acetate resulted in dominance of *Chlorobium* sp. or *Rhodopseudomonas* sp., respectively. Although the relative abundance of the two partner strains strictly relies on substrate availability, we were incapable of isolating *Chlorobium* sp. from the co-culture. Our study shows how substrate fluctuations shape microbial distribution and points toward potential community interactions that enable microbial survival.

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Chlorobium sp; phototrophy; metabolic flexibility; phototrophic iron(II) oxidation; *Rhodopseudomonas* sp

Introduction

Metabolic flexibility represents a survival strategy that allows microorganisms to adapt to changing environmental conditions. In littoral sediments, geochemical gradients fluctuate due to seasonal and diurnal variations (e.g., impact of sunlight and thus oxygen production, temperature, organic matter input) (Schmidt et al. 2010). Physical perturbations like wave movement or bioturbation impact geochemical conditions and/or displace microorganisms away from their preferred substrate sources (Cook et al. 2007; Huettel and Webster 2001). The excretion of metabolic products from organisms locally impacts the geochemistry, creates metabolic micro-niches and potentially complements a variety of different metabolisms (e.g., sulfur-reducing bacteria and methanogens contribute to the degradation of organic matter) (Plugge et al. 2011). The advantage of metabolic flexibility is to survive in environments that become temporarily depleted in electron acceptors or electron donors (Guerrero and Berlanga 2006).

Rhodopseudomonas palustris, a purple nonsulfur bacterium, represents one of the most metabolically versatile bacteria. This microorganism carries the ability to grow photoautotrophically, photoheterotrophically, chemoheterotrophically, as well as chemoautotrophically (Larimer et al. 2004). It can use light, inorganic electron donors or organic compounds to generate energy, and is able to fix nitrogen. Additionally, *Rhodopseudomonas palustris* is able to utilize electrical current as electron donor via extracellular electron transfer (Bose et al. 2014; Guzman et al. 2019), and electrons from the iron-mineral magnetite (Byrne et al. 2015). This exceptional metabolic flexibility might be the key for its commonness in various environments, including aquatic sediments, soils (e.g., soil-plant microbe associations) and wastewaters (Harwood and Gibson 1988; Kim et al. 2004; Lo et al. 2018). Microcosm experiments (Jiao et al. 2005; Melton et al. 2014), genomic (e.g., Larimer et al. 2004; Fixen et al. 2016) and proteomic studies (Bryce et al. 2018a) have been performed to explore the extent of versatility of metabolic modes involved in the survival of *Rhodopseudomonas palustris*.

One *Rhodopseudomonas palustris* strain has been described to be involved in the biogeochemical cycling of

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iron via its ability to grow photoferrotrophically (*R. palustris* TIE 1, Jiao et al. 2005). Photoferrotrophy describes the metabolic mode of using light as an energy source and ferrous iron (Fe(II)) as an electron donor while fixing CO_2 under anoxic conditions (Widdel et al. 1993). However, this ability is not commonly spread amongst all *Rhodopseudomonas palustris* strains (Table 1), creating a niche for alternative photoferrotrophic bacteria that can compete for substrate in the presence of *Rhodopseudomonas palustris*. Bryce et al. (2018b) summarized the biogeography of the few known purple and green sulfur photoferrotrophic bacteria that are involved in iron biogeochemical cycling. Although the metabolic versatility of *Rhodopseudomonas palustris* is well recognized in the scientific community, it is still unclear to what extent it employs and benefits from that mode in nature.

Compared to purple sulfur bacteria, green sulfur bacteria are more restricted with respect to metabolic flexibility (Table 1). Green sulfur bacteria are obligate anaerobic and obligate phototrophic bacteria belonging to the family Chlorobiaceae. They can use reduced sulfur species, H_2 or Fe(II) as an electron donor for anoxygenic photosynthesis, either strictly photolithoautotrophically or mixotrophically, assimilating simple organic compounds during photosynthetic sulfide oxidation (Canfield et al. 2005; Heising et al. 1999). Up to now four different *Chlorobium* strains that are involved in Fe(II) oxidation have been isolated from marine and freshwater environments (Table 1). However, two *Chlorobium* strains remain in co-culture and attempts at culture purification were unsuccessful (Heising et al. 1999; Schaedler et al. 2009; Walter et al. 2014). The question arises whether inseparability of the co-culture results from inappropriate isolation approaches or whether the microbial partnership offers nutritional or energetic benefits to either partner. Compared to purple sulfur bacteria, green sulfur bacteria are known for efficient light utilization (Table 1). Due to differences in their light harvesting pigments (Kappler et al. 2005) certain green sulfur bacteria can utilize light intensities down to 0.015 µmol quanta/m^2s (Manske et al. 2005) and down to 50 lux and lower (Hegler et al. 2008; Llirós et al. 2015; Overmann et al. 1992). The combination of physiological (utilization of low light intensity) and metabolic features (anoxygenic sulfide and Fe(II) oxidation) of green sulfur bacteria lead to the hypothesis that these microorganisms could have evolved in ancient anoxic ferruginous oceans, and thus represent one of the oldest microorganisms on Earth (Brocks et al. 2005; Bryant et al. 2012; Sadekar et al. 2006). Reasons for their abundance throughout Earth history might be related to the ability to grow at low light intensities, with low nutrient and high sulfide concentrations (Hegler et al. 2008; Manske et al. 2005; Thompson et al. 2017).

The goal of the present study was to evaluate the abundance pattern of phototrophic bacteria as a function of substrate availability. For this, we have enriched a phototrophic co-culture (*Rhodopseudomonas* sp. and *Chlorobium* sp.) from a freshwater lake sediment. The co-culture was supplied with a consecutive series of different substrates. Based on the characteristic absorption peaks of the two partner strains, we followed their individual abundance and show that substrate availability shifts the microbial dominance pattern.

Material and methods

Sample origin, enrichment and isolation

Littoral sediments for microbial enrichments were collected from a freshwater lake sediment (Lake Constance, Germany; N47°41′042.63, E9°11′040.29). A detailed description of the sediment geochemistry can be found in Schaedler et al. (2017). The phototrophic co-culture was enriched and cultivated in Hungate tubes (15 mL) containing 9 mL of anoxic liquid freshwater medium (FWM) with 10 mM $FeCl_2$ (referred to as Fe(II) in figures and following sections) and 10% inoculum. FWM contained the following salts per liter: 0.6 g KH_2PO_4, 0.3 g NH_4Cl, 0.025 g $MgSO_4$*$7H_2O$, 0.4 g $MgCl_2$*$6H_2O$, 0.1 g $CaCl_2$*$2H_2O$ with aliquots of 1 mL of each, a 7-vitamin solution (Widdel and Pfennig 1981), a trace element solution (Widdel 1983) and a selenite-tungstate solution (Widdel and Bak 1992). The medium was prepared with a headspace of N_2/CO_2 (90:10) and buffered with 22 mM bicarbonate. The pH of the media was adjusted to pH 7 with anoxic 1 M HCl and anoxic 0.5 mM H_2CO_3.

For enrichment, continuous dilution series (10^{-10} dilution) were performed. DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was added in order to prevent cyanobacterial growth. The samples were supplied with H_2 (headspace exchanged with 80% H_2/20% CO_2), and incubated at 20 °C in standard full wavelength light conditions (400–1000 nm) using a standard light bulb (2700 K, 46 W). After the third transfer, samples were cultivated under infrared light (IR; 760–980 nm) using a standard light bulb (2700 K, 46 W) with a light filter (Lee, UK; infrared filter 87).

For isolation of the individual partners of the phototrophic co-culture, agar shakes were prepared after Pfennig and Trüper (1981) using H_2 as the electron source. Dilution series were performed using one co-culture grown on acetate (dominated by *Rhodopseudomonas* sp.) and one grown on H_2 (dominated by *Chlorobium* sp.). The headspace of the Hungate tubes was flushed every 2-3 days with H_2 and incubated under full light conditions at 20 °C. After single colonies appeared (1 week after incubation), they were transferred into liquid FWM and supplied with H_2.

DNA was extracted from the isolated *Rhodopseudomonas* sp. culture (1.8 mL) and from a highly enriched *Chlorobium* sp. culture (1.8 mL) using the UltraClean® Microbial DNA Isolation Kit (MO BIO Labratories, Carlsbad, CA, US). 16S rRNA gene fragments were amplified using the 341 F (CCTACGGGAGGCAGCAG) and 907 R (CCGTCAATTC CTTTRAGTTT) primer pair (Muyzer et al. 1993) and resulting amplicons were sent for Sanger sequencing (Eurofins GATC biotech, Konstanz, Germany). Sequence results were analyzed using nucleotide BLAST (Basic Local Alignment Search Tool) to identify the closest relatives of both microbial partners.

Table 1. Compilation of published isolated *Chlorobium* sp. strains, their metabolic versatility and characteristic absorption maxima.

Strain	Origin	Metabolic versatility	Optimum light intensities -Characteristic absorption maxima	Reference
Chlorobium ferrooxidans strain KoFox[a]	Freshwater sediment	Fe(II), H_2, fumarate, pyruvate, acetate, **no growth on sulfide, thiosulfate, sulfur**	0.95 μmol/m^{2}s^1 –Chlorobactene (435,462,491 nm)$^{(++)}$	Heising et al. (1999) Hegler et al. (2008)
Chlorobium sp.[b] closest relative *C. ferrooxidans*	Freshwater sediment	see Table 2, **no growth on sulfide, thiosulfate**	3.9 μmol/m^{2}s^1 –460,750 nm$^{(+)}$	this study
Chlorobium sp.[c] closest relative *C. ferrooxidans*	Freshwater lake	Fe(II)	Not measured-not measured	Walter et al. (2014)
*Chlorobium phaeoferrooxidans**	Ferruginous lake	Fe(II)	0.6 μmol/m^{2}s^1 -major light harvesting pigment BChl e$^{(+)}$	Llirós et al. (2015) Crowe et al. (2017)
Chlorobium sp. Strain N1[d](closest relative *Chlorobium luteolum*)	Marine sediment	Sulfide, sulfur, H_2, yeast, sucrose, glucose, lactate, pyruvte, citrate, glycerol, mannitol, Fe(II)	8.9 μmol/m^{2}s^1-not measured	Imhoff (2003) Laufer et al. (2017) Bryce et al. (2019)
Chlorobium chlorochromatii[e]	Lake sediment	Sulfide, acetate, peptone, **no growth on thiosulfate, sulfur and Fe(II)**	10 μmol/m^{2}s^1 – 453,748 nm$^{(+)}$, 662,433 nm$^{(++)}$	Vogl et al. (2006)
Chlorobium limicola	Hot spring, cave	Sulfide, sulfur, H_2, thiosulfate, acetate, proprionate, H_2	Not measured –major pigments: BChl e, BChl c, isorenieratene$^{(+,\ ++)}$	Garrity et al. (2001)
Chlorobium phaeobacteriodes Chlorobium phaeobacteroides BS1 (extremely low light requirements)	Meromictic lake, Black Sea	Acetate, fructose, N_2 fixation, **no growth on H_2, sulfide**	0.25 μmol/m^{2}s^1-461,715 nm (BChl e), 803 nm (BChl a), 505 nm (isorenieratene)$^{(+)}$, 549,597,648 nm (BChl e), 770 nm (BChl a), 340n 460 nm$^{(++)}$	Pfennig (1968), Overmann et al. (1992)
Chlorobium phaeovibrioides[f]	Meromictic lake	Sulfide, sulfur, thiosulfate, N_2 fixation	Not measured-major pigments in green culture: BChl d, occasionally BChl c, chlorobactene; in brown culture: BChl e$^{(+,\ ++)}$	Pfennig 1968, Imhoff et al. (2003) Pfennig and Overmann (2001)
Chlorobium tepidum	Hot spring, anoxic sulfide rich waters and sediments	Sulfide (sulfide alone was not a good photosynthetic electron donor), thiosulfate, acetate, pyruvate, N_2 fixation	not measured-460,753 nm (BChl c, chlorobactene)$^{(+)}$, 436,669 nm (BChl c, chlorobactene), 771 nm (BChl a)$^{(++)}$	Wahlund et al. (1991), Eisen et al. (2002)
GSB 1	Hydrothermal vent	Sulfide, sulfur, acetate, propionate, peptone	Geothermal light-775 nm$^{(+)}$	Beatty et al. (2005)
Chlorobium clathratiforme comb. nov.	Freshwater lake	Sulfide, sulfur, thiosulfate, in presence of sulfide and bicarbonate photoassimilation	Not measured-major pigments in green culture: BChl c, chlorobactene, major pigments in brown culture: BChl e, isorenieratene	Szafer et al. (1911), Pfennig and Overmann (2001), Imhoff et al. (2003)
Rhodopseudomonas palustris strain TIE-1	Iron rich marine sediment	Fe(II), H_2, thiosulfate, acetate, lactate, succinate, pyruvate, malate, fumerate, benzoate, **no growth on sulfide, sulfur, sulfide, formate, glucose**	Not measured-590,805,871 nm$^{(+)}$ spheroidene, okenone, lycopene, rhodopin$^{(++)}$	Jiao et al. (2005)
Rhodopseudomonas sp. closest relative *Rhodopseudomonas palustris* strain BisA53	Freshwater sediment	see Table 3, **no growth on sulfide, Fe(II)**	7.4 μmol/m^{2}s^1 –580,805,860 nm$^{(+)}$	this study
Rhodopseudomonas palustris strain BisA53	Freshwater sediment	Carbon dioxide fixation, hydrogen production, nitrogen fixation, pollutant degrader	Not measured –810-820,890 nm$^{(+)}$	Oda et al. (2008)
Rhodopseudomonas palustris strain CGA009	Freshwater sediment, soil	Carbon dioxide fixation, pollutant degrader	Not measured –800,880 nm$^{(+)}$	Larimer et al. (2004), Oda et al. (2008)

For Rhodopseudomonas palustris three representative strains have been listed (TIE-1: strain that has been shown to be involved in Fe(II) oxidation; BisA53: strain that has been isolated and characterized in this study; CGA009: first strain from which the whole genome has been studied extensively). Highlighted in grey: the strains that have been shown to be involved in biogeochemical iron cycling. Characteristic absorption maxima are listed as published, either with the respective pigment (wavelengths for pigments listed below table) or the measured absorption maxima. (+) spectra measured from whole cells, (++) spectra measured cell extracts.

Bold texts highlights absences of growth.

Growth in co-culture:[a]*Geospirillum arsenophillum,*[b]*Rhodopseudomonas palustris BIS A 53,*[c]*Acidobacteria* sp.,[d]heterotrophic contamination was found based on genome,[e]isolated from a phototrophic consortium *Chlorochromatium aggregatum*,[f]produces H_2 from acetate in syntrophic co-culture with *Desulfuromonas acetoxidans,* **when first published strain was related to Chlorobium ferroxidans KoFox.*

Pigment absorption maxima: BChl a: 805, 830–890*nm, BChl c: 745–755*nm, Bchl d: 705–740, BChl e: 719–726*nm, chlorobactene: (450*nm), isorenieratene (450,505*nm), spheroidene (450,482,514*nm), okenone (521*nm), lycopene (443,471,502*nm), rhodopin (463,490,524*nm).

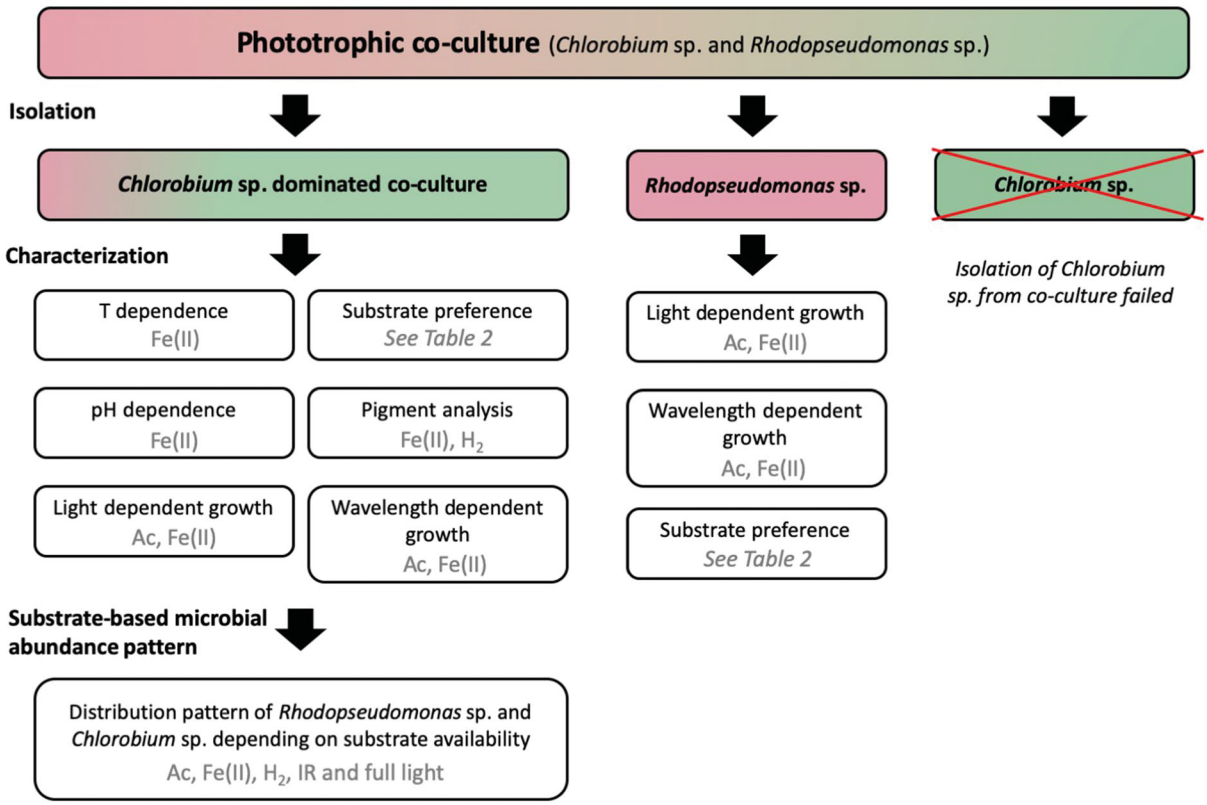

Figure 1. Overview of the experimental strategy. In gray: conditions used for experiments.

Experimental strategy

In order to monitor the distribution of the two partner strains in the enriched phototrophic co-culture, the spectrophotometric absorption pattern was followed as a function of time and substrate supply (Figure 1). The characteristic coloration of the two partner strains allowed individual quantification of the bacteria in the co-culture. For that, the extent of absorption of the individual partner strains (for *Chlorobium* sp.: co-culture highly enriched in *Chlorobium* sp.) was correlated to cell numbers. Cell numbers were quantified by qPCR.

The co-culture was grown on 3 different substrates for 3 transfers (acetate, H_2 or $FeCl_2$) and transferred consecutively two times to another substrate (acetate, H_2 or $FeCl_2$). The abundance of the individual partners was quantified as a function of time based on their specific absorption pattern (Figure 2). Isolation of the partners of the co-culture was attempted. Isolates and co-culture were characterized physiologically (Figure 1).

Cell quantification

As no specific molecular primers are available for both of the strains cell quantification in the inseparable co-culture was performed based on the specific cell coloration of the partner strains. This approach allowed quantification of the individual cell numbers of each strain based on the growth conditions. For that, samples of the co-culture were analyzed spectrophotometrically and the height of a specific absorbance peak of each partner strain was used to quantify

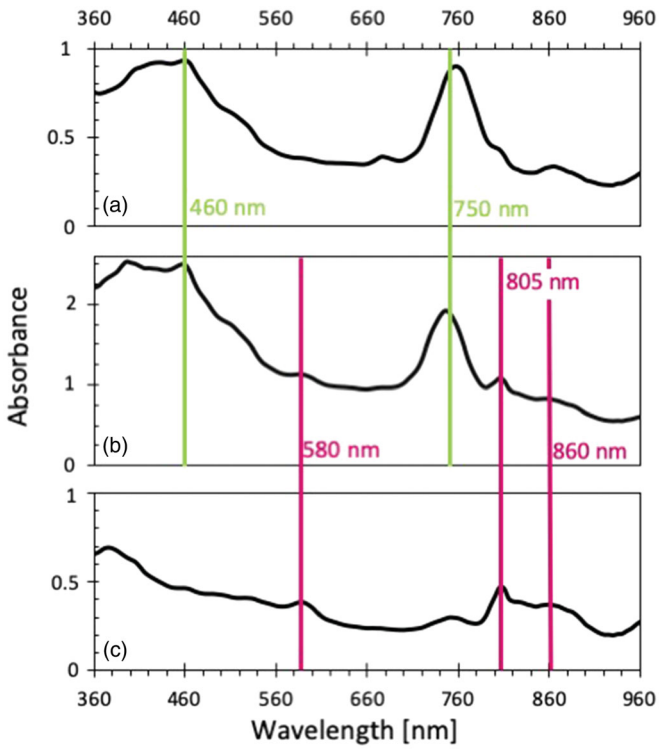

Figure 2. Absorbance spectra for (a) the co-culture dominated by *Chlorobium* sp., (b) the co-culture in which *Chlorobium* sp. and *Rhodopseudomonas* sp. are equally present, and (c) the isolated *Rhodopseudomonas* sp. culture. Lines indicate the characteristic absorption peaks for Chlorobium sp. and Rhodopseudomonas sp., respectively.

cell numbers (*Rhodopseudomonas* sp. 805 nm; *Chlorobium* sp. 750 nm) (Figure 2). Cell quantification based on the spectrophotometric measurement was cross-calibrated with cell quantification by qPCR. Growth experiments were performed where sampling for spectrophotometry and DNA extraction (for qPCR) were taken at the same time. The obtained calibration curve (Figure S1) was used to get quantitative data of cell numbers of the individual partner strains under different growth conditions. Flow cytometry was applied to determine the detection limit of the spectrophotometric cell quantification approach.

Spectrophotometric cell quantification

From cultures grown on H_2 and acetate, 200 µL sample aliquots were transferred to a 96-well microtiter plate and the absorbance was measured in the range of 230-1000 nm in 5 nm steps with a spectrophotometric plate reader (SkanIT RE for Multiskan GO 3.2, Thermo Fisher Scientific Germany BV & Co. KG, Braunschweig, Germany). Samples containing $FeCl_2$ were treated with 50 µL oxalate solution (0.23 M $(NH_4)_2C_2O_4$ $*H_2O$ and 0.17 M $C_2H_2O_4$)prior to analysis in order to dissolve interfering minerals. Peak heights were measured at 805 nm (*Rhodopseudomonas* sp.) and at 750 nm (*Chlorobium* sp.). These peaks were most prominent throughout the culture conditions and are distinct enough from each other to be analyzed individually. No peak shift was observed for the different culture conditions. The detection limit for this method was $3 \times 10^6 \pm 3 \times 10^5$ cells/mL^{-1} and $2 \times 10^4 \pm 1 \times 10^3$ cells/mL^{-1} for *Rhodopseudomonas* sp. and *Chlorobium* sp., respectively (Figure S2) based on results obtained from cell quantification by flow cytometry. For each measurement, triplicate samples (individual set-ups) have been analyzed and obtained data were averaged.

Quantitative PCR

For cell quantification via qPCR, 1.8 mL of samples for DNA extraction were kept frozen at −20 °C. DNA was extracted using the UltraClean®Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) and carried out according to the instructions provided by the producer. The quantity and quality of the extracted DNA was checked with the Nanodrop ND-1000 Spectrophotometer (NanoDrop™ 1000, Thermo Scientific, Waltham, MA, USA). Quantification of 16S rRNA genes was carried out by quantitative PCR using the iCycler iQ™Real-Time PCR Detection System and the iQ™ 5 Optical System software (Bio-Rad laboratories, Hercules, CA, USA). 16S rRNA was quantified with the 341 F (CCTACGGGAGGCAGCAG) and 797 R (GGACTACCAGGGTATCTAATCCTGTT) primer pair (Nadkarni et al. 2002). As standards, a dilution series with plasmids containing the target genes were used and quantified fluorometrically with Qubit 2.0 (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was carried out for all standards, samples and negative controls in triplicates in a 96-well plate with a reaction volume of 10 µl containing SsoAdvanced™ Universal SYBR® Green Supermix, 341 F (75 nM), 797 R (225 nM) and 1 µl DNA template. The thermal profile consisted of 2 min heating at 98 °C, followed by 40 cycles alternating between 5 sec at 98 °C and 12 sec at 60 °C. Then, 1 min at 95 °C and 1 min at 60 °C followed. In the last step, the temperature was increased in 0.5 °C steps every 10 sec starting at 60 °C to 95 °C. Successful qPCR was confirmed by the melt curves. Cell numbers were calculated using 2 gene copies per cell for Rhodopseudomonas and 1.7 gene copies for Chlorobium (values taken from the average of species found).

Flow cytometry

In order to rapidly follow growth in the cultures, the preferable method is to use optical density measurements; however, this optical density must be calibrated to cell numbers. Since these anaerobic phototrophs are difficult to grow on solid medium, we used total cell counts by flow cytometry to calibrate our optical density measurements. To do this, we prepared a dilution series of a stationary phase culture with 10 mM HCO_3^- buffer and measured each dilution by both flow cytometry and optical density. For flow cytometry, BacLight Green stain (Thermo Fisher Scientific, 1 µM stain/1 ml sample) was added to all samples. For measurements, samples were transferred in a 96 well plate and incubated for 20 minutes before cell quantification using a flow cytometer equipped with a 488 nm laser as an excitation source (Attune Nxt flow cytometer, Thermo Fisher Scientific). Cells were distinguished from noise or debris by gating based on their properties in the side scatter and BL1 channel (with emission filter 530/30 nm). Samples with Chlorobium were also analyzed with the YL 1 channel due to its autofluorescence (with emission filter 585/15 nm). The total number of events which show blue fluorescence in the side scatter region associated with cells was divided by the total volume of sample run to give a final cell concentration in cells per milliliter. The flow cytometer injects the sample using a syringe rather than by a peristaltic pump so it is not necessary to use counting beads to know the accurate volume of sample injected. All measurements were conducted in triplicates and the results reported as an average.

In the samples from the same dilution series used for the cell quantification by flow cytometry, we measured the absorbance spectra of the same dilution series using a spectrophotometer. The detection limit of the flow cytometer is significantly better than for optical density measurements. Therefore, we expect to be able to observe cells by flow cytometry even when the optical density is indistinguishable from the background. The lowest cell numbers where a distinctive peak for the relevant bacteriochlorophyll could be detected was defined as the 'detection limit' for our optical density measurements.

Iron quantification

Dissolved Fe(II) and total iron concentrations were determined spectrophotometrically applying the ferrozine assay (Stookey 1970).

Physiological characterization

Temperature-dependent Fe(II) oxidation

The co-culture was grown at 5 different temperatures (4, 10, 20, 26 and 37 °C) under full light conditions and placed 15–20 cm away from light source. It was incubated in 15mL Hungate tubes with 9 mL media and 1 mL culture. For each temperature condition, triplicates were prepared. Fe(II) oxidation was visually confirmed by a color change to orange due to the formation of orange Fe(III) oxyhydroxide minerals. Temperature-dependent growth was not tested for the isolated *Rhodopseudomonas* strain because this strain did not oxidize Fe(II).

pH-dependent Fe(II) oxidation

Growth of the co-culture was tested at different pH values (5.6, 6, 6.4, 6.6, 6.8, 7.1, 7.4, 7.6, 8 and 8.5) in triplicates. The culture was incubated at 20 °C under full light conditions. Samples were taken anoxically and analyzed for total Fe(II) oxidation using the ferrozine assay. pH-dependent growth was not tested for the isolated strain *Rhodopseudomonas* sp. as this strain did not oxidize Fe(II).

Light-dependent growth

The growth of the co-culture and the isolated *Rhodopseudomonas* sp. strain was tested at different light intensities (2.2 ± 1.0, 4.5 ± 1.0, 7.4 ± 1.0 and 10.0 ± 1.0 µmol/m^2 s) and wavelengths (blue light: 420–540 nm; green light: 480–580 nm; red light: 580–760 nm; IR light: 760–980 nm) in triplicates in 15-mL Hungate tubes. Light intensity was measured with a universal light meter (ULM-500, Heinz WALZ GmbH, Effeltrich, Germany). For growth dependence on light intensity, the samples were illuminated with a standard light bulb (2700 K, 46 W, 400–1000 nm). Wavelength ranges were measured with laboratory-made sensors connected to a detector (USB 400-XR1-ES, Ocean Optics, FL, USA). LEDs (3000 K, 18 W) were used for illumination with a wavelength filter for red and green light (Lee, UK; primary red filter 106 and primary green filter 139), whereas different LEDs (5500 to 6500 K, 15 W) were used for illumination with a filter for blue light (Lee, UK; dark blue filter 119). For illumination in the IR range a standard light bulb (2700 K, 46 W) with a light filter (Lee, UK; infrared filter 87,) was used. The distance of the culture bottles from the light source was adjusted to reach 18 µmol/m^2 s at the sample at 20 °C. Subsamples were taken under anoxic conditions and analyzed for cell growth (based on the typical absorption spectra). In setups containing $FeCl_2$, Fe(II) oxidation was quantified via the spectrophotometric Ferrozine assay.

Substrate preference

Substrate preference was tested for the co-culture, as well as for the isolated *Rhodopseudomonas* sp. strain in 15-mL Hungate tubes. The pre-culture of *Rhodopseudomonas* sp. was grown on acetate under full light conditions and transferred after acetate was completely used up to avoid transfer of the pre-substrate. The pre-co-culture was grown on H_2 under IR light conditions at 20 °C. Both cultures were tested for their light-dependent growth on organic compounds (acetate, lactate, fumarate, pyruvate, propionate, butyrate, citrate, formate, glycerol, mannitol, cysteine, D-glucose, succinic acid, sucrose) and a selection of inorganic compounds (thiosulfate, sulfide, Fe(II), H_2). Organic compounds were added to a final concentration of 4 mM, except for yeast extract, which was added to a final concentration of 10 mg/L. Thiosulfate and sulfide were added to a final concentration of 2 mM. Fe(II) ($FeCl_2$) was added to a final concentration of 10 mM and H_2 was supplied as H_2/CO_2 (80:20) gas to the headspace. Growth was observed through occurring turbidity and color change. The culture was transferred twice with the respective substrate.

Pigment analysis

Pigments were extracted from 10 mL co-culture that was highly enriched with *Chlorobium* sp., growing on H_2 and Fe(II) individually. Extraction was done according to the protocol of Bóna-Lovász (2013). Iron minerals were dissolved with 9 mL oxalate solution (0.23 M $(NH_4)_2C_2O_4$ *H_2O and 0.17 M $C_2H_2O_4$) and 1 mL $FeCl_2$. After centrifuging with 4000 rpm for 5 min the supernatant was discarded and the cells washed twice with MilliQ water. Samples grown on H_2 were centrifuged at 4000 rpm for 5 min and the supernatant was discarded. 1 mL methanol and 2 mL cyclohexane were added to the pellets of all samples and the mixture was vortexed for 2 min. Further phase separation was achieved by the addition of 1 mL MQ water to the mixture, followed by vortexing for 1 min and centrifugation at 1800 *g* for 20 min. The supernatant containing the pigments was transferred to a new vial, headspace was flushed with N_2 and closed with a butyl stopper. Extraction was carried out under N_2 atmosphere and under dim light conditions. UHPLC QToF-ESI/MS analysis was used for the detection of pigments with the main focus on carotenoids. Reversed phase chromatographic separation was achieved on a Waters Acquity BEH C18 column (Wörmer et al. 2013, 2016) with a Thermo Dionex Ultimate 3000RS UHPLC coupled to a maXis quadrupole time-of-flight mass spectrometer (Q ToF-MS, Bruker Daltonics, Bremen, Germany) by an electrospray ionization source. Detection of lipids was performed in positive ionization mode while scanning a mass-to-charge range from 150 to 2,000. MS^2 scans were obtained in data dependent mode, targeting the most abundant ions. Compound identification was achieved by screening of exact masses of molecular ions (present as either H^+, NH_4^+ adducts) in combination with characteristic fragmentation derived from MS^2 (Figure S6 for examples) as well as fragmentation as outlined by for example, Airs and Keely (2002), Caple et al. (1978), Garcia Costas et al. (2012), Rivera et al. (2014), van Breemen et al. (2012), as well as elution pattern.

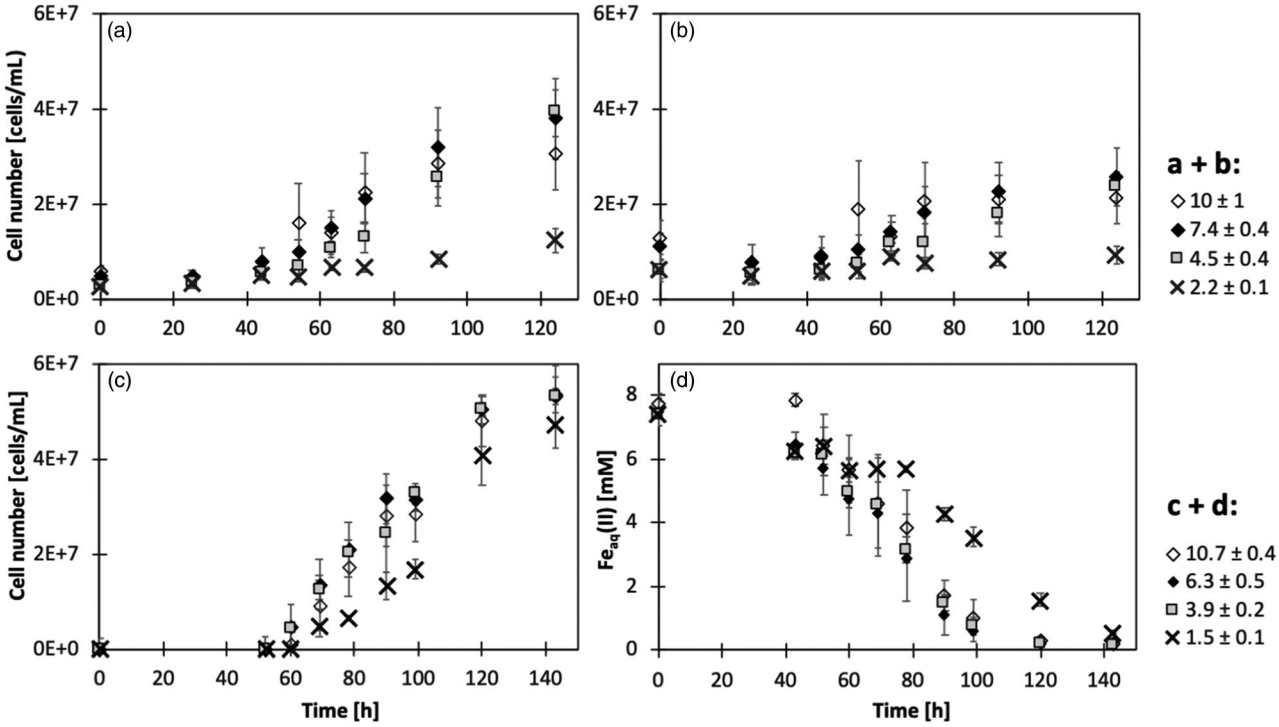

Figure 3. Light-dependent growth at light intensities (in µmol/m^{2}s^1 photons). (a) *Rhodopseudomonas* sp. grown on acetate, (b) Co-culture grown on acetate, (c) Co-culture dominated by *Chlorobium* sp. grown on Fe(II), and (d) the corresponding Fe(II) oxidation data. Data present average value from triplicate setups (standard deviation).

Results

Enrichment

The enrichment culture from the freshwater sediment samples was composed of a phototrophic co-culture containing *Rhodopseudomonas* sp. (99% similarity to *Rhodopseudomonas palustris* strain Bis A53) and *Chlorobium* sp. (99% similarity to *Chlorobium ferrooxidans*) based on 16S analysis. No other strains were detected. Isolation was successful for *Rhodopseudomonas* sp. but not for *Chlorobium* sp. We achieved isolation of *Rhodopseudomonas* sp. from the co-culture using agar shakes that were amended with H_2 and incubated in light (Figure S3). However, all attempts for isolation of *Chlorobium* sp (under IR and full light conditions, H_2 or Fe(II) as electron donor) failed to out dilute *Rhodopseudomonas* sp. which grew up when conditions were favorable (i.e., transferred to acetate).

Physiological characterization

Fastest Fe(II) oxidation of the co-culture was observed at 26 °C after 3 days (visual confirmation of orange coloration due to precipitation of orange Fe(III) oxyhydroxide minerals). After 9 days of incubation Fe(II) oxidation was observed at 10 °C and 4 °C, but no Fe(II) oxidation was observed at 37 °C within 36 days (Figure S4). Optimum pH for complete Fe(II) oxidation by the co-culture was between 7.4 and 7.6. Incomplete, i.e., no significant, Fe(II) oxidation occurred at pH 6.8 and 8.5 (Figure S5), whereas no Fe(II) oxidation was observed at pH 5.6.

The abundance of *Rhodopseudomonas* sp. and *Chlorobium* sp. was quantified based on their characteristic absorption pattern. The peak height was related to cell abundance (*Rhodopseudomonas* sp. 805 nm; *Chlorobium* sp. 750 nm (Figure 2)) after calibration qPCR vs. spectrophotometric analyses (Figure S1).

The isolated strain *Rhodopseudomonas* sp. grew up to $3 \times 10^7 \pm 1 \times 10^6$ cells/mL within 124 h at 7.4 µmol/m^2 s on 5 mM of acetate (Figure 3(a)). Cell growth was comparable at 10 and 7.4 µmol/m^2 s, lower cell growth was observed at 2.2 µmol/m^2 s. The phototrophic co-culture (initially dominated by *Chlorobium* sp.) grown on 5 mM acetate showed a similar pattern with comparable cell growth but turned pinkish during incubation (Figure 3(b)). Growth of the co-culture that was initially dominated by *Chlorobium* sp. under Fe(II)-oxidizing conditions revealed cell densities in the order of $5 \times 10^7 \pm 4 \times 10^6$ cells/mL independent on the light intensity (Figure 3(c) and 10 mM of Fe(II) (unfiltered medium) were completely oxidized (Figure 3(d)).

The *Rhodopseudomonas* sp. isolate grew best with 5 mM acetate under red light (580–760 nm) and poor growth, starting only after 600 h was observed under IR light conditions (760–980 nm) (Figure S6(a)). The co-culture grown on 5 mM acetate showed similar trends with slightly lower cell growth, but turned pinkish (Figure S6(b)). Under Fe(II)-oxidizing conditions the co-culture that was dominated by *Chlorobium* sp. reached cell numbers of up to $4 \times 10^7 \pm 3 \times 10^6$ cells/mL after 120 h (independent of the wavelength) (Figure S6(c)). No growth of *Rhodopseudomonas* sp. was detected under Fe(II)-oxidizing conditions.

Table 2. Substrate growth test for the phototrophic co-culture containing Chlorobium sp. and Rhodopseudomonas sp.

Substrate	Growth +/−	Substrate	Growth +/−
Organic acids		Sugars	
Acetate	+	D-glucose	−
Lactate	+	Sucrose	−
Fumarate	−		
Propionate	+	Amino acids	
Succinate	+	Cysteine	−
Pyruvate	+		
Butyrate	+	Complex substrates	
Citrate	−	Yeast extract	−
Formate	−		
		Inorganic e^- donors	
Alcohols		Thiosulfate	−
Glycerol	−	Sulfide	−
Mannitol	−	H_2	++
		Fe(II)	++

The growth of *Chlorobium* sp. (marked in ++) was verified based on their specific absorption pattern (750 nm; spectrophotometric measurement). For Fe(II)-amendment spectrophotometric measurement was impossible due to interfering Fe(III) minerals. Growth was marked positive for *Chlorobium* sp. as the *Rhodopseudomonas* sp. isolate was incapable of phototrophic Fe(II) oxidation. In case growth of co-culture was observed but *Chlorobium* sp. was not detectable spectrophotometrically, marked in +.

Table 3. Substrate growth test for the isolated phototrophic strain *Rhodopseudomonas* sp.

Substrate	Growth +/−	Substrate	Growth +/−
Organic acids		Complex substrates	
Acetate	+	Yeast extract	−
Lactate	+		
Propionate	+	Inorganic e^- donors	
Succinate	+	Sulfide	−
Pyruvate	+	H_2	+
Butyrate	+	Fe(II)	−

Substrate preference

The co-culture exhibited anaerobic photoheterotrophic growth with acetate, lactate, propionate, succinate, pyruvate and butyrate. However, *Chlorobium* sp. only grew phototrophically and dominated the co-culture when grown on Fe(II) and H_2 (Table 2). The cultures that were amended with organic substrates turned pinkish and were dominated by *Rhodopseudomonas* sp. (confirmed with spectrophotometric assay of characteristic absorption peaks). No phototrophic growth of the co-culture was measured when grown on yeast extract, fumarate, citrate, formate, glycerol, mannitol, cysteine, D-glucose, sucrose, thiosulfate or sulfide. Substrate tests for the isolated *Rhodopseudomonas* sp. strain showed phototrophic growth in the presence of acetate, lactate, succinate, pyruvate, propionate, butyrate, as well as for H_2 (Table 3). No phototrophic growth was found in the presence of sulfide, Fe(II) and yeast.

Response of co-culture to substrate fluctuation

In order to investigate how the co-culture responds to substrate fluctuation, the abundance of *Rhodopseudomonas* sp. and *Chlorobium* sp. (Percentage correlates to detectable signal for each strain).was monitored along transfers between acetate, Fe(II) or H_2 starting from different pre-culture conditions (acetate, Fe(II) or H_2).

Pre-co-culture grown on acetate

The pre-co-culture grown on acetate was dominated by *Rhodopseudomonas* sp. (100%) and *Chlorobium* sp. was below detection with the spectrophotometric assay (Figure 4). A transfer from acetate onto 10 mM Fe(II) revealed an increase of *Chlorobium* sp. ($7 \times 10^6 \pm 2 \times 10^6$ cells/mL) (*Rhodopseudomonas* sp. below detection limit) after 17 days under full light conditions. *Rhodopseudomonas* sp. only grew up ($4 \times 10^7 \pm 3 \times 10^6$ cells/mL) and dominated over *Chlorobium* sp. ($1 \times 10^7 \pm 2 \times 10^6$ cells/mL) when the culture was further transferred onto acetate. In comparison, the transfer from Fe(II) onto H_2 did not result in detectable growth of *Rhodopseudomonas* sp. under full or IR light conditions (after 35 days), but increased *Chlorobium* sp. growth to $2 \times 10^7 \pm 3 \times 10^6$ cells/mL and $1 \times 10^7 \pm 1 \times 10^5$ cells/mL under full and IR light conditions, respectively.

Pre-co-culture grown on Fe(II)

The pre-co-culture grown on Fe(II) was dominated by *Chlorobium* sp. (100%), whereas *Rhodopseudomonas* sp. was below detection with the spectrophotometric assay (Figure 5). A transfer to acetate revealed absolute dominance (100%) of *Rhodopseudomonas* sp. ($3 \times 10^7 \pm 3 \times 10^6$ cells/mL) after 17 days and a subsequent transfer to Fe(II) resulted in a decrease of *Rhodopseudomonas* sp. below detection and a dominance of *Chlorobium* sp. ($6 \times 10^7 \pm 5 \times 10^6$ cells/mL). In comparison, when the *Rhodopseudomonas* sp. dominating co-culture was transferred from acetate onto H_2, cell numbers decreased to $9 \times 10^6 \pm 2 \times 10^6$ cells/ml under full light conditions and below detection under IR light conditions after 35 days. No growth of *Chlorobium* sp. was detected in these setups. In contrast, a transfer from the Fe(II) grown pre-co-culture directly to H_2 revealed dominance of *Chlorobium* sp. over the entire incubation duration (78 days). The abundance of *Rhodopseudomonas* sp. increased from below detection, over 33% (35 days) to 41% (78 days) in this setup.

Pre-co-culture grown on H_2

The pre-co-culture grown on H_2 (grown under full light conditions) was dominated by *Chlorobium* sp. (70%), and *Rhodopseudomonas* sp. represented only 30% (Figure 6). A transfer to 5 mM acetate showed almost equally low growth for both, *Rhodopseudomonas* sp. ($7 \times 10^6 \pm 7 \times 10^5$ cells/mL) and *Chlorobium* sp. ($6 \times 10^6 \pm 7 \times 10^5$ cells/mL) after 17 days. A subsequent transfer to Fe(II) resulted in absolute dominance (100%) of *Chlorobium* sp. ($5 \times 10^7 \pm 8 \times 10^6$ cells/mL) after 35 days, whereas the transfer onto H_2 revealed a decrease in cell numbers for both strains (*Chlorobium* sp. below detection under full and IR light conditions). A direct transfer of the pre-co-culture from H_2 onto Fe(II) allowed growth of *Chlorobium* sp. ($2 \times 10^7 \pm 2 \times 10^6$ cells/mL) after 17 days (*Rhodopseudomonas* sp. below detection). A consecutive transfer to 5 mM acetate

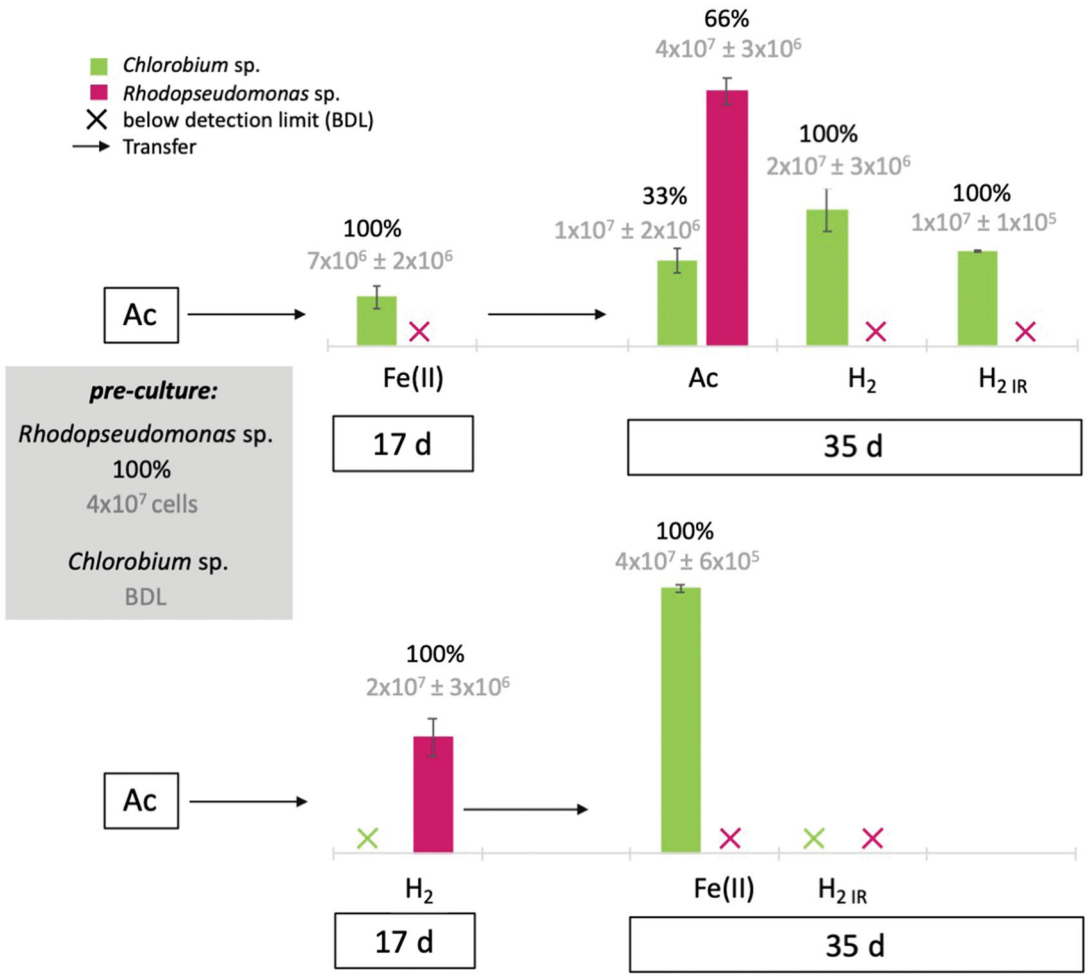

Figure 4. Microbial distribution pattern in the co-culture as a function of substrate availability (percentage values based on spectrophotometrically measured abundance). Displayed numbers represent quantification of *Rhodopseudomonas* sp. and *Chlorobium* sp. based on their specific absorption spectra (unit: cells/mL). The pre-co-culture was grown on acetate before transferring to the chosen variety of substrates (Ac: acetate; Fe: Fe(II); H2: hydrogen; H2 IR: hydrogen IR light). The pre-culture was transferred 3 times on the initial substrate (acetate) and grown for 3 weeks before transferring to Fe(II) or H2. If not stated differently incubations have been performed at full light conditions. Data present average value from triplicate setups (standard deviation). Percentage correlates to detectable signal. (BDL - below detection limit).

changed the abundance pattern to *Rhodopseudomonas* sp. dominance ($8 \times 10^7 \pm 8 \times 10^5$ cells/mL, *Chlorobium* sp. below detection).

A transfer from the *Chlorobium*-dominated co-culture from Fe(II) to H_2 revealed dominance of *Chlorobium* sp. after 35 and 78 days at both, IR and full light conditions (*Rhodopseudomonas* sp. below detection). The pre-co-culture that was grown on H_2, and was maintained on H_2 under full and IR light conditions showed no detectable growth after 17 days. Only after 78 days, we detected an absolute dominance (100%) of *Rhodopseudomonas* sp. ($4 \times 10^7 \pm 8 \times 10^6$ cells/mL) under full light conditions and *Chlorobium* sp. ($3 \times 10^8 \pm 1 \times 10^8$ cells/mL) under IR light conditions.

Pigment analysis

Chlorobactene was identified as the dominant carotenoid for Chlorobium in both samples of the co-culture that grew on Fe(II) and H_2 (Figure S7). Quinones were dominated by menaquinones with lower amounts of ubiquinones in both samples (Fe(II) and H_2). Bacteriochlorophyll c (BChl c) esterified with farnesyl at position C-17 represents the dominant bacteriochlorophyll (Figure S7), but could only be detected in samples grown with H_2. The corresponding bacteriopheophytins could be analyzed in both samples (Fe(II) and H_2). The obtained data are summarized in Figure S8 (additional information on pigment results in supplementary information).

Discussion

Potential benefit for chlorobium sp. for living in co-culture

An open question that arose during our study is why we were not able to isolate the *Chlorobium* sp. from the co-culture (separating it completely from the *Rhodopseudomonas* sp.). We performed several attempts including multiple subsequent dilution series, agar shakes, long-term incubation under full light and IR conditions with the amendment of Fe(II) or H_2 over many generations. Although we were not able to detect *Rhodopseudomonas* sp. in any of these setups with the spectrophotometric assay, it always grew to a dense

population as soon as we transferred the culture to its (*Rhodopseudomonas* sp.) most favorable conditions (i.e., acetate, incubation under full light conditions). Based on our data, we cannot fully conclude on whether we were technically not able to separate *Chlorobium* sp. from *Rhodopseudomonas* sp., or whether *Chlorobium* sp. requires the presence of *Rhodopseudomonas* sp. for physiological needs. Similar to our work, several studies reported on microbial consortia involving *Chlorobium* sp. with potentially beneficial interactions between the involving bacteria (Heising et al. 1999; Pfennig 1968; Walter et al. 2014) (see Table 1). Most recently Bryce et al. (2019) found genomic evidence that the culture that was previously considered to be a pure culture of the marine strain *Chlorobium sp. strain* N1 (Laufer et al. 2017), actually carried small amounts of a partner strain. However, Vogl et al. (2006) isolated *Chlorobium chlorochromatii* sp. nov. from a phototrophic consortium containing *Chlorochromatium aggregatum* (Mechsner 1957). Phototrophic consortia between green sulfur bacteria and chemotrophic betaproteobacteria have been found in numerous stratified lakes (Glaeser and Overmann 2004), where they can represent up to two-thirds of the total microbial mass in the chemocline (Gasol et al. 1995). Green sulfur bacteria grow photolithoautotrophically on sulfide within these consortia, but still seem to rely on organic compounds (Overmann and Schubert 2002). Although none of the partners in the recently isolated *Chlorobium* containing co-cultures (Table 1) belong to the class of betaproteobacteria, potential for beneficial interspecies relation is given and should be the focus of future research. The presence of *Rhodopseudomonas* sp. in our co-culture, even in low cell numbers, might provide organic substrates that have a stimulating effect on the growth and survival of *Chlorobium* sp. Several studies hint toward the co-assimilation of organic substrates via autotrophic bacteria. Zarzycki and Fuchs (2011) reported that an autotrophic green non-sulfur bacterium (*Chloroflexus aurantiacus*) benefits from organic cyanobacterial photorespiration and fermentation products and stated that co-assimilation of trace organic amounts together with CO_2 is beneficial for growth. Similar observations have been made for methanogenic bacteria (Whitman 2014). Acetate, cysteine and yeast extract have been shown to be stimulatory for *Methanobacterium*. Such examples for interspecies interaction relationships are widespread amongst several microbial groups and species. They might be a key for success in survival, they are considered to occupy a unique niche in nature, potentially carrying an important role in the carbon cycling in anoxic environments (Morris 2013). Such an interspecies relationship might be plausible as well for our phototrophic co-culture.

Generalist versus specialist species

The metabolic versatility of *Rhodopseudomonas* sp. has been shown in various studies (e.g., Jiao et al. 2005; Larimer et al. 2004; Oda et al. 2008). The flexibility toward substrates ranges from heterotrophy to carbon dioxide fixation, pollutant degradation, nitrogen fixation and hydrogen production, while carrying a broad range of light harvesting systems (see Table 1). It has been suggested that *Rhodopseudomonas* isolates are different ecotypes that evolved by migration into environments that differ in light and substrate availability, whilst maintaining a high degree of metabolic flexibility (Oda et al. 2004). *Rhodopseudomonas* sp. can thus be considered as generalists, whereas less metabolic flexibility makes *Chlorobium* a specialist. Specialists have a narrower niche compared to generalists, assumed to be less successful in abundance and survival. However, it has been shown that specialists have been more dominant than generalists along natural environmental gradients, suggesting that specialists are constrained by few factors (i.e., substrate availability) and as such can occupy the narrow niche space (Carbonero et al. 2014; Kassen 2002). When a niche is already occupied only little is left for generalist species. This might explain the presence of *Chlorobium* sp. in various sedimentary systems (Heising et al. 1999; Laufer et al. 2017) where these microbes occupy a niche that is well defined by substrate and light availability. Seasonal changes might lead to changes in substrate availability and consequently also influence the bacterial community. Other studies present that bacterial communities are highly diverse and change depending on the season. Only a few phyla were present throughout the year and depending on the season specific phyla grew to higher abundances and were not present throughout the other seasons (Gilbert et al. 2009; Gobet et al. 2012). As we saw in our experiment *Chlorobium* sp. became dominant if their niche substrate Fe was present. *Rhodopseudomonas* sp., present but under the detection limit, was growing again and reached 41% after 78 days and would dominate the co-culture after following its growth trend (Figure 5).

As physico-chemical gradients are established throughout the sedimentary column as a function of active biogeochemistry, a series of niches are established temporally (i.e., day-night induced photosynthesis affects oxygenation of top sediment layers) and provide living space for additional microorganisms. Thus, a co-existence of the generalist *Rhodopseudomonas* sp. and the specialist *Chlorobium* sp. is favored. However, the abundance of each species might vary (similar to our substrate fluctuation experiments, Figures 3–5), which could point toward the efficient survival strategies in being a specialist versus generalist. If we consider the specialist species as the most successful survivor, the hypothesis of *Chlorobium* sp. being one of the oldest organisms on Earth (Brocks et al. 2005; Bryant et al., 2012) increases in weight.

Niche differentiation between Rhodopseudomonas sp. and Chlorobium sp

Metabolic flexibility has been considered as an advantage for microorganisms, allowing them to survive when substrate and energy sources become temporarily depleted. The opportunity to survive on a variety of substrates has been viewed as a major reason for finding microorganisms in environments that do not fulfill their optimum substrate

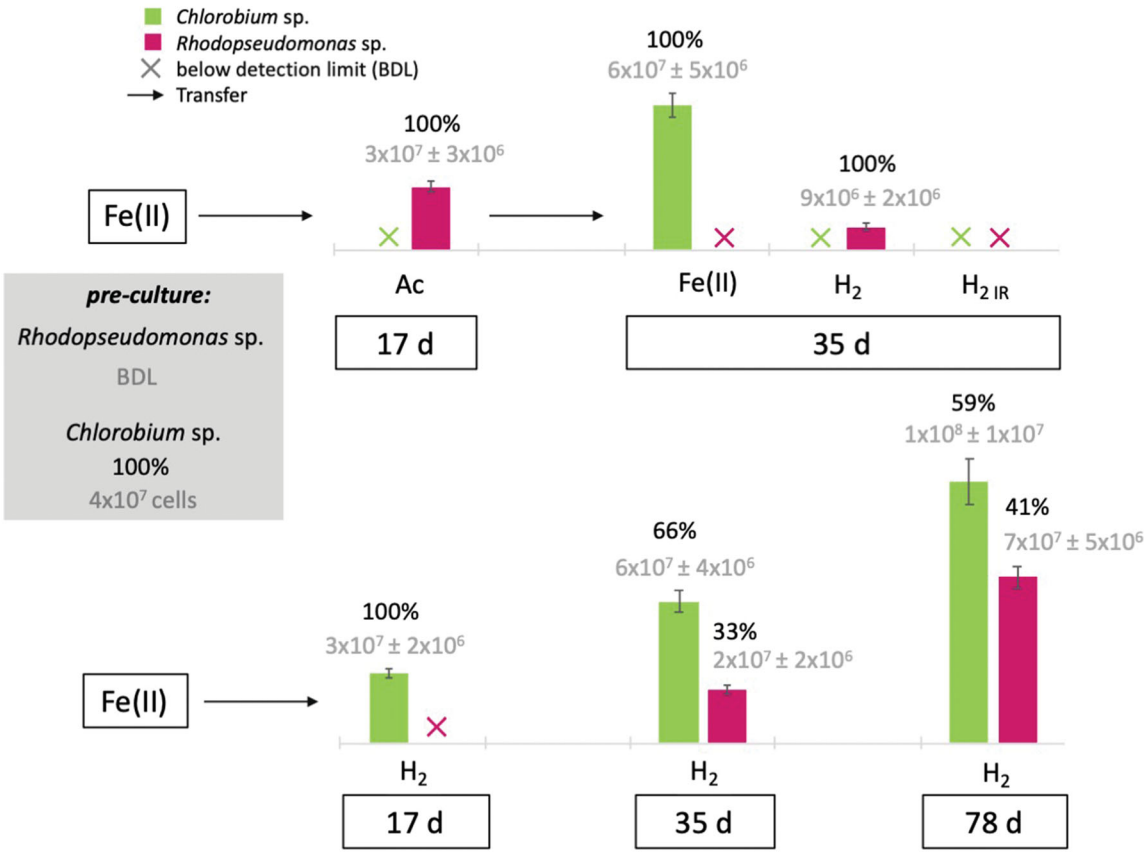

Figure 5. Microbial distribution pattern within the co-culture as a function of substrate availability (percentage values based on spectrophotometrically measured abundance). Displayed numbers represent quantification of *Rhodopseudomonas* sp. and *Chlorobium* sp. based on their specific absorption spectra (unit: cells/mL). The pre-co-culture was grown on Fe(II) before transferring to different substrates (Ac: acetate; Fe: Fe(II); H2: hydrogen; H2 IR: hydrogen at IR light). The pre-culture was transferred 3 times on the initial substrate (Fe(II)) and grown for 3 weeks before transferring to acetate or H2. If not stated differently incubations have been performed at full light conditions. The transfer from the Fe(II) pre-co-culture onto H2 was not further transferred, but let stand for longer time (78 d). Data present average value from triplicate setups (standard deviation). Percentage correlates to detectable signal. (BDL: below detection limit).

requirements (e.g., Guerrero and Berlanga 2006; Laufer et al. 2015; Plugge et al. 2011). The microbial distribution in sediments does not necessarily follow geochemical gradients (Laufer et al. 2015; Schaedler et al. 2017), which swings back and forth between the two ecological principles of 'everything is everywhere, but the environment selects' (Baas-Becking 1934) and 'all species are always absent from almost everywhere' (Begon et al. 1996). Our data move along these lines showing that the abundance pattern of *Rhodopseudomonas* sp. and *Chlorobium* sp. varies with environmental conditions, such as substrate and light availability. The relative abundance of *Rhodopseudomonas* sp. and *Chlorobium* sp. has been quantified based on DNA and RNA in the sediments sampled at the same location in a previous study (Otte et al. 2018). Although the DNA-based relative abundance of *Rhodopseudomonas* sp. and *Chlorobium* sp. was low (<0.01%), the authors could demonstrate microbial activity (based on RNA analysis) of the respective strains along the sediment depth of 0-15 mm (Otte et al. 2018). Even when no signal of the strains could be detected under particular substrate conditions (i.e., Fe(II)), *Rhodopseudomonas* sp. grew up and dominated the co-culture as soon as transferred to optimum growth conditions (i.e., acetate) (Figures 4–6).

As *Chlorobium* sp. does not grow on acetate (Tables 2 and 3), the co-culture was quickly dominated by *Rhodopseudomonas* sp. when transferred to acetate, independent of pre-culture conditions (Figures 4–6). In contrast, *Chlorobium* sp. dominated the co-culture that was transferred to H_2 in case the pre-co-culture was grown on Fe(II). This pre-co-culture was already dominated by *Chlorobium* sp. with *Rhodopseudomonas* sp. being below detection limit. Although *Rhodopseudomonas* sp. is able to grow on H_2 (Table 3) the *Rhodopseudomonas* sp. cell numbers were below detection in most setups (spectrophotometric measurements based on typical absorption peaks, Fig. 2) and only grew to higher cell numbers with longer incubation duration (78 days, Figure 4). Since the isolated *Rhodopseudomonas* sp. strain is not able to grow on Fe(II) (Table 3), the co-culture was dominated by *Chlorobium* sp. (*Rhodopseudomonas* sp. below detection) when transferred to Fe(II) (Figures 4–6). Based on these data we can define a substrate-based niche for each strain in the co-culture. In comparison to the highly metabolically flexible *Rhodopseudomonas* sp., the *Chlorobium* sp. can occupy niches that are rich in Fe(II) or H_2. *Rhodopseudomonas* sp. grew best under photoheterotrophic conditions (i.e., with acetate) or photoautotrophically on H_2. In natural sediments

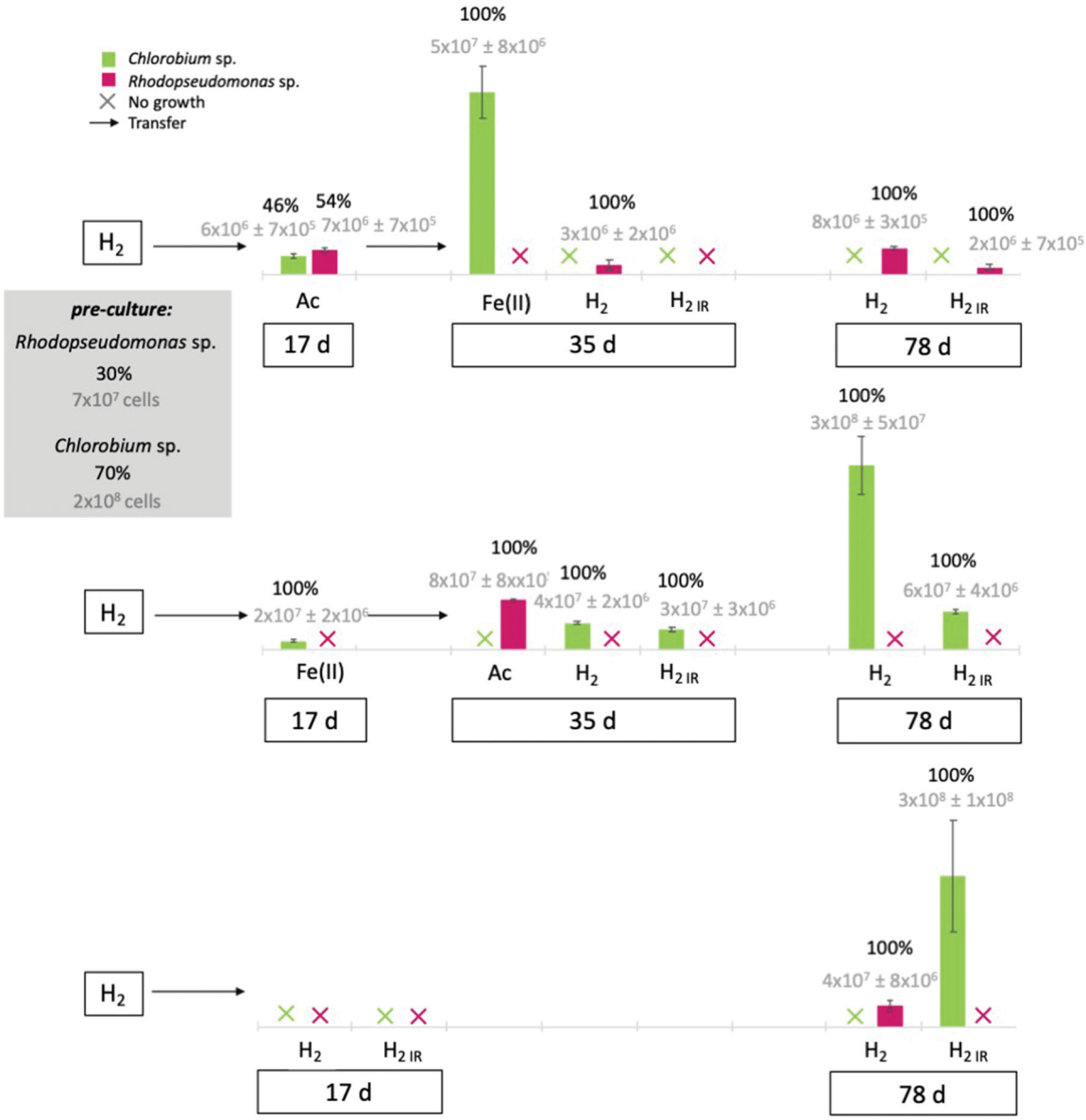

Figure 6. Microbial distribution pattern within the co-culture as a function of substrate availability (percentage values based on spectrophotometrically measured abundance). Displayed numbers represent quantification of *Rhodopseudomonas* sp. and *Chlorobium* sp. based on their specific absorption spectra (unit: cells/mL). The pre-co-culture was grown on H2 before transferring to the chosen variety of substrates (Ac: acetate; Fe: Fe(II); H2: hydrogen; H2 IR: hydrogen at IR light). The pre-culture was transferred 3 times on the initial substrate (H2) and grown for 4 weeks before transferring to Fe(II), H2 or acetate. If not stated differently incubations have been performed at full light conditions. The transfer cultures onto H2 were not further transferred, but let stand for longer time. Data present average value from triplicate setups (standard deviation). Percentage correlates to detectable signal. (BDL - below detection limit).

low-molecular-weight organic compounds such as acetate are produced during fermentation. Similarly, H_2 is potentially enriched in the top sediment layers, as it is produced during both photosynthesis and fermentation. *Rhodopseudomonas* sp. might therefore occupy niches that are located closest to the sediment-water interface. However, its high metabolic flexibility might also support survival in neighboring niches as well. Given that *Chlorobium* sp. grew best under Fe(II)- and H_2-oxidizing conditions, it can be expected to find its niche closest to a source of Fe(II) in the sediments, potentially along the diffusion gradient of Fe(II) resulting from the Fe(III) reduction zone located in deeper anoxic sediment layers (Schmidt et al. 2010) or closer to the sediment-water interface where Fe(II) is produced during Fe(III) photoreduction and diffuses downwards into the sediment (Lueder et al. 2020). This could also be a suitable habitat leading to cryptic Fe-cycling with phototrophic Fe(II)-oxidizers coupled to photoreduction (Peng et al. 2019).

Since *Rhodopseudomonas* sp. and *Chlorobium* sp. are both phototrophs, their niche is not only defined by the required substrate, but also by light intensity and quality (wavelength). When entering a water body or sediment, light splits into separate wavelengths, which are sequentially absorbed and therefore create individual niches for phototrophic life (e.g., Stomp et al. 2007; Vila and Abella 1994). In contrast to the water column, where short energy-rich wavelengths (blue) are absorbed last (i.e., penetrate deepest), short wavelengths are absorbed

first in sedimentary systems (Kühl et al. 1994; Pierson et al. 1990). Our *Rhodopseudomonas* sp. grew best in red and green light, whereas growth was hardly detectable under IR light conditions (Figure S6). Compared to the *Chlorobium* sp. strain in the co-culture, *Rhodopseudomonas* sp. preferred higher light intensities (7.4 µmol/m^2 s, Figure S6, Table 1). These data suggest that phototrophic growth of *Rhodopseudomonas* sp. would be favored in the top sediment layers, the same location where substrate availability (H_2, acetate) creates a favorable niche. Previous studies even reported that *Rhodopseudomonas* sp. is able to grow microaerobically in light, as well as aerobically in dark (Larimer et al. 2004; Oda et al. 2008); conditions that are again found closest to the sediment-water interface. In contrast, *Chlorobium* sp. grew well at highest and low light intensities (range $1.5 - 10.7$ µmol/m^2 s, Figure S6, Table 1), supporting the hypothesis that *Chlorobium* sp. might occupy a niche that is distinct from *Rhodopseudomonas* sp. The absorption peaks in the *Chlorobium* sp. enriched co-culture matched with the absorption peak at 460 nm for chlorobactene (Hegler et al. 2008), which was also identified by UHPLC QToF-ESI/MS analysis (Figures S7 and S8). Minor amounts of ubiquinones could be detected, confirming the presence of phototrophic purple bacteria (*Rhodopseudomonas* sp.) in the co-culture. The absorption pattern (spectrophotometric analyses) of our co-culture clearly distinguishes *Chlorobium* sp. from *Rhodopseudomonas* sp. (Figure 2), suggesting that each of them will be able to occupy its individual niche. *Rhodopseudomonas palustris* BisA53, the strain our isolate is most closely related to, has a unique absorption spectrum compared to other *Rhodopseudomonas* strains (Table 1), and as such is expected to have a competitive advantage (Oda et al. 2008). It carries a number of photoreceptors, which allow a response to changes in light intensity and quality (Larimer et al. 2004; Oda et al. 2008). In our co-culture, however, growth of *Rhodopseudomonas* sp. was outcompeted by *Chlorobium* sp. under IR light and Fe(II) (or H_2) substrate conditions (Figure S6, Figures 4–6).

Based on their substrate needs, the two strains of our co-culture, *Rhodopseudomonas* sp. and *Chlorobium* sp., find their individual niche close to each other. The strains might spatially co-exist although occupying and dominating individual substrate-based niches. Our data nicely show how the phototrophic co-culture responds to substrate fluctuations by decreasing or increasing the population density of the individual strains (Figures 4–6). Such substrate fluctuations occur constantly in natural sediments, where day-night cycles, or restructuring as a consequence of wave movement or animal activity (bioturbation) control the flux of nutrients and substrates for microbial growth. Geochemical gradients will create temporarily individual niches that offer optimal survival conditions for particular microorganisms and might influence their competitive advantage over other strains. However, the fact that certain organisms can switch between their metabolic mode releases competition pressure for substrate and potentially enforces microbial co-existence, instead of competition (Massé et al. 2002).

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