



# Archive of bacterial community in anhydrite crystals from a deep-sea basin provides evidence of past oil-spilling in a benthic environment in the Red Sea

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**Abstract.** In deep-sea sediment, the microbes present in anhydrite crystals are potential markers of the past environment. In the Atlantis II Deep, anhydrite veins were produced by mild mixture of calcium-rich hydrothermal solutions and sulfate in the bottom water, which had probably preserved microbial inhabitants in the past seafloor of the Red Sea. In this study, this hypothesis was tested by analyzing the metagenome of an anhydrite crystal sample from the Atlantis II Deep. The estimated age of the anhydrite layer was between 750 and 770 years, which might span the event of hydrothermal eruption into the benthic floor. The 16S/18S rRNA genes in the metagenome were assigned to bacteria, archaea, fungi and even invertebrate species. The dominant species in the crystals was an oil-degrading *Alcanivorax borkumensis* bacterium, which was not detected in the adjacent sediment layer. Fluorescence microscopy using 16S rRNA and marker gene probes revealed intact cells of the *Alcanivorax* bacterium in the crystals. A draft genome of *A. borkumensis* was binned from the metagenome. It contained all functional genes for alkane utilization and the reduction of nitrogen oxides. Moreover, the metagenomes of the anhydrites and control sediment contained aromatic degradation pathways, which were mostly derived from *Ochrobactrum* sp. Altogether, these results indicate an oxic, oil-spilling benthic environment in the Atlantis II basin of the Red Sea in ap-

proximately the 14th century. The original microbial inhabitants probably underwent a dramatic selection process via drastic environmental changes following the formation of an overlying anoxic brine pool in the basin due to hydrothermal activities.

## 1 Introduction

Deep-sea sediment is among the least explored biospheres on Earth. Indigenous microbes differ vastly in community composition and metabolic spectra at different depths and sites (Orcutt et al., 2011; Teske and Sorensen, 2007). The distribution of microbes in subsuperficial sediments is determined by the porosity, nutrient availability and geochemical conditions of the sediment (Parkes et al., 2000; Webster et al., 2006). In return, genomic features and the community composition of the indigenous microbial inhabitants may reflect the in situ conditions and serve as biomarkers containing the geochemical indicators. However, most of the biomarkers cannot be well preserved and will be degraded by biological and abiological activities. Although lipids and other organic carbons present in some minerals allow the interpretation of microbial activities to some extent (Brocks et al., 2005), the orig-

inal metabolic activities are difficult to retrieve in a comprehensive and precise manner.

Most of the dead microbes are damaged during the sedimentation process, but some can be maintained in almost their original shape (Taher, 2014; Benison et al., 2008). Evaporites, which mostly consist of halite and anhydrite ( $\text{CaSO}_4$ ) or gypsum ( $\text{CaSO}_4 \times 2\text{H}_2\text{O}$ , temperature  $< 38^\circ\text{C}$ ; Hill, 1937), are common microbialites with accretionary organosedimentary structures (Dupraz et al., 2011). Numerous dead bacteria, algae and metazoans have been detected in gypsum granules (Petrash et al., 2012; Trichet et al., 2001); bacterial mats growing on evaporites may become trapped and constitute much larger microbialites (Babel, 2004). Consequently, microbial inhabitants on the benthic surface may get trapped in the evaporites (Benison et al., 2008). Anhydrite facies are not found throughout deep-sea sediments. They usually form around hydrothermal vents in deep-sea environments (Janasch and Mottl, 1985). A strong deep-sea volcanic eruption may break the crustal basalts, resulting in a drastic emission of hydrothermal gases followed by the crystallization of anhydrites and the deposition of metal sulfides (Kristall et al., 2006). An alternative model is that mild hydrothermal activities lead to a slow influx of solutions into the overlying sediment at temperatures in the sub-seafloor ranging from 20 to 100 °C. This process also results in the formation of crystalline anhydrites in veins and around warm vents (Janasch and Mottl, 1985). The latter process may trap microbial inhabitants on the seafloor and within surface layers in anhydrites. Due to the mild temperature, the trapped bodies are better preserved as excellent biological evidence for past geochemical conditions.

A similar mild hydrothermal field is present in the Red Sea. Initially found in a deep-sea rift in the 1960s (Swallow and Crease, 1965; Girdler, 1970), the temperature of the Atlantis II brine pool has recently increased to 68 °C (Anschutz and Blanc, 1996). In 1972, several sediment cores were obtained from the southwestern region of the pool (DSDP Site226), and metal sulfides and evaporites were recognized as major mineral facies in this brine-filled basin (Shipboard Scientific Party, 1974). In particular, thick and well-crystallized anhydrite layers were found within the hematite and at the bottom of the cores. Two major anhydrite units were later defined by analysis of the adjacent core samples. The lower unit comprised anhydrite ranging from 12 to 70 wt % (Anschutz et al., 2000). The anhydrite in the sediments likely resulted from a geyser-type eruption of hydrothermal solutions into the Atlantis II brine pool followed by the mixing of calcium-rich solutions with dissolved sulfate-bearing brine and the precipitation of anhydrites during the cooling process (Ramboz et al., 1988). The discovery of veins containing sulfides and anhydrite in the sediment suggests that a mild hydrothermal eruption created the anhydrite facies in the Atlantis II sediment (Zierenberg and Shanks, 1983; Oudin et al., 1984; Missack et al., 1989). The formation of anhydrite facies in this manner would trap mi-

crobial cells and organic debris in the bottom water and surface sediment. These anhydrite layers probably contained important indigenous microbial inhabitants during the occurrence of the hydrothermal events on the deep-sea benthic floor of the Red Sea. Coupled with  $^{14}\text{C}$  markers to estimate age, the anhydrite facies contained a large quantity of information regarding past geochemical changes. The formation process of the Atlantis II brine pool is still controversial, largely because the source of the brine is uncertain (Schardt, 2016). The brine water had converted the bottom of the deep into a hot anoxic hypersaline environment. The microbes in the anhydrite facies may provide hints about the original benthic conditions and age of the pool. It is also an interesting question whether oil was generated in the sediments under the mild hydrothermal activities in the past. If yes, seeping hydrothermal solutions may bring oil into the seafloor of the deep, which might be documented by the microbes in the anhydrites.

In the present study, we sampled a sediment core near Site226 and detected an anhydrite layer. The dominant species were alkane- and oil-degrading bacteria indicating an oxic, oil-spilling benthic condition when the layer was formed. The present study sheds light on the importance of anhydrites in deep-sea sediment as an archive of microbial inhabitants that can serve as biomarkers of past geochemical events.

## 2 Materials and methods

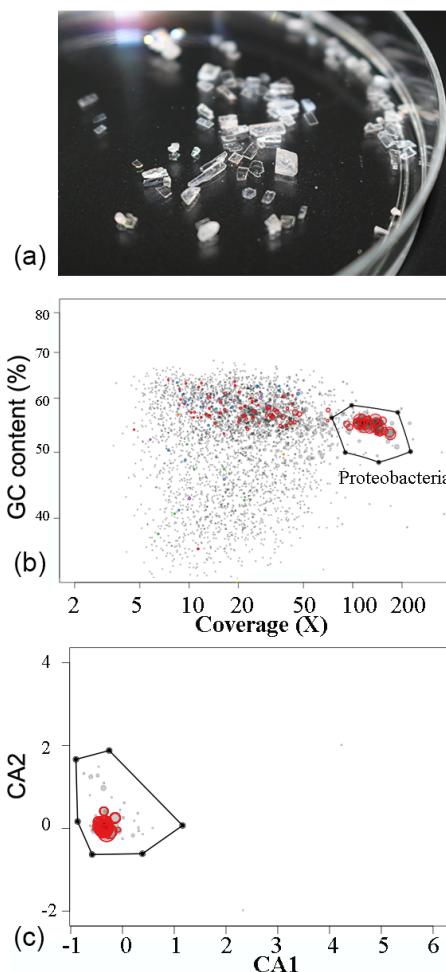
### 2.1 Physicochemical measurements of sediment layers

In 2008, a 2.25 m gravity sediment core was obtained from the southwestern basin (approximately 2180 m below sea level) of the Atlantis II Deep ( $21^\circ 20.76' \text{N}$ ,  $38^\circ 04.68' \text{E}$ ) in the Red Sea (Fig. S1 in the Supplement) (Bower, 2009). The core was frozen at  $-80^\circ\text{C}$  and then sliced aseptically into 75 sections of 3 cm each. Microbes from sediment slices of 12–15, 63–66, 105–108, 183–186 and 222–225 cm were first suspended in phosphate-buffered saline and shaken by a vortexer for 30 s. After 30 min, the supernatant was filtered through a  $0.22\ \mu\text{m}$  black polycarbonate filter. After 6-diamidino-2-phenylindole (DAPI) staining, the microbes from each layer were counted under an epifluorescence microscope ( $n = 3$ ) (Gough and Stahl, 2003). The pore water from the top five layers was collected by centrifugation. The concentration of dissolved organic carbon (DOC) in the pore water was determined using the combustion method (Trichet et al., 2001). The concentrations of ammonium, nitrite and nitrate were measured using a TNM-I analyzer (Shimadzu Corp., Kyoto, Japan). To separate large particles ( $> 63\ \mu\text{m}$ ) from small particles ( $< 63\ \mu\text{m}$ ), the sediment samples were passed through a  $63\ \mu\text{m}$  stainless steel sieve. The percentage of small particles (dry weight) was calculated for all slices.

The age of the sections was estimated with a radiometric dating method that utilizes the naturally occurring radioisotope  $^{14}\text{C}$ . The monospecific *Globigerinoides sacculifer* specimens, ranging in size from 250 to 350  $\mu\text{m}$ , were manually selected with caution and then subjected to  $^{14}\text{C}$  measurement in the National Ocean Sciences Accelerator Mass Spectrometry (AMS) Facility at the Woods Hole Oceanographic Institute, USA. The raw AMS  $^{14}\text{C}$  ages were converted to calendar ages using the CALIB 6.0 program (<http://calib.qub.ac.uk/calib/>) with the dataset Marine 09 (Reimer et al., 2009). A reservoir correction has been considered for the  $^{14}\text{C}$  difference between atmospheric and surface waters (Bard, 1988).

## 2.2 DNA extraction and amplification

The boundary of the anhydrite layer was determined by naked eye observation and particle size measurement. Crystals were manually collected from the layers, followed by ultrasonic cleaning. The homogenized crystals were then analyzed by X-ray diffraction (XRD) (Rigaku, Tokyo, Japan) using Cu K-alpha radiation of 40 kV and 30 mA. The following procedure was conducted for DNA extraction from the crystals with caution to avoid contamination. Surface contamination was removed by rinsing with 70 % alcohol in autoclaved distilled deionized water, followed by pulsed ultrasonic cleaning for 2 h. Anhydrite crystals (20 g) (Fig. 1a) of different sizes were treated with 1  $\mu\text{L}$  (2U) Turbo DNase I (Ambion, Austin, Texas, USA) for 30 m in a 37 °C incubation before being ground for DNA extraction in a sterile hood. The anhydrite powder was used for DNA extraction with the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, USA), followed by a purification step according to the manufacturer's instructions. The DNA concentration was quantified with a Quant-iT PicoGreen Kit (Invitrogen, USA). Of the raw DNA extract, 20  $\mu\text{g}$  was used for DNA amplification using a MALBAC kit (Yikon Genomics, Jiangsu, China) according to the manufacturer's manual (Zong et al., 2012). The MALBAC amplification method has been evaluated recently in metagenomic studies (Wang et al., 2016). Two MALBAC amplification assays were conducted using 21 PCR cycles to acquire a sufficient amount of DNA for subsequent sequencing. A negative control was also incorporated in the assay. The DNA concentration of the MALBAC-amplified sample and the negative control was measured with a Bioanalyzer (Agilent Technologies, CA, US). The products of the MALBAC amplification and negative control were examined by gel electrophoresis to confirm the size ranges of the amplicons. Three replicates of MALBAC amplifications for each sample were mixed and used for Illumina sequencing on a HiSeq2000 platform (Illumina, San Diego, US). As a control, 10 g of sediment from a position at 168 cm from the top of the core was used for DNA extraction. There were no recognizable anhydrite crystals in this layer. DNA sequencing was conducted as described above.



**Figure 1.** Anhydrite crystals and genome binning. Anhydrite crystals in a Petri dish (90 mm in diameter) (a) were used for DNA extraction. The amplified genomic DNA was sequenced and then reassembled. Based on the G + C content and read coverage, the binned contigs with high coverage levels (b) were selected for examination of the tetranucleotide frequency consistency in the PCA analysis (c).

## 2.3 Binning of metagenomes

The initial Illumina  $2 \times 110$  bp paired-end reads were subjected to quality assessments using the NGS QC Toolkit with default parameters (Patel and Jain, 2012). The Illumina sequencing data were deposited in the NCBI SRA database (accession number SRA356974). The 35 bp MALBAC adapters at the two ends of the sequencing reads were removed. Assembly of the trimmed Illumina  $2 \times 75$  bp paired-end reads was performed using SPAdes 3.5 (Nurk et al., 2013). The read coverage for the assembled contigs was calculated using SAMtools (Li et al., 2009). The 16S/18S rRNA genes in the contigs were identified using rRNA\_HMM (Huang et al., 2009). Using classify\_seqs command in mothur package (Schloss et al., 2009), taxonomic sorting of the 16S rRNA

genes was conducted against the SILVA database with a confidence threshold of 80 %. The relative abundance of the species in the metagenomes was roughly estimated based on the coverage of the 16S/18S rRNA genes. Binning of the draft genomes was performed based on the read coverage and G + C content of the contigs (Fig. 1b), followed by principal component analysis (PCA) of the tetranucleotide frequencies (TNF) of their respective contigs using a previously described pipeline (Fig. 1c) (Albertsen et al., 2013). The R scripts (R Core Team, 2013) for the binning process were obtained from <https://github.com/MadsAlbertsen/multi-metagenome>. To evaluate the completeness of the draft genome, conserved single-copy genes (CSCGs) were counted in the genome. The CSCGs were identified by searching the CDSs (coding DNA sequences) against a database of essential bacterial genes (107 essential genes) (Albertsen et al., 2013) using *hmmsearch* (3.0) with default cutoffs for each protein family (Krogh et al., 1994).

## 2.4 Genomic analyses

The CDSs of the draft genome were predicted using Prodigal (version 2.60) (Hyatt et al., 2010). KEGG annotation of the CDSs was performed using BLASTp against the KEGG database (Kanehisa et al., 2012) with a maximum *e*-value cutoff of  $1e-05$ . The KEGG pathways were reconstructed using the KEGG website (<http://www.kegg.jp>). CDSs were also annotated against the NCBI NR database, and MEGAN (MEtaGenome ANalyzer) was used for taxonomic affiliation and SEED/subsystem annotation of the CDSs (Overbeek et al., 2005). The draft genome was submitted to NCBI (accession number LKAP00000000). The average nucleotide identity (ANI) was calculated using the algorithm integrated in the web service of EzGenome (Goris et al., 2007). The DNA–DNA hybridization (DDH) estimate value was calculated using the genome-to-genome distance calculator (GGDC2.0) (Meier-Kolthoff et al., 2013; Auch et al., 2010a, b).

## 2.5 Detection and phylogeny of 16S ribosomal RNA (rRNA) genes

The 16S rRNA gene sequence was identified from the draft genome sequence. The closest relatives based on 16S sequence similarity were determined using the web service of EzTaxon (Kim et al., 2012). The neighbor-joining phylogenetic tree was constructed using MEGA version 5.0 (Tamura et al., 2011) with the Kimura 2-parameter model. The phylogenetic tree was supported with bootstrap values based on 1000 replications.

## 2.6 Fluorescence in situ hybridization (FISH) of *Alcanivorax* bacteria

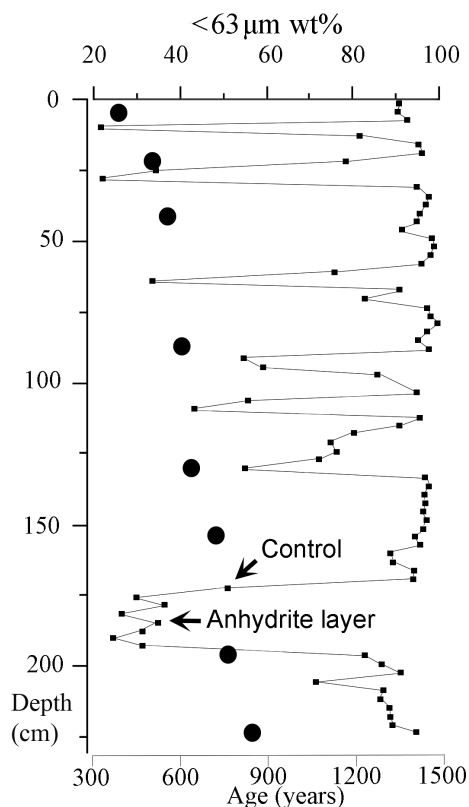
FISH probes for 16S rRNA gene of *Alcanivorax* bacteria were designed based on the 16S rRNA gene sequence extracted from the *Alcanivorax* draft genome. Two 16S

rRNA fragments, 5'-CCTCTAATGGGCAGATTC-3' and 5'-CCCCCTCTAATGGGCAGA-3', were selected as candidate probes with Probe\_Design in the ARB package (Ludwig et al., 2004). The coverage efficiency of the probes was then examined in the Silva database (Quast et al., 2013). The 6-FAM-labeled probe used to target the *alkB* gene was 5'-ATGGAGCCTAGATAATGAAGT-3' (Wang et al., 2010). A pure culture of *Alcanivorax borkumensis* Sk2 (Yakimov et al., 1998) was first used to examine the probes before performing the assay, and a culture of *Escherichia coli* was used as a negative control. Two grams of anhydrite crystals were sonicated for 30 min in 1 U DNase I solution (Takara, Dalian, China). The crystals were washed with deionized water and then ground into a powder with a BeadBeater in a germ-free environment. The supernatant was mixed with 37 % formaldehyde (final concentration, 1–4 %). To fix the cells in phosphate buffer saline solution, the sample was maintained at 4 °C for 3–4 h. After centrifugation at  $13\,000\text{ r min}^{-1}$  for 3 min, the supernatant was discarded. The remaining microbes were soaked in 200  $\mu\text{L}$  of PBS buffer, followed by the addition of 200  $\mu\text{L}$  of ethanol (Pernthaler et al., 2002). The sample was filtered through 3  $\mu\text{m}$  and 0.22  $\mu\text{m}$  membranes sequentially (diameter, 25 mm; Millipore, Massachusetts, USA). After dehydration of the membrane using alcohol, 2  $\mu\text{L}$  of dying solution containing oligonucleotide probes and 20  $\mu\text{L}$  of buffer (360  $\mu\text{L}$  of 5 M NaCl, 40  $\mu\text{L}$  of 1 M Tris / HCl, 700  $\mu\text{L}$  of 100 % formamide, 2  $\mu\text{L}$  of 10 % SD and water to a total volume of 2 mL) was added. The hybridization of the probes to the microbes was performed for 2 h at 46 °C. Rinsing buffer (700  $\mu\text{L}$  of 5 M NaCl, 1 mL of 1 M Tris / HCl, 500  $\mu\text{L}$  of 0.5 M EDTA, 50  $\mu\text{L}$  of 10 % SDS and water to a total volume of 50 mL) was used to remove free probes. For counterstaining, 50  $\mu\text{L}$  of 4',6'-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Massachusetts, USA) solution ( $1\text{ }\mu\text{g mL}^{-1}$ ) was added to the sample. After incubation for 3 min, the sample was washed in Milli-Q water (Millipore, Massachusetts, USA) and 96 % ethanol for 1 min (Pernthaler et al., 2002). The microscopic observation was conducted using an Olympus BX51 (Olympus, Tokyo, Japan).

## 3 Results

### 3.1 Physicochemical profile and cell counts

A thick anhydrite layer was present at the bottom of the sediment core based on naked-eye observation of the color and grain size. The anhydrite layer at depths ranging from 177 to 198 cm consisted of coarse, agglutinated crystals, which corresponded to the high percentage of large grains (78 wt % larger than 63  $\mu\text{m}$ ) (Fig. 2). The XRD analysis further confirmed that the crystals in this layer were anhydrite. In contrast, halite comprised the evaporites at depths of 12, 63, 105 and 222 cm. For the samples at different depths, the



**Figure 2.** Grain size and age of selected layers. The percentages of the small particles ( $<63\ \mu\text{m}$ ) in dry weight are shown for 75 slices of the sediment core (small squares on the line). The age estimates (black circles) of the selected layers were performed using radioisotope  $^{14}\text{C}$  of the monospecific *Globigerinoides sacculifer* specimens. Age errors ranged between 25 and 40 years. Anhydrite and control layers for metagenomic study were indicated by arrows.

DOC concentration was measured, and the highest value was recorded at 183 cm ( $80.9\ \text{mg L}^{-1}$ ), which was even higher than the surface layer at 12 cm (Fig. 3). In the 12 cm layer, the cell density was  $3.2 \times 10^5$  cells per  $\text{cm}^3$ , whereas in the layers at 63 cm, 105 and 222 cm, it was reduced by 88, 92 and 96 %, respectively (Fig. 3). The cell density was also calculated as the number of cells per gram of sediment. The results revealed a value of  $7.1 \times 10^5$  cells per gram at a depth of 12 cm, which declined more than 70 % in the deeper layers. Although the cell density in the 183 cm layer ( $6.7 \times 10^4$  cells  $\text{cm}^{-3}$ ) was markedly lower than that in the 12 cm layer, it was higher than those in the 105 and 222 cm layers.

The sediment as a whole is a highly reductive environment, as indicated by the low nitrate, low nitrite and extremely high ammonium concentrations (Fig. 3). To determine the time of the anhydrite layers at 177–198 cm, an age estimate was performed for several layers. The sediment ages were estimated based on the radioisotope  $^{14}\text{C}$  of *G. sacculifer*, assuming a linear increment from the top (Fig. 2).

**Table 1.** Age estimates of the sediment layers.

Layer (cm)	Age (year)	Age error (year)
3–6	320	25
21–24	475	35
45–48	490	30
90–93	500	25
129–132	560	35
153–156	750	30
198–201	770	30
222–225	880	30

Eight sediment layers were selected for the age estimates using radioisotope  $^{14}\text{C}$  of *G. sacculifer* collected from the respective layers. The age was corrected by the 400-year reservoir age with an error range.

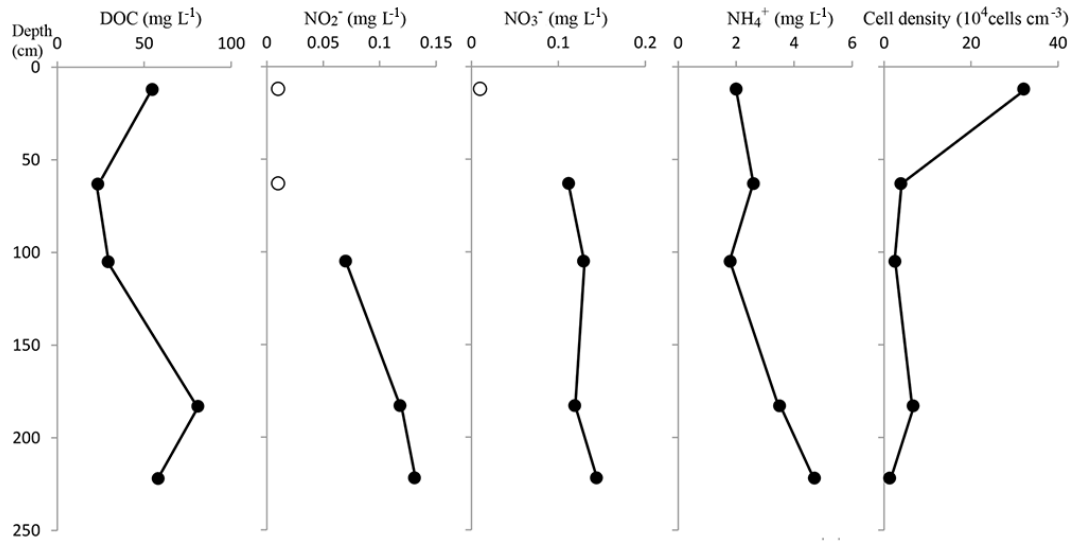
The results obtained for the layers above and below the anhydrite layer indicated a narrow range of 750–770 years between 153 and 198 cm (Table 1).

### 3.2 Draft genome of the dominant bacterial species in anhydrites

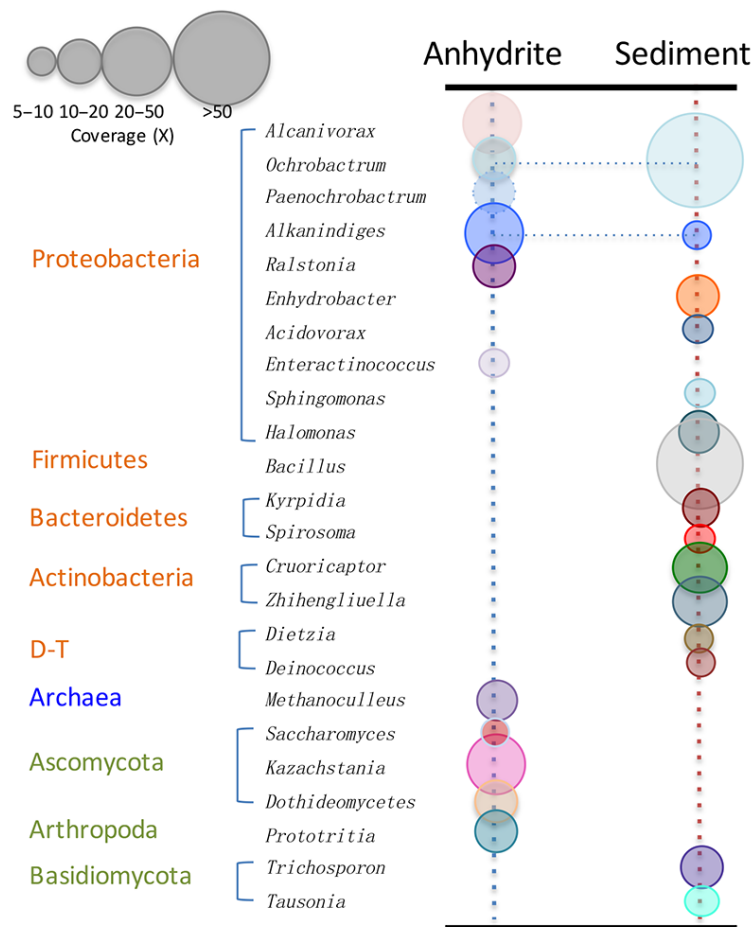
About 1.8 Gbp Illumina raw sequencing data were obtained for the anhydrite sample and 3.1 Gbp data were obtained for the adjacent control layer. The size of the anhydrite and control metagenomes was 59 and 84 Mbp, respectively, after assembly (Table S1 in the Supplement). The microbial communities differed remarkably according to the taxonomic assignment of the 16S/18S rRNA gene fragments in the two metagenomes (Fig. 4). At the genus level, only *Ochrobactrum* and *Alkanindiges* were common inhabitants in both samples. *Alcanivorax* and *Bacillus* were also dominant genera in the anhydrite and the control, respectively. At the phylum level, excluding the Proteobacteria, the two metagenomes had distinctive phyla. The anhydrite contained archaea that were represented by the methanogenic *Methanoculleus* (Barret et al., 2012) and fungi that consisted of the Ascomycota. In contrast, the control sediment contained mainly Firmicutes, Bacteroides, Actinobacteria, and Deinococcus-Thermus. At last, an invertebrate species, *Prototritia* sp. belonging to Arthropoda, was identified in the anhydrite.

### 3.3 Genome binning of an *Alcanivorax borkumensis* genome

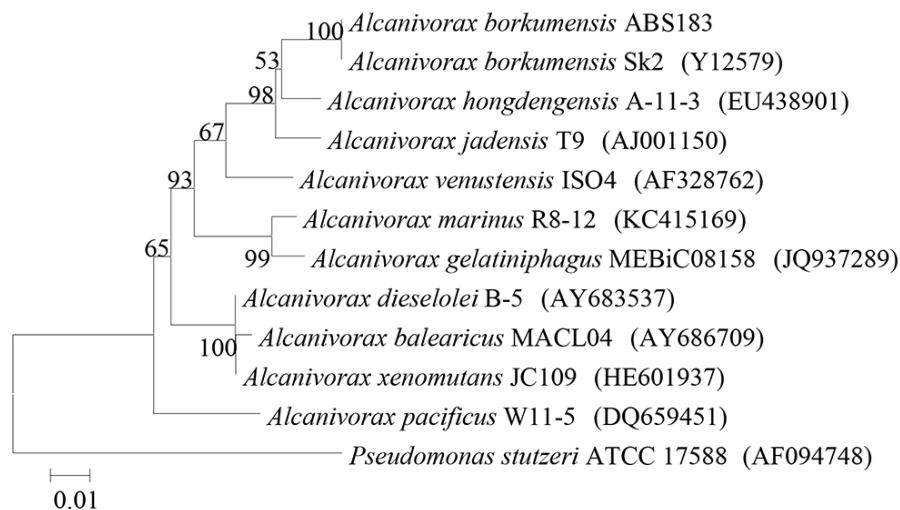
The binned draft genome from the anhydrite metagenome was 3 069 971 bp and comprised 77 contigs. A partial 16S rRNA gene sequence (805 bp) was extracted from the draft genome. Because the sequence was almost identical to that of *A. borkumensis* Sk2 (99.9 %) (see also genomic alignment in Fig. S2), we considered the binned draft genome to be from a strain of *A. borkumensis*. As shown in Fig. 5, a phy-



**Figure 3.** Nutrient measurements and cell counts in the different sediment layers. The pore water samples were analyzed for five layers of a sediment core obtained from the Atlantis II Deep (21°20.76' N, 38°04.68' E) in 2008. DOC: dissolved organic carbon.



**Figure 4.** Microbial communities in anhydrite crystals and neighboring control sediment. Phyla and genera in the anhydrite crystals and control layer were predicted using 16S/18S rRNA gene fragments extracted from the corresponding metagenomes (D-T: *Deinococcus-Thermus*). The relative abundance of the genera can be estimated by the coverage level (>5) of the 16S/18S rRNA fragments by reads.



**Figure 5.** Phylogenetic tree of 16S rRNA genes. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branches of the neighbor-joining tree.

logenetic tree based on the 16S rRNA gene sequences of the genus *Alcanivorax* indicated that the strain clustered with *A. borkumensis* Sk2, an exclusive and ubiquitous hydrocarbon-degrading bacterium (Schneiker et al., 2006; Sabirova et al., 2011). The strain name of the *A. borkumensis* in the sediment core was ABS183. It was the only microbial species that could be reliably separated from the metagenome.

The genome of *A. borkumensis* ABS183, despite containing gaps, was slightly smaller than that of *A. borkumensis* Sk2 (accession number NC\_008260; 3,120,143 bp), suggesting that the draft genome of *A. borkumensis* ABS183 was nearly complete. Also, there were not detectable alignment gaps between the two genomes (Fig. S2). The identification of a complete list of single-copy genes also supported the completeness of the genome. The DDH estimation between *A. borkumensis* ABS183 and Sk2 was  $97.1\% \pm 1.3\%$ , which was higher than the standard cut-off value of 70% for genome relatedness between pairs of species (Wayne et al., 1987). The ANI value between ABS183 and Sk2 was 99.9%, which was also higher than the standard ANI criterion for species identity (95–96%) (Richter and Rossello-Mora, 2009). These results further confirmed that ABS183 was a strain of *A. borkumensis*.

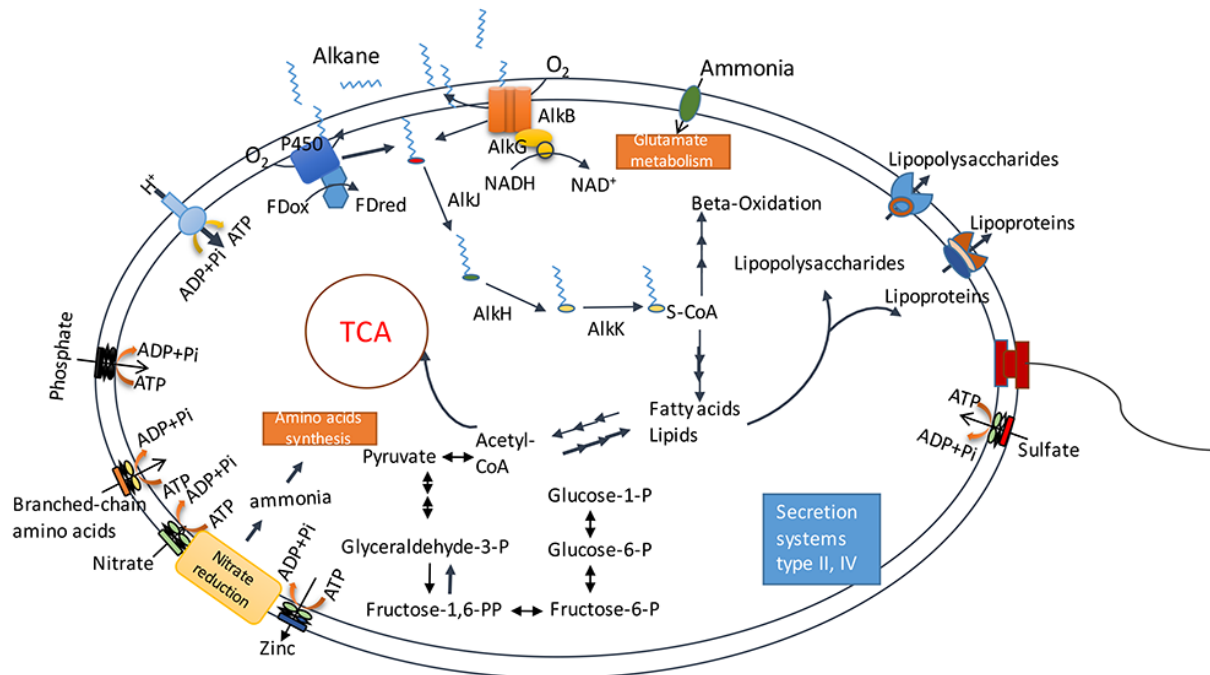
The genome of *A. borkumensis* ABS183 contains two copies of the alkane-1 monooxygenase gene (*alkA*; 10502\_28 and 2890\_35), which is an essential functional gene for alkane utilization by *Alcanivorax* bacteria (Fig. 6) (Schneiker et al., 2006). Neighboring the *alkA* genes, *alk-BGHJ* genes, a GntR family transcriptional regulator gene, and a rubredoxin gene were identified. The gene order of the related genes was consistent with that of the homologs in the genome of strain Sk2 (Schneiker et al., 2006). The *alk* genes were completely absent from the control metagenome. Moreover, the genome of *A. borkumensis* ABS183 contains genes

responsible for the reduction of nitrogen oxides (KEGG genes: K00370-K00374 and K00362-K00363; nitrate reductase I genes and nitrite reductase genes). The reduction process was believed to generate ammonia for the efficient synthesis of amino acids (Schneiker et al., 2006). Ammonia might be generated through nitrate reduction as indicated by the presence of the related genes encoding nitrate and nitrite reduction enzymes (Fig. 6). Ammonia might be imported by transmembrane transporters and assimilated into glutamate. A high demand for fatty acids was a characteristic of *A. borkumensis* to perform rapid energy and organic carbon storage. *A. borkumensis* ABS183 was probably able to synthesize long fatty acids because the *fas* and *fabBFGIKZ* genes responsible for the elongation of fatty acids were all present in its draft genome. In contrast, the essential *fas* gene (K11533) and other relevant genes were not found in the control metagenome. Crude oil generally contains aromatic compounds, and the current sediment at the sampling site also contained oil (Wang et al., 2011). As expected, the two metagenomes possessed a complete set of genes responsible for the degradation of aromatic compounds. Based on the homology of the genes, the *Ochrobactrum* and *Alkanindiges* species probably played a role in this degradation.

### 3.4 Detection of bacteria in anhydrite crystals by DAPI and FISH

To determine whether complete microbial cells could be maintained in the anhydrite crystals, DAPI and FISH assays were conducted to visualize the microbes. The DAPI results revealed the presence of complete cells that were released or embedded in the crystals (Fig. 7a, d and h). However, the FISH assay, which was used to detect *A. borkumensis* ABS183 with two probes specific to the 16S rRNA gene,





**Figure 6.** Schematic model of metabolism and cross-membrane transporters. The model was predicted based on the genes in the draft genome of *Alcanivorax borkumensis* ABS183.

showed some fluorescence-labeled microbes (Fig. 7b, e and i). These microbes could also be envisioned with the FISH assay using the *alkB* gene probe (Fig. 7f and j). The *alkB* is one of the functional genes that participate in alkane degradation (Schneiker et al., 2006). The rod shape of the fluorescent microbes is consistent with the microscopic features reported previously (Sabirova et al., 2011). These results indicated that some microbes in the microscopic fields were *A. borkumensis* ABS183, as revealed in the anhydrite metagenome.

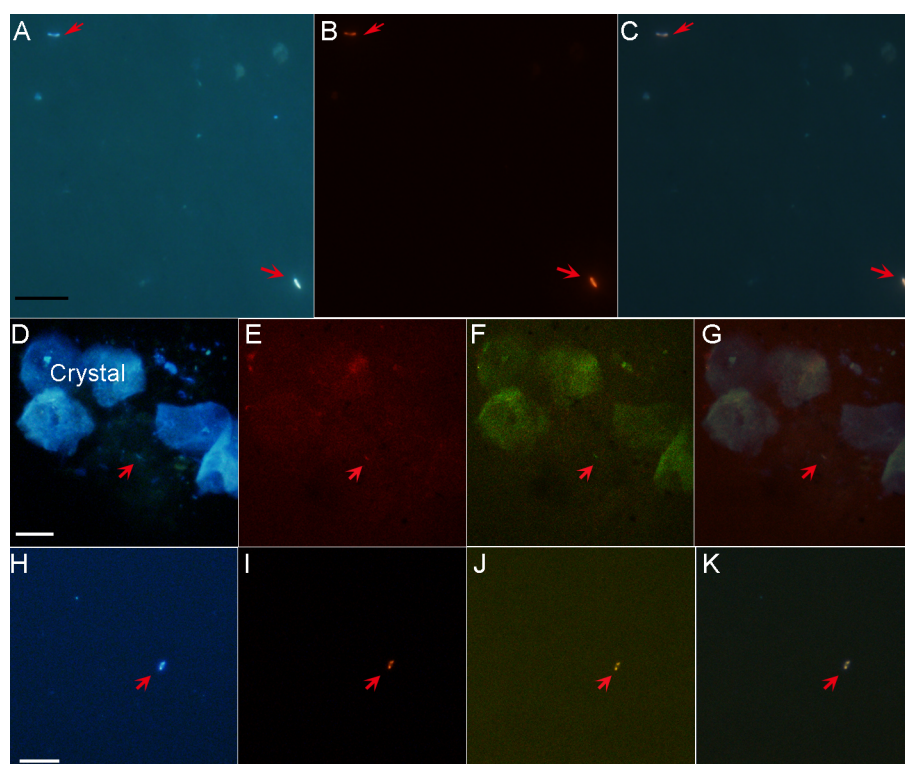
#### 4 Discussion

In the present study, we detected complete microbial cells and analyzed their metagenome in the anhydrite crystals from a deep-sea anoxic basin. The dominant bacterial species was *A. borkumensis* ABS183, an aerobic bacterium that is capable of degrading alkanes in crude oil. *Alcanivorax* is one of the bacterial indicators for the spilling of oil in waters and surface sediment (Yakimov et al., 2007). However, the Atlantis II brine pool is anaerobic and increasingly hydrothermal (Bougouffa et al., 2013b). The brine sediment in the basin was also found to be anoxic. Thus, *A. borkumensis* ABS183 could not be current inhabitants of the hydrothermal anoxic basin. This difference did not explain the stratification of microbial communities in the different sediment layers of the brine-filled basin. A recent study showed that *Alcanivorax* was not present in all sediment layers of a sediment core from the Atlantis II basin (Wang et al., 2015). A reason-

able explanation for this finding is that the anhydrite layer at 177–198 cm in the sediment core was formed at a previous benthic site when hydrothermal solution was injected into the seafloor. The organisms living in the benthic water and subsurface sediment were subsequently sealed and protected in the anhydrite crystals. Because the metabolism of *A. borkumensis* bacteria was specifically used for the degradation of alkanes and other hydrocarbons in crude oil (Yakimov et al., 2007), the benthic site in which the anhydrite layer formed was probably an oil-spilling or oil-forming environment in the Atlantis II basin. The current hot sediments in the basin are biogenic and abiogenic sources of crude oil (Simoneit, 1988). Seeping of the oil has resulted in proliferation of *A. borkumensis* bacteria in the bottom water. Similarly, oil-utilizing bacteria were nourished after the oil-spilling disaster in the Gulf of Mexico (Gutierrez et al., 2013). The *A. borkumensis* bacteria were important producers of organic carbons as they could convert alkanes and nitrate into organic matter. Fatty acids and lipopolysaccharides that were yielded by *A. borkumensis* bacteria were nutrients for the whole ecosystem.

Based on the results in the present study, we propose that mild eruptions of hydrothermal solutions injected calcium-rich solutions into the seafloor and produced anhydrite veins by mixing with sulfate in the bottom water of the Atlantis II rift basin. The anhydrite layer was then covered by sulfide minerals and biological debris such as the planktonic foraminifera *G. sacculifer*. In this study, we narrowed the age of the thick anhydrite layer to 750–770 years using the





**Figure 7.** Fluorescence in situ hybridization (FISH) of *Alcanivorax borkumensis* ABS183 embedded in anhydrite crystals. DAPI staining and FISH using 16S rRNA probes are shown in (a) and (b). The merged image of (a) and (b) is shown in Fig. 7c. DAPI staining and FISH were also performed using two samples that were filtered with 3 µm (d–g) and 0.22 µm (h–k) membranes, respectively. *Alcanivorax* bacteria were released from the large crystals filtered through the 3 µm membranes (d–g). The bacteria were stained with DAPI (d), *Alcanivorax borkumensis* ABS183 probes (f) and the *alkB* probe (f), respectively, and overlaid (g). Using a sample filtered through a 0.22 µm membrane, a dividing *Alcanivorax borkumensis* ABS183 cell was labeled using the same method and probes (h–j). The microscopic fields shown in (h)–(j) are merged in (k).

$^{14}\text{C}$  isotope of the *G. sacculifer* specimens. This result also indicates a relatively young sediment age and a high accumulation rate of precipitated metals in the Atlantis II basin. Because the upward movement of hydrothermal solutions might transfer some foraminifera specimens from lower layers to the anhydrite layer, we did not use the foraminifera between anhydrite crystals. In our previous study, we showed evidence of oil formation in the Atlantis II brine pool (Wang et al., 2011). The organic carbon content can be converted to aromatic compounds under the hydrothermal conditions in the pool based on chemical and metagenomic evidence (Wang et al., 2011). However, the bottom of the anoxic brine pool was not a habitat of *Alcanivorax* species (Bougouffa et al., 2013a; Blanc and Anschutz, 1995), suggesting that *Alcanivorax* flourished in the basin before the formation of brine water layers over the sediment (Blanc and Anschutz, 1995).

Although there were differences in microbial communities between the anhydrite crystals and the control sediment, *Ochrobactrum* sp. was one of the common inhabitants. Previous studies have shown that *Ochrobactrum* species could me-

tabolize aromatic compounds aerobically and anaerobically (Zu et al., 2014; Mahmood et al., 2009), which explains their presence in both metagenomes assessed in the current study. Moreover, we determined the concentrations of nitrogen oxides in the different sulfide layers, although only low concentrations were detected. *Ochrobactrum* species were potentially able to anaerobically degrade polycyclic aromatic compounds using nitrate as an oxygen donor (Mahmood et al., 2009; Wu et al., 2009). Such a chemolithoheterotrophic lifestyle is in accordance with the current in situ environment of the sediment in the Atlantis II. Regardless of the environmental changes indicated by the findings in the present study, the spreading of *Ochrobactrum* sp. was seemingly not affected. Although the metagenomes in the present study contained an abundance of essential genes for degrading a variety of aromatic compounds, the microbial degradation of these compounds might have been attenuated by a lack of oxygen and a high level of salinity (Klinkhammer and Lambert, 1989). Anaerobic degradation of compounds is more difficult than aerobic degradation, often requiring oxygen donors such as nitrate and sulfate (Mahmood et al., 2009;

Wu et al., 2009). Based on its ability to survive under anoxic conditions, *Ochrobactrum* sp. is probably able to maintain a higher level of fitness in the control sediment compared with *Alcanivorax*. In the present study, the *Alkanindiges* identified in both metagenomes was also a well-known alkane degrader (Klein et al., 2007; Bogan et al., 2003). Because of its presence in both anhydrites and the adjacent sulfide layer, we assumed that the *Alkanindiges* bacterium was also capable of surviving aerobically and anaerobically in the oil-producing sediment. Hence, the change from an oxic to an anoxic benthic environment caused a dramatic shift in the microbial communities, resulting in the extinction of the obligate aerobic alkane-utilizer *Alcanivorax* and continuous residency of anaerobic oil-degraders. The availability of nitrogen oxides and the dissolution of sulfate from anhydrite crystals were possibly critical to the metabolic activities of the anaerobes. In addition, the *Bacillus* and fungi present in the control sediment were probably present in the form of dormant spores. In a recent report, *Ochrobactrum* and *Bacillus* were confirmed to be dominant species in some upper sulfide layers in the Atlantis II (Wang et al., 2015). Altogether, in the present study, the current microbial inhabitants in the sulfide layers were largely different from those in the anhydrite crystals.

The geochemical data collected herein suggested that the sub-superficial anhydrite layer could release organic carbon contents into the sediment, as reported previously. Our measurement of DOC at  $80.9 \text{ mg L}^{-1}$  in the anhydrite layer was higher than the generally accepted maximum value of  $50 \text{ mg L}^{-1}$  for marine sediments (Cameron et al., 2006). The abnormally high DOC was considered a notable alteration of the local environments and probably resulted from the breakdown of anhydrite crystals. Anhydrites in the Atlantis II brine sediment were likely maintained by the high salinity and temperature and then slowly dissolved. This phenomenon may be explained by the slight undersaturation of the anhydrite in the Atlantis II sediment (Anschutz et al., 2000). Such anhydrite layers are widely distributed in Middle Eastern sediments (Alsharhan and Nairn, 1997). Hence, our findings shed light on the formation of micro-environments by anhydrite evaporites in the deep sediments. In this study, there was an inconsistency between the cell density and the DOC at the 12 cm depth layer, in which the DOC could not support a 10-fold higher biomass. This phenomenon probably resulted from the formation of petroleum compounds under the hydrothermal effects (Wang et al., 2011). In the petroleum, hydrophobic organic compounds (HOCs) consisting of polycyclic aromatic hydrocarbons (PAHs) could not be counted in our DOC measurements (J. Pearsons, personal communication, 2014). The nutrient supply is critical for microbes to survive in deep-sea sediment. Apart from the chemolithoautotrophic microbes, numerous other inhabitants take advantage of the buried organic matter. Importantly, the trapped organic matter serves as a nutrient supply following the dissolution of organic-rich anhydrite crystals. Therefore, our findings highlighted the importance of the nutrients

released from the anhydrite facies for microbes in deep-sea subsuperficial sediment.

## 5 Data availability

Illumina raw data will be accessible under SRA356974 in the NCBI SRA database. *A. borkumensis* ABS183 genome was deposited in the NCBI database: BioProject LKAP00000000.

**The Supplement related to this article is available online at doi:10.5194/bg-13-6405-2016-supplement.**

*Author contributions.* Yong Wang, Tie Gang Li and Pei-Yuan Qian were responsible for the study design. Data analysis was performed by Yong Wang, Tie Gang Li, Jiao Tao Li, Qi Liang Lai and Zhao Ming Gao. Meng Ying Wang conducted the FISH assays. The paper was prepared by Yong Wang with contributions from all co-authors.

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