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Direct and indirect effects of vertical mixing, nutrients and ultraviolet radiation on the bacterioplankton metabolism in high-mountain lakes from southern Europe

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Abstract

As a consequence of global change, modifications in the interaction among abiotic stressors on aquatic ecosystems have been predicted. Among other factors, UVR transparency, nutrient inputs and shallower epilimnetic layers could alter the trophic links in the microbial food web. Currently, there are some evidences of higher sensitiveness of aquatic microbial organisms to UVR in opaque lakes. Our aim was to assess the interactive direct and indirect effects of UVR (through the excretion of organic carbon – EOC – by algae), mixing regime and nutrient input on bacterial metabolism. We performed in situ short-term experiments under the following treatments: full sunlight (UVR+ PAR, > 280 nm) vs. UVR exclusion (PAR only, > 400 nm); ambient vs. nutrient addition (phosphorus (P; 30 μg P L^{-1}) and nitrogen (N; up to final N : P molar ratio of 31)); and static vs. mixed regime. The experiments were conducted in three high-mountain lakes of Spain: Enol [LE], Las Yeguas [LY] and La Caldera [LC] which had contrasting UVR transparency characteristics (opaque (LE) vs. clear lakes (LY and LC)). Under ambient nutrient conditions and static regimes, UVR exerted a stimulatory effect on heterotrophic bacterial production (HBP) in the opaque lake but not in the clear ones. Under UVR, vertical mixing and nutrient addition HBP values were lower than under the static and ambient nutrient conditions, and the stimulatory effect that UVR exerted on HBP in the opaque lake disappeared. By contrast, vertical mixing and nutrient addition increased HBP values in the clear lakes, highlighting for a photoinhibitory effect of UVR on HBP. Mixed regime and nutrient addition resulted in negative effects of UVR on HBP more in the opaque than in the clear lakes. Moreover, in the opaque lake, bacterial respiration (BR) increased and EOC did not support the bacterial carbon demand (BCD). In contrast, bacterial metabolic costs did not increase in the clear lakes and the increased nutrient availability even led to higher HBP. Consequently, EOC satisfied BCD in the clear lakes, particularly in the clearest one [LC]. Our results suggest that the higher vulnerability of bacteria to the damaging effects of UVR may be particularly accentuated in the opaque lakes and further recognizes the rele-

vance of light exposure history and biotic interactions on bacterioplankton metabolism when coping with fluctuating radiation and nutrient inputs.

1 Introduction

Among the organisms that constitute the pelagic community, bacterioplankton is an important compartment in the structure and function of ecosystems due to their key role in biogeochemical cycles (Cotner and Biddanda, 2002). Moreover, their biomass production and their trophic coupling to eukaryotes have a profound impact on elemental fluxes (Newton et al., 2011). However, due to their particular characteristics (small cell-volume, general lack of pigmentation and short generation times), bacterioplankton are especially sensitive to environmental perturbations (Garcia-Pichel, 1994; Paerl et al., 2003; Shade et al., 2012). This sensitivity is particularly relevant in a scenario of global change, characterized by increased mean atmospheric temperatures, alteration in precipitation regimes (Giorgi and Lionello, 2008; IPCC 2013) and higher frequency of extreme weather events (Graham and Vinebrooke, 2009). These changes, together with increased atmospheric depositions (Goudie, 2009) and increased levels of ultraviolet radiation (UVR, 280–400 nm) (Manney et al., 2011) are altering the natural characteristic of water masses (IPCC 2013; Häder et al., 2011).

Natural levels of UVR can be stressors, with significant negative effects on most aquatic microorganisms (see review of Helbling and Zagarase, 2003). The direct negative effects of UVR on bacterioplankton include the damage to the DNA molecule (Jeffrey et al., 1996; Hernandez et al., 2007), decrease in enzymatic activity (Herndl et al., 1993) and reduction of membrane permeability (Chatila et al., 2001; Buma et al., 2003). All these direct effects can negatively affect heterotrophic bacterial production (HBP; Conan et al., 2008; Bullock and Jeffrey, 2010). However, neutral or even stimulatory effects of UVR have also been reported (Aas et al., 1996; Sánchez et al., 2002; Durán et al. unpublished). These opposite results have been related either with the differential photoacclimation capacity of organisms (Pakulski et al., 1998) or with the

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interaction of UVR with other environmental factors (Häder et al., 2011; Ruiz-Gonzalez et al., 2013). For example, the addition of nutrients can reduce UVR photoinhibition (Medina-Sánchez et al., 2006) or, contrarily, unmask the damaging effect of UVR (Durán et al. unpublished). Also, dissolved organic matter (DOM) might contribute to explain the differences in the observed effects on aquatic organisms by reducing UVR penetration in the water column (Rose et al., 2009). Thus, high DOM contents can absorb UVR in upper water layers, therefore biota would receive less solar radiation (Williamson and Rose, 2010) and so they would reduce damage caused by these wavelengths. However, recent studies (Harrison and Smith, 2011; Helbling et al., 2013) showed that shade-adapted algae from ecosystems with high DOM content are more vulnerable to UVR under increased or fluctuating levels. Moreover, photochemical processes might influence organic C availability for bacteria (Ruiz-Gonzalez et al., 2013). Thus, recalcitrant DOM can be photolysed into more readily forms (Abboudi et al., 2008), while labile DOM can result into more recalcitrant forms (Bastidas Navarro et al., 2009).

Increases in temperature can modify the thermocline depth and accentuate the stratification of water column thus influencing UVR exposure of bacterioplankton within the upper mixed layer (UML) (Sahoo et al., 2010; de Senerpoint-Domis et al., 2013). Because the solar radiation spectrum have different penetration into the water column, with more rapid UV-B (280–315 nm) attenuation as compared to that of UV-A (315–400 nm) or photosynthetic active radiation (PAR, 400–700 nm; Hargreaves, 2003), organisms are not only affected to varying light intensities in the upper water layers but also to different light quality. As a consequence, the net effect of solar radiation on bacteria within an actively mixed water column will be a function of radiation exposure, with photoinhibition in near-surface waters, and physiological recovery when irradiance is attenuated in deeper waters (Ferrero et al., 2006). Moreover, a persistent stratification would determine vertical nutrient gradients with higher accumulation below the UML, which can generate nutrient limitation in the epilimnion (Sarmento et al., 2010; Song et al., 2013). However, nutrient limitation in the epilimnion can be counteracted by

an increase in wind speed and duration (Helbling et al., 2013) if water mixing reaches the nutricline (Fouilland et al., 2007). Also limitation by nutrients can be alleviated by the input of nutrients associated with atmospheric processes (Carrillo et al., 2008b; Bullejos et al., 2010; Lekunberri et al., 2010) or water runoff (Moss, 2012) which, may
5 also contain DOM (Evans et al., 2006). In turn, higher DOM concentration contribute to higher temperature in the upper layers and therefore to shallower thermocline depths in small non-eutrophic lakes (Caplanne and Laurion, 2008; Read and Rose, 2013).

Eventually, changes in the physical structure of the water column would determine the primary production levels within the UML, and thereby the release of organic car-
10 bon associated with photosynthetic activity (Grubisic et al., 2012; Helbling et al., 2013). Because heterotrophic bacteria, unlike phytoplankton, have a metabolism that is not directly dependent on solar radiation, part of the UVR effects (i.e. indirect) on bacterioplankton might be mediated by phytoplankton excretion of organic carbon (EOC; Carrillo et al., 2002; Medina-Sánchez et al., 2004). In fact, there are numerous studies
15 that recognize the importance of vertical mixing in affecting the responses of phytoplankton to UVR (e.g. Helbling et al., 1994, 2008, 2013; Neale et al., 1998). However, due to the logistic complexity in experimentally mimicking vertical mixing (Ruiz-Gonzalez et al., 2013) and the difficulties to discriminate between direct and indirect effects of fluctuating radiation on bacterioplankton, less studies have considered the
20 interactive effects of UVR and vertical mixing on bacteria (Jeffrey et al., 1996; Huot et al., 2000; Bertoni et al., 2011; Gali et al., 2013), and most of them indicated that fluctuating light tend to decrease the inhibition of HBP, altering the ratio of damaging to repair processes. Nevertheless it is known that constant UVR exposure, by favoring the uncoupling between photosynthesis and growth of phytoplankton, results in higher
25 EOC (Carrillo et al., 2008a; Korbee et al., 2012), which modulates the bacterioplankton responses to UVR in ecosystems with low organic-carbon content (Medina-Sánchez et al., 2002). Moreover, EOC also changed by fluctuant irradiance due to vertical mixing in high-mountain lakes (Helbling et al., 2013).

The aim of this study was to test the direct and indirect interactive effects of UVR, nutrient-addition instead of enrichment and mixing on the metabolism of bacterioplankton with different light histories. For this purpose we chose three oligotrophic high-mountain lakes from Spain with different biological and physical characteristics, with differences in UVR transparency as the most distinctive feature among them. We first analyzed how the joint action of mixing and nutrient-enrichment modified the effects of UVR on bacterioplankton during short-term exposures. Previous studies (Helbling et al., 2013) found a higher damage due to UVR exposure in autotrophic organisms of opaque lakes under mixing and after nutrient input (i.e., with higher inhibition of primary production and less carbon excretion), and a potential limitation of C for bacteria dependent on photosynthetic carbon. Therefore, with this background, we hypothesized that mixing, together with nutrient input would result in higher direct and indirect UVR damage on bacterioplankton in opaque as compared to clear lakes.

2 Methods

2.1 Study site

The study was carried out in three high-mountain lakes of Spain: Lake Enol (hereafter LE), located at Picos de Europa National Park at 1075 m a.s.l., and Lake Las Yeguas (hereafter LY) and Lake La Caldera (hereafter LC), both located in Sierra Nevada National Park at 2800 and 3050 m a.s.l., respectively. LE is an oligotrophic lake, with low light penetration in the water column and high influence of cattle activity (Velasco et al., 1999; Helbling et al., 2013) and has a mean chlorophyll a (Chl a) concentration at the surface of 2.1 µg Chl a L^{-1} (Helbling et al., 2013). LY and LC are two small and shallow lakes, highly transparent and oligotrophic (Medina-Sánchez et al., 2010). LY has water inlets and outlets, but they are absent in LC (Medina-Sánchez et al., 2010). Both Sierra Nevada's lakes have dissolved organic carbon (DOC) levels ≤ 1 mg L^{-1} (Reche et al., 2001) and are strongly P-limited, with a dissolved inorganic nitrogen:

total phosphorus (DIN : TP) ratio 30–90 (by mass) (Carrillo et al., 1996; Helbling et al., 2013). LY and LC have a mean surface Chl a concentration of 1.1 and 0.7 μg Chl a L^{-1}, respectively (Helbling et al., 2013). The three lakes receive pulses of nutrient from different origins: in LE they are mainly from cattle activity (López-Merino et al., 2011), whereas in LY and LC they come from atmospheric Saharan dust containing high P levels (Morales-Baquero et al., 2006).

2.2 Experimental design

Short-term experiments to assess the combined effects of vertical mixing, nutrient and UVR on HBP and bacterial carbon demand (BCD) were carried out in situ during summer of 2010: on 23 July in LE, on 10 September in LC, and on 13 September in LY. An acid-cleaned 6 L horizontal Van Dorn sampler was used to collect the water samples within the upper 3 m of the water column. Before performing the experiments, water samples were filtered through a 45 μm-pore size mesh to remove large zooplankton. The experiments had a 2 × 2 × 2 factorial design. Two radiation treatments (in triplicate) were implemented: (i) UVR+PAR (> 280 nm; treatment UVR): using uncovered quartz flasks and (ii) exclusion of UVR (> 400 nm; treatment PAR): covering the flasks with UV Opak 395 filter (Ultraphan, Difegra). The spectral characteristics of this filter are published elsewhere (Figueroa et al., 1997). Samples were also exposed to two nutrient conditions: (i) ambient nutrient concentration (NP-ambient) and (ii) nutrient addition (NP-added). The nutrient-added treatments were obtained by adding Na_2HPO_4 to a final concentration of 30 μg P L^{-1} and NO_3NH_4 to a final N : P ratio of 31. In this way, we simulated and kept the proportion of nutrients input caused by pulses of Saharan dust as reported by Morales-Baquero et al. (2006).

The flasks were put in two trays, one at a fixed depth (STATIC) and one moving vertically (MIXING). The STATIC treatment was put at a fixed depth that varied between 1.3 and 1.4 m (according to their PAR attenuation coefficient, k_{PAR}) to receive the mean irradiance of the upper 3 m of the water column (Helbling et al., 2013). Vertical mixing in the MIXING treatment was simulated by transporting a tray up and down to 3 m

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depth at a speed of 1 m every 4 min. This speed was previously determined the day before experimentation by doing measurements of the effective photochemical quantum yield (Y) at different depths 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 and back to the surface) were completed. The tray containing the 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 linear and constant transport rate on the flasks from the surface to the bottom. The whole setup was placed on a boat that was anchored in a deep section of the lakes and it did not receive any shadows.

2.3 Physical analyses

Vertical profiles of solar radiation and temperature in the water column were obtained at noon with a submersible BIC compact 4-channel radiometer (Biospherical Instruments Inc., CA, USA), which has three channels in the UVR region of the spectra (305, 320 and 380 nm) and one broad-band channel for PAR (400–700 nm) and a temperature sensor. Diffuse attenuation coefficients for downward irradiance (k_d) were determined from the slope of the linear regression of the natural logarithm of downwelling irradiance vs. depth for each wavelength range considered ($n > 200$ per profile). Temperature data were used to determine the strength of the thermocline and the depth of the epilimnion.

2.4 Analysis of abiotic and biotic structural variables

Water samples from the upper water layer were collected in triplicate in each lake to determine abiotic and biotic structural variables. Dissolved inorganic nitrogen (DIN) was considered as the sum of nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+), which were determined by UV-spectrophotometric screening, sulfanilamide and phenol-hypochlorite techniques, respectively (APHA,1992). Total phosphorus (TP) was measured by analyzing 50 mL aliquots with the acid molybdate technique after persul-

fate digestion (APHA, 1992). Blanks and standards were done in all procedures. Dissolved organic carbon (DOC) values were determined by filtering the samples through pre-combusted (2 h at 500 °C) glass-fiber filters (Whatman GF/F) and acidifying them with HCl. Samples were then measured in a total organic carbon analyzer (TOC-V CSH/CSN Shimadzu).

Phytoplankton abundance (PA) was obtained from samples that were put in 250 mL brown glass bottles and preserved with Lugol alkaline solution (1 % vol vol^{-1}) and then stored until analysis. A volume of 50 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).

Bacterial abundance was determined by flow cytometry techniques (FACScanto II, Becton Dickinson Biosciences, Oxford, UK) from samples of water (three replicates and two controls for each experimental treatment) fixed with 1 % paraformaldehyde and stained with SYBER Green I DNA stain (Sigma-Aldrich) to a 1 : 5000 final dilution of initial stock (Zubkov et al., 2007). Stained microbial cells were discriminated on bivariate plots of particle side scatter vs. green fluorescence. Yellow-green 1 μm beads (Fluoresbrite Microparticles, Polysciences, Warrington, PA, USA) concentration standard was added at known dilution to determine absolute cell concentration and fluorescence (Zubkov and Burkill, 2006; Zubkov et al., 2007).

2.5 Analysis of biotic functional variables

Heterotrophic bacterial production. Water samples for HBP measurements were placed in 10 mL-quartz flasks (three replicates and two blanks for each experimental treatment). Flaks were exposed in situ for 3 h under the different treatments previous to the radiotracer addition. HBP was determined by incorporating ^{3}H-thymidine (S.A = 46.5 Ci mmol^{-1}, Amershan Pharmacia) into the bacterial DNA (Fuhrman and Azam, 1982; Smith, 1992). ^{3}H-thymidine was added to each experimental flask to a final concentration of 16.6 nM (saturating concentration). Vials with the radiotracer were incubated during 1 h in situ under the different treatments. Therefore all flask sets were

horizontally held during the total 4 h of incubations (3 h without radiotracer followed by 1 h with radiotracer) around noon.

After incubation, the incorporation of ^{3}H-thymidine was stopped with the addition of tricholoracetic acid (TCA, 5 % final concentration). Blanks were killed with 5 % TCA before addition of the radiotracer. Extraction was performed in cold 5 % TCA by keeping the vials in the ice for 20 min, after which the precipitate was collected by centrifugation (1600 g for 10 min). Then, vials were rinsed twice with 1.5 mL of 5 % TCA to remove any residual unincorporated radioactivity. After that, scintillation liquid (Ecoscint A) was added for subsequent measurement in an autocalibrated scintillation counter (Beckman LS 6000TA). The conversion factor 1×10^{18} cell mol^{-1} (Bell, 1993) was used to estimate the number of bacteria produced per mol of incorporated thymidine. The factor 2×10^{-14} g C cell^{-1} (Lee and Fuhrman, 1987) was applied to estimate the amount of carbon.

Respiration rates. Water samples for total planktonic respiration (TPR) (in the < 45 μm fraction) were placed in 10 mL-quartz flasks (three replicates for each experimental treatment) and exposed in situ for 4 h under the different treatments as explained above. TPR was assessed by using optode sensor-spots (SP-PSt3-NAU-D5-YOP; PreSens GmbH, Germany) and an optic-fiber oxygen transmitter (Fibox 3; PreSens GmbH, Germany) connected to a computer (Warkentin et al., 2007). Data were recorded using the OxyView 3.51 software (PreSens GmbH) and measurements were made at the initial time and then every 8 h during 48 h. In order to estimate BR from TPR we used a potential maximum value of 75 % of TPR, which is based on data reported for oligotrophic waters (Lemeé et al., 2002) and a minimum value of 50 % of TPR, which is an average value based in previous studies in LC (Carrillo et al., 2014) and on data reported in other oligotrophic systems (Robinson et al., 2002; Robinson, 2008). The consumption rates of oxygen (μM O$_2$ h^{-1}) by the bacterial community were converted into carbon units by using a respiratory quotient of 1 described for bacterioplankton (del Giorgio and Cole, 1998). Bacterial carbon demand (BCD) was calculated as HBP + BR.

Bacterial carbon demand and their relationship with excreted organic carbon. To determine the supply of algal C for bacterioplankton, we considered the values of excreted organic carbon (EOC) reported by (Helbling et al., 2013) in LE, LY and LC. These data was obtained under the same experimental conditions and dates as in this study. In brief, to determine EOC, the experimental 50 mL-flasks (three clear and one dark per treatment) were incubated with 0.37 MBq of $NaH_{14}CO_3$ (SA: 310.8 MBq mmol^{-1}, DHI Water and Environment, Germany). After 4 h of incubation under the different treatments, the samples were filtered onto 0.7 pore size filters in Las Yeguas (LE) and 1 μm pore size filters in Las Yeguas and La Caldera (LY and LC); Nucleopore filters (25 mm diameter) under low pressure (< 100 mmHg). Fraction on the filters was used to determine primary production (see Helbling et al., 2013) and 4 mL subsamples of the filtrate were collected to determine EOC. After 24 h of acidification (100 μL of 1N HCl) in which vials stood open in a hood, scintillation cocktail (Ecoscint A) was added to the samples and disintegrations per minute (dpm) counted using a scintillation counter (Beckman LS 6000TA) equipped with an internal calibration source. For all EOC calculations, dark values were subtracted from corresponding light values.

To assess the bacterial limitation by EOC (%BCD : EOC) we quantified if EOC rates were enough for sustaining the potential BCD (Morán et al., 2002) as:

$$\%BCD : EOC = BCD \times EOC^{-1} \times 100 \tag{1}$$

2.6 Data treatment and statistics

The percentage of UVR-induced inhibition (UVR$_{inh}$) on HBP was calculated as:

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

where HBP$_{PAR}$ and HBP$_{UVR}$ represent the carbon produced in samples under the PAR and UVR treatments, respectively. The differences of UVR$_{inh}$ among treatments were evaluated by a *t* test. We used propagation errors to calculate the variance of the UVR-induced inhibition.

To determine significant interactions among the radiation, nutrients and mixing regimes for the different variables, a three-way analysis of variance (ANOVA) was used. When significant effects were determined, a post hoc Fisher LSD-test was used to determine significant differences among treatments. Data were checked for normal distribution with the Kolmorov–Smirnov test. Homocedasticity was verified with Cochran and Levene's test, and data were log-transformed when these conditions did not meet. Statistical analysis was performed using the program Statistica 7.0 for Windows (Statsoft, 2001).

The points presented in Fig. 3 (UVR-induced inhibition of HBP as a function of the attenuation coefficient) were based on the results obtained in this study and, in order to extend the relationship obtained in our model lakes, we also used data from previous published studies and also unpublished data obtained by our research group. We used data by (Bertoni et al., 2011) from their experiment 1 comparing static vs. moving incubations. Thus, we calculated the difference in the percentage of UVR_{inh} (calculated according to Eq. (2) from data reported in their Table 2) between values in mixing and static (i.e., 31.4 % (mixed) – 20.8 % (static)) treatments, and normalized it by a mean PAR irradiance of $259.2\,W\,m^{-2}$. We used the k_{d320} value of 0.425 reported in their Table 2. The second additional point is from experiments carried out by our research group in La Colgada Lake, in Ruidera Natural Park (Spain) (unpublished data). There, we obtained the difference between $\%UVR_{inh}$ (according to Eq. 2) in mixing and static treatments (i.e. 53.2 % (mixed) – −53.6 % (static)). The differences were normalized by a mean PAR of $264.3\,W\,m^{-2}$ and they were related with their k_{d320} (2.08) in the graph.

3 Results

3.1 Experimental conditions

The main initial physical, chemical and biological characteristics measured in the epilimnion of the three studied lakes are shown in Table 1. The high DIN : TP ratios for

the three lakes evidenced the strong likelihood of P-limitation in the upper layers (DIN : TP> 12 by weight, sensu, Morris and Lewis, 1988). However, the DIN : TP ratio in LE (~ 118) was ca. three-fold higher than in LY and LC due to the high concentration of DIN. Moreover, the DOC concentration (~ 2.2 mg L^{-1}) was 2-fold higher in LE as compared to the other two lakes. In regard to the radiation conditions (Fig. 1, Table 1), LC was the most transparent, having the lowest k_d value i.e., k_{dPAR} of 0.16 m^{-1}, followed by LY and LE (Table 1). Thus, and hereafter, we will refer LE as to the "opaque" lake and LY and LC as to the "clear" lakes. The highest mean surface irradiance during the experiments was received in LC, then followed by LY and LE (e.g. surface PAR irradiance: 1774, 1670 and 1558 µmol photons m^{-2} s^{-1} for LC, LY and LE, respectively). LE had the highest epilimnetic temperature value (Fig. 1a) with a marked epilimnion down to ca. 3.5 m depth, below which temperature steadily decreased to 9 °C at 12 m depth. In contrast, LY (Fig. 1b) was well mixed and temperature did not substantially vary in the water column; LC (Fig. 1c) showed a weak stratification with temperature decreasing from 14 °C at the surface to 12 °C at 1 m depth.

3.2 Joint effects of solar radiation, mixing and nutrient on bacterioplankton metabolism

In the clear lakes (LC and LY), interaction radiation × mixing × nutrient was found for HBP but not in the opaque lake (LE) (Table 2). LE had the highest values of HBP, followed by LY and LC (Fig. 2). In LE, samples exposed to UVR in static conditions, had significantly higher HBP values than those exposed only to PAR (Fig. 2a). Nutrient addition decreased HBP under UVR in both static and mixing treatments (Fig. 2a) as compared to values obtained without nutrient addition. While UVR stimulated HBP in static conditions regardless of the nutrient treatment, this effect decreased or almost vanished under mixing conditions (Fig. 2b). For LY, HBP was not affected by UVR exposure under static and ambient nutrient conditions, but HBP values increased in the PAR treatment in response to nutrient addition resulting in a high UVR inhibitory effect (UVR$_{inh}$ = 40 %; Fig. 2c and d). Under ambient nutrient conditions, mixing increased

HBP under PAR as compared to static conditions, leading to an UVR inhibitory effect of 21 % (Fig. 2c and d). Under nutrient addition, mixing reduced HBP value in PAR treatment as compared to static conditions (Fig. 2c). In LC (Fig. 2e), UVR did not significantly affect HBP in static ambient nutrient conditions as compared to PAR-exposed samples. The addition of nutrients under UVR increased HBP in both static and mixing regimes, but nutrient-addition stimulated HBP under PAR only in the mixing regime. Hence, nutrient addition resulted in a UVR-stimulatory effect on HBP up to 58 % under static conditions. Despite HBP values increased after nutrient-addition under mixing regime, UVR was responsible for a strong inhibitory effect of HBP (47 %) under mixing, with no significant differences between nutrient treatments (Fig. 2f).

Figure 3 shows the relationship between the UVR-transparency, as estimated by the attenuation coefficient at 320 nm (k_{d320}), and the effects of mixing on the inhibitory effect of UVR on HBP. We found a decreasing inhibitory trend of UVR towards lower k_{d320} values (Fig. 3). Consistently with our observations, the data obtained by Bertoni et al. (2010) and from our own group in La Colgada Lake showed that the inhibitory effect due to mixing was higher in the more opaque lakes (high k_{d320}) and decreased towards the more clear lakes (low k_{d320}; $r = 0.93$, p value < 0.01). It is worth noting that the decrease in the inhibitory effect of UVR is much more pronounced when nutrients were added.

For BR, no significant interactive effects between the three studied factors were found for any ecosystem (Table 2). As in HBP, BR showed higher values in LE as compared to the other lakes (Fig. 4). In LE, BR increased in response to nutrient addition regardless the radiation and mixing regimes, but BR was neither affected by mixing nor by UVR (Fig. 4a). In LY, BR was stimulated by nutrient addition under UVR regardless the mixing regime and under PAR only under mixing. As determined in LE, mixing and UVR did not affect BR (Fig. 4b). Finally, for LC, while the addition of nutrients reduced BR in static conditions regardless the radiation conditions (Fig. 4c), mixing only reduced BR under UVR. Noticeably, nutrient addition unmasked the UVR stimulatory effect in mixing and static regimes.

3.3 Joint effects of solar radiation, mixing and nutrient-addition on bacterial C demand and supply of algal C

In order to discriminate whether the effect of UVR on bacteria was mediated by their commensalistic interaction with algae, we compared bacterial carbon demands with the dissolved organic carbon excreted by algae (EOC). EOC was significantly higher in LE as compared to the other lakes (Fig. 5). There were significant interactive effects among radiation, mixing, and nutrient (Table 2) on EOC in the three lakes. In LE and under static and ambient nutrient conditions, EOC was higher in samples exposed to UVR (Fig. 5a), although nutrient addition reduced EOC in samples under UVR as compared to samples with no nutrient addition (Fig. 5a). There was no significant difference under mixing regime between PAR and UVR treatments (Fig. 5a). EOC was generally higher in static than in mixing regime in LY (Fig. 5b), whereas the opposite was observed in LC (Fig. 5c). In LY and LC, nutrient addition in the presence of UVR resulted in lowered EOC under static conditions, and higher EOC under mixing regime (Fig. 5b and c).

Figure 6 depicts the ratio between BDC and EOC, i.e. an estimation of whether EOC meets the potential bacterial carbon demands for growth and respiration. In LE, under UVR and ambient nutrient conditions, EOC exceeded BCD (static) or was enough (under mixing) to meet BCD (i.e. %BCD : EOC $\leq$ 100; Fig. 6a). In LY and LC, EOC exceeded BCD under simultaneous UVR, mixed and nutrient-added conditions. However, EOC did not meet BCD only under UVR and ambient nutrient and mixing conditions in LY (Fig. 6b), and under UVR and nutrient added and static conditions in LC (Fig. 6c).

4 Discussion

This study fills a gap of knowledge about how vertical mixing modulates the UVR effects on bacterioplankton in optically different high-mountain lakes. We quantified how the interaction of UVR, nutrient inputs, and the epilimnetic mixing regime affected the bacterial metabolism both directly and indirectly, this latter through the excretion of or-

ganic carbon by algae. The three selected model ecosystems, with a gradient of UVR transparency, enabled us to understand how their optical characteristics modulated the interactive effects that the three studied factors exerted on the algae–bacteria relationship. Our experiments were made under realistic experimental exposure conditions resembling the epilimnetic vertical mixing during the middle ice-free period when both UVR flux and nutrient inputs reach a maximum in high-mountain Iberian lakes (Bullejos et al., 2010; Villar-Argaiz et al., 2012; Mladenov et al., 2011).

Our study showed that, under static conditions, UVR alone stimulated HBP in the opaque, but not in the clear lakes. As a major finding, and in agreement with our initial hypothesis, mixing and nutrient addition resulted in higher negative UVR effect on the bacterioplankton metabolism as compared to static conditions in all systems and, this was more accentuated in the opaque system where mixing and nutrients annulled the strong UVR stimulation of HBP in opaque lake; by contrast, in the clear systems these factors unmasked an inhibitory effect of UVR on HBP. These results have important implications, as explained in the following paragraphs:

Firstly, our results challenge the long-standing assumption that vertical mixing (at ambient nutrient condition) counteracts the UVR-induced damage in aquatic ecosystems. This assumption is based on the modulating effect that mixing exerts on the ratio of UVR: PAR received by organisms which, consequently, reduced the damage to repair ratio from the upper water layers to the deeper ones (Neale et al., 2003). Our results contrast with those reported for bacteria in marine clear-waters of Mediterranean Sea, where mixing slightly reduced photoinhibition on HBP (Gali et al., 2013). Likewise, our findings are opposed to previous results showing the effect of vertical mixing decreasing photoinhibition established for autotrophic organisms in clear high-mountain lakes (Helbling et al., 2013; Carrillo et al. unpublished).

Secondly, our results do not fully support the paradigmatic proposal of DOC as the "ozone of the underwater world" (Williamson and Rose, 2010) protecting against harmful UVR. Thus, fluctuating radiation due to mixing in the opaque lake was responsible for the largest UVR negative effect in the experiments (Fig. 3). This result is in agree-

ment with previous studies that showed less acclimation of algae to cope with fluctuating UVR in high-DOC ecosystems (Villafane et al., 2004; Harrison and Smith, 2011; Helbling et al., 2013). However, by assuming that mixed conditions represented the most realistic climate of light affecting HBP in each lake (Ruiz-Gonzalez et al., 2013), bacteria of the opaque lake were the most protected as no UVR inhibition was observed. In contrast, UVR under mixing inhibited HBP on the clear lakes. This finding in clear lakes was due to the increase in HBP under PAR rather than to a direct effect of UVR. This result suggests that fluctuating PAR could contribute to the enhancement of HBP through light-dependent mechanisms. Similar results were reported by Bertoni et al. (2011).

Thirdly, our findings reveal a non-generalized direct positive effect of P-pulse on the bacterial metabolism. Particularly for the opaque lake P-addition decreased HBP regardless the radiation quality or mixing regime. While literature indicates a widespread occurrence of P limitation of HBP in nutrient-limited waters (Bertoni et al., 2011; Ogbebo and Ochs, 2008; Nelson and Carlson, 2011), we found that supplementation with P increased HBP only in clear lakes. This result suggests that a resource as C released by autotrophs might be key in controlling bacteria growth under the joint action of UVR, mixing regime and nutrient-addition. In fact, a large number of studies have reported the bacterial dependence on EOC in oligotrophic ecosystems (Baines and Pace, 1991; Carrillo et al., 2002; Medina-Sánchez et al., 2002, 2004; Morán et al., 2002; Pugnetti et al., 2010). The reduction of HBP after P addition, under mixing and UVR in the opaque lake is consistent with the higher metabolic cost due to respiration. The fact that EOC did not completely overcome C demands for growth strongly implicates that photosynthetic carbon acts as the main limiting resource for bacteria, despite the high DOC content in the lake, because of the reported preference of bacteria for the former (Medina-Sánchez et al., 2002; Kritberg et al., 2005). Moreover, the absence of P stimulus in simultaneous primary production measurements carried out under identical experimental conditions (Helbling et al., 2013) provides evidence to conclude that phytoplankton were not actively competing with bacteria for the available P. Thus, if P

uptake by algae would have been higher than that of bacteria, supplementation with P in our experiments would have not decreased, or at the most not stimulated, HBP. A contrasting pattern was found for the clear lakes, where EOC satisfied BCD under the joint action of UVR, mixing regime and nutrient-addition. Thus, under UVR and fluctuating regime, P supplementation increased HBP, and these effects were concomitant with steady bacterial respiration.

Based on the results obtained in our model ecosystems, we propose a conceptual graphical model (Fig. 7) where we consider environmental drivers acting under ambient conditions and under global change predicted conditions (i.e. increasing nutrient inputs, DOM, wind forcing, temperature). The ecological impact of these drivers of global change is predicted to become more important affecting solar radiation penetration and mixing in aquatic systems (Caplanne and Laurion, 2008; Sarmento et al., 2010). Our study indicates that this may cause alterations to the networks of biotic interactions by weakening the commensalistic relationship between algae and bacteria (i.e., %BCD : EOC) and accentuating bacterial limitation for the labile-C under predicted global change conditions (Fig. 7). Eventually bacteria vulnerability to UVR may affect the development of the microbial loop and particularly in the most opaque lakes.

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Table 1. Mean values of the main physical, chemical and biological variables of the studied lakes at the beginning of the experiment. Diffuse attenuation coefficients (k_d) of UVR at 305 nm (k_{d305}), at 320 nm (k_{d320}) and at 380 nm (k_{d380}) and PAR (k_{dPAR}); dissolved organic carbon (DOC); total phosphorus (TP); dissolved inorganic nitrogen (DIN); DIN/TP ratio expressed by weight; bacterial abundance (BA); phytoplanktonic abundance (PA).

Variable	Lake		
	LE	LY	LC
k_{d305} (m^{-1})	3.15	0.62	0.30
k_{d320} (m^{-1})	2.28	0.58	0.26
k_{d380} (m^{-1})	1.22	0.26	0.17
k_{dPAR} (m^{-1})	0.34	0.18	0.16
DOC (mg L^{-1})	2.23 ± 0.45	1.01 ± 0.11	1.07 ± 0.08
TP (mM)	0.11 ± 0.05	0.11 ± 0.003	0.128 ± 0.007
DIN (mM)	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
BA (cell m L^{-1}) $\times 10^5$	25 ± 19.2	4.14 ± 0.24	1.22 ± 0.22
PA (cell m L^{-1})	3599	2063	2000

Table 2. Result of the three-way analysis of variance (ANOVA) of the interactive effects of radiation, nutrient-addition, and mixing regime. Numbers in bold indicate significant effects. HBP: heterotrophic bacterial production; BR: bacterial respiration; EOC: excreted organic carbon; %BCD : EOC: relationship between the bacterial carbon demand (BCD) and supply by algal excretion.

|			HBP		BR		EOC		%BCD : EOC	
LE	$df1$	$df2$	$F_{df1,df2}$	p	$F_{df1,df2}$	p	$F_{df1,df2}$	p	$F_{df1,df2}$	p
Radiation	1	16	27.092	**0.000**	5.007	**0.040**	13.618	**0.002**	5.500	**0.032**
Nutrients	1	16	40.495	**0.000**	925.493	**0.000**	0.181	0.676	35.639	**0.000**
Mixing	1	16	2.572	0.128	18.478	**0.001**	15.095	**0.001**	9.949	**0.006**
Mixing × Radiation	1	16	11.179	**0.004**	1.944	0.182	0.816	0.380	0.929	0.350
Mixing × Nutrients	1	16	1.623	0.221	14.331	**0.002**	0.360	0.557	11.854	**0.003**
Radiation × Nutrients	1	16	2.274	0.151	3.157	0.095	20.792	**0.000**	5.983	**0.026**
Radiation × Nutrients × Mixing	1	16	0.166	0.689	0.415	0.528	25.257	**0.000**	15.685	**0.001**
LY										
Radiation	1	16	16.108	**0.001**	0.086	0.773	0.895	0.358	4.832	**0.043**
Nutrients	1	16	16.920	**0.000**	21.558	**0.000**	22.912	**0.000**	0.840	0.373
Mixing	1	16	0.094	0.764	3.538	0.078	72.986	**0.000**	4.832	**0.043**
Mixing × Radiation	1	16	0.463	0.506	0.319	0.580	3.570	0.077	6.327	**0.023**
Mixing × Nutrients	1	16	1.512	0.237	2.130	0.164	13.220	**0.002**	5.859	**0.028**
Radiation × Nutrients	1	16	10.738	**0.005**	0.917	0.353	45.253	**0.000**	55.459	**0.000**
Radiation × Nutrients × Mixing	1	16	25.373	**0.000**	0.016	0.901	24.965	**0.000**	50.450	**0.000**
LC										
Radiation	1	16	17.155	**0.000**	3.664	0.073	0.088	0.771	11.024	**0.004**
Nutrients	1	16	129.735	**0.000**	9.828	**0.006**	0.058	0.812	0.002	0.964
Mixing	1	16	12.347	**0.003**	2.420	0.139	86.126	**0.000**	17.553	**0.001**
Radiation × Mixing	1	16	49.149	**0.000**	0.258	0.618	83.738	**0.000**	25.783	**0.000**
Mixing × Nutrients	1	16	7.562	**0.014**	14.766	**0.001**	177.981	**0.000**	5.799	**0.028**
Radiation × Nutrients	1	16	0.139	0.714	9.865	**0.006**	2.624	0.125	20.387	**0.000**
Radiation × Nutrients × Mixing	1	16	25.076	**0.000**	0.460	0.507	0.978	0.337	1.646	0.218
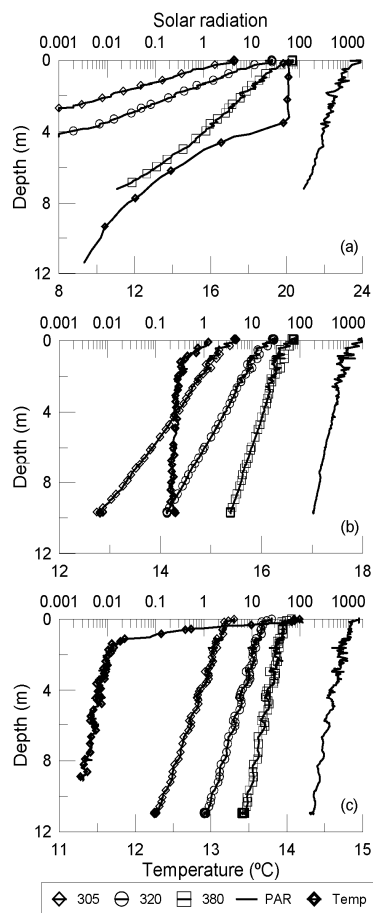
Figure 1. UVR irradiance and temperature as a function of depth in **(a)** Lake Enol (LE), **(b)** Las Yeguas (LY) and **(c)** La Caldera (LC). Irradiance data are expressed in µW cm^{-2}.
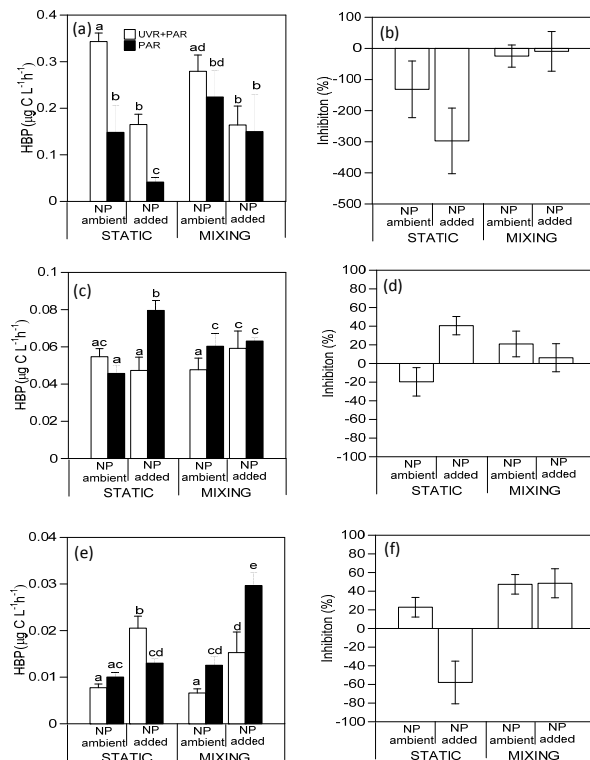
Figure 2. Heterotrophic bacterial production (HBP, in $\mu g\, C\, L^{-1}\, h^{-1}$) under different radiation (UVR+PAR vs. PAR), nutrient concentration (ambient vs. nutrient added) and mixing regime (static vs. mixing) in **(a)** Lake Enol (LE), **(c)** Las Yeguas (LY) and **(e)** La Caldera (LC). Percentage of inhibition of HBP (%) due to UVR under the different treatments in **(b)** LE, **(d)** LY and **(f)** LC. Negative values indicate stimulation by UVR and positive values indicate inhibition by UVR. Data are expressed as mean values ± SD ($n = 3$). Letters indicate significant differences among treatments.

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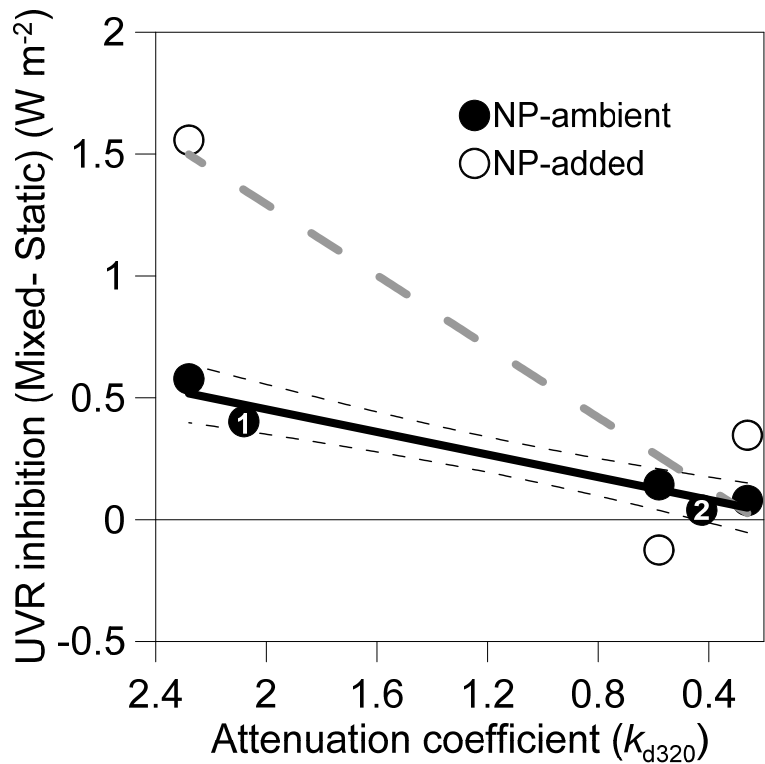
Figure 3. UVR inhibition of HBP in mixed minus static samples, normalized by the mean irradiance received, as a function of the attenuation coefficient (k_{d320}) for samples without nutrient addition (black symbols), and with nutrient addition (white symbols). The black line represents the linear regression fit for the five lakes without nutrient addition ($r^2 = 0.87$, $y = -0.0527 + 0.2418x$), and the dashed grey line represents the fit for the three lakes with nutrient addition. The dashed thin lines are the 95 % confidence for the fit of the five lakes without nutrient addition. The additional data were calculated from (1) Bertoni et al., 2011 and (2) from unpublished data in lakes of Ruidera Natural Park (Spain).
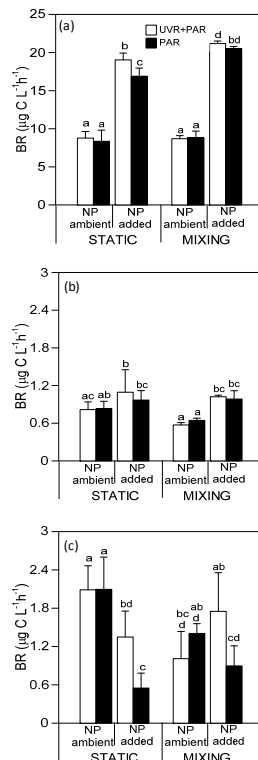
Figure 4. Bacterial respiration (BR; in $\mu g \, C \, L^{-1} \, h^{-1}$) under different radiation (UVR+PAR vs. PAR), nutrient concentration (ambient vs. nutrient added) and mixing regime (static vs. mixing) conditions in **(a)** Lake Enol (LE), **(b)** Las Yeguas (LY) and **(c)** La Caldera (LC). Only BR estimated as 50 % of total planktonic respiration (TPR) is represented in this figure, since significant differences among treatments are the same than for BR estimated as 75 % of TPR. Bars represent the mean BR values and the lines on top of them represent the standard deviation (SD; $n = 3$). Letters indicate significant differences among treatments.
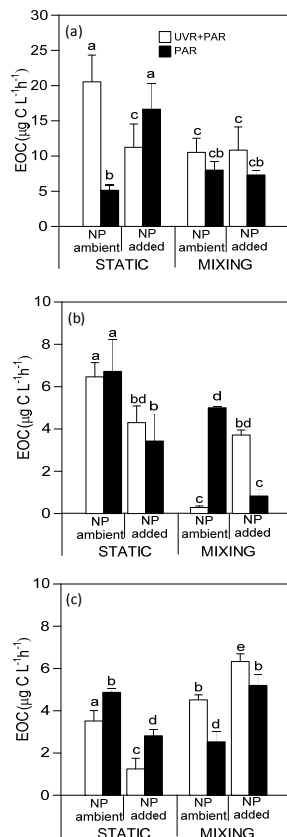
Figure 5. Concentration of excreted organic carbon (EOC, in μg C L^{-1} h^{-1}) under the different radiation (UVR+PAR vs. PAR), nutrient concentration (ambient vs. nutrient added) and mixing regime (static vs. mixing) in **(a)** Lake Enol (LE), **(b)** Las Yeguas (LY) and **(c)** La Caldera (LC). Data are expressed as mean values ± SD ($n = 3$). Letters indicate significant differences among treatments.

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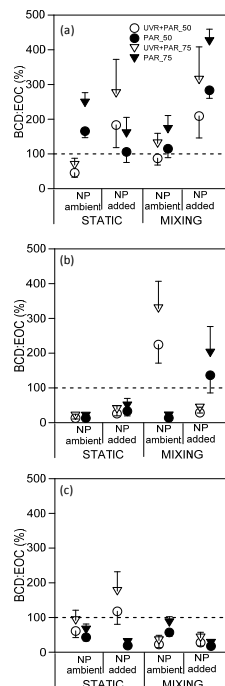
Figure 6. Relationship between the bacterial carbon demand (BCD) and supply of carbon by algal excretion (EOC) as percentage, measured under the different radiation (UVR+PAR vs. PAR), nutrient concentration (ambient vs. nutrient added) and mixing regime (static vs. mixing) in Lake Enol **(a)**, Las Yeguas **(b)** and La Caldera **(c)**. A value of 100 % means a similar carbon demand and carbon supply. Triangles represent the mean value of %BCD : EOC ($n = 3$) when considering bacterial respiration (BR) as 75 % of total planktonic respiration (TPR), whereas circles represent the mean value of %BCD : EOC ($n = 3$) when considering BR as 50 % of TPR. The displayed vertical error bars embrace from the lowest error value for BR from 50 % to the highest error value for BR from 75 %. Error bars were calculated with error propagation.
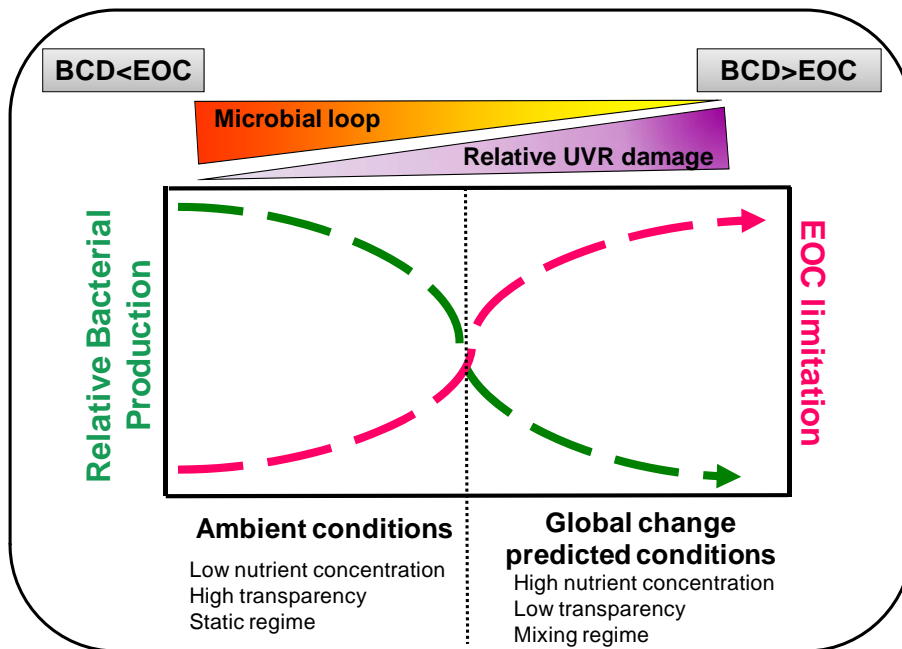
Figure 7. Conceptual graphical model of the interactive effects of increased inorganic nutrients, mixing regime and UVR transparency on the relative heterotrophic bacterial production (green line) and bacterial limitation by algal released C (red line). The green line represents the decreasing trend of HBP values under the interactive effects of the considered factors. BCD: bacterial carbon demand; EOC: excreted organic carbon.