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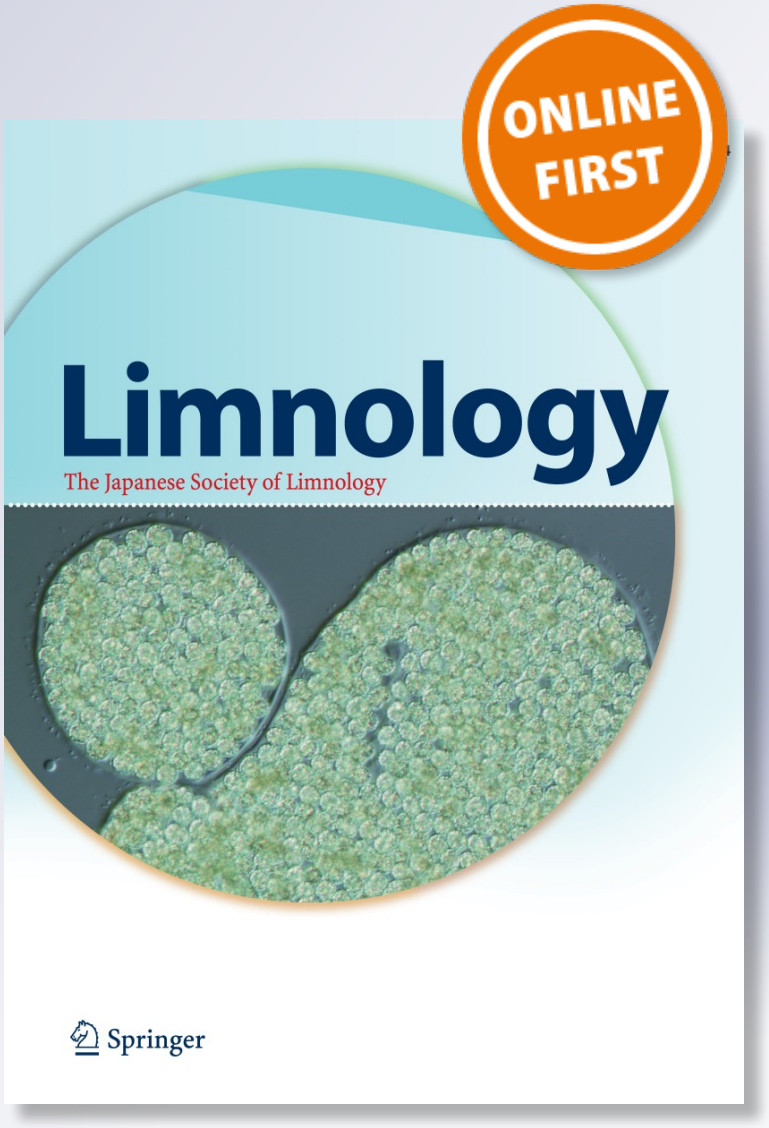
Limnology
The Japanese Society of Limnology

ISSN 1439-8621

Limnology
DOI 10.1007/s10201-014-0427-2



Springer

Limnology
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RESEARCH PAPER

Utilisation of organic compounds by osmotrophic algae in an acidic lake of Patagonia (Argentina)

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Received: 17 May 2013 / Accepted: 3 February 2014

Abstract We investigated whether algal osmotrophy in naturally acidic Lake Caviahue is an important process for acquisition of organic carbon and organic nitrogen. To accomplish this, we quantified algal assimilation of organic compounds, measured the specific growth rate and biomass yield, and documented incorporation of organic compounds by phytoplankton in situ using microautoradiography. Substrate uptake quantification and microautoradiographic investigations were performed using ^{3}H-leucine, ^{3}H-glucose, ^{3}H-thymidine, ^{14}C-aspartic acid, ^{14}C-acetic acid and ^{14}C-bicarbonates. The results showed that the most important species of the phytoplankton community, *Keratococcus rhaphidioides* and *Watanabea* sp., took up various sources of organic carbon and nitrogen under both light and dark conditions. They were also able to assimilate leucine, thymidine, aspartic acid and acetate under high levels of inorganic nitrogen and phosphorus, while they could use leucine, arginine, glutamine and glucose under low levels of nitrogen and phosphorus. The assimilation rates were higher in light than in darkness, and the algal specific growth rates increased when organic sources were added. We proposed that osmotrophy complements the main photosynthetic process of the phytoplankton in Lake Caviahue, which helps to overcome the scarcity of light and inorganic nitrogen and carbon in the water column.

Keywords Osmotrophy · Acidic lake · *Keratococcus*

Introduction

Organisms are generally photoautotrophs or heterotrophs (Jones 1994); however, mixotrophs are organisms able to facultatively supplement photoautotrophy with heterotrophic carbon intake (Falkowski and Raven 2007). Mixotrophy has been shown to play an important role in the microbial food web of aquatic ecosystems (Stoecker 1998). This may be due to the ability to use more resources for nutrient, carbon or energy requirements than a strict photo- or strict heterotrophic organism. When there are insufficient organic resources, a mixotrophic organism can switch to photosynthesis for its carbon and inorganic nutrients for its nutrition, then switch back to hetroptrophy under limited light and/or inorganic carbon conditions (Glibert and Legrand 2006; Flynn et al. 2013). Osmotrophy within algae has been confirmed by Granéli et al. (1999), Liu et al. (2009), Tittel et al. (2009) and Wan et al. (2011).

The extremely acidic upstream headwaters emanating from the Copahue Volcano flow into Lake Caviahue, Argentina, resulting in the lake water having a very low pH (~2.5) and high summer concentrations of metallic iron (0.3 mmol l^{-1}) and sulphates (1.4 mmol l^{-1}) (Pedrozo et al. 2002; Beamud et al. 2007). For a full description of the study area, see Pedrozo et al. (2001), Baffico et al. (2004), Beamud et al. (2007) and Pedrozo et al. (2008). Few planktonic organisms can tolerate these conditions, and those that can are known as extremophiles (Pick 1999). The metabolic extremophiles of Lake Caviahue thrive under a high annual

Handling Editor: Jotaro Urabe.

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Published online: 05 March 2014

mean phosphorus concentration (0.01 mmol P l^{-1}), low nitrogen concentration (5 μmol N l^{-1}) and relatively low annual mean concentration of pure carbon dioxide (0.01 mg C l^{-1}). Additionally, the mean concentration of dissolved oxygen is 0.5 mmol O_2 l^{-1} (on average) in the epilimnion and 0.4 mmol O_2 l^{-1} in the hypolimnion (Pedrozo et al. 2001; Beamud et al. 2007, 2010a). The phytoplankton of Lake Caviahue is characterised by a very low diversity of nano-microplanktonic unicellular species (Beamud et al. 2007). The small green algae, *Keratococcus rhaphidioides*, is the dominant species, representing 60–100 % of the total biomass with a fresh weight of 0.5–0.8 μg ml^{-1} (Beamud et al. 2010a). The following species are also found, but in lower abundance: chlorophytes *Watanabea* sp. (0–22 %), *Chlamydomonas* sp. (0–5 %) and *Palmellopsis* sp. (0–25 %) and euglenophyte *Euglena mutabilis* (0–40 %). Phytoplankton biomass is more or less uniform throughout the water column, but chlorophyll *a* values of 1.0–1.6 mg m^{-3} occasionally occur below the thermocline at the end of autumn and winter, forming a deep chlorophyll *a* maximum or layer (DCM or DCL) at 30 m (Beamud et al. 2007, 2010a, b). The coefficient of attenuation of photosynthetically active radiation penetrating the surface (Kd PAR) ranges from 0.29 to 0.39 m^{-1}, and the Secchi disk depth ranges from 5.5 to 2.2 m; consequently light is not available at below 15 m (Beamud et al. 2010a). At 30 m, where the maximum values of chlorophyll *a* occasionally occur, the light intensity ranges from 0.58 to 1.014 μmol photon m^{-2} s^{-1} (Beamud et al. 2010a). Most phytoplanktonic species present in Lake Caviahue had never been identified as mixotrophic or heterotrophic. Samples collected from depths of 50–70 m containing *Keratococcus rhaphidioides* were studied under epifluorescence microscopy, and the cells were found to be in a good physiological state, showing an intense red fluorescence due to the presence of chlorophyll *a*, suggesting that they have a strategy that enables their survival without light.

In this study, we investigated whether Lake Caviahue algae are able to use sources of organic carbon and organic nitrogen to supplement photosynthetic production, which would explain the homogeneous distribution of algal biomass with depth. To accomplish this, we conducted microautoradiographic (MAR) investigations in situ to demonstrate the osmotrophic capabilities of Lake Caviahue phytoplankton. Additionally, we present direct evidence of the nutritional strategies that the phytoplankton employ based on growth and uptake laboratory experiments.

Methods

We performed three sets of bioassays: (1) in situ experiments to test assimilation of radiolabelled substrates under lake conditions; (2) laboratory experiments to assess the assimilation of radiolabelled organic substrates by algae under nutrient-replete conditions and (3) laboratory experiments to measure the specific growth rate and biomass yield responses to organic carbon and organic nitrogen additions mimicking lake conditions.

The substrates used in the experiments comprise a fraction of the total dissolved organic carbon (DOC) pool in natural waters (Stumm and Morgan 1996) and have been employed in previous algae and bacteria experiments (Vincent and Goldman 1980; Grover 2000; Jones and Feuchtmayr 2002; Kamjunke and Tittel 2008). The nitrogen and carbon contents within the substrates selected for the experiments represent valuable resources in nitrogen-limited environments, as well as those with very low inorganic carbon concentrations such as Lake Caviahue.

Assimilation experiment (in situ)

We employed the MAR technique described by Hoppe (1976) and Carman (1993) to test the ability of algae to take up dissolved organic substrates under natural environmental conditions. Briefly, natural plankton samples collected from different depths of Lake Caviahue (0, 1, 5 and 10 m) during February 2007 were incubated with 370 kBq of ^{3}H-leucine and $NaH^{14}CO_3$ for 24 h. Following incubation, the samples were fixed with formaldehyde (2 %) and filtered through 0.2-μm Nuclepore filters. In a dark room, the filters were then placed upside down onto glass slides and freshly dipped in 43 °C photo emulsion for electron microscopy (Amersham Buchler). The slides were then exposed for 7 days while desiccated and refrigerated in the dark. Next, the slides were developed in Kodak D 19 developer and stabilised in Kodak fixer, after which they were treated with distilled water and counter-stained with pH-adjusted (6.6) acridine orange solution. Finally, the slides were treated with sodium citrate buffer for 6–10 min with descending pH (6.6, 6.0, 5.0 and 4.0) and a glycerine solution (1 %). The slides were subsequently dried and the filters were removed. Silver spots in the photo emulsion caused by the decay energy of radioactive substrate molecules and their association with cells were analysed under a Zeiss Axioplan microscope, using a combination of bright-field and epifluorescence illumination (1,000×). The phytoplankton species present in the lake samples were *K. rhaphidioides* and *Watanabea* sp.

Assimilation experiment (in laboratory)

Photosynthetic production and osmotrophic activity experiments were performed in May–June 2007 in the isotope laboratory of the Helmholtz Centre for Environmental Research GmbH-UFZ (Germany). Water samples

from Lake Caviahue were pre-incubated in WC medium (Guillard and Lorenzen 1972) acidified to pH 3 for 4 days under experimental conditions described below. The initial inoculum contained *Keratococcus rhaphidioides* (cell biovolume: 37 μm^3, maximum linear dimension (Reynolds 2006) MLD: 10 µm, abundance: 12,500 cell ml^{-1}, biomass: 0.52 µg ml^{-1} fresh weight), *Euglena mutabilis* (cell biovolume: 4,890 μm^3, MLD: 24 µm, abundance: 29 cell ml^{-1}, biomass: 0.1 µg ml^{-1} fresh weight) and bacteria (cell biovolume: 0.102 μm^3, abundance: 950,000 cell ml^{-1}, biomass: 0.023 µg ml^{-1} fresh weight). Biovolume was determined as the mean volume measured in 50 cells of both algal species and bacteria (Wetzel and Likens 1991).

The osmotrophic activity of the algal culture samples from the lake were quantified using tritium (^3H) and radiocarbon (^{14}C) labelled substrates (L-[4,5-^3H] leucine, D-[6-^3H] glucose, [methyl-^3H] thymidine, L-[U-^{14}C] aspartic acid and [1(2)-^{14}C] acetic acid sodium salt, acetate) according to Gocke (1977) and Fuhrman and Azam (1980). All substrates were supplied by Amersham Biosciences. Briefly, 100 nmol l^{-1} L-[4,5-^3H] leucine (67.5 kBq) solution (blend of radioactive L-[4,5-^3H] leucine and non-radioactive L-leucine; ratio of mixture 1:5.25), 500 nmol l^{-1} of D-[6-^3H] glucose (25.9 kBq), 20 nmol l^{-1} of [methyl-^3H] thymidine (9.5 kBq), 200 nmol l^{-1} of L-[U-^{14}C] aspartic acid (3.8 kBq) or 1,000 nmol l^{-1} of [1(2)-^{14}C] acetic acid sodium salt (5.2 kBq) was added to 2.5 ml of algal culture. All samples were conducted in triplicate with a control (algal culture without any labelled substrate). The concentrations of the added organic compounds were similar to the natural waters (leucine, thymidine and aspartic acid) or up to five times greater than the natural concentrations (acetic acid and glucose) (Jørgenssen 2009a, b). Incubation was carried out in the dark or under light conditions corresponding to the light intensity at a depth of 5–7 m in the lake at the end of summer (14:10 h light:dark cycle with a light intensity of 275 µmol photon m^{-2} s^{-1}; Baffico 2013). The vessels were continuously shaken using a IKA Labortechnik KS 501 digital shaker. Cultures were kept at 14 °C for 7 h, then preserved with 2 % formaldehyde, after which they were immediately filtered through Nuclepore polycarbonate filters with pore sizes of 0.2 and 2.0 µm to separate the bacteria (0.2–2 µm) from the algae (≥2 µm). The filter pore size selection was based on microscopic measurements of the MLD of each organism before running the experiments.

Photosynthetic production rates were determined according to Steemann Nielsen (1952). Briefly, 60 nmol l^{-1} (185 kBq) of $NaH^{14}CO_3$ was added to 5 ml of algal culture and incubated for 7 h in an incubator at 14 °C under a light intensity (PAR) of 275 µmol photon m^{-2} s^{-1}. All samples were conducted in triplicate with a dark control. During incubation, the vessels were closed to avoid out gassing of CO_2 and continuously shaken. Following the incubation period, samples were fixed with 2 % formaldehyde, and the photosynthetic activity was quantified with a liquid scintillation counter (Packard TriCarb-2300).

To visualise the substrate uptake on a cellular level and identify osmotrophic algae directly, we used the MAR technique with the following labelled substrates: L-[4,5-^3H] leucine, D-[6-^3H] glucose, [methyl-^3H] thymidine, L-[U-^{14}C] aspartic acid and [1(2)-^{14}C] acetic acid sodium salt acetate. The methodology and substrate concentrations used for MAR experiments were the same as those described above; however, algal cultures (1 ml) were incubated for 7 h at 14 °C in the dark.

Growth rate and biomass yield

Experiments were performed using an axenic monoculture of *Keratococcus rhaphidioides* isolated from the lake in July 2012. The monocultures were maintained in the laboratory in a Neoline N322 incubator at 10 °C under 200 µmol photon m^{-2} s^{-1} of continuous light.

The growth experiment was performed using lake water with the following amendments: control (untreated lake water), glucose (3.8 mmol C l^{-1}), glutamine (0.5 mmol C l^{-1}, 0.2 mmol N l^{-1}) and arginine (0.6 mmol C l^{-1} and 0.3 mmol N l^{-1}). The organic substrate sources and final concentrations added to each treatment were taken from Jones and Feuchtmayr (2002) and represent 10–100 times the natural water concentrations (Jørgenssen 2009a, b). One set of treatments was incubated under light conditions, while another second set was incubated under dark conditions. Each set of experiments had three replicates per treatment. Samples were incubated in Nunclon culture flasks (60 ml, angled neck) adapted for inverted microscopy, at 10 °C under a light:dark period of 12:12 h over 10 days. As in the assimilation laboratory experiments, the light intensity was selected according to the PAR values recorded for winter at a depth of 5 m. The axenic conditions were tested at the beginning and end of the experiment from aliquots analysed by epifluorescence microscopy using acridine orange (Woelfl and Whitton 2000). Lake water was collected in a Van Dorn bottle and filtered in the laboratory (Nuclepore, 0.22 µm) before being used in the culture experiments.

Table 1 shows the chemical, physical and biological parameters of Lake Caviahue from three separate studies. The organic and inorganic carbon is from samples collected during winter (Beamud et al. 2010b), the total organic nitrogen values are based on samples collected during summer (Pedrozo et al. 2008), and the remaining data were generated in this study.

Table 1 Chemical, physical and biological characteristics of the north arm of Lake Caviahue (*NA*)

Variables	Units	NA
[1]DOC	mmol l^{-1}	0.11 (±0.03)
[1]DIC	mmol l^{-1}	0.05 (±0.01)
[2]TON	µmol l^{-1}	2.4–8.2
[3]TP	mmol l^{-1}	0.01
[3]SRP	mmol l^{-1}	0.01
[3]NH_4^+	µmol l^{-1}	3
[3]Temp	°C	4.7
[3]O_2	mg l^{-1}	7.7–10.9
[3]pH		2.9
[3]Conductivity	µS cm^{-1}	1,093
[3]Transparency	m	5.6
[3]PAR	µmol photon m^{-2} s^{-1}	1,405
[3]Chl-*a*	mg m^{-3}	2.08
[3]*Kr* biomass	µg ml^{-1}	0.5
[3]*Kr* abundance	cell ml^{-1}	6,341

Data extracted from: [1]Beamud et al. (2010b) average data (± SD) from the water column during winter sampling, [2]Pedrozo et al. (2008) data range over the 2000–2004 during summer sampling (at 0 m depth), and [3]data from July 2012 samples collected from 1 m depth (this study)

DOC dissolved organic carbon, *DIC* dissolved inorganic carbon, *TON* total organic nitrogen, *TP* total phosphorus, *SRP* soluble reactive phosphorus, *NH4*$^+$ ammonium, *Temp* temperature, O_2 dissolved oxygen, *Transparency* Secchi disk transparency, *Chl-a* chlorophyll *a*, and Kr biomass: *Keratococcus rhaphidioides* fresh weight biomass

The cell abundance was determined using an inverted microscope at 400× (Utermöhl 1958) every day until day 5 and then every 2 days until the end of the experiment. Cell biovolume and biomass (fresh weight, µg ml^{-1}) were estimated according to Wetzel and Likens (1991). Specific growth rate was estimated by linear regression of the natural log of biomass versus time when algal growth was in the exponential phase as described by Maberly et al. (2002). Yield was estimated from the biomass on day 10.

In these experiments autotrophy was considered as the production of biomass under light conditions without added organic nutrients, while osmotrophy was defined as the use of organic compounds in the dark and mixotrophy was the use of organic compounds in the light.

Data analysis

Assimilation experiment

Since cultures were non-axenic, the incorporation rate of organic substrates was calculated based on the algae and bacteria fraction based on the difference between the total incorporation (0.2 µm) minus the algae fraction (0.2 to ≥2 µm). Two-way analysis of variance (ANOVA) was used to identify significant differences in incorporation rates between treatments. The two categorical factors were light (with or without) and organic substrates (leucine, aspartic acid, acetic acid, glucose and thymidine). ANOVA of the bacterial and algal fractions was conducted separately. A $p < 0.05$ was considered to indicate significance.

Specific growth rate and biomass yield

Differences in the average specific growth rate obtained using different organic substrates under light and dark conditions were identified by a *t* test for two independent samples. A $p < 0.05$ was considered to indicate significance.

Changes in the biomass and abundance values with time were determined by repeated measures ANOVA for all the treatments separately. Changes in biomass with time for the grouped treatments (autotrophy, osmotrophy, mixotrophy and dark control) were also analysed by repeated measures ANOVA. The differences in the abundance and biomass yield at the end of the experiment (day 10) were analysed by two-way ANOVA as described above using light conditions as one factor and organic solutions added as the other factor. A $p < 0.05$ was considered significant.

Results

In situ experiments

During the MAR experiments, the abundance of the dominant algal species *Keratococcus rhaphidioides* and *Watanabea* sp. ranged from 12,770 to 15,088 cell ml^{-1} and 393 to 2,790 cell ml^{-1}, respectively, in samples collected from 0 to 10 m. Radiolabelled cells from both species were found, indicating an ability to assimilate the added bicarbonate and leucine (Fig. 1A–G).

Assimilation in laboratory experiments

Figure 2 illustrates the assimilation of substrates according to cell size fractions. The algal uptake rates of acetate, aspartic acid and glucose were higher under light conditions than dark conditions, while those of thymidine and leucine were not. Overall, the incorporation rate of organic substrates was related to the availability of light during incubation ($p < 0.05$, $df = 3$). Under light conditions (Fig. 2A, B), the highest uptake rate was found after the addition of acetate (14.4 ± 0.16 nmol l^{-1} h^{-1}), followed by aspartic acid (1.99 ± 0.16 nmol l^{-1} h^{-1}), glucose (0.98 ± 0.04 nmol l^{-1} h^{-1}), leucine (0.13 ± 0.00 nmol l^{-1} h^{-1}) and thymidine (0.82 ± 0.17 pmol l^{-1} h^{-1}). Under dark conditions, the assimilation of organic sources was highest

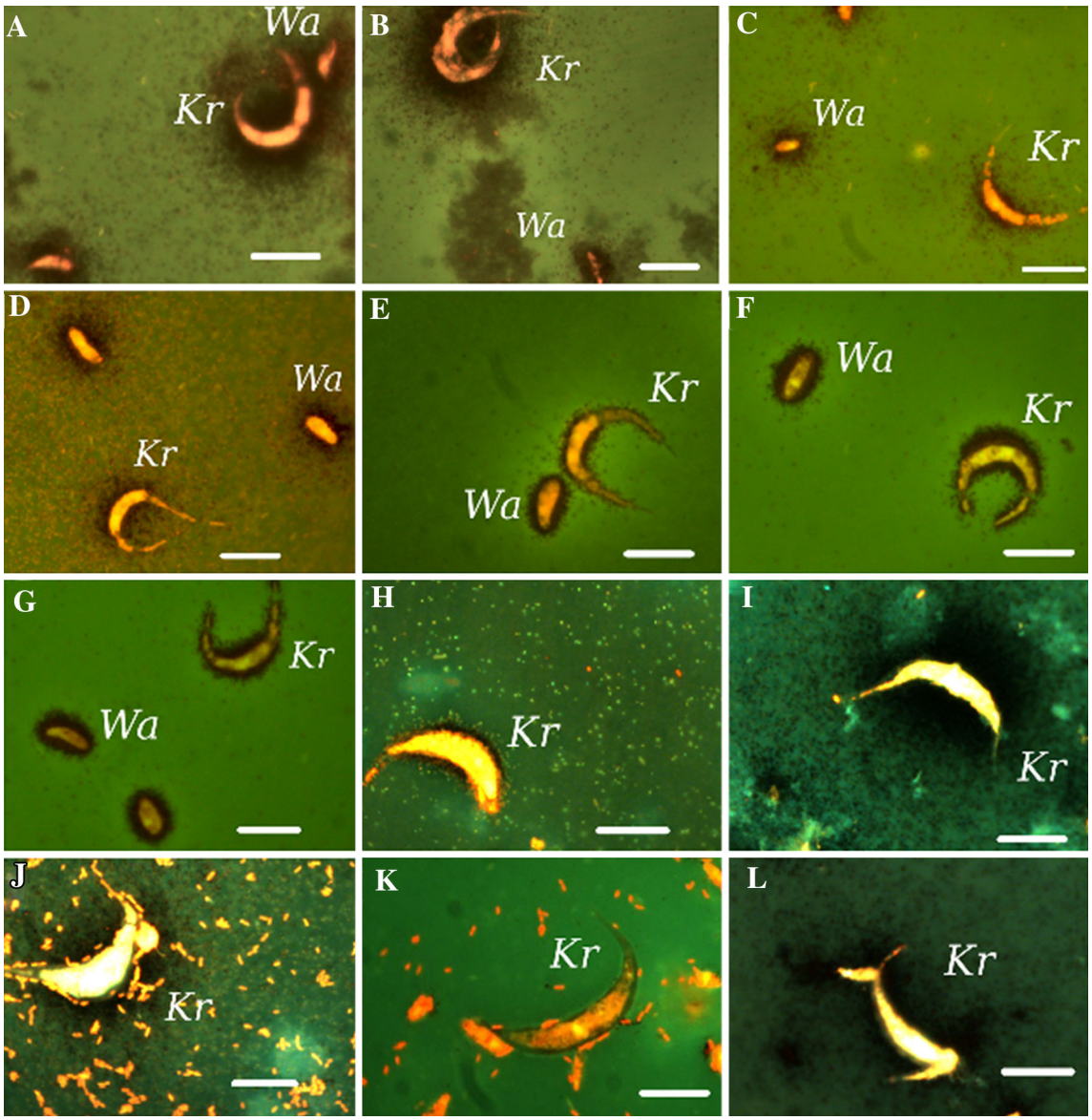

Fig. 1 Microautoradiographs from in situ (**A**–**G**) and assimilation laboratory experiments (**H**–**L**). In situ experiments in the north arm of Lake Caviahue, $NaH^{14}CO_3$ incubations at: **A** 0 m; **B** 1 m; **C** 5 m; **D** 10 m; and ^{3}H-leucine incubations at **E** 0 m; **F** 1 m; **G** 10 m. Assimilation laboratory experiments, dark incubations: **H** ^{3}H-thymidine; **I** ^{14}C-acetic acid; **J** ^{14}C-aspartic acid; and light incubations: **K** ^{3}H-thymidine; **L** ^{14}C-acetic acid. Magnification: ×1,000, *scale bar*: 10 µm. *Kr*, *Keratococcus rhaphidioides* cells, *Wa*, *Watanabea* sp. cells. Uptake is shown in labelled algal cells with black grains around them

for acetate (4.56 ± 0.31 nmol l^{-1} h^{-1}). The photosynthetic production rate (inorganic carbon assimilation) of algae (≥2 µm fraction) was 49.13 (±0.14 nmol l^{-1} h^{-1}). The results observed during incubation of the bacteria-sized fraction (Fig. 2C, D) were similar to those observed for algae. Specifically, the highest incorporation was found for acetate, followed by aspartic acid, glucose, leucine and thymidine. The only difference in patterns from the larger size fractions was that glucose incorporation under dark conditions (0.35 ± 0.02 nmol l^{-1} h^{-1}) was higher than that observed under light conditions (0.09 ± 0.01 nmol l^{-1} h^{-1}). The MAR results confirm the uptake of all substrates except glucose and aspartic acid by the algae under dark and light conditions (Fig. 1H–L).

Specific growth rate and biomass yield in laboratory experiments

The *t* test showed that the average specific growth rate µ (day^{-1}) after the addition of arginine, glucose and glutamine in the light treatments was higher than the specific growth rate in the dark treatments (Table 2). The highest specific growth rate (0.22 day^{-1}) was observed following glutamine addition under light conditions. This was

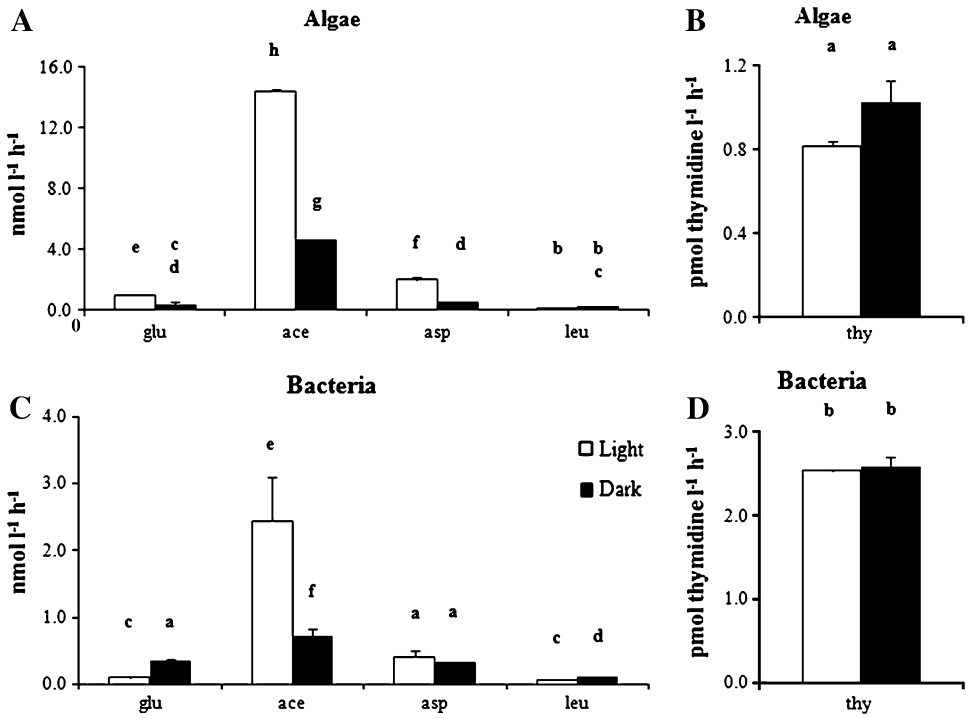

Fig. 2 Results of the laboratory assimilation experiment. Incorporation rate (nmol l^{-1} h^{-1}) of ^{3}H-glucose (glu), ^{14}C-aspartic acid (asp), ^{14}C-acetic acid (ace), ^{3}H- and ^{3}H-leucine (leu) under light (*white bars*) and dark (*black bars*) conditions. **A** Algae: incorporation in the size fraction ≥2 μm; **B** algae thymidine incorporation in the size fraction ≥2 μm (pmol l^{-1} h^{-1}); **C** bacteria: incorporation in the size fraction ≥0.2 to <2 μm; **D** bacteria thymidine incorporation in the size fraction ≥0.2 to <2 μm (pmol l^{-1} h^{-1}). Each value represents the mean ($n = 3$) ± the standard deviation. *a–h* Homogeneous groups between treatments ($p < 0.05$)

followed by that of arginine addition under dark conditions (0.12 day^{-1}). The changes in biomass and abundance over time for all treatments (Fig. 3A–D) depended on the organic source added and the availability of light during the experiment ($p < 0.05$, $df = 21$; Table 3). The abundance of *K. rhaphidioides* increased continuously under light conditions, while under dark conditions it grew during the first day, then remained almost constant until the end of the experiment (Fig. 3A, B). The final abundance at day 10 (Fig. 3A, B) recorded in the treatments with organic sources depended on the light conditions ($p < 0.05$, $df = 3$). The final abundance in the light-incubated treatments was the same (Fig. 3A). Under dark conditions, the arginine treatment showed the highest final abundance (19,947 ± 562 cell ml^{-1}), and this abundance was the same as that observed for treatments under light conditions (Fig. 3B). Control, glucose and glutamine treatments showed lower final abundance than arginine.

The biomass responses to substrate additions differed from the abundance in that higher biomass yield was measured at the end of the experiment in almost all organic substrate treatments under light conditions and in all such treatments under dark conditions (Fig. 3C, D). These findings reflect an increase in cell size and biovolume in response to organic substrate addition. The biomass yield for all treatments under light conditions was higher than treatments under the dark conditions and depended on the organic source added ($p < 0.05$, $df = 3$; Fig. 3C, D). The dark control showed the lowest biomass of all treatments at day 10 (0.41 ± 0.16 μg ml^{-1} fresh weight).

Table 2 Specific growth rate of *Keratococcus rhaphidioides* (days 4–8) calculated from biomass yield in the laboratory experiments

	Specific growth rate (μ, day^{-1})			
	Light	Dark	*t* test	*p* value
Control	0.18	0.00	3.83*	0.018*
Glucose	0.15	0.07	3.01*	0.039*
Glutamine	0.22	0.11	6.14*	0.003*
Arginine	0.18	0.12	2.85*	0.046*

t test, Statistical test for two samples comparing light versus dark conditions average specific growth rate, $n = 24$. *$p < 0.05$

The pattern of biomass according to nutritional strategy is shown in Fig. 4. Light incubations with glutamine, arginine and glucose were averaged in the mixotrophy strategy. Dark incubations with glutamine, arginine and glucose were averaged in the osmotrophy strategy. Incubation under light condition represented autotrophy, and the experiment control was the incubation under dark conditions. These biomass changes over time depended on the nutritional strategy (Table 3). The stimulatory effect of organic substrates on biomass began after day 4 (Fig. 4). After day 6 of the experiment, mixotrophy treatments showed the highest biomass values (1.56 ± 0.23 μg ml^{-1}), followed by autotrophy (1.14 ± 0.13 μg ml^{-1}) and osmotrophy (0.88 ± 0.20 μg ml^{-1}). The lowest values were found in the dark control treatment. This pattern in the

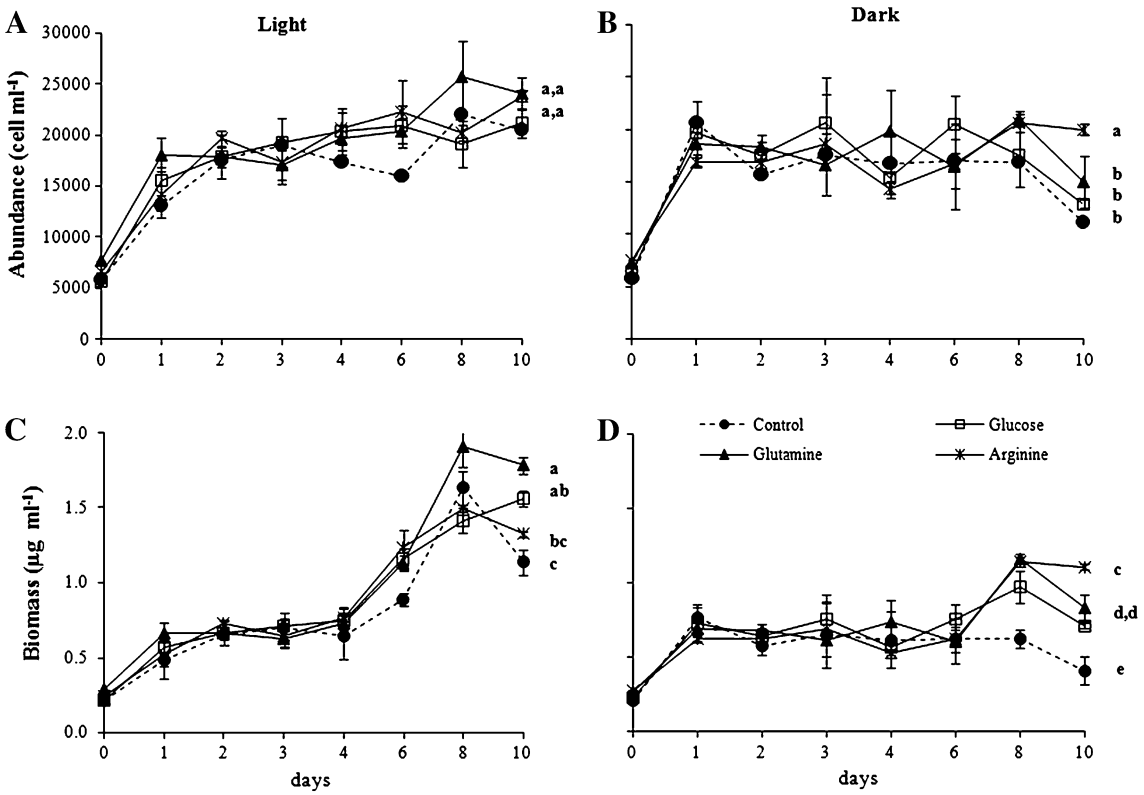

Fig. 3 Results of the specific growth rate and biomass yield laboratory experiments. Changes in **A**, **B** abundance (cell ml^{-1}) and **C**, **D** biomass (µg ml^{-1}) of *K. rhaphidioides* over time after the addition of organic carbon sources: glucose, arginine, glutamine and control (under light and dark conditions). *Each point* represents the mean biomass ($n = 3$) ± the standard deviation from day 0 to day 10. *a–e* Homogeneous groups between treatments ($p < 0.05$)

Table 3 Repeated measures ANOVA for changes in abundance (cell ml^{-1}) and biomass (µg ml^{-1}) of *Keratococcus rhaphidioides* over time in specific growth rate and biomass yield in laboratory experiments ($n = 192$)

FV	*dF*	*F*	*p* value
Abundance days × light × organic source	21	2.04	0.03311*
Biomass days × light × organic source	21	4.89	0.00005*
Biomass according to nutrition strategy days × light × organic source	21	15.73	0.00000*
Error	112		

* $p < 0.05$

biomass values remained steady until the end of the experiment (day 10).

Discussion

At Lake Caviahue, the high assimilation of inorganic carbon during photosynthetic production experiments indicated the good state of algae during incubation as well as their potential to produce biomass. The assimilation, specific growth rate and biomass yield experiments demonstrated that the algae could utilise the organic compounds offered under in situ conditions or laboratory conditions, without limitation of inorganic nitrogen and phosphorus.

Algae have been shown to exploit several components of the dissolved organic nitrogen pool (DON), either directly or after bacterial degradation (Wheeler et al. 1974; Lewitus et al. 2000). Some phytoplankton species are able to use amino acids through amino acid oxidases among other modes of organic N uptake located on the outer cell surface. These extracellular enzymes hydrolyse amino acids in the water column to ammonium, which can then be incorporated by the cells (Berman and Bronk 2003; Mulholland et al. 1998). This pathway appears to represent a potentially important source of inorganic nitrogen for phytoplankton, especially in oligotrophic waters, where inorganic nitrogen concentrations in the water are low (Mulholland et al. 1998). Even if phytoplankton cells cannot produce their own extracellular enzymes, they may still benefit from the pool of extracellular enzymes released directly by bacteria or indirectly by processes such as lysis of bacteria, phyto- and zooplankton cells (Karrasch et al.

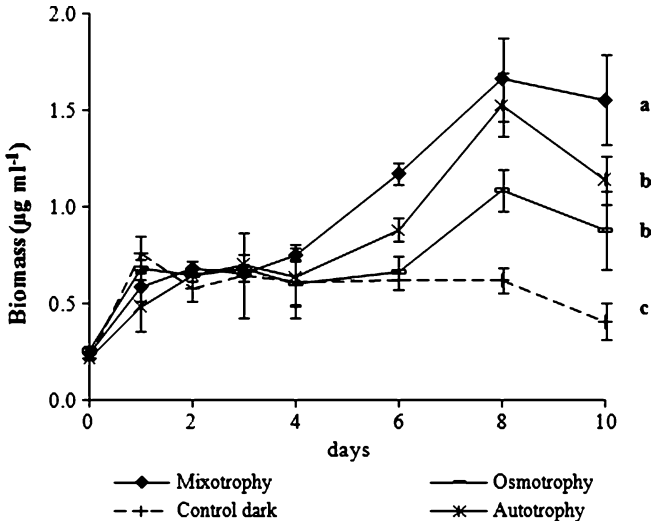

Fig. 4 Changes in biomass over time for the specific growth rate and biomass yield laboratory experiments with *K. rhaphidioides*, according to nutritional strategy. Mixotrophy: average glucose, glutamine and arginine under light conditions ($n = 9$, ± 1 SD); osmotrophy: average glucose, glutamine and arginine under dark conditions ($n = 9$, ± 1 SD); autotrophy: control treatment with light and no organic substrate addition ($n = 3$, ± 1 SD); control dark: no organic substrate addition ($n = 3$, ± 1 SD). *a–c* Homogeneous groups between treatments ($p < 0.05$)

2003a, b, 2006; Bronk et al. 2007). Amino acids may also be used as carbon storage compounds or sources of both organic carbon and organic nitrogen (Neilson and Lewin 1974; Rivkin and Putt 1987). Therefore, the low availability of inorganic carbon and inorganic nitrogen in Lake Caviahue would favour algae, which are capable of incorporating amino acids and perhaps other organic substrates such as glucose and acetate.

In all experiments with and without bacteria, the uptake rates, specific growth rates and biomass yield were higher under mixotrophy than osmotrophy conditions, with the exception of leucine and thymidine. The only comparable results from extreme environments were found in some algae of the Antarctic, where the highest leucine uptake occurs under low light conditions for most species (Rivkin and Putt 1987). The specific growth rate and biomass yield laboratory experiments enabled osmotrophy and mixotrophy to be distinguished. A slower specific growth rate and lower biomass yield were observed under osmotrophic incubation than under mixotrophic conditions. Some studies have shown that at irradiances below saturation, the mixotrophic-specific growth rate was approximately equal to the sum of osmotrophic and autotrophic-specific growth rates in most algae (Ogawa and Aiba 1981; Laliberté and De La Noüe 1993). In our experiments, the sum of osmotrophy and autotrophy processes was higher than that of mixotrophy for both specific growth rate and biomass. These results are in accordance with the idea that mixotrophic production in an individual organism is not a simple sum of phototrophy and heterotrophy (Flynn et al. 2013).

MAR illustrated the uptake of organic substrates by *Keratococcus rhaphidioides* and *Watanabea* sp. under in situ conditions and by *K. rhaphidioides* under laboratory conditions and indicated the potential uptake and metabolisation of the DOC and DON in this acidic lake. Particularly, *K. rhaphidioides* is described for the first time in an acidic environment as well as its ability to assimilate organic substrates. However, *Monoraphidium contortum*, a related species belonging to the same order (Chlorellales), also showed mixotrophic activity in Lake Tahoe (Vincent and Goldman 1980). The genus *Watanabea* was established by Hanagata et al. (1998) to separate some ellipsoid species of *Chlorella*. *Watanabea* species from Lake Caviahue can take up leucine in situ in the euphotic zone, and a species of *Chlorella* showed similar behaviour when grown with different forms of DON (Wheeler et al. 1974; Timperley et al. 1985; Granéli et al. 1999) and glucose under dark conditions in cultures (Droop 1974) and during light depth incubations in Lake Tahoe (Vincent and Goldman 1980). *E. mutabilis* was present in the inoculum in very low abundance in the laboratory assimilation experiment, but was not observed upon MAR analysis in the present study.

Stoecker (1998) reported that mixotrophy could be an important trophic mode in aquatic environments since it influences competition between bacteria and algae for limiting nutrients and among phytoplankton for limiting resources, while also affecting predator–prey relationships within the microbial food web. Thus, mixotrophs should represent an important link in the flux of materials through planktonic food webs (Jones 2000). Mixotrophic nutrition

could contribute to trophic dynamics not only through its impact on carbon supply, but also through its effects on nitrogen, phosphorus and iron supply (Hartmann et al. 2012; Flynn et al. 2013).

There are situations in which multiple sources of nitrogen (organic and inorganic) can be used concurrently, reflecting the microheterogeneity of aquatic systems (Berman and Bronk 2003). The assimilation of organic nutrients in addition to inorganic forms that are typically assimilated by algae shows the different strategies employed by phytoplankton for its nutrition. Osmotrophic and mixotrophic organisms are known to have advantages in extreme environments with low pH and in systems with low light levels, which are both present in Lake Caviahue. However, these conditions alone do not explain the homogeneous distribution of phytoplankton throughout the water column nor the presence of a DCM. The factors that determine vertical homogeneity and DCM of phytoplankton were discussed by Beamud et al. (2010b). Predation of phytoplankton by either zooplankton or other mixotrophic algae was not a consideration owing to the positive relationship between zooplankton and phytoplankton biomass, suggesting that *Philodina* sp. had little or no effect on *K. rhaphidioides* in the lake. Additionally, other zooplankton with the ability to prey on algae are not present in this lake. The thermal behaviour of the 90-m-deep lake Caviahue (Beamud et al. 2010a) is not enough to explain the homogeneous phytoplankton distribution throughout the year, because the water column is completely mixed during the coldest period (May–September), and thermal stratification occurs once a year during summer (January–March). However, the phytoplankton distribution might be a combination of three factors. Specifically, slow sedimentation rates of epilimnetic phytoplankton may influence their distribution. Moreover, decreased light intensity could produce an increase in chlorophyll *a* inside the cell, causing the phytoplankton to adapt their chlorophyll *a*:carbon ratio to the prevailing environmental conditions (Banse 1977; Kirk 1994). Finally, the use of recycled available nutrients in the meta-hypolimnion (nitrogen, phosphorus, carbon) might stimulate the sustained growth of algal populations beyond the euphotic zone. It should also be noted that the relevance of the microbial loop in this naturally acidic lake remains unknown.

The results of our laboratory experiments conducted using different sources of organic carbon and nitrogen in the light and/or in the dark confirm their capability of mixotrophy and osmotrophy. These findings indicate that mixotrophy is an efficient nutrition strategy for the dominant phytoplankton species of Lake Caviahue. From our results, we can conclude that photosynthesis would be the main source of carbon for algae in the lake, whereas osmotrophy and mixotrophy seem to supplement autotrophy according to the availability of nutrients and light. The balance among the three nutritional strategies may be an explanation for the homogeneity of phytoplankton abundance and biomass throughout the water column in Lake Caviahue.

Acknowledgments This study was supported by grants and fellowships from: ANPCyT PICT 38137, PICT 2008-1105 and PICT 2012 1389, Universidad Nacional del Comahue (Program 04/B001 and 04/B166), CONICET PIP 0013/10 and the ALE-ARG Cooperation Programme between the Ministry of Education, Science and Technology (Argentina) and the German Academic Exchange Service (Germany). We also thank Maren Mehrens from the UFZ Centre for Environmental Research in Magdeburg (Germany) for her laboratory assistance. Furthermore, we are grateful to Gustavo Baffico, Pepe DiGiusto, Mauricio Uguccioni and Pedro Temporetti for their field and computer task assistance. Finally, we thank Norbert Kamjunke for his valuable comments and corrections of the manuscript and the two anonymous reviewers for their valuable input.

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