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ORIGINAL ARTICLE

Unveiling microbial activities along the halocline of Thetis, a deep-sea hypersaline anoxic basin

Maria G Pachiadaki[1], Michail M Yakimov[2], Violetta LaCono[2], Edward Leadbetter[1] and Virginia Edgcomb[1]
[1]*Woods Hole Oceanographic Institution, Woods Hole, MA, USA and* [2]*CNR–Institute for Coastal Marine Environment, Messina, Italy*

Deep-sea hypersaline anoxic basins (DHABs) in the Eastern Mediterranean Sea are considered some of the most hostile environments on Earth. Little is known about the biochemical adaptations of microorganisms living in these habitats. This first metatranscriptome analysis of DHAB samples provides significant insights into shifts in metabolic activities of microorganisms as physicochemical conditions change from deep Mediterranean sea water to brine. The analysis of Thetis DHAB interface indicates that sulfate reduction occurs in both the upper (7.0–16.3% salinity) and lower (21.4–27.6%) halocline, but that expression of dissimilatory sulfate reductase is reduced in the more hypersaline lower halocline. High dark-carbon assimilation rates in the upper interface coincided with high abundance of transcripts for ribulose 1,5-bisphosphate carboxylase affiliated to sulfur-oxidizing bacteria. In the lower interface, increased expression of genes associated with methane metabolism and osmoregulation is noted. In addition, in this layer, nitrogenase transcripts affiliated to uncultivated putative methanotrophic archaea were detected, implying nitrogen fixation in this anoxic habitat, and providing evidence of linked carbon, nitrogen and sulfur cycles.
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Introduction

Microorganisms exhibit an incredible diversity of physiologies and behaviors that allow them to exploit virtually all habitats on our planet. These habitats include many that seemed uninhabitable, such as those characterized by physicochemical parameters with values close to the limits known to support life. Deep-sea hypersaline anoxic basins (DHABs) are considered to be some of the most hostile environments because of their combination of extreme physicochemical features, including nearly saturated salt concentration and corresponding low water activity, high hydrostatic pressure, anoxia and high sulfide concentration. Typical DHABs of Eastern Mediterranean are >3000 m below sea level; their origin is associated with the Miocene Messinian salinity crisis 5.59–5.33 million years ago. The genesis of the brine bodies on the sea floor of the Eastern Mediterranean Sea occurred 2000–176 000 years ago through either the *de novo* dissolution of buried Messinian evaporitic deposits or the ejection into seafloor depressions of ancient interstitial evaporated sea water entrapped in those deposits (Cita, 2006 and references therein). The extreme salinities (high densities) of DHABs act as a barrier to seawater mixing and salt diffusion, physically isolating them from other marine habitats, selecting for organisms adapted to multiple 'stressors' and probably preventing dispersal of those organisms. An interface layer, with a sharp oxycline, redoxcline and halocline, separates the hypersaline brine body and the oxygenated sea water.

Thus far, DHABs in the Mediterranean and Red Sea have provided exciting new insights into novel microbial diversity and have extended our knowledge of environmental factors that define the limits of life (Eder *et al.*, 1999, 2001; Hallsworth *et al.*, 2007). Studies of Discovery, L'Atalante, Urania, Thetis and Bannock basins revealed that DHAB interfaces harbor abundant and diverse microbial communities that include numerous novel candidate divisions that are more productive than most pelagic marine systems (see, for example, Sass *et al.*, 2001; Van Der Wielen *et al.*, 2005; Yakimov *et al.*, 2007; Edgcomb *et al.*, 2009; Stock *et al.*, 2012). Functional analyses of these communities based on PCR amplification of key functional genes as well as activity measurements revealed sulfur cycling and methanogenesis to be dominant prokaryotic metabolic processes supporting life in DHABs and contributing to observed elevated biomass in DHAB haloclines (Daffonchio *et al.*, 2006; Yakimov *et al.*, 2007; Borin *et al.*, 2009). Recent advances in RNA isolation, amplification and high-throughput

Correspondence: MG Pachiadaki or V Edgcomb, Woods Hole Oceanographic Institution, 266 Woods Hole Road, MS #8, 02543 Woods Hole, MA, USA.
E-mail: mpachiadaki@whoi.edu or vedgcomb@whoi.edu
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sequencing make it possible to acquire millions of sequences of transcribed genes from microbial communities. Metatranscriptomics is a powerful tool for examining microbial community activities that enables inferences about the relative expression of diverse metabolic pathways based upon transcript abundance as a proxy for gene expression (Frias-Lopez *et al.*, 2008; Stewart *et al.*, 2011).

Thetis, discovered in 2008, is among the best-studied deep-sea hypersaline basins. Chemical analysis of the brine body shows it is almost nine times more saline than sea water, with an ion composition reflecting late-stage evaporites. Based on this, the Thetis brine is argued to originate from ancient trapped interstitial brines rather than dissolution of halite (La Cono *et al.*, 2011). A pronounced stratification of both prokaryotic and eukaryotic communities was observed over the ~2 m thick interface, as was a distinction between halocline and brine communities (La Cono *et al.*, 2011; Stock *et al.*, 2012). A recent metagenomics study of the Thetis brine and halocline (Ferrer *et al.*, 2012) provided increased understanding of the metabolic potential of microbial communities of these habitats. However, [^{14}C]-bicarbonate assimilation rates and reverse transcriptase-PCR of a few functional genes in samples from the same Thetis habitats detected activities not suggested by the metagenome (La Cono *et al.*, 2011), including the expression of ribulose 1,5-bisphosphate carboxylase. Such inconsistency points to the need for metatranscriptomic information on a wider range of metabolic activities for a better understanding of community processes.

To elucidate active metabolic pathways of microorganisms in the Thetis interface, we applied whole community metatranscriptomics to samples from two distinct positions along the halocline that differ in salinity and oxygen concentration. This study presents the first transcriptome analysis of microbiota from a DHAB halocline, and provides the first insights of expressed genes other than ribosomal RNA from the eukaryotic microbial community.

Materials and methods

Sampling of Thetis halocline

Sampling of Thetis (34° 40.158 N, 22° 08.703 E) was conducted from the *R/V Urania* on 20 September 2012. The seawater/brine interface of Thetis is at a depth of 3258 m below sea level and the brine maximum thickness is ~157 m. The control sample (salinity 3.87% and oxygen 203 μmol l^{-1}) from 2222 m depth (36° 29.565 N, 15° 39.593 E) was sampled on 28 September. Samples were collected using 12 l Niskin bottles housed on a rosette (General Oceanics, Miami, FL, USA) equipped with a SBE- 911plus conductivity-temperature-depth sensor (Sea-Bird Electronics, Miami, FL, USA). Determination of oxygen concentration at selected depths was carried out using the Winkler method with an automatic end point detection burette (716 DNS Titrino, Metrohm AG, Herisau, Switzerland). Water from two distinct horizons (each representing a distinct range of salinity and oxygen between the top and bottom of each Niskin bottle) was collected from the Thetis interface; the upper interface (UI) layer corresponding to 7.0–16.3% salinity, and the lower (LI) layer with 21.4–27.6% salinity. Oxygen in the UI sample ranged from 9.5 μmol l^{-1} to undetectable, and remained undetectable in the lower sample. From each horizon, ca. 12 l of water was collected on Durapore membranes (47 mm, 0.65 μm, Millipore, Millford, MA, USA) under gentle pressure (~100 ml min^{-1}), using a peristaltic pump (Ecoline ISM 1079, Ismatec, Germany). The filters were stored in RNA Shield (Zymo Research, Orange, CA, USA) at −80 °C until analysis.

RNA extractions

Two replicate RNA extractions were processed per depth representing a total of 12 l of water per horizon studied. The liquid RNA Shield and the filters were extracted separately. The RNA Shield was transferred to 50 ml tubes and centrifuged at 4000 *g* for 3 min to remove particulates. An equal volume of 100% ethanol was added to the supernatant, mixed, transferred to an RNeasy Midi Kit (Qiagen, Hildesheim, Germany) column and processed following the manufacturer's recommendations. Contaminating DNA was removed by TURBO DNAse treatment (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA was purified with the MEGA Clear Kit (Life Technologies) as directed and suspended in 100 μl dH_2O.

The filters were transferred into Lysing Matrix E tubes (MP Biomedicals, Solon, OH, USA). Then, 4 ml of RNeasy Midi Kit Buffer RTL (Qiagen) was added, homogenized for 60 s at 4.0 m s^{-1} using a FastPrep-24 (MP Biomedicals) and centrifuged for 10 min at 4000 *g*. Liquids were transferred to clean tubes, 1 volume of 70% ethanol was added to each tube and extracts were processed following the RNeasy kit instructions. Extractions were treated with DNase and purified with the MEGA Clear kit as described above. Fluid and filter extracts were combined for each sample and concentrated by ethanol precipitation. Absence of DNA was confirmed by 40 cycles of PCR using the general bacterial small subunit ribosomal RNA (SSU rRNA) gene primer 8F and the universal primer 1492R. A control deep-sea oxic sample was processed in a similar manner. More details about the control sample site and the processing of this sample are described in Edgcomb *et al.* (2014).

For each extraction of total RNA (two replicates per depth horizon), complementary DNA was synthesized using the Ovation RNA-Seq System V2 Kit (NuGEN Technologies, San Carlos, CA, USA) following the manufacturer's instructions.

Finally, complementary DNAs were purified with the MinElute Reaction Cleanup Kit (Qiagen) and sent for paired-end sequencing. One lane of Illumina (San Diego, CA, USA) HiSeq 2×100 bp was requested for each sample (two replicates per lane). Sequences are deposited to the NCBI Short Read Archive (ID pending).

Analysis of transcriptome data

Forward and reverse reads were paired and filtered for quality control using CLC Genomics Workbench 5.0 (CLCBio, Cambridge, MA, USA) and a minimum quality score of 28, a minimum read length of 94 bp, allowing no sequences with ambigious nucleotides. The same platform was used to perform assembly of contigs and mapping of reads to contigs. The Rapid Analysis of Multiple Metagenomes with a Clustering and Annotation Pipeline (Weizhong, 2009) was used through the CAMERA platform (Sun *et al.*, 2011) to assign contigs to clusters of orthologous gene (COG) families, gene ontologies (GO) and protein families (Pfam) using four translation tables (Supplementary Table S1) and an *e*-value of 10^{-5}. Although the sequencing effort requested for each library was the same, different numbers of reads were recovered and survived quality trimming (Supplementary Table S2). To account for this variation (and the variation caused by difference in assembled reads and annotated contigs) and to compare abundances of transcripts among libraries, each library was normalized by expressing the number of annotated reads assigned to each function as a percentage of reads annotated to DNA-directed RNA polymerase subunit B (Stewart *et al.*, 2011).

The degree of variation in gene expression profiles observed between each of the two replicates for each interface layer as well as the variation in expression profile between the two interface layers were assessed using R (R Development Core Team, 2008) version 3.0.2, and the DEGSeq package using a MA-plot-based method with Random Sampling (Wang *et al.*, 2009).

The rRNA prediction and annotation was performed by BLASTn (Altschul *et al.*, 1990) against the SILVA 115 database (Quast *et al.*, 2013) with an *e* cutoff value of 10^{-10}, whereas the taxonomic affiliation (binning) of the functional genes was done by BLASTx (Altschul *et al.*, 1990).

Results and discussion

This is the first study using a metatranscriptome approach to examine microbial community activities within a DHAB habitat. The recently discovered Thetis DHAB was selected because a detailed description of the environmental setting, the diversity of its prokaryotes and eukaryotes, selected functional activities, and the genetic potential of the prokaryotic communities existed (La Cono *et al.*, 2011; Ferrer *et al.*, 2012; Stock *et al.*, 2012). For the present study we examined the UI, from 7.0% to 16.3% salinity, and dissolved oxygen from 9.5 μmol l^{-1} to 0, and the anoxic LI, with a salinity of 21.4% to 27.6%. The topography of Thetis Basin and the depth profiles of oxygen, salinity and sulfide are shown in Supplementary Figure S1. Additional environmental parameters and cell counts are included in Supplementary Table S3.

Relative transcript abundance does not necessarily correlate directly with *in situ* activity levels, because potential biases can be introduced at the nucleic acid extraction step and during sample recovery using Niskin bottles that expose water samples and the microorganisms in them to significant environmental changes before filtration and preservation. Comparisons of metatranscriptomes for closely located samples (in time and space) subjected to the same potential biases can still provide valuable insights into ecologically relevant processes within the targeted habitats (Moran *et al.*, 2013).

The taxonomic classification of partial gene transcripts is a major challenge for metatranscriptomic analyses. Several published taxonomic annotation tools were utilized, and given inconsistent annotations, we do not present taxonomic assignments for all metabolic gene transcripts. SSU rRNA reads were extracted from each data set to provide a separate taxonomic overview of the communities. Although our approach is free of PCR-related biases, relative abundances should be interpreted with caution because of differences in cell ribosome contents, potential biases at the level of RNA extraction and because our reverse transcription protocol intentionally biases against rRNA recovery. Consistency in the methodology applied to all samples, and good consistency observed between replicate samples for individual water features, enable us to compare results for the different water samples. The transcripts that showed significant ($P < 0.05$) difference expression levels between the UI and LI, as well as their normalized abundance in each one of the replicates, are reported in Supplementary Table S4.

Taxonomic composition of microbial communities along the interface of Thetis lake

The archaeal groups dominating UI rRNA transcripts were Marine Group I (Thaumarchaeota) followed by Halobacteriales (Euryarchaeota), whereas the highest abundance of reads in the LI was affiliated with Methanomicrobiales (Euryarchaeota) (Figure 1). Within bacterial rRNA reads, Proteobacteria were the most abundant in both halocline layers. Signatures of Deltaproteobacteria and Gammaproteobacteria were the most abundant in UI libraries, whereas gammaproteobacterial signatures dominated the LI (Figure 1). We recovered signatures of Mediterranean Sea Brine Lake (MSBL) groups MSBL-2 and MSBL-8 (affiliated with

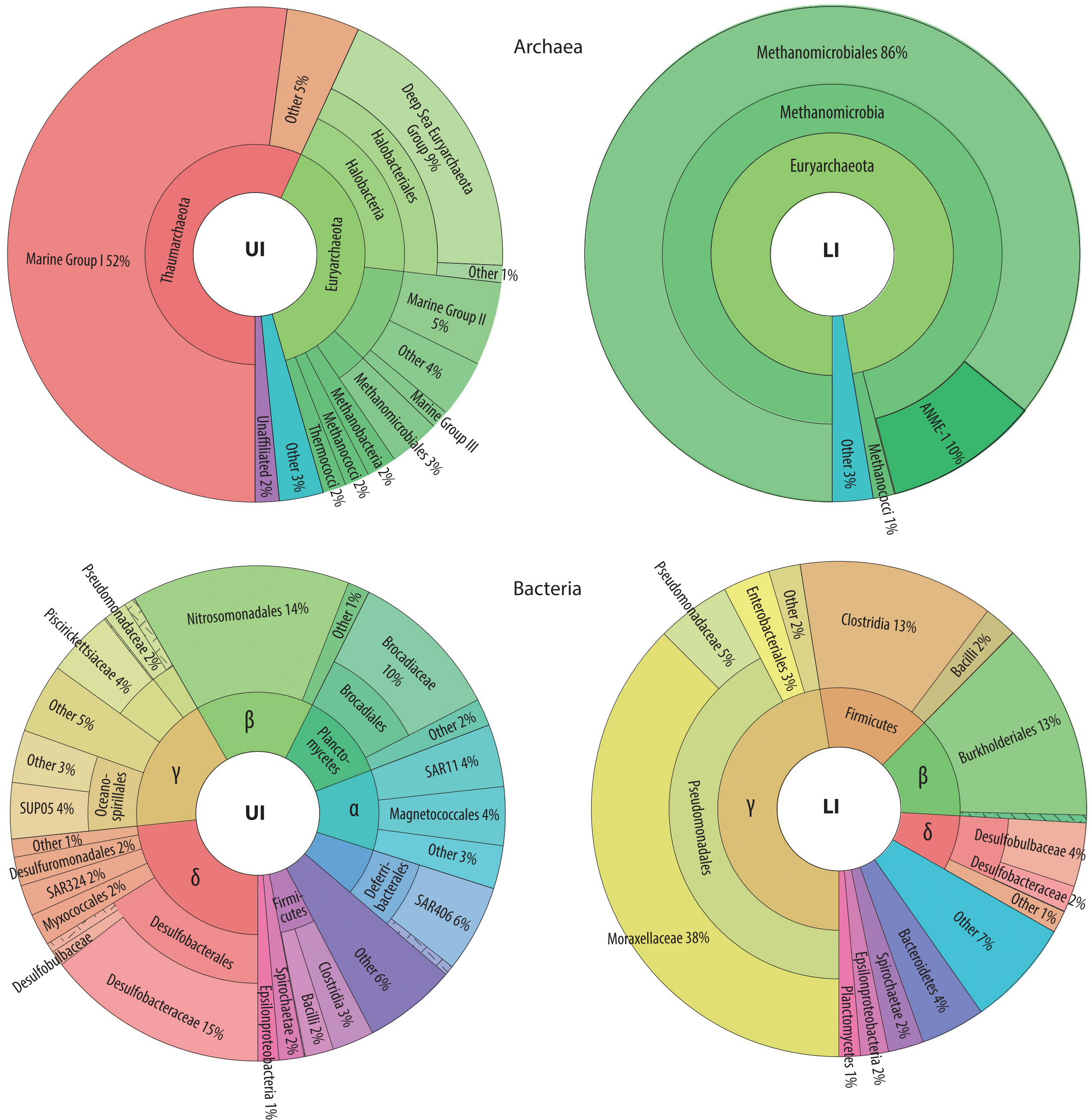

Figure 1 Relative abundance of rRNA reads of the major taxonomic groups of Archaea and Bacteria in the upper (UI) and lower (LI) interface of Thetis. 'Other' indicates taxa with relative abundance <1%.

Spirochaetes), MSBL-5 (affiliated with Chloroflexi), MSBL-6 (affiliated with Fusobacteria), MSBL-9 (affiliated with Planctomycetes) and MSBL-7 (affiliated with Deltaproteobacteria) from both interface layers, whereas MSBL-3 (affiliated with Lentisphaerae) signatures were only detected in the UI. Archaeal and bacterial rRNA profiles are generally consistent with previous studies (Yakimov *et al.*, 2007; Borin *et al.*, 2009; La Cono *et al.*, 2011), although there were some differences, including the low abundance of epsilonproteobacterial and Candidate Division KB1 signatures in our study. This may be attributed to differences in methodologies applied. A previous study of Thetis indicates that abundances (fluorescence in situ hybridization counts) of bacteria are higher than archaea in both UI and LI, 13.5 and 5.7 times, respectively (Supplementary Table S1).

The majority of unicellular eukaryotic reads in the UI were assigned to Ciliophora, whereas the LI was co-dominated by ciliate and fungal signatures (Figure 2). Euglenozoan, jakobid, rhizarian and

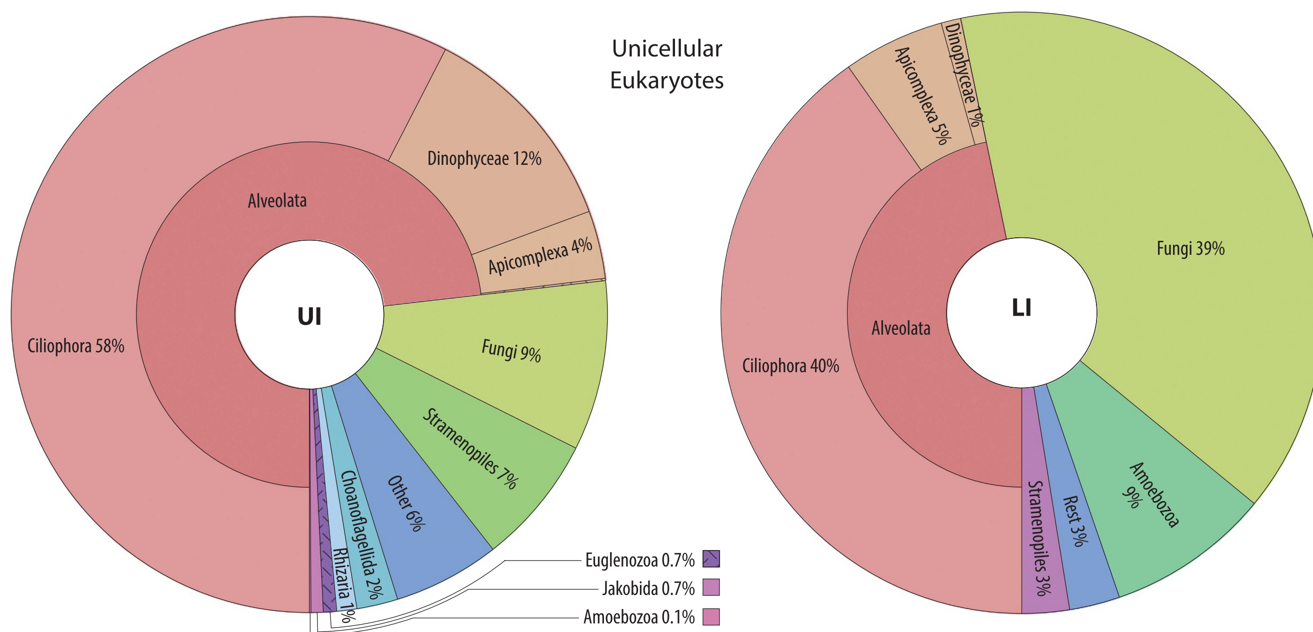

Figure 2 Relative abundance of the rRNA reads of the major taxonomic groups of the unicellular eukaryotes in the upper (UI) and lower (LI) interface of Thetis.

choanoflagellate signatures were only detected in the UI, indicating a decrease in diversity in the anoxic and more hypersaline layer. Other studies have found ciliates to dominate the eukaryotic microbial community in upper DHAB haloclines (Alexander *et al.*, 2009; Edgcomb *et al.*, 2009), but we observed a decrease in the relative abundance of ciliate rRNA as environmental conditions become more challenging in the LI, where Fungi appear equally abundant.

Autotrophy (CO_2 fixation)

In contrast to a previous metagenome study of Thetis interface (Ferrer *et al.*, 2012), we detected transcripts for ribulose 1,5-bisphosphate carboxylase (RuBisCo), the indicative enzyme for the Calvin–Benson–Bassham cycle (CBB) in the UI. Transcripts for RuBisCo were also retrieved to a lesser extent from the LI (Figure 3a). UI transcripts were assigned to sulfur-oxidizing chemolithotrophs (Supplementary Table S5). Transcripts encoding RuBisCO were reported in the L'Atalante halocline, a DHAB with similar ionic concentrations (Yakimov *et al.*, 2007), in the UI of Thetis (La Cono *et al.*, 2011), and RuBisCO genes were detected in the athalassohaline Discovery basin (Van Der Wielen, 2006). Even though not predicted by the metagenome study of Thetis, the CBB cycle appears to be an important carbon fixation pathway in the UI. Elevated expression of RuBisCO coincides with high abundances of rRNA transcripts affiliated with *Thiomicrospira* and unassigned genera of the family Piscirickettsiaceae, as well as the SUP05 group within Gammaproteobacteria. RuBisCO-mediated CO_2 fixation is known among *Thiomicrospira* species (Dobrinski *et al.*, 2005) and SUP05 (Jost *et al.*, 2008; Walsh *et al.*, 2009), and hence these taxa are likely among the key carbon fixers in the upper Thetis interface. The binning of RuBisCO contigs from the anoxic LI indicated they were derived from different (compared with the UI) groups. Only two groups were highly similar to known taxa, one to a methylotrophic, methanogenic archaeon and the second to an uncultivated bacterium from saline soils (Supplementary Table S5). Because carbon fixation activity was not detected in the LI (La Cono *et al.*, 2011), a functioning RuBisCO involved in carbon fixation using nitrate is unlikely. These transcripts may be participating in adenosine 5'-monophosphate metabolism, a distinct function of classical RuBisCO (Sato *et al.*, 2007). The CBB cycle is considered to be important in the mesopelagic and bathypelagic dark ocean (Swan *et al.*, 2011). The high abundance of RuBisCO transcripts found in our deep-sea control data set supports this.

CO dehydrogenase/acetyl-CoA synthase catalyzes the reversible reduction of CO_2 to CO and the subsequent synthesis/cleavage of acetyl-CoA (Pezacka and Harland, 1984) and is used as an indicator of autotrophic carbon fixation through the reductive acetyl-CoA (r acetyl CoA) pathway (Hügler and Sievert, 2011). In the control sample only the corrinoid Fe-S (delta) subunit was detected, whereas transcripts for all subunits of this enzyme complex were found in the UI and the LI. The majority of detected contigs in the UI were affiliated to bacteria, two to the metagenomes of uncultured Anaerobic Methane oxidizers Group 1 (ANME-1) and one to the methanogen *Methanoregula formicica* (Supplementary Table S5).

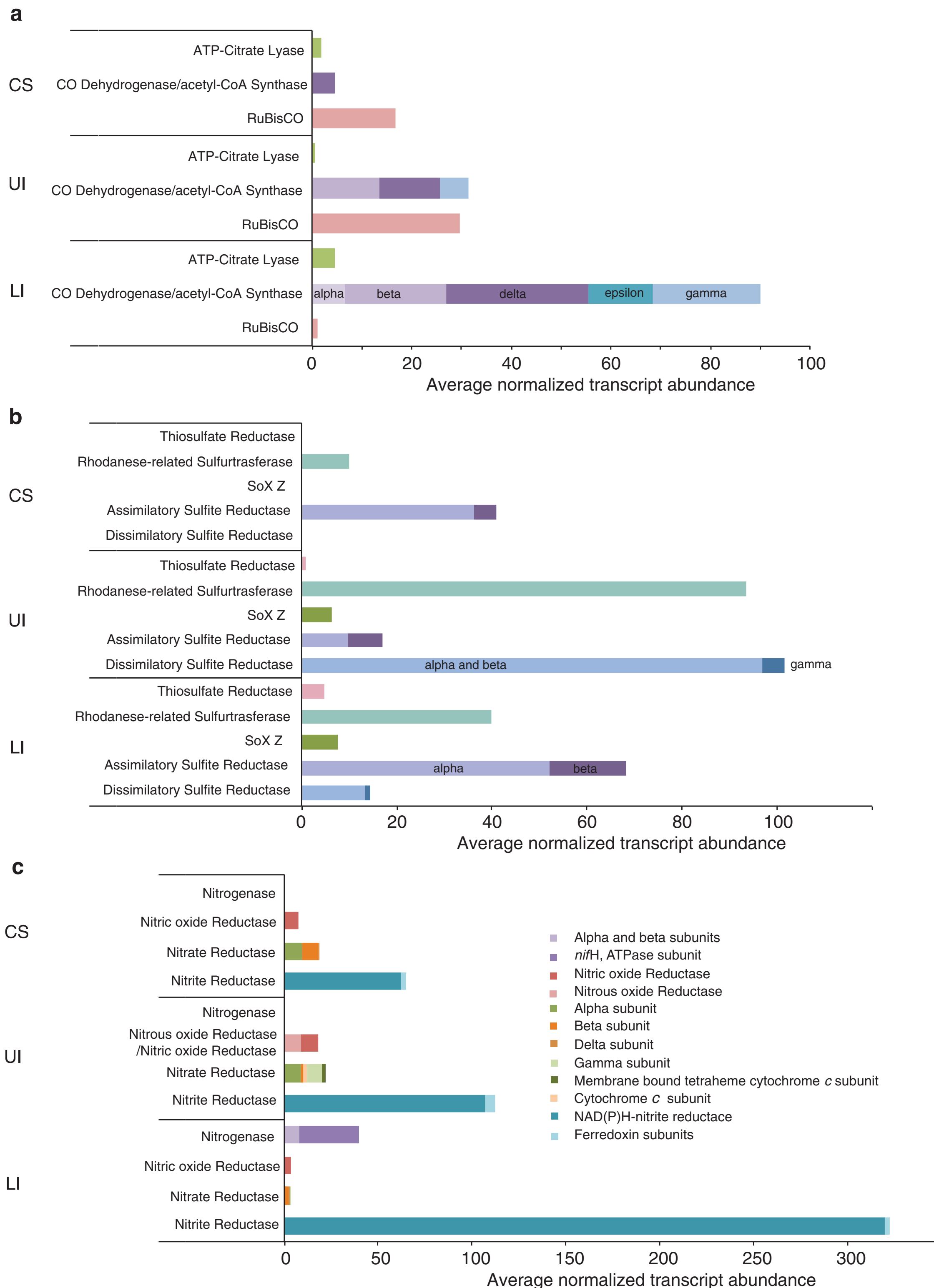

Figure 3 Average normalized transcript abundance of genes involved in the (**a**) carbon cycle (**b**) sulfur cycle and (**c**) nitrogen cycle. CS, control sample. For multi-unit enzymes in (**a**, **b**), each color represents a different subunit and the name of the subunit is indicated.

The bacterial contigs are closely related to taxa of the sulfate-reducing Desulfobacteracea family (*Desulfospira*, *Desulfotignum*, *Desulfobacula* and *Desulfococcus*). The CO dehydrogenase/acetyl-CoA synthase can be operating in either the oxidative heterotrophic or reductive CO-dehydrogenase pathway in these taxa (Kuever *et al.*, 2001). In the LI, the majority of the CO dehydrogenase/acetyl-CoA synthase contigs were closely related (Supplementary Table S5) to the ANME-1 from the anaerobic oxidation of

methane (AOM) system in Eel River Basin (Hallam *et al.*, 2004).

Evidence for the presence of an active reductive tricarboxylic acid cycle was inconclusive. No contigs encoding the key enzyme 2-oxyglutarate synthase were detected, and only a few contigs for the other key enzyme, ATP-citrate lyase, were retrieved. No contigs for key enzymes of either 3-hydroxypropionate/4-hydroxybutyrate or 3- hydroxypropionate bicycle were found.

Methanogenesis and AOM

Although methanogenesis was thought to be a predominant process occurring in DHAB anoxic habitats (Borin *et al.*, 2009), the Thetis metagenome did not suggest this (Ferrer *et al.*, 2012). However, the transcriptome shows that the key enzyme of methanogenesis/AOM, methyl coenzyme M reductase, was highly expressed in both interface layers. This enzyme catalyzes the final reaction in biogenic methane production and its amino acid sequence is highly conserved among all methanogenic archaea (Ellermann *et al.*, 1988; Grabarse *et al.*, 2000). In AOM it is presumed to function in reverse (Hallam *et al.*, 2003; Krüger *et al.*, 2003; Wang *et al.*, 2013). Transcripts for all subunits were significantly more abundant in the LI (Supplementary Figure S2), and were absent in the oxic control sample. Sequences exhibited high identity with methyl coenzyme M reductase sequences from the methanotrophic group ANME-1 (Supplementary Table S5). The absence of transcripts for coenzyme F_{420}-dependent N_5,N_{10}-methenyltetrahydromethanopterin dehydrogenase, which is lacking in ANME (Hallam *et al.*, 2004), supports the hypothesis that ANMEs, performing AOM, are the key players here. The ANME-1 archaea were assumed to be obligatory methanotrophs, but recent studies provide evidence of co-occurrence of AOM and methanogenesis (Lloyd *et al.*, 2011; Bertram *et al.*, 2013; Wang *et al.*, 2013). ANMEs were shown to assimilate inorganic carbon (Kellermann *et al.*, 2012), but also to be capable of growing on acetate, pyruvate or butyrate using thiosulfate as an electron acceptor (Jagersma *et al.*, 2012). The acetate-metabolizing potential in ANME-2a was supported by the identification of the ion-motive electron transport complex Rnf that is independent of H_2 (Wang *et al.*, 2013). Previous studies of microbiota in this and other DHABs suggest autotrophy is precluded by high salinity in the LI and brine (Yakimov *et al.*, 2007; La Cono *et al.*, 2011); hence, the ANME community may function through a pathway that does not involve assimilation of inorganic carbon.

The sulfate concentration in Thetis brine is 10-fold higher than in sea water (La Cono *et al.*, 2011). Although ANME-1 archaea have been found most frequently in sulfate-depleted environments (Yanagawa *et al.*, 2011), they are known to be adapted to hypersalinity (Lloyd *et al.*, 2006; Maignien *et al.*, 2013). This suggests salinity is a stronger selective force for this group than sulfate concentration.

Metabolism of sulfur compounds

High expression of sulfite reductase (assimilatory type) transcripts was observed in the LI, whereas in the UI expression appeared reduced relative to the LI and even relative to oxic deep-sea water (Figure 3b). Transcripts from all other enzymes in the assimilatory sulfate reduction pathway, ATP sulfurylase, 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase (PAPS reductase) and adenosine-5'-phosphosulfate-3'-phosphokinase (APS kinase), were detected in the LI. Evidence for dissimilatory sulfate reduction was apparent in both interface samples but not in the control deep-sea oxic sample. The abundance of dissimilatory sulfite reductase (desulfoviridin) transcripts was higher in the UI, in parallel with high abundance of rRNA transcripts from sulfate-reducing Deltaproteobacteria. The most represented family in the UI was Desulfobacteraceae that was reported previously in DHAB interfaces (Van Der Wielen and Heijs, 2007; Borin *et al.*, 2009); their members are believed to be oxygen tolerant. In the LI, expression of dissimilatory sulfite reductase and rRNA of deltaproteobacterial sulfate reducers was reduced (Figure 3b). The Desulfobulbaceae appear to be the primary sulfate reducers in the LI. This family is known to be present in both interfaces and brines of DHABs (Van Der Wielen and Heijs, 2007; Borin *et al.*, 2009) and other hypersaline environments (Ollivier *et al.*, 1991; Kjeldsen *et al.*, 2007).

Only low numbers of reads mapped to contigs encoding enzymes from the Sox (sulfur oxidation) enzyme system in interface samples, and these were not detected in the oxic control sample. Signatures of putative epsilonproteobacterial sulfide and sulfur oxidizers were in low abundance, but putative sulfide/sulfur-oxidizing Gammaproteobacteria were well represented, and were found to be involved in CO_2 fixation through the CBB cycle in the UI. Sulfide/sulfur oxidation may be underestimated as this process is likely taking place in the uppermost layers of the interface where dissolved oxygen is available. The UI examined here corresponds to salinity 7.0–16.3% and oxygen content of 9.5 to 0 $\mu mol\, l^{-1}$. We may have missed the peak expression of sulfide oxidation. Detected Epsilonproteobacteria were mostly affiliated with *Arcobacter* (Campylobacteriaceae) and *Sulfurimonas* and *Sulfurovum* (Helicobacteraceae). These taxa have been detected in both oxic and anoxic DHAB layers previously, and their potential role, apart from sulfur transformations, was thought to be associated with manganese reduction (Borin *et al.*, 2009). Finally, evidence for nonreducing thiosulfate cleavage driven by RhoD rodanese-like thiosulfate:cyanide sulfurtransferases was detected in both interface layers as well as

evidence for the reductive thiosulfate/thiosulfonate dismutation to sulfide by MopB-like thiosulfate reductases.

Metabolism of nitrogen compounds

The presence of transcripts for several different pathways within the nitrogen cycle was examined (Figure 3c). Transcripts indicative of denitrification and nitrous and nitric oxide reductases were detected in all samples. Expression levels in the LI were lower than in the UI and control sample. Similarly, transcripts for nitrate reductase subunits were less abundant in the LI compared with both UI and control samples. The abundance of NAD(P)H-nitrite reductase transcripts showed an increase from the oxic deep sea to the LI. Finally, transcripts for nitrate/nitrate transporters and ammonia permeases were more abundant in the interface samples compared with the control.

No evidence for ammonia oxidation—apart from the high abundance of MG-1 Thaumarchaeota—or anaerobic ammonium oxidation (anammox) was found in either the UI or LI of Thetis. Anammox was shown to be active in the Black Sea oxycline (Kuypers *et al.*, 2003) and in Atalante DHAB upper interface only up to 9.2% salinity (Borin *et al.*, 2013), but no evidence of anammox activity was observed in the Thetis UI metatranscriptome. The redoxycline/halocline is so sharp that anammox might be active in an upper interface layer, but not captured.

Transcripts for the nitrogenase complex (α and β chains, as well as the ATPase component) were exclusively detected in the LI. This is the first time that evidence for nitrogen fixation activity in a DHAB habitat is reported. In ammonium-rich environments such as the DHAB interfaces and brines (Daffonchio *et al.*, 2006), dinitrogen fixation is expected to be inhibited (Madigan *et al.*, 2008), but some organisms are capable of maintaining high N_2 fixation rates in the presence of a high level of ammonium. This was observed in isolates (Okoronkwo *et al.*, 1989) and in natural populations from deep anoxic waters of the Baltic Sea (Farnelid *et al.*, 2013). The binning of nitrogenase transcripts from the LI were to Archaea, including methanogens and ANME. Methanogens are considered to be among the nitrogen-fixing free-living anaerobes (Madigan *et al.*, 2008), and recently it was demonstrated that deep-sea ANME from ammonium-replete sediments fix N_2 and share the products of nitrogen fixation with sulfate-reducing bacterial partners (Dekas *et al.*, 2009; Offre *et al.*, 2013). We do not have an explanation for why methanogens/ANME compensate for the energetic burden of diazotrophy in an ammonium-rich environment. In the LI, the high relative abundance of transcripts for ammonia permease indicates that other pathways of nitrogen assimilation co-occur. It is possible that LI prokaryotes are fixing nitrogen and assimilating ammonia in order to synthesize osmoprotectants via glutamine synthase and glutamate synthase pathways.

Osmolytes

Two strategies are used by microorganisms for coping with the osmotic stresses associated with low water activity in hypersaline environments. They can achieve osmotic stability using inorganic ions such as KCl or, more commonly, microorganisms can produce and accumulate low-molecular-mass osmolytes. These include organic compounds such as amino acids and derivatives, alcohols, polyols (for example, sugars, manitol, arabitol, glycerol) and their derivatives, betaines and thetines and ectoines that protect enzymes and macromolecular structures from inactivation, inhibition and denaturation under low water activity (Grant, 2004; Stan-Lotter and Fendrihan, 2013).

These first insights into strategies for osmoprotection in DHABs via osmolyte synthesis show that differential expression of putative osmoprotectants, such as glutamine, glutamate, proline, ectoine and 2-sulfotrehalose, was detected in halocline vs control samples (Supplementary Figure S3). Glutamine synthetase transcript abundance was similar in the deep-sea oxic water sample and in the UI, but showed an increase in the LI, suggesting this mechanism becomes increasingly important as salinity increases. The same pattern was observed for proline biosynthesis pathway transcripts. Significantly higher abundance of proline biosynthesis pathway transcripts for pyrroline-5-carboxylate reductase, glutamate 5-kinase and glutamate-5-semialdehyde dehydrogenase were observed in the LI vs UI. Transcripts for choline dehydrogenase, a key enzyme for glycine betaine biosynthesis, and for lysine 2,3-aminomutase showed a similar pattern. Consistent with synthesis of putative osmoprotectants, abundances of transcripts for osmoregulator transporters, including the choline–glycine betaine transporter, were higher in the LI. Glutamate synthase expression was higher in both interface layers compared with the control sample. Among sugar and polyol compounds that are also used as osmoprotectants, transcripts of trehalose-6-phosphate synthase were 10 times more abundant in the LI than in the UI and the control. Similarly, myo-inositol-1-phosphate synthase was expressed at levels 20 times higher in LI relative to UI. These findings indicate that multiple adaptive mechanisms for coping with high salinity are utilized by microorganisms inhabiting the UI and LI, and that expression of associated genes increases with salinity along the halocline.

Ion transporters

Transcripts for several types of Na^+ and K^+ transporters were recovered that can provide insight

about strategies for osmoprotection via regulation of ion concentrations. Most were more abundant in the interface layers relative to the oxic deep-sea water. Nha-type Na^+/H^+ antiporter and K^+ transporter transcripts were more expressed in the UI relative to the control (Supplementary Figure S4A). In both LI and UI, expression of the NhaC Na^+/H^+ antiporter was similar, but a substantial increase in expression of the NhaP and Mnh systems was observed in the LI, as was expression of all K^+ transport systems, particularly Kdp (Supplementary Figures S4A and B). This implies that LI communities adapt to hypersalinity using the 'salt-in', rather than 'salt-out' adaptation strategy, consistent with generally accepted statements about energetic constraints associated with life at high salinity (Oren, 2013). Ion concentrations are two to four times higher than in sea water in the UI, and seven- to eight-fold higher in the LI. Different groups of organisms are probably using different osmoregulation mechanisms, but at the community level both strategies, 'salt-in' and osmolyte synthesis, seem to be active in DHAB interfaces. Finally, transcripts of Rnf were enriched in the LI (~150 times higher than in sea water). The presence of sodium-motive ferredoxin:-NAD oxidoreductase (Rnf) subunits may indirectly indicate the presence of organisms capable of completely relying on a sodium ion potential for energetic reactions (Biegel *et al*., 2011; Poehlein *et al*., 2012). The Rnf membrane-associated electrogenic antiporter is apparently involved in generating sodium ion gradients. This gradient may be further used for ATP synthesis under highly reduced conditions at redox below −320 mV.

Eukaryotic signatures

As we did not base our metatranscriptome libraries on exclusively polyA-selected transcripts, it is difficult to unequivocally assign many metabolic gene transcripts to eukaryotes as many have close homologs in prokaryotes. We therefore refrain in this study from reporting on specific eukaryotic metabolic activities. To assess their relative activity in the Thetis UI and LI, transcripts for two structural proteins were used as indicators. Actin and tubulin proteins from eukaryotes have only weak amino acid similarity compared with the prokaryotic cytoskeletal proteins (Desai and Mitchison, 1998). The abundances of transcripts were more than four times higher in the UI compared with the control site, whereas there was a significant drop of the abundances in the LI (Supplementary Figure S5). This is in accordance with a previous study (Edgcomb *et al*., 2011b) reporting high numbers of unicellular eukaryotes in the UI layers of DHABs. The LI, a high-saline totally anoxic habitat, appears to be more challenging for eukaryotes.

Extremely halophilic heterotrophic protists that are distinct from marine or freshwater forms have been observed in solar salterns with salinity of 29% (Cho *et al*., 2008). A diversity of other heterotrophic protists were isolated from nearly saturated brines (⩾30% salinity) that could not grow at salinities of <7.5% (Cho *et al*., 2008; Park *et al*., 2006; Park *et al*., 2007, 2009). Based on SSU rRNA gene transcripts recovered within our metatranscriptome libraries, the eukaryotic community appears to be dominated by ciliates and dinoflagellates in the UI, and by ciliates and fungi in the LI (Figure 2). Previous studies of DHABs, including Thetis, based on SSU rRNA and microscopic observations have detected heterotrophic protists (particularly ciliates, dinoflagellates and fungi) in up to 36% salinity (see, for example, Alexander *et al*., 2009; Edgcomb *et al*., 2009; Stock *et al*., 2012). Phagotrophic protists are known to be successful along pelagic oxyclines where prokaryotes are abundant (see, for example, Behnke *et al*., 2006; Edgcomb *et al*., 2011a) and hence it follows that they are also successful within these haloclines. Fungi are active remineralizers of organic material, and given the known accumulation of organic detritus at these haloclines, and their known presence in other types of hypersaline habitats such as solar salterns (for example, Cantrell *et al*., 2006), it is not surprising that their signatures dominate in the lower halocline.

Recovery of transcribed genes from a slightly broader taxonomic representation of eukaryotes in the UI vs LI is consistent with previous findings of rich and diverse communities in moderately saline systems (Hauer and Rogerson, 2005). Signatures from apicomplexans and stramenopiles were also found in the UI and LI, and unique to the UI, we detected signatures from jakobids, euglenozoans and choanoflagellates. All these groups have been detected in previous SSU RNA- and DNA-based molecular surveys of DHABs (for overview see Edgcomb and Bernhard, 2013). Further elucidation of the specific adaptive strategies and ecological roles and impacts on nutrient cycling of these protists is a fascinating avenue for future research.

Conclusion

In this first metatranscriptomic study of a deep-sea redoxcline/halocline, we find that microbial communities of the upper—hypoxic to anoxic—halocline are shaped by transformations of sulfur compounds and inorganic carbon assimilation, occurring primarily through the CBB cycle. In the lower anoxic, more saline habitat, it is likely that AOM rather than methanogenesis may be the dominant process. Transcripts for dinitrogen fixation were detected in the same habitat, where ammonium concentrations are considered prohibitive for such a process. Laboratory incubations of ammonium-replete CH_4 seep sediments demonstrated that methanotrophic archaea are capable of dinitrogen fixation (Dekas *et al*., 2009). This finding,

although it requires confirmation, suggests new sources of fixed nitrogen in nontraditional, 'unexpected' marine habitats, linking the function of carbon, nitrogen and sulfur cycles.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

MGP and VE designed the study and collected the samples. MGP conducted data analysis and manuscript preparation with input from EL and VE. VL performed chemical analyses reported here. MMY was the chief scientist on the *R/V Urania* cruise, and assisted with sample collection and manuscript preparation.

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