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Biogeosciences Discussions

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Interactive Comment

Interactive comment on "Increases in the abundance of microbial genes encoding halotolerance and photosynthesis along a sediment salinity gradient" *by* T. C. Jeffries et al.

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Final author comments

We thank Dr. Gerhard Herndl for handling our manuscript entitled 'Increases in the abundance of microbial genes encoding halotolerance and photosynthesis along a sediment salinity gradient' and thank Dr. Aharon Oren and an Anonymous Referee for their insightful and helpful comments. Below we have addressed each specific Referee comment. We have also described changes to a revised manuscript that we feel have enhanced the quality of the paper making it suitable for consideration for publication in Biogeosciences. Each numbered original Referee comment is provided, with

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our response detailed below each point.

Response to Dr. A. Oren

1. Referee comment: My main problem with the Jeffries et al. paper is the mode of sampling of the sediments at the four salinities studied. The authors have sampled from each site 10 g of sediment from a core representing the upper 10 cm of the sediment. Although the authors did not provide any information about the vertical structure of the sediment, it may be expected that the samples included aerobic surface sediment as well as anaerobic reducing mud. This is clear from the abundance of different groups of methanogenic Archaea, Clostridia, and (possibly sulfate-reducing) Deltaproteobacteria. Each sample is thus composed of a complex mixture of different microbial communities that had developed along the vertical gradients at each site. It is difficult to believe that the aerobic/anaerobic boundary at the four sampling sites was located at exactly the same depth. Therefore the changes detected in the community structure along the salinity gradient, as illustrated in Fig. 4, may well be due to factors unrelated to the salinity of the pore water: metagenomic analysis of a sample that consists for 90% of aerobic surface sediment will show dominance of very different taxa than a sample largely consisting of anaerobic mud. The study by Kunin et al. (2008) of a microbial mat at 90 PSU in Guerrero Negro showed sharp differences in microbial community structure on a millimeter scale. Jeffries et al. state that no such layered mats were evident in the Coorong lagoon, but still an understanding of the chemical gradients in the upper 10 cm of the sediments sampled is essential for a proper evaluation of the results. By pooling the upper 10 cm of the sediments much information about the structure of the microbial communities in the sediments was lost, and it therefore becomes very difficult to compare the metagenomic sequence data from the four sites in a meaningful way.

Author response: The reviewer makes a fair point regarding the likely vertical chemical and biological heterogeneity in the sediment samples at each site. However, our motivation for homogenizing the 10 cm sediment sample was to combine the complex

mixture of different microbial communities that have developed along the vertical gradients at each site. In doing so we deliberately sampled the average, or bulk, community in the surface layers of our sediment samples, so that we could adequately assess regional-scale, rather than microscale, patterns. Within the context of the focus of this study we feel this approach is justified because:

1. As the precise nature (e.g. depth of chemical layers) of the vertical gradients would vary between sites, as the Reviewer indicates, it would not be possible to directly compare sediment samples from the different environments by sampling at a given depth or interval. Our study aimed to determine the bulk metabolic potential, and thus the potential biogeochemical influence, of the top 10cm of sediment in contact with the water column. Therefore, we wished to summarize the metabolic diversity contained in the entire microbial community of these cores, rather than to describe the heterogeneity within each core, which addresses a different question. By homogenizing the upper 10cm of sediment we have profiled the entire metabolic potential of the sediment, inclusive of both oxic and anoxic zones, which is a more suitable approach for determining overall regional-scale shifts in functional potential along the salinity gradient that we have profiled.

2. From a practical perspective, pooling the upper 10cm of sediment allowed us to have enough DNA to sequence the metagenomes from each site. Sampling smaller volumes would not have provided enough DNA for a shotgun pyrosequencing approach, without introducing potential biases using whole genome amplification. We note that studies regarding microscale vertical heterogeneity, such as a microscale investigation of mat heterogeneity (Kunin et al., 2008), still require pooling of horizontal samples to gain enough DNA for sequencing.

We do not believe that our sampling approach makes meaningful comparison of the metagenomes difficult. Many metagenomic studies to date (e.g. Dinsdale et al., 2008b; Gilbert et al., 2010; Rusch et al., 2007) have sequenced the bulk condition of an environment rather than describing the fine-scale heterogeneity within each sampling unit.

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These studies have still allowed for meaningful comparison between samples on the larger scales relevant to the hypothesis of the study. In reality our approach is similar to studies that compare ocean metagenomes, which involve the filtration of tens of litres of seawater. Within these large volumes of seawater there will be multiple microscale niches (attached to organic particles etc) where the local chemical conditions and microbial communities differ substantially. This microscale heterogeneity is ultimately averaged out when the large volume sample is filtered. However, meaningful comparisons between environments, in relation to large-scale environmental variability, can still be made. We agree that the taxonomic shifts, such as those that we present in Fig. 4, may to some extent be influenced by factors unrelated to porewater salinity, such as differences in the aerobic and anaerobic boundary in the cores. Despite this, our taxonomic profiles still describe taxa which are able to tolerate the porewater salinity of each given site indicating that salinity tolerance is still an important factor, which controls community composition along the gradient, as demonstrated by Figure 5 of our paper. This is also reflected in metabolic function of genes encoded by these taxa (Figures 2 and 3), which can be specifically related to salinity variability indicating that, overall, salinity is an important factor in determining the genetic composition of these sediment communities. While other environmental variables undoubtedly shape the microbial communities studied here, the shifts in metabolic potential along the Coorong lagoon, and the biogeochemical consequences of these shifts remain within the context of salinity variability along the gradient.

In light of the Reviewers comments we have added the following to the discussion of the revised manuscript: "Taxonomic shifts may also reflect variation in other variables such as the relative amount of oxic and anoxic sediment present in each core, however the nature of metabolic shifts along the gradient indicate that salinity is a dominant factor, as does the increased representation of halophiles along the gradient". We have also added the following to the discussion: "Sediment cores on this scale demonstrate strong vertical gradients in Oxygen, Nitrogen, Carbon, and Sulfur (Paerl and Pickney, 1996). As our focus was on regional-scale rather than micro-scale shifts it was nec-

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essary to incorporate all of this heterogeneity in our sample to characterize the bulk metagenomic potential of the upper surface sediment, in a similar fashion to which water metagenomic studies (e.g. Dinsdale et. al., 2008b; Rusch et. al., 2007) and sediment 16S rDNA studies (e.g. Hollister et al; 2010) combine spatially heterogeneous samples to investigate regional scale shifts".

We have also added the potential consequences of homogenizing the 10cm core to the methods section as follows: "This sampling approach averages out the vertical heterogeneity present in the sample, combining chemical gradients and pooling both oxic sand and black anaerobic mud. In each sample approximately 7cm of the core was dark grey and black mud overlayed by approximately 3cm of pale sand "

We thank the reviewer for highlighting this issue as we believe discussion of this topic has improved the quality of the manuscript.

2. Referee comment: Metagenomics is an excellent tool to obtain information about the structure of microbial communities in different ecosystems, but it should always be combined with different, independent techniques. Jeffries et al. find evidence for an increased abundance of phototrophic microorganisms, especially cyanobacteria, at the elevated salt concentrations in the lagoon. One does not need metagenomics to draw such a conclusion: simple extraction of chlorophyll and its spectrophotometric or fluorimetric quantification is the first method of choice to compare the abundance of photosynthetic microorganisms. Microscopy can also add much important information.

Author response: We agree that we could have identified the increase in cyanobacteria with the aid of other techniques, including microscopy, but microscopy would not have allowed us to simultaneously examine other features of the microbial community, including shifts in non-phototrophic members of the community or changes in the frequency of specific gene-groups. The advantage of metagenomics is that it allows us to characterise multiple taxonomic and metabolic features of the community within the one analysis. To obtain the level of information obtained using metagenomics would re-

quire the coupling of a huge amount of different analyses, which is unfeasible. Over the last five years metagenomics has become a routine tool to investigate microbial community composition and many studies (e.g. Rusch et al., 2007;Gilbert et al., 2010;Dinsdale et al., 2008a;Kunin et al., 2008) and the molecular identification of microorganisms is typically more informative and precise than microscopic identification of organisms. We agree that microscopy and chlorophyll concentration can add useful information, but we do not believe they are necessary to support the conclusions of the paper.

3. Referee comment: The metagenomic analysis showed apparent abundance of three groups of cyanobacteria in the sedimentsat the highest salinities: Nostocales, Chroococcales and Oscillatoriales. The latter two groups are the common types in hypersaline cyanobacterial mats (Oren, 2000 [erroneously cited as Oren, 2002 by Jeffries et al.]), but abundance of Nostocales, a group of heterocystous cyanobacteria, it not expected in hypersaline environments. Blooms of Nodularia occur the south arm of Great Salt Lake, Utah, at 60-100 PSU (Roney et al., 2009), but to the best of my knowledge massive occurrence of Nostocales was never reported at higher salinities such as found in the Coorong lagoon.

Author response: Whilst Nostocales have also been observed in saline Antarctic lakes (Jungblut and Neilan, 2010) and in the hypersaline stromatolites of Shark Bay, Australia (Burns et al., 2004), there are indeed no reports that we could find of high abundance Nostocales in samples as hypersaline as ours. Thus our data could either describe a novel group of halophilic Nostocales, or potentially our Nostocales sequences are derived from other filamentous cyanobacterial taxa which are closely related to Nostocales, for which there are no examples in the SEED database used for taxonomic classification.

We have added this caveat to the discussion of our manuscript as follows: "The occurrence of abundant Nostocales sequences in our metagenomes is unexpected as while Nostocales have also been observed in saline Antarctic lakes (Jungblut and Neilan, 2010) and in the hypersaline stromatolites of Shark Bay, Australia (Burns et al., 2004),

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there are indeed no reports that we could find of high abundance Nostocales in samples as hypersaline as ours. Our data could either describe a novel group of halophilic heteroscystous Nostocales, which would require further microscopic analysis and detailed molecular taxonomic classification to confirm, or potentially our Nostocales sequences are derived from other filamentous cyanobacterial taxa which are closely related to Nostocales, for which there are no examples in the SEED database used for taxonomic classification".

We do not feel that this reduces the accuracy or significance of discussion regarding grazing and the biogeochemical influence of filamentous cyanobacterial taxa. We apologize for the erroneous citation and have corrected this in the revised manuscript.

4. Referee comment: Heterocystous filamentous cyanobacteria can be easily recognized by their morphology, and a simple microscopic examination of the samples could have confirmed the occurrence of members of the Nostocales. Observation of heterocysts would have further strengthened the discussion about the possible role of the benthic cyanobacteria in the nitrogen cycle in the Coorong lagoon.

Author response: We agree that this data would have strengthened our discussion, but unfortunately samples for microscopic analysis from the time of sampling are not available. We have added a statement regarding microscopic observation of Nostocales and hetrocysts to our discussion. Please see point 3.

5. Referee comment: In view of the apparent abundance of cyanobacteria in the more saline samples, a search for genes involved in glucosylglycerol metabolism may be of interest as well. The genes that participate in glucosylglycerol biosynthesis and degradation have been well characterized in model organisms (Hagemann, 2011).

Author response: We found no evidence of these genes in our samples based on BLAST searches against the KEGG and SEED databases.

6. Referee comment: In their discussion about the different organic compounds in-

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volved in osmotic adaptation and the genes for their biosynthesis or accumulation, Jeffries et al. also mention the glucans accumulated by some Proteobacteria in the periplasm under osmotic stress (Bohin, 2000). Whether or not the detection of genes potentially involved in the synthesis of such glucans may tell us anything about the mode of osmotic adaptation along the salinity gradient is not clear. Such genes are found in many non-halophiles, and appear to be especially important for bacteria such as Rhizobium, Agrobacterium, and related taxa that have to interact with a eukaryotic host, something not relevant in hypersaline sediments. I am not aware of any indications that genes for production of such glucans are prominently present in the genomes of salt-tolerant bacteria.

Author response: We thank the Reviewer for his insight and have removed discussion of periplasmic glucans from our revised manuscript.

7. Referee comment: Therefore the finding of gene sequences assigned to the Halobacteriaceae in the hypersaline part of the Coorong lagoon is of interest and deserves a more indepth discussion. The finding is not unique, and it may be interesting to compare the sequences obtained with those reported in a metagenomic study of Hamelin Pool, Shark Bay, Western Australia, an environment with a salinity about twice that of sea water (Allen et al., 2009), and with the 16S rRNA genes of Haloferax and Halococcus species recovered from that site (Allen et al., 2008; Goh et al., 2006).

Author response: We thank the reviewer for this suggestion and to address this we have added the following to our discussion: "The presence of sequences matching the Halobacteriacea, which contain the pigment bacteriorhodopsin, indicates an adapted halophilic community as these Archaea tend to be found at the highest salinities and generally use a 'high-salt in' strategy (Oren, 2008) which suggests that this mode of salt adaptation is also present in our samples. Sequences and isolates matching the Halobacteriales have been observed in the hypersaline microbial mats of Shark Bay, Australia (Goh et al., 2006;Burns et al., 2004;Allen et al., 2008) indicating that they are an important constituent of benthic microbial communities that exist at more moderate

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hypersaline conditions as well as at the extremes of salt saturation”

Comparison of individual Halobacteriaceaea 16S rDNA sequences from the Shark Bay datasets would indeed be interesting however is outside the scope of the current manuscript which has not specifically targeted the 16S rDNA gene. We also note that the study of Hamelin Pool (Allen et al., 2009) is not specifically a metagenomic study but a 16S rDNA survey.

8. Referee comment: Some interesting features relating to the apparent loss of metabolic functions at elevated salt concentrations deserve to be discussed. One is the elevated ammonium concentration in the high-salinity sediments, which may be caused by the inability of autotrophic nitrifying bacteria to function at high salt. Another interesting, and unexpected, observation is the apparent enrichment of methanogenic Archaea of the ‘Methanomicrobia’ (not a validly published name) at 136 PSU as compared to the 37 PSU sample. Like the Methanobacteria and Methanococci, the members of the order Methanomicrobiales produce methane almost exclusively from hydrogen and carbon dioxide, a process that ceases to function already at a relatively low salinity. At high salinity I rather expect to find enrichment in the Methanosarcinales, many of which metabolize trimethylamine and other methylated amines, formed as breakdown products of glycine betaine.

Author response: We thank the reviewer for this insight and have added the following to our discussion regarding taxonomic shifts along the Coorong gradient: “Another interesting trend in our data is the higher level of ammonium in hypersaline sediments compared to the 37 PSU sediment (Supplement Fig. S1). This is potentially related to the potential negative influence of high salt concentrations on nitrifying bacteria, such as the genera Nitrosomonas and Nitrosococcus, which are both present in our data”.

Methanomicrobia refers to the class containing the orders Methanomicrobiales and the Methanosarcinales within the National Centre of Biotechnology Information taxonomy (http://www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy) and SEED database (Over-

beek et al. 2005). Our data contain abundant matches to both of these orders within the class Methanomicrobia. Thus we have added the following to our discussion when referring to the potential significance of organic solute degradation “Members of the class Methanosarcinales, which is over-represented in the most hypersaline metagenome, contain the order Methanosarcinales which metabolize methylated amines, formed from the breakdown of the osmoprotectant glycine betaine (Kendall and Boone, 2006), potentially influencing the rates of methane flux in the sediment”

We have thanked Dr. A. Oren in the acknowledgements of our revised manuscript for these insightful comments.

9. Referee comment: It may be worthwhile to calculate the isoelectric point distribution of the proteins encoded by the DNA from the four sites of different salinities studied by Jeffries et al. and to compare the result with the data published by Kunin et al. (2008) and Rhodes et al. (2010). Such an analysis may well yield interesting new insights into the adaptation of the microbial communities in the Coorong lagoon to the increasing salinity along the gradient.

Author response: We agree that such a comparison may be interesting, but feel that such an analysis is outside of the scope of this study.

10. Referee comment: The names of the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria that appear in Fig. 4 were validly published in 2006 (Euzéby, 2006), and therefore these names are to be preferred over _-Proteobacteria, _-Proteobacteria, -Proteobacteria, _-Proteobacteria, and "-Proteobacteria, used in the text of the article.

Author response: We have made this change to the revised manuscript.

Reviewer #2 – Anonymous

11. Referee comment: Based on this dataset it remains unproven if salinity really determines microbial composition and functions. Other physicobiochemical parameters

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have to be taken into account, too. The potential sedimentary redoxcline which has very probably been sampled is, for instance, often a much more important determinant than salinity.

Author response: Please refer to our response to point 1 above.

12. Referee comment: Statistical analyses were used to determine significant differences between sediments of increasing salinity. In fact, statistics is a powerful tool for the interpretation of environmental datasets; however, especially in ecology independent replicates are essential for significant statistical analyses. If I got it right one single sample, mayb even only a single core was taken for each of the four sediment types. From this, one metagenomic library was generated per sediment. Thus, we have no idea on the mean or variances of gene abundances (how can confidence intervals be presented based on one sampling?). This is simply insufficient for statistical analyses and the whole statistical part has to be removed from the manuscript. It is for sure that metagenomics in general generates large numbers of reads often leading to the assumption that this could be of relevance, but statistics is a question of random sampling, not of the amount of reads in one sample.

Author response: We agree that independent replicates are desirable when applying ecological statistics, however due to the prohibitive cost and large computational requirements necessary to analyse metagenomic datasets the vast majority of metagenomic studies performed to date have not employed replication. However, the application of several specific statistical tests have been shown to be effective for comparing un-replicated metagenomes. For example, Fisher's exact test has been implemented to determine statistically significant differences between single individual metagenomes in several studies (Lamendella et al., 2011;Biddle et al., 2011;McCarthy et al., 2011;Dinsdale et al., 2008a); and the conceptually similar G-test has been used to determine significant differences in metabolic processes between metagenomes from four coral reef environments, which consisted of one sample per reef (Dinsdale et al., 2008b). Several software packages (Parks and Beiko, 2010;Kristiansson et al.,

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2009;White et al., 2009) are specifically designed for the pair-wise comparison of unreplicated metagenomes so we argue that this is a routine statistical approach for comparative metagenomics. Indeed the application of the Fisher's exact test within the STAMP software we used in our study to two example datasets, consisting of a pair of unreplicated metagenomes each, found statistically significant differences between metagenomes (Parks and Beiko, 2010).

Regarding the Reviewer's question about how we determined confidence intervals without replicates, we clarify that our confidence intervals display the range of values of the effect size statistic, in our case the difference in single proportions of a feature, for which the true value will occur for 95% of permutations. A detailed description of the application of this test and the nature of the confidence intervals obtained is provided in the paper by Parks and Beiko (2010) and by Newcombe (1998).

In order to clarify that our statistical techniques are appropriate to our methodology we have added the following to the methods section of our revised manuscript: "Fisher's exact test uses a hypergeometric distribution of sequences drawn without replacement from a pair of metagenomic samples to generate a statistical significance value in a computationally efficient manner (Parks and Beiko, 2010) and is thus ideal for the pair-wise comparison of metagenomes. Fisher's exact test has been routinely applied to observe statistically significant differences between single metagenomic profiles (e.g. Lamendella et al., 2011;McCarthy et al., 2011;Biddle et al., 2011). "

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