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Biogeosciences Discussions

Interactive comment on "Short scale variations in nutrients, ectoenzymatic activities and bottom-up effects on bacterial production and community structure during late summer-autumn transition in the open NW Mediterranean Sea" *by* F. Van Wambeke et al.

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Response to E. Vazquez-Dominguez (Referee)

After reading the manuscript, however, I find some difficulties into have a clear idea the short term scale effect on the structure and function of the heterotrophic bacteria in the microcosms, and how the findings are comparable to the short term changes that can be found in the samples withdrawn during the cruise.

We are aware that short term changes observed during enrichment experiments could give a different information - a difference in intensity of response, but not a difference in the time lag of response (only few days) - on what should happens *in situ* (e.g. see an *in situ* fertilization like CYCLOPS (Thingstad et al., 2005) or a natural Saharan dust pulse in Pulido-Villena et al. (2008). However, it allows considering different responses along the vertical water column (5m versus 80 m depths). This has been discussed below and in the revised version of the ms. As suggested by referee 1 and 2, we have changed the title.

Since my point of view, there are some small problems in the results to see clearly the effect of the nutrient amendment on the function and structure of heterotrophic bacterioplankton. Besides the problem of the lack of replicates, there is the lack of the NxP interaction. If this treatment was not performed, you could not really say that the effect of the NxPxG interaction was related to G and not to the interaction of the other two inorganic nutrients (NxP).

We agree on this point. Few studies examined all the 8 combinations (C, P, N, G, NP, NG, PG, NPG) in Mediterranean enrichment experiments. When it was done, different situations can be obtained. In a study in western Med in September (Sala et al., 2002) bacteria were stimulated by P alone on the same degree than GP or NP treatment, whereas the NPG addition resulted in a much higher bacterial growth. In June, however, possible co-limitation PG was evoked, as P alone was not responding. In a study in Aegean Sea in March (Christaki et al., 1999), bacterial production responded on the same degree in P alone than in NP (x 3 compared to the control), whereas it increased much more in PG and NPG (x 7). Further complication is the interpretation of the G source. Is it a source of C or of energy? This can have consequence on the potential study of limitation by N if only nitrate is used as a nitrogen source, and not ammonium.

The sentences discussing on that point were modified (page 19 lines 29-32) “Although the absence of NxP combination precludes to respond firmly to what

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were the second limiting factors in surface at the beginning of the cruise (N, G or both N and G), these results showed that the apparent severity of limitation of P is thus related to the availability of the other limiting factors after P (Pinhassi et al., 2006)."

In addition, the ectoenzymatic function is related as positive, negative, and zero effect during the cruise and in the microcosms. However, the community structure is represented as a three with a cluster approach. I think it could be much easier to see both results as a three to compare them visually (see for example Alonso-Saez et al. figure 6).

In the discussion you have to consider that Alonso-Saez et al. (2008) did not found an agreement between carbon use and community structure in NW Mediterranean coastal waters and you find such a relation. I think that you have to discuss such contrasting findings.

Alonso-Saez et al. (2008) followed the surface layers of a NW Mediterranean Bay during one year and did not observe a similar grouping of seasonal samples based on a non-metric multidimensional scaling (MDS) analysis between community structure (based on DGGE) and carbon use (based on measurements of bacterial production, bacterial respiration and bacterial growth efficiency). During our study, bacterial diversity (studied with SSCP), came from 4 enrichment experiments (21 Sept, 4 October, 5m and 80 m depths) and 2 profiles whereas the bacterial abundance, ectoenzymatic activity, and bacterial production came from up to 55 vertical profiles analyzed during the month survey. Thus, only bacterial production (which was the single parameter tracking carbon cycling in enrichment experiments) could be paralleled to SSCP profiles of enrichment experiments.

MDS analysis is an ordination method very close to cluster analysis representation. Both methods are based on the same similarity matrix among samples, but present the results in different ways. They generally give the same tendencies, so that our results may be comparable to Alonso-Saez et al. (2008). The idea of representing our data the same way Alonso-Saez et al. (2008) is then possible, but it may confuse the

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reader and poorly improve the information already given by other figures. First, our data are not only based on DNA but also on RNA, that gives a better view of the total and active bacterial community structure changes during enrichment. On an MDS plot, such results would visually give two data on the same sample (DNA and RNA) that the reader will have to make coincide with only one data for the corresponding activities. Moreover, our results may not appear as clear as for Alonso-Saez et al. (2008) on an MDS plot, because their study was based on 11 samples whereas ours is a compilation of 64 samples. Second, our manuscript is already composed of 3 tables and 7 figures. The proposition of the reviewer would be redundant with the information given on figure 6 and 7 that clearly showed the influence of NPG or P amendments on bacterial activity (Figure 6), and its clear relation with changes in bacterial community structure (Figure 7). Such additional representation would not bring any new information than already visible on figures 6 and 7, and it could not substitute to the raw data presented in the figures 6 and 7.
Finally, a comparison of environmental parameters (nutrients, temperature, DOC, lipid characteristics) and community structure (SSCP analysis, but only on DNA levels), based also on a MDS analysis, but exploring only in situ data (and not enrichment experiments) was made in a companion paper (Ghiglione et al., 2008). These authors demonstrated that clusters of bacterial communities were separated based on vertical structure, but not the temporal variability, and were related to distribution of environmental parameters (nutrients, temperature, DOC, lipid biomarkers in the dissolved fraction, class of pigments). Two clusters (0-40 m and 60-150 m) were clearly separated. Our enrichment experiments confirmed these findings.

In addition, the authors have to consider that the nutrient deficit for certain groups could not really be a deficit for others. For example, Zohary et al. (2005) have found for the eastern Mediterranean that while P was the limiting nutrient for heterotrophic bacterioplankton, N and P were simultaneously limiting for phytoplankton.
Yes it is possible; yes it was not discussed in our paper because we did not follow

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response of phytoplankton in our enrichment experiments. Comparative parameters available between these 2 studies are nutrient concentrations, chlorophyll stocks and picophytoplankton abundances. During CYCLOPS experiment, nitrate and SRP concentration in the nutrient-depleted surface layer were below the detection limits (2 nM) of the nanomolar techniques for SRP and nitrate down to 60 m depth whereas NH4 was in the range 0.06-0.08 μM N (Zohary et al., 2005). At the opposite, it was 0.030 μM SRP for 0.030 (C1) to 0.092 (C4) μM nitrate in our study (with NH4 around 0.01 μM). Thus nutrient deprivation was more important during CYCLOPS and N/P of these nutrients more elevated during CYCLOPS. However, unfortunately the study of concentration alone cannot be conclusive about the status of limitation, particularly owing to the fact that phytoplankton populations were not the same. In our study higher abundances of Synechococcus, euglenophytes and dinoflagellates in the 0-20 m depth samples were noticed (with up to 2 x 10^5 Synechococcus cells ml^{-1}) whereas higher levels of pelagophytes and diatoms were characteristics of the deeper 60-150 m layer (Ghiglione et al., 2008; Denis, per comm.). During CYCLOPS experiments, information was available only for the 0-20 m layer with much lower abundances of Synechococus (4000 ml^{-1}). Chla stocks were also much lower in the surface layers deprived of nutrients during CYCLOPS experiment (0.018 μg Chla ml^{-1}, Psarra et al., 2005, versus 0.1 to 0.2 in our study, Marty et al., 2008). All these differences between these 2 situations suggests that the status of limitation of phytoplankton encountered during our study could be different that the one observed during CYCLOPS, and unfortunately we cannot conclude what should have been factors limiting phytoplankton.

We must notice also that, dealing on heterotrophic bacterial production data, the absence of any response after single enrichments suggested also a multiple limitation of bacterial production during the first half of our cruise.

Some information about this has been added in the ms page 19 lines 17-23: " Indeed, regeneration process and DOM fuelling by high-sized organisms was still present, because samples were unfiltered. Thus, stimulation of leucine incorporation rate, if

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any, is a consequence of both direct and indirect effects. We did not follow response of phytoplankton in our enrichment experiments and thus it is possible, like described in Zohary et al. (2008) that phytoplankton and heterotrophic bacteria could not be limited by the same elements (or combination of elements) ”

Finally, the authors state that the bottle effect may lead to higher changes in the RNA and DNA fingerprinting than the effect of depth. Couldn't be more interesting to add directly nutrients into the sea? (i.e. Krom et al. 2005).

As far as I know, there were no study of the diversity structure of heterotrophic prokaryotic bacteria in the CYCLOPS experiment and a large scale P enrichment studies in Mediterranean like CYCLOPS was made only once. Previous experience exists, however, on iron enrichment experiments, mainly in southern hemisphere. In some of these studies, bacterial diversity was studied within and out of the fertilized patch (with terminal restriction fragment length polymorphism (T-RFLP) and some differences were seen in ectoenzymatic activity but not in bacterioplankton community (Arietta et al., 2004). Indeed, the more activity of a group increase, the more a change of diversity of that group is expected. Changes in activity and diversity of heterotrophic bacteria are more expected in systems where activity and diversity of phytoplankton also varied a lot (Pinhassi et al., 2004), although this is not systematic (Hutchins et al., 2001). Thus, more changes are expected in a system were the amended nutrient stays longer in the euphotic zone, thus in conditions with a narrow mixed layer (Boyd et al., 2007), or in conditions of permanent fertilization (West et al., 2008). Small bottle volumes are not ideal restitution of nature, but it allows multiplication of measurements, as well as multiplication of depth layers investigated, and thus help to confirm reproducibility of results in different time or space scales (different depths, short time scale for instance).

Some information about this has been added page 21 lines 22-34, page 21 lines 1-2.

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The main points reached are: a) surface NW Mediterranean waters are limited mainly by P, which is in general agreement with other studies of the area, and b) deep bacterioplankton could be limited by the availability of fresh organic carbon. Then, the structure and function of heterotrophic bacterioplankton could be influenced by inorganic nutrients in surface waters and organic carbon in deep waters. It is necessary a more conclusive discussion about the short term variability and the relation between what happens in the addition experiments and in the field.
This is the message in our conclusion where we pointed out the vertical distribution of bacterial communities.

The title is good but the discussion should be improved to match better the title.
As suggested by referee 1, we have changed the title. In accordance to reviewer 2, we enlighten the monthly variations instead of daily variations to make this time scale clearer for the reader.

The abstract provides a concise and complete summary, except in the part that the authors are talking about the second half of the cruise. You didn't mention in the same abstract a first half of the cruise.
The sentence was modified.

Response to referee 2

1. GENERAL COMMENTS

The title does not fully reflect the conclusions since little is discussed on short-term variations in the paper and that part of the title could be removed. Additionally, the title is too specific and long. My suggestion would be: "Bottom up effects on

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bacterioplankton growth and composition during summer-autumn transition in the open NW Mediterranean Sea"
We changed the title as suggested by the referee

2. SPECIFIC COMMENTS
Quality of figures is often poor in my copy. I suspect this is due to file format and for example, in fig 5 and 6, X-axes are incomplete.
The scale was added on figure 5b. It is the same as on figure 5a.
We did not added scales between the two lines of figure 6 to simplify presentation. The legends on the top of the figures of the second line are the same as for the top of the first line, and the legend on the bottom of the figures of the first line is the same as for the bottom of the second line. This was added in the legend.
For the "leucine stimulation factor" (last figure - this legend is know at the bottom of the second line, which is more logical-), there are no units.

Also, same symbols in Fig 4 for total and attached bacteria.
The symbols were not the same in a colour version (blue and black). We modified the colour code so that a difference could be seen even in a B&W version.

Fig 2 shows mean vertical distributions of the main parameters along the water column. The main players in the story are bacteria so profiles of bacterial abundance should be shown here. Data are available since the authors used it for correlations and regression. Bacterial abundance profiles will strongly help the reader to draw a general picture of the studied ecosystem.
We added vertical distribution of total abundances on figure 2 and 6.

The incubation experiments were carried out under close to in situ light and water was

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not prefiltered, phytoplankton was also present in the bottles. It would be interesting to know about the evolution of phytoplankton during this 24h. If phytoplankton grows, competition with bacteria for inorganic nutrients can be expected; if phytoplankton died due to confinement, this could provide an uncharacterized source of both inorganic and organic nutrients for bacterioplankton. If the authors followed phytoplankton abundance or chlorophyll concentration, it would be desirable to include a paragraph with their observations and possible influence of those in their results.

Phytoplankton parameters were not followed during enrichment experiments. However the conditions of confinement (transparent polycarbonate bottles incubated in *in situ* simulated conditions (running sea water baths), and the duration of incubation (24h) are the same as those used routinely for measuring primary production. We then suppose that most phytoplankton did not die. However, confinement effect implies new source of nutrient and DOM, at least partly due to some breakage of fragile cells and/or rupture of long food chain (high-sized organisms not statistically represented in small bottles) as well as an increase of the surface/volume ratio. This confinement effect induces a small increase of BP which is known by microbial ecologists since a long time (Zobell and Anderson, 1936). However, all our amendment effects are corrected from the response in the control. Finally, as it is probable that phytoplankton also responded to N or P, or both enrichments (because these nutrients were limiting in surface) then yes, response of heterotrophic bacteria are due to the sum of direct and indirect effects. We felt more interesting to keep intact trophic levels to explore bacterial responses to enrichments because it would be very difficult to investigate only bacterial fraction. Indeed prefiltration had many disadvantages. This was discussed in a paper focusing on limiting nutrients in the South Pacific (Van Wambeke et al., 2008). We added information on this page 19 lines 17-23.

P690 L2. Recent papers have observed consumption of dissolved organic matter by other organisms, such as phytoplankton (Vila-Costa et al. 2006, Science 314: 652-654), therefore I suggest replacing only by main.

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OK, this is done.

The symbol for mole is mol, so the term mole should be replaced along the ms in text and figs.
OK, this is done.

Only attached bacteria were retained in 0.8 um filters? What percentage of free living bacteria could be found on the filter?
Microscopic observations showed that free-living bacteria retained on the 0.8μm filters represented less of 5% of the >0.8μm fraction (Mével et al., 2008).

There is a broad spectrum of substrates to investigate lipase activity in the literature. Why did the authors choose MUF-palmitate?
Lipase is defined as a glycerol ester hydrolase (E.C. 3.1.1.) and consequently there is a variety of substrates that can be used to track its activity. MUF-derived molecules generally used in marine and freshwater microbial ecology are MUF-palmitate (saturated C16), MUF - stearate and MUF-oleate (mono-unsaturated C18) having long linear fatty acids chain; as well as substrate with smaller fatty acid chain (MUF-butyrate C4, MUF-heptanoate C7). Generally on extracted single lipase enzyme, enzymatic activity tested on a substrate with a short-chain length (C4-C7) is greater than with long-chain length substrates (up to C18) (Ruiz et al., 2002), but these smaller molecules are also susceptible to attack by broader esterases. More generally, different lipases exhibit specific preferences for their triacylglycerol substrates, depending on chain length, optical and positional isomers, and the characteristics of the lipid/water interface. Thus, depending on the choice of the fatty acid chain probably different information in terms of Vmax and Km is expected due to the different specificity of ectoenzymatic “lipase”. But intercomparative measurements are scarce in environmental microbiology. Palmitic acid, stearic acid and oleic acid belong to the

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major constituents of chains of tri- di- and mono-glycerides as well as fatty acids in vegetals and animals (Volkman et al., 1989) and this is why we choose a MUF-analog with a long-chain length In addition, on must be aware that the natural triacylglycerol behaves differently than the equivalent MUF-substrate (Bourguet el al., 2003). This is why in our study only the relative information was used.
Few words about this were added page 16 lines 12-16.

I suggest the authors to make the exercise of compiling a unique dendogram to check if all amendments cluster together independently of the date of the experiment. Enrichment generally decrease the number of bacteria OTUS, probably due to the response of a taxa to the enrichment. It would be interesting to identify which bacteria respond to the enrichments but I wonder if this is possible with SSCP.
. Compilation of all data on a same dendrogram do not show clear clusters organised by amendments independently of the date of the experiment and such representation would have made the figure more complex for the reader with poor information added. The reviewer underlines that enrichment, and especially NPG enrichment, decrease the number of bacteria OTUs. Indeed, NPG treatment "present the lowest number of CE-SSCP peaks" for 21 September enrichment experiment (page 14, line 8) and "a slight decrease in the number of CE-SSCP peaks compared to the unamended control" for 7 October bioassays (page 14, line 19). However, by looking more closely to the CE-SSCP profiles, we did not found specific peaks associated to NPG amendment independently of the date of the experiment, suggesting that different species were selected from the initial communities at the different dates. Such difference may be due to the difference observed in the initial communities, an hypothesis that was confirmed in this paper by clear differences in NPG amended bacterial community structures originated from different communities sampled at 5 m and 80 m (see fig. 7).
As wondered by the reviewer, it is technically not possible to identify which bacteria respond to the enrichments by the CE-SSCP technique. As explained in the in the Discussion (page 20 lines 13-16) "Although molecular fingerprinting is not as com-

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plete technique for describing microbial diversity as cloning and sequencing approach, it offers the best compromise between the need to process a significant number of samples and the information generated (Muyzer et al., 1998)."

3. TECHNICAL CORRECTIONS

Thanks to the reviewer for providing these technical corrections.

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