Granada, Spain, 19 August, 2014

Dear Dr. Tom Battin,

Please find below our detailed responses to the suggestions and concerns raised by the reviewers with regards to the manuscript entitled **"***Direct and indirect effects of vertical mixing, nutrients and ultraviolet radiation on the bacterioplankton metabolism in high-mountain lakes from southern Europe"* by C. Durán, J. M. Medina-Sánchez, G. Herrera, M. Villar-Argaiz, V. E. Villafañe, E. W. Helbling, and P. Carrillo. Their very thoughtful contributions were very constructive and helpful in completing the revision of the manuscript.

Sincerely,

Cristina Durán (corresponding author)

REFEREE #1

This manuscript reports results from a single experiment done in 3 high mountain lakes of different water transparency to understand the single and interactive effect of solar UV radiation, nutrients enrichment and mixing. The topic is interesting and relevant to the **Biogeosciences** Journal. The authors have published an accompanying paper on this topic but showing the effects on primary producers. So, some of the information is somehow here repeated. Though the idea of the experiment as I mentioned above is interesting, I have several concerns on the experimental approach used and the conclusions extracted.

Reviewer: The authors have published an accompanying paper on this topic but showing the effects on primary producers. So, some of the information is somehow here repeated.

Author response: The reviewer is correct in that both studies share the same experiment. However, both have completely distinct goals. While the allied paper by Helbling et al. examined the role of UVR, vertical mixing and nutrients on primary producers, this work deals with how these factors and their interaction affect bacterial metabolism. Although the reviewer found some overlapping information, this is rather collateral to the core of the MS. It is our opinion that a potential reader needs to have prompt access to relevant information (radiation, temperature profiles, etc.) without having to search for the already published MS.

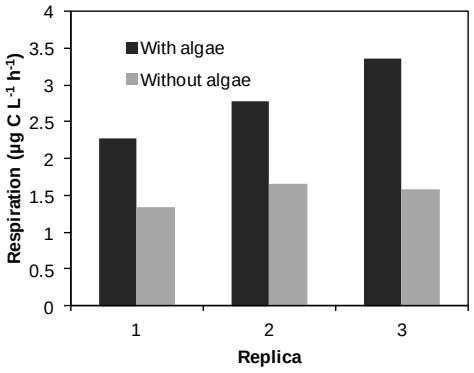
Reviewer: For example, I am not convinced that a model adjusted for an eutrophic, turbid, shallow tropical lake (Viallafañe et al. 2007) and used to define the speed of circulation (1 m every 4 min) in the experiment can be directly extrapolated to high mountain lakes. Will that PQY follow the same pattern? I think this need to be discussed.

Author response: The reviewer is certainly right when he/she questions the extrapolations of our results from an eutrophic lake in China to our model lakes. We chose to follow this same approach in order to determine a new model and speed of circulation for the lakes in our study. Once calculated for Lake Enol, this mixing speed was extended to the other models lakes for comparative purposes.

Reviewer: Similarly, I am concerned about the lack of direct measurements of bacterial respiration and therefore about the derived calculations on BCD and bacterial carbon limitation that are essential to the study. It is unacceptable that no direct measurements were done because the conclusions extracted have a large question mark.

Author response: We fully agree with the Reviewer that direct measurements of bacterial respiration are the best approach to calculate BCD. However, there are certain circumstances in which this is not possible, as it was the case for Lake Enol (LE). In this lake, autotrophic picoplankton and bacteria coexist and thus it is impossible to physically separate them in order to measure only bacterial respiration (i.e., without the authotrophic picoplankton). Of course, one may try to evaluate the contribution of both groups to picoplanktonic respiration based on their abundances, but this would have added further uncertainty to the interpretation of the results. A more valid alternative to us was to estimate BR based on previously published contributions of BR with respect to the values of total plankton respiration (TPR) measured in this study. In doing so, BR lie within two limits: a conservative value of 75% of TPR (Lemeé et al. 2002), and a potential minimum value of 50% of TPR (Robinson et al. 2002; Robinson 2008).

We were able to determine bacterial respiration for the rest of the lakes (LC and LY), although in a different set of experiments. Measurements in these lakes indicated that BR comprised between 50 and 70% of TPR (see figure below). These values do not significantly differ from those proposed by Robison and Lemeé, what further justified the range used in our calculations.



Respiration in presence and absence of algae show that bacterial respiration accounted between 50 and 70% of total respiration in LC. Water samples come from the upper water layer (0.5m).

Although real BR measurements would have been desirable, our measurements are congruent with the fact that the pattern of BCD:EOC ratio above or below the

100% threshold using the different percentages of TPR to calculate BR did not change our conclusions. Thus, we trust that our approach is adequate for this study.

Reviewer: Sampling: What does it exactly mean "water samples within the upper 3m of the water column"? How many samples were taken? Was the water pooled from different water layers?

Author response: Water samples were collected at four discrete depths within the first three meters of the lake. Thus, the samples were taken at 0m, 1m, 2m and 3m.

Action taken:

The text (page 7, lines 10-13) "An acid-cleaned 6 L horizontal Van Dorn sampler was used to collect the water samples within the upper 3m of the water column. Before performing the experiments, water samples were filtered through a 45 μm-pore size mesh to remove large zooplankton."

Now read:

For the experiments, lake water from four depths (0, 1, 2, and 3 m), collected with an acid-cleaned 6-L horizontal Van Dorn sampler and screened through 45 μm mesh to remove zooplankton, was used to fill two containers (10 L).

Reviewer: Another concern is on the exp. procedures: One cannot really follow how the experiment was done. Were all parameters measured from the same bottle or were parameters such as bacterial production and total respiration measured separately? It seems the authors used just small volume quartz tubes that were treated in the same way, but I am not sure. Also how were nutrients added?

Author response: Parameters were assessed for each flask (glass or quartz-flasks depending on the radiation treatment), which were all incubated under the same experimental conditions. Nutrients were added to one of the two containers in which water from the upper three meters was collected. The container was shaken and water left for an acclimation time of 90 min exposed to full sunlight in the lakes; finally, this nutrient-added subsample water was used to fill the flasks assigned for this treatment.

Action taken: We revised the first paragraph of the subsection "Experimental design" (page 7, lines 8-13) and we added new information (in bold the part referring to this concern).

Now read:

"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. For the experiments, lake water from four depths (0, 1, 2, and 3 m), collected with an acid-cleaned 6-L horizontal Van Dorn sampler and screened through 45 μm mesh to remove zooplankton, was used to fill two containers (10 L). **One of the**

containers received P (as Na_2HPO_4) to give a final concentration of 30 μgP 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). After nutrient addition, the container was shaken and left for an acclimation time of 90 min exposed to sunlight in the lake before being used to fill the experimental flasks. "

Reviewer: More important is the lack of rationale on why samples were moved surface-down to 3 m and back to the surface when the temperature profiles indicate that in LE the thermocline was at 4 m depth and in the other two lakes the epilimnion was even shallower. If the objective of the study was to compare the effects on communities with different light conditions, why the real mixing depth and attenuation of light were not considered to determine the extent of the vertical mixing simulation. In my opinion, this is in strong contrast with the statement by the authors in the discussion that these experiments were made under realistic experimental exposure conditions resembling the epilimnetic vertical mixing…

Author response: The depths of incubations were determined both based on temperature profiles obtained in this study and on previous studies conducted in LY and LC (Delgado-Molina et al. 2009). The aim was to keep similar experimental conditions in the three studied ecosystems with the objective of comparing how fluctuating radiation (result of vertical mixing regime) would affect bacterial metabolism. The intensity and quality of the radiation depend on the concentration and characteristics of the CDOM in each lake.

There are evidences for microstratification and vertical mixing processes in Sierra Nevada lakes associated to strong wind episodes (Rueda et al. 2007), which support the limit for the mixing depth of >3 m used in our experiments.

Reviewer: The text is unclear in many parts (see some examples below), but the Introduction is particularly difficult to read and lacks clear structure. Also many citations that are not always referred to the type of lake studied are used, which is confusing. So one ends without a clear picture of which factors are really relevant for those high mountain lakes.

Author response: The introduction was structured according to the following criteria:

_ The first paragraph deals with the importance of bacterioplankton for ecosystem functioning and its sensitivity to different global change stressors.

_ The second paragraph deals with the effects of UVR on bacterioplankton and how the interaction with environmental factors such as nutrients or dissolved organic carbon content (CDOM) can explain the lack of consistence of these UVR effects.

_ In the third paragraph we analyze how UVR exposure is related to the physical structure of the water column, in turn affected by global warming. The variations of the upper mixing layer depth as result of the increase in temperature, wind speed or storm events determine how bacteria cope with UVR fluctuation and nutrient input.

_ The fourth paragraph deals with how fluctuating radiation regime affect phytoplankton metabolism (e.g. carbon release) and bacterioplankton, and

whether carbon release may modulate the bacterial response to UVR exposure under fluctuating mixing regime.
_ Finally, the objective and hypothesis are posed.

We recognized that some sentences in the introduction were misleading and adhered to the reviewer's recommendation of reorganizing some arguments. Specifically, the fourth paragraph has been rewritten to highlight the gap of knowledge in how bacteria might respond to fluctuating radiation regime based on their dependence on algal carbon.

Action taken: We revised the fourth paragraph (page 5, lines 8-28) and the beginning of fifth paragraph (page 6, lines 1-3) of the Introduction. The old paragraphs:

"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 carbon 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 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 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 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."

Now read:

"In the high mountain lakes of southern Europe, it is known that under static UVR exposure, by favoring the uncoupling between photosynthesis and growth of phytoplankton, results in higher 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 (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). Although numerous studies recognize the importance of vertical mixing in the response of phytoplankton to UVR (e.g. Helbling et al., 1994, 2008, 2013; Neale et al., 1998), studies dealing with the interactive effects of UVR and mixing on bacteria are spare (Jeffrey et al., 1996; Huot et al., 2000; Bertoni et al., 2011; Galí et al., 2013) largely due to the logistic complexity of these experiments, particularly in high-mountain lakes, and the difficulty to discriminate between direct and indirect effects.
The aim of this study was to test the direct and indirect interactive effects of UVR, nutrient-addition and mixing on the metabolism of bacterioplankton with different light histories in oligotrophic high-mountain lakes."

Reviewer: Overall in text: what is defined here as opaque? To what wavelengths do the authors refer?

Author response: "Opaque" vs. "clear" was defined according to UVR transparency; therefore "opaque" refers to a low transparency to UVR.

Action taken: Page 13, lines 5-7.

The sentence:

"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 0.16m^{-1}, followed by LY and LE (Table 1). Thus, and hereafter…"

has been replaced by:

"In regard to the radiation conditions (Fig. 1, Table 1), LC was the most transparent, having the lowest k_d value **for PAR (e.g., k_{dPAR} of 0.16m^{-1}) particularly for UVR (e.g., k_{d305} of 0.30 m^{-1}), followed by LY and LE (Table 1)**. Thus, and hereafter…"

Reviewer: Is a lake located at 1075 m.a.s.l. a high mountain lake? I am not sure though I did not find a clear definition of what "high" is.

Author response: As a criteria, a "high-mountain lake" was located above the tree line, a feature shared by the three studied lakes. As a consequence, Enol lake also above tree line, was considered a high-mountain lake despite its low altitude compared to the other two lakes.

Reviewer: p.5, l.5. Write CDOM instead of DOM because only the colored or chromophoric DOM will have that effect

Author response: We agree with the Reviewer.

Action taken: page 5, line 5. Changed as suggested.

CDOM (Evans et al., 2006). In turn, higher **C**DOM concentration contribute to higher temperature

Reviewer: p. 6, l.2, the sentence including "nutrient-addition instead of enrichment" is awkward.

Author response: This was a typesetting error which is now amended.
Action taken: page 6, lines 1-3.

The sentence:
"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."

has been replaced by:

"The aim of this study was to test the direct and indirect interactive effects of UVR, nutrient-**addition** and mixing on the metabolism of bacterioplankton with different light histories. "

Reviewer: p.6, l.7, "Previous studies" is just one study.
Author response: Reviewer is certainly right in that there is only one study by Helbling and coworkers (2013) that deals with the interactive effect of UVR, mixing regime and nutrient inputs on primary producers in ecosystems with different transparency to UVR.
Action taken: Page 6, line 7.

The sentence:
"Previous studies (Helbling et al., 2013) found…"

has been replaced by:
"A previous study (Helbling et al., 2013) found…"

Reviewer: p. 6, l. 13, revise use of "nutrient input"
Author response: We here interpreted that Reviewer suggested changing "input" by "addition", as this may better reflect the factor manipulated in the experiments.

Action taken: Page 6, line 13; "nutrient input" has been replaced by "**nutrient addition**"

Reviewer: p.7, describe type of quartz flasks used
Author response: We provide a more detailed description of the quartz flasks by including their shape and manufacturer.
Action taken: Page 7, line 15.

The text "**spherical** quartz flasks **(Trallero and Schlee, Barcelona, Spain)**" was added in the following location (indicated in bold):

"(i) UVR+PAR (> 280 nm; treatment UVR): using uncovered **spherical** quartz flasks **(Trallero and Schlee, Barcelona, Spain)** and…"

Reviewer: p. 7, l 13, "filtered through a 45 µm-pore size mesh" not really a pore
Author response: We agree with the Reviewer.
Action taken: Page 7, line 13.

The text:
"filtered through a 45 µm-pore size mesh"

has been replaced by:
"filtered through **45 µm mesh"**

Reviewer: what is "UV-spectrophotometric screening" in the case of nitrate? Are units for PAR also in µW cm^{-2}?

Author response: This has to do with the standard method use to analyze nitrates (APHA 1995). Unfortunately, we omitted units for PAR. These are µmol photons m^{-2} s^{-1}.

Action taken: Page 29. Figure 1 caption.

The figure caption:
"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 µWcm^{-2}."

Has been replaced by:
"Solar irradiance and temperature as a function of depth in (a) Lake Enol (LE), (b) Las Yeguas (LY) and (c) La Caldera (LC). **UVR** data are expressed in µWcm^{-2}; **PAR data are expressed in µmol photons m^{-2} s^{-1}. Each dot represents the mean for 20 data points."**

Reviewer: p. 9, l14 "SYBER" is wrong

Author response: Yes!, this was a typo error.

Action taken: Page 9, line 14. We have replaced "SYBER" by "**SYBR**"

"and stained with **SYBR** Green I DNA stain (Sigma-Aldrich)"

Reviewer: p. 9 l24, Revise meaning of "incorporating" in "HBP was determined by incorporating ^{3}H-thymidine (S.A= 46.5Cimmol^{-1}, Amershan Pharmacia) into the bacterial DNA". By the way, it is Amersham!

Author response: Again, we are grateful to the reviewer for pointing at this typo error, now amended in the revised version of the MS.

Action taken: Page 9, line 24.

We have replaced the sentence:
"HBP was determined by incorporating ^{3}H-thymidine (S.A= 46.5Cimmol^{-1}, Amershan Pharmacia) into the bacterial DNA"

by the sentence:
"HBP was determined by **incorporation of** ^{3}H-thymidine (S.A= 46.5Cimmol^{-1}, Amersham Pharmacia) into the bacterial DNA"

Reviewer: p. 11 Revise "were filtered onto 0.7 pore size filters"

Author response:

Action taken: Page 11, line 8.

We have replaced the text:
…"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);"

by:

"the samples were filtered onto 0.7 **µm** pore size filters in **LE (Whatman GF/F filters, 25 mm diameter)** and 1 µm pore size filters in **LY and LC (Nucleopore filters; 25 mm diameter)**

REFEREE #2

This is an interesting work on the effect of UVR on bacterial production in mixing layers, carried out through experiments in three different mountain lakes in the Iberian Peninsula. The idea of carrying out incubations in moving devices is not new, but still has quite a lot of new information we can get from that kind of experiments. Although in a general view the work is well designed and organized, I have some major concerns.

Reviewer: The first one is that each lake showed different physical features (temperature gradient, mixing layer depth, transparency to PAR and to UVR, etc) but all the experiments were set up equally, that is the mixing device moved from 0 to 3 m depth independent of the lake and its characteristics.

Author response: Our aim was to keep similar experimental conditions in the three studied lakes to compare how vertical mixing regime, and hence fluctuating radiation, would affect bacterial metabolism. The depths of vertical mixing were determined both based on temperature profiles obtained in this study and on previous studies and inter-annual observations in LY and LC. There are evidences for microstratification and vertical mixing processes in Sierra Nevada lakes associated to strong wind episodes (Rueda et al. 2007), which support the limit for the mixing depth of ~3 m used in our experiments.

Reviewer: Another one is that all three lakes are oligotrophic, so the generalization to eutrophic one is not straightforward. For example, Helbling et al (cited in the text) showed that UV protected cell (in opaque waters) are more sensitive to UV exposures. This means that in a eutrophic lake, the effect of mixing can have more negative effect than for an oligotrophic system. This could happen because in an eutrophic lake the water column will protect most of the time from UVR, and when the cells are dragged to the upper layers, this short time exposed to UV may be much more damaging.

Author response: This study did not aim at comparing between oligo- vs. eutrophic lakes. Also, the generalization to more eutrophic conditions was not pursued in this study since only oligotrophic lakes were included here. Besides, it is well known that transparency to UVR is not so much related to the trophic state of the system but rather to the concentration and optical characteristics of the dissolved organic carbon (e.g. oligotrophic humic lakes).

Reviewer: It is surprising that authors did not use 0.2 μm pore size (or at least 0.45) for estimating dissolved compounds (DOC, EOC, etc) but used 0.7 or 1 μm, since in 1 μm poro size many particles (including bacteria) may pass through the filter.

Author response: We measured DOC in order to chemically characterize the study ecosystems. The methodology used here is extensively used in the literature (pre-combusted GF/F filters) and particularly recommended by previous research in these ecosystems (see Reche et al. 2005; Mladenov et al. 2011)

With regards to EOC, the use of a 0.7 μm pore size filter to obtain this fraction was well justified by the fact that the smallest planktonic fraction in this ecosystem (i.e. picoplankton) ranged between 0.7 and 3 μm. Therefore filtration through 0.7 would retain plankton and allow EOC measurement of filtrates.

In contrast, the reasons for using a 1 μm pore size filter in lakes LY and LC include: 1) the absence of autotrophic picoplankton in these lakes, and 2) most of the heterotrophic bacteria passed through the 1.0 μm pore size filter. Therefore, filtrates < 1.0 μm corresponded to the photosyntehtic exudates by phytoplankton (EOC), including both the dissolved fraction and the share incorporated by bacterioplankton (< 1μm). This methodology has been widely use in other studies by our group and is extensively described in previous references (see Carrillo et al. 2002; Korbee et al. 2012).

Reviewer: Finally, and probably my major concern, respiration experiments were carried out as bulk respiration, and bacterial respiration was calculated as a constant fraction of it. Although this estimation is not wrong as a general approach, considering that the manuscript deals specifically with bacteria production is a very weak point. Authors should have carried out the respiration experiments in 2 μm filtered water following Del Giorgio (many papers of Del Giorgio deals with respiration experiments) so authors should reduce the emphasis and analyses on BR. There are several works were authors estimate Bacterial respiration rates, without applying this kind of approximations.

Author response: We agree with the Reviewer in that because we are specifically dealing with bacterial production, direct measurements of bacterial respiration would have been the best approach to calculate BCD. Unfortunately, size overlapping between autotrophic picoplankton and bacterioplankton prevented the physical separation between both groups (see reviewer 1´s comment and response). The reviewer is correct in that there are studies to calculate BR from bacterial production assessment by applying equations and without any direct measurement of respiration (at least total planktonic community respiration [TPR]). However, we considered more realistic the estimations based on true assessments of planktonic community respiration under the different treatments assayed in this study. By applying Del Giorgio´s approach we would have obtained a single value for each treatment, and yet, this would have been no more than just another estimation. In our approach, BR would plausibly lie within the upper limit of 75% and the lower limit of 50% of TPR, calculated from results in the literature and from our own results in parallel experiments (see response to Reviewer 1). Whereas we recognize the limitations of dealing with estimates, we provide with a realistic range in which real BR values may lay into. In addition, the estimation of BR is consistent with the

pattern of BCD:EOC ratio above or below the 100% threshold, which did not change regardless the different percentages used to calculate BR from TPR. We therefore, believe that the approach use to estimate BR is appropriate as it provides a realistic range for bacterial metabolism in these ecosystems.

Reviewer: Symbols in Figure 1 should be more clearly presented; it is very difficult to recognize each component of the figure with such small and clumped symbols.

Author response: Changed as suggested.