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Carbon dioxide fixation in the dark by photosynthetic bacteria in sulfide-rich stratified lakes with oxic–anoxic interfaces

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Abstract

Carbon fixation was analyzed in a series of stratified lakes with oxygen–sulfide interfaces following ¹⁴C-bicarbonate incorporation into oxygenic phototrophic, anoxygenic phototrophic (photosynthetic sulfur bacteria; PSB), and chemolithoautotrophic guilds (dark fixation processes), respectively. One of the lakes (Lake Cisó, Banyoles) was studied in more detail using microautoradiography of ¹⁴C-labeled cells and microscopic observations during day and night. Dark carbon fixation was high for many of the depths sampled in all the lakes studied and there was a correspondence between carbon (C) fixation in the dark and abundance of photosynthetic purple sulfur bacteria (PSB). After in-situ dark incubations, microautoradiographies revealed labeled cells actively incorporating inorganic carbon belonging to both unidentified rod bacteria but also to PSB (*Amoebobacter* sp. and *Thiocystis* sp.). The concentration of labeled rod bacteria was very similar in light and dark incubations, as well as in day and night samplings, and had a tendency to increase in the anoxic dark hypolimnion. Surprisingly, PSB consistently incorporated inorganic carbon in dark incubations at high rates during the day but much less at night. This suggests that photosynthetic organisms are capable of substantial dark C fixation after being subjected to light, and that “light dependent” dark carbon-fixation activity is an issue that has to be carefully addressed in future experiments.

Synthesis of organic carbon by primary producers is one of the essential functions in any ecosystem. The biological activity of a given ecosystem will depend on the flow of organic carbon through the different food web compartments. In aquatic ecosystems, both freshwater and marine,

there is a widespread knowledge that carbon dioxide (CO₂) fixation occurs in the dark (heretofore referred to as dark carbon fixation), but it is usually assumed to be negligible compared to photosynthesis carried out by algae and cyanobacteria. However, when autotrophic bacterial processes (other than those of cyanobacteria) have been specifically addressed, their contribution has been shown to be significant in many habitats (e.g., Casamayor et al. 2001a and references therein) and could exceed even that of the phytoplankton (e.g., Culver and Brunskill 1969, Takahashi and Ichimura 1970, García-Cantizano et al. 2005).

In stratified freshwater environments both light–sulfide and oxic–anoxic interfaces provide environments favorable for the growth of photo- and chemolithoautotrophic sulfur-oxidizing bacteria, respectively. Photosynthetic purple sulfur bacteria (PSB) use hydrogen sulfide (H₂S) as an electron donor in photosynthesis. Chemolithoautotrophic bacteria oxidize reduced inorganic compounds to obtain both energy and reducing power for fixing inorganic carbon. Anoxygenic photosynthesis (AnoxyPh) is a primary production process that has been measured together with

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Table 1. Data from the depths selected for in situ incubations in the lakes studied. Carbon fixation has been partitioned into oxygenic photosynthesis (OxyPh), anoxygenic photosynthesis (AnoxyPh), and in the dark.

Lake and date	Depth (m)	T (°C)	Conduct. ($\mu\text{S cm}^{-1}$)	O ₂ (mg L ⁻¹)	H ₂ S ($\mu\text{mol L}^{-1}$)	Chl <i>a</i> ($\mu\text{g L}^{-1}$)	BChl <i>a</i> ($\mu\text{g L}^{-1}$)	OxyPh	AnoxyPh	Dark
								($\mu\text{g C L}^{-1} \text{ h}^{-1}$)		
Cisó 13 Nov 90	0.10	12.0	1,500	1.3	752	180	73.3	0.0	11.4	5.4
	0.25	12.0	1,500	0.0	799	184	74.7	3.0	11.7	5.9
	0.50	11.8	1,525	0.0	787	215	87.3	2.5	6.6	7.4
Cisó 05 Feb 91	0.00	8.0	1,250	3.1	90	11	8.2	0.7	7.5	13.0
	0.20	7.6	1,250	0.0	159	95	201.3	2.5	25.7	11.7
	0.40	7.0	1,250	0.0	197	102	213.2	4.2	22.1	12.0
	0.60	6.0	1,250	0.0	206	104	176.5	2.7	7.7	19.2
Cisó 11 Jun 91	0.40	18.0	1,270	5.8	0	7	1.5	0.2	0.1	0.1
	0.80	17.0	1,200	6.2	0	326	22.8	7.7	1.6	7.2
	1.15	14.0	1,200	0.0	227	236	148.7	0.4	0.2	2.5
	1.75	12.0	1,150	0.0	728	228	102.4	0.0	0.0	1.7
Vilar 03 Apr 91	1.00	13.8	965	12.0	0	7	0.0	10.5	0.0	2.3
	4.50	13.6	1,073	12.4	0	13	0.2	4.5	0.9	1.2
	7.00	13.7	1,820	1.3	1	5	0.1	3.2	0.0	2.9
	8.00	14.1	1,860	1.0	2	4	0.3	0.0	0.0	4.2
Vilar 19 Sep 91	2.00	25.0	1,005	8.3	0	12	0.0	3.9	0.0	0.0
	5.50	23.5	1,100	1.7	0	48	315.2	1.5	2.0	22.8
	7.50	19.8	1,450	0.0	683	13	11.8	0.0	1.5	0.0
Estanya 15 Oct 91	10.00	17.2	1,950	8.9	0	3	0.4	0.5	0.3	0.0
	11.50	11.8	1,650	0.3	0	18	2.9	2.8	0.2	0.0
	12.00	10.2	1,550	0.1	23	29	581.4	9.7	35.8	17.6
	12.50	9.6	1,500	0.0	195	8	78.9	1.5	0.0	12.5
Massona 28 May 91	0.50	21.0	1,800	9.6	0	19	0.0	7.1	0.4	0.0
	4.00	16.0	5,800	2.7	0	30	0.0	0.6	0.0	0.0
	4.50	14.0	10,000	1.5	0	8	0.0	0.0	0.7	0.0
	5.50	12.5	23,500	0.8	19	56	7.6	0.3	0.7	0.5
Massona 25 Sep 91	1.50	23.2	3,780	5.8	0	52	1.2	61.9	0.8	0.3
	5.50	23.1	5,000	2.2	0	45	1.0	61.9	0.1	0.6
	6.25	21.1	17,000	0.0	237	n.d.	n.d.	2.9	2.8	0.4
	7.00	17.0	21,700	0.0	338	8	1.3	0.6	0.0	0.1

n.d., not determined.

oxygenic photosynthesis (OxyPh) in several holomictic and meromictic lakes (García-Cantizano et al. 2005 and references therein). Dark incorporation, on the other hand, has been traditionally simply subtracted from carbon incorporation in light bottles or just ignored. The importance of dark carbon fixation, though, has been shown not only for oxic–anoxic interfaces but also for anoxic waters in lakes (Culver and Brunskill 1969, Jorgensen et al. 1979, García-Cantizano et al. 2005 and references therein) and seas (Tuttle and Jannasch 1979, Juniper and Brinkhurst 1986, Jorgensen et al. 1991).

In a recent paper (García-Cantizano et al. 2005) we presented a detailed study on partitioning of CO₂ incorporation in Lake Cisó into the three autotrophic guilds: OxyPh, AnoxyPh (PSB), and chemolithoautotrophs (dark carbon-fixation processes), at different depths along the vertical profile, throughout diel cycles, and seasonally. Annual data indicated that most of the CO₂ fixation in the lake was due to dark incorporation processes, whereas diel variations of CO₂ incorporation showed values relatively low at night and higher and quite uniform throughout the light period. In the present work we extend these studies to other freshwater lakes with oxygen–sulfide interfaces to look for a confirmation of the overall pattern in dark CO₂

incorporation (dark carbon fixation), going beyond single case studies and complementing the previously published results. In addition, we use microautoradiography to check both whether dark incorporation could be attributed, at least in part, to some of the morphologically distinct bacteria inhabiting the lake and to look with more detail at the “light-dependent” dark carbon fixation observed.

Materials and Methods

Description of the systems—We have included in this study three karstic lakes (Lake Vilar, Lake Cisó, and Lake Estanya) and a coastal lagoon (La Massona) located in northeastern Spain. These stratified aquatic ecosystems have bottom waters rich in sulfate with high sulfide concentrations and oxic–anoxic interfaces located in the water column. Therefore, for these types of systems, the results presented here can be extrapolated. The four ecosystems were sampled several times and at different seasons in 1990 and 1991, and incubations were carried out covering the noon period (maximal irradiance for these lakes). More details are given in Table 1.

Lakes Vilar and Cisó are in the Banyoles karstic area (42°8'N, 2°45'E) (Casamitjana et al. 2006) and the

microbial communities inhabiting these systems have been extensively studied (*for a review see* Pedrós-Alió and Guerrero 1993, and references therein). Lake Vilar is a meromictic lake formed by two basins with a maximum depth of 9 m and a surface area of 11,000 m². 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 around 4–6 m, where dense populations of *Thiocystis minor* and *Chorobium phaeobacteroides* develop (Casamayor et al. 2002, Casamayor et al. 2007). Lake Cisó is a small monomictic lake (650 m²), 1 km away from the former, with a maximum depth of 6.5 m. The thermocline is at 1.5 m, where dense populations of the PSB *Thiocystis* sp. and *Amoebobacter* sp. develop (e.g., Casamayor et al. 2000a). The lake becomes anoxic during winter holomixis (complete mixing) and high sulfide concentrations (up to 500 $\mu\text{mol L}^{-1}$) are present in the entire water column at this time of the year (Pedrós-Alió and Guerrero 1993).

Lake Estanya (42°02'N, 0°32'E) is a sulfide-rich holomictic lake (maximal H₂S concentrations around 600 $\mu\text{mol L}^{-1}$), located 670 m above sea level in the Pre-Pyrenees area, 10 km SE of the town of Benabarre, Huesca. It is constituted by two basins of 12 m and 22 m maximal depth connected by a shallow sill that dries up during summer. The lower water masses are rich in dissolved sulfate and carbonates brought by subsurface incoming waters. We sampled the deepest basin (Lake Grande de Estanya, southwest position) that had a thermocline located between 12 m and 14 m. Only a few limnological and microbiological studies have been published from this lake (Ferrera et al. 2004, and references therein), reporting blooms of the PSBs *Chromatium okenii* and *Thiocystis minor* (*Chromatium minus* in former reports) 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'Empordà Natural Park. Some limnological studies have been carried out in this lagoon (Riera and Abellà 1991 and references therein). 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 chemocline 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 (Riera and Abellà 1991).

Sampling and analyses—Water temperature and conductivity were measured in situ using a submersible probe (YSI-33 S-TS; Yellow Springs Instruments). Light penetration was measured with a submersible spherical quantum meter (QSP-170; Biospherical Instruments). Samples for biological and chemical analyses were taken from

different depths using a battery-driven pump connected with tubing to a conical polyvinyl chloride structure to improve laminar sampling at the interface and were measured as reported (Casamayor et al. 2001a, García-Cantizano et al. 2005). For sulfide measurements, 10-mL subsamples were first alkalized by adding 100 μL of 10 mol L⁻¹ sodium hydroxide (NaOH) and then chemically fixed by adding Zn-acetate to a final concentration of 0.1 mol L⁻¹, and oxygen was measured as previously reported (García-Cantizano et al. 2005). For total cells counts, 10-mL subsamples were fixed by addition of formaldehyde to a final concentration of 4% (vol.:vol.). Counts of 4',6-diamidino-2-phenylindole (DAPI)-stained cells were taken using an epifluorescence Olympus BH microscope. Standard deviation was <10% for the cell counts. Morphologically distinguishable phototrophic bacteria were identified by microscopy, and some taxonomically valuable characters, such as motility and presence of gas vesicles, were observed under phase-contrast with live samples (Casamayor et al. 2000b). Chlorophyll *a* (Chl *a*) and bacteriochlorophyll *a* (Bchl *a*) were determined spectrophotometrically as reported (Casamayor et al. 2001c) on samples filtered through membrane filters (Sartorius, 0.45 μm) and extracted overnight in 90% acetone (saturated with magnesium carbonate).

Carbon dioxide incorporation experiments—Carbon dioxide incorporation was estimated using the procedure of Steemann Nielsen (1952) modified for oxic–anoxic interfaces as described in detail in a recent paper (García-Cantizano et al. 2005). Briefly, incubations were carried out using 13-mL or 22-mL, screw-capped tubes where radioactive sodium bicarbonate (NaH¹⁴CO₃) was added at a final concentration of 0.25 $\mu\text{Ci mL}^{-1}$. Neither the oxygen present in the small volume of tracer injected (only a few microliters) nor the air present in the small headspace of the tubes (that would react chemically with the sulfide present in high concentrations) would significantly increase dark CO₂ incorporation measured or inhibit the anoxygenic photosynthesis carried out by strictly anaerobic bacteria. Conversely, sulfide concentrations present at the oxygen–sulfide interface (2–20 μmol) would have decreased during water manipulation and would have led to an underestimation of chemoautotrophic activity. Four different treatments were done with replicates (two replicate tubes per treatment). 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 dark carbon-fixation processes. To a third set of samples incubated in the light, 3-(3', 4'-dichlorophenyl)-1,1'-dimethyl urea (DCMU) was added at a final concentration of 2 $\mu\text{mol L}^{-1}$. DCMU inhibits photosystem II and thus, photosynthesis by algae (Bishop 1958). Finally, nontreated samples were also incubated in the light. The disintegrations per minute (dpm) assimilated by OxyPh were calculated by subtracting dpm incorporated in clear tubes with DCMU from dpm incorporated in clear tubes. The dpm incorporated by AnoxyPh were determined by

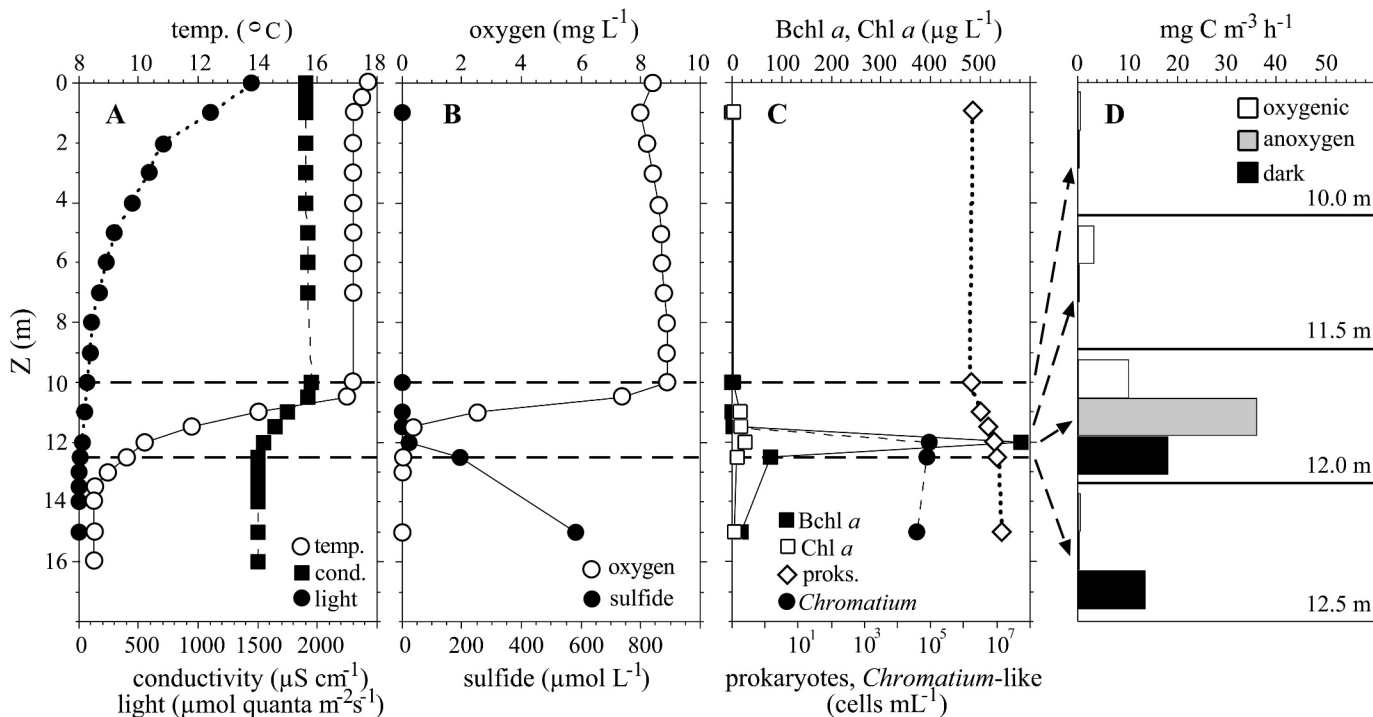


Fig. 1. Vertical profiles for different physico-chemical and biological parameters in Lake Estanya in October 1991. (A) Vertical distribution of temperature, conductivity, and light; (B) distribution of oxygen and sulfide; (C) distribution of photosynthetic pigments; and (D) CO_2 incorporation values at four different depths within the metalimnion.

subtracting the dpm incorporated in dark tubes from dpm incorporated in clear tubes with DCMU. Finally, the dpm incorporated in the dark were calculated by subtracting dpm incorporated in the killed control from dpm incorporated in dark tubes. Incubations were carried out for a period of 4 h at the same depths where the samples had been taken. At the end of the incubation the content of the tubes was quickly filtered through glass fiber filters (Whatman GF/F). After a 20-min exposure to hydrogen chloride (HCl) fumes, the filters were immersed in scintillation fluid (Optiphase Hisafe II) and kept there for 10–12 h prior to counting in a liquid scintillation counter (LKB Company, Austria). Carbon-uptake rate and carbon incorporation per unit area (integrated values) for selected parts of the lake (mostly metalimnion, and therefore not extrapolated to the whole lake) were made as previously reported (García-Cantizano et al. 2005). To properly estimate daily incorporation values when only noon incubations are done, several diel cycles for each lake should be carried out as we did previously in one lake (García-Cantizano et al. 2005). Therefore, activity results are presented here per hour instead of daily rates.

Microautoradiography—The method of Tabor and Neihof (1982) as modified in Pedrós-Alió and Newell (1989) was used, except for the staining procedure. Microautoradiographies (AUs) were carried out with aliquots taken from both control and experimental tubes at the end of the incubation period. These subsamples were fixed with formaldehyde (4% final concentration) and kept in an ice bath and in the dark. Microautoradiograms were

prepared with 2-mL subsamples filtered through polycarbonate filters with 0.2- μm pore diameter. Filters were rinsed twice with saline solution (0.9% NaCl) to remove excess radioactivity and formaldehyde precipitates and kept at 4°C until processing in the dark room. There, microscope slides were dipped in a melted Kodak NTB3 nuclear emulsion, and bacteria were directly deposited onto the wet emulsion (face to face with the filter). Exposure took place during 10 d at 4°C in complete darkness. Optimal exposure conditions were determined in a series of methodological experiments (see Results). After exposure, slides were developed in Kodak D-19 developer and fixed in Valca Fi-val fixer. Air-dried slides were stained with a few drops of DAPI (10 $\mu\text{g L}^{-1}$) for 10 min, rinsed, and dipped in 1% glycerol solution. Once the slides were dried, the filters were carefully peeled off, and autoradiograms were observed at 1,000 \times in an Olympus BH epifluorescence microscope. We observed that most of the cells were transferred to the slide, but a variable fraction remained on the filter. Because biased losses against one type of bacteria were not expected, we used the percentage of labeled cells calculated from the total cells transferred to the slides. Total cells and labeled cells were counted in 2–4 replicates. Controls without radioactivity were carried out occasionally and yielded negative results. Photographs were taken with 100 ASA Kodak Ektachrome film.

Results

The importance of dark carbon incorporation in different water masses—All the basins studied showed well-defined

Table 2. Estimations of carbon fixation rates ($\text{mg C m}^{-2} \text{h}^{-1}$) valid for the range of depths prospected (mostly metalimnia, and therefore not extrapolated to the whole lake) in different lakes and periods by oxygenic photosynthesis (OxyPh), anoxygenic photosynthesis (AnoxyPh), and in the dark. Percentage values of each activity are indicated between parentheses.

Lake and date	OxyPh	AnoxyPh	Dark	Total
	(mg C m ⁻² h ⁻¹)			(mg C m ⁻² h ⁻¹)
Cisó 13 Nov 90 (Depths 0.1–0.5 m)	1.8 (13%)	6.8 (50%)	5.0 (37%)	13.6 (100%)
Cisó 05 Feb 91 (Depths 0.0–0.6 m)	2.7 (9%)	15.3 (52%)	11.5 (39%)	29.5 (100%)
Cisó 11 Jun 91 (Depths 0.4–1.75 m)	1.6 (31%)	0.4 (8%)	3.1 (61%)	5.1 (100%)
Vilar 03 Apr 91 (Depths 1.0–8.0 m)	37.5 (63%)	3.2 (5%)	18.9 (32%)	59.6 (100%)
Vilar 19 Sep 91 (Depths 2.0–7.5 m)	12.9 (13%)	9.8 (10%)	74.1 (77%)	96.8 (100%)
Estanya 15 Oct 91 (Depths 10.0–12.5 m)	9.9 (22%)	21.5 (47%)	14.0 (31%)	45.4 (100%)
Massona 28 May 91 (Depths 0.5–5.5 m)	15.3 (92%)	1.2 (7%)	0.1 (1%)	16.6 (100%)
Massona 25 Sep 91* (Depths 1.5–7.0 m)	24.1 (94%)	1.1 (4%)	0.5 (2%)	25.7 (100%)
Ebro River salt wedge† (Depths 3.0–4.8 m)	1.7 (48%)	0.2 (3%)	1.8 (48%)	3.6 (100%)

* Incubations carried out in the laboratory, room temperature, and $20 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$.

† Data from Casamayor et al. 2001a.

vertical stratification at the time of sampling. Lake Estanya, for example, had a thermocline at a 10-m depth (Fig. 1). The epilimnion was aerobic with oxygen concentrations homogeneously distributed at $\sim 8 \text{ mg L}^{-1}$. The hypolimnion was anaerobic with high concentrations of sulfide ($\leq 600 \mu\text{mol L}^{-1}$), and the metalimnion showed opposite gradients of oxygen and sulfide. Light reaching the metalimnion was a small percent of that incident on the surface, but it was still significant ($\sim 1\%$). Vertical distribution of phototrophic microorganisms was indicated by their main pigments, i.e., Chl *a* for oxygenic and Bchl *a* for anoxygenic phototrophs (PSB). As can be seen, both accumulated at the metalimnion (e.g., Chl *a* was one order of magnitude higher here than in the epilimnion). Prokaryotes without characteristic morphologies (PROKS), on the other hand, showed a more or less uniform distribution with depth with higher concentrations in the metalimnion and hypolimnion ($\leq 10^7$ cells mL^{-1}). Four depths were selected for incubations with ^{14}C -bicarbonate to cover the metalimnion in detail; we assumed that most activity would be concentrated where most of the organisms were present. OxyPh increased from the upper part of the metalimnion down to 12 m and decreased again below this depth (Table 1). AnoxyPh was only significant precisely at 12 m, and dark incorporation was detected from 12 m downward. Thus, the three processes had different vertical distributions but co-occurred at 12 m. When these values were integrated for the metalimnion, OxyPh, AnoxyPh, and dark incorporation accounted for 22%, 47%, and 31% of total CO_2 incorporation carried out in this water compartment, respectively (Table 2).

Representative data for the different aquatic systems sampled are presented in Table 1. For some depths, no H_2S

was detected, but anoxygenic photosynthesis was measured. This is because PSB store elemental sulfur inside the cells as a transient product of sulfide oxidation to sulfate and can migrate upward, where light is available to use this sulfur. The same general patterns could be observed in most systems for the two light-dependent processes. However, dark incorporation showed very different patterns in different systems. It was detected both in oxic and anoxic water masses; it did not necessarily show maximal activity at the oxic–anoxic interface, and it had a tendency to increase with depth in some cases (Table 1, see e.g., 05 February 1991 in Lake Cisó and 03 April 1991 in Lake Vilar). In addition, the relative importance of the three autotrophic processes changed dramatically from one system to another (Table 2). For example, the three processes showed similar contributions in the metalimnion of Lake Estanya (between 22% and 47%), but AnoxyPh was almost irrelevant in La Massona (1%), and dark carbon fixation was the most important process (77%) in Lake Vilar on 19 September 1991.

Both AnoxyPh and dark carbon fixation showed a bimodal distribution with respect to sulfide concentrations (Fig. 2A). Activity was higher at sulfide concentrations $< 250 \mu\text{mol L}^{-1}$ (corresponding to the metalimnia) and at concentrations $> 700 \mu\text{mol L}^{-1}$ (corresponding to values from Lake Cisó in winter and to some of the hypolimnia). Additionally, dark carbon fixation showed a positive correlation with the concentration of Bchl *a*, but not with that of Chl *a* (Fig. 2B). Overall, dark carbon fixation was an important CO_2 -fixation process in most of the environments studied, and this phenomenon is not just a peculiarity of the previously studied Lake Cisó. Variability among systems and between depths was very high and

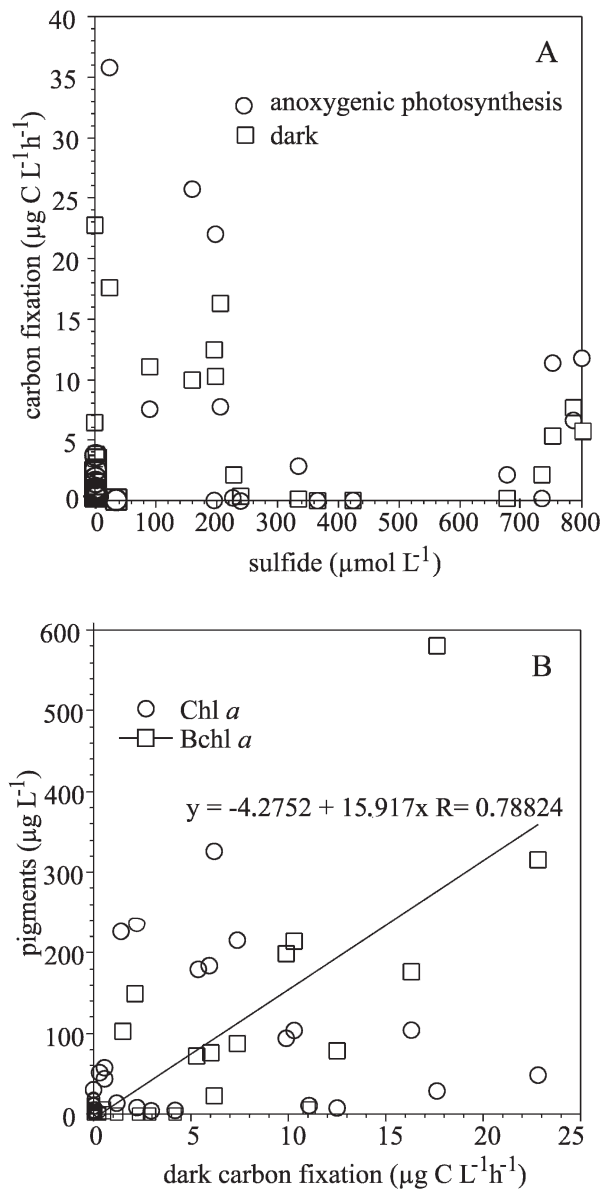


Fig. 2. (A) Carbon fixation rates along different sulfide concentrations in the different lakes and depths analyzed, and (B) relationship between photosynthetic pigments (Chl *a* and Bchl *a*) and dark carbon fixation in the different samples.

suggests that different metabolisms were involved. We used microautoradiography to look in more detail at one of the lakes (Lake Cisó).

Microautoradiography optimization experiments—In order to determine the optimal conditions for autoradiography (AU), we carried out an experiment in the laboratory with water from 2-m depth from Lake Cisó taken on 03 May 1990. This sample had high concentrations of the three main phototrophic microorganisms: *Cryptomonas* (5.2×10^4 cells mL^{-1}), *Amoebobacter* (6.8×10^4 cells mL^{-1}), and *Thiocystis* (4.2×10^4 cells mL^{-1}). We tested two incubation times (4 h and 8 h), three periods of exposure to the nuclear emulsion (5 d, 10 d, and 19 d), and two conditions (light and dark). Previously, incorpo-

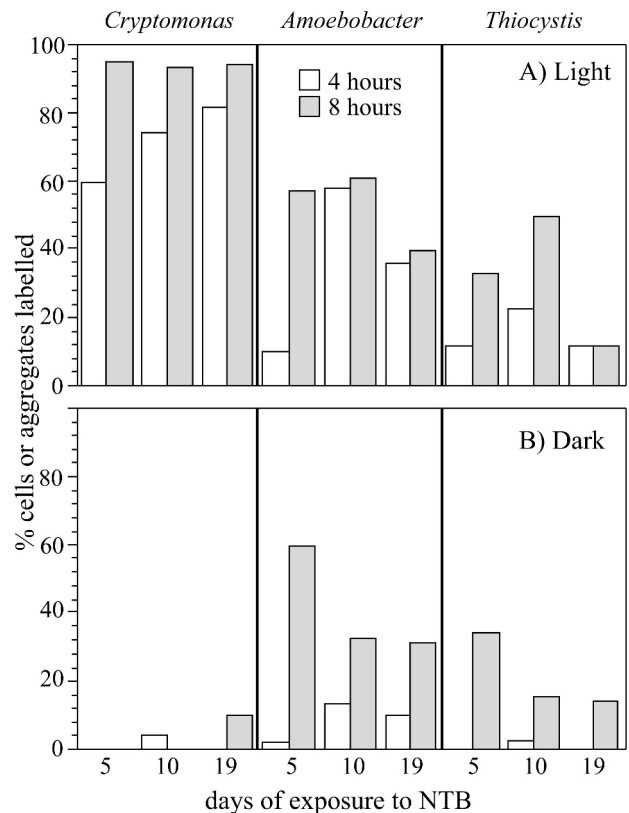


Fig. 3. Percent of labeled cells in autoradiograms prepared after different incubation times in situ (A) in the light and (B) in the dark, and days of exposure to photographic emulsion.

ration of ^{14}C -bicarbonate was shown to be linear for up to 8 h (García-Cantizano et al. 2005).

In essentially all conditions, the percentage of labeled cells was higher after 8 h than after 4 h of incubation (Fig. 3). Therefore, it would appear that 8 h was the appropriate incubation time. In the field, however, light conditions change considerably in 8 h. For Lake Cisó, for example, we determined that a period of 4 h around noon received 80% of the sun radiation for the whole day (see fig. 3 in García-Cantizano et al. 2005). Radiation reaching the lake before and after this period decreased dramatically due to trees shading the lake. Moreover, long incubations might cause undesirable bottle effects as well as exhaustion of products for photosynthesis. For these reasons, we used 4-h incubation periods.

A second factor to consider is the time that the photographic emulsion is exposed to the radioactivity in the sample. Usually, short exposure times underestimate the number of labeled cells, and long exposure times also tend to underestimate this number due to chemical fading processes. For the three organisms in the light, the percent of labeled cells was either not significantly different among exposure times or, more frequently, percentages were higher after 10 d of exposure than after 5 d or 19 d (Fig. 3). We repeated the experiment in the field, and we found that these findings were reasonable under more realistic in situ conditions. Therefore, 4 h of incubation and 10 d of exposure were taken as the best compromise among the several factors

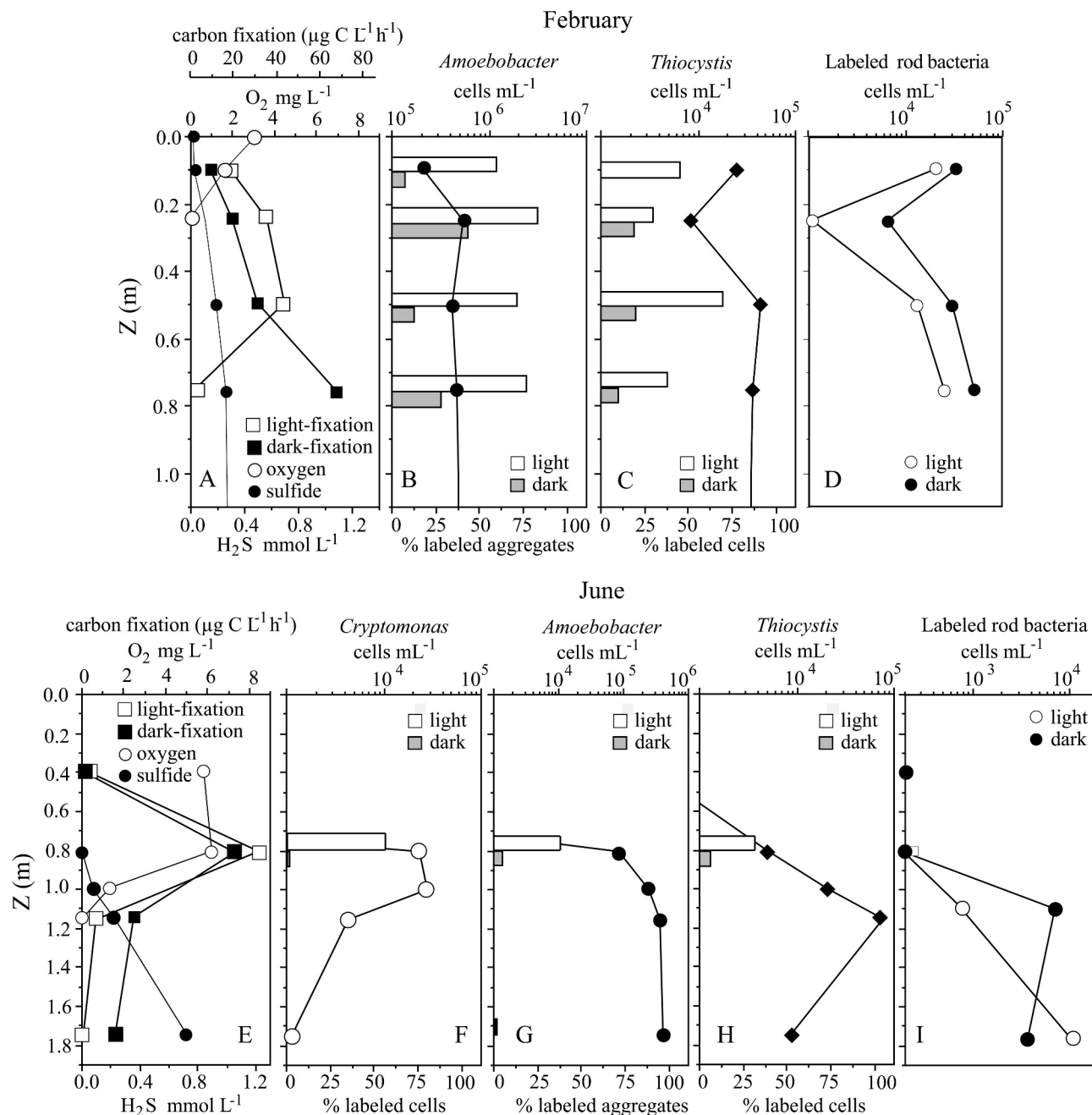


Fig. 4. Vertical profiles of the light and dark carbon fixation carried out in Lake Cisó for the winter mixing (19 Feb 91; A–D) and stratification periods (11 Jun 91; E–I). Total cells and percentage of labeled cells (in bars) for the oxygenic phototrophic algae *Cryptomonas* and the anoxygenic phototrophic bacteria *Amoebobacter* and *Thiocystis*. *Cryptomonas* were below detection limit in winter. Total labeled rod bacteria are shown on panels D and I for light and dark incubations, respectively.

involved in these incubations. Consequently, our numbers of labeled cells are likely to be underestimates.

Microautoradiography in the field—AU experiments were carried out in four additional samplings in Lake Cisó (05 Feb 91 and 19 Feb 91 during holomixis, 12 Mar 91, and 11 Jun 91 during permanent stratification). Figure 4 shows the percentage of ^{14}C -labeled microorganisms detected after light and dark incubations for two of those vertical profiles corresponding to winter (19 Feb 91) and summer

(11 Jun 91) conditions, respectively. Examples of labeled and unlabeled cells in AU are shown in Fig. 5, and the complete data set is presented in Table 3. The lake became anoxic during winter and labeled microorganisms were exclusively bacteria of the types *Amoebobacter*, *Thiocystis*, and PROKS (Fig. 5A, B). Since *Amoebobacter* formed aggregates under in situ conditions, counts for this bacterium have been expressed as a percentage of labeled aggregates. In February, the lake showed what we had defined as surface stratification (*see* García-Cantizano et al.

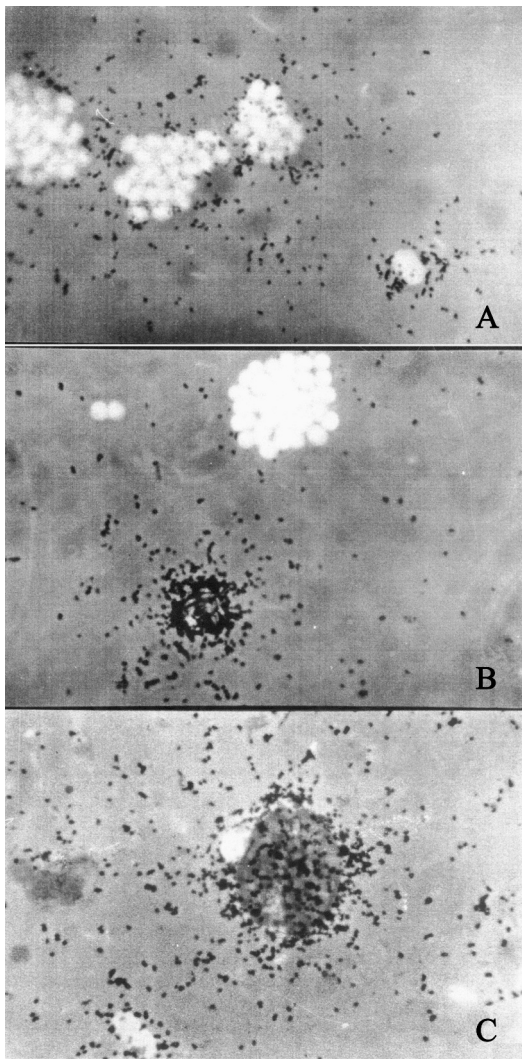


Fig. 5. Microautoradiograms from Lake Cisó. Dark spots indicate incorporation of $\text{NaH}^{14}\text{CO}_3$ along the incubation period. (A) Labeled *Thiocystis* and aggregates of *Amoebobacter* in the light at 0.5-m depth on 19 February 1991; (B) labeled unidentified rods in the dark, at 0.5-m depth on 19 February 1991; and (C) simultaneous labeling of the oxygenic phototrophic algae *Cryptomonas* and the anoxygenic phototrophic bacteria *Thiocystis* in the light, at the metalimnion of the lake.

2005). This slight stratification affected the upper centimeters only and exclusively during the day (Fig. 4A). The lake was completely mixed again at night.

Large percentages of *Amoebobacter* aggregates were labeled in light incubations during the two winter profiles, and the numbers did not change much with depth (Fig. 4B; Table 3). In the case of *Thiocystis* the percentages were lower on 05 February 1991 than on 19 February 1991, but again they did not change very much with depth (Fig. 4C; Table 3). During permanent stratification, on the other hand, both PSB species showed significant numbers of labeled cells at only one or two depths (Fig. 4G,H; Table 3), and labeled cells of *Cryptomonas* also appeared at one depth (Figs. 4F, 5C; Table 3). These depths concentrating most of the active cells were always found

Table 3. Microautoradiography data for experiments carried out in Lake Cisó at different depths for winter mixing and spring stratification.

Lake Cisó sampling date	Incorporation		<i>Cryptomonas</i>				<i>Amoebobacter</i>				<i>Thiocystis</i>				Other PROKS cells labeled			
	Z (m)	(mg C m ⁻³ h ⁻¹)	counts × 10 ² (cells mL ⁻¹)		% Labeled cells		Counts × 10 ⁴ (cells mL ⁻¹)		% Labeled cells		Counts × 10 ³ (cells mL ⁻¹)		% Labeled cells		Counts × 10 ⁶ (cells mL ⁻¹)		Counts × 10 ⁶ (cells mL ⁻¹)	
			Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
05 Feb 91	0.00	8.2	13.0	0	0	0	0	33.0	55	24	10.0	13	47	9.0	0	0	0	0
	0.20	28.2	11.7	0	0	0	0	50.0	51	0	6.7	6	0	11.0	380	2,300	0	0
	0.40	26.3	12.0	0	0	0	0	60.0	65	25	4.9	5	4	11.0	250	1,100	0	0
19 Feb 91	0.60	10.3	19.2	0	0	0	0	39.0	17	20	12.0	0	11	10.0	0	0	0	0
	0.10	15.8	4.5	0	0	0	0	48.0	59	8	2.5	46	0	2.5	21,000	33,000	0	0
	0.25	35.1	19.5	0	0	0	0	54.0	82	42	8.4	31	18	4.1	—	6,500	0	0
12 Mar 91	0.50	44.0	31.6	0	0	0	0	51.0	71	13	2.6	70	19	3.8	13,000	30,000	0	0
	0.75	3.2	68.2	0	0	0	0	55.0	77	28	1.5	37	10	4.2	25,000	51,000	0	0
	0.25	3.0	0.0	0	0	0	0	1.3	0	0	0	0	0	2.9	0	0	0	0
11 Jun 91	0.50	10.2	0.0	7	0	0	0	1.3	3	0	3.8	11	0	4.7	0	0	0	0
	0.80	58.9	28.7	0	0	0	0	79.0	6	2	1.4	0	0	4.6	0	5,900	0	0
	1.25	5.8	12.8	0	0	0	0	36.0	0	0	1.4	0	0	3.5	8,400	2,000	0	0
11 Jun 91	0.40	0.3	0.1	0	0	0	0	0.0	0	0	0.4	0	0	0.6	0	0	0	0
	0.80	9.5	7.2	230.0	56	2	0	8.6	38	5	5.0	32	7	1.4	0	0	0	0
	1.15	0.6	2.5	48.0	0	0	0	37.0	0	0	77.0	0	0	1.0	740	7,400	0	0
	1.75	0.0	1.7	11.0	0	0	41.0	0	1	8.5	0	0	1.6	9,900	5,000	0	0	

at the top of the layer of accumulation of biomass, indicating that most of the phototrophic biomass is not very active due to self-shading. Overall, PSB were active at all depths in surface stratification (winter) and only at the top of the bacterial layer during permanent stratification. AU thus confirms previous results from bulk activity measurements (Pedrós-Alió and Guerrero 1993).

We observed that the PSBs *Amoebobacter* and *Thiocystis* were also labeled after dark incubations. Percentages of labeled PSB were always higher in the light incubation (up to 82% of total cells) than in the dark incubations, and in the dark it was higher for *Amoebobacter* (range: 8–42% of aggregates) than for *Thiocystis* (range: 10–18% of cells). The maximal percent of cells labeled in the dark always coincided with the maximal percent in the light (Table 3). We did not observe labeled cells in the dark for those depths with the lowest bulk carbon fixation value in the vertical profile. This is related to the specific activity of the cell because a certain amount of radioactivity should be incorporated into the bacterium to react with the nuclear emulsion and produce a visible silver grain.

Concentration of labeled PROKS was slightly higher in dark incubations than in the light, and it tended to increase with depth (Fig. 4D,I; Table 3). These prokaryotes were morphologically undistinguishable from the heterotrophic bacteria present along the vertical profile (range: $4.8\text{--}9 \times 10^6$ cells mL⁻¹) and represented between 0.3% and 2% of total counts (PSB excluded). It is remarkable that neither the point of maximal dark CO₂ incorporation nor that of maximal percent of labeled PROKS coincided with the oxygen–sulfide interface. Therefore, metabolisms other than aerobic sulfur-dependent chemolithoautotrophy must be involved.

An additional experiment was carried out in Lake Cisó with samples taken on 11 June 1991 at night and incubated under in situ dark conditions for 6 h. Carbon incorporation experiments were carried out at 0.4-m depth, 0.8-m depth, 1-m depth, and 1.30-m depth. The total carbon incorporation profile was very homogeneous along the water column sampled ($1.1 \mu\text{g C L}^{-1} \text{ h}^{-1}$, $1.7 \mu\text{g C L}^{-1} \text{ h}^{-1}$, $1.8 \mu\text{g C L}^{-1} \text{ h}^{-1}$, and $1.8 \mu\text{g C L}^{-1} \text{ h}^{-1}$, respectively), and autoradiograms showed PROKS as the main labeled microorganisms (data not shown). The concentration of labeled cells tended to increase through the metalimnion from $<10^3$ cells mL⁻¹ at the top to 10^4 cells mL⁻¹ at 1.30 m (anoxic, high-sulfide waters). The percent of labeled *Amoebobacter* aggregates was only 1% compared to the 38% found in dark incubations during the day. This experiment revealed that PSB incorporated CO₂ at night at a considerably lower specific activity per cell.

In order to check whether phototrophic bacteria would also incorporate CO₂ in the dark in culture, we carried out experiments with strains *Amoebobacter* M3 and *Chlorobium limicola* isolated from Lake Cisó. Subsamples were incubated at 28°C either in the dark or under $60 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ of continuous illumination. *Amoebobacter* M3 incorporated carbon in the dark but at a rate $20\times$ lower than in the light. In the case of *Chlorobium* some activity was also detected in the dark incubations but up to $100\times$ lower than the control incubated in the light.

Discussion

The lakes surveyed in the present work are stratified and have peculiar chemistry, with a wide repertoire of reduced compounds that coexist with oxidants and light at the oxic–anoxic interfaces. Such interfaces concentrate most of the autotrophic activity of the lake with usually slow-growing populations and large biomasses (Pedrós-Alió and Guerrero, 1993 and references therein). In marine environments, oxic–anoxic interfaces are also common, with important dark carbon-fixation activity in the same range that we have described here (Casamayor et al. 2001a, and references therein). The importance of autotrophic processes other than OxyPh has also been described in other freshwater nonsulfurous environments (Gorlenko et al. 1983), suggesting that alternative CO₂ incorporation processes may be more widespread than is currently thought. In addition, dark incorporation in open marine waters has been reported to contribute significantly to the whole system of carbon fixation (between 30% and 60% of total photosynthetic carbon uptake, Prakash et al. 1991). Altogether, dark carbon fixation seems to be a widespread process involving a significant portion of energy and matter fluxes in marine and freshwater ecosystems.

The ¹⁴C-labeling method described by Steemann Nielsen (1952) is a generally accepted and applied technique to estimate carbon fixation by phytoplankton. However, questions have always remained with respect to the significance of the often-high ¹⁴C dark uptake measured in the control experiment (Prakash et al. 1991 and references therein). Therefore, the measurement of dark carbon fixation is a controversial issue not yet satisfactorily solved by plankton ecologists. Unexpectedly, in our study, PSB (mostly *Amoebobacter* but also *Thiocystis*) appeared as one of the main players in dark carbon fixation at the oxic–anoxic interfaces. Other authors had previously reported high rates of dark fixation at the same depth that PSB layers developed (Cohen et al. 1977, Camacho et al. 2001). Probably this is a common trait for natural PSB layers. Actually, PSB strains have shown a wide range of metabolic capabilities in laboratory experiments and the ability to incorporate inorganic carbon under aerobic conditions in the dark (see references in a review by van Gemerden and Mas 1995). Our work, however, is the first experimental evidence that PSB fix carbon in the dark in situ.

Intriguingly, the phototrophic bacteria *Amoebobacter* and *Thiocystis* were able to actively incorporate carbon in the dark mostly during the day and at much lower rates during the night, suggesting that a fraction of this dark fixation could be a result of previous exposure to light. This is congruent with the lower rates of dark carbon fixation at night than during the day detected consistently in Lake Cisó in a former study (García-Cantizano et al. 2005). The possibility exists that fixation fuelled by light would continue for a short while after samples were under dark conditions. How long this process would last in darkness is, however, difficult to determine with our data. What we observed was a quite linear relationship in carbon incorporation after 4-h to 8-h incubation in the dark

(Fig. 3). However, keeping samples acclimated for several hours under dark conditions before injecting the tracer should be considered carefully because long incubations might cause undesirable bottle effects as well as exhaustion of products for energy supply and oxidant reactions, specially at the oxic–anoxic interfaces. Due to this process of light-stimulated CO₂ fixation, it would not be possible for current approaches to distinguish quantitatively the two underlying processes and the guilds of chemolithoautotrophs and photolithoautotrophs for dark CO₂ fixation.

Alternatively, photosynthetic bacteria would have an energy supply metabolism (e.g., storage products produced during photosynthesis such as intracellular sulfur globules) that could be used in the dark (van Gemerden and Mas 1995) and act as real chemoautotrophs. In any case, this methodological concern if demonstrated would have an effect on the quantitative distinction of photo- and chemolithoautotrophic guilds with implication for the interpretation of dark incubations carried during the day in previous studies (García-Cantizano et al. 2005), whereas the possible existence of alternative metabolisms in photosynthetic bacteria would lead to a re-conceptualization of the guild concept for bacteria because different individuals within the same population may use different energy sources and electron acceptors and may simultaneously contribute to both autotrophic guilds.

Certainly, specific experiments should address this point in future dark carbon-fixation measurements because it is a key methodological issue to identify “light-dependent dark carbon fixation” due to delayed metabolism if that is the case. Whether this “light-dependent dark carbon fixation” in photosynthetic bacteria is an alternative strategy for better dealing with environmental perturbations or not also deserve further study. In fact, a laboratory comparison of both sulfide affinity and maximal growth rates of PSB with those of the specialist thiobacilli (sulfur oxidizers chemolithoautotrophs) shows 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).

Our data indicated that photosynthetic organisms were capable of substantial dark carbon fixation after being subjected to light. Thus, in future measurements, a “light-dependent” dark carbon-fixation activity has to be carefully considered before looking for a general pattern for dark fixation in the photic zones of general aquatic ecosystems. For the algae *Cryptomonas*, present in Lake Cisó we did not consistently observe carbon fixation in the dark (only in one of the samples did we observe a 2% of the labeled cells incubated in the dark), but we cannot discard the fact that other photosynthetic bacteria such as marine cyanobacteria may use this strategy in the field or have this methodological limitation in the ¹⁴C-incorporation experiments carried out so far. This would provide an explanation of both the high dark fixation rates and the spatial and temporal variations often reported among different oceans (Prakash et al. 1991 and references therein) that deserves further research.

In the case of stratified aquatic ecosystems with oxic–anoxic interfaces such as those studied here, the existence of

substantial dark carbon fixation under fully in situ anoxic and dark conditions at the bottom of the lakes, and the fact that both the depths of maximal dark CO₂ incorporation and those of maximal percent of labeled PROKS were found far from the oxygen–sulfide interface suggest that other metabolisms may have taken part in the process as well. The reduced inorganic compounds generated in the sediments and mobilized to the water column, (such as sulfide, ammonia, ferrous iron, methane, molecular hydrogen, etc.) may act as energy sources and electron donors for planktonic bacteria or even archaea (Casamayor et al. 2001b), which fix CO₂ in the dark (Shively et al. 1998; Casamayor et al. unpubl.). The complexity of the ecologies and physiologies acting under anoxic conditions and darkness needs to be explored in detail. The full range picture of the metabolic capabilities in such microorganisms will offer consistent clues on the biogeochemical cycling in freshwater ecosystems with oxygen–sulfide interfaces.

Altogether, dark incorporation could not be easily assigned to one specific group of microorganisms, but still remains a complex, open issue to deal with both at the ecophysiological level and at the methodological level, where differing underlying processes are responsible at different temporal and spatial scales within aquatic ecosystems.

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