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Effects of phosphorus enrichment and grazing snails on modern stromatolitic microbial communities

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SUMMARY

1. The effects of phosphorus enrichment and grazing snails on a benthic microbial community that builds stromatolic oncolites were examined in an experiment at Rio Mesquites, Cuatro Ciénegas, Mexico. Chemical analyses of stream water samples indicated that overall atomic ratios of total nitrogen (N) to total phosphorus (P) were approximately 110, indicating a strong potential for P-limitation of microbial growth.
2. Phosphorus enrichment involved addition of 5 μmol Na_2HPO_4 L^{-1} to streamside microcosms receiving intermittent inputs of stream water while grazer manipulation involved removal of the dominant grazer, the snail *Mexithauma quadripaludium*. After 7 weeks, we examined responses in organic matter content, C : N : P ratios, metabolism (P removal, primary production, dark respiration, and calcification), and microbial community structure using molecular fingerprinting of 16S rRNA genes.
3. Manipulation of snails did not affect response variables measured in these treatments (organic matter, C : P ratio, P removal rate). However, P enrichment significantly decreased the C : P and N : P ratios of surficial materials in the oncolites (organic matter content was unchanged), increased net and gross photosynthesis (oxygen consumption in the dark was unchanged), increased rates of calcification, and increased diatoms relative to cyanobacteria. Heterotrophic Eubacteria and Archaea were only modestly affected. Thus, our results indicate weak grazing effects but strong impacts of P in this benthic system.
4. We hypothesise that a state of severe P-limitation is imposed on autotrophic production in this food web due, at least in part, to co-precipitation of phosphate during calcite deposition. This produces severe P-limitation of the benthic algae and cyanobacteria, resulting in high C : P ratio of microbial mats relative to the biomass of photoautotrophs (phytoplankton, terrestrial foliage) in other ecosystems. In turn, this high C : P ratio is likely to generate severe stoichiometric constraints on the herbivores, thus limiting their populations and resulting in weak overall grazing impacts.

Keywords: nutrient limitation, phosphorus, snails, stoichiometry, stream, stromatolites

Introduction

Microbial mats, which are benthic organosedimentary communities built by photosynthetic microbes and containing diverse microbial flora, are common in aquatic habitats on the modern Earth (Cohen & Rosenberg, 1989; Paerl & Pinckney, 1996; Stal, 2000)

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and have been widespread throughout Earth's history (Grotzinger & Knoll, 1999). Of particular interest, and found less frequently in modern times, are microbial mats that actively generate mineralised structures either through trapping and binding of mineral particles or through an active process of biologically mediated mineralisation (i.e. calcification), as this type of mat is thought to be responsible for the formation of fossil stromatolites that were the dominant form of life from the late Archaean through the later Proterozoic periods (Grotzinger & Knoll, 1999; Stal, 2000). Mineralised mats, which are referred to as microbialites in the geological literature, are common in extreme environments such as in hypersaline and highly erosive marine settings and in a variety of freshwater systems, ranging from hot springs to lakes with high levels of dissolved salts. In a few cases, these mats form the basis of benthic food webs that include metazoan consumers such as gastropods, microcrustaceans, insect larvae and vertebrates (tadpoles, fishes). The impact of grazing on benthic mats is especially interesting in a palaeoecological context, as the microbial mats that formed stromatolites were largely spared from grazing prior to the appearance of metazoans ca 500 million years ago. Indeed, the appearance of metazoans has been suggested as a major factor causing the demise of stromatolite-forming mats at the Precambrian–Cambrian boundary (Garrett, 1970; Awramik, 1971; Stanley, 1976), although this idea has been contested (Riding, 2000).

Previous research in benthic ecology has examined the impact of various factors, such as disturbance (Resh *et al*., 1988), solar radiation (Hill, Ryon & Schilling, 1995), salinity (Garcia-Pichel *et al*., 1999), nutrient supply (Francoeur, 2001), and grazing (Hillebrand, 2002), on benthic communities, with a particular emphasis on the response of autotrophic components. The importance of limiting nutrients such as nitrogen (N) and phosphorus (P) has been demonstrated repeatedly for benthic autotrophs in both lotic and lentic environments (Francoeur, 2001; Hillebrand, 2002), including studies of microbial mats. Addition of limiting nutrients can alter productivity (Francoeur, Espeland & Wetzel, 2003), biomass (Fong, Zedler & Donohoe, 1993; Pinckney, Paerl & Fitzpatrick, 1995; Hillebrand *et al*., 2002), species composition (Hillebrand *et al*., 2002; Camacho & de Wit, 2003), N fixation (Pinckney *et al*., 1995; Camacho & de Wit, 2003) and elemental composition (Frost & Elser, 2002a; Hillebrand & Kahlert, 2002; Stelzer & Lamberti, 2002; Camacho & de Wit, 2003) of benthic communities. Grazers can also have a major impact on the species composition, biomass and productivity of benthic communities (Feminella & Hawkins, 1995; Hillebrand, 2002), although little work has been done on effects of grazing on microbial mats *per se* (see Wickstrom & Castenholz, 1985 for an example). Nevertheless, Hillebrand's (2002) meta-analysis indicates that the impacts of nutrient supply and grazing on periphyton biomass are relatively equal in magnitude, although there is much variation in both 'top down' and 'bottom up' effects in different ecosystems and during different seasons and years.

As in the rest of ecology, increasing efforts in benthic ecology are being expended to better understand these simultaneous interactions among nutrients, autotrophic microbes and grazers. In pelagic ecosystems, these complex relationships have come to be analysed within the framework of ecological stoichiometry (Sterner & Elser, 2002), an approach that considers the transactions of energy and multiple chemical elements in ecological systems. Work with planktonic food webs has shown that phytoplankton growing under nutrient limitation develop high C : nutrient ratios in their biomass, resulting in stoichiometric constraints on the growth and reproduction of zooplankton herbivores (Sterner & Hessen, 1994) as well as reduced rates of consumer-driven recycling of the limiting nutrient (Elser & Urabe, 1999). Periphyton also develops increased C : nutrient ratios when grown under nutrient limitation (Hillebrand & Sommer, 1999) and similar stoichiometric constraints on consumers have been demonstrated for benthic grazers feeding on nutrient-limited periphyton (Frost & Elser, 2002b; Stelzer & Lamberti, 2002). Nevertheless, stoichiometric studies in the benthos are still relatively limited (Frost *et al*., 2002), although papers in this volume expand the scope of benthic stoichiometric analysis (Frost, Cross & Benstead, 2005a).

In this study we examine the effects of a potentially limiting nutrient (P) and grazing on calcifying microbial mats at Cuatro Ciénegas, Mexico. This system is of particular interest because the waters are highly mineralised and thus these mats form a diversity of laminated structures, which may be considered to constitute modern stromatolites. This locality is also of note because, unlike other places where modern stromatolites form in the absence of metazoans

because of extreme hypersalinity (e.g. Shark Bay, Australia; Monty, 1984; Riding, 2000), a variety of metazoan grazers actively feed on these stromatolites. Thus, to the extent that these stromatolites are similar to those that dominated early Earth, experimentation at Cuatro Ciénegas may shed light on factors regulating food webs at the Precambrian-Cambrian transition. In particular, we were interested in testing a newly proposed hypothesis suggesting that extreme P-limitation of microbial autotrophs in stromatolite-forming mats imposed a stoichiometric constraint on the success and evolution of metazoans (Elser, 2003). This would occur if P-limited photoautotrophs in early benthic communities had very high C : P ratios, resulting in severe P-limitation of higher consumers leaving them unable to execute RNA-intensive (and thus P-intensive) developmental programmes characteristic of metazoans (Elser *et al*., 2000c). In this paper we focus on responses of stromatolite biomass, elemental composition, productivity, calcification and microbial community structure to a factorial manipulation of P supply and presence or absence of grazing snails. An accompanying paper (Elser *et al*., 2005) reports the responses of the snails to the P fertilisation.

Methods

Study site and environmental characterisation

Our experiment was performed using detached stromatolitic oncolites (ovoid lamination-forming structures) found in the Rio Mesquites, a stream fed by thermal springs in the Cuatro Ciénegas basin (CCB hereafter), Coahuila, Mexico. These oncolites range in size from 3 to 25 cm in major dimension and can be found strewn continuously on the stream bottom, some imbedded in soft-bottom substrates, some concreted to each other or to hard bottom areas, but most are found aggregated in detached form. Because of high biodiversity and a significant number of endemic species, the CCB is an area designated as an 'Area de Protección de Flora y Fauna,' administered by the Mexican Instituto Nacional de Ecologia. A substantial, 60-year literature exists on the natural history of the CCB and its environs (Minckley, 1994). A large number (>300) and diversity of springs and spring-fed aquatic habitats can be found within this small (approximately 500 km^2) intermontane basin at the eastern edge of the Chihuahuan Desert (Minckley, 1969). The area is hot and arid, with most of its <15.0 cm of annual precipitation falling in summer (Shreve, 1944; Muller, 1947). Geothermal waters are associated with a major north–south fault bisecting the basin. Springheads are small to large (<1.0 m^2 surface area to >1.0 ha), with surface outflows varying from seepage to approximately 1.0 $m^3\ s^{-1}$, temperatures from 24 to approximately 40 °C, total dissolved solids (TDS) from 0.3 to 2.0 mg L^{-1} and chemistry varying from $CaSO_4$ to $Ca(HCO_3)_2$-dominated. Of particular interest for our studies is the presence in the CCB of more than a dozen hydrobiid snail species (Hershler, 1985), of which the endemic species *Mexithauma quadripaludium* (Taylor) is especially germane, as it grazes primarily on hard surfaces such as rocks and stromatolites. Also relevant is *Nymphophilus minckleyi* (Taylor), found primarily on the surfaces of aquatic macrophytes but also co-occurring with *M. quadripaludium* on hard substrata.

Physical-chemical parameters in the Rio Mesquites were assessed on an approximately quarterly schedule during 1998–2003 and on a regular basis (approximately weekly) during the 2003 experiment. In general, measurements were made on surface water collections. Temperature, pH and conductivity were measured using a Beckman 255 combination electrode (Beckman Coulter Inc., Fullerton, CA, U.S.A.) and dissolved oxygen was measured using an YSI Model 85 temperature–oxygen meter (Yellow Springs Instruments Inc., Yellow Springs, OH, U.S.A.). On limited dates, temperature data at the stream bottom and in the experimental containers were recorded at 15-min intervals using submersible Hobo™ temperature loggers (Onset Computer Corporation, Pocasset, MA, U.S.A.). Light intensity at the surface of the stream and at the bottom at the site of stromatolite collection was measured on several occasions using a Licor LI-1000 2*pi* submersible quantum sensor (LI-COR Biosciences, Lincoln, NE, U.S.A.) for comparison with measurements made in the experimental containers. Intensive *in situ* light data in the stream and in the enclosures were also acquired at 15-min intervals using Hobo™ square box light-intensity loggers (Onset Computer Corporation). Alkalinity was quantified by Gran alkalinity titration (APHA, 1999). Calcium concentrations were assessed by atomic absorption spectrometry using a Varian SpectrAA 400 flame atomic absorption spectrometer (Varian Inc., Palo Alto, CA, U.S.A.). Sulphate was quantified using Quickchem 8000

(Latchat Instruments, Loveland, CO, U.S.A.) auto-analyser. Concentrations of various forms of nitrogen (N) and phosphorus (P), including soluble reactive phosphorus (SRP), ammonium (NH_4^+), and nitrate + nitrite (NO_3^- + NO_2^-) were quantified spectrophotometrically following standard procedures (APHA, 1999). In most cases, dissolved inorganic pools were analysed within 24 h while analyses of total P and total N were performed on frozen samples within 4 months of collection.

Experimental set-up

The experiment was run streamside at the Rio Mesquites between 26 June and 10 August 2002 with a total duration of 7 weeks. Oncolites of relatively uniform dimensions (10-cm diameter) were collected from oncolite fields at a depth of approximately 1.5 m along a 50-m stretch of the stream. They were held in individual plastic containers with stream water while being prepared for the experiment. Each stromatolite was photographed and weighed and then initial samples were taken. These samples consisted of surficial scrapings of material (used for organic matter and C : P analysis) and removal of 1–2 cm branch-like 'florets' (used for DNA fingerprinting; see Wade & Garcia-Pichel, 2003). Surficial scraping consisted of isolating a relatively smooth approximately 1 cm^2 patch of stromatolite surface and scraping to a standardised depth of approximately 0.5 mm. The same person prepared all such samples across treatments and replicates to reduce variability and remove possible bias. Some samples were placed in vials and dried for later analysis of organic matter or P content while the latter samples (DNA analyses) were taken with alcohol-sterilised forceps, immediately submerged in liquid nitrogen, and kept frozen at −80 °C until analysis. In addition, the stromatolites were inspected for the presence of snails; all snails were removed and held for later use. Snails removed from stromatolites were enumerated to determine their average ambient density (9.3 snails per oncolite ± 0.1 SE); *M. quadripaludium* and *N. minckleyi* were present in a proportion of approximately 4 : 1. Only *M. quadripaludium* individuals were used in the experiment to simplify interpretation of eventual outcomes.

The experiment consisted of a 2 × 2 fully factorial manipulation of P fertilisation and presence or absence of snails with six replicates of each treatment combination: 'controls' (no enrichment, with snails: −P+S), 'control removals' (no enrichment, no snails: −P−S), 'fertilised grazing' (P-fertilised, with snails: +P+S), 'fertilised removals' (P fertilised, no snails: +P−S). After sampling, two randomly chosen oncolites were placed in each of 24 20-L white plastic buckets containing 18 L of fresh stream water. Each stromatolite was suspended in the bucket 5 cm above the bottom using nylon string; this prevented snails from leaving the stromatolites (and thus not consuming stromatolite biomass nor experiencing P-amended stromatolite biomass). Half of these buckets were randomly assigned to the +S treatments. To create the +S treatments, collected *Mexithauma* were first arranged according to size by visual ranking. Size-ranked snails were sorted into three arbitrary categories with equal numbers of snails in each category. Then, four snails were randomly chosen from each category and combined into a single group of 12 that was then placed on each of the stromatolites in the +S treatments. Thus, snail densities in the +S treatments were somewhat higher than ambient levels (12 versus 9.3 snails per stromatolite). Over the course of the experiment snails occasionally fell off the stromatolite and were returned randomly to one of the two stromatolites. Half of the +S buckets and half of the remaining buckets lacking snails (−S) were randomly assigned to the P-fertilisation treatment (+P). P-fertilised buckets received a spike of Na_2HPO_4 sufficient to raise the concentration of P in the stream water by 5 μmol L^{-1}. These waters are highly buffered and no change in stream water pH was observed after P enrichment. This spike was added whenever fresh stream water was replenished in the buckets. Water replenishment occurred three times daily during the first several days but then a regular regime of twice-daily water replacements (approximate 10–14 h intervals) was implemented for the remainder of the experiment. Preliminary measurements determined that a 5-μmol L^{-1} spike was sufficient to prevent depletion of PO_4^{3-} during a 12-h replacement interval. In experiments with stream biota it is important to avoid the creation of inordinately large viscous benthic boundary layers that would limit diffusive mass transfer between benthic organisms and overlying water (Wieland *et al*., 2001). Because of electrical power constraints in the remote field locality, we could not circulate the water continuously. Instead,

water in each bucket was circulated intermittently using individual battery-powered pumps in which pumps were alternately turned on and off during the period between replacement of bucket water with fresh stream water. Pumps were operating approximately 50% of the time under this procedure. Possible impacts of this intermittent circulation are discussed below. Buckets were held streamside under a shade structure to prevent excessive light exposure and temperature increases. Deployment of temperature and irradiance data loggers indicated that water temperatures in the buckets generally diverged by only 5–7% from values in the Rio Mesquites itself while light intensity was only approximately 4% lower than levels measured at the stream depth where the stromatolites were collected.

Organic matter and C : P ratio

Biomass and elemental composition (C and P) of surficial biomass for each stromatolite was estimated by triplicate scraping of approximately 100 mg of surface material from different areas on the surface of the stromatolite and combining the scraped material for later analysis. This sampling occurred at the end of the 7-week period. In addition, several freshly collected stromatolites from the stream were also analysed at the beginning of the experiment. The organic matter content of the samples was estimated following the method of Hedges & Stern (1984): the sample was dried for 12 h at 60 °C, weighed, and then combusted for 2 h at 525 °C using a muffle furnace, re-equilibrated to room temperature for 30 min to account for loss of hydration by $CaCO_3$, and re-weighed. The loss of mass upon combustion was converted to units of organic C by determining the %C of organic material directly using a CHN analyser. Phosphorus content was estimated by dissolving the postcombusted sample in 5 N H_2SO_4 for 30 min, bringing the sample to a known volume with deionised water, and then analysing the solution for PO_4^{3-} using spectrophotometry (APHA, 1999). Biomass C : P was estimated by dividing the %C by %P values for each sample and then converting to an atomic ratio by multiplying by 2.54. This method likely underestimates the C : P ratio of organic materials, as some unknown fraction of the P measured in this way may have been bound in the inorganic matrix and thus not associated with organic matter before acid hydrolysis.

P removal

Preliminary measurements of SRP removal rates were taken during the daytime on 5 July during the second week of the experiment; these initial measures involved two replicates from the +P+S treatment combination and one replicate from the +P−S combination. A more thorough study was made of both night and day removal on 30–31 July. On this occasion, all six replicates in both +P treatment combinations (+P−S, +P+S) were used. As in the normal enrichment routine, spikes of dissolved Na_2HPO_4 were added to create target concentrations of approximately 5 μmol L^{-1}. Following spike addition, water in the buckets was mixed with submersible aquarium pumps for 5 min, at which point initial SRP samples were collected. Stromatolites were then incubated for approximately 9 h with intermittent pump recirculation, at which point final samples were collected. This procedure was repeated after nightfall to obtain estimates of night-time P removal. All water samples were immediately refrigerated and analysed within 24 h of collection as described above. Rates of SRP removal were calculated by subtracting final from initial SRP concentrations, converting that to an absolute quantity of SRP removed by correcting for the volume of water in the bucket, and then dividing by incubation time. Removal rates were then normalised to the wet mass of the stromatolites used in each replicate.

Photosynthesis and respiration

Rates of net photosynthesis (NP) and gross photosynthesis (GP) and of dark respiration were determined by assessing changes in oxygen concentration under light versus dark conditions. Note that we use 'dark respiration' to refer to the import of oxygen into the mat in the dark; this may not equate with biological respiration to the extent that other oxygen-consuming processes, such as oxidation of sulphides, are taking place. Thus, estimates of GP may also be influenced by impacts on non-metabolic oxygen consumption. Because of limitations on the number of samples that could be processed at the end of the experiment and because preliminary observations indicated that snails generally had weak effects on the stromatolites, oxygen metabolism was measured only for stromatolites from the −S treatments. Six

individual stromatolites from the +P–S and –P–S treatments, along with three stromatolites freshly sampled from the Rio Mesquites, were immersed individually in 1.5-L sealable translucent plastic containers filled with Rio Mesquites water. Initial dissolved oxygen levels in the containers were measured using a calibrated and temperature-corrected YSI Model 85 oxygen meter and the containers were then sealed to exclude air bubbles. For determination of photosynthesis, stromatolites were incubated in partial sunlight equivalent to the light intensity in the incubation buckets and dissolved oxygen concentrations were measured at 15, 45 and 90 min. Following the incubation for NP, incubations for respiration were initiated using fresh Rio Mesquites water. Stromatolites were incubated in the dark and oxygen measurements were made after 2, 8 and 18 h. It was necessary to open the containers to make these measurements but the effect of this is mostly taken into account by comparison with the control chambers lacking a stromatolite. Incubation times were adjusted to avoid supersaturation in the chambers. Rates of NP and respiration (in terms of O_2 generation and consumption) were calculated for each replicate container by fitting a line to observations made at the three time intervals (15, 45 and 90 min for NP; 2, 8 and 18 h for respiration). The slopes of these lines for each stromatolite were then used as estimates of its NP or respiration rates, respectively. In all cases, these individual relationships were statistically significant ($P < 0.01$, $R^2 > 0.90$). Gross primary production rate for each stromatolite was then calculated by adding the respiration rate to the NP rate for that stromatolite.

Calcification

As for oxygen metabolism, calcification rates were estimated for stromatolites from the –S treatments only (–P–S, +P–S) because of logistical limitations on the numbers of stromatolites we could process simultaneously at the end of the experiment. Net calcification was assessed by measuring the change in bulk concentrations of Ca^{2+} in water containing oncolites relative to control water lacking a stromatolite. Individual stromatolites were placed in 2-L plastic containers with Rio Mesquites water in a 4.5 : 1 ratio of water to stromatolite volume. This was to increase the sensitivity of the measurement as it magnified the change in Ca^{2+} concentration that occurred. In the case of +P–S oncolites, the P concentration used during the previous incubations (approximately 5 μmol L^{-1}) was maintained. Carbonate dissolution in the dark does not occur to measurable extent in these oncolites on the time-scale of our incubations and therefore dark measurements were not needed (Garcia-Pichel *et al.*, 2004). Containers were held for 24 h at approximate ambient temperature of the Rio Mesquites under constant illumination in the laboratory. Water in the containers was continuously circulated to reduce establishment of excessively thick boundary layers. Samples from all containers, both with and without stromatolites, were taken initially and after 12 and 24 h. Calcium removal was relatively linear and thus only data from 24 h were used in calculating calcification rate. Water samples were acidified and then frozen until analysis by atomic absorption spectrometry. The bulk water method used here to estimate calcification rates provides results similar to those obtained using calcium microelectrodes at the stromatolite–water interface (Garcia-Pichel *et al.*, 2004).

DNA extraction and isolation

Replicate sample florets from a single stromatolite were combined, pulverised using mortar and pestle, transferred aseptically into individual Bead Solution tubes of UltraClean Plant DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, U.S.A.), homogenised by vortexing, and submitted to seven to 10 freeze-thaw cycles. DNA was then isolated according to kit instructions and quantified by agarose gel electrophoresis against a mass ruler after ethidium bromide staining using a Bio-Rad Fluor-S^{TM} Multi-Imager system (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Polymerase chain reaction amplification of microalgal 16S rDNA

Oligonucleotide primers CYA359F and CYA781R (Nübel *et al.*, 2000), specific for cyanobacteria and eukaryotic plastids (i.e. oxygenic photoautotrophs), were used for polymerase chain reaction (PCR) amplification of approximately 450 bp-long 16S rRNA gene segments. The 5′ end of primer CYA359F included a GC-rich sequence (‘clamp’) to prevent

complete denaturation of the amplicons during DGGE (see below). Thirty cycles of 1 min each at 94 °C (denaturation), 60 °C (annealing) and 72 °C (extension) were performed and the reaction finished with a final extension at 72 °C for 9 min. The PCRs began with denaturation at 95 °C for 5 min (hot-start) followed by the addition of 2.5 units of Takara Ex Taq DNA polymerase (PanVera Corporation, Madison, WI, U.S.A.) to each reaction at 80 °C. Each 100-μL PCR contained the following: 10 μL of 10× Takara Ex Taq PCR buffer, 8 μL of Takara dNTP mixture (2.5 mM each), 50 pmol of each primer (Operon Technologies, Inc., Alameda, CA, U.S.A.), 200 mg of bovine serum albumin (BSA; PanVera), 20 μL of 5× Eppendorf TaqMaster PCR-enhancer (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.) and 10 ng of template DNA. Quantification of PCR product was done as described above.

Denaturing gradient gel electrophoresis fingerprinting of the microalgal community

Denaturing gradient gel electrophoresis (DGGE) fingerprinting (Muyzer, de Waal & Uitterlinden, 1993; Muyzer *et al*., 1996) was used mainly to evaluate changes in the relative abundances of photoautotrophic microbial taxa present in the microbial mats. PCR-amplified 16S rRNA fragments were separated according to Nübel *et al.* (2000) and quantified as described above for agarose gels. DGGE uses a gradient of chemical denaturants to separate DNA segments of equal length but different sequence (Muyzer *et al*., 1993, 1996). More intense bands may indicate a higher relative abundance of that ribotype in the overall community (Brüggemann *et al*., 2000; Abed & Garcia-Pichel, 2001; Sekiguchi *et al*., 2001; Abed *et al*., 2002). While PCR-based methods of community structure characterisation are subject to a variety of biases because of differential amplification efficiency and because of the fact that molecular methods in general are dependent on the extraction efficiency of DNA which can vary for different community members (see Muyzer, 1998 for a review of such biases), DGGE offers a fast method to detect major community changes because of externally imposed treatments (e.g. Abed & Garcia-Pichel, 2001; Abed *et al*., 2002). Each band was excised using a sterile scalpel and DNA allowed to diffuse out for at least 3 days at 4 °C in 50 mL of 10 mM Tris buffer. One microlitre of the solution was PCR amplified using the same primers (minus a GC-rich clamp), reaction mixture, thermocycling conditions, and product quantification as above. A kit was used to purify PCR product (Qiagen, Inc., Valencia, CA, U.S.A.) and 150 ng was commercially sequenced in two separate reactions (5′ to 3′ and 3′ to 5′). Complementary sequences were matched, aligned, and edited using Sequence Navigator (Applied Biosystems, Foster City, CA, U.S.A.) and a BLAST search (http://www.ncbi.nlm.nih.gov) was used to find the most similar sequences traceable to a known microbe in culture. DNA sequences obtained from DGGE bands A to R have been deposited in GenBank with accession numbers AY541040 to AY541057, respectively.

The DGGE analysis is particularly amenable to use in the computation of richness and diversity indices (Nübel *et al*., 1999a,b, 2000) and, in the particular case of benthic photoautotrophs, typically yields results congruent with those obtained using alternative methods of community analyses not based on PCR (Nübel *et al*., 1999b). We used quantification of the relative abundance of each allele in a fingerprint to compute the overall diversity using Simpson's index ($D = 1/\sum pi^2$), which takes into the account the relative proportion (p_i) of the total community contributed by particular ribotypes (Magurran, 1988). As preliminary data indicated no effect of snails on stromatolite characteristics and the analysis is relatively costly, DGGE analysis was confined to a randomly selected set ($n = 3$) of stromatolites from the −P−S and +P−S treatments. The relative abundances of different taxa in initial and final samples were determined by quantitative image analysis of the gels as above.

Terminal restriction fragment length polymorphisms analysis of archaeal and eubacterial 16S rDNA

Using DNA extracted from stromatolite florets as above, 16S rDNA for terminal restriction fragment length polymorphisms (T-RFLP) analysis was PCR amplified with fluorescently labelled universal primers 515F and 1492R. Cycling conditions were as follows: 4 min at 94 °C; 35 cycles of 1.5 min at 92 °C, 1.5 min at 60 °C, 2 min at 72 °C; final extension for 10 min at 72 °C. Approximately 50 ng of the purified PCR product of 16S rDNA was digested in a 20-μL reaction volume with 5 units of restriction enzyme

AluI for 3 h. The aliquots were electrophoresed in a polymer-filled capillary in an ABI 3100 sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.). Profiles from sister samples (before and after the experiment) were analysed using Genescan analytical software v202 (Applied Biosystems).

Results

Physical-chemical conditions in the Rio Mesquites

In general (Table 1) the waters were warm (average temperature 27 °C), neutral to alkaline (average pH approximately 7.6), well-buffered (average alkalinity approximately 4 meq L^{-1}), and high in conductivity (average approximately 2900 μS cm^{-1}). Concentrations of dissolved inorganic nitrogen (DIN; here NO_3^- + NH_4^+; nitrite levels are generally very low) were high, with an average NO_3^- concentration of approximately 20 μmol L^{-1} (Table 1). NO_3^- levels never fell below 6 μmol L^{-1}. However, P levels were low (Table 1): total P (TP) concentration was approximately 0.6 μmol L^{-1} with P primarily in the form of SRP. The average SRP concentration was somewhat higher than the TP concentration, although this trend was not statistically significant and primarily indicates that little of the stream TP was in particulate form. The DIN : SRP and TN : TP ratios were high (>35 and >100, respectively, on average), indicating a strong likelihood of P-limitation of microbial growth (Table 1). Calcium and sulphate levels (Table 1) were also consistently high (average approximately 309 mg L^{-1} and 1200 mg L^{-1}, respectively). High Ca^{2+} concentrations and alkalinity promote very efficient and rapid photosynthesis-driven calcification such that oncolites at Cuatro Ciénegas have areal calcification rates similar to those found in corals (Garcia-Pichel *et al.*, 2004).

Organic matter and C : P ratio

The organic matter content of surficial material was relatively low (<5%) and did not respond to P enrichment ($F_{1,20} = 0.025$, $P > 0.87$ in a two-way ANOVA) or manipulation of snails ($F_{1,20} = 0.054$, $P > 0.82$) during the experiment (Fig. 1a). However, organic C : P ratio responded very strongly to P-enrichment (Fig. 1b; $F_{1,20} = 126$, $P < 0.001$), declining from initial values of approximately 750 to <150 by the midpoint (data not shown) and <100 at the end of the experiment. Manipulation of snails had no effect on the surficial C : P ratio, with or without P enrichment (Fig. 1b; $F_{1,20} = 0.01$, $P > 0.85$). C : N ratios were uniform across all treatments and thus N : P ratios of surficial biomass directly tracked the trends shown for C : P (data not shown). There were

Table 1 Physical-chemical conditions in the Rio Mesquites, Cuatro Cièenegas, Coahuila, Mexico. Values are shown for year-round sampling (approximate quarterly intervals) during 1998–2003 as well as for the experimental period in summer 2002. In addition to physical parameters, data are given for total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), nitrate (NO_3^-), ammonium (NH_4^+), ratio of dissolved inorganic nitrogen (NO_3^- + NH_4^+) to SRP, ratio of TN to TP, buffering capacity (alkalinity), calcium (Ca^+) and sulphate (SO_4^{2-}). All ratios are given as atomic ratios. The number of observations for each parameter is also indicated (*n*).

	1998–2003				Summer 2002			
Parameter	Mean ± SD	*n*	Minimum	Maximum	Mean ± SD	*n*	Minimum	Maximum
Temperature (°C)	26.1 ± 3.6	5	19.8	28.5	27.9 ± 1.7	2	27.3	28.5
pH	7.7 ± 0.2	5	7.4	8.1	7.5 ± 0.2	2	7.4	7.7
Conductivity (μS cm^{-1})	2966 ± 155	5	2811	3165	2812 ± 1.4	2	2811	2813
TP (μM)	0.54 ± 0.22	19	0.18	1.06	0.57 ± 0.21	16	0.18	1.06
SRP (μM)	0.59 ± 0.1	25	0.1	1.47	0.77 ± 0.32	18	0.25	1.46
TN (μM)	58.5 ± 14.9	19	15.05	69.2	62.0 ± 12.8	16	14.9	69.2
NO_3^- (μM)	20.1 ± 10.6	24	6.2	45.1	19.4 ± 7.8	18	9.9	45.2
NH_4^+ (μM)	0.64 ± 0.98	21	0.13	3.47	0.31 ± .36	18	0.13	1.41
DIN : DIP	64.2 ± 103.3	20	9.2	441.6	35.4 ± 39.1	18	9.2	183.8
TN : TP	11 9.0 ± 44.4	20	26.8	180.1	113.8 ± 44.3	16	26.8	180.1
Alkalinity (meq L^{-1})	3.94 ± 0.39	5	3.44	4.29	–		–	–
Ca^{2+} (mM)	7.97 ± 1.62	26	6.32	13.7	7.42 ± 0.4	18	6.47	8.1
SO^{2-} (mM)	13.5 ± 1.76	21	11.7	19.1	13.0 ± 1.31	17	11.7	17.3

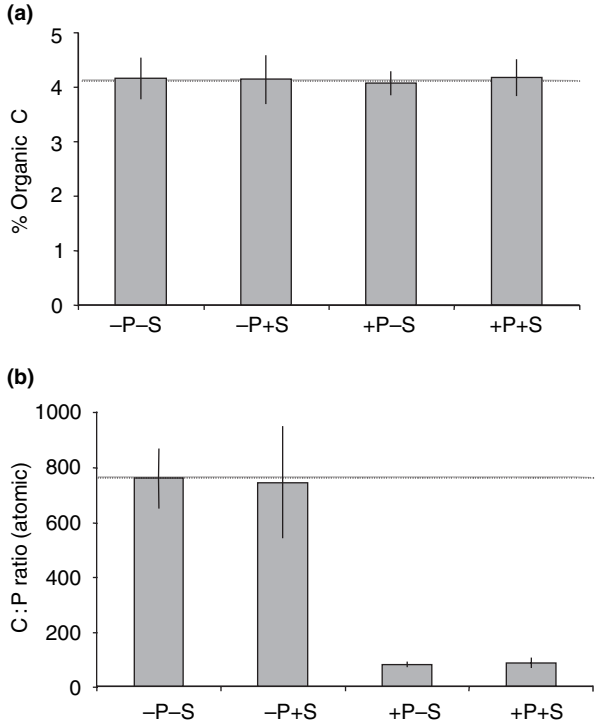

Fig. 1 Effects of P-fertilisation and snail removal on (a) organic matter content and (b) C : P ratio of surficial materials from stromatolites at the end of the 7-week experiment. The mean value for a subset of stromatolites at the start of the experiment is also shown as the horizontal dotted line. Error bars indicate ±95% confidence limits.

no significant two-way interactions ($P > 0.72$) between snail and P effects for any of these parameters.

P removal

Stromatolites contained in the buckets were capable of removing approximately 2.5 μmoles of SRP every hour (normalised to stromatolite mass, approximately 0.015 μmoles P g^{-1} h^{-1}). Analysis of variance indicated no effect of snail manipulation on P-removal rate ($F_{1,19} = 0.71$, $P > 0.41$), nor was there any difference in P uptake between day and night ($F_{1,19} = 0.33$, $P > 0.57$). However, it did appear that there was a general temporal decline in the P uptake capacity of the stromatolites. A *t*-test comparing the normalised uptake rates for the three measurements on 5 July (two replicates from +P–S and one from +P+S) and the combined daytime measurements from 30 July (11 replicates in all; chemical data for one of the 12 replicates was lost) indicated a significant difference

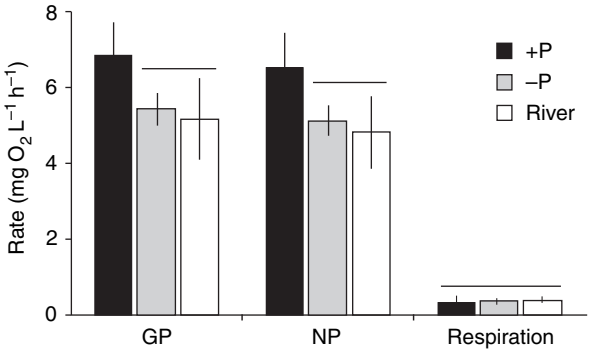

Fig. 2 Effects of P-fertilisation on rates of gross (GP) and net (NP) photosynthesis and dark respiration at the end of the experiment (–S treatments only). Overall treatment effects were significant ($P < 0.02$) for both GP and NP but not for respiration. Error bars indicate ±95% confidence limits. The horizontal bars join mean values that were not significantly different according to *post hoc* comparison (Scheffé's test). Values are also given for stromatolites that were freshly collected from the river.

(0.025 versus 0.012 μmol P g^{-1} h^{-1}, $t = 2.48$, d.f. $= 12$, $P < 0.03$).

Photosynthesis and respiration

Phosphorus enrichment had no effect on stromatolite dark respiration rate (Fig. 2; $t = 0.37$, d.f. $= 10$, $P > 0.71$). However, as shown in Fig. 2, P enrichment significantly stimulated NP ($t = 3.01$, d.f. $= 10$, $P < 0.02$) and, as a consequence, GP was significantly different as well ($t = 2.99$, d.f. $= 10$, $P < 0.02$). GP increased from approximately 5 mg O_2 L^{-1} h^{-1} for –P and fresh stromatolites to approximately 7.5 for stromatolites in the +P treatment. Rates of photosynthesis and respiration did not differ for unenriched stromatolites relative to similar stromatolites freshly collected from the stream (Fig. 2; $P > 0.54$ in *t*-tests), suggesting that incubation in the containers did not substantially alter these metabolic processes.

Calcification

Concentrations of dissolved Ca^{2+} declined steadily from approximately 430 to 380 mg L^{-1} in containers with stromatolites during the 24-h calcification incubations while concentrations in control containers lacking stromatolites remained relatively constant. For each stromatolite measured, we calculated the rate of calcification based on this change in concentration and normalised the rate to the size (wet mass) of the

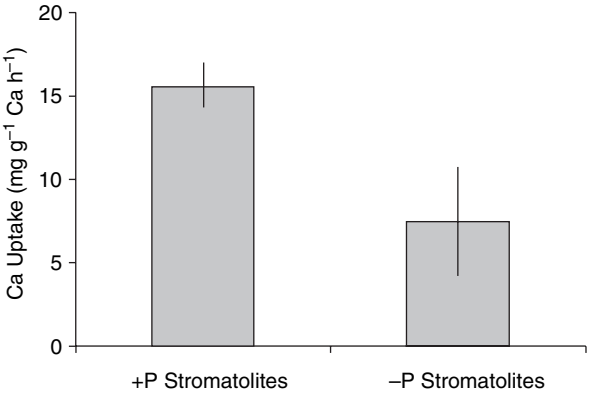

Fig. 3 Effects of P-fertilisation on net rates of calcification at the end of the experiment. Error bars indicate ±95% confidence limits. The mean values are statistically different according to a *t*-test ($P = 0.01$). Data are for stromatolites in the −S treatments only.

stromatolite employed. P enrichment significantly stimulated calcification rate (Fig. 3; $t = 4.51$, d.f. $= 4$, $P < 0.01$).

Microbial community structure

Eighteen repeatable bands were identified in the DGGE fingerprints (Fig. 4a; Table 2). In general, in all three −P−S stromatolites, the number of bands (i.e. richness) increased and a relatively even distribution of abundance among the different taxa was maintained such that the overall diversity index *D* increased during the incubation period (Fig. 4b,c). In contrast, in all three of the +P−S stromatolites the number of bands declined, the distribution shifted strongly in favour of certain bands (e.g. B and especially A), and the diversity index *D* consequently decreased during the experimental period (Fig. 4b,c). Thus, P enrichment had a statistically significant impact on the temporal dynamics of autotrophic community diversity (Fig. 4c; $t = 3.82$, d.f. $= 4$, $P < 0.02$). Sequencing results (Table 2) indicated that bands A and B represented diatom plastids (closest match to those of the genera *Asterionella* and *Haslea*) and thus it appears that P fertilisation resulted in a major increase in the relative abundance of diatoms. Bands representing cyanobacteria (bands F–R) were relatively stable during the experiment and their absolute intensity did not appear to respond strongly to P enrichment (Fig. 4a), although their relative contribution did change because of the increase in diatom bands. Thus, the main effect of P-enrichment on photoautotrophic community structure appeared to be a general increase in diatom abundance, leading to a subsequent decline in the relative dominance of cyanobacteria. We do not have quantitative data on the relative abundance of different algal types by microscopic analysis but qualitative inspections supported this major trend.

The T-RFLP analysis of DNA extracted from whole stromatolite florets and focusing on members of the Eubacteria and Archaea showed that the diversity of these taxa generally declined during the incubation period in both control and +P treatments (Fig. 5). Analyses were confined to peaks greater than 80 units to avoid effects of fluorescence noise (Blackwood *et al.* 2003). Using chi-square test we observed an overall significant difference ($\chi^2 = 22.3$, d.f. $= 5$, $P < 0.001$) in the number of peaks higher than 80 units of fluorescence when comparing stromatolites at the beginning and end of the experiment. Thus, all samples showed a strong reduction in the total diversity of prokaryotic microbes due either to seasonal decline or to handling and container effects. In general, peaks representing Archaea taxa (e.g. peak no. 49 in Fig. 5) showed the strongest reduction during the incubation period. However, these changes were not influenced by P enrichment, as there was no difference between treatments in the number of peaks at the end of the incubation ($\chi^2 = 5.45$, d.f. $= 5$, $P = 0.36$).

Discussion

It is apparent from our results that P supply has major impacts, and that grazing has minor impacts, on various ecological characteristics of the oncoid stromatolites at Cuatro Ciénegas, Mexico. However, before discussing these results it is important to consider sources of potential artefacts that might be present in the experimental systems. While we sought to re-create environmental conditions as similar to those in the stream as possible, and indeed insolation and temperature measurements indicated close correspondence, we were unable to use a more realistic flow-through design because we could not reasonably nor ethically continuously enrich high volumes of stream flow water with PO_4^{3-} in the Cuatro Ciénegas protected area. Thus, we used closed chambers with twice-daily water exchange

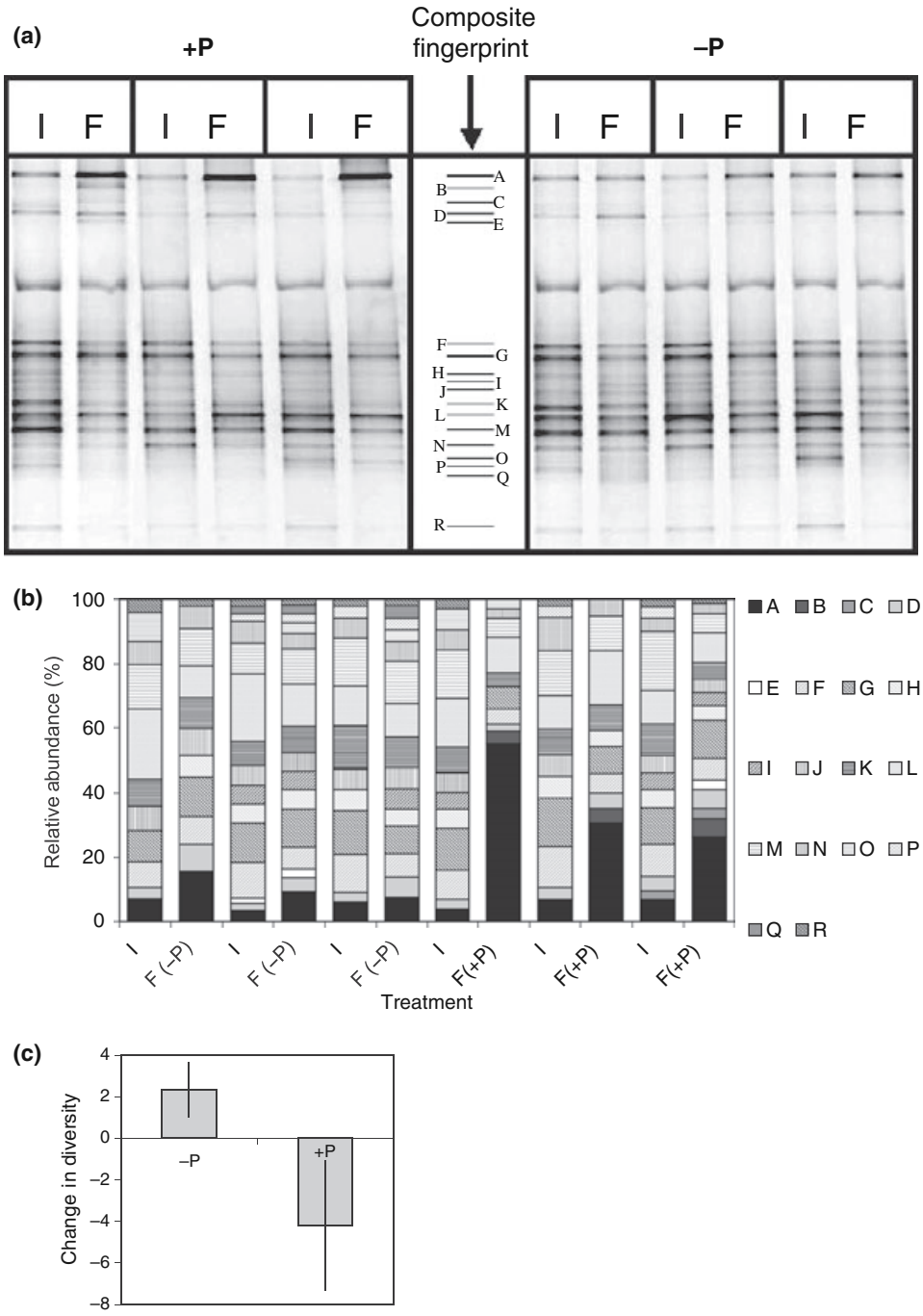

Fig. 4 Effects of P fertilisation on community structure of autotrophic microbes at the end of the 8-week experiment as determined by DGGE analysis (only −S treatments were analysed). (a) Composite image (negative) of the DGGE separation of microalgal 16S rRNA gene segments acquired after PCR-amplification of DNA extracted and isolated independently from three stromatolites from control removal (−P−S) and fertilised removal (+P−S) treatments for initial (I) and final (F) samples. In the centre, the 18 variants (A–R) chosen for sequencing and analysis in statistical treatments are indicated. Identifications of the bands are given in Table 2. (b) Relative abundance of dominant DGGE bands representing different photoautotrophic ribotypes at the beginning (I) and end (F) of the experiment in unenriched (−P) and P-enriched (+P) treatments. Bands A–E are diatoms while bands F–R are cyanobacteria. The dotted line connecting initial and final data for individual replicates separates diatom taxa (below) from cyanobacteria (above). (c) Changes in taxon diversity during the experimental period as indexed by Simpson's diversity index (*D*) in +P−S and −P−S treatments. Error bars indicate ±95% confidence limits. There was a significant effect of P enrichment on the change in *D* during the experiment ($P < 0.02$).

Table 2 Phylogenetic affinities of microalgal 16S rDNA fragments obtained by BLAST analysis of sequences re-amplified from DGGE bands in Fig. 4

DGGE Band	Closest related ribotype(s)	% Similarity
A	*Haslea, Asterionella*	98
B	*Haslea*	98
C	*Haslea, Gyrosigma, Pleurosigma*	98
D	*Haslea, Gyrosigma, Pleurosigma*	98
E	*Haslea*	98
F	LPP-group, *Phormidium, Leptolyngbya*	92
G	*Tolypothrix, Hapalosiphon, Anabaena, Anabaenopsis, Spirirestis, Nodularia*	93
H	*Tolypothrix, Anabaena, Anabaenopsis, Aphanizomenon, Fischerella, Coleodesmium*	93
I	*Leptolyngbya*	93
J	*Leptolyngbya, Halomicronema*	94
K	*Halomicronema*, LPP-group	95
L	*Acaryochloris*	96
M	*Leptolyngbya, Halomicronema, Oscillatoria*	92
N	*Leptolyngbya*	95
O	*Leptolyngbya*	94
P	*Leptolyngbya, Phormidium*	92
Q	*Leptolyngbya*	94
R	*Phormidium*	92

The term ribotype is used here in place of a taxonomic epithet to better reflect the ribosomal information source. Only database ribotypes obtained from cultures are listed; environmental clones are not included. Ribotypes represented by bands A–E match diatom plastids, while F–R match cyanobacteria. Generic epithets listed for database ribotypes are those given in GenBank®, and we did not prove their validity. Identifications were based on a comparison of 406–410 DNA bases.

and internal water circulation. Water circulation was not continuous, however, because of limitations on our field power supplies but measurements of water velocities during water circulation indicated that *average* water flows in the buckets (approximately 2.2 m s^{-1}, averaged over times when the pumps were on versus off) were probably somewhat higher than flows experienced by stromatolites in the Rio Mesquites (approximately 2.5 m s^{-1} at the stream surface, 0.8 m s^{-1} 10 cm above the bed). Thus, hydrodynamic conditions in the enclosures perhaps *over*-supplied nutrients to the stromatolites, a conservative bias relative to our finding of strong nutrient response of stromatolites in the buckets. In addition, various measurements we made indicated that effects of enclosing the stromatolites were not severe. For example, metabolic rates as well as organic matter content and C : P ratios were similar for control stromatolites from the buckets at the end of the experiment and stromatolites collected from the river (Figs 1 and 2). Thus, we conclude that our results are likely to have relevance for the *in situ* effects of P and grazing on stromatolites in the Rio Mesquites itself.

Phosphorus enrichment had large and highly significant effects on nearly all response variables considered (Figs 1–4), with the exception of overall organic matter content (Fig. 1a) and prokaryotic diversity as indicated by T-RFLP analysis (Fig. 5). These strong responses are in agreement with observations made in other freshwater oncolites (Pentecost & Whitton, 2000), suggesting a generalised limitation of oncolite communities by P. Camacho & de Wit (2003) also observed no response of mat organic matter content to manipulations of nutrients despite major changes in other measured parameters in their study of a laminated mat in a hypersaline lake. Certainly there are limitations on the suitability of using organic matter content as an index of the overall biomass response. For example, P stimulation of calcification might have cancelled out the effects of increased net organic matter production as captured in the percentage of organic matter in the overall sample. Nevertheless, the lack of biomass response we observed is understandable if one considers that only a small fraction of the total organic matter in a benthic biofilm mat is likely to be active biomass (Frost, Hillebrand & Kahlert, 2005b). Thus, P-enrichment

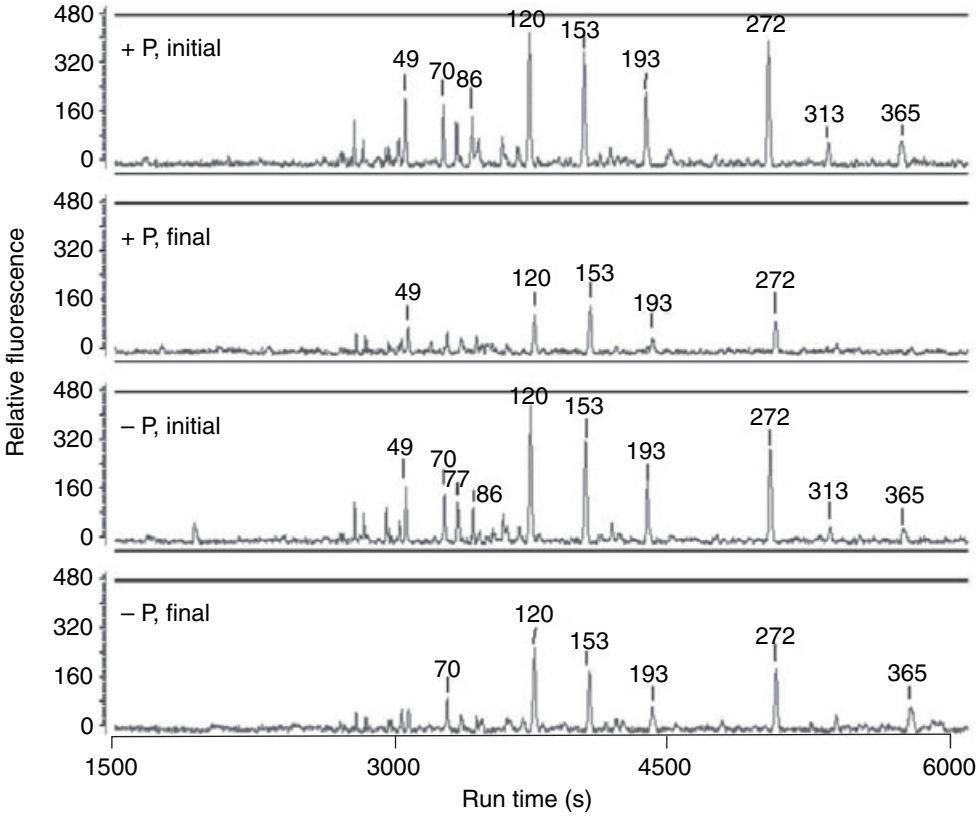

Fig. 5 Community structure of microheterotrophs (Eubacteria and Archaea) in the stromatolites before and after the experimental incubation with (+P, top panels) and without (–P, bottom panels) PO_4^{3-} enrichment (–S treatments only). Representative examples (of three replicates for each treatment) for the two treatments are shown. The figure shows the relative fluorescence detected as a function of elution time, with different peaks representing distinct taxa based on amplification and subsequent restriction endonuclease digestion of 16S rRNA gene fragments. Peaks were labelled if fluorescence exceeded 80 units. While there was a significant overall decline in total diversity during the experiment ($P < 0.001$, χ^2-test), P enrichment had no effect on the number of taxa detected in the samples ($P = 0.36$).

likely altered properties directly related to the living components of the mat (P sequestration, primary production, calcification, microbial community structure) without influencing the total organic matter present during the time scale of the experiment. However, it seems reasonable to expect that accumulation of effects of added P would eventually lead to detectable changes in overall standing biomass.

Stromatolites in the Rio Mesquites had high biomass C : P ratios relative to values reported for autotrophic components in other ecosystems; we are not aware of any published measurements of overall C : P ratio in microbial mat biomass. Stromatolites upon collection and in the unenriched treatments during the study had average C : P ratios ranging from 500 to 1000 (Fig. 1b); even higher values were observed for Rio Mesquites stromatolites studied in 2001 (see Elser *et al.*, 2005). These considerably exceed mean values for freshwater seston of approximately 310 and are comparable with values seen for terrestrial foliage (mean of 877; Elser *et al.*, 2000a). These ratios are well above previously published thresholds thought to induce P-limitation of animals (Sterner & Elser, 2002) and thus one would expect strong limiting effects of P on snails under these conditions (see Elser *et al.*, 2005). P-fertilisation drastically reduced the C : P ratios of surficial stromatolite biomass (Fig. 1b) to values (<100) lower than seen in the great majority of freshwater seston samples and even lower than the Redfield ratio of approximately 106 seen in marine particulate matter (Elser & Hassett, 1994). As C : N ratios did not change with P enrichment, N : P ratios also declined, similar to responses to P enrichment in the study of Camacho & de Wit (2003). Under

conditions favouring P-limitation, lower C : P and N : P ratios are consistent with more rapid growth rates of algae for both planktonic (Goldman, McCarthy & Peavey, 1979) and benthic (Hillebrand & Sommer, 1999) forms. More rapid rates of growth and metabolism in response to P-fertilisation are consistent with the increased rate of primary production that we documented in our oxygen measurements (Fig. 2a).

It is interesting to note that P fertilisation had its primary effect on diatoms with little impact on cyanobacteria (Fig. 4). It is important to remember that the DGGE analyses were performed on samples of whole 'florets' rather than materials obtained from the immediate stromatolite surface. Similar to the mats studied by Camacho & de Wit (2003), the phototrophic community of these stromatolites is highly structured along the vertical dimension, with diatoms and Rivulariaceae (tapering filamentous cyanobacteria) being the dominant forms in the outermost, easily scraped layers and with thin filamentous cyanobacteria allied to the morphogenera *Leptolyngbya/Phormidium* in deeper, cemented layers (Wade & Garcia-Pichel, 2003; Garcia-Pichel *et al*., 2004). Thus, diatoms had ready access to the added P while many of the cyanobacteria may never have actually experienced increased P supply in the +P treatments. This is supported by a limited set of DGGE fingerprints of samples obtained by surficial scraping (data not shown). These were very strongly dominated by diatom bands for a +P stromatolite and most major cyanobacteria bands were minor or missing. Like the cyanobacteria, heterotrophic microbes, as revealed by T-RFLP analysis, responded relatively modestly to the P enrichment. These results suggest that there is considerable variation among members of the microbial mat in the nature of the key resources influencing community structure: P appears to be playing a major role for certain, but not all, photoautotrophs whereas microheterotrophs are apparently not affected.

With respect to the photoautotrophs, the overall picture that emerges is that P enrichment favoured the proliferation of diatoms in the surface layers, perhaps at the expense of cyanobacteria and especially N-fixers. This result is at odds with previous enrichment studies examining nutrient effects on community structure in microbial mats. For example, in studies manipulating the supplies of nitrogen and phosphorus, it has been consistently shown that addition of N stimulates diatoms at the expense of cyanobacteria while addition of phosphorus favours cyanobacteria over diatoms (Fong *et al*., 1993; Marks & Lowe, 1993; Camacho & de Wit, 2003), a result that is consistent with resource ratio competition theory (Tilman, 1982; Smith, 1983) in which diatoms are generally thought to be superior P competitors (and thus likely to be N-limited) while N-fixing cyanobacteria are favoured under low N conditions because of their ability to fix atmospheric N (and thus are likely to be P-limited). However, most of these previous studies have been performed under conditions in which overall N and P availability were present in more balanced ratios than at the CCB, where overall N : P ratios were extremely high. Thus, in the previous studies, local supply ratios likely maintained diatoms under N-limitation and cyanobacteria under P-limitation. However, the extremely unbalanced N and P supplies at the CCB seem to have produced a situation where all members of the community had P-limited growth and thus P-enrichment stimulated all taxa that had access to the P enrichment.

As noted earlier, there is considerable debate among benthic ecologists, and among ecologists in general, about the importance of 'top down' grazer impacts on primary producers (Strong, 1992; Polis, 1999; Chase, 2000). Indeed, meta-analyses have indicated considerable variation among different ecosystems, both within and beyond the benthos, in the relative impacts of herbivores (Cebrian, 1999; Hillebrand, 2002). A variety of hypotheses have been proposed to explain that variation, including well-documented impacts of food-web structure via cascading trophic interactions (Pace *et al*., 1999). Among those hypotheses is the idea that in some habitats, primary producers are of such poor nutritional quality for herbivores that, regardless of food-web structure, herbivore impacts are muted because herbivores are maintained at very low levels by the poor food base (Polis, 1999). Indeed, elemental imbalance between autotrophs and consumers has been invoked (Sterner & Elser, 2002) in explaining the divergent outcomes of food-web manipulation in two lakes differing in algal C : P ratio, in which strong top-down effects were seen in a P-rich lake with a low algal C : P ratio (Elser *et al*., 2000b) but a lack of a trophic cascade was seen in a low-P lake with a high algal C : P ratio (Elser *et al*., 1998). We could not detect any impacts of grazing snails on any of our

response variables, despite the fact that we returned snails to the stromatolites at densities that were approximately 30% higher than ambient levels. As discussed above, stromatolite biomass had a very high C : P ratio and thus the weak snail impacts are consistent with the idea that stoichiometric constraints on herbivores can restrict the impact of grazing on primary producers in food webs. This conclusion of weak snail effects is tempered somewhat by the observation that snails in the +P+S treatment experienced high rates of mortality (see Elser *et al*., 2005) and thus grazing levels in that treatment were likely lower than normal. However, there was no apparent effect of snails on stromatolites in the unfertilised treatments where snails had high rates of survivorship and may even have produced offspring (see Elser *et al*., 2005). Thus, we conclude that grazers probably have little influence on the stromatolitic microbial mats in the Rio Mesquites.

Biogeochemical studies in other Ca^{2+}-rich ecosystems suggest that there may be strong and reciprocal interactions between the deposition of carbonate in mineralised waters and P-limitation of primary production. This occurs because phosphate can become bound in calcified materials via formation of various intermediate mineral substances (Corbett *et al*., 2000; Koch, Benz & Rudnick, 2001). In this way, high rates of calcification in mineral-rich waters such as those at Cuatro Ciénegas may scavenge PO_4^{3-} from the water column, reducing P availability to the biota (Erftemeijer *et al*., 1994; Corbett *et al*., 2000). Thus, P-limitation in the microbial mats at Cuatro Ciénegas is consistent with similar observations in other carbonate-rich habitats, such as seagrasses in Florida Bay (Short, Dennison & Capone, 1990; Fourqurean, Zieman & Powell, 1992). In this way, mineralogical trapping of PO_4^{3-} because of biologically enhanced calcification may generate a feedback system that maintains these stromatolite-based ecosystems in a permanent state of severe P-limitation that might only be broken by massive and sustained alterations in P supply.

In conclusion, our study indicates that the supply of phosphorus has large effects on the composition and metabolism of the community comprising the oncoid stromatolites at Cuatro Ciénegas. However, the impacts of ambient densities of grazing snails are negligible. These two outcomes may be linked by a single mechanism: intense calcification in these waters, because of their inherently high alkalinity and the action of the photosynthetic microbes themselves, may strip PO_4^{3-} from the water, resulting in extremely high TN : TP ratios, severe P-limitation of the microbial community and consequently high C : P ratios of microbial biomass. Thus, the end product is a food web-base that is stoichiometrically inadequate for grazing animals and that renders the herbivores at the base of the food web strongly responsive, both positively and negatively, to P enrichment, as shown in the accompanying paper (Elser *et al*., 2005).

Acknowledgments

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