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Interactive effects of vertical mixing, nutrients and ultraviolet radiation: in situ photosynthetic responses of phytoplankton from high mountain lakes of Southern Europe

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Global change, together with human activities had resulted in increasing amounts of organic material (including nutrients) received by water bodies. This input further attenuates the penetration of solar radiation leading to the view that opaque lakes are more “protected” from solar ultraviolet radiation (UVR) than clear ones. Vertical mixing, however, complicates this view as cells are exposed to fluctuating radiation regimes, which effects have in general been neglected. Even more, the combined impacts of mixing, together with those of UVR and nutrients input are virtually unknown. In this study, we carried out in situ experiments in three high mountain lakes of Spain (Lake Enl in Asturias, and lakes Las Yeguas and La Caldera in Granada) to determine the combined effects of these three variables associated to global change on photosynthetic responses of natural phytoplankton communities. The experimentation consisted in all possible combinations of the following treatments: (a) solar radiation: UVR + PAR (280–700 nm) versus PAR alone (400–700 nm); (b) nutrient addition (phosphorus (P) and nitrogen (N)): ambient versus addition (P to reach to a final concentration of $30 \mu\text{g P l}^{-1}$, and N to reach a N:P molar ratio of 31) and, (c) mixing: mixed (one rotation from surface to 3 m depth (speed of  every 4 min, total of 10 cycles) versus static. Our findings suggest that under in situ nutrient conditions there is a synergistic effect between vertical mixing and UVR, increasing phytoplankton photosynthetic inhibition and EOC from opaque lakes as compared to algae that received constant mean irradiance within the epilimnion. The opposite occurs in clear lakes where antagonistic effects were determined, with mixing partially counteracting the negative effects of UVR. Nutrients input mimicking atmospheric pulses from Saharan dust, reversed this effect and clear lakes became more inhibited during  ng, while opaque lakes benefited from the fluctuating irradiance regime. These climate change-related nutrients input and increased mixing would not only affect photosynthesis and production of lakes, but might also further influence the microbial loop and trophic interactions via enhanced EOC under fluctuating UVR exposure.

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1 Introduction

Increased global temperature, changes in precipitation, enhanced levels of solar ultraviolet radiation (UVR, 280–400 nm) together with other anthropogenic activities (e.g. increased inputs of nutrients due to land or agricultural use) had significantly altered natural conditions of aquatic bodies causing direct and indirect effects on phytoplankton (Hallegraeff, 2010; Häder et al., 2011; United Nations Environmental Programme, 2012). Solar UVR, either at normal and enhanced levels (due to the depletion of the ozone layer) is generally a stressor for phytoplankton, affecting growth and productivity rates and causing DNA damage, among others effects (see reviews by Buma et al., 2003; Villafañe et al., 2003 and references therein). However, it can also have positive effects as UV-A energy is used to photorepair DNA damage (Buma et al., 2003) or it can be used for photosynthesis when PAR is limited (Barbieri et al., 2002; Gao et al., 2007). Under conditions of global warming, it is expected that the temperature increase would produce a stronger (i.e. higher gradient at the thermocline) and shallower epilimnion which may have profound effects on organisms and ecosystems especially by exposing them to higher radiation levels and / or different fluctuating radiation field (Häder et al., 2011). Also, and under this scenario, nutrients would be used-up and depleted from the epilimnion (as mixing with deep waters would be prevented) thus productivity might decrease (Beardall et al., 2009; Raven et al., 2011) and the impacts of UVR might be greater (Litchman et al., 2002; Shelly et al., 2005). In other cases however, nutrients would not be limiting, as their inputs rather occur via atmosphere or carried by rivers as shown for various lakes and coastal environments (Xenopoulos et al., 2002; Carrillo et al., 2008; Hessen et al., 2008).

Within any ecosystem different variables can interact in complex non-additive (synergistic or antagonistic) ways (Folt et al., 1999; Crain et al., 2008) and therefore exceeding or attenuating the additive effect of single variables. For example, Christensen et al. (2006) showed that changes in biomass of plankton consumers and producers in boreal lakes over a 23-yr period were best explained by the interaction between

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warming, drought, and acidification, rather than by the sum of their individual effects. In another study (Helbling et al., 2003) it was found that the negative effects of UVR on phytoplankton photosynthesis were highly reduced and even reversed under conditions of fast mixing. Carrillo et al. (2008) found that phosphorus (P) inputs from Saharan dust unmasked the deleterious effects of UVR on algae from a high mountain lake. Furthermore, P inputs increased algal biomass and both, P inputs and UVR exposure reduced the seston carbon-to-phosphorus (C : P) ratio (Xenopoulos et al., 2002; Carrillo et al., 2008; Hessen et al., 2008) enhancing consumer growth by simultaneously improving food quantity and quality (Villar-Argaiz et al., 2012).

Overall, it is expected that significant interactions between mixing, solar radiation and nutrients would occur and thus it is not possible to predict or extrapolate responses of organisms or ecosystems based on single-variable studies. Moreover, evaluating multifactor interactions influencing ecosystem structure and functioning is critical to understand their response to global change (Crain et al., 2008; Hallegraeff, 2010). This is particularly urgent in southern latitudes of Europe where droughts, atmospheric inputs of nutrients from desert areas or the raise of temperature are increasingly frequent traits (Bullejos et al., 2010). High mountain lakes have been considered “sentinels of change” (Williamson et al., 2009), and increasing inputs from of terrigenous material (i.e. dissolved organic matter, DOM) have been determined in lakes over a wide range of freshwater environments (Findlay, 2005; Evans et al., 2006). These findings lead to the view that cells in the water column would receive less solar radiation, thus reducing the impact of UVR on the biota of surface waters (Williamson and Rose, 2010). However, previous studies (Villafañe et al., 2004) determined higher rates of UV-B-induced DNA damage in opaque as compared to clear lakes, hinting for the crucial role of vertical mixing by exposing dark-acclimated cells to surface irradiances.

With the aim to assess the effects of multiple variables on phytoplankton photosynthesis we performed experiments in three high mountain lakes of Spain. These lakes have different biological and physical characteristics that allowed for a wide range of responses under relatively similar experimental conditions, so we used them as model

ecosystems to respond to a question that now deserve major attention: how mixing within the epilimnion modifies UVR-inhibition of primary producers under inorganic nutrient-pulsed (related to global change) in two contrasting lake scenarios: transparent vs. opaque. Hence, we are testing the following hypothesis: fluctuating irradiance (i.e. due to vertical mixing of the water column), together with nutrient inputs, will increase UVR-induced damage (i.e. assessed through measurements of photosynthetic efficiency and activity) on phytoplankton from opaque lakes. This, in turn, would result in higher amounts of carbon released by these UVR-stressed organisms.

2 Methods



2.1 Model ecosystems

This study was done in three high mountain lakes of Spain: (a) Enol (43° 16' N; 4° 59' W) (hereafter LE), located in a calcareous massif at Picos de Europa National Park at 1075 m a.s.l. in the Asturias Province. This lake (maximum depth: 12 m) is oligotrophic, with low light penetration in the water column (Velasco et al., 1999) and the lake watershed area is highly influenced by cattle activity; (b) Las Yeguas (37° 02' N; 3° 22' W) (hereafter LY), is a small (2530 m²), shallow (maximum depth: 8 m), with inlets and outlets, and highly transparent oligotrophic lake (Medina-Sánchez et al., 2010) located at 2800 m a.s.l., in the Granada Province and, (c) La Caldera (37° 03' N; 3° 19' W) (hereafter LC), is a remote high mountain lake (maximum depth: 12 m) located above the tree-line (3050 m a.s.l.) in the Sierra Nevada National Park (Granada Province) with not visible inlets or outlets. The lake is highly transparent (Bullejos et al., 2010) with dissolved organic carbon (DOC) levels generally < 1 mg l⁻¹ (Reche et al., 2001). The pelagic community is strongly P-limited, with DIN to total phosphorus (TP) ratio of 30–90 (by mass) (Carrillo et al., 1996; Villar-Argaiz et al., 2001) and molar algal N : P ratio > 30 (Delgado-Molina et al., 2009).

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The three lakes selected for experimentation share some characteristics that are essential to answer our main question. The three of them receive pulses of nutrients inputs from the surface; however, while in LE these nutrients originate mainly from cattle activity (López-Merino et al., 2007), LY and LC experience frequent inputs of atmospheric Saharan dust containing high P levels, with TN to TP ratio ranging from 10 to 50 in dust deposition (Morales-Baquero et al., 2006). Previous studies (Medina-Sánchez et al., 1999) as well as data presented here indicate that none of the lakes develop a deep chlorophyll maxima (DCM) and phytoplankton is normally equally distributed in the water column. It should be noted that in this study we are not considering deep clear lakes with a DCM (that normally occurs below the epilimnion) as any potential impact of the variables that we are addressing in this study would be indirect on the DCM. In addition, as mentioned before, climate change would drive the water column towards a shallower and more stable epilimnion. Thus in deep clear lakes (with a DCM) this would result in more isolation of the DCM from the upper (stratified) part of the water column.

The experiments carried out in LE were done from 22 to 28 July 2010 whereas those done in LY and LC were done from 10 to 13 September 2010. Before experimentation, the initial physical, chemical, optical and biological characteristics of the water column were determined (see below) and water samples were collected for carrying out measurements of photosynthetic activity and efficiency as described below.

2.2 Experimentation

To assess the combined effects of vertical mixing, nutrient and ultraviolet radiation (UVR) on carbon incorporation/excretion of natural phytoplankton communities, water samples were taken within the upper 3 m of the water column and dispensed into 50 ml quartz round vessels (three clear and one dark per radiation treatment) and incubated with 0.37 MBq of $\text{NaH}^{14}\text{CO}_3$ (DHI Water and Environment, Germany)/specific activity of 310.8 MBq mmol^{-1} . Two radiation treatments were implemented: (a) PAR + UV-A + UV-B (280–700 nm) uncovered vessels (treatment UVR) and, (b) only PAR (400–700 nm)

(treatment PAR), vessels covered with UV Opak 395 filter (Ultraphan, Digefra); the spectral characteristics of this filter is published elsewhere (Figuroa et al., 1997). As mentioned before, UV-B and UV- A could have both beneficial and negative impact; however, in our study we did not separate the single effects of each waveband due to space constraints in our in situ incubation system. Nevertheless, the two radiation treatments that we implemented allowed us to determine the overall effects of UVR (i.e. a balance between the negative and positive effects produced by UV-B and UV-A).

Samples were also exposed to two nutrients conditions: (a) Natural (ambient) nutrient concentration and, (b) Nutrients addition: phosphorus (as Na_2HPO_4), to final concentration of $30 \mu\text{gPI}^{-1}$, and nitrogen (as NO_3NH_4) to reach a N to P molar ratio of 31. In this way, we simulated and kept the proportion of nutrient inputs caused by pulses of Saharan dust as previously shown by Morales-Baquero et al. (2006). We did not use a long-term (days) pre-acclimation of the samples to these enhanced nutrient conditions, as we were interested in assessing any rapid algal response (hours) to the nutrient pulses in combination with the other variables considered (i.e. mixing and UVR exposure).

The vessels were distributed in two round trays, with one of them put at a fixed depth (static samples–fixed irradiance) that varied between 1.3 and 1.4 m (according to their K_{PAR}) to receive the mean irradiance of the upper 3 m of the water column (Helbling et al., 1994). The other tray was moved vertically up and down from the surface to 3 m depth at a speed of 1 m every 4 min (mixed samples–fluctuating irradiance). The depths of incubations were determined either based on temperature profiles obtained in this study, and on previous studies conducted in LY and LC (Delgado-Molina, 2008). The speed of mixing was selected the day before experimentation by doing measurements of the effective photochemical quantum yield (Y) at different depths in the water column and at the surface and applying the model described in Villafañe et al. (2007). The incubations lasted 4 h therefore, a total of 10 cycles (surface – down to 3 m – surface) was completed. In the case of LE, we performed an additional experiment by measuring Y under a slower circulation speed of 1 m every 16 min (one cycle). The whole set up

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was deployed from a boat that was anchored in a deep section of the lakes, so it did not receive shadows or any type of inputs from the shoreline.

The tray (i.e. mixed samples) was vertically moved in the water column by a custom-made mixing simulator using a frequency-controlled DC motor (Maxon motor, Switzerland) to impose a sinusoidal transport rate on the vessels. In this moving device, a UVR-transparent bag containing surface water lake (10 l) was also moved up and down together with and counterbalancing the previously described tray. The samples inside this bag received full solar radiation, and the water was pumped (in a close circuit) from the bag to a pulse-amplitude modulated (PAM) fluorometer for measurements of Chl *a* fluorescence (see below); however, and due to space limitations, no measurements were performed under other different radiation treatments. A custom-made darkened flow-through measuring quartz cuvette (5 ml) was connected to a pump that continuously pumped water containing phytoplankton from and to the bag via a dark 4 m silicon tube (5 mm diameter). The flow rate was ca. 250 ml min⁻¹, so it was enough to keep cells a minimum time (< 1 min) in the silicon tube before been measured. Fluorescence of Chl *a* data was acquired at a frequency of 1 datum every 10 s during 2 h of incubation.

2.3 Analyses and measurements

The following measurements and analyses were done during these experiments:

Solar radiation: incident solar radiation was continuously monitored using a BIC Compact 4-channel deck radiometer (Biospherical Instruments Inc., CA, USA) that has three channels in the UVR region (305, 320, and 380 nm) and one broad-band channel for PAR (400–700 nm). Vertical profiles of solar radiation in the water column were also obtained at noon with a BIC Compact 4-channel underwater radiometer (Biospherical Instruments Inc., CA, USA) that the same channels as described before, in addition to temperature and depth sensors. The mean PAR irradiance within the epilimnion was calculated as:

$$I_{m(\text{PAR})} = I_{0(\text{PAR})} [1 - \exp(-k_{d(\text{PAR})}z)] / k_{d(\text{PAR})}z \quad (1)$$



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where $I_{0(\text{PAR})}$ is the mean surface irradiance, $k_{d(\text{PAR})}$ is the attenuation coefficient for PAR, and z is the depth to where the samples were mixed (3 m). In the case of fixed samples the mean PAR was calculated as the mean irradiance received during the incubation period at 1.3 or 1.4 m depth.

Physical and chemical characteristics of the water column: temperature, conductivity, dissolved oxygen, and pH were determined using a multiparameter probe (Turco Water Quality Analysis T-611). Vertical profiles of these variables were daily obtained at noon; temperature data was used to determine the strength of the thermocline and the depth of the epilimnion in the water column.

The total CO_2 concentration in the lakes was calculated from alkalinity and pH measurements (APHA, 1992). Spectrophotometric techniques were used to determine nitrate (NO_3^-) and soluble reactive P (SRP) (APHA, 1992). Samples for total phosphorus (TP) and total nitrogen (TN) were digested using potassium persulfate at 120°C for 30 min, and then analyzed as SRP or NO_3^- , respectively (APHA, 1992). Seston samples were collected in triplicate by filtering 300–500 ml onto pre-combusted filters (Whatman GF/F 25 mm diameter). The samples were analyzed for C and N using a CNH analyser (Perkin-Elmer Model 2400) and for P content by a colorimetric method after persulfate oxidation (APHA, 1992). The C : N : P ratios were calculated on molar basis.

Incorporated and excreted carbon: after the 4 h of in situ exposure to solar radiation, the samples were immediately filtered, under low pressure (< 100 mmHg) to minimize cell breakage, and fractionated in size fractions as follows: samples from LE were filtered through $3\ \mu\text{m}$ Whatman GF/D filters (25 mm diameter) and then through $0.7\ \mu\text{m}$ Whatman GF/F filters (25 mm diameter) to determine the presence of picoautotrophs ($3\text{--}0.7\ \mu\text{m}$) that might have incorporated some carbon. However, samples from LC and LY were filtered only through $1\ \mu\text{m}$ Nuclepore filters (25 mm diameter) as previous studies (Carrillo et al., 2002) already confirmed the absence of picoautotrophs. The filters were put in 20 ml scintillation vials, and inorganic carbon was removed by adding 100 μl of 1 N HCl and allowing the vial to stand open in a hood for 24 h (no bubbling) (Lignell, 1992). Excreted organic carbon (EOC) was measured on 4 ml sub-samples, collected

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from the 0.7 or 1 μm filtrate, that were put in 20 ml scintillation vials, together with 100 μl of 1 N HCl to eliminate the excess of inorganic radiocarbon. After acidification, scintillation cocktail (Ecoscint A) was added to both, samples for carbon incorporation and excretion, and counted using a scintillation counter (Beckman LS 6000TA) equipped with an internal calibration source. The carbon incorporated and excreted was calculated based on the CPMs, and in all calculations the dark values were subtracted from the light values.

Chl a fluorescence parameters: Chl *a* fluorescence parameters were obtained using a pulse amplitude modulated fluorometer (Water PAM, Walz, Germany). Because the time between sampling and the saturating light pulse was in the order of a few seconds, the photochemical effective quantum yield of PSII (*Y*) in the light was determined (Maxwell and Johnson, 2000). These fluorescence measurements were done every 10 s during the vertical movement of the samples. The measurements were not affected by pumping phytoplankton from and to the cuvette as previously determined by comparing pumped versus water taken directly with a syringe.

Chl a concentration: at the beginning of each experiment aliquots of 250–1000 ml of sample were filtered onto Whatman GF/F filters (25 mm diameter); the filters were then frozen at -20°C until analyses. Once in the laboratory, samples were thawed and put in centrifuge tubes (15 ml) with 5 ml of absolute methanol (Holm-Hansen and Riemann, 1978). The tubes containing the methanolic extract and filters were put in a sonicator for 20 min and then in the dark (4°C) for at least 1 h. After the extraction period the sample was centrifuged (15 min at 1750 *g*) and scanned (250–750 nm) in a UV-VIS spectrophotometer (Lambda 40 Perkin Elmer, USA) using a 5-cm path-length cuvette; Chl *a* concentration was calculated using the equation of Porra (2002). The same sample was also used to calculate Chl *a* concentration from the fluorescence of the extract (Holm-Hansen et al., 1965) before and after acidification (1 N HCl) using a spectrofluorometer (LS 55 Perkin Elmer, USA). There were no significant differences between the Chl *a* concentration obtained by these two methods so we used the fluorometric measurements in all calculations.

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Taxonomic analyses: samples for identification and enumeration of phytoplankton were put in 250 ml brown glass bottles and fixed with Lugol's reagent (1 % vol vol⁻¹). A variable amount of sample (50–100 ml) was allowed to settle for 48 h in Utermöhl chambers (Hydro-Bios GmbH, Germany) and species were enumerated and identified using an inverted microscope (Leitz Fluovert FS, Leica, Wetzlar, Germany) following the technique described by Villafañe and Reid (1995). Phytoplankton biovolumes were estimated from measurements of 20–30 cells of each species using image analysis (Inverted microscope Axio Observer A1, Zeiss – High resolution microscopy camera AxioCam HRc, Zeiss); biovolumes were calculated according to Carrillo et al. (1995). Cell volume was converted to carbon using the conversion factors reported by Rocha and Duncan (1985).

2.4 Data treatment and statistics

The data are reported as mean and standard deviation and the inhibition of photosynthesis due to UVR was calculated as:

$$UVR_{inh} (\%) = 100[(C_{PAR} - C_{UVR})]/C_{PAR} \quad (2)$$

where C_{PAR} , and C_{UVR} represent the carbon fixed in samples under the PAR only, and PAR + UV-A + UV-B treatments, respectively.

A three-way ANOVA test was used to determine significant interactions among the three factors: radiation treatment, nutrients addition and mixing regime. When significant differences were determined, a post hoc Fisher LSD test was used to determine significant differences among and within the different factors.

3 Results

The initial conditions (i.e. just before experimentation) of inorganic, total and seston carbon (C), nitrogen (N) and phosphorus (P) in surface waters are presented in Table 1.

The most evident feature was the higher nitrate and DIN concentration (and TN) in LE as compared to LC and LY, further reflected in the DIN to TP ratio that was 3–4 times higher in the former. Also, the DOC concentration in LE doubled that of LC and LY. Other chemical variables e.g., TP and SRP were rather similar among the lakes.

The underwater radiation profiles (Fig. 1) indicated that LE was the darkest, followed by LY whereas LC was the most transparent (e.g., K_{320} of 2.28, 0.58, and 0.26 m^{-1} (Fig. 1b), and K_{PAR} of 0.34, 0.18, and 0.16 m^{-1} , respectively (Fig. 1d)). Solar radiation conditions were in general of clear skies over LY and LC, while high variability due to cloud cover predominated over LE (data not shown). The mean PAR irradiances during the experimental periods were 1558 (SD = 271), 1670 and 1774 (SD = 14) $\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ for LE, LY and LC, respectively.

Temperature profiles (Fig. 2a) showed different stratification conditions: LE had the highest surface temperatures (20°C) and a marked epilimnion down to ca. 3 m depth (stratified condition) below which temperature decreased continuously to reach 9°C at 12 m. In LC a weak stratification was observed, with surface temperature of 14°C and decreasing to $\sim 12^\circ\text{C}$ at 1 m depth; below it the temperature remained constant. LY was well mixed, having a rather constant temperature ($\sim 14.5^\circ\text{C}$) from surface to bottom. The Chl *a* concentration in the water column increased with depth in LE, but remained rather constant in LY and LC (Fig. 2b); the mean Chl *a* concentrations at surface waters were 2.1, 1.1 and $0.7\ \mu\text{g Chl a l}^{-1}$ in LE, LY and LC, respectively. The concentration of phytoplankton cells (Fig. 2c) was higher in LE ($\sim 3600\text{ cells ml}^{-1}$ in surface waters) as compared to the other two lakes that had similar abundances ($\sim 2000\text{ cells ml}^{-1}$ in surface waters) and depth distributions. Phytoplankton biomass, expressed as carbon concentration (Fig. 2d) had high values at the surface in LY ($51\ \mu\text{g C l}^{-1}$), followed by LE ($33\ \mu\text{g C l}^{-1}$) and low values ($6.1\ \mu\text{g C l}^{-1}$) in LC.

Taxonomic analyses (Table 2) revealed important differences among the lakes, with Chlorophyceae dominating (in terms of abundance) in LE (e.g., *Dictyosphaerium chlorelloides* (Nauman) Komárek and Perman), *Bacillariophyceae* in LY (e.g., *Cydotella minuta* (Skvortzov) Antipova), and *Chrysophyceae* (e.g., *Chromulina nevaden-*

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sis P. M. Sánchez) in LC. However, when considering the share of carbon to the total community, *Bacillariophyceae* dominated in LE and LY (67 and 87 %, respectively) whereas in LC *Chrysophyceae* contributed for the bulk of the biomass (68 %). By comparing both the abundance and biomass of each taxonomic class (Table 2) is obvious the differences in cell size and carbon content per cell among the lakes: for example, in LE diatoms had a mean carbon content of 24 pgC cell⁻¹, but in LY they were larger i.e. 38 pgC cell⁻¹.

For the three lakes, there were no significant differences ($P > 0.05$, $n > 700$) in the photochemical effective quantum yield (Y) in the upper 3 m of the water column, so the mean values are presented in Fig. 3a. In LE there were significant differences in Y ($P < 0.05$, $n = 700$) among experiments carried out during cloudy and sunny conditions under the same mixing regime (Fig. 3a). There was also a significant difference in Y during sunny conditions when samples were subjected to the two mixing regimes, so that at a fast mixing speed (i.e. 1 m every 4 min) Y was significantly lower ($P < 0.05$, $n > 250$) than at a speed of 1 m every 16 min (Fig. 3a). Moreover, and at the speed of 1 m every 4 min, Y at LY was significantly higher ($P < 0.05$) than at the other two lakes, but there were no significant differences ($P > 0.05$) in Y when comparing with LE samples moving at 1 m every 16 min. The mean irradiance conditions in the upper 3 m of the water column are shown in Fig. 3b: LY and LC received the highest PAR levels due to a combination of high surface irradiances and low K_d whereas the lowest irradiance was determined in LE under cloudy conditions.

The rate of phytoplankton carbon fixation was highly variable not only among lakes, but it also depended on the experimental conditions imposed to the samples (Fig. 4). In LE there were no significant differences between radiation treatments or nutrient addition in the $> 3 \mu\text{m}$ fraction under the mixing condition, but in the $3\text{--}0.7 \mu\text{m}$ fraction samples receiving UVR had significant lower carbon fixation than those exposed only to PAR (Fig. 4a). The carbon fixed in the $> 3 \mu\text{m}$ fraction was significantly higher ($P < 0.05$) than in the $3\text{--}0.7 \mu\text{m}$ fraction, with the exception of both UVR treatments in static conditions. In LY (Fig. 4b) significant interactive effects between the three studied

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factors were determined (Table A1). All samples exposed to UVR had significantly lower carbon fixation than those exposed only to PAR. In addition, samples in the mixing regime had in general, significant higher carbon fixation as compared to the static ones. Also, and under mixing conditions, samples with addition of nutrients had higher carbon fixation than those without it for the same radiation treatment. In static samples, nutrient addition increased carbon fixation under UVR but not in samples receiving only PAR. Finally, in LC (Fig. 4c) there were interactive effects between mixing regime, radiation treatments and nutrient addition (Table A1). Carbon fixation in the samples with addition of nutrients was significantly higher than those without it, in both static and mixing regimes. Also, nutrient addition “highlighted” the UVR effects under mixing conditions – with significantly higher carbon fixation in the PAR + P as compared to UVR + P, but there were no significant effects of UVR when nutrients were not added. In static samples, UVR had significant effects regardless the nutrient treatment.

The UVR-induced inhibition of carbon fixation in LE (Fig. 5a) was ca. 20% under mixing in the 3–0.7 μm fraction, whereas under static conditions negative inhibition values were obtained (i.e. photosynthesis was enhanced by UVR). The effects of UVR and nutrients were especially evident in the $> 3 \mu\text{m}$ fraction under the static conditions, with negative inhibition (–20%) in samples without nutrient addition whereas it reached 48% in those that did receive them. In LY (Fig. 5b) significant inhibition was determined in all treatments (60–80%) but samples with nutrient addition and under static conditions were those having the lowest values of 34%. Finally, phytoplankton from LC had UVR-induced inhibition ranging from 30 to 90% in samples without nutrient addition under mixing and static conditions, respectively (Fig. 5c).

The total amount of excreted organic carbon (EOC) was significantly higher in LE as compared to the other lakes (Fig. 6). There were significant interactions between mixing regimes, radiation treatments and nutrient addition (Table A2). In particular, and for LE (Fig. 6a) the amount and percentage of EOC in the PAR-only exposed samples during the mixing regime were similar than those receiving UVR. In the static conditions, the addition of nutrients resulted in less EOC in the samples exposed to

UVR as compared to  samples in which no nutrients were added. In LY (Fig. 6b) samples under mixing conditions had lower amounts of EOC than in the static ones, while in LC (Fig. 6c) the opposite occurred. In general, the addition of nutrients resulted in significant less amount of EOC in LY and LC under static conditions (Fig. 6b and c).
5 In regard to the percentage of EOC, the general pattern was of higher values in LC (> 50%), followed by LY (> 35%) and then LE (> 20%).

In order to relate the results obtained in the three lakes we plotted the UVR inhibition of carbon fixation and EOC as the difference between mixed and static conditions (Fig. 7). Since the experimentation in the three lakes was conducted on different
10 dates we normalized the inhibition values by the irradiance received by the cells. These values were plotted as a function of the attenuation coefficient (k_{320}), considering independently the treatments with or without nutrient addition. It should be noted that the logistics involved to conduct in situ mixing experiments precluded us to repeat the experiments in more lakes under similar conditions; thus, in Fig. 7 we included data
15 derived from studies conducted in other lakes where mixing experiments (or models obtained based on them) were conducted. The reader should be aware that in doing this approximation we are comparing data that were obtained in different conditions, not only of solar radiation, but also in mixing speeds, mixing depth, etc. In order to derive the data to be used in Fig. 7 we took from these published work (see caption
20 of Fig. 7) the inhibition of primary production in mixing and static samples either from their figures/tables or, as stated in the text, at their experimental design that was closest to our own. In spite of the variability observed between these studies (and our own), a significant trend for the inhibition of carbon fixation in samples without addition of nutrients was observed (Fig. 7). In opaque lakes (i.e. with high k_{320}) the inhibition was
25 greater under mixed than under static conditions, and it decreased towards clear lakes with negative values, indicating that inhibition  was higher in the static conditions and that mixing counteracted the negative effects of UVR. Moreover, increased EOC values were also observed in opaque lakes under mixing conditions, and they decreased towards clear lakes (Fig. 7). An interesting result is that the addition of nutrients seemed

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to reverse the pattern, with mixing either enhancing or having no effect in the clear lakes, but significantly counteracting the negative effects of UVR in the opaque LE. No relationship was observed for EOC when nutrients were added (data not shown).

4 Discussion

In our study we used three high mountain lakes as model ecosystems to evaluate the impact of climate change variables on photosynthetic responses. These high mountain lakes are of key interest to predict climate change as they have been considered as “sentinels of change” (Williamson et al., 2009). We are aware of the limited number of test lakes used however, we consider that they are representative of different areas as their characteristics are shared by other lakes (e.g. see Rose et al., 2009). Our study led us to propose a conceptual model (Fig. 8) showing how UVR-induced photosynthetic inhibition is conditioned by climate change variables in these high mountain lakes. Briefly, what we show is that under nutrient limitation (mainly of P) there is a synergistic effect between vertical mixing and UVR on phytoplankton from opaque lakes, resulting in higher photosynthetic inhibition and EOC than if the cells received constant mean irradiance within the epilimnion. The opposite occurs in clear lakes where antagonistic effects are determined and mixing partially counteracted the negative effects of UVR. The input of nutrients (as may occur due to Saharan dust carried by the winds) reversed this effect and clear lakes become more inhibited during mixing, while opaque lakes benefited from the fluctuating irradiance regime counteracting the negative UVR impact.

It has been hypothesized that the increased amounts of DOM carried into lakes would decrease the penetration of solar UVR affecting the quality of the water, as some pathogens remained in the water column as they were not killed by UVR exposure (Connelly et al., 2007; Williamson and Rose, 2010). However, mixing conditions and the increase of UVR due to the formation of a shallower epilimnion (as predicted in a scenario of global change) together with the effects of an extra supply of nutrients,

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further complicates this view. It is obvious from our results that the responses observed not only among treatments, but also among lakes, depend on several factors. The water column structure and characteristics (Figs. 2 and 3, Table 1) together with the differences in dominance of species and size structure of the communities (Table 2) resulted in different acclimation to solar radiation. For example, phytoplankton from LE seemed to be light-limited as in the static experimental condition samples under the UVR treatment had higher carbon fixation than those under PAR only (with the exception of UVR + P in the > 3 μm, Fig. 4a) resulting in negative UVR-induced inhibition (Fig. 5a). Furthermore, mixing during a cloudy day resulted in lower in situ *Y* than during a sunny day under slow speeds (Fig. 3) indicating a less effective use of solar energy. The fact that phytoplankton can use UVR as source of energy for photosynthesis under light limitation was previously demonstrated for marine environments (Barbieri et al., 2002; Helbling et al., 2003; Gao et al., 2007). In a context of global change, an increase in precipitation is expected over the Northern Hemisphere (IPCC, 2007) therefore, higher inputs of organic matter from water run-off is expected in lakes (Williamson and Rose, 2010). This input, on the one hand, would bring more nutrients into the system, while on the other hand will decrease the penetration of solar radiation (Osburn and Morris, 2003) thus creating a new balance damage/utilization of UVR.

It is evident that climate change related variables play a crucial role in aquatic ecosystems: increased temperature would decrease the depth of the epilimnion, while increasing wind speed and duration would not only counteract this effect by deepening the epilimnion (Beardall et al., 2009; Raven et al., 2011) but also by distributing nutrients carried via atmosphere (Escudero et al., 2005; Bullejos et al., 2010) into the water column (i.e. nutrients reaching different depths due to vertical mixing transport). Within certain epilimnion, the decrease in penetration of solar UVR will decrease the mean irradiance received by the cells (Helbling et al., 1994) and thus would “force” cells to acclimate to a “dark” condition; however, the opposite would occur in clear lakes, provided that the irradiance conditions at the water surface are similar. The acclimation changes would further condition the response of phytoplankton to UVR under

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fluctuating or fixed irradiances or to different concentration of nutrients (Helbling et al., 2003; Beardall et al., 2009).

In our conceptual model (Fig. 8) we have opaque lakes (like LE) receiving low mean irradiances, and clear lakes such as LY and LC (which receive high irradiances). Increased temperature would create a strong thermocline and this would isolate the epilimnion from the deeper and colder water column, thus precluding nutrients from below to reach the photoactive zone (Beardall et al., 2009; Neale et al., 2003). However, with increasing wind speed, land use or nutrient transport through the atmosphere, more nutrients from above are carried into the epilimnion and thus productivity might increase (Fig. 4, López-Merino et al., 2011; Carrillo et al., 2008; Morales-Baquero et al., 2006). Mixing, due to increased wind forcing, causes cells to be exposed to a fluctuating radiation field that goes from low irradiance (at the base of the epilimnion) to maximum irradiance at the surface. In clear lakes, with cells acclimated to high irradiance conditions, the impact of UVR due to this fluctuating irradiance is minimum (Fig. 5, Villafañe et al., 2004) as cells, provided they have enough time to acclimate, have different mechanisms to cope with UVR as previously demonstrated (Buma et al., 2009; Helbling et al., 1996). In the case of opaque lakes however, mixing will result in damage of cells when they are at the surface, and depending on the rates of damage/repair, this could be cumulative causing increased photosynthetic inhibition and higher EOC as observed in these systems. A rather similar behavior in regard to photosynthetic inhibition was observed in Antarctic phytoplankton, but that was mostly related to the lack or slow repair due to low temperature (Helbling et al., 1994; Neale et al., 1998). The higher sensitivity of phytoplankton from opaque lakes as compared with clear ones was also observed when evaluating UVB-induced DNA damage in several Andean lakes (Villafañe et al., 2004).

In summary, and according to our hypothesis, and in contrast to the expected protecting effect of increased allochthonous DOM, we found that imposing a fluctuating irradiance regime (i.e. mimicking the mixing due to global warming in the upper layer of stratified lakes) promotes UVR-inhibition of primary producers in opaque ecosystems,

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which is reversed after nutrient-pulses. The opposite responses were found in clear lakes. To the best of our knowledge, the interaction of these three factors (i.e. nutrient addition, mixing, and UVR) has not been previously considered, and more data needs to be gathered to fully understand these responses by autotrophic organisms. These changes in nutrient pulses, together with fluctuating radiation conditions, would differentially affect clear and opaque lakes; hence, and due to their key role in carbon flux, variables such as mixing, UVR and nutrients may act in a synergistic or antagonistic way depending on the previous light acclimation of algae. This would not only affect the photosynthesis and production of lakes but it also might influence the microbial loop and trophic interactions via enhanced EOC (Medina-Sánchez and Villar-Argaiz, 2006) under fluctuating UVR exposure.

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Table 1. Mean concentrations (and SD) of inorganic, total and sestonic carbon (C), nitrogen (N) and phosphorus (P) in surface waters of lakes Enol (LE), Las Yeguas (LY) and La Caldera (LC). TP: Total phosphorus (μM); SRP: Soluble reactive phosphorus (μM); TN: Total nitrogen (μM); NO_3^- : Nitrate (μM); DIN: Dissolved inorganic nitrogen (μM); DOC: Dissolved organic carbon (mg l^{-1}). The ratio DIN to TP is expressed by weight.

Lake Variable	LE	LY	LC
TP	0.110 ± 0.054	0.109 ± 0.003	0.128 ± 0.007
SRP	0.075 ± 0.006	0.051 ± 0.003	0.047 ± 0.003
TN	26.68 ± 1.18	11.46 ± 2.15	12.65 ± 1.52
NO_3^-	27.68 ± 0.28	6.59 ± 0.23	10.95 ± 0.91
DIN	25.01 ± 0.44	6.67 ± 0.2	11.78 ± 0.53
DIN/TP	117.87 ± 12.53	27.64 ± 0.27	41.71 ± 0.4
DOC	2.238 ± 0.45	1.01 ± 0.11	1.078 ± 0.08
C : P seston	513 ± 123	401 ± 12	460 ± 32
N : P seston	58 ± 11	30 ± 1	34 ± 6

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Table 2. Surface phytoplankton composition and abundance (in cells ml⁻¹) and carbon biomass content (in µgC l⁻¹) in lakes Enol (LE), Las Yeguas (LY) and La Caldera (LC).

Lake Family	LE		LY		LC	
	Abundance	Biomass	Abundance	Biomass	Abundance	Biomass
<i>Chlorophyceae</i>	2270	6.06	676	4.13	382	1.03
<i>Cryptophyceae</i>	211	3.62	94	1.34	29	0.33
<i>Chrysophyceae</i>	64	0.91	113	1.03	1559	4.22
<i>Desmidiaceae</i>	9	0.15				
<i>Bacillariophyceae</i>	916	22.11	1176	44.89	2	0.05
<i>Dinophyceae</i>	129	0.13	4	0.14	28	0.51
TOTAL	3599	32.98	2063	51.73	2000	6.14

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Table A1. Results from statistical analyses showing probabilities when significant differences in carbon incorporation were established. The labels indicate: S = static; M = Mixing; U = UVR + PAR; P = PAR; np = No nutrients; p = nutrients added. For example, MUnp indicates the treatment Mixing – UVR + PAR – no nutrients added. LE: Lake Enol; LY: Lake Las Yeguas; LC: Lake La Caldera.

Lake Treatment	LE > 3 μm		LE 3–0.7 μm		LY		LC
Mixing							0.033
Radiation			0.023		0.000		0.000
Nutrients			0.000		0.005		0.000
Mix*Rad			0.000				
Mix*Nut			0.000		0.037		
Rad*Nut	0.005				0.001		0.000
Mix*Rad*Nut	0.003				0.000		0.000
LE > 3 μm	MUnp	MUp	MPnp	MPp	SUnp	SUp	SPnp
SUnp	0.048						
SUp	0.023	0.008	0.006	0.004	0.000		
SPp	0.034				0.000		
LE 3–0.7 μm							
MPnp	0.020	0.028					
SUnp	0.000	0.000	0.002	0.000			
SUp			0.005	0.029	0.000		
SPnp			0.048		0.000		
SPp	0.001	0.001	0.000	0.000	0.000	0.004	0.000
LY							
MUp	0.040						
MPnp	0.000	0.000					
MPp	0.000	0.000	0.004				
SUnp	0.038	0.000	0.000	0.000			
SUp	0.008		0.000	0.000	0.000		
SPnp	0.000	0.000	0.021		0.000	0.000	
SPp	0.000	0.000	0.100	0.000	0.000	0.000	0.001
LC							
MUp	0.009						
MPnp		0.039					
MPp	0.000	0.000	0.000				
SUnp		0.001		0.000			
SUp	0.035			0.000	0.003		
SPnp		0.047		0.000			
SPp	0.000	0.000	0.000	0.008	0.000	0.000	0.000

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Table A2. Results from statistical analyses showing probabilities when significant differences in excreted organic carbon – EOC (as percentage as well as absolute values) were established. The labels indicate: S = static; M = Mixing; U = UVR + PAR; P = PAR; np = No nutrients added; p = nutrients added. For example, MUnp indicates the treatment Mixing – UVR + PAR – no nutrients added. LE: Lake Enol; LY: Lake Las Yeguas; LC: Lake La Caldera.

Lake Treatment	LE EOC (%)	LY EOC (%)	LC EOC (%)	LE EOC ($\mu\text{Cl}^{-1}\text{h}^{-1}$)	LY EOC ($\mu\text{Cl}^{-1}\text{h}^{-1}$)	LC EOC ($\mu\text{Cl}^{-1}\text{h}^{-1}$)
Mixing		0.000	0.001		0.000	0.000
Radiation		0.000	0.000	0.018		
Nutrients		0.010	0.000		0.000	
Mix*Rad		0.001	0.006			0.000
Mix*Nut		0.000	0.000		0.002	0.000
Rad*Nut	0.009	0.000	0.015	0.007	0.000	
Mix*Rad*Nut	0.008	0.000	0.017	0.004	0.000	0.016

LE EOC (%)			LE EOC ($\mu\text{Cl}^{-1}\text{h}^{-1}$)											
E%	MUnp	MUp	MPnp	MPp	SUnp	SUp	SPnp	MUnp	MUp	MPnp	MPp	SUnp	SUp	SPnp
SUnp								0.009	0.011	0.002	0.001			
SUp												0.002		
SPnp	0.023	0.028			0.006	0.092						0.000		
SPp		0.044	0.007	0.005		0.012	0.000					0.039		0.015

LY EOC (%)			LY EOC ($\mu\text{Cl}^{-1}\text{h}^{-1}$)										
MUp	MPnp	MPp	SUnp	SUp	SPnp	SPp	MUp	MPnp	MPp	SUnp	SUp	SPnp	SPp
0.000							0.000						
0.000							0.000						
	0.000	0.000						0.000	0.000				
0.000	0.000	0.000	0.000				0.000	0.001	0.039	0.000			
0.000				0.000	0.000		0.000			0.000	0.004		
0.000				0.000	0.000		0.000	0.000	0.018	0.000		0.002	
0.000	0.011		0.000	0.000	0.007		0.000		0.027	0.001	0.000		0.000

LC EOC (%)			LC EOC ($\mu\text{Cl}^{-1}\text{h}^{-1}$)										
MUp	MPnp	MPp	SUnp	SUp	SPnp	SPp	MUp	MPnp	MPp	SUnp	SUp	SPnp	SPp
0.028							0.000						
0.006							0.000	0.000					
0.000	0.000	0.000						0.004	0.000				
0.000	0.001	0.000	0.000				0.008	0.000	0.009	0.000			
0.000	0.000	0.000		0.000			0.000	0.000	0.001	0.000	0.000		
0.000		0.042	0.000	0.016	0.000		0.000	0.000			0.001	0.000	
0.000	0.000	0.000	0.009	0.000	0.014	0.000	0.000	0.000		0.000	0.050	0.000	0.000

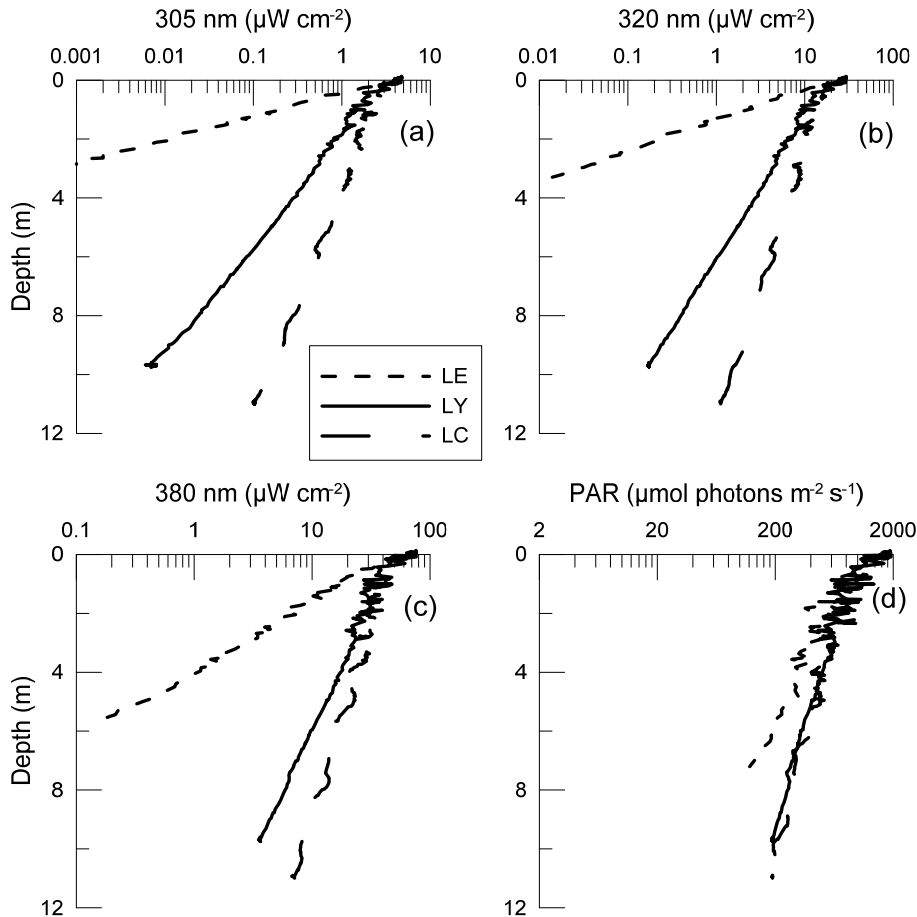


Fig. 1. Solar irradiance as a function of depth in lakes Enol (LE), Las Yeguas (LY) and La Caldera (LC): **(a)** 305 nm; **(b)** 320 nm; **(c)** 380 nm and, **(d)** PAR (400–700 nm). Irradiance data in the UVR range are expressed in $\mu\text{W cm}^{-2}$, PAR is in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

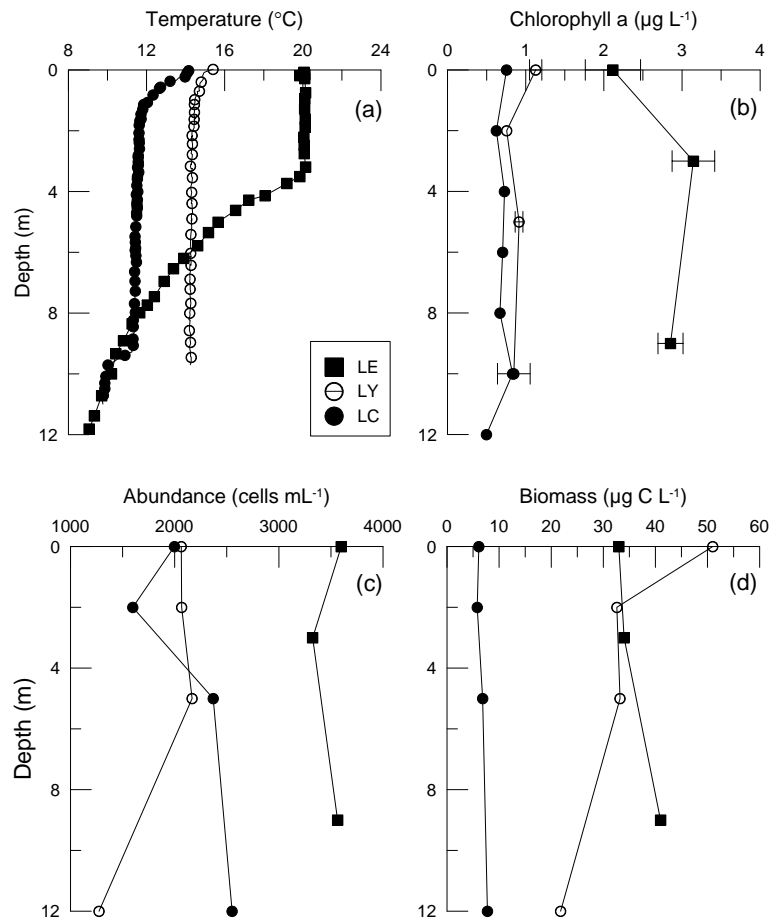


Fig. 2. Water column structure and phytoplankton distribution as a function of depth in lakes Enol (LE), Las Yeguas (LY) and La Caldera (LC). **(a)** Temperature (in °C); **(b)** Chlorophyll *a* (in µg l⁻¹); **(c)** Phytoplankton abundance (in cells ml⁻¹); and **(d)** Phytoplankton biomass (in µg C l⁻¹).

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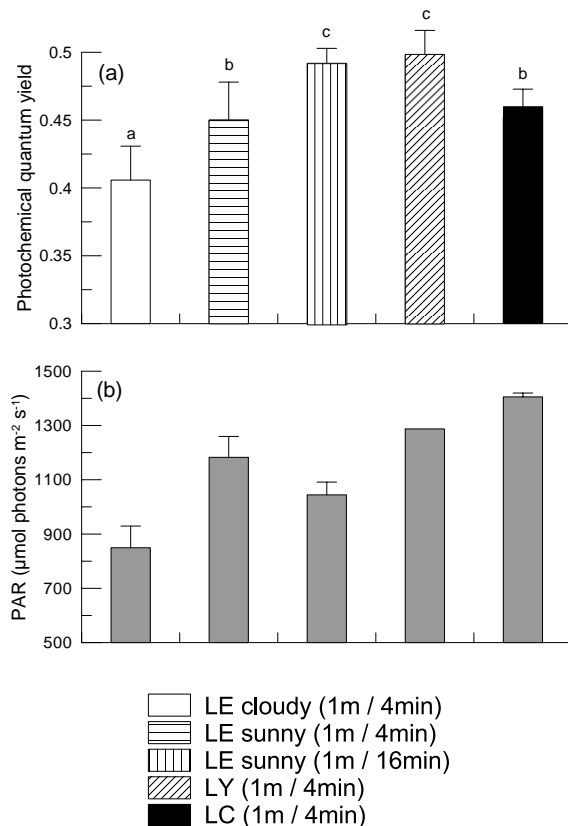


Fig. 3. (a) In situ effective photochemical quantum yield (Y) and, (b) Mean PAR irradiance (in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in the upper 3 m of the water column during measurements carried out in lakes Enol – LE (cloudy and sunny days, under different mixing speeds), Las Yeguas (LY) and La Caldera (LC). The lines on top of the bars are the standard deviation whereas the letters indicate differences among conditions.

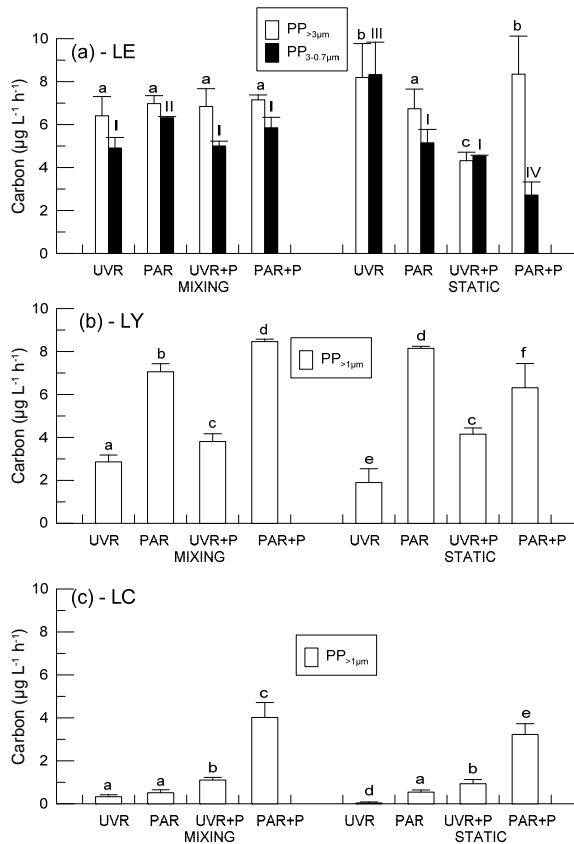


Fig. 4. Carbon incorporation (in $\mu\text{g C l}^{-1} \text{ h}^{-1}$) under different radiation conditions (UVR versus PAR alone), phosphorus concentration (ambient versus $30 \mu\text{g PI}^{-1}$) and mixing regime (mixing versus static) in: **(a)** Lake Enol (LE); **(b)** Lake Las Yeguas (LY) and, **(c)** Lake La Caldera (LC). The lines on top of the bars are the standard deviation whereas the letters and numbers indicate differences among treatments.

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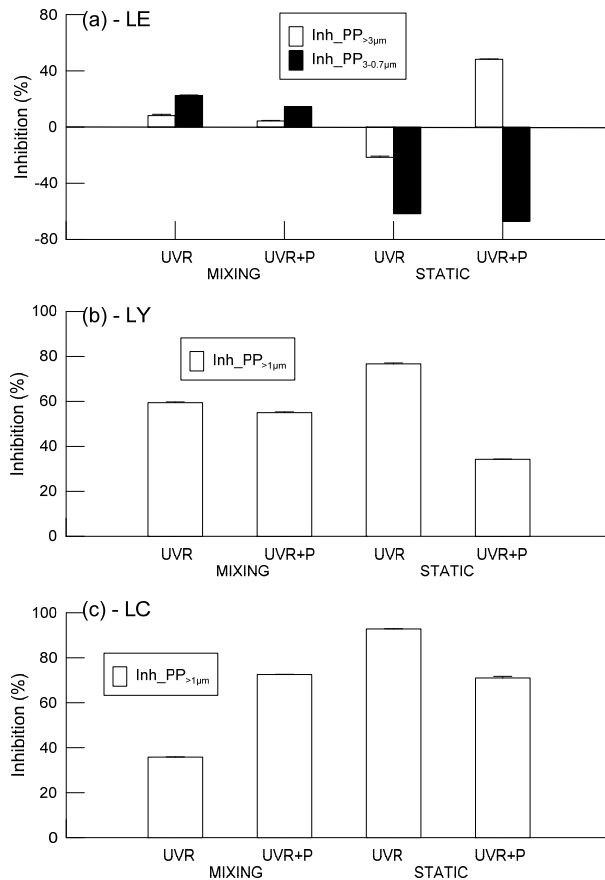


Fig. 5. Percentage inhibition of carbon fixation (%) due to UVR under different phosphorus concentration (ambient versus $30 \mu\text{g PI}^{-1}$) and mixing regime (mixing versus static) in: **(a)** Lake Enol (LE); **(b)** Lake Las Yeguas (LY) and, **(c)** Lake La Caldera (LC). The lines on top of the bar are the standard deviation.

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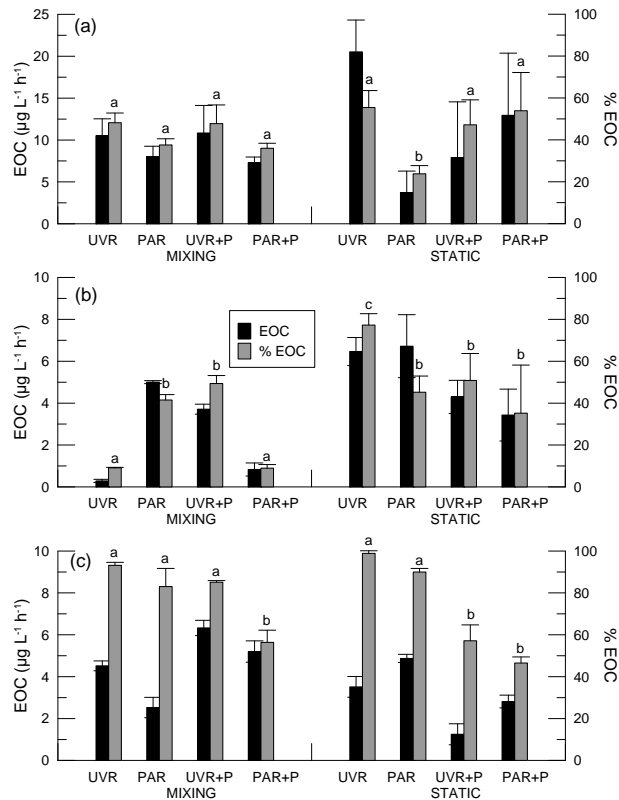


Fig. 6. Amount of excreted organic carbon (EOC, in $\mu\text{g Cl}^{-1} \text{h}^{-1}$) and percentage EOC (in %) under different radiation (UVR versus PAR alone), phosphorus concentration (ambient versus $30 \mu\text{g PI}^{-1}$) and mixing regime (mixing versus static) in: **(a)** Lake Enol (LE); **(b)** Lake Las Yeguas (LY) and, **(c)** Lake La Caldera (LC). The lines on top of the bars are the standard deviation whereas the letters indicate differences among treatments.

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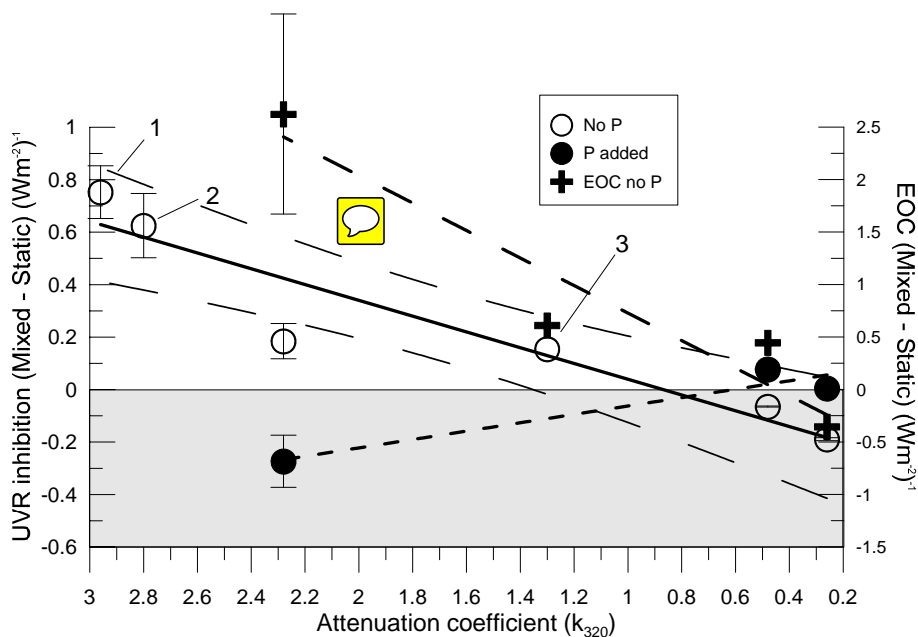


Fig. 7. UVR inhibition and EOC of mixed minus static samples, normalized by the mean irradiance received by the cells, as a function of the attenuation coefficient for samples with nutrient addition (black circles) and without (open circles and crosses) nutrient addition. The solid line represent the fit of linear regression, and the dashed thin lines the 95 % confidence limits; the darker broken lines represent the trend of the data. Additional data calculated from: (1) Villafañe et al. (2007); (2) Hiriart-Baer and Smith (2005); and (3) Köhler et al. (2001).

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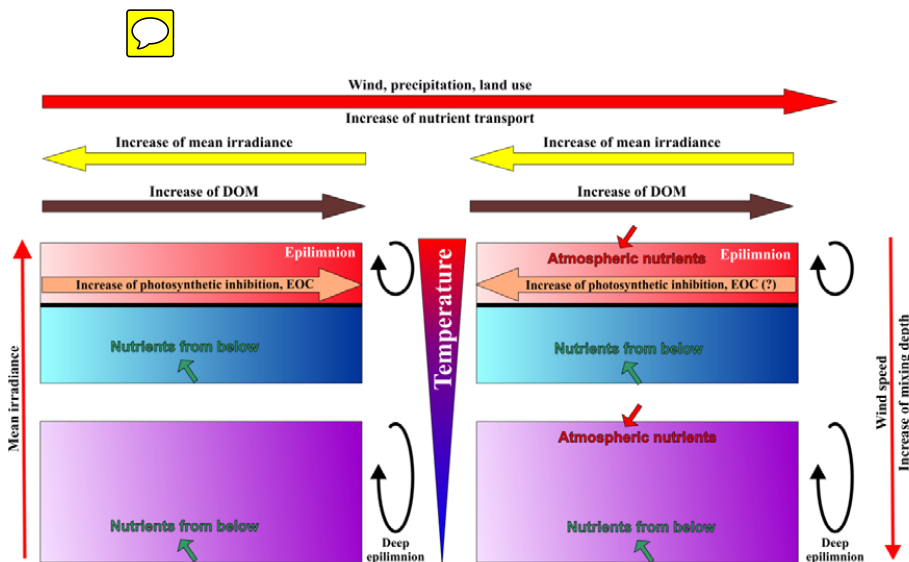


Fig. 8. Conceptual graphical model of photosynthetic responses (uptake and EOC), evaluated as damage, when subjected to interactions among vertical mixing, nutrients and exposure to fluctuating UVR. Color gradients indicate water color transparency. Arrows indicates nutrients input from below the epilimnion (green) or from atmospheric deposition or land use (red).

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