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***Interactive comment on* “Factors limiting heterotrophic bacterial production in the southern Pacific Ocean” by F. Van Wambeke et al.**

F. Van Wambeke et al.

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Response to referees on “Factors limiting heterotrophic bacterial production in the southern Pacific Ocean ”

We are thankful to the two referees for their objective and clear review as well as the detailed technical corrections. We have addressed below most of their comments and suggestions:

- Statistical tests were corrected. We used non parametric tests. This gave slightly different results than in the bgd version, but not inferring our main conclusions.
- We discussed the fact that we manipulated the whole sea water instead of pre-filtered sea water (referee 2) and corrected the first part of the discussion accordingly.

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Response to referee 1

1 - 2 *“the ms lack a description of statistical methods”*

We added a section in MM. Statistical tests have been added not only when comparing control to any amended sample, but also when comparing the level of stimulation of different enriched samples. With few data points (triplicates, sometimes duplicates only), the formal test has no power to discriminate between Gaussian and non-Gaussian distributions. We thus used non parametric tests: the Mann-Whitney test in TMC experiments (because this test lacks statistical power with small samples, we used it only when triplicate data were available in order to maintain comparison purpose). We used the Wilcoxon signed-rank test in non TMC experiments.

3 *“how the author can conclude that a labile carbon source limit BP after the relieving the N limitation in the GYR site from their TMC experiment. ”*

and related remarks: p 3811, line 16-17 *“I cannot understand how you can conclude that stimulation of bacterial production by nitrate + ammonium is direct”* and p 3811, lines 22-23. *“a labile carbon source limits BP after relieving the N limitation”*

When using the non parametric test in the TMC experiments, it appears that the leucine incorporation rate at the GYR site is statistically higher in N and G enriched sample than in the control (x 4.6 and x 3 enrichment factor at 24h, x 8.9 and x 3.5 at 48h, respectively, $p < 0.05$, Table 4). The growth after N addition is not higher than that after G addition at 24h, but it is at 48h ($p < 0.05$). Thus, based on a 48h response, it seems that N addition provides better conditions for heterotrophic bacteria to grow than addition of glucose alone. This trend is confirmed from the non TMC experiments made along different stations inside the gyre (STB6-STB15). In these experiments, the increase of leucine incorporation rates after glucose addition is lower than the one obtained after N addition (Wilcoxon signed-rank test, $p = 0.038$, $n = 9$ pairs of comparison, box plots figure 4b). These results suggest that the limitation of bacterial

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production by N is direct within the gyre.

Other observations also suggest that labile carbon is rapidly a second limiting factor and/or both labile carbon and N are co-limiting factors.

First, the increase of leucine incorporation rates between N, N+Fe and all enriched samples suggested that heterotrophic bacteria could benefit from the increase of DOC due to the stimulation of autotrophic activities. This hypothesis, based only on TMC results, is risky because, and like the referee said, there was not clear regular increase of PP or chlorophyll from N to FeN to all amended samples. In addition, due to the large variability between triplicates, the increase of leucine activity observed from N to FeN to all was not confirmed by Mann-Whitney test, particularly at 48h (Figure 2). We assumed that the absence of visible increase of PP or Chl was due to the top-down control, because HNAN increased at 24h in all (x2) compared to N and FeN (x1.1), and increased at 48h from N (x1.4) to FeN (x1.6) to all (x1.8) at 48h. An increase in grazer biomass suggests an overall carbon flux through the food chain, which is probably source of increased DOC source. However, these factors of enrichment are also not supported by statistical results (no triplicates available for HNAN). Second, in the surface waters of stations STB6 to STB15 (within the gyre), addition of glucose alone did not stimulate leucine incorporation rates (Wilcoxon signed-rank test between C and G enriched samples, $n=9$ pairs of comparison, $p=0.07$), but addition of N alone did (comparison C-N, $p<0.01$). However, leucine incorporation rates increased statistically much more in NPG amended samples than in N amended samples (Fig. 4, median of increasing factor switched from x 4.2 to x 37, Wilcoxon signed-rank test between N and NPG enriched samples, $n=9$ pairs of comparison, $p<0.01$). Considering further that P never stimulated bacteria, we infer that labile carbon was the second limiting factor after N (or co-limited with N) within the Gyre. The discussion was modified accordingly.

Specific comments

1) *p 3800 line 7* Yes, part of the sentence was missing. We corrected the sentence as follows: “From 141°W (Marquesas plateau) to approx. 125°W, bacteria were not bottom-up controlled”

2) *p 3802 line 1* Yes, we meant both heterotrophic and photoautotrophic. The sentence is corrected?

3) *p 3802 line 12 15. “how to achieve the TMC conditions or give relevant references”*
The following sentence was added in the text:

“All experimental setups were performed using strict trace metal clean techniques (Bruland et al., 1979) inside a clean container. Briefly, seawater was collected at 30-meter depth using a Teflon pump system and dispensed into acid-washed (Suprapur Merck HCl) transparent polycarbonate bottles. Under a laminar flow hood, nutrients were added alone and in combination to final concentrations of... ”

4) *p 3803 line 3-4 “compare temperature on deck with in situ temperature”*

For TMC experiments, water was pumped at 30 m. Surface seawater used to refrigerate on deck incubators was pumped by the ship at about 5 m depth. This resulted in no difference in temperature conditions at 3 sites (27.7° C at MAR and HN, 22.1° C at GYR) and only 1 ° C difference at EGY (18.05° C at 5 m, 17.05° C at 30 m). We thus consider that potential effects of temperature are negligible.

On the opposite, for the non TMC experiments, there could have been potential effects of temperature when vertical profiles of bioassays were made, as already emphasised in the bgd version (p 3803 lines 25-28).

5) *p 3804 line 1-3 “bacteria are unable to take leucine - change in population misinterpreted as a change in activity level - have other substrates been tested?”*

Leucine technique is largely used instead of thymidine since the pioneer studies

on the field by Kirchman et al. (1987). The labelling of proteins by leucine is indeed more specific than the labelling of DNA by thymidine (Torréton and Bouvy, 1991), and some species lack the permease for thymidine (see for instance Davis, 1989). The increasing information coming from micro-autoradiography coupled to hybridization in situ techniques, however, suggests that in oligotrophic environment all bacteria in the field do not assimilate leucine. For instance within surface water at the GYR site, only 25% of the “DAPI-labelled bacteria” showed distinct silver grains after MICRO-FISH (Obernosterer et al., 2007). However, the absence of a silver grain do not necessarily means absence of the permease, but simply an inactive state of the cell.

When adding the limiting factor, bacteria recovered stoichiometric equilibrium and/or energy capacity which gave them the capacity to assimilate leucine again. Thus an increase of activity is not immediately a change in population. But after some time, we can observe some changes in the diversity structure (this has been described in some enrichment experiments: Eilers et al., 2000; Fuchs et al., 2000; Lebaron et al., 2001; Massana et al., 2001). Thus we agree with the referee that a change in activity levels (like those observed in the enrichment experiments) is almost always associated with a change in the diversity of heterotrophic bacteria. (We discussed about that in the bgd ms p 3809 line 19, as after 48h we noticed an increase in percentage of gamma-proteobacteria). We did not test other substrates than leucine.

6) *p3805 line 17* We added “Fig 2” at the end of the sentence as suggested.

7) *p 3806 line 21-22*. “*the sentence is not in absolute agreement with Table 4*”

Yes, we agree with the referee. We described general trends, not absolute rules. The sentence was modified accordingly in the revised ms.

8) *p 3806, line 16* For us, “after 24 h” meant “at 48 h”. The sentence was nevertheless corrected because it was confusing.

9) *p 3808, line 18*. We added statistical results in the sentence.

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10) p 3809, line 10. Yes, we corrected “x 2” to “x 1.2”

11) p 3810, line 5. Yes, we corrected “Table 4” to “Table 5”

Response to referee 2

“Consequence in interpretation of filtered versus whole water experiments”

Different approaches have been equally used in the literature: whole seawater incubated with simulated in situ light conditions (Kirchman, 1990; Hutchins et al., 1998; Zohary and Robarts, 1998; Van Wambeke et al., 2002; this study), or in the dark (Pomeroy et al., 1995), and prefiltered seawater (0.6-0.8 μm) diluted or not with 0.2 μm , and incubated in the dark (Carlson and Ducklow, 1996; Cherrier et al., 1996; Palkuski et al 1996; Torr ton et al., 2000; Donachie et al., 2001). Both approaches are not completely satisfying, offering advantages and disadvantages.

By pre-filtering the samples, all material belonging to the high size fraction is removed. The consequence is the suppression of the top down control (by removing predators) but also all regeneration processes (excretion, lysis, degradation of particles). Bacteria staying in the filtrate are not anymore regulated by grazing, the fast-regenerated resources are removed, and theoretically only bottom up effects control bacterial growth. This situation should be ideal to test the potentially limiting nutrient on bacterial growth.

However, particularly in oligotrophic environments, organic material devoted to bacterial growth is provided continuously through regenerating processes by higher size-class organisms, so that “bottom up” control is influenced by “preying” organisms. This is particularly evidenced at GYR site where the “f ratio” estimated from 15N measurements is close to zero, but becomes slightly positive only when considering atmospheric di-nitrogen fixation (Raimbault et al., 2007). In this case, the response of all members of the community is needed to fully appreciate the complexity of changes in trophic regulations and nutrient transfers resulting from addition of nutrients. Another

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problem is the representativeness of the activity associated to a pre-filtered bacterial fraction. Indeed, it has been shown that within the gyre, on average 36% of the leucine activity was associated to the $>0.6 \mu\text{m}$ fraction.

Finally, all the manipulations necessary to maintain TMC conditions are huge, particularly when a pre-filtration of sea water is needed. We choose to maintain whole seawater samples to keep the possibility to study simultaneously effect of amendments on both autotrophic (Bonnet et al., 2007) and heterotrophic compartment (this study) and to explore potentially direct versus indirect effects. We agree with the referee that this has the disadvantage to mask partly bottom-up effects, so the effect of any amendment is seen only by stronger responses than those which should be obtained using pre-filtered seawater.

Presentation of the data

Statistical treatment has been changed (non parametric tests), which modified slightly the results (Table 4).

Now small factors of increase (around 2-9) becomes significant (at MAR, Chl in Fe and N at 24h; at HNL leu and specific leu in Fe, N and FeN at 24h, leu and specific leu in Fe, N, FeN and G at 48h; at GYR, leu in N, G and GF at 24h, specific leu in GF at 24h; at EGY leu and specific leu in GF at 24h, leu and specific leu in N, FeN and all at 48h). These changes do not have high consequences on our main interpretations. Bacterial production is not limited by any nutrient at MAR (considering the particularly high growth in the control). Bacterial production is limited by labile carbon in HNL (growth after G addition is higher compared to that after Fe, N and FeN additions, $p < 0.05$ in all 3 pairs of comparison). Within the Gyre, bacterial production is primarily controlled by N, and immediately after by labile carbon. Within EGY bacterial production is limited by labile carbon.

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“I would like to see the table includes standard deviations”.

Means and standard deviations of leucine data are drawn in Figure 2. Means and standard deviations of specific primary production are drawn in Figure 2 of Bonnet et al (2007). We did not add those of abundance, because they changed poorly, nor those of specific leucine, because they mainly reflected those of leucine. We feel thus unnecessary to present figures on PP and Chl data which would load much this ms, focusing on heterotrophic bacteria.

“results of STB 18 extrapolated to other experiments”

We preferred to compensate the absence of systematic triplicate measurements by a high number of stations investigated at each area. We compared leucine incorporation rates after different types of enrichment using a Wilcoxon signed-rank test (each pair of data corresponded to one enrichment experiment not replicated at a different station). We clustered stations like those stated on Figure 4. Results are as follows:

For MAR - STB5 (6 stations): only the comparison time zero - Control unamended after 24 h of incubation is significant ($p < 0.05$).

For STB6 - STB 15 (9 stations): the 3 comparisons time zero - Control unamended after 24 h of incubation, Control unamended after 24 h of incubation - N addition and Control unamended after 24 h of incubation - NPG addition are significant ($p < 0.01$ for the 3 comparisons).

For EGY - UPW (8 stations): the 3 comparisons time zero - Control unamended after 24 h of incubation, Control unamended after 24 h of incubation - G addition and Control unamended after 24 h of incubation - NPG addition are significant ($p < 0.05$ for the 3 comparisons).

These results confirmed our main conclusions, i.e.

- no effect except confinement within MAR site (stations MAR to STB5)

- nitrogen limitation of BP in the gyre (stations STB6 to STB15)
- labile carbon limitation of BP within the EGY- upwelling area (stations EGY to UPW)

Specific comments

1) *"NH₄ was not included in N enrichments MAR HNL Table 2"*

We apologize for this error. In fact for N enrichments nitrate + ammonium were always both added. Table 2 was corrected accordingly.

2) *"add more details in the methods"*

Few lines were added for each methodology.

3) *p 3804 results*, Yes, we corrected "table 1" to "table 3"

4 *p 3805 line 20 "the stock did not always increase less than the flux"*

The sentence was corrected.

5) 6) 7) typos errors corrected.

References not cited in the ms

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