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Community structure and photosynthetic activity of epilithon from a highly acidic (pH ≤ 2) mountain stream in Patagonia, Argentina

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Abstract We explored a benthic community living on stones in an acidic (pH ≤ 2) stream of active volcanic origin from Patagonia, Argentina, by combining in situ measurements (temperature, pH, conductivity, dissolved oxygen), photosynthesis of intact biofilms (measured with microsensors by the light–dark shift method), pure-culture experiments on isolated algae, and confocal laser scanning microscopy on the biofilms. The epilithon of the Agrio River was dominated (99% of total biomass) by one species: *Gloeochrysis* (Chrysophyceae). This species was observed as brown, mucilaginous, 200-μm-thick films on stones, growing in clumps in a dense matrix of fungal hyphae, bacteria, and inorganic particles held together by extracellular polymeric substances. *Gloeochrysis* was isolated and cultivated. The photosynthetic rate measured at saturation irradiance was 120 μmol oxygen (mg chlorophyll a)$^{-1}$h^{-1} under laboratory conditions, and the saturation rate of photosynthesis by carbon dioxide was 90 μmol oxygen (mg chlorophyll a)$^{-1}$ h^{-1} for oxygen evolution. Photosynthetic activity of the biofilm was light-dependent and saturated above 200 μmol photons m^{-2} s^{-1}. In the dark, the stone surface became anoxic. Our data suggest that primary production in the Agrio River was not limited by light, carbon, or phosphorus but instead, nitrogen-limited.

Keywords Acidic stream · *Gloeochrysis* · Oxygen fluxes · pH · Photosynthetic rate

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Introduction

Naturally acidic rivers are scarce worldwide (Hutchinson 1957) and are found mainly in volcanically active areas. Such systems have been studied in Japan, the former Soviet Union, New Zealand, and a few other regions (see details in Delmelle and Bernard 2000). However, the majority of limnological studies of acidic waters have focused on streams or lakes affected by acid mine drainage (Lackey 1939; Whitton and Diaz 1981; Geller et al. 1998) or by acid rain (Stumm 1985). Basically, the same ecological effects observed in acid mine drainage should be expected in naturally acidic rivers. Both systems are characterized by high concentrations of sulfuric acid, which originates from pyrite oxidation or volcanic activity. Very high concentrations of heavy metals are often also associated with extremely acidic waters.

Generally, the information available about periphyton communities in acidic aquatic environments comes from studies conducted on lakes (natural or contaminated). A few studies, however, have been carried out on streams. The changes in species composition, biomass, and structure of a periphyton community, following experimental acidification, were studied in a small Canadian stream (Planas et al. 1989). In addition, Satake et al. (1995) described several aspects of naturally acidic rivers from Japan.

Many acidic environments are characterized by benthic algal communities with low diversity (Whitton and Diaz 1981) and high productivity (Sheath et al. 1982; Planas et al. 1989). Both in natural or contaminated environments, the most widespread algal species are *Euglena mutabilis* Schmitz, followed by naviculoid diatoms [*Pinnularia acoricola* Hustedt, *Eunotia exigua* (Bréb. ex Kütz.) Rabh., *Nitzschia* spp.], *Gloeochrysis turfosa* (Pascher) Bourrelly, *Zygogonium*, and *Chlamydomonas* spp. (Lackey 1939; Whitton and Diaz 1981; Sheath et al. 1982; DeNicola 2000). In the littoral zone of experimentally acidified lakes, periphyton communities are mainly dominated by filamentous green algae from the family Zygnemataceae (e.g., Turner et al. 1987).

Furthermore, phototrophs in acidic aquatic environments all seem to be subject to carbon limitation (e.g., Schindler and Holmgren 1971; Turner et al. 1987; Gross 2000). Because of the low pH (<4), carbon dioxide exists as molecular carbon dioxide and carbonic acid which, when regulated by Henry's law, will not exceed concentrations of 0.31 mg carbon l^{-1} (Satake and Saijo 1974). At such low carbon concentration, autotrophic growth is potentially limited, and the advantages of a mixotrophic nutritional strategy are enhanced (Nixdorf et al. 1998).

In some acidic habitats, the high metal concentrations in the water seem to limit algal development due to toxicity. It was also noted that metal hydroxide deposition could change the physical conditions of the substrate, leading to a reduction of periphyton growth on rocks (Niyogi et al. 1999).

With respect to the evolution and adaptation of the organisms, natural and contaminated environments differ. Naturally acidic waters are older ($>10{,}000$ years) than contaminated sites (100 years) and, consequently, in natural systems a close coadaptation between organisms could have occurred. For example, Nakatsu and Hutchinson (1988) described a mutualism between *E. mutabilis* and associated yeast, growing in naturally acidic tundra ponds ($pH \leq 2$). This mutualism confers benefits to both partners because they are more resistant to the heavy metal concentrations and, thus, able to live in an environment that is toxic to each organism individually.

In this paper, the distribution and ecology of phototrophic organisms living in a naturally extreme acidic river ($pH \leq 2$) were studied. We report the physical conditions in the river, the concentration of the major nutrients and some elements, and the photosynthetic activity of the epiphytic community. The relationship between the water chemistry of the Agrio River and the main algal species growing on the rock bed and stones is discussed, presenting original information from a poorly understood aquatic environment.

Materials and methods

Study area

The Agrio River is located in the Copahue-Caviahue Provincial Park (37°53′S, 71°02′W) in the Andean area of the north-central zone in the Province of Neuquén, Argentina (Fig. 1). It is one of the main tributaries of Lake Caviahue, and it is the source of the lake acidity (Pedrozo et al. 2001). The headwaters of the Agrio River (2,740 m a.s.l.) are located on the slopes of the Copahue Volcano (2,953 m a.s.l.) and form a delta when joining Lake Caviahue (1,610 m a.s.l.). The river is 13.5 km in length (slope = 8.37%), and its mean annual discharge is 2.24 $m^3 s^{-1}$. The three main tributaries of Agrio River are (data from a single measurement in January 2000) the Rojo River ($pH = 3.63$, conductivity = 890 $\mu S\ cm^{-1}$) and the Jara River ($pH = 5.81$, conductivity = 445 $\mu S\ cm^{-1}$), both on the north bank, and the Blanco River ($pH = 5.93$, conductivity = 290 $\mu S\ cm^{-1}$) on the south bank.

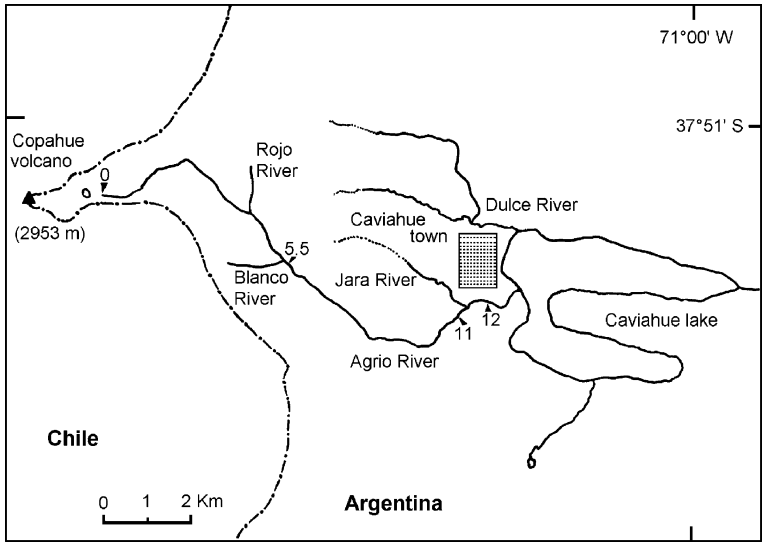

Fig. 1 Location of the study sites along the Agrio River. *Numbers* on the river indicate kilometers from the source of the river. Sampling sites: *11* 11 km, *12* 12 km

Sample collection

In April 2000, taking into account the information provided by the survey made during January 2000 (Pedrozo et al. 2002), two sites along the Agrio River (11 and 12 km from its source, Fig. 1) were selected for in situ measurements, water sampling, and epilithon collection. Measurements of temperature, conductivity (conductimeter Orion 135, Orion Research, USA), and pH (pHmeter Orion 265) were made. Conductivity was corrected to 25°C. This correction function differed markedly from standard functions (e.g., APHA 1992), but it was determined by direct measurement of triplicate samples, following the protocol of Schimmele and Herzsprung (2000). All water samples were stored in plastic bottles and kept in the dark and cold (4°C) until analysis. Only the time series for electrical conductivity, pH, dissolved oxygen, and temperature were measured in January 2000 (OCEAN SEVEN 316 multiparameter probe, Idronaut, Italy).

Epilithon samples were collected in the main channel of the Agrio River. A 10-cm^2 surface area ($n = 2$) on selected stones was scraped using a sampler (Snoeijs and Snoeijs 1993) and immediately preserved with formalin (4%). All samples were transported from the field site and processed within a few hours.

Laboratory analyses

The analyses of sulfate, chloride, soluble-reactive phosphorus, heavy and trace metals were conducted on samples prefiltered through 0.2-μm (cellulose acetate) membrane filters (Schleider and Schuell, Germany). Heavy and trace metals were subsequently fixed with 60 μl concentrated HNO_3. Samples for silicates, nitrates, nitrites, ammonium, and total nitrogen analysis were fixed with $HgCl_2$ (1%), and those for total phosphorus were fixed with H_2SO_4 (1:4). Nutrients were determined colorimetrically by a segmented flow analyzer; chloride and sulfates by ion chromatography; aluminum by ICP-OES; cadmium by ICP-MS; and iron, manganese, zinc and nickel by total reflection X-ray fluorescence spectroscopy (Schwenke et al. 1999).

In order to quantify algal density, 50 μl of the sample from the stones was placed between a slide and coverslip and counted under an epifluorescence microscope (model BX40, Olympus, Japan), using blue-light excitation (filter WU: excitation light 420–490 nm, 50 W mercury light source; Booth 1993). Because of the highly patchy distribution of the algae on stones, six cross sections of the coverslip (length of the cover, 32 mm) were evaluated. The counting was carried out in duplicate and density was expressed in cell cm^{-2}.

Cell volume was calculated using BIOVOL, version 2.1, software (created by D. Kirschtel, University of Vermont), using 15 measurements of cell dimensions for each species, determined microscopically (at 1,000× magnification, model BX 50, Olympus). Biomass was estimated as biovolume and expressed as fresh weight (μg cm^{-2}), using a conversion factor of 10^6 $\mu m^3 = 1$ μg (Wetzel and Likens 1991).

A strain of *Gloeochrysis* Pascher (1925) was isolated from the stones collected from the Agrio River and maintained in sterile (pH∼2) Satake Medium (Satake and Saijo 1974). The cultures were grown under aeration at 20°C and constant illumination (∼90 μmol photon $m^{-2}s^{-1}$). Photosynthetic rate was measured in two experiments (three replicates each) as the change in oxygen concentration, using a membrane-covered polarographic oxygen electrode (Clark-type oxygen electrode, Digital Model 10, Rank Brothers, Bottisham, Cambridge, UK). Photosynthetic light-response curves were measured at the following PAR: 5, 10, 15, 20, 25, 30, 40, 60, 80, 100, 140, and 200 μmol photons m^{-2} s^{-1}. Photosynthetic capacity and initial slope of the *P* versus *I* curve (α) were estimated by least squares fitting of the Michaelis–Menten equation (Solver software, Excel). Net oxygen evolution versus carbon dioxide was measured for incremental predetermined amounts of $NaHCO_3$ 3 mM. For chlorophyll a determination, 2 ml culture suspension used in the photosynthesis measurements was processed following Maberly et al. (2002), and fluorescence was read in a Turner Design 10-AU fluorometer (Turner Designs, Sunnyvale, Calif., USA). Apparent photosynthetic half-saturation constant (K_m) was estimated by the least squares method.

Microelectrode measurements

Photosynthetic activity at the surface of stones was measured with oxygen microelectrodes, using the light–dark shift technique (Revsbech et al. 1981). Stones were taken from the river and incubated in a bucket containing river water. The water was continuously aerated and mixed by an aquarium air pump. The microelectrodes (tip diameter < 30 μm, MASCOM, Bremen, Germany) were positioned using a micromanipulator and a dissection microscope. A cold light source (model KL200, Zeiss, Jena, Germany) was used for illumination. The decrease in oxygen concentration after shading the sample was recorded by a strip chart writer and converted to a photosynthetic rate in mmol $dm^{-1}h^{-1}$. This volume-related rate was converted to an area-related rate by multiplying by the biofilm thickness. The thickness of the biofilm was determined with a confocal laser scanning microscope. Due to the limited spatial resolution of the photosynthesis measurements (100 μm) (Revsbech and Jørgensen 1983) and the thickness of the biofilm (200 μm), it was not possible to measure photosynthesis at different depths within the biofilm. Instead, the electrode tip was positioned as near as possible to the stone surface. Oxygen gradients at the stone surface under light and dark conditions were measured by moving the electrode stepwise away from the stone (Table 1). From the oxygen gradients in the diffusive boundary layer above the surface of sediment and

Table 1 Oxygen fluxes under light and dark conditions and gross photosynthesis calculated from oxygen microprofiles and light–dark shift measurements (nmol cm^{-2} s^{-1}). The light intensity for photosynthesis measurements was 200 μmol photons m^{-2} s^{-1}. *ND* Not determined

Site	Oxygen flux—light	Photosynthesis	Respiration light	Oxygen flux—dark
Stone 1, Agrio River	0.003 ± 0.001	0.061	0.064	ND
Stone 2, Agrio River	0.006	0.071	0.077	0.005 ± 0.001

stones, oxygen fluxes were calculated (Revsbech and Jørgensen 1986) according to:

$$J_D = -D(dC/dz)$$

J_D = diffusive flux (μmol cm^{-2} s^{-1}), D = Diffusion coefficient for oxygen in water = 2.3×10^{-5} cm^2 s^{-1} (Lerman 1988), dC/dz = the measured concentration gradient (μmol l^{-1} cm^{-1}).

Oxygen consumption in the light was taken as the sum of gross photosynthesis and oxygen flux into the sample in the light. Light was measured using a LICOR quantum sensor (LI190SB, Logan, USA). All light measurements were made outside the incubation vessel and were carried out at 17°C, which represented a typical in situ summer afternoon temperature.

Confocal laser scanning microscopy

The biofilms on the stones were examined by confocal laser scanning microscopy (CSLM) in the fully hydrated state without fixation or embedding. After selecting stones with an appropriate indentation, a reservoir was made by using Scrintec 600 silicon rubber (Roth, Karlsruhe, Germany). All stains were performed in the small reservoir on the stones. In order to apply fluorochromes, the pH of the river water was adjusted to pH 3 with NaOH.

The nucleic acid-specific stain SYTO 9 (Molecular Probes, Eugene, Ore., USA) was used at a concentration of 1 μl ml^{-1} to record bacterial cell distribution. CellTracker Green (Molecular Probes) was employed at a concentration of 1 μl ml^{-1} to stain fungi. The tetramethyl rhodamine isothiocyanate (TRITC)-labeled lectin from *Urtica dioica* (EY Laboratories, San Mateo, Calif., USA) was used at a concentration of 0.1 mg ml^{-1} to stain glycoconjugates according to Neu and Lawrence (1999).

CSLM was performed with a TCS SP (Leica, Heidelberg, Germany) attached to an upright microscope and equipped with an argon, krypton and helium–neon laser. The biofilms were observed with 20×0.6 NA and 63×0.9 NA water-immersible lenses. Reflection images were taken at 568 nm excitation and recorded at 568 nm. The following settings were used: in the green channel for CellTracker Green, excitation = 488 nm and emission = 515–545 nm; for SYTO 9, excitation = 488 nm and emission = 500–550 nm; in the red channel for the TRITC-lectin, excitation = 568 nm and emission = 580–625 nm; in the far-red channel for the autofluorescence of chlorophyll-containing organisms, excitation = 647 nm and emission = 650–800 nm. Furthermore, the settings of the red and far-red channel in combination were used to record the autofluorescence of cyanobacteria.

Micrographs were prepared using the standard software ScanWare, version 5.1A (Leica), and Imaris, version 3.03 (Bitplane, Zurich, Switzerland). The optical sections were presented as maximum intensity projection or XYZ presentation and printed from PhotoShop 5.0 (Adobe, Edinburgh, UK).

Results

Data from in situ measurements are shown in Fig. 2. It can be seen that there is a high daily variability not only in temperature, but also in all of the environmental variables measured. In situ oxygen concentration was inversely related to temperature. In contrast, conductivity was more closely dependent on the daily variation in water flow than on the temperature. When the flow decreased (at night and during the morning), mineral deposition occurred at the banks of the river. When the flow increased (due to snowmelt) the mineral deposits resolubilized and, consequently, conductivity increased. The pH variation was also related to water flow. Because the deposition and resolubilization in the Agrio River are mainly due to sulfate, sulfuric acid formation could be responsible for the observed drop in pH when conductivity reached its maximum.

High phosphorus and low nitrogen concentrations, mainly as ammonium (mass total nitrogen:total phosphorus < 0.1), were measured in the Agrio River waters (Table 2). Sulfate was the dominant ion, followed by chloride. The major elements were aluminum and iron, as well as trace metals (e.g., manganese and zinc; Table 2).

Gloeochrysis was observed only as brown mucilaginous films on stone surfaces. Under the light microscope, the algae were observed growing in clumps with numerous fungal hyphae and organic matter. Cells were spherical to ellipsoidal in shape with a parietal, single, cup-like chloroplast. Cells were mostly 2.5–4.5 μm wide and 3–6 μm long, with an average biovolume of 44 μm^3. Chrysophyceae abundance and biomass were lower just before the entrance of the Jara River tributary (11 km) compared to the downstream site (12 km). The mean densities (± standard deviation) of *Gloeochrysis* were 373 (± 112)×10^3 cell cm^{-2} at the 11 km site and 2,103 (± 361)×10^3 cell cm^{-2} at the 12-km site, while the biomass was 16.3 (± 4.9) μg fresh wt. cm^{-2} and 92.1 (± 15.8) μg fresh wt. cm^{-2}, respectively.

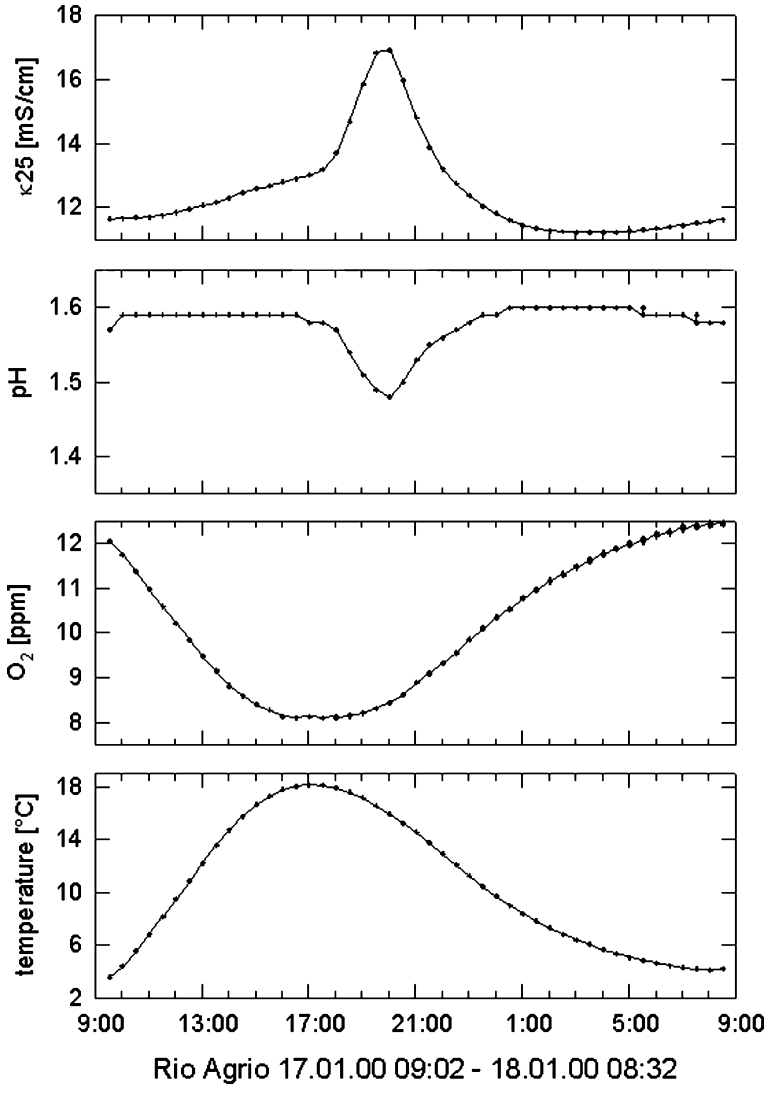

Fig. 2 Time series of electrical conductivity ($\kappa 25$), pH, dissolved oxygen, and temperature in the Agrio River upstream of the Jara River in January 2000

Table 2 Macro- and micronutrient concentrations in mg l^{-1} (except acidity in mmol l^{-1}) of Agrio River water at both sampling sites (referred) as distance from the source

	11 km	12 km
Total phosphorus	2.8	2.3
Soluble reactive phosphorus	2.8	2.3
Total nitrogen	< 0.2	< 0.2
Nitrate	0.027	0.024
Nitrite	< 0.005	< 0.005
Ammonia	0.086	0.069
Dissolved inorganic carbon		0.21
Acidity	72	72
Sulfate	2,460	2,190
Chloride ions	421	378
Aluminum	137	125
Iron	57	53
Silicon	26.4	25.8
Manganese	2.61	2.60
Zinc	0.16	0.13
Nickel	0.06	0.05
Cadmium	$4.8\ 10^{-3}$	$4.1\ 10^{-3}$

The photosynthesis–light response curve under laboratory conditions (Fig. 3) was constructed by plotting the biomass-specific rate of net oxygen exchange against irradiance and showed a light-limited region until an irradiance of 80 μmol photons $m^{-2}s^{-1}$ and initial slope (α) of 2.5. The maximum rate of light-saturated photosynthesis was 110 μmol oxygen (mg chlorophyll a)$^{-1}$ h^{-1}, presenting photoinhibition over 150 μmol photons m^{-2} s^{-1}. The net oxygen evolution strongly depended on the carbon dioxide concentration in the external medium (Fig. 4). Saturation of photosynthesis by carbon dioxide was reached between 60 and 100 μM carbon dioxide, where the rate was 90 μmol oxygen (mg chlorophyll a)$^{-1}h^{-1}$ for oxygen evolution. The apparent K_m uptake was found to be about 7.5 μM carbon dioxide.

Microelectrode measurements

We were able to measure photosynthesis at the surface of the stones. The oxygen concentration measured at the

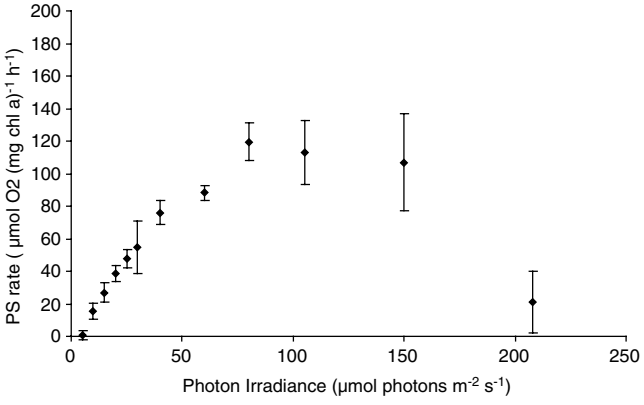

Fig. 3 Photosynthetic rate vs. photon irradiance of *Gloeochrysis* in the laboratory, isolated from Agrio River. *Error bars* show one standard deviation

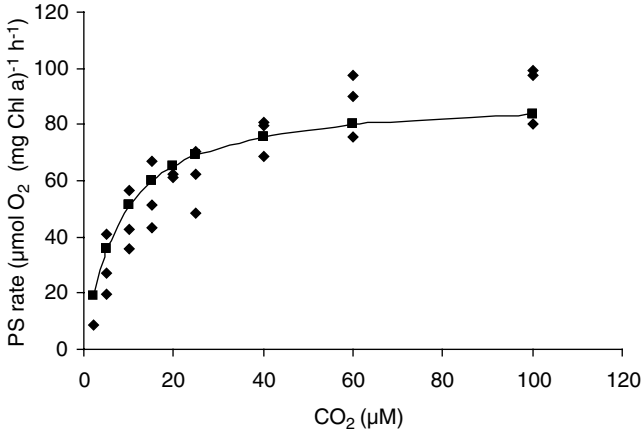

Fig. 4 Photosynthetic rate (*PS rate*) vs. carbon dioxide (CO_2) for *Gloeochrysis*. Curve fitting was performed by least squares fitting of the Michaelis–Menten equation

surface of the stones immediately decreased linearly when placed in the dark, indicating that the sensor tip was indeed positioned in the photosynthetic active biofilm (Revsbech and Jørgensen 1983). Photosynthetic activity was light dependent and saturated above 200 µmol photons $m^{-2}s^{-1}$ (Fig. 5). Triplicate measurements on two different stones gave similar results. On the stones, the oxygen consumption in the light was approximately one order of magnitude higher than in the dark. In the dark, the stone surface became anoxic (Fig. 6). However, it was difficult to see the exact position of the sensor tip at the stone surface; thus, the nearest position of it to the stone was not zero, but some micrometers above the stone surface. This means that the anoxic zone was somewhat larger than shown in Fig. 6.

CLSM

Direct CSLM without staining in the reflection and fluorescence mode showed biofilm layers in the range of

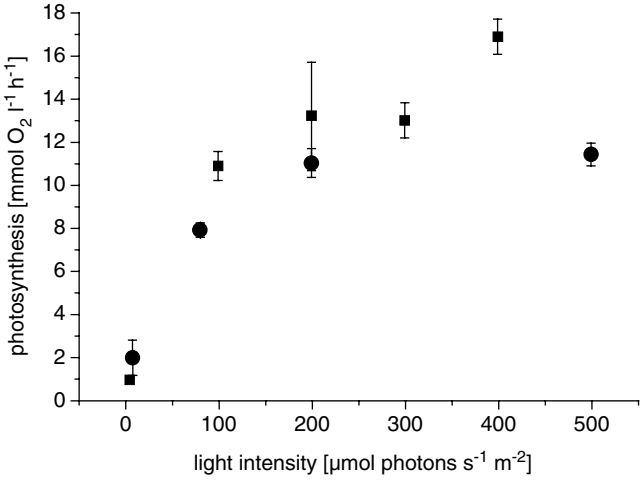

Fig. 5 Photosynthetic activity in the epilithic biofilm of two different stones (*filled circles* and *filled squares*) under different light intensities. Data are the mean and standard deviation of at least three replicate measurements

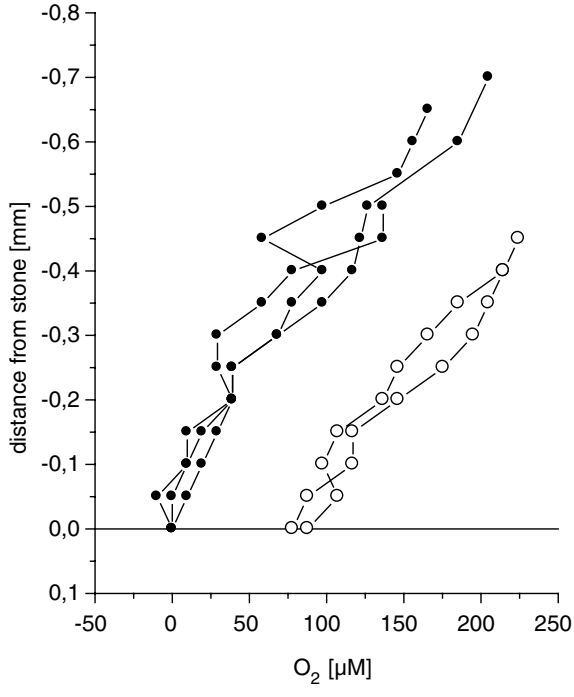

Fig. 6 Concentration of oxygen at different distances from the stone surface under light (*open circles*) and dark (*filled circles*) conditions

30–60 µm, with a significant mineral component. Within these layers, distinctive autofluorescence signals from algal colonies could be detected in the far-red channel. The algal colonies were densely packed and embedded in the mineral particles (Fig. 7a). Often smaller cells were scattered throughout the biofilm, showing a pink autofluorescence signal due to the overlay from the signals of the red (red-colored) and far-red (blue-colored) channel

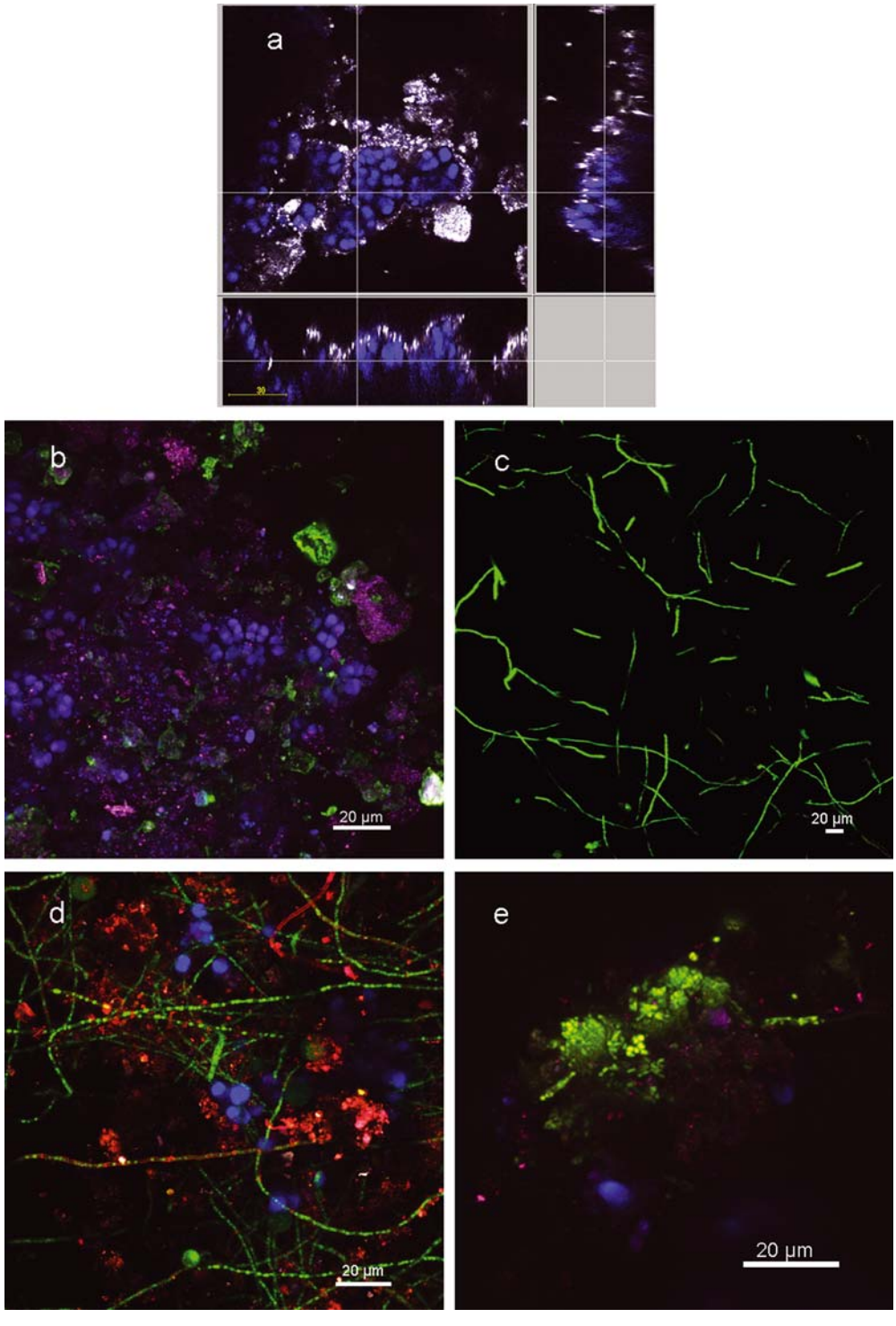

Fig. 7a–e Confocal laser scanning microscopy of living, fully hydrated epilithic biofilms from the highly acidic (pH < 2) Agrio River. **a** XYZ presentation of algal microcolonies embedded in mineral particles. Color allocation is reflection (*white*) and chlorophyll autofluorescence (*blue*). **b** Maximal intensity projection showing the autofluorescence of different phototrophic microcolonies (*blue* and *pink*). The green signal may originate from the autofluorescence of mineral particles. **c** Maximal intensity projection demonstrating fungal filaments at low magnification after staining with CellTracker Green. **d** Maximal intensity projection showing the microbial community consisting of algae due to their autofluorescence (*blue*), fungi after staining with SYTO 9 (*green*), and glycoconjugates stained with *Urtica dioica* lectin (*red*). **e** Maximal intensity projection of bacterial colonies stained with SYTO 9 (*green*), the other signals derive from the autofluorescence of phototrophic cells (*blue* and *pink*)

(Fig. 7b). Furthermore, the filamentous distribution of mineral particles indicated filamentous structures, possibly fungi. CellTracker Green, a stain that undergoes a glutathione S-transferase reaction inside the cell, clearly showed filamentous, branched structures (Fig. 7c). In addition, the nucleic acid-specific SYTO 9 stain confirmed that fungi formed a substantial part of the biofilms (Fig. 7d). The fungal filaments extended up to 100–200 µm from the stone surface (Fig. 7c, d). The lectin stained glycoconjugates within the extracellular polymeric substance (EPS) matrix (Fig. 7d), as well as the surface of some fungal filaments (data not shown). SYTO 9 was intended to stain bacterial cells; however, only a few bacterial microcolonies were detected within

the complex arrangement of biofilm constituents (Fig. 7e).

Discussion

According to the available information and our results, it is clear that sulfate, originating from sulfuric acid generated in the Copahue Volcano, is the main factor controlling the acidity of the water in the Agrio River (Pedrozo et al. 2001). This is an extreme environment not only due to the low pH value but also to the high concentration of heavy metals. Wide daily fluctuations in the physical and chemical variables (Fig. 2) also have a profound effect on the algal composition, biomass, and productivity.

Environmental conditions for algae are highly variable, not only in the longitudinal direction of the river (unpublished data), but also daily (Fig. 2) and, of course, seasonally. Along the lower stretch of the Agrio River (stream >1 km), the diurnal variability may be higher than the variability within the stream itself at a certain time of day. This means that the environmental parameters observed during sampling, compared to those at another site, only partly reflect local characteristics and may be due to the time of sampling. Furthermore, seasonal climate variations and the relationship between acidic volcanic outflow and snowmelt play a significant role.

Heavy metal concentrations measured during this study agreed with levels for acidic water bodies in volcanic areas presented in the literature (Sheath et al. 1982), but in general they were higher than the values recorded in acidic mine tailings or mining lakes (Nixdorf and Kapfer 1998; Lessmann et al. 1999; Niyogi et al. 1999). When compared with the mean heavy metal concentrations of Patagonian neutral lakes (Markert et al. 1997), those in the Agrio River are several times higher (between 20 and 2,000, depending on the element). Some heavy metals are essential trace nutrients for algae, but at high concentrations, they can be toxic.

In the Agrio River, the concentration of ammonia was three times higher than nitrate. Nitrification was likely inhibited by the low pH as described in other acidic lakes or streams (Schindler 1985; Rudd et al. 1988). However, the Agrio River has an unusually high phosphorus concentration—mainly dissolved—and thus, the algae are more likely to be nitrogen-limited (Beamud 2001). The high phosphorus concentration is a consequence of the local geology (predominant rocks are andesites) and the volcanic activity in the catchments area (Pedrozo et al. 2001).

It is assumed that in acidic aquatic systems, algal growth is limited by inorganic carbon due to its low solubility (Ohle 1981; Stumm and Schnoor 1995; Nixdorf et al. 1998). In an acidic, volcanic lake studied by Satake and Saijo (1974), carbon concentrations were from 5–40 times higher than expected from solubility calculations. The extra carbon coming from fumarole activity, microbial decomposition of organic matter, and fungal respiration explained the oversaturation. The value of the dissolved inorganic carbon concentration (17.5 μmol carbon l^{-1}) in the Agrio River was slightly higher than the calculated concentration of free carbon dioxide (14 μmol carbon l^{-1}, in equilibrium with the atmosphere, at 10°C and an altitude of 1,600 m). That is more than double of what *Gloeochrysis* needs to reach the concentration of carbon dioxide ($K_m = 7.5$ μM carbon dioxide) where the photosynthetic rate is half of its maximum value, under laboratory conditions. Our microscopic observations confirmed the abundant presence of fungal hyphae coupled with algae, suggesting another possible carbon source from this association. Although further research should be performed to clarify if carbon is a limiting factor of the primary production in the Agrio River, there are some indications that inorganic carbon is unlikely to limit growth rate.

Ambient light intensities on a cloudy day were around 250 μmol photons $m^{-2}s^{-1}$ at the water surface in the Agrio River. Therefore, photosynthesis was not limited by light. Overall, our results suggest that carbon, phosphorus, or light might not limit algae in the Agrio River.

The biofilm on the surface of the stones in the Agrio River was dominated by the chrysophyte *Gloeochrysis* sp. According to the literature, the taxonomic identification of this species from the Agrio River does not match (cell size and pirenoid absence) any of the four known species of this genus: *G. pyrenigera* Pascher, *G. turfosa*, *G. apyrenigera* Geitler, or *G. montana* Kalina (Pascher 1925, 1931; Geitler 1967; Kalina 1969). Of these species, only *G. turfosa* was reported to occur in highly acidic waters (pH = 1.8) and was associated with decomposing matter (Whitton and Diaz 1981). The Chrysophyceae biomass in the Agrio River was one order of magnitude lower than the total epilithon biomass in acidified (pH = 5) Lake 302S (Turner et al. 1991).

Shear stress is very high on stone surfaces in mountain streams. As a consequence, the epilithic biofilm observed was thin and tightly packed. The observed close association of algae, fungi, and mineral particles we observed provides a rigid matrix that can withstand these shear forces. Staining with a specific lectin applicable at low pH showed the presence of glycoconjugates. These may be involved as part of the EPS matrix in the stability of the biofilm as well as in sorption of dissolved nutrients. On the other hand, the entrapment of particulate organic matter is probably not an important nutrient source under such extreme conditions. We assume that the carbon supply from organic substrates is not as ideal as in the sediment (Wendt-Potthoff and Koschorreck 2002); thus, conditions for bacteria are not very good at the surface of stones.

Gloeochrysis in the Agrio River is growing in association with fungi (Fig. 7c,d), which may favor the development of both organisms in such an extreme environment. The association could be of double benefit for the algae, because a microenvironment is created that has a low concentration of metal ions (Nakatsu and Hutchinson 1988) and an increased carbon dioxide availability for photosynthesis (Satake and Saijo 1974; Turner et al. 1987). The algae can take up carbon dioxide that is excreted by the heterotrophic fungi directly. This prevents loss of carbon and other nutrients from the epilithic biofilm. The data suggest a close coupling between autotrophic and heterotrophic processes in the Agrio River. This coupling was not only functional but also structural, as indicated by the laser microscopy data. This interpretation differs somewhat from the common view of the function of epilithic biofilms because "normal" streams are high in total inorganic carbon, but are low in dissolved organic carbon. Under such conditions, autotrophic algae provide organic substrates for heterotrophic organisms in the biofilm (Haack and McFeters 1982). In the Agrio River, autotrophic carbon fixation was low in the epilithic biofilm. The gross photosynthetic rates measured on the surface of the stones were low compared to other habitats (Glud et al. 1992). The main reason for the low values measured may be the fact that the biofilms were no thicker than 200 μm. CLSM showed a signal in the red (e.g., phycocyanin, phycoerythrin) and far-red (chlorophyll) channel, resulting in a pink overlay (Fig. 7b), indicating the possibility of cyanobacteria presence. However, this has not been reported before, and these cells may have their origin from other neutral side arms of the Agrio River (e.g., the Jara River).

We never observed a net production of oxygen on the stones, as was expected and noted in the only published study of oxygen fluxes at stone surfaces (Glud et al. 1992). Oxygen consumption at the stone surface always exceeded oxygen production, resulting in a net uptake of oxygen by the biofilms on the stones. We have no field data to compare oxygen fluxes for the stone biofilms with laboratory measurements. The thickness of the diffusive boundary layer, and thus the flux of oxygen, depended strongly on the flow velocity of the water (Jørgensen and Revsbech 1985). The flow environment around the stones in the river was very heterogeneous. To discuss the flux data from the stones, we require additional measurements under in situ conditions or at the stone surfaces under different flow velocities. Nevertheless, we could show that under low flow conditions, the surface of the stones became anoxic. Such conditions might exist in zones of very low current at the banks of the river. Fluctuations of the oxygen concentration in the biofilm were further enhanced by the strong diurnal temperature fluctuations, which resulted in a fluctuating oxygen concentration in the river water. Under such conditions, facultative anaerobes have an advantage. It is also possible that a diurnal redox cycling of, for example, iron, occurs in the biofilms. Under oxic conditions Fe^{2+} can be oxidized to Fe^{3+} by iron-oxidizing bacteria, while in the dark, iron is reduced by iron-reducing anaerobes. Further research is necessary to clarify whether anoxia at the stone surface occurs in situ.

The epilithic algae appeared to be adapted to the light conditions in the river. Saturation of photosynthesis occurred at 150 μmol photons $m^{-2}s^{-1}$ for the isolated species in culture conditions and 200 μmol photons $m^{-2}\ s^{-1}$ for the biofilm, which is slightly below ambient light intensity on a cloudy day.

In the Agrio River, the oxygen consumption was much higher in the light than in the dark. There are several possible explanations for these observations and all probably play a role: (1) respiration is limited by oxygen diffusion, (2) during photosynthesis much more oxygen is available, (3) in the dark the oxygenated zone is smaller and thus some organisms are not in contact with oxygen, and (4) photorespiration and/or light induced chemical oxidation of organic matter.

Phototrophic primary production in the Agrio River was probably not limited by carbon dioxide. There may be a close cycling of carbon within biofilm communities, which partially uncouples the high carbon turnover in the biofilm from the carbon cycle in the running water. An extra input of carbon can be expected from the decomposition of organic matter and respiration of organisms. The activity of algae is almost certainly more important for the ecosystem function than can be seen from nutrient measurements in the water. The fact that the biofilms at the surface of stones were a net sink of oxygen even under illumination suggests that the Agrio River could be primarily a chemotrophic system, which depends on the allochtonous input of organic matter and/or reduced iron as energy source.

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