Vol. 215: 49–56, 2001 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published May 31

Primary production in estuarine oxic/anoxic interfaces: contribution of microbial dark CO_2 fixation in the Ebro River Salt Wedge Estuary

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ABSTRACT: The present study assesses the contribution of dark carbon fixation to the primary production in the oxic/anoxic interface of a shallow estuarine environment that develops a salt-water wedge with the presence of sulfide. Primary production was partitioned into oxygenic photosynthesis, anoxygenic photosynthesis and dark fixation. The results show the importance of dark fixation in the oxic/anoxic interface with values higher than 5 mg carbon fixed per cubic meter and per hour in some cases. The average rate of primary production in the dark during the anoxic season for the Ebro River salt wedge resulted in 42 mg C m^{-2} d^{-1} in the interface. This represents at least twice the contribution of oxygenic photosynthesis to the primary production in such interface. Because this process is probably important in other salt-wedge or highly stratified estuaries with oxic/anoxic interfaces containing sulfide, the estimates of carbon fixation made so far for these systems may have been underestimated, and should therefore be revised taking into account the contribution of dark processes.

KEY WORDS: Primary production · Dark fixation · Thiobacilli · Salt wedge · Estuary · Sulfide · Oxic/anoxic interface

INTRODUCTION

Primary production in marine environments depends on light and nutrient supply. In the turbid region of estuaries and salt marshes, suspended matter strongly limits light availability and, therefore, photosynthetic activity (Cole et al. 1992, Fishez et al. 1992, Irigoien & Castel 1997). In these systems, continuous mixing of water masses with rather different characteristics occurs, and allochthonous materials originating from soil erosion and river-borne phytoplankton detritus are predominant (e.g. Relexans et al. 1988). High heterotrophic bacterial activity has been reported in the maximum turbidity zone of estuaries (Crump et al. 1998, Goosen et al. 1999) and net heterotrophic balance with high levels of CO_2 emissions to the atmosphere characterize these systems (Frankignoulle et al. 1998).

In some cases, the estuarine area shows a stratified water column, with an upper layer of freshwater flowing on top of a salt-water wedge separated by a sharp interface. Examples are the Ebro, Rhone, Po, Swan and Mississippi River estuaries, among others. The dynamics of this saline intrusion, and the extent of its development depend mainly on river discharge, although the topography of the river bottom and tidal and non-tidal changes in sea level can play, in some cases, an important role (Muñoz & Prat 1989, Ibàñez et al. 1997). Salt-wedge estuaries can also be present in areas with high tidal regimes when the ratio of river discharge to the width of the estuary is high (Geyer & Farmer 1989). Because water in the salt wedge can remain isolated at the bottom of the river for several months during the year, decomposition of organic matter promotes oxy-

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gen depletion and the establishment of anoxic conditions with the production of hydrogen sulfide.

The oxygen-sulfide interface zone between fresh- and saltwater provides an environment favorable for the growth of chemolithoautotrophic bacteria. Chemolithoautotrophic bacteria oxidize reduced substances to obtain both energy and reducing power for fixing inorganic carbon. Therefore, they are primary producers in the dark. Dark CO_2 fixation in marine oxic/anoxic interfaces has been previously reported in a few environments such as stagnant marine basins (Sorokin 1972, Tuttle & Janasch 1979, Jørgensen et al. 1991, Detmer et al. 1993), fjords (Indrebø et al. 1979) and fjord-like embayments (Juniper & Brinkhurst 1986). Data available so far in the literature reporting such activity are scarce and, in addition, estuarine systems that do not fit classical fjord or basin bathymetry have not been considered as sites of chemolithoautotrophic activity. In the present work, we evaluate the contribution of dark CO_2 fixation to the primary production in the oxic/anoxic interface of a shallow estuarine environment that develops a temporal anoxic salt-water wedge. The results show the importance of dark fixation in this interface. Dark CO_2 fixation, therefore, should be taken into account in studies that evaluate primary production in environments with similar characteristics.

MATERIALS AND METHODS

Description of the environment. The Ebro River estuary is on the Mediterranean coast of Spain (40° 35′ N, 0° 41′ E). It was sampled at noon on 14 July 1989, 19 July 1990, 18 July 1991 and 18 May 1992. The sampling site was located some 15 km from the river mouth. The Ebro River is 928 km long and drains 85 550 km^2 with a mean discharge of 424 $m^3 s^{-1}$ (average of the last 30 yr). In summer, the discharge usually ranges from 70 to 200 $m^3 s^{-1}$, whereas in spring it reaches its highest values, ranging from 500 to 2000 $m^3 s^{-1}$. The river is strictly regulated by big reservoirs that enable massive growth of algae due to sewage coming from cities, farming and industries (Prat & Ibàñez 1995, Ibàñez et al. 1996). Thus, the river water carries considerable amounts of algae (many of which actually develop in the reservoirs), and these cause strong sedimentation in the salt wedge. The maximum extent of the salt wedge is 30 km upstream from the river mouth, but its most frequent extent is only 18 km, up to a sill (Gràcia Island) which prevents the salt wedge from advancing when the water discharge is higher than 100 $m^3 s^{-1}$. Decomposition leading to anoxia and sulfate reduction takes place along most of the salt wedge during summer, but never near the river mouth (Ibàñez et al. 1997, 1999).

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 quantum meter. Samples for biological and chemical analyses were taken using a battery-driven pump. The pump was connected to a length of tubing ended with a conical PVC structure built according to Jørgensen et al. (1979), to improve laminar sampling at the interfaces. Samples for cell counts, hydrogen sulfide and oxygen were fixed immediately. Oxygen was measured using the Winkler titration method modified according to Ingvorsen & Jørgensen (1979) to avoid sulfide interference. Sulfide was measured by the colorimetric method of Pachmayr described in Strickland & Parsons (1972). Samples for pigment analyses and MPN (most probable number) determination were kept in the dark, in coolers with ice, until analysis a few hours later. Samples for nutrient analyses were quickly frozen to determine nitrate, nitrite, ammonium and orthophosphate using a Technicon autoanalyzer (Strickland & Parsons 1972). Nutrients were determined by C. Ibàñez and N. Prat (University of Barcelona).

Biological analyses. Chlorophyll *a* and bacteriochlorophyll *a* were determined spectrophotometrically according to Strickland & Parsons (1972). Samples were filtered through membrane filters (Sartorius 0.45 µm) and extracted overnight in 90% acetone (saturated with magnesium carbonate). For the determination of the total number of bacteria, samples were fixed with formaldehyde (4% final concentration), concentrated on 0.2 µm polycarbonate filters (Nuclepore), and stained with DAPI (Porter & Feig 1980). Counting was made with an epifluorescence microscope (Olympus BH) following the statistical recommendations of Kirchman et al. (1982).

Chemolithoautotrophic sulfur bacteria (thiobacillus-like) were enumerated using the MPN technique in a selective medium (Visscher et al. 1991, 1992) containing carbonate (19 mM) as the only carbon source, thiosulfate (10 mM) as the only electron donor, and bromocresol blue to indicate acidification of the medium. Incubations were performed at room temperature for a period of up to 12 wk with 3 replicates per incubation. Samples were scored as positive when acidification occurred.

Carbon dioxide incorporation was estimated using the procedure of Steemann Nielsen (1952) modified for oxic/anoxic interfaces as described in Pedrós-Alió et al. (1993). Incubations were carried out using 13 ml screw-capped tubes to which $NaH^{14}CO_3$ was added at a final concentration of 0.25 µCi ml^{-1}. Four different treatments were applied. In one treatment formaldehyde (4%, vol./vol., final concentration) was added to the samples to correct for abiotic incorporation. A sec-

ond 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, DCMU (3-[3′, 4'-dichlorophenyl]-1,1′-dimethyl urea) was added at a final concentration of 2 µM. (DCMU inhibits the Photosystem II and, thus, photosynthesis by algae.) Finally, non-treated samples were also incubated in the light. The carbon assimilated by oxygenic photosynthesis was calculated by subtracting dpm incorporated in 'light' tubes with DCMU from dpm incorporated in 'light' tubes with no DCMU. The carbon incorporated by anoxygenic photosynthesis (photosynthetic sulfur bacteria and some cyanobacteria) was determined by subtracting the dpm incorporated in 'dark' tubes from that incorporated in 'light' tubes with DCMU. Finally, the carbon incorporated in the dark was calculated by subtracting dpm incorporated in the killed control from dpm incorporated in the 'dark' tubes. Incubations were carried out for a period of 4 h at the same depths as those from which the samples had been taken. At the end of the incubations, the contents of the tubes were quickly filtered through glass-fiber filters (Whatman GF/F). After an exposure of 20 min to HCl fumes, the filters were immersed in scintillation fluid (Optiphase Hisafe II) and kept there for 10 to 12 h prior to counting in a scintillation counter (LKB). Carbon uptake rate was calculated as described above (Pedrós-Alió et al. 1993).

RESULTS

The Ebro River estuary consists of 2 water masses with different physico-chemical characteristics and separated by an interface at around 3 m depth. Table 1 shows average values for several parameters measured in the 2 layers over the 4 yr study period, and the range of variability in the data. The freshwater layer showed higher temperatures than water from the saline wedge, and oxygen concentrations were usually close to saturation. Nitrate was the prevailing nitrogen species here and phosphate was on average 1.5 µM. In contrast, water in the wedge was of oceanic origin. It was colder, with higher conductivity and oxygen-depleted in summer. Sulfate and chloride concentrations were between 10 and 20 times higher than in the freshwater layer. Ammonium was the prevailing nitrogen species in the salt wedge. This layer was 10 times richer in phosphate because of redissolution from the sediment under anoxic conditions. Sulfide appeared here mainly as a biological product of sulfate respiration. Nitrite was found only at low concentration in both layers. Irradiance quickly declined through the freshwater layer. Incident irradiance decreased by about 90% during the first 1.5 m and the anoxic layer was essentially in darkness.

Table 1. Average values and range in variability (maximum and minimum values in parentheses) of several parameters in the 2 layers of the Ebro River from 1989 to 1992. nd: not detected

Parameter	Freshwater layer	Saline wedge
Depth of interface (m)	2.95	
Salinity (g l^{-1})	4.0 (2–5)	34.2 (31–36)
Temperature (°C)	25.1 (19–26)	21.2 (15–23)
Oxygen (mg l^{-1})	7.5 (6–10)	0.4 (0–5)
Sulfide (µM)	0.0 (nd)	8.0 (0–20)
Phosphate (µM)	1.5 (0.5–3.5)	11.0 (3.5–20)
Nitrate (µM)	178.0 (100–208)	10.0 (4–16)
Nitrite (µM)	1.8 (1–11)	0.7 (0.6–1.2)
Ammonia (µM)	7.6 (2–18)	69.3 (15–74)
Chloride (µM)	0.9	17.5
Sulfate (µM)	0.2	2.5
Chlorophyll *a* (µg l^{-1})	31.5 (20–35)	10.6 (7–12)

Fig. 1 shows vertical profiles of some physico-chemical variables in July 1990 and May 1992. Vertical profiles from the summers of July 1989 and 1991 (data not shown) were similar to that for 1990. In summer 1990, the saline wedge was well established. The upper freshwater layer had a temperature of approximately 25°C, a conductivity of 2000 µS cm^{-1} and oxygen at saturation level. In the saline wedge, temperature was around 22.5°C, conductivity 50000 µS cm^{-1}, and oxygen absent below 5 m depth. Sulfide concentration was around 20 µM, and the oxic/anoxic interface extended from 3 to 5 m. In spring 1992, the wedge was well-established, but anoxia had not yet fully developed. Surface temperatures were lower (19°C), and oxygen was 10 mg l^{-1} in the freshwater layer, but only half this in the saline layer. Sulfide was not detected. Irradiance, as in previous years, was unmeasurable below the freshwater layer.

Cell abundance and pigment concentrations are given in Fig. 2 for July 1990 and in Fig. 3 for May 1992. The abundance of chemolithoautotrophic sulfur bacteria (i.e. thiobacillus-like) was estimated by viable MPN counts, whereas oxygenic and anoxygenic phototrophic organisms were quantified both through the analysis of specific pigments in the water column and by epifluorescence microscopy. Chlorophyll *a* showed higher concentrations in the freshwater layer; the concentration in the wedge probably arose from moribund organisms sinking out of the freshwater layer. Microscopic examination checked for the possible presence of green sulfur bacteria (autofluorescent, non-motile small rods), but none were detected. Bacteriochlorophyll *a* (from purple sulfur bacteria) was also not detected. However, viable counts of chemolithoauto-

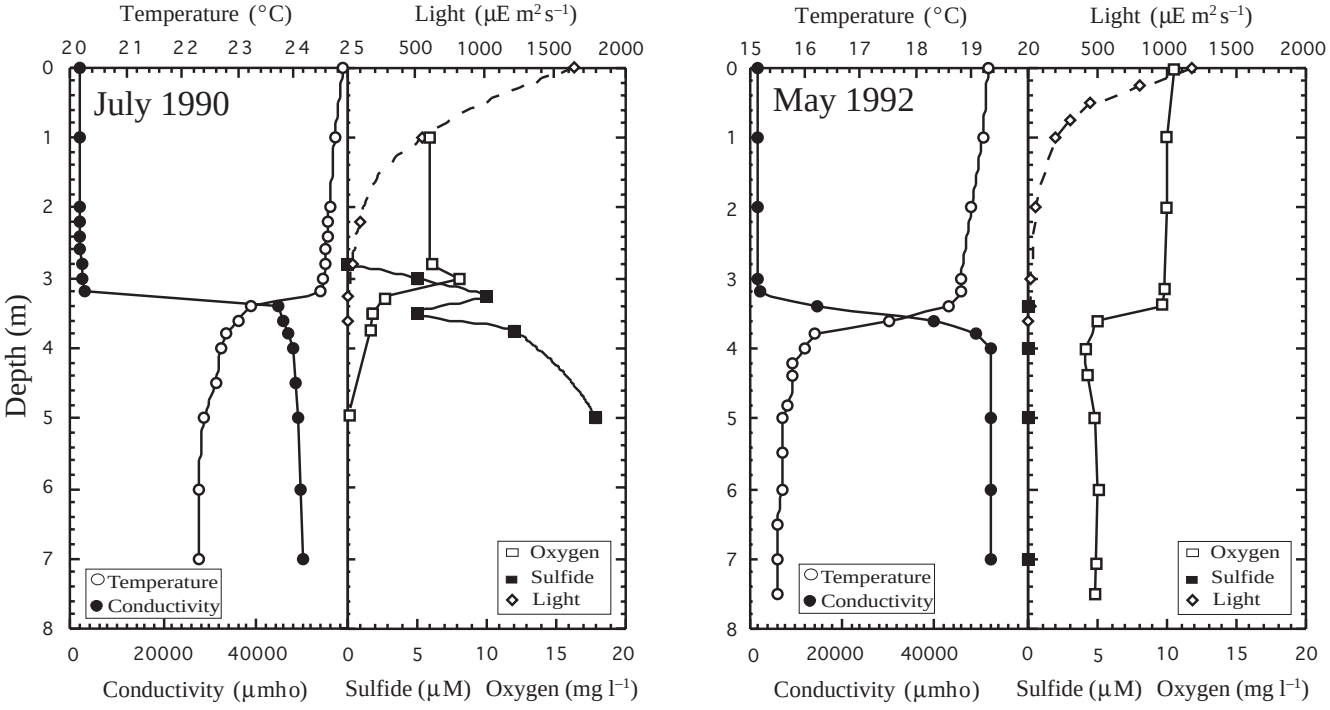

Fig. 1. Vertical profiles of temperature, conductivity (1 µmho = 1 µS cm^{-2}), sulfide concentration, oxygen concentration and light penetration in summer of 2 years (July 1990 and May 1992) in the Ebro River estuary

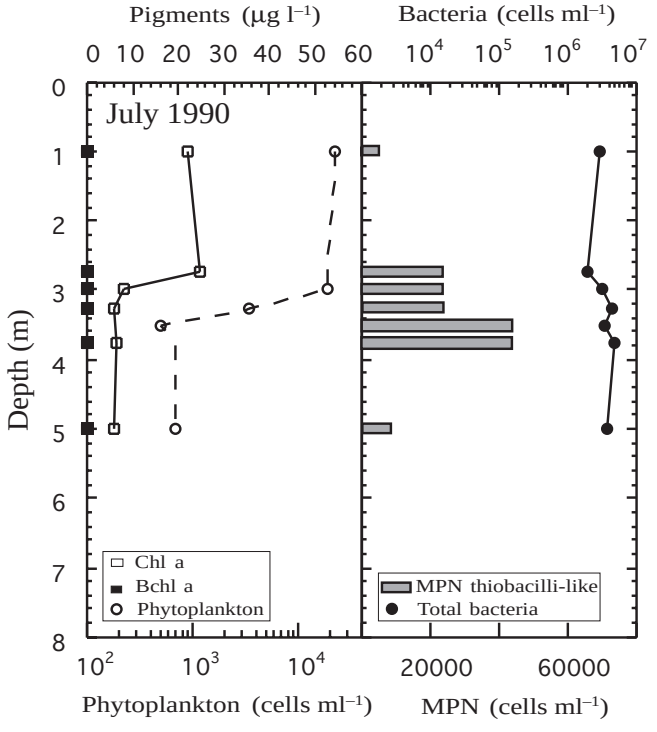

Fig. 2. Abundance of cells (phytoplankton and bacteria) and pigment concentration in July 1990. Abundance of chemolithoautotrophic sulfur bacteria is indicated as viable most probable number (MPN) counts. Bchl: bacteriochlorophyll

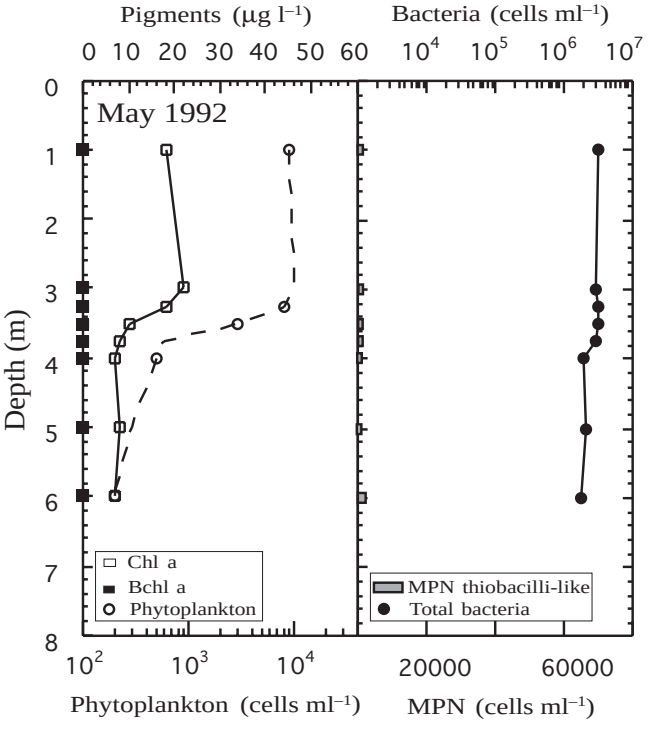

Fig. 3. Abundance of cells (phytoplankton and bacteria) and pigment concentration in May 1992. Abundance of chemolithoautotrophic sulfur bacteria is indicated as viable MPN counts

Table 2. Carbon dioxide fixation at the oxic/anoxic interface of the Ebro River measured during July 1989 and 1990. Activity has been partitioned into oxygenic, anoxygenic and dark. Values of several relevant environmental variables are also provided as a reference. nd: not determined

Date	Depth (m)	T (°C)	Oxygen (mg l^{-1})	Sulfide (µM)	Light (µE m^{-2} s^{-1})	Oxygenic (mg C m^{-3} h^{-1})	Anoxygenic (mg C m^{-3} h^{-1})	Dark (mg C m^{-3} h^{-1})
July 14 (1989)	3.2	24.5	5.7	nd	21.0	6.41	0.61	0.64
	3.8	23.5	0.6	nd	7.8	<0.2	<0.2	0.23
	4.8	22.5	0.0	nd	0.0	0.00	0.00	1.68
July 19 (1990)	3.0	24.7	10.0	4.9	nd	5.73	<0.2	0.29
	3.5	23.0	2.3	4.2	nd	<0.2	0.00	5.16
	4.0	22.8	2.1	12.0	nd	0.00	<0.2	1.09

trophic sulfur bacteria showed that these were present in the system and that they accumulated selectively at the oxic/anoxic interface (4×10^4 viable cells ml^{-1}). Counts in May 1992 revealed concentrations lower than 10^2 viable cells ml^{-1} (Fig. 3).

Microbial incorporation of CO_2 was measured at selective depths within the oxic/anoxic interface and was partitioned into oxygenic, anoxygenic and dark fixation (see 'Materials and methods'). The results for 1989 and 1990 are summarized in Table 2. Data from May 1992 are not shown because the oxic/anoxic interface was not yet established at that time, and carbon fixation in the dark was under detection limits. As expected from light-penetration profiles, CO_2 fixation by oxygenic phototrophs was significant above the interface (around 6 mg C m^{-3} h^{-1}), and was practically zero below the interface. Dark CO_2 fixation made a significant contribution at some depths, with values of >5 mg C m^{-3} h^{-1}. The integrated carbon fixation and the percent contribution of the different metabolisms to total carbon fixation at the oxic/anoxic interface are given in Table 3. On average, the hourly contribution of photosynthetic and chemosynthetic metabolisms to total carbon incorporation at the interface was similar. The dark CO_2 fixation rates measured at the oxic/anoxic interface ranged from 5.5 to 124 mg C m^{-3} d^{-1}. Thus, the average rate of primary production in the dark during the anoxic season in the Ebro River salt wedge was 42 mg C m^{-2} d^{-1} (Table 4). Because this is a light-independent process, it will proceed also during the night and, thus, dark fixation represents at least twice the contribution made by photosynthesis.

Table 3. Integrated carbon fixation (mg C m^{-2} h^{-1}) at the oxic/anoxic interface of the Ebro River and percent contribution to total fixation (in parentheses)

Date	Oxygenic	Anoxygenic	Dark	Total
14 July (1989)	1.4 (37%)	0.1 (2%)	2.3 (61%)	3.8
19 July (1990)	1.9 (58%)	0.2 (5%)	1.2 (37%)	3.3
Average	1.7 (48%)	0.2 (3%)	1.8 (48%)	3.6

DISCUSSION

The Ebro River salt wedge has a tendency to become anoxic as the spring season advances, resulting in the establishment of an oxic/anoxic interface in summer. This interface remains until the period of heavy rains, typical at the end of summer in the Mediterranean region, that increase the river discharge. Thus, the anoxic 'season' of the Ebro River normally lasts not longer than 4 mo (June to September). However, permanently low river flows were observed from July 1988 to April 1990, and this caused the continuous presence of a salt wedge for 18 mo (Ibàñez et al. 1996). Because of the highly turbid river waters, light limitation of photosynthetic activity was expected. However, under anoxic conditions reduced inorganic compounds are generated in the wedge and are mobilized to the oxic/anoxic interface. Here, these compounds may act

Table 4. Average dark carbon fixation at the oxic/anoxic interface of different oceanic basins, fjords and estuarine environments

System	Max. depth (m)	Oxic/anoxic interface interval (m)	Dark fixation (mg C m^{-2} d^{-1})	Source
Black Sea	2000	135–180	2.2	Sorokin (1972)
Cariaco Trench	1350	250–350	0.6	Tuttle & Jannasch (1979)
Saanich Inlet	228	110–140	9.2	Juniper & Brinkhurst (1986)
Saelenvann estuary	24	6.0–6.5	72	Indrebø et al. (1979)
Ebro River salt wedge	8	3.0–5.0	42	This study

as energy sources and electron donors for chemolithoautotrophic bacteria, which fix CO_2 in the dark and contribute to the primary productivity in the system. Microscopic and pigment observations indicated that phototrophic sulfur bacteria do not play a significant role in this environment. The absence of photosynthetic sulfur bacteria is probably due to the extremely low irradiance in the anoxic layer and to the low concentrations of sulfide. In the Ebro River salt wedge, dark fixation occurred at a rate of 42 mg C m^{-2} d^{-1}. Since this process continues during the night, dark fixation was the main process for primary production at the interface.

Aerobic oxidation of inorganic reduced sulfur compounds by chemolithoautotrophic sulfur bacteria has been reported as the main process linked to dark fixation in the chemocline of marine basins (Black Sea: Jørgensen et al. 1991). In the Ebro River salt wedge, sulfur- and nitrogen-reduced compounds are the most likely sources of reducing power. Viable counts lower that 10^2 cells ml^{-1} have been reported for autotrophic nitrogen-oxidizing bacteria in estuarine, coastal and oceanic waters (Billen 1975, Ward 1982, Owens 1986). We carried out viable counts for ammonium-oxidizing bacteria in May 1992. Unfortunately, the salt wedge had not yet developed anoxic conditions at that time and these bacteria were not detected (data not shown). Autotrophic sulfur-oxidizing bacteria, on the other hand, accounted for up to 4×10^4 cells ml^{-1} in the salt wedge during July 1990. This concentration of cells represented 1 to 2% of the total bacterial counts, a similar percentage to those reported for the Black Sea interface (Jørgensen et al. 1991). Because MPN counts underestimate the number of viable cells in the field by 1 to 3 orders of magnitude (Baker & Mills 1982), we can expect a higher contribution of sulfur-oxidizing bacteria to the natural assemblage at the oxic/anoxic interface. In fact, it is well known that many aerobic sulfur-oxidizing bacteria display positive chemotaxis toward such interfaces, and that they are able to form massive accumulations (Sorokin 1972, La Rivière & Schmidt 1982). It has been shown in cultured strains that specific growth rate, specific oxidation rates, carbon fixed per mole substrate oxidized, and affinity for oxygen are lower in ammonia oxidizers (Horrigan & Springer 1990) than in aerobic sulfur oxidizers (Kuenen & Bos 1987). If data from laboratory strains can be extrapolated to natural populations, sulfur oxidizers would easily outcompete ammonia oxidizers in marine oxic/anoxic interfaces (Ward 1984). In fact, field data have shown that the contribution of nitrifiers to dark CO_2 fixation decreased considerably in oxic/anoxic interfaces containing sulfide, accounting for less than 10% of the total dark fixation (Indrebø et al. 1979).

Coincidence between the maximal accumulation layer of aerobic sulfur oxidizers and the maximal dark CO_2 fixation activity, together with a minimum in the vertical concentration of sulfide (Fig. 1), strongly indicate that aerobic sulfur-oxidizing bacteria were the main organisms responsible for dark CO_2 fixation in the Ebro River. Coincidence between dark CO_2 assimilation and a minimum in the distribution of sulfur-reduced compounds has been also found in the oxic/anoxic interface of the Cariaco Trench (Tuttle & Jannasch 1973). This strongly suggests that dark CO_2 fixation is linked to utilization of reduced sulfur compounds in these interfaces. We cannot discard, however, a certain contribution (probably small) of autotrophic ammonia oxidizers (Indrebø et al. 1979) and heterotrophic aerobic and anaerobic bacteria (Tuttle & Jannasch 1979). In addition, phytoplankton can fix carbon in the dark through beta-carboxylation, and this process can be stimulated by ammonium in nitrogen-deficient algal cultures (Morris et al. 1971, Granum & Myklestad 1999). However, the distribution of dark carbon fixation was closer to the oxygen/sulfide distribution and the MPN counts of thiobacillus-like cells than to the distribution of phytoplankton. Moreover, the algae actually developed in the upstream freshwater reservoir. Therefore the phytoplankton contribution to dark fixation in the saline wedge is probably small.

Significant contributions of dark carbon fixation to primary productivity had been reported before for both marine basins and permanently stratified coastal systems. The rates measured in the Ebro River salt wedge (dark fixation up to 124 mg C m^{-3} d^{-1}) are the highest reported so far for the water column in marine oxic/anoxic interfaces. This can probably be explained by the fact that we used a special device for sampling such interfaces that allowed recovery of populations sharply stratified on the scale of centimeters. This high activity is limited strictly to the interface and results in an average rate of dark primary production during the anoxic season of 42 mg C m^{-2} d^{-1}. This value is higher than those reported for other marine systems with oxic/anoxic interfaces where dark fixation has been measured (Table 4), e.g. the Cariaco Trench and the Black Sea stagnant oceanic basins (Tuttle & Jannasch 1979, Jørgensen et al. 1991) and the permanently stratified but intermittently anoxic fjord-like embayment of Saanich Inlet (Juniper & Brinkhurst 1986). The permanently stratified Saelenvann estuary (Indrebø et al. 1979) yielded, on average, the highest integrated value. More data are needed in order to obtain a more robust estimation of dark fixation, but the values are well within the range reported for similar oxic/anoxic interfaces containing sulfide. In such interfaces, sharply stratified and highly active populations can be

missed when the sampling strategy is not on a fine enough scale. Because this process is likely to be important in other salt-wedge or highly stratified estuaries, the estimates of carbon fixation made so far for these systems may have been underestimated, and should therefore be revised taking into account the contribution of dark processes.

Acknowledgements. This work was financed by DGICyT grant PB87-0183 from the Spanish Ministerio de Educación y Ciencia. J.G.-C. was supported by a FPI scholarship from the Spanish Ministerio de Educación y Ciencia. During the writing of this work, E.O.C. was supported by MIDAS (MIcrobial Diversity in Aquatic Systems, MAS3-CT97-0154) from the European Union. We are grateful to N. Prat and C. Ibáñez from the Departament d'Ecologia, Universitat de Barcelona, for providing sampling facilities and nutrient data. The authors thank J. I. Calderón-Paz, R. Massana, J. Mir and C. Ibáñez for help during field work. The comments and suggestions from anonymous referees are greatly appreciated.

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Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Submitted: June 30, 2000; Accepted: November 11, 2000
Proofs received from author(s): May 30, 2001