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Vertical distribution of planktonic autotrophic thiobacilli and dark CO_2 fixation rates in lakes with oxygen–sulfide interfaces

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ABSTRACT: Vertical distributions of viable (most probable number, MPN) aerobic chemoautotrophic thiobacilli-like sulfur-oxidizing bacteria (ca. 70 samples in triplicate for MPN counts) and dark ^{14}C-bicarbonate incorporation rates were analyzed in a series of sulfide-rich lakes. A special device for sampling sharply stratified populations on the scale of a few centimeters was used. Detailed analyses focused on the oxic-anoxic transition zone where aerobic sulfur-oxidizing bacteria should display positive chemotaxis, and in both fully oxic epilimnia and sulfide-rich anoxic hypolimnia. Kinetics of sulfide and thiosulfate potential oxidations in the presence of oxygen were followed in microcosm enrichments in one of the lakes. The highest MPN counts (>10^4 to 10^5 cells ml^{-1}) were observed at the oxic-anoxic interfaces and in the depleted hypolimnia (1.3 ± 4.4 × 10^4 cells ml^{-1}), whereas 1 order of magnitude lower concentrations were detected in the epilimnia (1.0 ± 2.3× 10^3 cells ml^{-1}). Dark ^{14}C-bicarbonate incorporation rates were higher at the oxic-anoxic interface (11.4 ± 9.5 µg C l^{-1} h^{-1}) than in the hypolimnia (6.4 ± 5.9 µg C l^{-1} h^{-1}) and epilimnia (1.0 ± 2.1 µg C l^{-1} h^{-1}). A lack of correspondence between abundance of MPN thiobacilli, location at the sulfide interface, and dark carbon fixation rates was, however, consistently observed in a correlation analysis. Patterns of *in situ* potential aerobic thiosulfate oxidation did not match dark carbon fixation rates or MPN vertical distributions. The chemoautotrophic guild of these lakes emerged as a metabolically complex, taxonomically diverse group of aerobic, microaerophilic, and anaerobic microorganisms coexisting in the same lake. Thiobacilli may actively fix CO_2 at certain depths but the question of which types of bacteria contribute most to dark CO_2 fixation in the investigated lakes is still open, and the application of culture-independent molecular tools and single-cell analyses should be used to substantiate and further explore these findings.

KEY WORDS: Thiobacilli · Karstic lake · Sulfide · Oxic-anoxic interface · Most probable number · Dark CO_2 fixation

INTRODUCTION

Chemolithoautotrophic bacteria oxidize reduced inorganic compounds to obtain both energy and reducing power for fixing inorganic carbon in the dark. Usually they need to simultaneously combine reduced compounds with strong oxidants such as oxygen and nitrate. In stratified aquatic ecosystems with oxygen–sulfide interfaces, aerobic thiobacilli-like sulfur-oxidizing chemolithoautotrophic bacteria display positive chemotaxis towards the oxic-anoxic transition zone where sulfide, thiosulfate, and other reduced sulfur compounds coexist with oxygen (Sorokin 1972). Physiology and ecology of thiobacilli-like bacteria have been extensively studied in benthic ecosystems, and a large body of knowledge exists (Jørgensen 1982, Kuenen et al. 1992, Visscher et al. 1992, Van den Ende et al. 1996, Brinkhoff et al. 1999 and references therein, Jonkers et al. 2003). In the redoxclines of marine waters (the redox gradient around the oxic-anoxic interface), chemoautotrophy can be a significant midwater source of organic carbon (Detmer et al. 1993,

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Casamayor et al. 2001a, Taylor et al. 2001). In acidic industrial and natural environments, thiobacilli-like bacteria are important microorganisms associated with bioleaching processes (Demergasso et al. 2005). Thiobacilli in the plankton of stratified freshwater lakes, in turn, have not been so well studied; only very few data are available on their vertical distribution in the water column (Gorlenko et al. 1983, Sattley & Madigan 2006), and it is not well known whether or not the significance of thiobacillus-like bacteria in the biogeochemistry of the plankton is equivalent to their benthic counterparts.

Stratified aquatic ecosystems with oxygen–sulfide interfaces are more widespread than commonly realized. They can be found in microbial mats, fjords, stagnant marine basins, fjord-like embayments, coastal lagoons, estuaries, lakes and reservoirs, eutrophic shallow forest ponds, and sediments in flooded areas, among other areas (Gorlenko et al. 1983). Therefore, they appear not only in marine environments but also in a substantial amount of freshwater sites. Expected climatic scenarios of increasing water temperature, rising of the sea level, and larger marine intrusions would strengthen stratification processes and likely increase oxic-anoxic interfaces worldwide. At the oxic-anoxic interface, microbial populations are finely adapted to vertical physico-chemical gradients (light, oxygen, sulfide, and other reduced inorganic compounds) and this favors the coexistence of different autotrophic metabolisms through the water column. The importance of dark carbon fixation in stratified aquatic ecosystems has been shown not only for the oxic-anoxic interfaces, but also for anoxic waters in lakes (Culver & Brunskill 1969, Jørgensen et al. 1979, García-Cantizano et al. 2005 and references therein) and seas (Tuttle & Jannasch 1979, Juniper & Brinkhurst 1986, Jørgensen et al. 1991, Jost et al. 2008).

In karstic lakes rich in sulfide, most of the CO_2 incorporation can be related to dark incorporation processes (58% of total annual fixed carbon in Lake Cisó, Banyoles, Spain), making dark carbon incorporation one of the key processes in the autotrophic metabolism of this type of lake (García-Cantizano et al. 2005). In the metalimnia of other studied lakes and salt wedge estuaries, carbon fixation is also a relevant process (Gorlenko et al. 1983, Camacho et al. 2001, Casamayor et al. 2001a, 2008). The composition of the microbial guild responsible for dark incorporation in these environments, however, has mostly remained unknown. Recently, unexpected players such as photosynthetic sulfur bacteria have been identified (Casamayor et al. 2008). The present study explored, using a large set of data and correlational analysis, whether the high rates of dark carbon incorporation measured *in situ* could be related to the abundance of the viable cells with the most likely metabolism (aerobic oxidizers of reduced sulfur compounds) determined by most probable number (MPN) counts in a specific growth medium. In addition, the present study has one of the first data sets showing detailed vertical abundance and distribution of thiobacilli-like bacteria in the plankton of karstic lakes and a coastal lagoon rich in sulfide.

MATERIALS AND METHODS

Studied systems. Four karstic lakes (Banyoles, Vilar, Cisó, and Estanya) and a coastal lagoon (La Massona) located in NE Spain were studied. Three independent basins were studied in Lake Banyoles (Basins III, IV, and VI). A detailed location map and bathymetries can be found in Guerrero et al. (1987). These stratified aquatic ecosystems have bottom waters rich in sulfate with high sulfide concentrations, and oxic-anoxic interfaces located in the water column. The complete set of environments was sampled along the vertical gradient and incubations for carbon fixation were carried out covering the noon period (maximal irradiance for these lakes) (see Table 1). A marine benthic sample from an extensively studied laminated microbial ecosystem (microbial mat) located in the supralittoral of the Ebro River Delta (Mir et al. 1991) was added in the MPN analysis to test the performance of the analyses. MPNs were evaluated after slicing, sonication, and serial dilution (1:10 steps) of the first 5 cm of a sediment sample, and correcting salinity in the incubation medium with a concentrated NaCl solution. Results are shown in Appendix 1 (Table A1).

Lakes Banyoles, Vilar, and Cisó are in the Banyoles karstic area (42° 8′ N, 2° 45′ E) and the microbial communities inhabiting these systems have been extensively studied (for a review see Pedrós-Alió & Guerrero 1993 and references therein). Lake Banyoles is a gypsum karst spring area constituted by 6 main basins covering a surface area of 1.1 km^2. The 3 basins studied here are located in the northern area of the lake. Basins III and IV are meromictic with 25 and 18 m maximal depth, respectively, and incoming water seeps through bottom springs. The redoxcline oscillates between 16 and 21 m for Basin III and between 12 and 17 m for Basin IV, depending on the season. Basin VI is 17 m deep and holomictic, with an interface situated around 12 to 13 m depth, and does not have bottom seepage. Blooms of the photosynthetic bacteria *Chlorobium phaeobacteroides* and *Thiocystis minor* (formerly *Chromatium minus*) are present in the 3 basins. Lake Vilar is a meromictic lake formed by 2 basins with a maximum depth of 9 m and a surface area of 11 000 m^2. High sulfide concentrations are found during the entire year, although sulfide is restricted to the deeper, high-

conductivity waters. The oxic-anoxic interface is found at around 4 to 6 m depth, where dense populations of *T. minor* and *Chlorobium phaeobacteroides* develop. Lake Cisó is a small monomictic lake (650 m^2), 1 km away from Lake Vilar, with a maximum depth of 6.5 m. The thermocline is at 1.5 m, where dense populations of the photosynthetic sulfur bacteria *Chromatium* spp. and *Amoebobacter* sp. develop. The lake becomes anoxic during winter holomixis (complete mixing) and high sulfide concentrations (up to 500 µmol l^{-1}) are present in the entire water column, even reaching the surface (Pedrós-Alió & Guerrero 1993).

Lake Estanya (42° 02′ N, 0° 32′ E) is a sulfide-rich holomictic lake (maximal H_2S concentrations are around 600 µmol l^{-1}), located at 670 m above sea level in the Pre-Pyrenees area, 10 km SE of the town of Benabarre, Huesca. It is constituted by 2 basins of 12 and 22 m maximal depth, connected by a shallow sill subaerially exposed during summer. The lower water masses are rich in dissolved sulfate and carbonates brought by subsurface groundwater discharge. Here the deepest basin was sampled (Lake Grande de Estanya, SW position) with a thermocline located between 12 and 14 m. Only a few microbiological studies from this lake have been published (Ferrera et al. 2004 and references therein, Casamayor et al. 2008, Martínez-Alonso et al. 2008), reporting blooms of the purple sulfur bacteria (PSB) *Chromatium okenii* and *Thiocystis minor* at the light-sulfide interface.

Finally, La Massona is a meromictic coastal lagoon located on the southern part of the Bay of Roses (42° 13′ N, 3° 08′ E), within the protected marsh area of Aiguamolls de l'Emporda Natural Park. The lagoon is located between the mouths of the rivers Muga and Fluvià. It has a conical part separated from the sea by a 150 m wide sand bar, and an elongated part inland, connected to River Fluvià through a freshwater channel. Average depth is 1.5 m and the maximal depth of 10.5 m is found in the conical part, where a redoxcline separates freshwater from saltwater between 3.5 m and 6 m depending on the season. The upper part shows an intense algal development whereas high sulfide concentrations are present in the bottom waters. Green sulfur bacteria are abundant whereas PSB have never been described as quantitatively important.

Sampling and analyses. Water temperature and conductivity were measured *in situ* using a submersible probe (YSI-33 S-TS; Yellow Springs Instruments). Samples for biological and chemical analyses were taken from different depths using a battery-driven pump connected with tubing to a cone-shaped polyvinyl chloride laminar sampling structure (Miracle et al. 1992) which minimizes both turbulence and disturbance of the fine layering of microbial populations. For sulfide measurements, 10 ml subsamples were first alkalinized by adding 100 µl of 10 mol l^{-1} NaOH and then chemically fixed by addition of Zn acetate to a final concentration of 0.1 mol l^{-1}, and oxygen was measured with the Winkler titration method modified for oxic-anoxic interfaces as previously reported (García-Cantizano et al. 2005). Thiosulfate was analyzed spectrophotometrically by the cyanolytical method of Kelly et al. (1969).

For total cell counts, 10 ml subsamples were fixed by addition of formaldehyde to a final concentration of 4% (vol/vol). Counting of DAPI-stained cells was done using an epifluorescence Olympus BH microscope. Standard deviation was below 10% of cell counts. Morphologically distinguishable phototrophic bacteria (e.g. *Chromatium* spp., *Amoebobacter* sp.) and the algae *Cryptomonas* sp. were identified by microscopy, and some taxonomically valuable characteristics, such as motility and the presence of gas vesicles, were observed under phase contrast with live samples when needed (Casamayor et al. 2000). Chlorophyll *a* (chl *a*) and bacteriochlorophyll *a* (Bchl *a*) were determined spectrophotometrically (as reported in Casamayor et al. 2001a) on samples filtered through membrane filters (Sartorius 0.45 µm) and extracted overnight in 90% acetone (saturated with magnesium carbonate).

Aerobic chemolithoautotrophic sulfur bacteria (thiobacillus-like) were enumerated using the MPN technique in a selective medium containing carbonate (19 mmol l^{-1}) as the sole carbon source, thiosulfate (10 mmol l^{-1}) as the sole electron donor, and bromocresol blue to indicate acidification of the medium (Casamayor et al. 2001a). Incubations were carried out in the dark, at room temperature, and aseptically exposed to air oxygen for a period of up to 12 wk in 3 replicates. Samples were scored as positive when acidification occurred (color change of the pH indicator). The highest positive dilutions were checked microscopically by epifluorescence microscopy. In addition, some of these were further examined by scanning electron microscopy (SEM). SEM samples were filtered on 0.2 µm pore size polycarbonate filters, fixed with 2% buffered glutaraldehyde, and processed with ethyl alcohol dehydration and critical point drying (Paerl & Shimp 1973). Small pieces of the filters were mounted on SEM stubs coated with a gold-palladium layer, and viewed with a Hitachi S-570 SEM at accelerating voltage of 15 kV.

CO_2 incorporation experiments. CO_2 incorporation was estimated as previously described (García-Cantizano et al. 2005, Casamayor et al. 2008). Incubations were carried out using 22 ml screw-capped tubes where $NaH^{14}CO_3$ was added at a final concentration of 0.25 µCi ml^{-1}. Four different treatments were done with replicates (2 replicate tubes $treatment^{-1}$). The standard error of the mean between replicates was

<10%. In the first treatment, formaldehyde (4%, vol/vol, final concentration) was added to the samples to correct for abiotic incorporation. A second set of samples, incubated in the dark, allowed the estimation of light-independent CO_2 fixation. To a third set of samples incubated in the light, 3-(3,4-dichlorophenul)-1,1-dimethylurea (DCMU) was added at a final concentration of 2 µmol l^{-1}. DCMU inhibits photosystem II and, thus, photosynthesis by algae. Finally, non-treated samples were also incubated in the light. Incubations were carried out for a period of 4 h at the same depths as those from which the samples had been taken. Sample processing and carbon uptake rates were calculated as previously reported (García-Cantizano et al. 2005, Casamayor et al. 2008).

Sulfide and thiosulfate oxidation experiments. The potential rate of aerobic oxidation of reduced sulfur compounds was measured in freshly collected samples incubated at room temperature in the dark in 2 l glass Erlenmeyer flasks stirred with a magnetic bar to allow air diffusion under sterile conditions. Sulfide chemically reacts with oxygen and, therefore, formaldehyde-fixed controls (0.6% final concentration vol/vol) were run in parallel for abiotic consumption. Formaldehyde, however, showed strong interferences with the chemical oxidation of sulfide (Appendix 1, Fig. A1). Samples and controls for the sulfide oxidation experiment were supplemented using a neutralized stock solution of calcium carbonate and sodium sulfide (Casamayor et al. 2007). Thiosulfate is chemically stable in the presence of oxygen and is predominantly consumed by biotic processes, and dead controls showed no consumption of thiosulfate (data not shown). Thiosulfate was added (0.7 mM final concentration from a sterile concentrated solution of $Na_2S_2O_3$) on freshly collected samples previously treated with sterile (0.2 µm filtered) nitrogen gas for 1 h to get rid of the dissolved sulfide initially present. Thiosulfate is the product of a spontaneous chemical reaction between sulfide and oxygen, and is normally found at the oxic-anoxic interfaces (Chen & Morris 1972).

RESULTS

All lakes studied showed well-defined oxygen–sulfide interfaces at the time of sampling. Lake Cisó, for example, had a thermocline at ca. 1.5 m depth in summer (Fig. 1A), and the oxic-anoxic interface moves to the top of the lake during winter holomixis (Table 1). La Massona coastal lagoon showed a redoxcline at ca. 5 m depth and a deep layer of anoxic saline water (Table 1). The epilimnia of the lakes were in general well oxygenated without traces of reduced sulfur com-

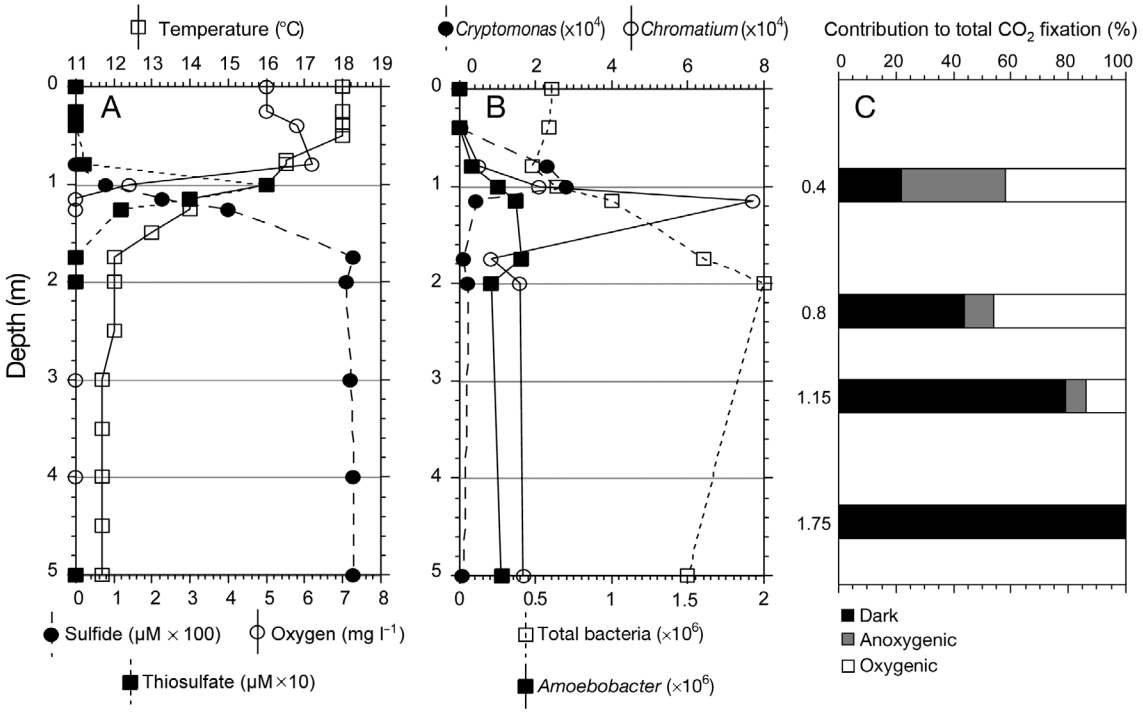

Fig. 1. Physico-chemical and biological profiles of Lake Cisó on June 11, 1991. (A) Thermal stratification and vertical distribution of oxygen, sulfide, and thiosulfate, (B) total bacteria, the algae *Cryptomonas* spp., and the anoxygenic photosynthetic sulfur bacteria *Amoebobacter* sp. and *Chromatium* sp. (all in cells ml^{-1}), and (C) relative contributions to oxygenic photosynthesis, anoxygenic photosynthesis, and dark fixation to total microbial CO_2 incorporation. Absolute values for total CO_2 incorporation were 0.36 (0.40 m), 14.09 (0.80 m), 2.71 (1.15 m), and 1.49 (1.75 m) µg C l^{-1} h^{-1}

Table 1. Temperature, conductivity, oxygen, sulfide, and pigments (chl *a* and bacteriochorophyll [Bchl] *a*) for the depths selected for most probable number (MPN) incubations (aerobic sulfur-oxidizing thiobacilli-like bacteria [Aer. sulf. ox.]) in the complete set of lakes studied

Lake and date (dd/mm/yy)	Depth (m)	Temp. (°C)	Conductivity (µS cm^{-1})	O_2 (µM)	H_2S (µM)	Chl *a* (µg l^{-1})	Bchl *a* (µg l^{-1})	Aer. sulf. ox. (cells ml^{-1})
Cisó	0.00	12.0	1500	40.6	752	180	73.3	7330
(13/11/90)	0.25	12.0	1500	0.0	799	184	74.7	4590
	0.50	11.8	1525	0.0	787	215	87.3	733
	0.75	11.8	1525	0.0	794	183	75.5	11500
	2.00	11.3	1550	0.0	795	176	72.3	1050
	5.00	11.6	1590	0.0	809	86	29.7	45900
Cisó	0.00	8.0	1250	96.9	90	11	8.2	10500
(05/02/91)	0.20	7.6	1250	0.0	159	95	201.3	733
	0.40	7.0	1250	0.0	197	102	213.2	733
	0.60	6.0	1250	0.0	206	104	176.5	212
	1.00	6.0	1250	0.0	204	102	189.9	1150
	4.00	6.0	1250	0.0	206	107	187.6	212
Cisó	0.40	18.0	1270	181.2	0	6	1.5	2100
(11/06/91)	0.80	17.0	1200	193.7	0	261	22.8	195
	1.00	16.0	1200	43.7	81	342	58.5	17300
	1.15	14.0	1200	0.0	227	179	148.7	621
	1.75	12.0	1150	0.0	728	170	102.4	764
	2.00	12.0	1150	0.0	710	128	79.1	5690
	5.00	11.7	1200	0.0	730	125	80.0	73300
Vilar	0.00	14.0	940	375.0	0	7	0.0	115
(03/04/91)	1.00	13.8	965	375.0	0	7	0.0	46
	3.00	13.1	1032	375.5	0	6	0.0	115
	4.50	13.6	1073	387.5	0	13	0.2	46
	7.00	13.7	1820	40.6	1	5	0.1	15
	8.00	14.1	1860	31.2	2	4	0.3	18
	9.00	16.8	1558	15.6	10	4	0.3	733
Vilar	2.00	25.0	1005	259.4	0	12	0.0	0
(19/09/91)	5.50	23.5	1100	53.1	0	48	315.2	0
	7.50	19.8	1450	0.0	683	13	11.8	0
	9.00	17.5	1700	0.0	2251	12	8.0	0
	9.50	17.3	1740	0.0	2625	18	14.3	73
Banyoles-III	1.00	17.0	1242	165.6	0	2	0.0	129
(29/10/91)	11.50	14.5	1320	221.9	0	3	0.0	102
	12.50	13.0	1340	171.9	0	2	0.0	133
	15.00	12.1	1357	62.5	0	2	0.0	116
	17.00	12.0	1352	43.7	9	4	2.5	0
	18.50	12.2	1449	28.1	18	3	1.7	0
	19.50	13.1	1968	0.0	362	3	0.2	571
	20.00	13.5	2330	0.0	380	1	0.0	46
	22.00	13.6	2350	0.0	423	1	0.0	71
Banyoles-IV	12.00	16.8	1969	34.4	0	7	0.0	102
(19/09/91)	12.50	16.4	2160	28.1	0	5	0.0	733
	13.00	16.3	2356	0.0	0	2	0.0	173
	13.50	16.3	2476	0.0	0	1	1.7	102
	14.00	16.3	2476	0.0	29	3	13.0	105
	16.00	16.3	2585	0.0	67	1	2.5	713
Banyoles-VI	9.00	21.0	1283	206.2	0	4	0.0	4590
(03/10/91)	10.00	17.4	1417	140.6	16	4	0.0	73300
	11.00	15.9	1482	62.5	4	5	0.0	45900
	12.00	14.5	1541	43.7	4	4	0.0	7690
	13.00	13.8	1571	34.4	0	6	0.0	13700
	14.00	13.4	1589	34.4	35	8	3.8	250000

Table 1. continued

Lake and date (dd/mm/yy)	Depth (m)	Temp. (°C)	Conductivity (µS cm^{-1})	O_2 (µM)	H_2S (µM)	Chl *a* (µg l^{-1})	Bchl *a* (µg l^{-1})	Aer. sulf. ox. (cells ml^{-1})
Estanya	1.00	17.2	1900	250.0	0	2	0.0	0
(15/10/91)	10.00	17.2	1950	278.1	0	3	0.4	582
	11.00	14.0	1750	78.1	0	17	0.2	99
	11.50	11.8	1650	9.4	0	18	2.9	37
	12.00	10.2	1550	3.1	23	29	581.4	36
	12.50	9.6	1500	0.0	195	8	78.9	210
	15.00	8.5	1500	0.0	584	4	17.6	18
Massona	0.50	21.0	1800	300.0	0	19	0.0	73
(28/05/91)	3.00	18.0	3200	168.7	0	26	0.0	459
	4.00	16.0	5800	84.4	0	30	0.0	105
	4.50	14.0	10000	46.9	0	8	0.0	4590
	5.00	13.5	15000	46.9	0	10	0.9	46
	5.50	12.5	23500	25.0	19	56	7.6	18
	9.00	12.0	24000	0.0	343	4	0.5	18
Massona	5.50	23.1	5000	68.7	0	45	1.0	10200
(25/09/91)	6.25	21.1	17000	0.0	237	12	1.0	370
	7.00	17.0	21700	0.0	338	8	1.3	1160

pounds, whereas the hypolimnia were anaerobic with high concentrations of sulfide (up to 2.6 mM in Lake Vilar; Table 1). The metalimnia showed opposite gradients of oxygen and reduced sulfur compounds with concentrations usually of a few µM of sulfide. Natural concentrations of thiosulfate were measured in summer in Lake Cisó; the highest concentrations (50 µM at 1.00 m depth) were found at the metalimnion, and at the sulfide-rich hypolimnion the concentration was 0 µM (Fig. 1A). The complete set of data showing the abundance and distribution of thiobacilli-like bacteria in the plankton of the sulfide-rich karstic lakes and coastal lagoon analyzed is shown in Table 1. MPN ranged widely among lakes. Some of them, such as lakes Cisó and Banyoles-VI, had large numbers ranging between 10^3 and 10^5 cells ml^{-1}. The highest numbers found were 2.5×10^5 viable cells ml^{-1} in Lake Banyoles-VI. Other systems, such as lakes Vilar, Estanya, Banyoles-III, and Banyoles-IV, had low abundances ranging between undetectable and 10^2 viable cells ml^{-1}. Finally, La Massona had both low and high numbers on different dates.

An example of the vertical distribution of microorganisms is shown in Fig. 1B. Both the algae *Cryptomonas* sp. and the PSB *Amoebobacter* sp.- and *Chromatium* spp.-like bacteria accumulated at the metalimnion. Thiobacilli-like MPN showed maximal concentrations both at 1.00 m depth (1.7×10^4 cells ml^{-1}, where thiosulfate had the highest concentration) and in the fully anoxic bottom (7.3×10^4 cells ml^{-1} at 5.00 m, where sulfide had the highest concentration) (see Table 1). Most of the morphologies observed by SEM were large bacilli and curved bacilli, and microscopic inspections of the highest positive dilutions ruled out that PSB were present in the MPN estimations (Fig. 2; inoculation from Lake Cisó on June 11, 1.0 m depth, after 12 wk incubation). Additional data for chl *a* of oxygenic and Bchl *a* of anoxygenic phototrophs (PSB) are shown in Table 1 for those depths where MPN were also evaluated.

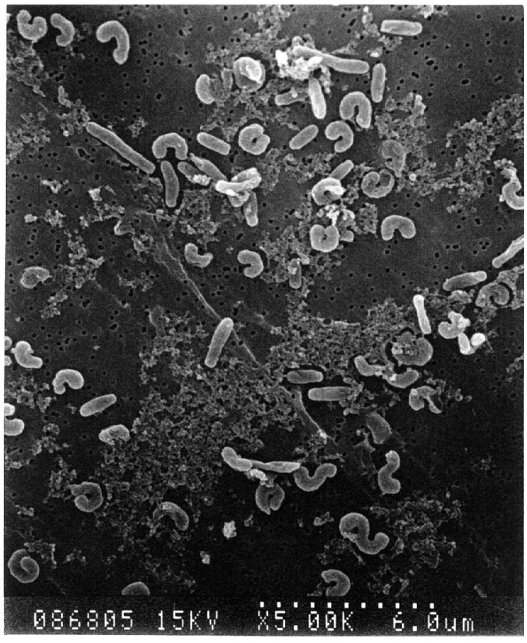

Fig. 2. Scanning electron microscope (SEM) of a most probable number (MPN) enrichment obtained from the oxic-anoxic interface of Lake Cisó (June 11, 1991; depth 1.0 m) after 12 wk laboratory incubation in the dark. See scale bar (dotted line) in bottom right-hand corner

Four depths were selected in Lake Cisó in summer for incubations with ^{14}C-bicarbonate to cover the metalimnion in detail, assuming most activity of thiobacilli-like bacteria would be concentrated at the oxic-anoxic interface (Fig. 1C). A fine segregation in the relative contributions of oxygenic, anoxygenic, and dark fixation processes was observed within a few centimeters, although the 3 processes co-occurred for most of the depths. Curiously, this analysis showed that dark fixation was quantitatively more important than anoxygenic photosynthesis. However, at the place of the highest fixation rate (14.09 µg C l^{-1} h^{-1} at 0.80 m) with up to 40% conducted for dark processes, the MPN showed the lowest concentrations of thiobacilli (1.9×10^2 cells ml^{-1}). Below this depth dark processes were dominant in relative terms (but at lower rates). Other investigated lakes (e.g. Vilar and Estanya) and data in the literature had also shown a peak of dark carbon fixation rates in the chemocline (Gorlenko et al. 1983, Camacho et al. 2001 and references therein).

These results in Lake Cisó suggested what was further confirmed when the complete set of lakes was analyzed. (1) The highest MPN counts detected (i.e. $>10^4$ to 10^5 cells ml^{-1}) were observed in samples with both very low or even zero sulfide concentrations (close to the redoxcline) but also with very high natural sulfide values close to 1 mM (Fig. 3A). (2) The highest rates of dark carbon fixation did not match the highest abundances of thiobacilli (Fig. 3B; R^2 = 0.013, best curve fit). More details of carbon fixation by photosynthetic bacteria in Lake Cisó have been published elsewhere (García-Cantizano et al. 2005, Casamayor et al. 2008).

Overall, the vertical distribution of thiobacilli within systems was quite variable. Initially, MPNs were expected to be higher at the oxic-anoxic interfaces. When the whole distribution of thiobacilli among systems was normalized to the distance to the oxygen–sulfide interface, the highest variability was observed at the different hypoliminia and metalimnia, and the lowest at the epiliminia (Fig. 4). Overall, higher MPN counts were observed both at the oxic-anoxic interfaces (median 1.3 ± 2.5 × 10^4 cells ml^{-1}) and in the oxygen-depleted hypolimnia (median 1.3 ± 4.4 × 10^4 cells ml^{-1}) than in the fully oxic epilimnia (median 1.0 ± 2.3 × 10^3 cells ml^{-1}). In parallel, dark ^{14}C-bicarbonate incorporation rates were higher in the oxic-anoxic interface (11.4 ± 9.5 µg C l^{-1} h^{-1}) than in the hypolimnia (6.4 ± 5.9 µg C l^{-1} h^{-1}) and epilimnia (1.0 ± 2.1 µg C l^{-1} h^{-1}) (n = 34, set of data available in Table 1 in Casamayor et al. 2008). A lack of correspondence between the abundance of MPN thiobacilli and dark carbon fixation rates for the whole data set was observed (p = 0.060).

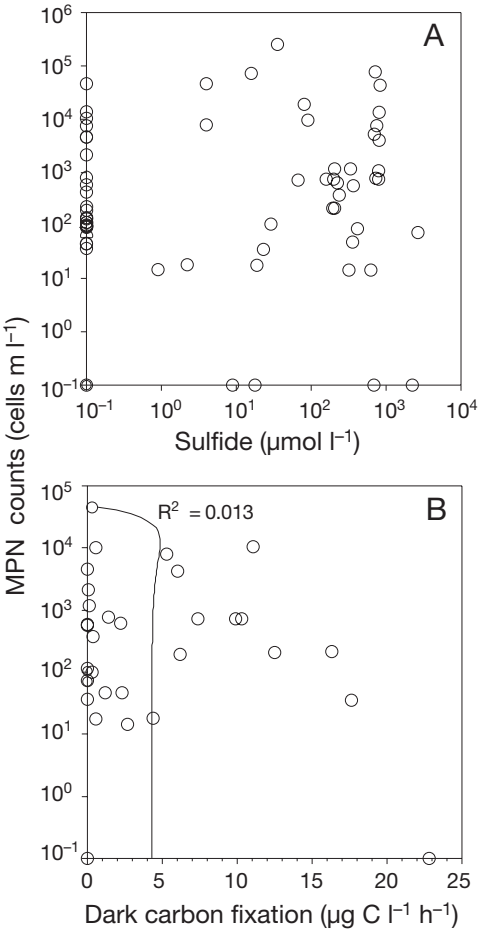

Fig. 3. Distribution of most probable number (MPN) along a gradient of (A) sulfide and (B) dark carbon fixation (best curve fit shown) in all lakes studied. Sulfide concentrations <1 µM are below detection limits and are presented on the *y*-axis. *In situ* dark carbon fixation incubations were carried out for approximately half of the total MPN samples

The potential rates of aerobic oxidation of reduced sulfur compounds in the dark were measured in the laboratory with freshly collected samples from Lake Cisó in duplicate. Initially, kinetics of aerobic sulfide oxidation showed consistent differences between fresh samples (biotic plus chemical oxidation) and a formaldehyde-fixed (0.6% vol/vol) control that apparently measured the chemical oxidation (Fig. A1). However, additional controls with sterile ultrapure Milli-Q water showed strong interferences of formaldehyde with the spontaneous chemical oxidation of sulfide. These experiments were therefore rejected. The experiments were repeated, adding thiosulfate along the vertical profile of Lake Cisó on June 11 for 6 selected depths located at the epi-, meta-, and hypolimnion, respectively (Fig. 5). Consumption rates ranged between 6 and 43 µM h^{-1} (calculated from beginning to end incubation times) with the highest rates at 1.15, 1.00, and

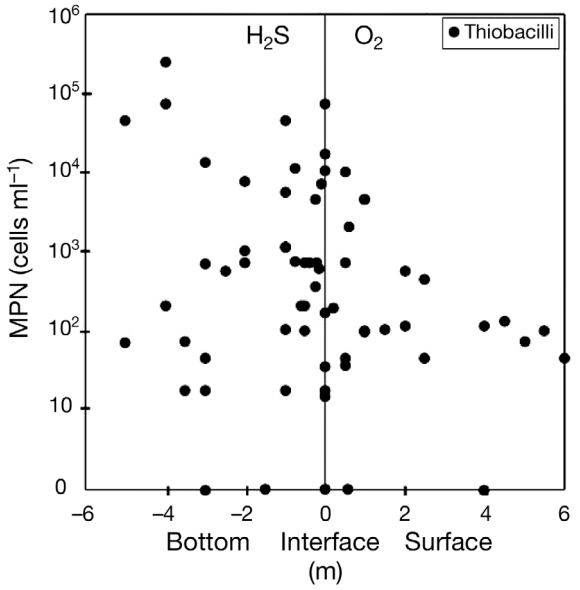

Fig. 4. Normalized positioning of chemoautotrophic thiobacilli vs. the oxygen–sulfide interface (relative depth = 0) in all lakes studied. MPN: most probable number

5.00 m, respectively. Vertical patterns in potential aerobic thiosulfate oxidation did not match dark carbon fixation rates ($p = 0.099$) or MPN distributions ($p = 0.242$) measured for the same samples, but were significantly related to the *in situ* abundance of photosynthetic PSB ($p = 0.006$).

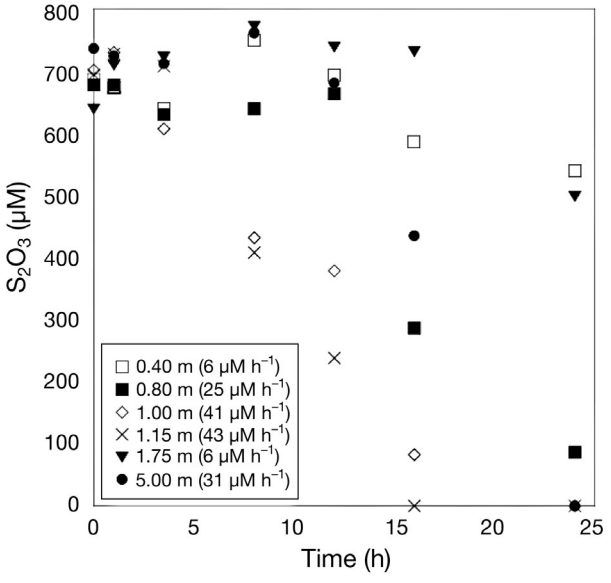

Fig. 5. Kinetics of thiosulfate oxidation using aerated fresh samples along the vertical profile of Lake Cisó on June 11, 1991 in duplicate. Consumption rates for each depth are shown in parentheses along a 24 h interval (calculated from beginning to end incubation times). Standard error of the mean between replicates was <10%

DISCUSSION

Characteristically, the vertical distribution of thiobacilli in microbial mats as determined by the MPN counts show the highest concentrations (up to 10^8 to 10^{10} cells cm^{-3}) restricted to a narrow zone, typically 1 to 5 mm thick, in which the organism's requirement for oxygen and sulfide is fulfilled, whereas a decreasing oxygen concentration with depth may inhibit growth (Visscher et al. 1992). These organisms exhibited diurnal migration patterns, and positioned themselves in narrow bands where the prevailing conditions are optimal for their development (Jørgensen 1982). In planktonic ecosystems, however, thiobacilli have to deal with daily turbulences and water mixing processes, especially in shallow lakes like those studied here (García-Cantizano et al. 2005), and positioning correctly in the water column is not trivial. Thiobacilli have to compete with spontaneous chemical oxidation by oxygen, biological oxidation by photosynthetic PSB, and heterotrophic bacteria that co-metabolize thiosulfate (Mason & Kelly 1988, Labrenz et al. 2005). Oxygen and sulfide can spontaneously react at a rate depending on the concentrations of both components, yielding a wide range of more stable sulfur compounds (e.g. thiosulfate, elemental sulfur, sulfite) (Chen & Morris 1972, Millero 1986). PSB, together with the use of sulfide as electron donor in the anoxygenic photosynthesis and the internal cell storage of sulfur globules, are able to aerobically use reduced sulfur compounds, both sulfide and thiosulfate, in the dark and grow chemolithotrophically (De Wit & Van Gemerden 1987 and references therein). The capacity for chemolithotrophic growth is quite common among members of the Chromatiaceae, and it has been shown for several species of *Amoebobacter*, *Chromatium*, *Thiocapsa*, and *Thiocystis*. In fact, a laboratory comparison of both sulfide affinity and maximal growth rates of PSB with those of the specialist thiobacilli showed that once the phototrophs have built up a dense population (bloom) in the light, they may successfully compete with the thiobacilli for sulfide in the dark, and even outcompete them (Kuenen 1989). This may explain why potential thiosulfate oxidation rates in Lake Cisó were better related to PSB than to thiobacilli distributions. In addition, PSB of Lake Cisó have shown substantial dark carbon-fixation activity *in situ* (Casamayor et al. 2008) and daily vertical migrations of up to 35 cm (Pedrós-Alió & Sala 1990). Altogether, it seems that the motile PSB are better adapted to the intrinsic characteristics and natural perturbations of the plankton realm.

MPN were grown in a minimal medium with carbonate and thiosulfate as the sole carbon and energy sources, respectively. The cell numbers reported here using MPN are likely to underestimate the actual num-

ber of aerobic sulfur oxidizers. This may be due to culture biases introduced by the composition of the growth medium and the incubation conditions. The medium used in the MPN analyses discriminates against those, for example, that were unable to use thiosulfate as an electron donor and/or bicarbonate as an inorganic carbon source. In addition, MPN only shows the most likely number of organisms present in a sample, and is therefore a purely statistical approximation. However, numbers around 10^2 to 10^3 cells ml^{-1} have been shown using fluorescent antibodies in surface and groundwater springs near the Black Sea, and by MPN in an Antarctic lake (Sattley & Madigan 2006 and references therein). Additionally, when the performance of the analyses carried out was tested in a benthic laminated microbial ecosystem in the Ebro Delta (Table A1), the MPN concentrations were up to 10^8 cells cm^{-3}, fitting well within previously reported values for such ecosystems (Visscher et al. 1992). Thus the estimates of cell numbers may not be that far from actual numbers, and probably MPN study provides an acceptable profile of the cultivable, thiosulfate-oxidizing, chemolithoautotrophic bacteria. Of course, the possibility exists that additional aerobic freshwater sulfur-oxidizing populations were not detected by the environmental conditions imitated in the MPN culture. For instance, *Thiotrix* spp.-like *Gammaproteobacteria* and a few *Thiobacillus* spp.-like *Betaproteobacteria* have been obtained from Lake Estanya (Ferrera et al. 2004), and *Thiomonas* spp.-like 16S rRNA gene sequences have been described in the plankton of a stratified rain forest reservoir (Dumestre et al. 2002). *Epsiloproteobacteria*, in turn, appeared as one of the main sulfur oxidizers in marine redoxclines (Grote et al. 2008 and references therein). However, none of these populations have been detected in the 16S rRNA gene surveys subsequently carried out in some of these lakes as predominant community members (Casamayor et al. 2000, 2002, Martínez-Alonso et al. 2008). The presence of MPN of 10^4 to 10^5 cells ml^{-1} close to the oxic-anoxic interface suggests that under certain conditions freshwater thiobacilli may actively fix CO_2. But the same concentrations found at the fully anoxic conditions of the hypolimnia are intriguing, and it is unclear whether these cells are maintaining active metabolism at these depths or are sinking cells in a dormant state until conditions become more favorable. These issues could be further addressed on a more focused and limited set of samples by single-cell analyses recently available such as fluorescence *in situ* hybridization (FISH) combined with microautoradiography or with ion mass spectroscopy (Musat et al. 2008) using specific probes.

In fact, on many occasions dark incorporation was seen to occur at a distance from the oxygen–sulfide interface. This has been previously observed (Culver & Brunskill 1969, Jørgensen et al. 1979, 1991, Tuttle & Jannasch 1979, Juniper & Brinkhurst 1986, García-Cantizano et al. 2005, Jost et al. 2008). In anoxic waters, facultative thiobacilli could use nitrate as the electron acceptor instead of oxygen (Kelly 1981). However, nitrate concentration in the hypolimnia of these lakes is practically zero (Miracle et al. 1992, Camacho et al. 2001). This may indicate that the viable thiobacilli recovered from the different lakes were either mostly inactive *in situ* at the time of sampling or they were able to carry out a more complex metabolism than expected. In fact, acidophilic counterparts of autotrophic thiobacilli are able to use elemental sulfur as an electron acceptor to oxidize molecular hydrogen (Ohmura et al. 2002), 2 components often present in anaerobic sulfurous hypolimnia. If dark incorporation at a short distance away from the oxygen–sulfide interface was carried out by thiobacilli, mechanisms for transport or *in situ* production of oxygen and sulfide gases should also be found. The oxygen and sulfide profiles are the result of many physical, chemical, and biological processes operating simultaneously. Oxygen diffuses downwards from the epilimnion. It is released *in situ* by oxygenic photosynthesis, mainly by *Cryptomonas phaseolus*. But oxygen is consumed by aerobic respiration of heterotrophic bacteria and ciliates, and may react chemically with sulfide. Sulfide diffuses upwards from the hypolimnion. It may be generated *in situ* from anaerobic sulfate respiration. Consumption of sulfide is caused by anoxygenic photosynthesis, chemolithoautotrophy, and chemical reaction with oxygen. Light-dependent processes either produce oxygen or consume sulfide. Thus, during the day, the interface is displaced downwards. On the contrary, sulfide-producing and oxygen-consuming processes predominate at night, and the interface is displaced upwards. This movement of the oxic-anoxic interface, due to physiological processes, is magnified by the vertical migration of the organisms themselves, especially of *C. phaseolus*, which migrates 40 cm up and down daily (Gasol et al. 1992). In the morning, the organism moves up and, at noon, it starts to descend. At dusk, *C. phaseolus* stops photosynthesis and swims into the sulfide-rich depths of the lower metalimnion. Early in the morning, the cells move back up again, reaching aerobic layers at dawn. These movements pull oxygen-rich water down at dusk and sulfide-rich water up at dawn. It has been calculated that the migration of *C. phaseolus* alone may explain a transport of these 2 gases several orders of magnitude higher than diffusive transport (J. G. Mitchell pers. comm.). Thus, oxygen and sulfide could be available, albeit at undetectable concentrations, to sulfur-dependent chemolithoautotrophs a few centimeters away from the interface. However, on several occasions the existence of dark carbon fixa-

tion was found several meters away from the interface and under fully oxic and fully anoxic conditions. This suggests that the microbes carrying out this process were able to thrive in very different environmental conditions, that the metabolisms involved in dark carbon fixation are larger than those traditionally known from aerobic chemolithoautotrophic bacteria, and that probably part of the large repertory of uncultured freshwater archaea recently detected may also participate in dark carbon fixation (Casamayor et al. 2001b, Auguet & Casamayor 2008, Auguet et al. 2008, Llirós et al. 2008).

The hourly rates of carbon fixation activity presented were more than 1 order of magnitude higher than those reported for marine anoxic basins, but within the ranges previously found for other stratified high sulfurous lakes. For instance, in Lake Cadagno the reported rates were between 10 and 74 mg C m^{-3} h^{-1} through the chemocline and hypolimnion, and up to 94 mg C m^{-3} h^{-1} in the oxic-anoxic boundary, whereas high values have also been found in lakes Mekkojarvi and Kinneret and the Big Soda Lake (Camacho et al. 2001 and references therein). The rates in the present study are within the normal range in the published literature (see reviews by Camacho & Vicente 1998, Wetzel 2001). This fact is related to the high biomasses that accumulated in the redoxcline and hypolimnia of these lakes, usually >1 order of magnitude higher than in marine sites. Indeed, estimations of *in situ*-specific growth rates and doubling times in Lake Cisó combining the number of cells and the carbon fixation rates indicated slow-growing populations with very large biomasses (García-Cantizano et al. 2005) within the range reported for other aquatic environments.

Altogether, the chemoautotrophic guild of these lakes may be composed of a metabolically complex, taxonomically diverse group of aerobic, microaerophilic, and anaerobic microorganisms coexisting in the same lake (García-Cantizano et al. 2005), and where photosynthetic sulfur bacteria may also actively participate (Casamayor et al. 2008). In this complex mixture of CO_2-fixing microorganisms, planktonic thiobacilli may actively fix CO_2 at certain depths but probably play a less relevant role than their benthic counterparts at the ecosystem level. Probably because thiobacilli have to deal with daily turbulences and local water mixing processes — mainly in shallow lakes like those studied here — and are less competitive in the plankton realm than motile photosynthetic sulfur bacteria that store sulfur intracellularly. The question of which types of bacteria contribute most to dark CO_2 fixation in the investigated lakes is still open, and the application of culture-independent molecular tools and single-cell analyses should be used to substantiate and further explore these findings.

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Appendix 1

Table A1. Data for depths selected for most probable number (MPN) incubations (aerobic sulfur-oxidizing thiobacilli-like bacteria [Aer. sulf. ox.]) in the benthic sample located in the supralittoral of the Ebro River Delta (NW Mediterranean) on July 16, 1990. Surface water data: 5 cm water layer; temperature: 28°C; conductivity: 92 000 µS cm^{-1}; salinity: 6.2%; pH: 8.74; oxygen: 315 µM; sulfide: 0 µM. Bchl: bacteriochorophyll

Depth (mm)	O_2[a] (µM)	H_2S[a] (mM)	Chl *a*[b] (µg cm^{-3})	BChl *a*[b] (µg cm^{-3})	Aer. sulf. ox. (cells cm^{-3})
0–5	135	1.50	310	140	1.68×10^8
5–10	0	7.50	0	0	1.70×10^6
10–15	0	10.12	0	0	1.08×10^6
15–20	0	9.87	0	0	8.38×10^4
20–30	0	10.20	0	0	3.09×10^4
30–40	0	10.07	0	0	2.19×10^3
40–50	0	8.75	0	0	1.77×10^5

[a]Interstitial water (Mir 1997)
[b]Averaged values (Martinez-Alonso 1997)

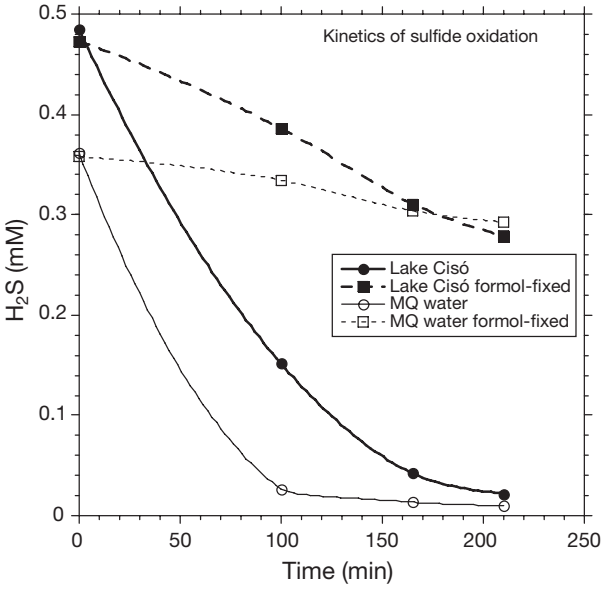

Fig. A1. Kinetics of sulfide oxidation in aerated fresh samples from the oxic-anoxic interface of Lake Cisó (biotic + chemical oxidations) and a formaldehyde-fixed (0.6% vol/vol) control sample (chemical oxidation) in duplicates. Additional controls with sterile ultrapure Milli-Q (MQ) water showed strong interferences of formaldehyde with the spontaneous chemical oxidation of sulfide. Standard error of the mean between replicates was < 10%

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