Responses to Anonymous Referee #2

R#2: The study by Vaquer-Sunyer describes the effects of wastewater treatment plants effluent inputs on different microbial parameters. I find the topic interesting and relevant for publication in BG. However, the approaches used are not well explained and therefore it is hard to judge the results. Moreover, I miss precise definitions and statements in the discussion. Therefore the manuscript seems in the current status premature.

Comment (C): The manuscript has changed considerably since its first version thanks to the comments by 3 reviewers, including yours. We believe that the manuscript has improved significantly and hope you will find it suitable for publication in its present form. Please, see detailed comments below.

Introduction
R#2: Line 50 ff. The introduction (and in the discussion) gives a very anthropogenic view about hypoxia. Various prokaryotic groups' life in oxygen minimum zones and often exists only at those conditions (read for example "Microbial ecology of expanding oxygen minimum zones" Jody J. Wright, Kishori M. Konwar & Steven J. Hallam). From a microbial point of view the biological diversity therefore increases by the presence of oxygen minimum zones. Moreover, oxygen minimum zones in the central Baltic Sea are connected to the presence of a halocline that prevents water mixing in spring and autumn and therefore a typical phenomenon in the Baltic Sea. The authors mean coastal water hypoxia (as in the cited Conley 2011 publication) that should become clearer in the introduction (and discussion).

Comment (C): We meant eutrophication-driven hypoxia, typical in coastal waters.

Action (A): We include some sentences to make clear that Baltic Sea suffers from eutrophication-driven hypoxia. We also acknowledge that in oxygen minimum zones prokaryotic diversity could increase and refer to the above-mentioned paper. The text now reads (lines 51-52 and 55-59): "The Baltic Sea has the largest area affected by **eutrophication-driven hypoxia** (Conley et al., 2011). (…) The lack of oxygen in marine waters causes death of the marine organisms and catastrophic changes in marine **metazoan** communities. Thus, hypoxia is emerging as a major threat to marine biodiversity (Vaquer-Sunyer and Duarte, 2008), although prokaryotic diversity can increase in oxygen minimum zones (Wright et al., 2012)."

Methods:
R#2: Line 105-105: what salt solution was used?

A: We now refer to the paper used to make the salt solution (Søndergaard et al., 2003).

R#2: Line 131-133. Use space between unit and number.

A: We have done so

R#2: Line 136: please give a short description who the calculation of the metabolic rates were performed. If I understand correctly, CR was estimated from change in oxygen over night and GPP from the sum of NCP and CR. Since DOC is produced during daytime the CR can differ between night and day. Typically incubations are performed at light and dark conditions in parallel to estimate CR and GPP. Please discuss that your approach gives comparable results as the parallel incubations.

C: We agree with the reviewer that a description on how metabolic rates were calculated will improve the manuscript. Dark incubations can underestimate CR for two reasons: (i) respiration during daylight is probably higher than at night and (ii) under dark conditions phytoplankton growth is suppressed, so contribution of phytoplankton to community respiration is limited. Here, we assume equal respiration rates during day and night, as it is done by incubations under light and dark conditions. However, as communities are incubated under conditions mimicking natural conditions, phytoplankton respiration will contribute more to community respiration than under light/dark incubations.

A: We have included a short description on how the metabolic rates were calculated. The text

now reads (lines 140-144): “NCP was estimated as the changes in dissolved oxygen content during 24 hours intervals (dDO/dt). CR was calculated from the rate of change in DO during the night from half an hour after lights went of to half an hour before light went on. CR was assumed to be the same during light and dark. NCP in darkness equals CR during night. GPP was estimated as the sum of NCP and CR (GPP = NCP + CR).” We have also included discussion about the possible differences in CR during day and night and comparability with light/dark incubations (lines 148-156): “As incubations were performed following a natural light regime to mimic natural conditions, results may differ from incubations performed at light and dark conditions in parallel. Both approaches assume equal respiration rates under light and dark conditions. This assumption may lead to underestimate CR and GPP, as respiration rates are probably higher during daylight than at night (Grande et al., 1989; Pace and Prairie, 2005; Pringault et al., 2007), but it does not affect NCP estimates (Cole et al., 2000). In incubations performed under dark conditions, phytoplankton growth is suppressed, decreasing phytoplankton respiration contribution to community respiration.”

R#2: Line 151: The 341-805r primers were designed for bacteria the protocols in Hugerth et al are for eukaryotes. If I understand correctly a two step PCR were performed, please describe the protocol in more detail, especially if the PCR contained several independent PCR cycles. This is important information since this can introduce a strong bias into the abundances estimates for bacteria.

C: The 341f-805r primers are designed for bacteria and were first used for bacteria in: Herlemann, D. P., et al. (2011). "Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea." ISME J. However, that study was made with 454-pyrosequencing. The description in Hugerth et al. is for Illumina Miseq designed primers using standard Nextera primers as template. The PCR program applied has two steps to limit biases such as described previously for pyrosequencing: Berry, D., et al. (2011). "Barcoded Primers Used in Multiplex Amplicon Pyrosequencing Bias Amplification." Applied and Environmental Microbiology 77(21): 7846-7849, and recently used in e.g.: Savio, D., Sinclair, L., Ijaz, U. Z., Parajka, J., Reischer, G. H., Stadler, P., Blaschke, A. P., Blöschl, G., Mach, R. L., Kirschner, A. K. T., Farnleitner, A. H. and Eiler, A. (2015), Bacterial diversity along a 2600 km river continuum. Environmental Microbiology, 17: 4994–5007. In the latter paper the two-step PCR was applied for the Illumina Miseq platform. Thus, in our work, the first step in the two-step PCR uses the main primer set 341f-805r to amplify the correct fragment. The second step is applied to attach the handles and indexes needed to run the 16S Illumina Miseq run and for tagging/barcoding individual samples.

A: We have now clarified the PCR method in Material and Methods section as follows (lines 201-205: “…with some modifications. We thus performed a two-step PCR: (i) amplification with the main forward and reverse primers 341F-805R to amplify the correct fragment within the V3-V4 hypervariable region of the 16S rRNA gene; (ii) amplification using template from the first PCR to attach the handles and indexes needed to run the Illumina Miseq run and for barcoding individual samples.”

R#2: Line 119: What happened to the data of the DPA measurements?

A: DPA concentration for effluent and seawater are given in tables 1 and 2, and for each experiment are included in table S1. As DPA did not explain variation in metabolic rates or bacterial community composition it is not discussed in the results or discussion sections.

Result

R#2: Line 190 221 table 1 and 2: How was DOC and TOC measured (replicates, sensitivity,. . .)?

C: We only measured DOC as all samples have been filtered. Samples were taken in duplicate.

A: We have better explained how DOC was measured. The text now reads (lines 172-179 and 182-185): “Samples for chlorophyll a (*Chl.a*), dissolved organic carbon (DOC) and nutrients were taken on days 0, 1, 3, 5 and 7 from the two 2.3 L bottles for each treatment incubated simultaneously than the bottles used to monitor oxygen content changes to calculate metabolic rates. Samples were taken in duplicate. For the last day of the experiment (day 7) the 2 bottles used to monitor oxygen content were used to sample *Chl.a* and nutrient content. Samples for nutrient determination were filtered using pre-combusted (450ºC, 4 h) glass-fiber (GF/F Whatman) filters and 0.2 µm membrane filters and frozen until analysis. All equipment used for handling the samples was acid washed.” “DOC was measured on a Shimadzu TOC V-CPN in non-purgeable organic carbon (NPOC) mode on

acidified samples (HCl to pH <2). The instrument was calibrated daily with potassium hydrogen phthalate. DOC concentrations were calculated from the average area of 3 injections, with an area covariance of less than 2%."

R#2: Line 273: How was the change in the number of OTUs?

A: For comparison we have now added text and a new supplementary Figure 2 that show variation in observed number of OTUs (Fig. S2A) and Chao.1 richness index (Fig. S2B). The text now reads as follows (Line 341-345): "Moreover, we analyzed the richness and found that the observed number of OTUs ranged between 206-946 ± 171 and Chao.1 index values ranged between 306-1273±220. Richness was generally lower in effluent amended treatments compared to controls, except for in the April experiment."

Discussion

R#2: Line 345: It is unclear what the abbreviation DOM stands for. Typically it is used for dissolved organic matter (which was not measured in this study), but in the manuscript it reads rather that N-rich dissolved organic matter is meant. Please clarify. The results suggest that certain concentrations of nutrients (Figure 2; figure 4, Figure 5 1:5; IN) cause a change in the GPP, CRR and BP compared to the control treatment. Moreover the statistics give often DOC and CHla as the variances explaining the variability (line 224, line 234, line2 44) and only for bacterial production a correlation with NO3. Therefore the statement: " OM significantly increased bacterial production, whereas it decreased gross and net primary production and community respiration rates" seems not justified.

C: We used DOC as a proxy for DOM. Mixed effects models showed that DOC was significantly related to BP, GPP, NCP and CR, with high R^2 values.

A: We have included a sentence to explain that we used DOC as proxy for DOM. The text now reads: (Lines 222-230): "Metabolic rates data from the four experiments were combined to test the relationship between the given metabolic rates and physicochemical parameters (Table 1) by mixed effects models. Physicochemical parameters were selected avoiding collinearity. Selected variables were DOC, DON, nitrate and phosphate concentration. We used DOC as a proxy for dissolved organic matter (DOM). Variables were selected according to its significance. Variables were removed following its p value (i.e. variables with higher p value were removed first) until all parameters were significant). To account for pseudo-replication we used incubation day nested to season (i.e. experiment) as a random factor." (…) Lines 415-418: "DOM significantly increased bacterial production, whereas it decreased gross and net primary production and community respiration rates, as showed in the results of the mixed effects models where DOC is used as a proxy for DOM."

R#2: Line 413. Alpha diversity is not only expressed in the Shannon index. I think you mean Shannon index here, but it would be interesting to see how the total number of OTUs (richness) change.

A: We have now changed the text to also accommodate our new analysis of species richness (observed number of OTUs and Chao.1 index). Lines 538-542: "The observed effect of species loss, i.e. lower richness (observed number of OTUs and Chao.1 index) and Shannon diversity index, may be closely linked with the functioning of microbial communities and could potentially render the whole community more sensitive to environmental perturbations (Allison and Martiny, 2008; Bell et al., 2005; Loreau, 2000, 2004; Shade et al., 2012)."

R#2: Please discuss also the artifacts that can arise from long term bottle incubations especially for t7.

A: We have added a paragraph on the "bottle effect" to address this issue (lines 549-562). For further detail, see reply to comment by reviewer 1 on bottle effect.

R#2: P has also a strong impact on the eutrophication in the Baltic Sea since many Cyanobacteria are able to fix N (read for example "Andersson, A., Höglander, H., Karlsson, C., and Huseby, S. (2015). Key role of phosphorus and nitrogen in regulating cyanobacterial community composition in the northern Baltic Sea. Estuar. Coast. Shelf Sci. 164, 161–171."). Since P was also measured in the experiments, and found to have a strong influence on the BCC (Line 271), I wonder why P is rarely

discussed.

A: We now include a sentence in the discussion section including reference to the above-mentioned paper. The text now reads (lines 507-5349: “"Apart from the influence of temperature in structuring the bacterial communities in the present paper, shifts in bacterioplankton community composition was highly correlated with changes in phosphate concentrations. In agreement, previous findings show that phosphate is a driver of shifts in community structure in the Southern Californian coast and Baltic Sea (Fuhrman et al. 2006; Andersson et al. 2010). For example, Andersson and colleagues (2010) suggested that limiting conditions due to a decline in phosphate during the summer Cyanobacterial bloom promote selection in the bacterioplankton community where specific OTUs can proliferate. Moreover, in an adjacent area of the Baltic Sea Proper opportunistic cyanobacteria, including N_2-fixers and picocyanobacteria, proliferated despite low phosphorus concentrations and may instead have been fueled by bioavailable nutrients from filamentous Cyanobacteria (Bertos-Fortis 2016). Recent evidence suggests that availability of phosphorus has a substantial impact on eutrophication in the Baltic Sea since many Cyanobacteria are able to fix nitrogen (Andersson et al. 2015). In the present study phosphate concentrations showed small variations between treatments within each experiment and we observed primarily seasonal oscillations between experiments. Absolute shifts in composition among the groups Bacteroidetes, Betaproteobacteria and Alphaproteobacteria were positively correlated with absolute changes in phosphate whereas shifts in Planctomycetes, Verrucomicrobia and Cyanobacteria were negatively correlated with variation in phosphate. Nevertheless, changes in phosphate concentrations significantly explained variation in community structure within the July experiment. Hence, the communities responded to effluent inputs by shifts in species composition and the influence of seasonal changes in phosphorus concentrations was outweighed by the simulated environmental disturbance investigated here. Thus, long-term changes in phosphorus resulting from natural seasonal variation or climate change related effects accompanied by episodic short-term effluent inputs may form a synergistic permanent impact on the structure of bacterioplankton communities with severe consequences for ecosystem services.”

Figures
The legend of figure 8 is almost not readable. The colors in the plot are not easy to distinguish the abbreviations should also be given in the legend

A: We have now modified the figure to make it more clear and we have increased font sizes.

R#2: Figure 2 and Figure 4: they are based on the same data, whereas one contains subtracted CR? Therefore I think one of them is sufficient.

C: Although the reviewer is right regarding that GPP and NCP are derived from same data and NCP equals GPP – CR (with CR being positive), we do believe that including both figures is important to understand metabolic rates dynamics. Net community production gives information on whether planktonic communities are heterotrophic (if NCP is < 0) or autotrophic (when NCP > 0) o if there is metabolic balance.