Authors' Reply to Anonymous Reviewer #2

General Comments:

The paper by Obernosterer et al. reports new data on Fe and C limitation of heterotrophic bacteria in the Southern Ocean. It addresses an important scientific question and provides the first strong support for the co-limitation hypothesis of Tortell et al.(1996; 1999). Other tests of this hypothesis in different regions of the sea by Church et al 2000 and Kirchman et al 2000 showed bacteria were C-limited and did not re-spond to Fe addition alone. A few additional studies have also tested the co-limitation hypothesis and found support for it (or not) and these need to be acknowledged. The submitted manuscript does a poor job of crediting the research and ideas of other scientists who have contributed to this field of study (see Technical Comments).

Reviewer Query 1)

One of the most serious shortcoming of the paper is the lack of information about the Fe uptake measurements, which make it impossible to understand what was actually done and how to interpret the results. I would rate the scientific significance, good; the scientific quality, poor; and the presentation quality, fair.

Authors' Response :

We thank the Reviewer for the time invested and the numerous comments that helped improve our manuscript. We appreciate the Reviewers' overall positive feedback on the importance of the scientific objective addressed in the present study. The Reviewer raises 2 major critical comments.

1) **Lack of information on the Fe uptake rates.** We would like to clarify a misunderstanding. The Fe uptake data presented in Fig. 2 of the initially submitted manuscript were determined by Fourquez et al. (2014), and they are not part of the core results of the present manuscript. The companion paper by Fourquez et al. (now accepted for publication in the Special Issue KEOPS2 in BG) is entirely dedicated to the Fe uptake by the microbial community in the study region. It describes in detail the experimental setup and methods applied, and it discusses the results in the context of previous studies, in particular the papers highlighted by the Reviewer. Even though we have mentioned this in our manuscript, the full reference of Fourquez et al. (2014) could not be provided, because the manuscript was still in the editorial processing. We recognize that the use of these data without a complete reference was to some extent misleading. Upon the Reviewers' comment, we have re-considered the use of the Fe uptake data for the discussion of our findings in the present manuscript, as it might not be straightforward to understand these data without having read the paper by Fourquez et al. (2014). We have therefore modified Fig. 2 in the revised version of the manuscript (see also more specific comment below).
2) **Discussion of relevant literature.** We produced Table 2 to review previous results on similar types of incubation experiments, and thereby set the context for our own study. We consider this a suitable way, because the information is easily accessible for any reader. We are, however, aware, that such an exercise of a « Review Table » contains the risk of missing the one or other published study. We thank the Reviewer for pointing out the publication by Agawin et al (2006), which, together with a publication in press (Jain et al. 2015) has now been included in Table 2 in the revised

version. As suggested by the Reviewer, we now mention more often the conclusions from these previous studies in the main text.

Specific Comments:

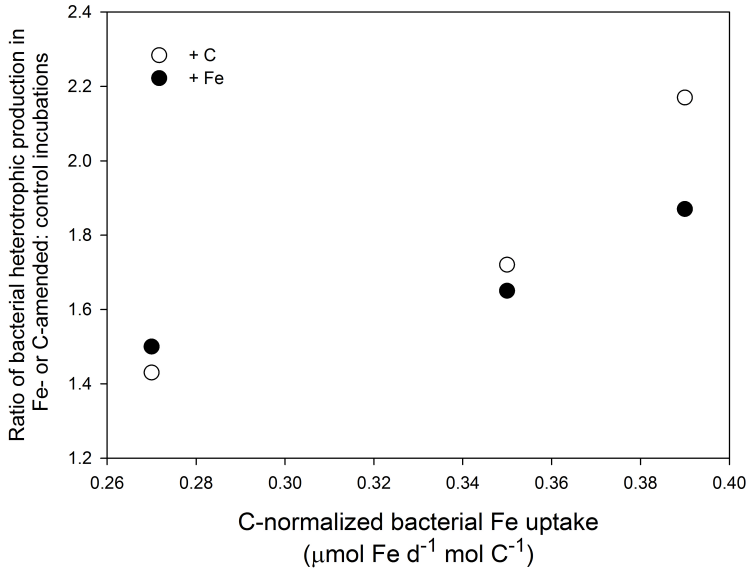
Reviewer Query 2)

There is no way to evaluate what the Fe uptake measurements mean. I think that the wrong paper has been cited here (page 15740, line 2: Fourquez?) (and elsewhere), but even if it is correct, readers need to know some details about the method and how it was applied. Figure 2 reports water-column integrated Fe uptake rate – but over what depth (ML?) and how many sample depths? Why would the maximum extent of stim-ulation (MEOS) of bacterial production (BP) by Fe and C be related to water-column integrated Fe uptake? I would have thought that the MEOS should be compared to Fe uptake of samples taken from the same depth (a volumetric rate)?? Some justification is required. The Fe uptake rates are also referred to as in situ rates – but what does this mean? Was the 55Fe complexed to some ligand or added in the inorganic form? I suspect that Fe uptake was measured by adding 55Fe at a total Fe concentration equal to or higher than the in situ concentration, but this is not reported. The rates are unlikely to be true in situ rates and are probably closer to saturated rates, but not enough information is provided for readers to judge. Knowing which of these rates was actually measured will completely alter how the MEOS results are interpreted.

Authors' Response :

We acknowledge that the Fe uptake rates are not straightforward to understand without having read the companion paper by Fourquez et al. (2014). We consider a full description of the 55Fe uptake measurements redundant with the paper by Fourquez et al. (2014), and we have therefore eliminated these data and the observed trend from the manuscript. A revised version of the Figure 2 now shows the extent of stimulation vs DFe concentration (see answer to Reviewer Query 4).

The seawater for the Fe uptake measurements and our incubation experiments were taken from the same cast in the surface mixed layer. Fe-uptake rates were then determined at different irradiance levels. In response to the Reviewers' comment, we present below the extent of stimulation vs the bacterial Fe uptake rates, normalized to cell biomass, on a volumetric basis (determined at 1% light level). We basically obtain the same trend as for the integrated values. The aim of this figure was to illustrate the potential relationship between these two independent measures of bacterial activity related to Fe and C, and thereby to point to the idea of the strong coupling between these cycles through microbial activity.



Reviewer Query 3)

As it stands now the bacteria Fe uptake rate is not normalized to bacteria density, which varies by a factor of 2 among sites. Since the water column integrated rate will depend on the uptake rate per cell and the bacterial abundance, then shouldn't this be factored in? In a co-limited community, Fe uptake rate per cell should somehow be related to the degree to which bacteria are limited by Fe and C which influences the MEOS.

Authors' Response :

In the above figure, the Fe uptake rates are normalized to cell biomass, based on cell abundances and a carbon conversion factor of 12.4 fg C cell $^{-1}$ (Fukuda et al. 1998).

Reviewer Query 4)

Reporting the MEOS seems completely arbitrary and potentially biased. We have no way of knowing whether the values are really the maxima, since samples were only taken at days 2 and 5 and if I understand correctly, some of the values plotted in Figure 2 are from samples that were taken at d2 and others at d5. What if the maximum stimulation occurred on d3 or d4 at Station E-4W for example? Then the true maximum would be missed (look at the data from E-3 which shows a peak at d2 and then decline by d5, so that the MEOS can vary quite substantially). We could be completely misled if the maximum was not measured at all stations. I think the only way to circumvent this problem is to construct this graph using BP measured either at d2 or d5 for all stations. Since water temperature is the same at all sites the kinetics of the bacteria metabolic response should be similar and so shouldn't confound the results.

Authors' Response :

The term « maximum extent of stimulation » was intended to provide a relative indication for the responses to a given treatment in the incubation experiments. We agree, in absolute terms,

this is not an appropriate term. In response to the Reviewers' concern, this term is not used any more in the revised version of the manuscript.

We propose a different way of looking at our data, which does not change the overall conclusion presented in the initially submitted manuscript. We now use the ratio of bacterial production in the Fe- or C-amended treatments to the controls for the time points when significant differences were detected for the first time in the cultures. This was the case after 2 days of incubation at Stations E-3 and E-5, and after 4d of incubation at Station E-4W. The rationale behind this is that it takes into consideration the differences in the time lag of the bacterial communities to respond to Fe- or C- additions at the different sites. We consider these different temporal dynamics of the microbial community part of the response to the question of whether and to what extent they are C- or Fe-limited. These variable responses are most likely driven by the initial environmental conditions. We have listed the parameters that appear of importance in this context, such as concentrations of Chla, DOC and DFe and in situ bacterial heterotrophic production. The combination of these and other factors are likely to set in part the temporal evolution in the incubation experiments. For this reason, and also because the time that separates the sampling is not exactly the same among experiments, we consider it not appropriate to choose only one time point for all incubation experiments for the calculation of the extent of stimulation.

The corresponding paragraph and Figure 2 have been moved to the Discussion Section, and we refer to the « extent of stimulation ». As an aside, for a given experiment, the extent of stimulation, whenever significant differences are observed, does not vary substantially between time points (<10%).

Reviewer Query 5)

Although the paper claims that the MEOS "was also positively related to in situ DFe concentrations", I can't believe that this is correct. The author's will need to report statistical analyses to back this up, although as I suggested in comment 3 the approach is currently flawed. Using the DFe concentration reported in Table 1 the values are: 0.13 nM Fe (1.4, 1.65-fold increase); 0.06 nM Fe (1.4, 1.5); 0.17 nM Fe (1.6, 1.6); 0.35 nM Fe (1.85, 2.05).

Authors' Response :

Fig. 2 of the revised version of the manuscript shows the extent of stimulation vs DFe concentration. The aim of this figure is to illustrate a tendency between these parameters, not to show a correlation. Due to the low number of observations, we purposefully did not calculate any correlation coefficient in the initially submitted and revised version of the manuscript. We agree with the Reviewer, the term « related » is not appropriate here. In the revised version, this term is replaced by "observation of a trend or tendency". The results for Station R2 are not shown on this graph, because single additions did not reveal a significant difference to the control (see answer below).

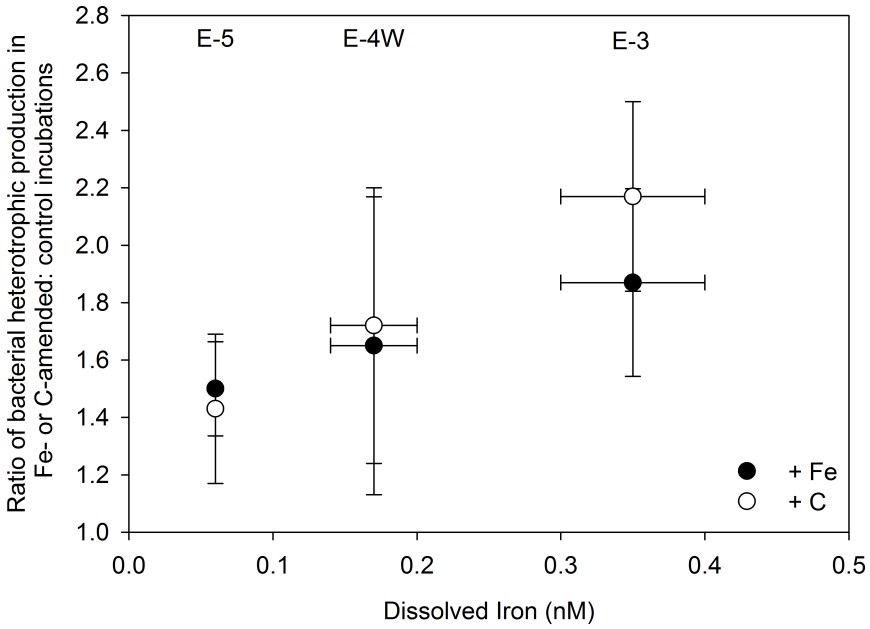


Fig. 2_revised. Extent of stimulation of bacterial heterotrophic production by Fe (+Fe) - or C (+ C)-addition and in situ dissolved iron (DFe) concentrations. Ratios of bacterial production in the Fe- or C-amended treatments to the controls correspond to the time points when significant differences were detected for the first time in the cultures (see Fig. 1), and the error bars denote the cumulated error of the bacterial production measurements in the control and the Fe- or C- amended treatments, respectively. For DFe, mean values ±SD of the surface mixed layer are given (see Table 1).

Reviewer Query 6)

I fail to see how the ratio of DFe:DOC "clearly identifies Fe as a potentially limiting resource for heterotrophic bacteria". Since we don't know the fraction of DOC or Fe that is utilizable, this ratio is not very informative. The authors acknowledge the problems with bulk DOC analysis in the next sentence. Delete.

Authors' Response :

The objective of this paragraph is to provide some ideas on why the addition of both Fe and C could stimulate bacterial heterotrophic metabolism. We do so by comparing the molar ratios of Fe:C of bacterial cells to those of their resource that are DFe and DOC, similar to what is commonly done for N:P-ratios for phytoplankton. We entirely agree with the Reviewer, the bioavailable fractions of both DFe and DOC are unknown, and thereby this ratio is not as straightforward to apply as for inorganic nutrients. However, we still consider this an interesting exercise, and the similarity in the molar ratios provides some clues on the potential limitation of Fe and C in this environmental context.

In response to the Reviewers' comment, we have rewritten this paragraph in the revised version of the manuscript, pointing out the concern raised by the Reviewer.

Reviewer Query 7)

It doesn't look like the t-tests were corrected for multiple comparisons – a 2-way ANOVA with time and treatment as fixed factors would be more appropriate or perhaps a repeated-measures ANOVA.

Authors' Response :

As suggested by the Reviewer, we re-analyzed our results using a different statistical test. The main aim of the statistical analyses was to identify the treatment effect at a given time point during the incubation. So, for each time point there is only one factor in question, which is the treatment. We therefore performed a one-way ANOVA and a post hoc Tukey test. The results from these analyses overall confirm our results, with the exception of Station R2 (See Fig. 1). In the revised version of the manuscript, we have slightly changed the presentation of these statistical analyses. We highlight only the treatments that are significantly different to the control (at 95% confidence interval).

The Reviewer suggests to perform a two-way ANOVA, using time and treatment as factors. However, days cannot be used as a factor level, because measurements taken on a given day are not independent of those taken already on the previous sampling date. Time is therefore not an independent factor. For the same reason, it is not appropriate to perform a repeated measures ANOVA.

Reviewer Query 8)

Page 15741, lines 17-22. There is no way to evaluate whether these hypotheses have any merit because of the lack of information about Fe uptake.

Authors' Response :

We do not present bacterial Fe uptake rates in the revised version of the manuscript, and these ideas have been reformulated accordingly.

Technical Comments:

Reviewer Query 9)

Page 15735, line 5 – Schmidt and Hutchins 1999 and Tortell et al. 1996 should be given credit here as they were the first (along with Maldonado et al.) to quantify the relative rates of Fe uptake by heterotrophs.

Authors' Response :

These references were added in the revised version of the manuscript.

Reviewer Query 10)

Line 7 – some citation is needed here to support this statement (Ducklow, Kirchman?).

Authors' Response :

A citation has been added in the text.

Reviewer Query 11)

Line 20 – Kuparinen et al. 2011 presented results that showed a positive effect of Fe addition and argued for C and Fe co-limitation – I'm surprized it has not been referenced here. It must be included. Agawin et al. 2006 also looked at Fe and C co-limitation in dark incubations in the subarctic Pacific Ocean, it too should be cited –if not here in the text, then in the Table.

Authors' Response :

This brief overview of bacterial responses to Fe addition refers only to incubation experiments performed in the dark. The reason for this is that in incubations in the light, the direct effect of Fe cannot be distinguished from the indirect effect due to Fe-stimulated production of phytoplankton-DOM. Kuparinen et al. (2011) have performed light incubations only. The publication by Kuparinen et al. (2011) is cited in Table 2. The publication by Agawin et al. (2006) and a report in press (Jain et al. DSR) have been added to the Introduction and also to Table 2.

Reviewer Query 12)

Line 21 – As far as I can tell, a single seawater sample was collected using a Niskin bottle and then dispensed into replicate sample bottles – are these then pseudoreplicates or true replicates? A more powerful analysis of the effect of Fe and C enrichment would be to consider the results from each station as truly independent samples and then combine the stations to test for a significant treatment effect. Some normalization of the data may be required for this sort of analysis.

Authors' Response :

We consider these samples as true replicates, as they represent independent biological incubations. Our experimental protocol respects the most fundamental rules of replication for any addition experiment one can conceive and this consists in 3 replicates.
We do not quite understand the approach suggested by the Reviewer, because we test the treatment effect (+C, + Fe or +C+Fe) compared to a control, and each of the unamended controls is representative only for a given site.

Reviewer Query 13)

Page 15736, line 1 – Queroue et al., 2014 is missing from the references.

Authors' Response :

The manuscript by Quéroué et al. is part of the KEOPS2 Special Issue. At the time when we submitted our manuscript, the paper of Quéroué et al. was not submitted, and it could therefore not be cited. It has been added to the References in the revised version of the manuscript.

Reviewer Query 14)

Line 14 – Bowie et al., 2014 is missing from the references.

Authors' Response :

The manuscript by Bowie et al. is part of the KEOPS2 Special Issue. At the time when we submitted our manuscript, the one of Bowie et al was not submitted, and it could therefore not be cited. It has been added to the References in the revised version of the manuscript.

Reviewer Query 15)

Line 15 - "The Niskin bottles were transferred to a trace-metal clean container" – I'm not sure if you mean lab instead of container?

Authors' Response :

The sentence is correct as it is: The Niskin bottles were transferred to a trace-metal clean container. The container had two sections, separated from each other: one where the Niskin bottles were sampled, and one where the analyses and incubations were performed. This latter part of the container could be considered as a trace-metal clean lab. Details of the trace-metal clean work are provided in the manuscript by Bowie et al. (2014).

Reviewer Query 16)

Line 18 – "dispensed" would be better than "dispatched"

AR : DONE

Reviewer Query 17)

Line 20 – consisted "of"

AR : DONE

Reviewer Query 18)

Page 15739, line 6 – awkward wording please change "the most contrasted station"

AR : DONE

Reviewer Query 19)

Page 15740, second paragraph. The idea of Fe and C co-limitation was originally advanced by Tortell et al. (1996, 1999) and needs to be referenced here. Kuparinen et al. 2011 should also be included, since they obtained some support for this hypothesis in field experiments in the sub-Antarctic. Church et al. 2000 also observed a Fe/C interaction in enrichment experiments. This part of the discussion minimizes the contributions of other researchers and makes it sound like the idea of Fe/C co-limitation has its origins here. It would make sense to introduce the co-limitation hypothesis in the Introduction.

Authors' Response :

As stated earlier, the overview Table 2 was made with the intention to appreciate previous reports on C and Fe enrichment experiments in various ocean regimes, and the publications highlighted by the Reviewer (Kuparinen et al. 2011 ; Church et al. 2000) are cited in this

Table. To address the Reviewers request, we have now added Tortell et al. (1996, 1999) and more citations in the text that was modified accordingly.

Reviewer Query 19)

Page 15742, line 18 – A temperature and organic substrate interaction was originally advanced by Pomeroy and colleagues in the late 80's and I fail to see how the proposal made here is any different than the original idea. Cite them.

Authors' Response :

This rather short paragraph aimed to briefly discuss the observation that combined additions did not yield significantly higher bacterial production rates than single additions, as observed in several previous studies. We refer now to a study that demonstrates an increase in the bacterial response to nutrient amendment at higher temperatures.
The studies by Pomeroy and Deibel (1986) and Wiebe et al. (1992) suggest that bacteria require higher concentrations of labile organic matter at low temperatures, which does not exactly reflect the idea presented in this paragraph.

Reviewer Query 20)

Page 15743, line 8. Here again the authors need some appropriate citations. The idea that the relief of Fe limitation of phytoplankton could increase the flow of C to bacteria has been around for some time.

Authors' Response :

This sentence was accompanied by a citation.

Reviewer Query 21)

Table 1 reports that bacterial production (ng C/L/h) is roughly equal at the E stations and 10 times lower at the R station. Yet, in Figure 1 the relative production values (here reported as leucine uptake) are quite different. If the same conversion factor was applied to compute the C rates, then something is odd. The leucine rates at the E stations differ by a factor of 3 and the R station is not too different from E-4W. Comment please.

Authors' Response :

We present BHP rates in ng C 1^{-1} h^{-1} in Table 1, because these data are from Christaki et al. (2014) and we wanted to maintain the same units as in the initial paper. By contrast, in our incubation experiments, leucine incorporation was used as a measure to determine the bacterial response to C, Fe and C+Fe additions, and we used this measure only in a comparative manner among treatments. We therefore consider it more appropriate to present the leucine uptake rates prior to the use of a carbon conversion factor.

When calculating our leucine uptake rates in carbon units, we obtain similar results (at time zero) as those given in Christaki et al. (2014) for 3 sites: Station R-2: 3.6±0.2 vs 2.6±0.5 ng C 1^{-1} h^{-1} ; Station E-4W: 26.5±3.9 vs 29.1±3.9 ng C 1^{-1} h^{-1} ; Station E-5: 19.9±4.1 vs 27.4±1.3

ng C 1^{-1} h^{-1}. At Station E-3, our values 7.7±0.7 ng C 1^{-1} h^{-1} are indeed lower than those given in Christaki et al. (2014) 24.9±1.7 ng C 1^{-1} h^{-1}.

Given that the two independent bacterial production measurements were done on water samples collected from different CTD casts, the overall coherent results point out an excellent reproducibility of the production measurement. The different values obtained at one site could be attributed to the differences in the collection of seawater (trace-metal clean vs common Niskin bottle), which could have affected instantaneous rates of BHP at this site. We consider, however, that this difference in rates of BHP does not affect the interpretation of our results in the incubation experiments.