

Abstract

Biogeochemical cycles are driven by the metabolic activity of microbial communities, yet the environmental parameters that underpin shifts in the functional potential coded within microbial community genomes are still poorly understood. Salinity is one of the primary determinants of microbial community structure and can vary strongly along gradients within a variety of habitats. To test the hypothesis that shifts in salinity will also alter the bulk biogeochemical potential of aquatic microbial assemblages, we generated four metagenomic DNA sequence libraries from sediment samples taken along a continuous, natural salinity gradient in the Coorong lagoon, Australia, and compared them to physical and chemical parameters. A total of 392483 DNA sequences obtained from four sediment samples were generated and used to compare genomic characteristics along the gradient. The most significant shifts along the salinity gradient were in the genetic potential for halotolerance and photosynthesis, which were more highly represented in hypersaline samples. At these sites, halotolerance was achieved by an increase in genes responsible for the acquisition of compatible solutes – organic chemicals which influence the carbon, nitrogen and methane cycles of sediment. Photosynthesis gene increases were coupled to an increase in genes matching Cyanobacteria, which are responsible for mediating CO₂ and nitrogen cycles. These salinity driven shifts in gene abundance will influence nutrient cycles along the gradient, controlling the ecology and biogeochemistry of the entire ecosystem.

1 Introduction

Biogeochemical cycles, over geological time, have fundamentally determined the chemical nature of the Earth's surface and atmosphere. Due to their high abundance and metabolic activities, microorganisms drive many global biogeochemical processes including the carbon, oxygen, nitrogen, hydrogen, sulfur and iron cycles (Falkowski et al., 2008; Fuhrman, 2009). The biochemical potential of the microbial inhabitants

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of an environment is determined by the community structure – the types of organisms present and their relative abundance, which is in turn largely determined by the physico-chemical conditions of the habitat, such as the need for cells to survive in highly saline environments by adjusting their internal salt concentrations (Oren, 2009).

5 How microbial communities respond to and contribute to chemical gradients is a central question of microbial ecology and is essential to our understanding of biogeochemical cycling and biological adaptation to global change.

Salinity has an important influence on the global distribution of bacterial diversity (Lozupone and Knight, 2007). Salinity gradients occur in a wide variety of ecologically important habitats such as estuaries, wetlands, salt marshes & coastal lagoons. Many of these habitats are under increasing pressure from climate change, due to increased evaporation, reduced freshwater flows, and rising sea levels (Scavia et al., 2002; Schallenburg et al., 2003).

15 In high salinity environments, microbes must maintain their cellular osmotic balance via the acquisition of charged solutes (Roberts, 2005; Oren, 2009). This fundamental physiological requirement has led to the evolution of halotolerant specialists, with several studies in hypersaline habitats demonstrating that microbial diversity decreases with salinity (Estrada et al., 2004; Schapira et al., 2010; Pedrós-Alió et al., 2000; Benlloch et al., 2002) with halotolerant and halophilic taxa becoming dominant in more extreme salinities. Shifts in microbial community structure have also been observed along estuaries (Bouvier and del Giorgio, 2002; Oakley et al., 2010; Bernhard et al., 2005) and in saline sediments (Swan et al., 2010; Hollister et al., 2010), with changes in the abundance of specific functional groups, such as ammonia-oxidizing (Bernhard et al., 2005) and sulfate-reducing bacteria (Oakley et al., 2010), and overall composition (Hollister et al., 2010; Swan et al., 2010; Bouvier and del Giorgio, 2002), suggesting the important selective role of salinity. However, it is not known how these taxonomic shifts will change the functional gene content involved in biogeochemical processes, with the majority of studies focusing on taxonomic marker genes or specific functional groups.

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Metagenomics allows for the elucidation of the biochemical potential of microbial genomes present in a given environmental sample via direct sequencing of community DNA (Hugenholtz and Tyson, 2008; Wooley et al., 2010). Several metagenomic studies (Kunin et al., 2008; Rodriguez-Brito et al., 2010) have focused on specific hypersaline environments, but there has been no assessment of metabolic shifts along salinity gradients. Additionally, the majority of non-metagenomic studies have investigated either estuarine habitats that do not exceed 50 PSU salinity or extreme hypersaline environments (e.g. solar salterns).

In this context, the Coorong lagoon, in South Australia provides a unique model system of a continuous, natural salinity gradient from estuarine to hypersaline salinities (Lester and Fairweather, 2009; Schapira et al., 2009), which provides an opportunity to investigate shifts in the biogeochemical potential and function of microbial communities.

The Coorong lagoon is one of Australia's most significant wetlands and is listed under the Ramsar convention as a wetland of international significance (Kingsford et al., 2011). The 150 km long, 2 km wide system is contained between the last interglacial dune before the ocean and a modern peninsula that has been established from the mid-holocene. The system receives water inputs at one end from the Southern Ocean and the Murray River, Australia's largest freshwater system. These combined inputs result in an estuarine system at the mouth of the lagoon that becomes hypersaline along the gradient due to evaporation. In recent decades, reduced freshwater inputs due to agricultural practices and anthropogenic barriers, coupled with climate driven increases in evaporation and decreases in rainfall, have resulted in increasingly hypersaline conditions within the lagoon (Lester and Fairweather, 2009). This has led to a shift in the biogeochemical status of the system with increased nutrient levels, acidification, and degradation of the overall ecological condition of the wetland (Lester and Fairweather, 2009; Kingsford et al., 2011). A better knowledge of the response of microbial communities to these conditions is essential from the perspective of both (i) ecosystem management and (ii) as a model to understand the effect of increased salinity levels on

microbially mediated biogeochemical cycles. While microbial and viral abundance and activity has been shown to increase along this salinity gradient (Schapira et al., 2009, Pollet et al., 2010; Schapira et al., 2010), the identity and metabolic potential of the bacteria that drive particular steps in a biogeochemical cycle have not been characterized in this system.

We conducted a metagenomic survey of the Coorong lagoon as a model for continuous natural salinity and nutrient gradients, and describe the shifts in gene content of sediment microbial metagenomes along the salinity gradient from marine to hypersaline conditions. This provides a model for how environmental gradients can drive shifts in the biogeochemically important metabolic processes involved in salinity tolerance and in taxonomic groups involved in photosynthesis and nitrogen cycling.

2 Materials and methods

2.1 Study sites and sample collection

Sampling was conducted at four reference stations along the Coorong lagoon, South Australia, in January 2008, during the Austral summer. Salinity varied by 99 practical salinity units (PSU) across stations. The sites were named by their salinity and defined by their GPS coordinates, which were as follows: 37 PSU (−35.551 S, 138.883 E), 109 PSU (−35.797 S, 139.317 E), 132 PSU (−35.938 S, 139.488 E) & 136 PSU (−36.166 S, 139.651 E). Ammonia concentrations at these sites ranged between 0.21 (±0.09) and 3.10 (±0.84) mg N/L, phosphate concentrations ranged between 0.05 (±0.01) and 0.27 (±0.09) mg P/L (Supplement Fig. S1). Heterotrophic bacteria and virus like particles in porewater, as determined by flow cytometry (Marie et al., 1995; Seymour et al., 2005), increased from 4.8×10^6 (± 6.3×10^5) to 1.5×10^8 (± 1.4×10^7) bacteria per ml and 1.5×10^7 (± 5.8×10^6) to 4.2×10^8 (± 3.1×10^7) viruses per mL along the salinity gradient (Supplement Fig. S1).

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At each site, 10 g of sediment, submerged in approximately 2 m deep water was sampled using a sterile corer. This equated to a core containing the upper 10 cm of sediment. Samples were stored on ice prior to DNA extraction which was performed within 8 h of collection.

2.2 DNA extraction and sequencing

DNA was extracted from 10 g of homogenized sediment using a bead beating and chemical lysis procedure (Powersoil, MoBio). Four shotgun metagenomic libraries were generated and sequenced using 454 GS-FLX pyrosequencing technology (Roche) at the Australian Genome Research facility. This sequencing yielded 68888 DNA sequences in the 37 PSU metagenome, 101003 sequences in the 109 PSU metagenome, 114335 sequences in the 132 PSU metagenome and 108257 sequences in the 136 PSU metagenome, with an average read length of 232 bp.

2.3 Bioinformatics and statistical analysis

Unassembled DNA sequences (environmental sequence tags) from each site were annotated using the MG-RAST pipeline (Meyer et al., 2008). MG-RAST implements the automated BLASTX annotation of DNA sequencing reads to the SEED non redundant database which is a database of genome sequences organized into cellular functions termed subsystems (Overbeek et al., 2005). Within MG-RAST, metabolic assignments were annotated to the SEED subsystems database (Overbeek et al., 2005) and taxonomic identification was determined based on the top BLAST hit to the SEED taxonomy. The SEED is organized in three hierarchical levels for metabolism and six for taxonomy and allows for data to be exported at each level. The heat map function of MG-RAST version 3.0 was used to display the normalized abundance of sequences matching different categories with the Euclidian distance between profiles being displayed as a ward-based clustering dendrogram. Taxonomic and metabolic reconstructions generated using MG-RAST version 2.0 with an E-value cutoff of 1×10^{-5}

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and a 50 bp minimum alignment length were imported into the STatistical Analysis of Metagenomic Profiles (STAMP) package to test for statistically significant abundance differences in taxonomic and metabolic groupings (Parks and Beiko, 2010). These were investigated at the second and third level of the MG-RAST metabolic hierarchy and the third level of the MG-RAST taxonomic hierarchy. Fisher's exact test was used to determine the most significantly different categories, with a Storey's FDR multiple test correction applied (Agresti, 1990; Storey and Tibshirani, 2003). Confidence intervals were determined using a Newcombe-Wilson method (Newcombe, 1998). Results were filtered to display only categories with a q-value of <0.05.

The Salinity tolerance of identified taxa were determined within the MEGAN software package (Huson et al., 2009) using the NCBI prokaryotic attributes table to display the results of a BLASTX search of our datasets against the NCBI non redundant database using CAMERA (Sun et al., 2011).

3 Results

3.1 Overall shifts in metagenomic profiles

To investigate the influence of salinity on the composition of the Coorong sediment metagenomes, we compared the abundance profiles of the metabolic potential (Fig. 1a) and the taxonomic identity of genes (Fig. 1b) sampled along the gradient. In both cases the metagenomic profiles demonstrated shifts in structure along the gradient. Metagenomes derived from hypersaline sites showed a higher degree of similarity to each other than to the 37 PSU metagenome for both function and taxonomic identity. The signature for metabolic potential was more conserved between samples than that for the phylogenetic identity of genes.

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3.2 Shifts in functional potential along the salinity gradient

We further investigated shifts in the functional gene content of microbial communities along the salinity gradient using STAMP (Parks and Beiko, 2010) to determine which finer level metabolic processes were statistically over-represented in the hypersaline metagenomes relative to the 37 PSU metagenome (Fig. 2). This was investigated at the second level of the MG-RAST metabolic hierarchy.

Genes responsible for the synthesis of cell membrane bound ABC transporter proteins, predominantly composed of branched chain amino acid and oligopeptide transporters (Fig. 3a), were over-represented in the hypersaline metagenomes (Fig. 2), as were ATP synthase enzymes (Fig. 2a and c) and pathways responsible for the cellular response to osmotic stress. Osmotic stress genes were primarily involved in the synthesis and transport of the osmoprotectants choline, betaine, ectoine and periplasmic glucans (Fig. 3b). DNA metabolism genes and the genes responsible for the metabolism of di- and oligosaccharide sugars were also significantly more abundant in the hypersaline metagenomes than in the 37 PSU metagenome.

Sequences related to photosynthesis and pigment synthesis were over-represented in all hypersaline metagenomes relative to the 37 PSU metagenome (Fig. 2). Specifically, the abundance of sequences matching tetrapyrrole synthesis (chlorophyll) and photosynthetic electron transport and photophosphorylation pathways were significantly higher in the hypersaline metagenomes than in the 37 PSU metagenome.

3.3 Shifts in taxonomic identity of genes along the salinity gradient

We further investigated the taxonomic identity of genes along the salinity gradient using STAMP (Parks and Beiko, 2010) to determine which finer level taxonomic groups were statistically different in abundance between the 37 PSU metagenome and the hypersaline metagenomes (Fig. 4, Supplement Fig. S2). The cyanobacterial classes Nostocales, Oscillatoriales and Chroococcales were found to be over-represented in the most hypersaline metagenome (136 PSU) relative to the 37 PSU sample (Fig. 4),

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as was the photoheterotrophic bacterial class Chloroflexi, which contains the green non-sulfur bacteria.

Several archaeal taxa were over-represented in the 109 PSU, 132 PSU and 136 PSU metagenomes relative to the 37 PSU sample. Of these, the class Methanomicrobia was the most over-represented in all cases. The halophilic class Halobacteria were over-represented in the 136 PSU and 109 PSU metagenomes showing the highest increase in proportion in the most hypersaline metagenome (136 PSU) (Fig. 4; Supplement Fig. S2).

We also observed shifts in the structure of the Proteobacteria. The class δ/ϵ -Proteobacteria were over-represented in hypersaline metagenomes, while the relative abundance of γ -Proteobacteria, β -Proteobacteria and α -Proteobacteria were significantly higher in the 37 PSU metagenome. The classes Bacteroidetes and Plactomycetacia were also strongly over-represented in the 37 PSU metagenome.

To investigate how these shifts in taxon abundance were reflected in the salinity tolerance of members of the microbial community, we used MEGAN (Huson et al., 2009) to summarize taxonomic assignments of sequencing reads in NCBI's microbial attributes table. We found that the proportion of reads matching moderate halophiles and extreme halophiles increased by 5 % and 6 % respectively, and that the total number of moderate and extreme halophilic taxa increased from 15 to 32 in the 136 PSU metagenome relative to 37 PSU metagenome (Fig. 5). Overall the majority of identifiable taxa in both of these communities were mesophilic and moderately halophilic.

4 Discussion

Our results comprise the first metagenomic survey of a model continuous natural salinity gradient and describe the shifts in gene content of sediment microbial metagenomes along the gradient from marine to hypersaline salinities. Overall shifts in the genetic composition of the metagenomes highlighted the substantial influence of salinity on the metabolic potential of microbial communities, which in turn has biogeochemical

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consequences. The most significant differences along the gradient can be categorized into two biogeochemically important categories: osmotic stress tolerance, via acquisition of compatible solutes, and photosynthesis. Our data allows us to form several new hypotheses relating to how microbial communities may respond to increasing salinity levels in the environment, and influence the biogeochemistry of salinity gradient habitats.

4.1 Salinity tolerance via compatible solute acquisition and its influence on carbon and nutrient cycling

Many of the metabolic pathways over-represented in the hypersaline metagenomes (Fig. 2) are potentially involved in cellular halotolerance. Microorganisms can overcome the osmotic stress caused by increased salt concentration by two mechanisms: the accumulation of KCl, which requires heavy modification of the enzyme content of the cell, or by accumulating organic compatible solutes which requires less proteomic modification and allows adaptation to a broad salinity range (Oren, 2008). It is this 'organic solutes in' strategy that seems most prevalent in our data. Osmotic stress functional categories were over-represented in hypersaline metagenomes and these were largely composed of pathways responsible for choline, betaine and ectoine transport and synthesis, and the acquisition of periplasmic glucans. These solutes are common osmoprotectants in halotolerant and halophilic microorganisms. In particular, ectoine and betaine are important osmolytes in a wide range of taxonomic groups (Oren, 2008; Roberts, 2005) and betaine is an important characteristic of halotolerant Cyanobacteria and other phototrophic bacteria (Welsh, 2000). Choline is a precursor for betaine synthesis and its concentration has been shown to be salt dependant in halophilic bacteria (Roberts, 2005; Canovas et al., 1998). Periplasmic glucans have been proposed to play a role as osmoprotectants in the cellular intermembrane space (Bohin, 2000).

Consistent with the osmoregulated accumulation of solutes, di- and oligosaccharide functional categories were over-represented in both hypersaline metagenomes (Fig. 2) and the biosynthesis of other sugars (galactoglycans/lipopolysaccharide) was

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also enriched in the most hypersaline metagenome. Many sugars act as osmoprotectants (Oren, 2008; Roberts, 2005) for example trehalose is a common compatible solute in a variety of halotolerant and halophilic microorganisms, and sucrose in halotolerant cyanobacteria and proteobacteria (Roberts, 2005). The presence of elevated sugar biosynthesis has the biogeochemical implications that microbially mediated cycling can occur at higher salinities and that there will be more energy available in the form of sugars to stimulate the metabolism of biogeochemically active heterotrophic bacteria.

Genes responsible for the synthesis of cell membrane bound ATP binding cassette (ABC) transporter proteins were over-represented in both hypersaline metagenomes and also potentially play a role in salinity tolerance. In our data, these enzymes were largely dominated by those involved in the transport of branched chain amino acids. Amino acids are common compatible solutes (Oren, 2008) and a branched chain amino acid ABC-transporter has been transcriptionally up-regulated during salt adaptation in the sediment bacteria *Desulfovibrio vulgaris* along with other ABC transporters responsible for betaine transport (He et al., 2010). The over-representation of sequences for ATP synthase enzymes is also potentially explained by halotolerance as these membrane bound pumps are up regulated in salt stressed yeast (Yale and Bohnert, 2001) and a novel form of this enzyme plays a role in salinity tolerance in halotolerant Cyanobacteria (Soontharapirakkul et al., 2011).

In addition to providing survivability to the increasing biomass present in the hypersaline samples, which is reflected in the increase in halotolerant and halophilic taxa along the gradient, the increased synthesis and uptake of compatible solutes also has direct consequences for the nutrient cycling and greenhouse gas emissions of the sediment. The extent to which compatible solute metabolism influences primary production and provides key substrates for heterotrophic nutrition is still to be determined (Oren, 2009), but the release of osmoprotectants via diffusion, lysis and grazing provides a significant source of carbon, nitrogen and sulfur to heterotrophic microorganisms (Welsh, 2000; Howard et al., 2006). This process appears to be particularly important

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in hypersaline sediments and mats where the utilization of high concentration glycine-betaine, trehalose and sucrose represent a significant carbon source for microorganisms and where glycine betaine can represent up to 20% of the total nitrogen of the surface layers (Welsh, 2000; King, 1988). The potentially increased catabolism of betaine is particularly significant in hypersaline sediment where anaerobic degradation of this compound may result in methane as an end product (Welsh, 2000). Additionally, the climate regulating gas dimethylsulfide (DMS) precursor dimethylsulfoniopropionate (DMSP) is a structural analogue to betaine and shares a cellular transport system (Welsh, 2000), thus the increased abundance of betaine transport potential with salinity could also result in an increase in the accumulation of this solute which is central to global scale climate and sulfur cycles. Thus, the observation that metabolisms related to compatible solute metabolism are over-represented in hypersaline metagenomes directly links the halotolerant metabolic potential of the community to global scale nutrient cycles and climate processes, and suggest that with increasing salinity, this influence will become further exaggerated.

4.2 Photosynthesis

The over-representation of sequences matching tetrapyrrole synthesis (chlorophyll) and photosynthetic electron transport and photophosphorylation pathways in the hypersaline metagenomes is consistent with the overrepresentation of Cyanobacteria in the 136 PSU metagenome. Cyanobacteria are abundant in hypersaline systems (Javor, 1989; Oren, 2002) particularly in the form of benthic microbial mats which drive primary productivity in hypersaline environments between 100 and 200 g l^{-1} salinity (Oren, 2009). The Cyanobacteria over-represented in our most hypersaline metagenome represent filamentous Cyanobacteria. Many taxa comprising mats are filamentous (Oren, 2002, 2009), however the sediment we sampled in this study did not show the laminated structure characteristic of cyanobacterial mats, but was sandy sediment dominated by non photosynthetic taxa. Our data indicate that increasing salinity could potentially increase the presence of filamentous Cyanobacteria without precipitating

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the transformation of porous sediment into laminated mats. Mats are associated with photosynthesis and nitrogen cycling but our results indicate that these processes occur significantly in sediments without the visual presence of stratified mat communities.

Salinity often co-varies with other parameters such as nutrient concentration and microbial/viral abundance (Schapira et al., 2009) thus other gradients in the system can be expected to influence the abundance of Cyanobacteria and determine their morphology, such as the increase in ammonia and phosphate concentration observed in our data (Supplement Fig. S1). Larger cells with small surface to volume ratios, such as colonial and filamentous cyanobacteria, preferentially grow at higher nutrient concentrations and find a niche when protozoan grazing is high (Cotner and Biddanda, 2002; Pernthaler et al., 2004). Reduced grazing due to high salinity also facilitates the development of stratified mats (Oren, 2009) however grazing is still prevalent in the hypersaline Coorong (Newton, personal communication, 2011) potentially limiting the formation of these structures, but favoring filamentous morphologies.

The increase of photosynthetic metabolisms and taxa in the most hypersaline metagenome (136 PSU) has implications for the exchange of nutrients and CO₂ between the benthic and pelagic systems within the lagoon. Photosynthetic microbial mats and similar environments release dissolved organic carbon and oxygen to the environment and act as a sink for CO₂ (Ford, 2007). Photosynthetic benthic surfaces also provide energy for nitrogen fixation in underlying sediments as well as capturing phosphorous and sulfur from the overlying water (Ford, 2007). Whilst the extent of these environments in the Coorong remain unknown and the overall influence of salinity on production rates and nutrient flux remains undetermined (Ford, 2007), our data indicate that this habitat could become more common with further increases in salinity, which have been predicted to occur in environments such as the Coorong due to climate change (Hughes, 2003), altering the primary productivity and nutrient levels of lagoons and potentially altering mineral precipitation via changes in DOC concentration (Javor, 1989).

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4.3 Concluding remarks

Our study comprises the first metagenomic characterization of a model hypersaline, continuous and natural salinity gradient and describes the shifts in gene content of sediment microbial metagenomes in the system. Shifts in the biochemical potential and identity of the microorganisms controlling the potential can be summarized as an increase in halotolerant and benthic photosynthetic forms with salinity. This data provides the first direct observation of an increase in genes responsible for the acquisition of compatible solutes in a natural hypersaline environment as opposed to in culture. The biogeochemical implications of an increase in compatible solute acquisition and increased benthic photosynthesis potentially represent important drivers of the ecosystem biogeochemistry. Given the ecological and biogeochemical importance of salinity gradients and increased pressure on these systems from climate change and its associated effects, understanding microbial adaptation to increasing salinity at the community level is crucial to predicting how the biogeochemistry of aquatic habitats will change over space and time.

Supplementary material related to this article is available online at:
[http://www.biogeosciences-discuss.net/8/7551/2011/
bgd-8-7551-2011-supplement.pdf](http://www.biogeosciences-discuss.net/8/7551/2011/bgd-8-7551-2011-supplement.pdf).

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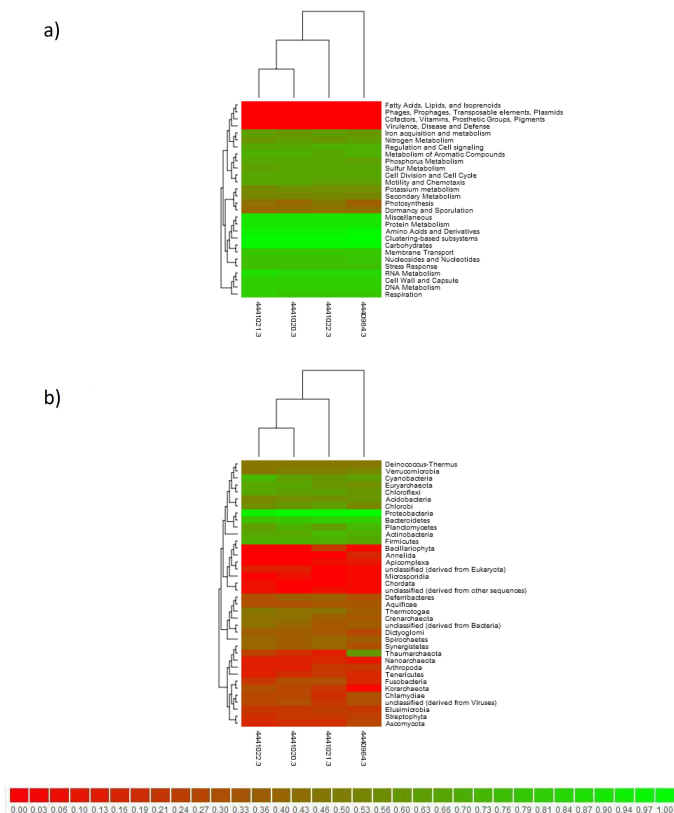


Fig. 1. Disimilarity between metagenomic profiles. **(a)** Functional potential **(b)** Taxonomic composition. 4440984.3 = 37 PSU, 4441020.3 = 109 PSU, 4441021.3 = 132 PSU, 4441022.3 = 136 PSU. Colour gradient represents proportion of sequences.

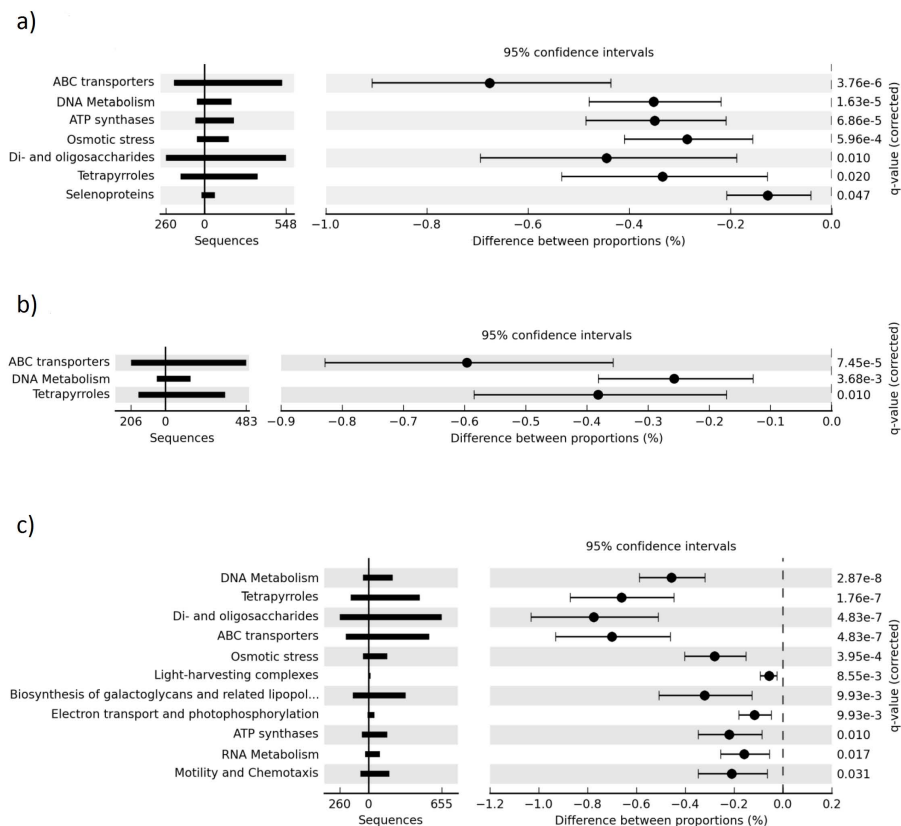


Fig. 2. Metabolic processes over-represented in hypersaline metagenomes relative to the 37 PSU metagenome. **(a)** 109 PSU **(b)** 132 PSU **(c)** 136 PSU. Corrected P-values were calculated using Storey's FDR approach.

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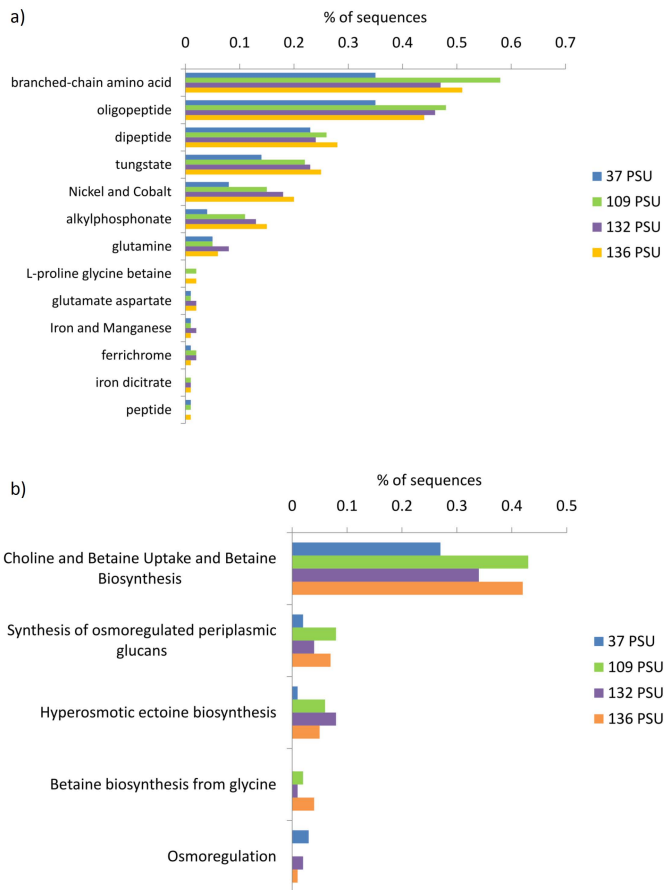


Fig. 3. Breakdown of subsystem contribution to **(a)** ABC transporter and **(b)** osmotic stress categories. Subsystems are the third level of organization within the MG-RAST hierarchy.

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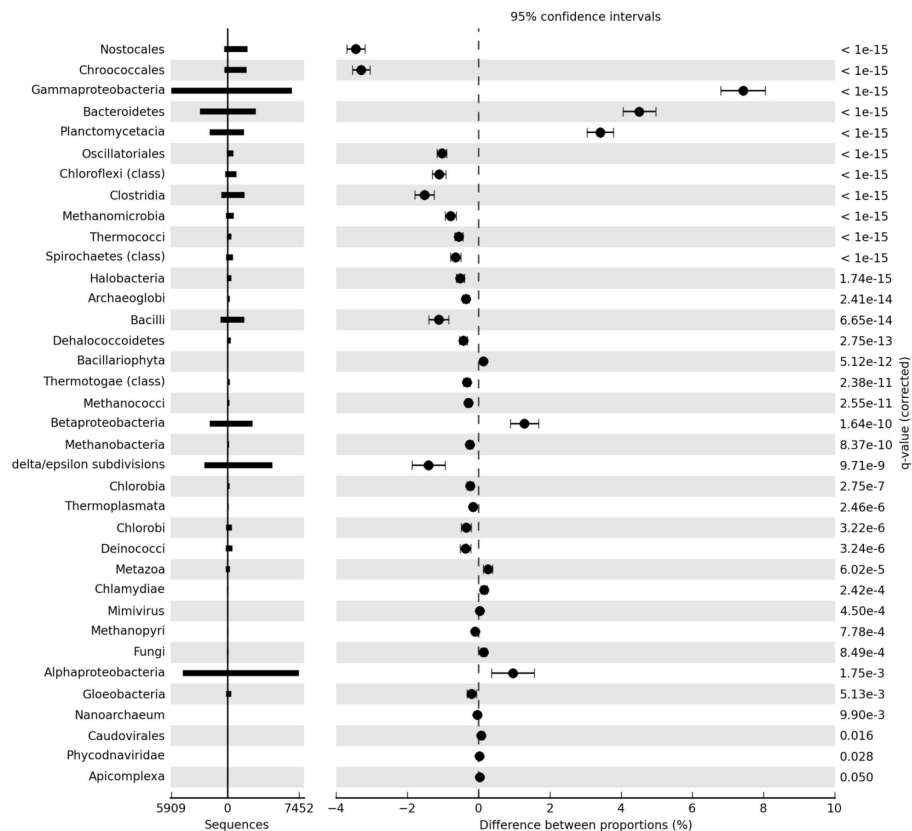


Fig. 4. Taxa enriched or depleted in the 37 PSU and 136 PSU metagenomes. Corrected P-values were calculated using Storey's FDR approach. Taxa enriched in the marine (37 PSU) metagenomes have positive differences between proportions.

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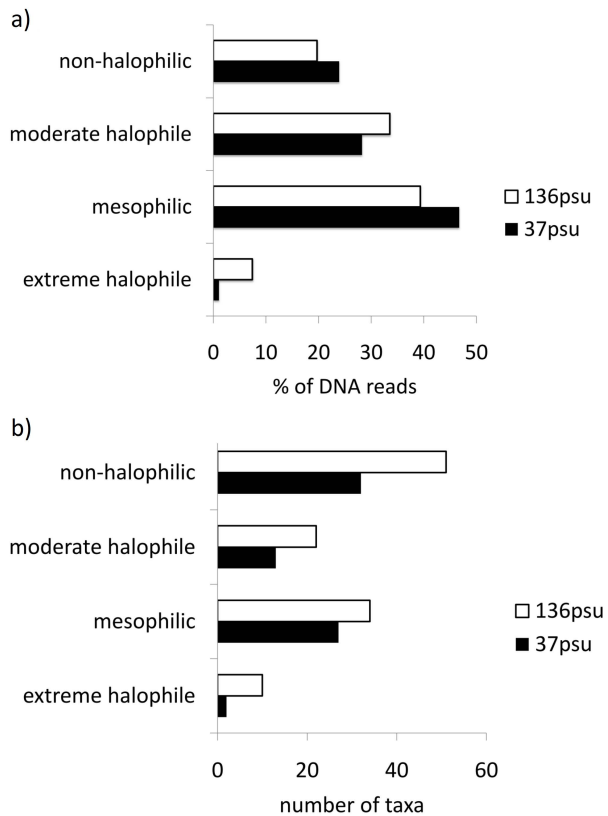


Fig. 5. Representation of halophilic taxa in the 37 PSU and 136 PSU metagenome. **(a)** % DNA reads matching taxa with a defined salinity tolerance **(b)** number of taxa with a defined salinity tolerance