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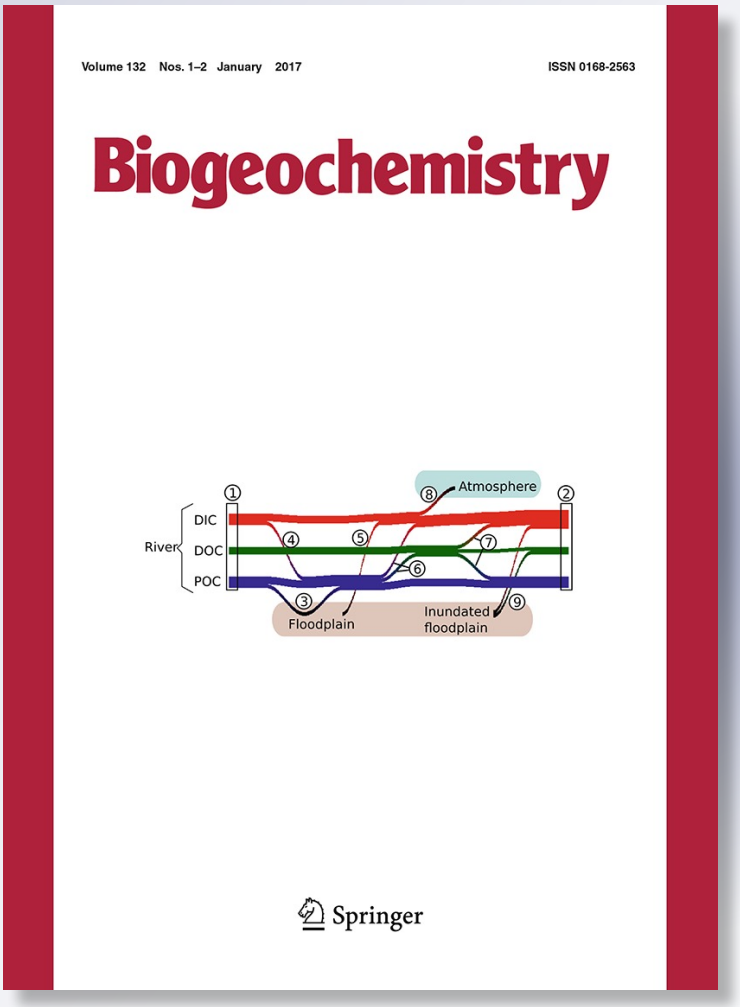

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Ecoenzyme activity ratios reveal interactive effects of nutrient inputs and UVR in a Mediterranean high-mountain lake

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Abstract To understand how atmospheric dust deposition and ultraviolet radiation (UVR) can affect remote, freshwater ecosystems through changes in their microbial metabolism, it is important to have tools that allow us detecting alterations and anticipating potential shifts in the functioning of microbial communities. Ecoenzyme activities (EA) are easy to measure and their ratios can be used to assess system microbial metabolism of freshwater bodies, thus evaluating the effects of global change stressors. We carried out an in situ full factorial experiment to determine how the interaction between the addition of C and P, and UVR affect the microbial metabolism of a Mediterranean high-mountain lake. Overall, activities of five ecoenzymes involved in the degradation of C-compounds and in the acquisition of N and P revealed that, under natural conditions, the growth of heterotrophic prokaryotes was dependent on organic compounds released by algae, which is consistent with a higher constraint of bacterial carbon production by C than by P or N, as suggested by EA ratios. Accordingly, the addition of a labile C source did not lead to any significant response of microbial communities, but the addition of P provoked a clear change in the microbial metabolism of the lake, promoting the growth of phytoplankton and leading heterotrophic prokaryotes to be more constrained by P, and to a lesser extent by N, in relation to C. UVR played a secondary role, probably because microbial communities inhabiting high-mountain lakes possess several evolutionary adaptations to high UVR levels. Changes in the microbial metabolism of our model lake under different scenarios of nutrient inputs and UVR can therefore be evaluated by EA ratios.

Keywords Ecoenzyme activity (EA) ratios · Nutrient inputs · UVR · High-mountain lakes · Mediterranean · Energy/nutrient limitation

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Introduction

Inland water bodies are of great importance for assessing changes in global ecological processes

because they comprise biodiversity hot-spots and are interconnected with terrestrial ecosystems (Williamson et al. 2008). It is widely accepted that they are directly related with global biogeochemical cycles, constituting metabolically active ecosystems and not only matter or energy storage compartments (Cole et al. 2007; Williamson et al. 2008). Several global change stressors can affect freshwater ecosystems through changes in their metabolism, especially when ecological processes (e.g., primary production, decomposition, etc.) are maintained by a few species. Among these stressors, the ultraviolet radiation (UVR), which inhibits the growth of organisms, particularly in upper mixing layers (UML) of lakes (Carrillo et al. 2015a), and the increase of atmospheric dust deposition (Bullejos et al. 2010; Cabrerizo et al. 2016), a source of organic matter rich in phosphorus (P) and carbon (C) compounds (Mladenov et al. 2009) which might alter the coupling between algae and bacteria in lakes by modifying their availability (Durán et al. 2016), have received special attention during last years (IPCC 2013). High-mountain lakes have been recognized as sentinels of global change because they are located outside of direct human influence and respond rapidly to climate and land-use change through shifts in the ecological structure and functioning of their microbial communities (Mladenov et al. 2011; Medina-Sánchez et al. 2013; Vinebrooke et al. 2014). In the Mediterranean area, probably the most vigorous place on the face of our planet for interactions between different societies, the capacity of freshwater bodies to act as sensors of global change is particularly valuable.

Ecoenzymes, a general term that encompasses all the enzymes located outside the confines of intact cell membranes, regardless of whether they are produced by secretion or lysis, facilitate the generation of labile substrates to cover energetic demands and/or for building biomass in osmotrophic microorganisms (Sinsabaugh and Follstad-Shah 2012). Ecoenzyme activities (EA) are therefore an important link between dissolved organic matter (DOM) and biogeochemical processes driven by osmotrophic microorganisms in aquatic environments, playing a central role in the recycling of C and nutrients, and in the transfer of energy among trophic compartments (Chróst 1992). Several works have assessed the effects of UVR (Boavida and Wetzel 1998; Scully et al. 2003; Tank et al. 2005; Santos et al. 2011; Sereda et al. 2011; Korbee et al. 2012; Janssen and McNeill 2015) and nutrient inputs (Cotner and Wetzel 1992; Foreman et al. 1998; Stepanauskas et al. 1999; Sala et al. 2001; Sirová et al. 2006; Wang et al. 2014; Burpee et al. 2016) on EA. However, despite the importance to understand the effects of both nutrient inputs and UVR on microbial metabolism in aquatic ecosystems, there are only a few studies reporting interactive effects of these two global change stressors in aquatic environments (Korbee et al. 2012; Proia et al. 2012; Carrillo et al. 2015b).

In addition, EA play a central role in the intersection of metabolic and stoichiometric theories in ecology and, consequently, they are considered to be as an expression of the microbial production that mediates energy/resource consumption and carbon/nutrient limitation (Sinsabaugh et al. 2009, 2010). EA ratios are very useful for assessing carbon/nutrient limitations in natural ecosystems (Sinsabaugh and Follstad-Shah 2012). The basis of EA ratios is organismal control over enzyme expression based on environmental signals to maximize both N- and P-assimilation efficiency and C production in microbial communities (Sinsabaugh and Follstad-Shah 2012). When availability of carbon and nutrients is in balance with the microbial growth rate, microbial communities tend to reach a steady state in which C, N and P are co-limiting bacterial carbon production (BCP), and ecoenzymatic C:N:P ratios tend to be constant at 1:1:1 (Sinsabaugh et al. 2009). Deviations in these ratios, i.e., variations in the stoichiometry of N and P acquisition relative to C, may indicate that microbial communities have not reached that state, which suggests that microbial growth is limited by the element in short supply, and that this limitation will propagate from cells to ecosystems (Sinsabaugh et al. 2009; Hill et al. 2014). Relative carbon/nutrient limitation associated with C, N and P acquisition can also be assessed by vectors defined by ratios of ecoenzyme activities according to Moorhead et al. (2013). The length and angle of these vectors represent relative microbial investments in C (length) or N and P (angle) acquisition (see Moorhead et al. 2013).

With this background, the goals of the present study were (1) to assess the energy (C) and/or nutrient (N or P) status of microbial communities of a remote high-mountain lake by using EA ratios, (2) to comprehend the potential effects of two global change stressors (nutrient inputs and UVR) on the biogeochemical

processes driven by microbial communities in a pristine Mediterranean lake by using EA ratios, and (3) to evaluate the usefulness of EA ratios to describe the microbial metabolism in an oligotrophic clear-water model system. Because EA ratios can track C:N:P relationships in the lake, reflecting a *microbial perception* of the environment (Álvarez and Guerrero 2000; Ayuso et al. 2011), and not only the activities of the microbial community, we hypothesized that both nutrient inputs and UVR will have negative effects on prokaryotic EA, inhibiting them, thus modifying EA ratios as a response to shifts in energy (C) or nutrient (N and P) limitation in microbial communities. To test our hypothesis, and to reach our goals, we carried out an in situ experiment manipulating nutrient (C, P) availability and UVR.

Materials and methods

Study area and experimental design

La Caldera Lake is a remote, oligotrophic and relatively small and shallow (surface area of around 0.02 km^2 and mean depth of 4.3 m) high-mountain water body situated above the tree line (3050 m above sea level) in Sierra Nevada National Park (SE Spain) (supplementary Fig. 1). Located on a siliceous bedrock in a glacial cirque at the feet of the tallest mountain of the Iberian Peninsula (Mulhacén Peak), the lake has no visible inlets or outlets, and macrophytes, littoral vegetation and fish are absent (Medina-Sánchez et al. 2006).

The experiment consisted of a full $2 \times 2 \times 2$ factorial design with UVR (full sunlight, 280–700 nm, +U, and photosynthetic active radiation (PAR), 400–700 nm, −U), carbon (+C and −C) and phosphorus (+P and −P) factors. The experiment was conducted in situ for 19 days (August, 31st–September, 18th 2011). Mesocosms consisted of semi-spherical, transparent polyethylene bags (mesocosms) filled with 100 L of water taken in the upper layers of the lake and filtered through a 45 μm-mesh to remove large zooplankton. Three mesocosms per treatment (replicates) were attached to floating PVC frameworks that allowed the incubation of polyethylene bags (approximately 1 m below the water surface) to reproduce future predictions of UVR irradiances due to shallower UML. Thus, mean irradiances at this depth were 2–3× higher than those received by organisms in deeper layers (mean irradiances at 1 m $\simeq$ 1.54, 11.04 and 34. 66 μW cm^{-2} nm^{-1} for UVR (305, 320 and 380 nm, respectively) and 908.60 μmol photons m^{-2} s^{-1} for PAR; mean irradiances in the water column $\simeq$0.52, 4.06 and 15.90 μW cm^{-2} nm^{-1} for UVR (305, 320 and 380 nm, respectively) and 468.86 μmol photons m^{-2} s^{-1} for PAR). In total, 24 mesocosms were installed. PVC frameworks were anchored to the bottom of the lake. Mesocosms of the −U treatments were covered with Ultraphan 395 plastic filters to block UVR, whereas those of the +U treatments were covered with Ultraphan 290 plastic filters (Digefra, GmbH, Munich, Germany, EU). Sucrose was added as a source of labile carbon in the +C treatments (3480 μC L^{-1}, final concentration), and Na_2HPO_4 was added as a source of phosphorus in the +P treatments (30 μg P L^{-1}, final concentration). We added, on day 0 of the experiment, 406 μg N–NH_4NO_3 L^{-1} as a source of N to reach a N:P molar ratio of 30 in +P treatments, mimicking the N:P ratio values observed in dust depositions on Sierra Nevada National Park area (see Morales-Baquero et al. 2006). All nutrients were prepared in fresh, double-distilled, autoclaved water, and added as a unique pulse on day 0. To summarize, we setup four different treatments per level of the UVR factor: +UK (control, −C − P), +UC (carbon, +C − P), +UP (phosphorus, −C + P) and +UCP (carbon and phosphorus, +C + P) for the +U treatments (full sunlight), and −UK (control, −C − P), −UC (carbon, +C − P), −UP (phosphorus, −C + P) and −UCP (carbon and phosphorus, +C + P) for the −U treatments.

Sampling method

Water was carefully sampled through a plastic tube installed in each mesocosm by using an electrical water pump. Water samples were taken 1, 2, 3, 4, 7, 10, 13, 16 and 19 days after the experimental setup. Samples were transported cold (4 °C) and dark every day to the laboratory within 4 h, then filtered, and appropriately conserved and stored until analyzed, normally during the next 10 days after sampling.

Physicochemical water variables

Vertical profiles of solar radiation in the water column were obtained at noon during the day 1 of the experiment by using a submersible BIC Compact

4-channel radiometer (Biospherical Instruments, San Diego, CA, USA) with three channels in the UVR region (305, 320, and 380 nm) and one broad-band channel for PAR light (400–700 nm). Diffuse attenuation coefficients in the water column (k_d) were determined from the slope of the linear regression of the natural logarithm of measured irradiance versus depth for each wavelength range considered. Temperature and dissolved oxygen profiles were recorded during the first day using a multiparametric probe (Hanna Instruments, Woonsocket, RI, USA).

DOC concentrations were measured by using the high-temperature catalytic oxidation method in a Shimadzu 5000 TOC analyzer (Shimadzu Corporation, Kyoto, Japan) (Benner and Strom 1993). Chemical variables (TP, total phosphorus; TDP, total dissolved phosphorus; SRP, soluble reactive phosphorus; TN, total nitrogen; TDN, total dissolved nitrogen; NO_3^-, nitrate) were spectrophotometrically measured according to standard methods (APHA et al. 1999).

Structural biological variables

Chlorophyll *a* (chl *a*) was spectrofluorometrically measured by using standard methods (APHA et al. 1999). Chl *a* content was determined during days 4, 7, 10, 13, 16 and 19. Briefly, water samples were filtered through Whatman GF/C glass-fiber filters (Whatman, Little Chalfont, England, UK, EU) and then, filters were frozen at −20 °C. To extract chl *a*, filters were thawed and placed in 15 mL centrifuge tubes with 5 mL of 90% acetone during 24 h in the dark and at 4 °C. After this, samples were centrifuged and the fluorescence of the supernatant measured with a Perkin Elmer LS 55 fluorometer (Perkin Elmer, Waltham, MA, USA).

Bacterial abundance (BA) was determined by using a FACSCanto II flow cytometer (Becton–Dickinson Biosciences, Oxford, England, UK, EU). Algal abundance (AA) was determined by counting cells at 400× and 1000× in water samples preserved with lugol solution, and allowed to settle in Utermöhl chambers for 24 h, using a Zeiss Axio Observer A1 inverted microscope (Zeiss, Oberkochen, Germany, EU). BA was determined during days 1, 2, 3, 4, 10 and 19. AA was estimated during days 7, 10, 13, 16 and 19 of the experiment. For more details, see Dorado-García et al. (2014).

Functional biological variables

For excreted organic carbon (EOC) measurements, samples with phytoplankton were placed in 50 mL quartz flasks (three clear and one dark per radiation treatment), inoculated with 0.37 MBq of $NaH^{14}CO_3$ (310.8 MBq $mmol^{-1}$; DHI Water and Environment, Berlin, Germany, EU), and exposed in the upper layers of the lake (approximately 1 m below the water surface) to light treatments during 4 h (around noon) in days 4 and 19 of the experiment. The total organic carbon (TOC) produced was determined in 4 mL aliquots before filtration. EOC was measured on 4 mL aliquots from the filtrates (<0.2 μm). Aliquots were placed in 20 mL scintillation vials and acidified with 100 μL of 1 N HCl for 24 h (no bubbling), to remove inorganic ^{14}C, before the addition of liquid scintillation cocktail to the vials. For more details, see Carrillo et al. (2002).

Bacterial carbon production (BCP) was estimated through the incorporation of ^3H-leucine into cellular proteins over time (Kirchman 1993). 1.5 mL of water (in 2 mL micro tubes) and the radiotracer (^3H-leucine, L-[3,4,5-^3H(N)]-leucine, 40–60 Ci/mmol; Perkin Elmer, Waltham, MS, USA) were incubated at in situ temperature during 60 min in the dark. Extraction was made with 5% cold trichloroacetic acid (TCA). In all the calculations, data were corrected by blanks, i.e., bacteria were killed with 5% cold TCA before the addition of the radiotracer. The factor 1.55 kg mol^{-1} was used to convert the incorporated ^3H-leucine into carbon (Simon and Azam 1989). BCP was measured during days 1, 2, 3, 4, 10 and 19.

Water samples for estimating ecoenzyme activities (EA) were stored in 50 mL polyurethane bottles, and frozen (−20 °C) until analyses began. Freezing is a procedure recommended for samples on which EA cannot be determined immediately (Chróst and Velimirov 1991). In the laboratory, samples were air-thawed, filtered through Whatman GF/C glass-fiber filters (Whatman, Little Chalfont, England, UK, EU), and then assayed for the activity of five different enzymes involved in the degradation of labile C compounds (α-1,4-D-glucosidase, ALPHA, EC 3.2.1.20, and ß-1,4-D-glucosidase, BETA, EC 3.2.1.21) or recalcitrant C-compounds (phenol oxidase, PHO, EC 1.10.3.2) to assimilate C, and in the acquisition of N (leucine aminopeptidase, LAP, EC 3.4.11.1) or P (alkaline phosphatase, AP, EC 3.1.3.1).

ALPHA, BETA, PHO, LAP and AP activities were determined by using fluorogenic or chromogenic model substrates according to Pind et al. (1994), Sinsabaugh and Foreman (2001) and Sinsabaugh (2010). All enzyme analyses were conducted in black (fluorometry, APHA, BETA, LAP and AP) or transparent (colorimetry, PHO) 96-well microplates at saturating substrate concentrations to facilitate comparison of total enzyme activities among treatments (0.2 mM, final substrate concentration). Therefore, the measured values should be considered assays of potential activities. Five technical replicates (per mesocosm) were used for EA analyses. Substrates for ALPHA, BETA, PHO, LAP and AP were 4-methylumbelliferyl-α-D-glucopyranoside, 4-methylumbelliferyl-ß-D-glucopyranoside, L-3,4-dihydroxyphenylalanine (DOPA), L-leucine-7-amido-4-methylcoumarin, and 4-methylumbelliferylphosphate, respectively (Sigma-Aldrich, Saint Louis, MO, USA). Substrate solutions (1 mM, final concentration) were prepared in autoclaved sodium bicarbonate buffer (pH 8.0) and stored at 4 °C in the dark. Fresh DOPA was always used. Fluorescence readings for ALPHA, BETA, LAP and AP were recorded under optimized conditions of a 365-nm wavelength slit width excitation filter and a 450-nm wavelength slit width emission filter. Absorption readings for PHO were recorded at 460 nm. The microplates were kept in the dark at 20 °C and shaken continuously to reduce cell adhesion to well walls. Quenching was estimated by comparing the fluorescence of methylumbelliferone (MUF) or 7-amido-4-methyl coumarin (COU) standards, or the absorbance of DOPA standards, mixed with lake water to the fluorescence, or absorbance, of 50 µL of standard solution mixed with 200 µL of a 5 mM sodium bicarbonate solution. Fluorescence or absorbance readings were recorded every hour during 24 h with a Biotek Sinergy H1 Multimode Reader (Biotek Instruments, Winooski, VT, USA).

Ecoenzyme activity ratios

According to Sinsabaugh et al. (2009) and Sinsabaugh and Follstad-Shah (2012), ratios of EA can be linked to both microbial metabolism and environmental energy/resource availability through the following equations:

$$\mathrm{GLU/LAP} \sim (\mathrm{TER_{C:N}}/\mathrm{B_{C:N}}) = (\mathrm{A_N}/\mathrm{GE}) \quad (1)$$

$$\mathrm{GLU/AP} \sim (\mathrm{TER_{C:P}}/\mathrm{B_{C:P}}) = (\mathrm{A_P}/\mathrm{GE}) \quad (2)$$

where GLU is the activity of enzymes related with the degradation of labile C compounds (ALPHA + BETA), LAP is the activity of enzymes related with N acquisition (LAP), AP is the activity of enzymes related with P acquisition (AP), $\mathrm{TER_{C:N}}$ and $\mathrm{TER_{C:P}}$ are the threshold elemental ratios for C:N and C:P respectively, $\mathrm{B_{C:N}}$ and $\mathrm{B_{C:P}}$ are the elemental C:N and C:P ratios of microbial biomass, $\mathrm{A_N}$ and $\mathrm{A_P}$ are the microbial assimilation efficiencies for N and P, and GE is the microbial growth efficiency with respect to C. Because it is difficult to measure simultaneously all the variables included in Eqs. (1) and (2), EA ratios can be seen as indicators of energy fluxes (C) and potential nutrient limitations (N, P) in aquatic ecosystems. We also determined energy/nutrient limitation by using vector analysis (length, vector L, VL; angle, vector L, VA) of EA ratios according to Moorhead et al. (2013) and Hill et al. (2014):

$$\text{vector L} = \sqrt{\left(\frac{\mathrm{GLU}}{\mathrm{LAP}}\right)^2 + \left(\frac{\mathrm{GLU}}{\mathrm{AP}}\right)^2} \quad (3)$$

$$\text{vector A} = \frac{\tan\left(\left(\frac{\mathrm{GLU}}{\mathrm{LAP}}\right), \left(\frac{\mathrm{GLU}}{\mathrm{AP}}\right)\right) \times 180}{\pi} \quad (4)$$

where GLU, LAP and AP are the same as in Eqs. (1) and (2). In Eqs. (1), (2), (3) and (4), GLU, LAP and AP activities were always ln-transformed prior to their use.

Data analyses

We analyzed the effects of C, P and UVR on EA over time by using linear mixed-effects (LME) models. We fitted different LME models for each EA in which UVR, C, P and time were treated as fixed effects, and the mesocosms were treated as a random effect. The structure of these LME models allowed us to avoid temporal pseudo-replication (Faraway 2006). Diagnostic plots did not reveal apparent deviations from homoscedasticity and normality in these models; *p* values were obtained by likelihood ratio tests. Relationships among physicochemical water variables, and both structural and functional biological variables were explored using Spearman rank order correlation coefficients to avoid problems with non-normal distribution of data (Zar 1998). Differences

among the linear regressions of ln-transformed GLU/LAP, GLU/AP and LAP/AP among treatments were tested using ANCOVA (Zar 1998). We analyzed vector L and vector A data using LME models in a repeated measures structure where C, P, UVR and time were considered to be fixed effects; we included the factor ID (the identity of each mesocosm) as a random effect in the analysis. Visual inspection of residual plots did not reveal apparent deviations from homoscedasticity and normality in these models; p values were obtained by likelihood ratio tests. A p value of 0.05 was set as the significant threshold for all statistical analyses. All statistical analyses and plots were completed in *R* (R Development Core Team 2016). Specifically, all LME models were fitted using the "nlme" package (Pinheiro et al. 2016), whereas Tukey's HSD posthoc tests, used to perform pairwise comparisons among treatments after LME and ANCOVA models, were computed using the "lsmeans" package (Lenth 2016).

Results

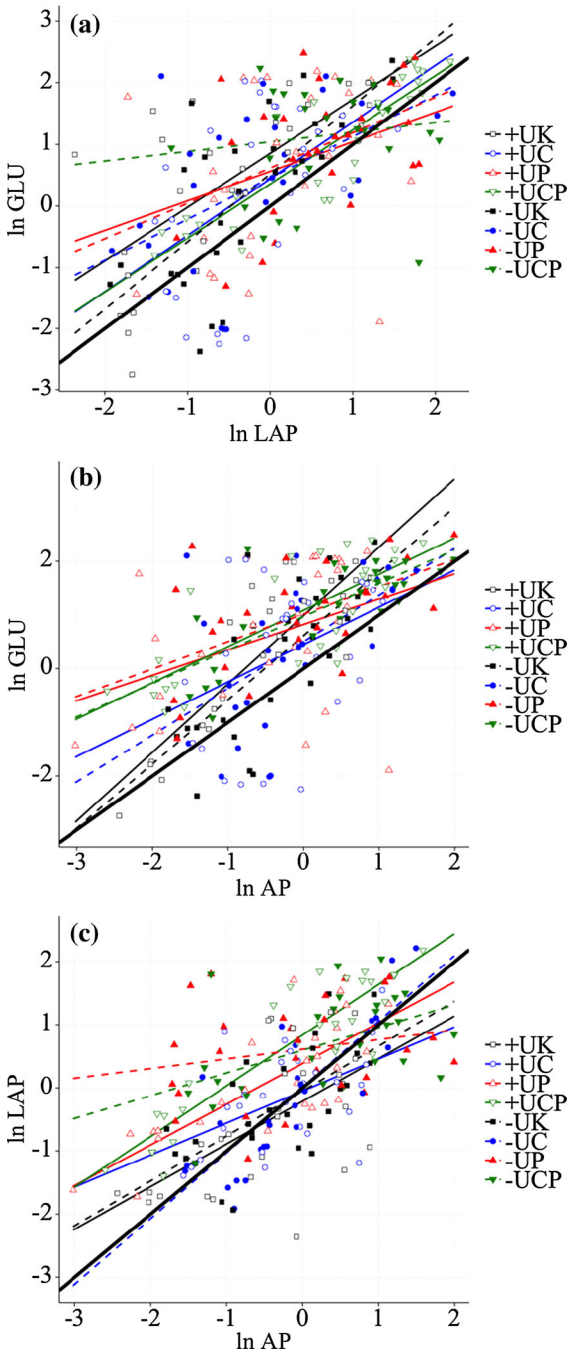
Physicochemical and structural and functional biological variables

The water temperature in the lake was rather homogeneous through the vertical profile and along the experiment. Upper layers of the lake were always well oxygenated. Vertical coefficients of attenuation for the different regions of the UVR spectrum showed low values, with ca. 90% of incident UVR in the first 2 m, where mesocosms were placed. Similarly, the penetration of PAR was very high, with more than 10% of incident PAR reaching the bottom of the lake. Initial conditions of the lake (August, 30th 2011) are shown in supplementary Table 1. Changes in physicochemical and both structural and functional biological variables over the experiment are reported elsewhere (Dorado-García et al. 2014; Medina et al. in preparation). All these variables are used in this study for correlation analyses.

Ecoenzyme activities

PHO activities were negligible, almost zero in all cases, whereas glucosidase activities (ALPHA and BETA) showed variable values depending on treatments (supplementary Fig. 2a, b). Thus, ALPHA activities varied between 0.03 ± 0.09 μmol L^{-1} h^{-1} (+UK treatment on day 1) and 5.87 ± 1.84 μmol L^{-1} h^{-1} (+UP treatment on day 13) (supplementary Fig. 2c, d). The addition of P had significant stimulatory effects on ALPHA activities (LME model, $p < 0.05$, supplementary Table 2) (HSD tests, $p < 0.05$). ALPHA activities positively correlated with NO_3^-, chl *a*, BA, AA and BCP (supplementary Table 3). BETA values varied between 0.04 ± 0.01 and 6.94 ± 0.70 μmol L^{-1} h^{-1}, showing their minimum values in the −UC treatment on day 2 and their maximum values in the +UCP treatment on day 7 (supplementary Fig. 2e, f). We obtained significantly higher BETA activities when P was added (LME model, $p < 0.05$, supplementary Table 2) (HSD tests, $p < 0.05$). We found a significant UVR × P interaction effect on BETA activities (LME model, $p < 0.05$, supplementary Table 2): +P treatments showed higher BETA values than −P treatments, although significantly higher only when UVR was blocked (HSD tests, $p < 0.05$, supplementary Fig. 3a). BETA activities positively correlated with NO_3^-, chl *a*, AA, EOC and BCP (supplementary Table 3).

LAP activity values were lowest in the +UK treatment on day 1 (0.18 ± 0.00 μmol L^{-1} h^{-1}) and highest in the −UCP treatment on day 4 (5.92 ± 0.47 μmol L^{-1} h^{-1}) (supplementary Fig. 4a, b). The addition of P had a significant stimulatory effect on LAP activities (LME model, $p < 0.05$, supplementary Table 2) (HSD tests, $p < 0.05$). LAP values positively correlated with all N and P forms, as well as with chl *a*, BA, AA, EOC and BCP (supplementary Table 3). AP activities ranged from 0.10 ± 0.18 μmol L^{-1} h^{-1} (+UP treatment, day 1) to 6.16 ± 3.52 μmol L^{-1} h^{-1} (−UCP treatment, day 7) (supplementary Fig. 4c, d). All factors had significant effects on AP activities (LME model, $p < 0.05$, supplementary Table 2). The presence of UVR negatively affected AP activities (HSD tests, $p < 0.05$), whereas the addition of C and/or P enhanced them (HSD tests, $p < 0.05$). There was a significant UVR × P interactive effect on AP activities (LME model, $p < 0.05$, supplementary Table 2): again, +P treatments showed higher AP values than −P treatments, although significantly higher only when UVR was blocked (HSD tests, $p < 0.05$, supplementary Fig. 3b). AP activities positively correlated with NO_3^-, chl *a*, EOC and BCP,



◀**Fig. 1** Scatterplots showing the linear regression lines of transformed to ln GLU versus transformed to ln LAP (**a**), transformed to ln GLU versus transformed to ln AP (**b**), transformed to ln LAP versus transformed to ln AP (**c**), under each of the eight treatments (*GLU*, sum of *ALPHA* (activity of α-1,4-D-glucosidase) and *BETA* (activity of ß-1,4-D-glucosidase) activities; *LAP* activity of leucine aminopeptidase; *AP* activity of alkaline phosphatase). *Thick*, *solid*, *black lines* denote 1:1 lines. Slopes, intercepts, and both R^2 and p values are shown in Table 1 (−*U*, PAR light; +*U*, full sunlight; *K*, control; *C* addition of carbon; *P* addition of phosphorus; *CP* addition of both carbon and phosphorus)

whereas negatively correlated with TDN and SRP (supplementary Table 3).

Ecoenzyme activity ratios

Results for relative energy/nutrient availabilities, measured by linear relationships of ln-transformed GLU/LAP, GLU/AP and LAP/AP among treatments, are shown in Fig. 1. Models were always significant for the GLU/LAP, GLU/AP and LAP/AP linear relationships in all treatments (Table 1). Generally speaking, the addition of P considerably modified EA ratios, but the addition of C (without P), or the UVR conditions, did not markedly change them when compared to the −UK treatment (with no nutrients added and UVR blocked). We found significant differences in GLU/LAP ratios among treatments (ANCOVA model, $p < 0.05$, Table 2). In +UK and −UK treatments, GLU/LAP ratios showed values close to 1 (Table 1), with no significant differences in their slopes (HSD tests, $p > 0.05$), although the slope in the −UK treatment was slightly higher (Table 1; Fig. 1a). In +P treatments, GLU/LAP slopes were lower than in +UK and −UK treatments, although +UP and +UCP treatments did not show significant differences when compared to the +UK treatment (HSD tests, $p > 0.05$), whereas only the −UCP treatment showed a significantly lower slope than the −UK treatment (HSD tests, $p < 0.05$) (Table 1; Fig. 1a). GLU/LAP ratios negatively correlated with DOC/TP, TN/TP, DOC/TDP and TDN/TDP (supplementary Table 3). As in the GLU/LAP ratios, significant differences in slopes among treatments were found in GLU/AP ratios (ANCOVA model, $p < 0.05$, Table 2). In +UK and −UK treatments, slopes of GLU/AP ratios were >1 and always higher than in any other treatment (Table 1; Fig. 1b). In +C treatments, GLU/AP slopes lowered, but no significant differences were found when compared to the slopes of both control treatments (HSD tests, $p > 0.05$) (Table 1; Fig. 1b). In +P treatments, GLU/AP slopes were significantly lower than in both control treatments (HSD tests, $p < 0.05$) (Table 1; Fig. 1b), but we did not find significant differences in the slopes due to the

Table 1 Results of linear regression analyses for transformed to ln ecoenzyme activity (EA) ratios (GLU, sum of ALPHA (activity of α-1,4-D-glucosidase) and BETA (activity of ß-1,4-D-glucosidase) activities; LAP, activity of leucine aminopeptidase; AP, activity of alkaline phosphatase; SE, standard error; −U, PAR; +U, full sunlight; K, control; C, addition of carbon; P, addition of phosphorus; CP, addition of carbon and phosphorus) (bold-face indicates significant values, $p < 0.05$)

Dependent variable	Independent variable	Treatment	Slope (SE)	Intercept (SE)	r^2	p
Ln GLU	Ln LAP	+UK	0.87 (0.18)	0.85 (0.21)	0.56	**0.000**
		+UC	0.91 (0.26)	0.44 (0.20)	0.35	**0.000**
		+UP	0.47 (0.21)	0.56 (0.19)	0.29	**0.006**
		+UCP	0.87 (0.17)	0.35 (0.22)	0.89	**0.000**
		−UK	1.10 (0.22)	0.52 (0.20)	0.67	**0.000**
		−UC	0.66 (0.18)	0.45 (0.20)	0.38	**0.000**
		−UP	0.52 (0.25)	0.61 (0.24)	0.30	**0.002**
		−UCP	0.15 (0.25)	1.04 (0.26)	0.29	**0.009**
Ln GLU	Ln AP	+UK	1.27 (0.20)	0.98 (0.21)	0.84	**0.000**
		+UC	0.79 (0.25)	0.44 (0.19)	0.25	**0.006**
		+UP	0.47 (0.17)	0.82 (0.20)	0.26	**0.006**
		+UCP	0.67 (0.14)	1.08 (0.19)	0.70	**0.000**
		−UK	1.19 (0.23)	0.61 (0.20)	0.61	**0.000**
		−UC	0.87 (0.22)	0.50 (0.19)	0.39	**0.000**
		−UP	0.51 (0.17)	1.01 (0.18)	0.40	**0.003**
		−UCP	0.62 (0.17)	0.96 (0.19)	0.67	**0.000**
Ln LAP	Ln AP	+UK	0.67 (0.14)	0.00 (0.14)	0.44	**0.000**
		+UC	0.50 (0.17)	0.04 (0.13)	0.35	**0.004**
		+UP	0.64 (0.12)	0.37 (0.14)	0.72	**0.000**
		+UCP	0.74 (0.10)	0.83 (0.13)	0.92	**0.000**
		−UK	0.71 (0.16)	0.05 (0.14)	0.43	**0.000**
		−UC	0.94 (0.16)	0.01 (0.13)	0.75	**0.000**
		−UP	0.15 (0.12)	0.61 (0.13)	0.35	**0.008**
		−UCP	0.36 (0.12)	0.59 (0.13)	0.34	**0.009**

UVR factor when C and P treatments were compared (HSD tests, $p > 0.05$) (Table 1; Fig. 1b). GLU/AP ratios negatively correlated with DOC/TP, DOC/TDP, TN/TP and TDN/TDP ratios (supplementary Table 3). We also observed significant differences in LAP/AP ratios among treatments (ANCOVA model, $p < 0.05$, Table 2). In +UK and −UK treatments, LAP/AP ratios were <1 (Table 1; Fig. 1c). +C treatments also showed LAP/AP ratios <1, but with statistically similar slopes than these for +UK and −UK treatments (HSD tests, $p > 0.05$) (Table 1; Fig. 1c). +P treatments showed lower LAP/AP slopes than these for both control treatments (+UK and −UK), although the differences were significant only in −U treatments (HSD tests, $p < 0.05$) (Table 1; Fig. 1c). LAP/AP ratios negatively correlated with DOC/TP, TN/TP, DOC/TDP, TDN/TDP, DOC/SRP and NO_3^-/SRP (supplementary Table 3).

The effects of UVR, C and P factors on vectors VL and VA values are shown in Table 3. Overall, the addition of P significantly modified VL and VA values when compared to treatments with no nutrients added (+UK and −UK), regardless of UVR conditions. Among the main factors, P had a significant effect on VL (LME model, $p < 0.05$, Table 3): −P treatments showed higher VL than +P treatments (HSD tests, $p < 0.05$). All factors significantly affected VA (LME model, $p < 0.05$, Table 3): +U treatments showed significantly higher VA than −U treatments, +C treatments displayed significantly higher VA than −C treatments, and we found significantly lower VA in +P treatments than in −P treatments (HSD tests,

Table 2 Results of analysis of covariance (ANCOVA) for ecoenzyme activity ratios (EA ratios)

Treatments are the independent factor for each ecoenzyme pair (bold-face indicates significant values, $p < 0.05$)

Dependent variable	Independent variable	*df*	*F*	*p*
Ln GLU	Ln LAP	1	115.25	**0.000**
	Treatments	7	2.41	**0.023**
	Ln LAP × treatments	7	3.01	**0.012**
Ln GLU	Ln AP	1	124.70	**0.000**
	Treatments	7	2.12	**0.043**
	Ln AP × treatments	7	2.25	**0.031**
Ln LAP	Ln AP	1	187.90	**0.000**
	Treatments	7	8.86	**0.000**
	Ln AP × treatments	7	4.17	**0.000**

Table 3 Results of ANOVA after linear mixed-effects (LME) models showing the effects of fixed factors, and their interactions, on vector L and vector A (UVR, ultraviolet radiation factor; C, carbon factor; P, phosphorus factor) (bold-face indicates significant values, $p < 0.05$)

	Vector L			Vector A		
	df	*F*	*p*	*df*	*F*	*p*
Intercept	1	170.16	**0.000**	1	191,179.36	**0.000**
UVR	1	0.87	0.364	1	124.29	**0.000**
C	1	0.00	0.977	1	37.96	**0.000**
P	1	74.51	**0.000**	1	135.97	**0.000**
Time	8	12.33	**0.000**	8	21.11	**0.000**
UVR × C	1	0.49	0.493	1	1.26	0.125
UVR × P	1	1.06	0.318	1	112.78	**0.000**
C × P	1	1.48	0.241	1	0.45	0.510
UVR × time	8	0.79	0.609	8	5.94	**0.000**
C × time	8	1.16	0.326	8	0.65	0.734
P × time	8	1.98	0.053	8	3.86	**0.000**
UVR × C × P	1	2.18	0.158	1	0.95	0.343
UVR × C × time	8	4.84	**0.000**	8	0.22	0.986
UVR × P × time	8	0.96	0.466	8	4.93	**0.000**
C × P × time	8	3.57	**0.000**	8	4.14	**0.000**
UVR × C × P × time	8	3.95	**0.000**	8	5.59	**0.000**

$p < 0.05$). Interestingly, we found a significant UVR × P interactive effect on VA (LME model, $p < 0.05$, Table 3): +P treatments significantly lowered VA values when compared to −P treatments, and this decrease was more evident when UVR was blocked (HSD tests, $p < 0.05$, supplementary Fig. 3c).

Discussion

This work evaluates, for the first time, the simultaneous effects of two important global change-related stressors (nutrient inputs and UVR) on the activities of five ecoenzymes involved in the biogeochemical cycles of C, N and P in a Mediterranean high-mountain lake. Our results indicate that both single EA and EA ratios can detect shifts in the microbial metabolism of oligotrophic aquatic ecosystems provoked by the interaction of these stressors. We have summarized these changes in a functional diagram that compares our model system under natural nutrient and UVR conditions, as well as under future scenarios of dust deposition and higher UVR incidence (Fig. 2). As discussed below, the information obtained after analyzing single EA is complemented by EA ratios and vector analysis of EA ratios, which are normally used to assess microbial energy/nutrient limitation in aquatic ecosystems because they can reflect a *microbial perception* of mineral nutrients (N and P) with respect to energy (C). In that way, this work provides

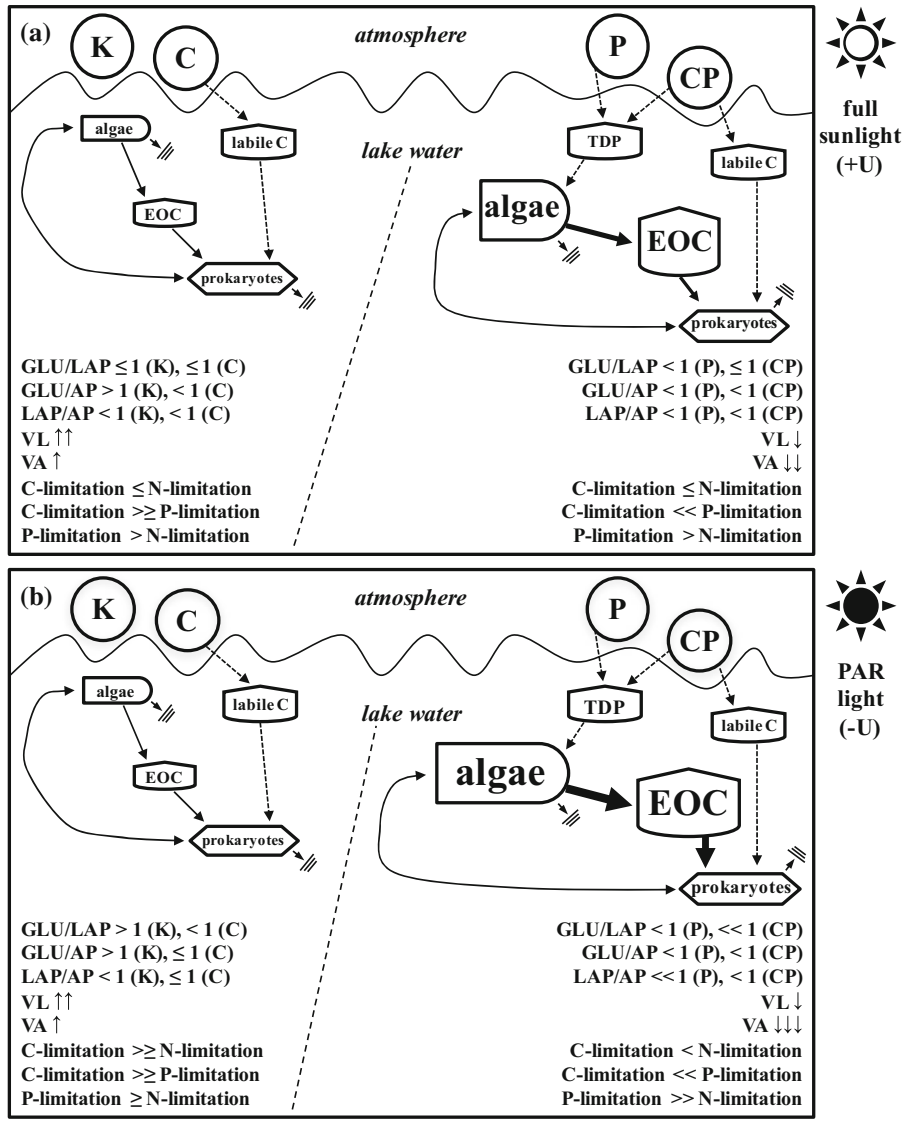


Fig. 2 Functional diagram based on Odum and Barrett nomenclature (2001) describing the lake metabolism driven by microbial communities (epilimnetic phytoplankton-prokaryotes relationships) under full sunlight (**a**, $+U$ treatments) or under PAR light conditions (**b**, $-U$ treatments). On the *left side* of both sections, natural nutrient conditions of the lake (K) or conditions after the addition of a labile C source (C) are shown, whereas on the *right side* of both sections, conditions after the addition of P (P) or C + P (CP) to the lake are shown. In each section, under the different nutrient scenarios studied (K, C, P and CP), the energy (C) or nutrient limitation (P or N) in the growth of prokaryotic communities are defined (last *three lines*) according to EA ratios (GLU/LAP, GLU/AP and LAP/AP, first *three lines*) or vector analysis of EA ratios (VL and VA, *middle two lines*) (*K* control; *C* addition of carbon; *P* addition of phosphorus; *CP* addition of carbon and phosphorus; *EOC* excreted organic carbon; *TDP* total dissolved phosphorus; *GLU* sum of *ALPHA* (activity of α-1,4-D-glucosidase) and *BETA* (activity of ß-1,4-D-glucosidase) activities; *LAP* activity of leucine aminopeptidase; *AP* activity of alkaline phosphatase; *VL* vector L; *VA* vector A; ↑ or ↓, high or low VL or VA values; ↑↑ or ↓↓, very high or very low VL or VA values; ↓↓↓, particularly low VA values)

observational support to the theoretical models that relate metabolic and stoichiometric theories in ecology through EA ratios, thus connecting theory to observation.

Ecoenzyme activities

Our PHO activities were low in all treatments (supplementary Fig. 2a, b). Because PHO is an

enzyme that transforms a broad spectrum of phenolic compounds (Sinsabaugh 2010; Ylla et al. 2013) and usually shows high activity values in aquatic ecosystems rich in lignin and humic acid compounds (Sinsabaugh 2010; Sinsabaugh and Follstad-Shah 2011), we have enough support, when PHO activities are considered, to propose that there are no major external C sources to this lake, probably due to the absence of littoral vegetation or shrubs in its small basin (Medina-Sánchez et al. 2006). By contrast, ALPHA and BETA activities were within the range as those reported for other clear-water lakes (supplementary Fig. 2c–f) (Karrasch et al. 2011; Sereda et al. 2011; Burpee et al. 2016). The fact that both carbohydrolases positively correlated with chl *a* and AA (BETA correlated with EOC too) suggests that algal exudates, which are rich in carbohydrates, can be degraded by prokaryotic glucosidases to obtain C as an energy source. Although correlations must be interpreted with caution, our results agree with an already reported prokaryotic growth dependence on C compounds released by algae in La Caldera Lake (Medina-Sánchez et al. 2002; Durán et al. 2016). It is well known that LAP has a dual role on DOM decay, being important in both C degradation and N acquisition by breaking down complex macromolecules such as polypeptides (Sala et al. 2001; Proia et al. 2012; Sinsabaugh and Follstad-Shah 2012). The fact that there is (1) a positive relationship between LAP activities and NO_3^- (i.e., LAP was not inhibited by the presence of NO_3^-) (supplementary Table 3), (2) a positive relationship between LAP activity and total nitrogen forms, chl *a*, AA, and EOC (supplementary Table 3), and (3) an absence of N limitation in La Caldera Lake (Carrillo et al. 2008a), even in +P treatments where N was added to reach a N:P molar ratio of 30 at the beginning of the experiment, corroborates the idea that LAP is more active in C processing than in N acquisition in our clear-water lake. Finally, our finding of a negative correlation between AP activity and SRP (supplementary Table 3) is in agreement with several studies carried out in oligotrophic aquatic ecosystems indicating that high SRP concentrations can inhibit the action of AP (Hoppe 2003; Cao et al. 2010).

After analyzing the effects of both global change stressors on single EA, it is very clear that the functioning of the microbial food web in this model ecosystem deeply changed after the addition of P. Contrarily to our hypothesis, inorganic P inputs produced a generalized positive effect on all EA values, including AP (supplementary Table 2). Consequently, the presence of P apparently triggered prokaryotic activities in the lake. But the addition of labile C compounds did not affect ALPHA, BETA or LAP activities (supplementary Table 2); the presence of some essential nutrients in the algal exudates, which can fulfill the trophic requirements of prokaryotic communities, could explain this striking response. UVR did not affect EA, although controlled AP activity values (supplementary Table 2). Results of single EA values are consistent with (1) an apparent prokaryotic P limitation in this lake (Medina-Sánchez et al. 2013), (2) the lack of prokaryotic growth response when labile C compounds alone are added in oligotrophic ecosystems with an important organic carbon pool, such as humic (Smith and Prairie 2004; Dorado-García et al. 2016) and autotrophic lakes (Dorado-García et al. 2014), and (3) a decrease in AP activity values under high UVR levels (Boavida and Wetzel 1998; Hoppe 2003; Tank et al. 2005; Santos et al. 2011; Sereda et al. 2011; Janssen and McNeill 2015). However, the absence of significant UVR effects on AP activities when P was added (supplementary Fig. 3b) seems to indicate that this enzyme may be resistant to UVR, and/or that prokaryotic communities of high-mountain lakes are well acclimated to high UVR levels (Korbee et al. 2012; Santos et al. 2014) in the presence of P. Supporting this observation, Medina-Sánchez et al. (2006) showed that P addition can attenuate the negative effects of UVR on bacterioplankton activities in high-mountain lakes, probably because excreted organic matter by algae can partially reduce harmful effects of UVR on EA (Janssen and McNeill 2015).

Ecoenzyme activity ratios

In line with the prokaryotic growth dependence on C compounds released by algae in La Caldera Lake that we saw after analyzing single EA, our GLU/AP ratios suggest that BCP is principally limited by the availability of energy (C) in relation to P under natural conditions, regardless of UVR (+UK and −UK treatments), whereas P constrains BCP much more than N, following our LAP/AP ratios results (Table 1; Figs. 1b, c, 2). GLU/LAP ratios also support the idea that N is not a major limiting nutrient for bacterial

growth in this lake, according to some previous results (Carrillo et al. 2008a) (Table 1; Figs. 1a, 2). These findings totally agree with the patterns of C and P limitation described by Medina-Sánchez et al. (2010), who demonstrated that BCP is largely limited by C sources towards the oligotrophic end of a trophic gradient in Mediterranean inland waters.

The addition of P significantly lowered GLU/AP slopes, especially in the absence of UVR, indicating that P will limit the prokaryotic growth in relation to C under future scenarios of Saharan dust deposition rich in P compounds (Morales-Baquero et al. 2006; Mahowald et al. 2008) (Table 1, Figs. 1b, 2). These results are in line with the apparent P limitation of prokaryotes in La Caldera Lake after P-addition as suggested by the analysis of single EA, and also with the work by Burpee et al. (2016), which propose that prokaryotic communities of pristine Greenland lakes receiving DOC inputs (rich in P compounds) will become P-limited and would support an increasing P limitation after atmospheric dust deposition rich in soluble P compounds. Moreover, lower GLU/LAP ratios that we observed after adding P also indicate that BCP was potentially constrained by N in relation to C (Table 1; Figs. 1a, 2). However, this potential N limitation was partially mitigated by UVR, which suggests an antagonistic UVR × P effect on N limitation in relation to C (Table 1; Figs. 1a, 2). This result reinforces the idea that N will not limit the growth of prokaryotic communities, even under future scenarios of dust deposition and high UVR incidence. However, it is important to note again that prokaryotes use LAP to obtain not only N but also C in aquatic ecosystems (Findlay and Sinsabaugh 2003; Sinsabaugh et al. 2010; Godwin and Cotner 2015). Negative relationships found between GLU/AP and DOC/TP and DOC/TDP, as well as between LAP/AP and TN/TP and TDN/TDP (supplementary Table 3), emphasize our interpretations, support the idea of considering EA ratios as a potential measure of the *microbial perception* of C:P and N:P ratios (Álvarez and Guerrero 2000; Ayuso et al. 2011), and are in agreement with some previous results (Hill et al. 2006; Liao et al. 2014).

Finally, the energy/nutrient status in the microbial communities of our model system was also analyzed by using vector analyses of EA ratios according to Moorhead et al. (2013) and Hill et al. (2014). Overall, and consistently with EA analyzed individually and EA ratios values, our vector analyses of EA ratios suggest that, under control conditions with no nutrients added (+UK and −UK treatments), prokaryotic communities in La Caldera Lake seem to have a greater energy (C) than P and N demand, as suggested by our high VL values (Fig. 2). VA values were also higher under control conditions, consistent with low P demands (Fig. 2). However, when P was added, VL and VA values tended to decrease, which suggests both a decrease in C demand and a greater P versus N prokaryotic demand. Shantz et al. (2016) propose that the activities of heterotrophs that are highly-dependent on photosynthetically-derived carbon compounds, will be particularly vulnerable to nutrient pollution and will decline because of the lack of substrates excreted by primary producers to fuel prokaryotic metabolism. However, algae of La Caldera Lake, well-adapted to UVR conditions, can grow and excrete organic carbon at high rates when P is present (Medina-Sánchez et al. 2002; Korbee et al. 2012), which can explain why VA values decreased under P-added conditions, regardless of UVR conditions (supplementary Fig. 3c).

Conclusion

In summary, our results demonstrate that EA ratios can be a valuable tool in assessing energy/nutrient limitation in microbial communities of Mediterranean high-mountain lakes. Under a future scenario of dust deposition with organic matter rich in P (and C) compounds, and a higher incidence of UVR, only the presence of labile P compounds in the aerosols will have a noteworthy effect on the microbial metabolism of high-mountain lakes in the Sierra Nevada National Park area. At high P concentrations, the growth of phytoplankton, which are superior competitors than prokaryotic heterotrophs for P compounds when the P content is high (Cotner and Wetzel 1992; Dorado-García et al. 2014; Shantz et al. 2016), would remove labile P compounds from the water, thus constraining BCP and, consequently, prokaryotic communities would become more limited by essential nutrients (P and, to a lesser extent, N) than by energy (C), thus provoking changes in the matter and energy fluxes between autotrophs and heterotrophs in the lake (Medina-Sánchez et al. 2006; Carrillo et al. 2008b) (Fig. 2). In contrast, the forecasted increase of UVR

irradiance in the area (Häder et al. 2007) will not produce such clear changes and, consequently, we conclude that UVR will play a secondary role. Evolutionary adaptations to high UVR levels, and/or a metabolism that quickly divert resources towards reparation strategies (Santos et al. 2014), could explain the lack of clear effects of UVR on ecological processes driven by microbial communities in high-mountain lakes. Finally, we have demonstrated that it is feasible to describe the coupling between primary producers and heterotrophic prokaryotes of La Caldera Lake by using EA ratios. Our functional model, depicted in Fig. 2, is complementary to other models of energy and matter fluxes previously published (Carrillo et al. 2006, 2008b; Korbee et al. 2012; Carrillo et al. 2015a), which suggests that similar results can be obtained by measuring only the activities, and their ratios, of five ecoenzymes that play a key role in C, N and P biogeochemical transformations in this kind of aquatic ecosystems.

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