Reviewer 1:
The paper by Wang et al describes the microbial community associated with anhydrite crystals in a deep sea sediment basin located at more than 2 km depth in the Red Sea. According to the authors the microbial communities detected could verify past oil-spilling events to a relatively good accuracy. The microbial communities were investigated using metagenomic tools and the authors found that alkane-degrading Alcanivorax species were dominant in the metagenomes coinciding with the past oil-spill. Several metabolic alkane-degradation pathways were detected. The microbial community of the anhydrite crystals were investigated using fluorescent in situ hybridization for identification of Alcanivorax cells that contained alkB genes. The chemistry and biological parameters of the sediment core from which the anhydrite crystals originated, differed with depth. A clear peak in organic carbon content and a significant peak at specific depth corresponding to the oil-spill. The authors conclude that the organic carbon stored in the sediments, e.g. as anhydrite crystals, is slowly released for the benefit of the whole sediment microbial community. The text as a whole is quite clear and the subject is interesting. The materials and methods could benefit from more information. I would also like to see some mote of the genome of the Alcanvorax and some metabolic pathway maps. The fact that a new uncultured Alcanivorax has been detected could be discussed more as well as its role in carbon cycling in deep sediments.
Response: Thanks for the comments. We have put more details in the M&M. In the Results, we made a schematic map for the Alkanivorax bacteria. Regarding the role of Alcanivorax, we put several lines in Discussion, lines 451-453.

Specific comments; L115, how did you get the supernatant? Did you let the solids sedimentate first or did you use centrifugation?
Response: we put the sample for 30 min and did not centrifuge. We modified the corresponding place.
L175, give more information about
what you did with R and which packages you used.
Response: yes, we inserted the linkage of the R scripts.
L178, what is HMM
Response: HMM means hidden markov model. We have deleted it.
L203, what label did you use? Did you have a nonsense probe to check for unspecific labeling?
Response: actually we used E. coli to examine the probe as a negative control. We have inserted this in the new version.
L223, MQ water?
Response: we have spelled out.
L234-251, check the figures. I thing the reference to Fig 2 should be Fig 3 and vice versa.
Response: yes, modified.
L255 ->, did you get any sequences from the blanks and if so,

Response: The sequences in blanks were dimers of the primers for the amplification, which can be justified from the size of the smear. There were not any long amplified sequences from contaminated DNA.

Results&Discussion, I would like to see some figures with the Alcanivorax genome and relevant metabolic maps. How does the Alcanivorax interact with the rest of the microbial community?

Response: yes, we inserted a metabolic map for the Alcanivorax bacterium. Regarding the interaction, we inserted lines in Discussion, (lines 451-454). They are supposed to a convertor between alkanes and organic carbons and nitrogen.

Typos; L58, change 'were' to 'are' L81, do you mean 'found', not 'located'? L137, correct 'grounded' to 'ground' or 'homogenized'

Response: yes, all are corrected.

Reviewer 2

The manuscript submitted by Wang et al. describes the diversity of organisms in anhydrite crystals and further states a high abundance of Alcanivorax microbes. The authors see the specific community composition of the 750 years old crystals as indicator of an oxic oil-spilling benthic environment. Overall, the approach and results are highly interesting and definitely worth reporting in Biogeosciences.

I, however, suggest revising and clarifying several parts of the manuscript before final publication.

General remarks:

While I do not doubt the results of the metagenomic investigation per se, I am honestly wondering what the meaning of the quantification of microbes based on traces of DNA is and how you could find intact cells being preserved for 750 years in crystals, which are still traceable using FISH.

Response: Since 2009, we have been working on this sediment layers for several years to answer the question regarding the elevated organic carbons in the ABS183 layer. The hypothesis is the preservation of cells in the crystals as we also revealed eukaryotic and archaeal sequences from metagenome. It was very surprising for us to find intact microbes in the crystals.

I am missing a detailed explanation and discussion on
that topic as also for me the microscopic pictures are not entirely satisfying.

Response: we have to apologize for the quality of the pics. We selected these pictures to illustrate the presence of the microbes from hundreds of pictures. Although the quality is still not satisfying for you, the result is almost our best for readers. As we can understand, these microbes had been preserved for hundreds of years. It's difficult to find out well-preserved and well-labeled microbes.

Additionally

I would like to see some proof of the assembled genome and a metabolic map.

Further, the conclusion on past oil-spilling is a bit too loosely connected to the other parts of the story, both in the abstract and the results and discussion parts. This could

be done by introducing geochemical data earlier and by rephrasing some of the respective text parts. The references are not in Copernicus style.

Response: thanks for these comments. We have finished a new schematic map for the metabolism based on the gene profile. About the context of oil-spilling, we have rephrased the Abstract and inserted several lines in the Introduction (lines 139-157) to introduce the geochemical background in the oil-producing hydrothermal sediment.

Specific comments:

Abstract:

First sentence should be rephrased as a hypothesis.

Response: yes

l.33: which binning tool? Replace separated by assembled

Response: we assembled the reads and then conducted binning of the contigs for a draft genome.

l. 35. Remove 'the'

l.33-l.37: you are jumping back and forward between the metagenome and the cell identification

Response: yes, these sentences are reorganized.

l. 40 sentence is misplaced, here. Should probably be moved upwards.

Response: yes

l. 41-45: As written this seems to be highly speculative

Response: yes, we have changed the tone.

Introduction

L. 54: Repetition of previous sentence.

Response: yes , deleted.

l. 57: I don't understand this sentence.

Response: rephrased!

l. 59: replace 'prediction' by ' interpretation'

Response: yes

l. 60: replace 'have been' by 'are'

Response: yes

l. 63: The previous sentence already started with 'although'- replace.

Response: yes, used 'but' now.

l. 64: I would like to see a reference for this.

Response: yes, two refs inserted.

l. 69: replace 'become' by ' may get' and add a reference for this statement.

Response: yes.

l. 79: the bodies are the biomarkers? Actually lipids, DNA or also pigments may be used as biomarkers. Not sure whether the word 'body' is really the right expression dead microbial cells.

Response: yes, it was replaced with 'evidence'. Line 118

l. 81: similar to what?

Response: changed. Line 120

l. 95: 'Hence' is contradictory to 'probably'

Response: yes.
l. 97: Which other markers? Why probably? This would actually be the place to explain the geochemical background.
Response: yes, we inserted several lines for the introduction.
l. 100-104. This belongs to the methods part
Response: this part was simplified.
Material and methods
l. 110: A map of the sampling location would be beneficial.
Response: we used a suppl figure S1.
l. 138: Commonly, all abbreviations should be introduced when used for the first time.
Response: yes.
l. 146: this is a very low amount of DNA, how representative is this for community analysis? In how far is the quantification of OTUs and the genome identification trustworthy based on such low amounts of DNA? What about different degradation patterns of different organisms?
Response: We did not use up all the extraction. In total, there was not 1ng of DNA, so we have to use the amplification. The DNA might be used for many amplifications actually. The kit may allow for unbiased amplification of all sorts of DNA. We have a publication to support the linear amplification of microbial genomic DNA.
l. 150: Is this a random amplification?
Response: the theoretical basis and our test support the random amplification for the trace DNA.
l. 156: Which chemistry and protocol?
Some statistics on the sequencing (how many reads per run, how many of the identifiable, how many reads related to 16S rDNA, etc.) would be desirable.
Response: we should have put all the information. The sequencing was done in a service centre. We don't know how many sequences were produced in one run. Perhaps there were many samples in one runs.
l. 169: How?
Response: we inserted the command for this work (line2 239-240).
l. 169: Based on the low amount of DNA and amplification steps in between, I doubt the quantitative aspects of the analysis.
Response: we admit that this step is a brief statistics of the community. The abundance of the species could not be quantified in an accurate manner.
l.175: remove gap after). Rstudio will not make any visualization without a prober script, so please provide details on your script. Also, provide the correct reference instead of the link to the homepage.
Response: yes. We inserted a linkage for the R scripts. See lines 247-248.
l. 181: It is unclear how the draft genome was assembled. What is the completeness? A genome plot would increase the credibility.
Response: the assembly work was introduced at line 236. The assembly made the reads into long contigs. Then the binning process grouped the contigs according

to their distinctive coverage levels and tetranucleotide frequencies. The completeness was assessed firstly by the number of single-copy genes. We have used Fig. S2 to illustrate the genomic alignment between Sk2 and ABS183. From the alignment, there are no notable gaps between the two genomes, indicating the high genomic completeness of the ABS183 strain.

l. 207: replace 'examine' by 'validate'

Response: yes

l. 223 MQ- please spell

Response: yes

I doubt that FISH produces a reliable signal if you only get 20pg of DNA out of the crystals.

Response: actually we obtained far more than 20pg of DNA. We concentrated the cells into a membrane, so that the microbes from many crystals were released and condensed on one membrane.

Results

l.232, 236, 238, 246, 249 : Check numbering of figures. Also, where do you refer to Fig. 5?

Response: yes, there are mistakes in the figures. The figure 5 was described in line 364.

l. 262: archaea, fungi without capital letters.

Response: yes.

l. 265: Why is that surprising?

Response: deleted.

l. 269 onwards: this needs a genome plot and a Kegg metabolic map.

Response: we described the metabolic map of Figure 6. Lines 392-403.

l. 278: I don't buy the quantitative aspect, here.

Response: we deleted it.

l. 346: postulate seems to be a bit strong for the line of evidence provided, here.

Response: we used 'proposed' now.

l. 346 ff: This information is what you need to put into the abstract to make it convincing

Response: this is a nice comment. See the second sentence in the Abstract.

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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Keywords: *Alcanivorax*; metagenome; anhydrite; Atlantis II brine pool; hydrothermal sediment

Running title: Archive of microbial inhabitants in anhydrites

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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 750-770 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 approximately 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.

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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 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 geochemical conditions of the sediment (Parkes et al., 2000;Webster et al., 2006). In return, 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 will be degraded by biological and abiological activities. Although lipids and other organic organic carbons present in some minerals allow the interpretation of microbial activities to to some extent (Brocks et al., 2005), the original metabolic activities are difficult to retrieve retrieve in a comprehensive and precise manner.

Most of the dead microbes are damaged during the sedimentation process, but some can be be maintained in almost their original shape (Taher, 2014;Benison et al., 2008). Evaporites, Evaporites, which mostly consist of halite and anhydrite ($CaSO_4$) or gypsum ($CaSO_4 \cdot 2H_2O$, ($CaSO_4 \cdot 2H_2O$, temperature <38℃ (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 et al., 2001); bacterial mats growing on evaporites may become trapped and constitute much much larger microbialites (Babel, 2004). Consequently, microbial inhabitants on the benthic benthic surface may get trapped in the evaporites (Benison et al., 2008). Anhydrite facies are are not found throughout deep-sea sediments. They usually form around hydrothermal vents vents in deep-sea environments (Jannasch and Mottl, 1985). A strong deep-sea volcanic eruption may break the crustal basalts, resulting in a drastic emission of hydrothermal gases 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 slow influx of solutions into the overlying sediment at temperatures in the sub-seafloor ranging from 20-100℃. This process also results in the formation of crystalline anhydrites anhydrites in veins and around warm vents (Jannasch and Mottl, 1985). The latter process

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may trap microbial inhabitants on the seafloor and within surface layers in anhydrites. Due Due to the mild temperature, the trapped bodies are better preserved as excellent biological biological evidence for past geochemical conditions.

A similar mild hydrothermal field is present in the Red Sea. Initially found in a deep-sea rift rift in the 1960s (Swallow and Crease, 1965;Girdler, 1970), the temperature of the Atlantis Atlantis II brine pool has recently increased to 68℃ (Anschutz and Blanc, 1996). In 1972, several sediment cores were obtained from the southwest region of the pool (DSDP Site226), Site226), and metal sulfides and evaporites were recognized as major mineral facies in this this brine-filled basin (Party, 1974). In particular, thick and well-crystalized anhydrite layers layers were found within the hematite and at the bottom of the cores. Two major anhydrite anhydrite units were later defined by analysis of the adjacent core samples. The lower unit unit comprised anhydrite ranging from 12 to 70 wt% (Anschutz et al., 2000). The anhydrite 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 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 in the sediment suggests that a mild hydrothermal eruption created the anhydrite facies in the 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 microbial 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 at the deep-sea benthic floor of the Red Sea. Coupled with ^{14}C markers markers to estimate age, the anhydrite facies contained a large quantity of information regarding the 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 anoxic, hypersaline and hot environment. The microbes in the anhydrite facies may provide hints for 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 deep. If yes, seeping

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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. 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 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-meter gravity sediment core was obtained from the southwest basin (approximately 2180 meters below sea level) of the Atlantis II Deep (21°20.76' N, 38°04.68' E) in the Red Sea (Fig. S1) (Bower, 2009). The core was frozen at -80℃ and then sliced aseptically into seventy-five 3-cm sections. Microbes from sediment slices of 12-15 cm, 63-66 cm, 105-108 cm, 183-186 cm, and 222-225 cm were first suspended in phosphate-buffered saline and shaken on a vortexer for 30 s. After 30 minutes, the supernatant was filtered through a 0.22-μm black polycarbonate filter. After 6-diamidino-2-phenyloindole (DAPI) staining, the microbes from each layer were counted under an epifluorescence microscope (n = 3) (Gough and Stahl, 2003). The pore water from the above 5 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 (Simadzu, Kyoto, Japan). To separate large particles (>63 μm) from small particles (<63 μm), the sediment samples were passed through a 63-μm 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}C. The monospecific *Globigerinoides sacculifer* specimens ranging in size from 250 to 350 μm were manually selected with caution and

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then subjected to ^{14}C measurement in the National Ocean Sciences Accelerator Mass Spectrometry (AMS) Facility at the Woods Hole Oceanographic Institute, USA. The raw AMS ^{14}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}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 hours. Anhydrite crystals (20 g) (Fig. 1A) of different sizes were treated with 1 µL (2U) Turbo DNase I (Ambion, Austin, Texas, US) for 30 m in a 37℃ 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. Twenty picograms of the raw DNA extract was used for DNA amplification using a MALBAC kit (Yikang, 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 twenty-one 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, 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 Hiseq2500 platform (Illumina, San Diego, US). As a control, 10 g of

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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 amplification and sequencing were conducted as described above.

2.2 Binning of metagenomes

The initial Illumina 2×110-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 start of the sequencing reads were removed. Assembly of the trimmed Illumina 2×75-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 (http://www.mothur.org), 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 for the binning process were obtained in 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 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.

2.3 Genomic analyses

The coding DNA sequences (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

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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 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) with the alignment method of BLAST+ (Meier-Kolthoff et al., 2013;Auch et al., 2010a;Auch et al., 2010b).

2.4 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 neighbour-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.

2.5 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 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. 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 PBS buffer, the sample was maintained at 4℃

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for 3-4 hr. After centrifugation at 13,000 r/min for 3 min, the supernatant was discarded. The remaining microbes were soaked in 200 μL of PBS buffer, followed by addition of 200 μL of ethanol (Pernthaler et al., 2002). The sample was filtered through 3-μm and 0.22-μm membranes sequentially (diameter, 25 mm; Millipore, Eschborn, Germany). After dehydration of the membrane using alcohol, 2 μL of dying solution containing oligonucleotide probes and 20 μL of buffer (360 μL of 5 M NaCl，40 μL of 1 M Tris/HCl, 700 μL of 100% formamide，2 μL of 10% SDS，and water to a total volume of 2 mL). The hybridization of the probes to the microbes was performed for 2 h at 46℃. Rinsing buffer (700 μL of 5 M NaCl, 1 mL of 1 M Tris/ HCl, 500 μL of 0.5 M EDTA, 50 μL of 10% SDS and water to a total volume of 50 mL) was used to remove free probes. For counterstaining, 50 μL of 4', DAPI solution (1 μg/mL) was added to the sample. After incubation for 3 min, the sample was washed in Milli-Q water (MetaPhor Bioproducts, Rockland, Maine) 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 177-198 cm consisted of coarse, agglutinated crystals, which corresponded to the high percentage of large grains (78 wt% larger than 63 μm) (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 cm, 63 cm, 105 cm and 222 cm. For the samples at different depths, the DOC concentration was measured, and the highest value was recorded at 183 cm (80.9 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 3.2×10^5 cells per cm^3, whereas in the layers at 63 cm, 105 cm 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 7.1×10^5 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

329 (6.7×10^4 cells/cm^3) was markedly lower than that in the 12 cm layer, it was higher than
330 those in the 105 cm and 222 cm layers.
331
332 The sediment as a whole is a highly reductive environment, as indicated by the low
333 nitrate, low nitrite and extremely high ammonium concentrations (Fig. 3). To determine
334 the time of the anhydrite layers at 177-198 cm, an age estimate was performed for several
335 layers. The sediment ages were estimated based on the radioisotope ^{14}C of *G. sacculifer*
336 assuming a linear increment from the top (Fig. 2). The results obtained for the layers
337 above and below the anhydrite layer indicated a narrow range of 750-770 years between
338 153 cm and 198 cm (Table 1).
339
340 **3.2 Draft genome of the dominant bacterial species in anhydrites**
341 DNA was extracted from the anhydrite crystals and the adjacent layer separately. After
342 amplification, about 16 Gbp Illumina sequencing data were obtained and then assembled.
343 The size of the anhydrite and control metagenomes was 59 and 84 Mbp, respectively,
344 after assembly. The microbial communities differed remarkably according to the
345 taxonomic assignment of the 16S/18S rRNA gene fragments in the two metagenomes
346 (Fig. 4). At the genus level, only *Ochrobactrum* and *Alkanindiges* were common
347 inhabitants in both samples. *Alcanivorax* and *Bacillus* were also dominant genera in the
348 anhydrite and the control, respectively. At the phylum level, excluding the Proteobacteria,
349 the two metagenomes had distinctive phyla. The anhydrite contained archaea that were
350 represented by the methanogenic *Methanoculleus* (Barret et al., 2012); and fungi that
351 consisted of the Ascomycota. In contrast, the control sediment contained mainly
352 Firmicutes, Bacteroides, Actinobacteria, and Deinococcus-Thermus. At last, an
353 invertebrate species, *Prototritia* sp. belonging to Arthropoda, was identified in the
354 anhydrite.
355
356 **3.3 Genome binning of an *Alcanivorax borkumensis* genome**
357 The binned draft genome from the anhydrite metagenome was 3,069,971 bp and
358 comprised 77 contigs. A partial 16S rRNA gene sequence (805 bp) was extracted from
359 the draft genome. Because the sequence was almost identical to that of *A. borkumensis*

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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 Figure 5, a phylogenetic 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%±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, *alkBGHJ* 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

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might be generated through nitrogen fixation as indicated by the presence of the related genes (Fig. 6). 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.

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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, 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 7J). 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 reasonable 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 proposed 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 ^{14}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

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foraminifera between anhydrite crystals. In our previous study, we have shown 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 metabolize 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 abundant 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

496 anaerobically in the oil-producing sediment. Hence, the change from an oxic to an anoxic
497 benthic environment caused a dramatic shift in the microbial communities, resulting in
498 the extinction of the obligate aerobic alkane-utilizer *Alcanivorax* and continuous
499 residency of anaerobic oil-degraders. The availability of nitrogen oxides and the
500 dissolution of sulfate from anhydrite crystals were possibly critical to the metabolic
501 activities of the anaerobes. In addition, the *Bacillus* and fungi present in the control
502 sediment were probably present in the form of dormant spores. In a recent report,
503 *Ochrobactrum* and *Bacillus* were confirmed to be dominant species in some upper sulfide
504 layers in the Atlantis II (Wang et al., 2015). Altogether, in the present study, the current
505 microbial inhabitants in the sulfide layers were largely different from those in the
506 anhydrite crystals.
507
508 The geochemical data collected herein suggested that the sub-superficial anhydrite layer
509 could release organic carbon contents into the sediment, as reported previously [12,13]. Our
510 measurement of DOC at 80.9 mg L^{-1} in the anhydrite layer was higher than the generally
511 accepted maximum value of 50 mg L^{-1} for marine sediments (Cameron et al., 2006). The
512 abnormally high DOC was considered a notable alteration of the local environments,
513 probably resulted from the breakdown of anhydrite crystals. Anhydrites in the Atlantis II
514 brine sediment were likely maintained by the high salinity and temperature, and then
515 slowly dissolved. This phenomenon may be explained by the slight undersatuation of the
516 anhydrite in the Atlantis II sediment (Anschutz et al., 2000). Such anhydrite layers are
517 widely distributed in Middle Eastern sediments (Alsharhan and Nairn, 1997). Hence, our
518 findings shed light on the formation of micro-environments by anhydrite evaporites in the
519 deep sediments. In this study, there was an inconsistency between the cell density and the
520 DOC at the 12-cm depth layer, in which the DOC could not support a 10-fold higher
521 biomass. This phenomenon probably resulted from the formation of petroleum
522 compounds under the hydrothermal effects (Wang et al., 2011). In the petroleum,
523 hydrophobic organic compounds (HOCs) consisting of polycyclic aromatic hydrocarbons
524 (PAHs) could not be counted in our DOC measurements (personal communication with J.
525 Pearsons). The nutrient supply is critical for microbes to survive in deep-sea sediment. Apart
526 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.

Author contributions. Y. Wang, T.G. Li, and P. Y. Qian were responsible for the study design. Data analysis was performed by Y. Wang, T.G. Li, J. T. Li, Q. L. Lai, and Z. M. Gao. M.Y. Wang conducted FISH assay. The manuscript was prepared by Y. Wang with contributions from all co-authors.

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Data Accessibility

Illumina raw data will be accessible under SRA356974 in the NCBI SRA database. *B. borkumensis* ABS183 genome was deposited in the NCBI under BioProject LKAP00000000 and will be public on October 31, 2016.

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}C of *G. sacculifer* collected from the respective layers. The age was corrected by the 400-year reservoir age with an error range.

Figures

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).

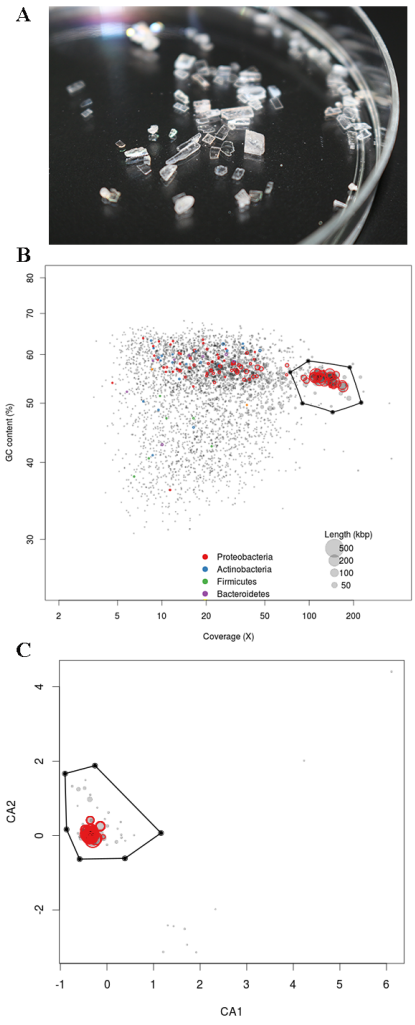
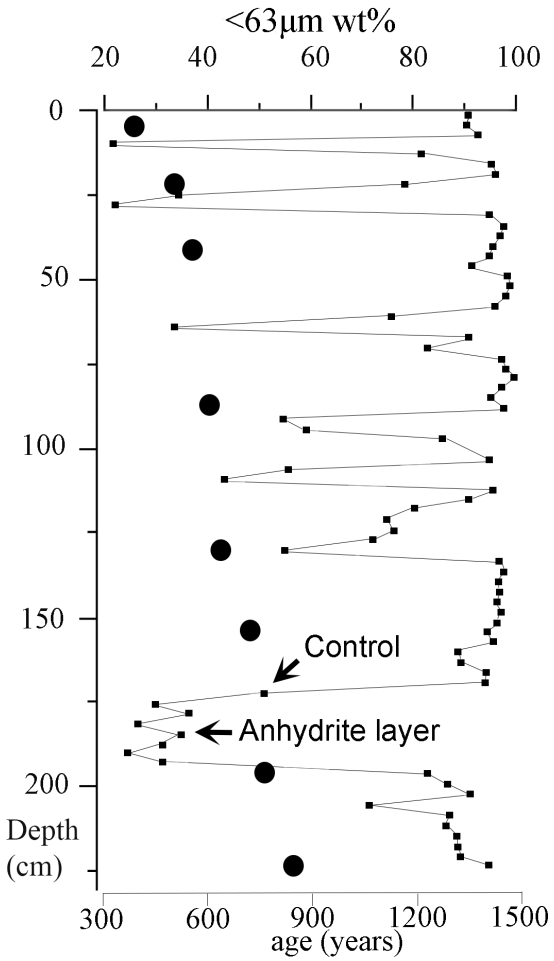


Figure 2. Grain size and age of selected layers

The percentages of the small particles (<63μm) 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 14C of the monospecific *Globigerinoides sacculifer* specimens. Age errors ranged between 25 to 40 years. Anhydrite and control layers for metagenomic study were indicated by arrows.



Microsoft Office 用户 2016/9/27 4:18 PM
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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.

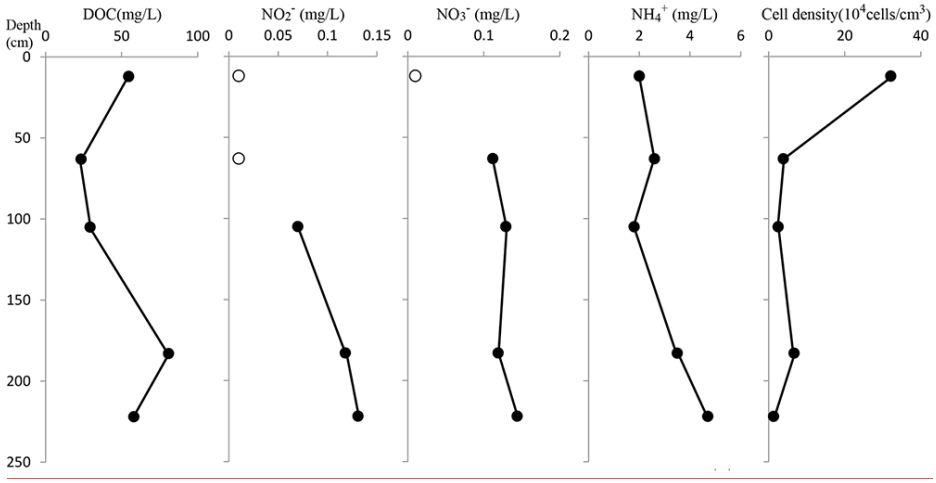

Figure 4. Microbial communities in anhydrite crystals and neighboring control sediment.

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 of the 16S/18S rRNA fragments by reads.

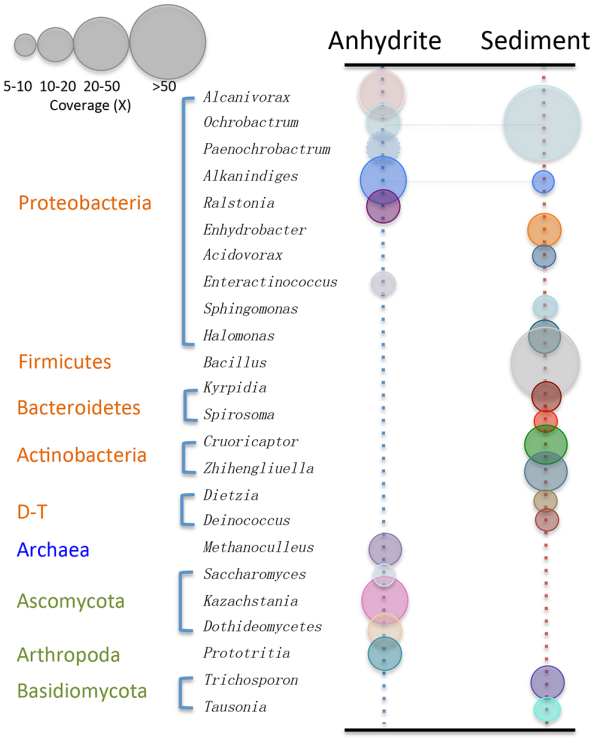

Figure 5. Phylogenetic tree of 16S rRNA genes.

Bootstrap values (expressed as percentages of 1000 replications) are shown at the branches of the neighbor-joining tree.

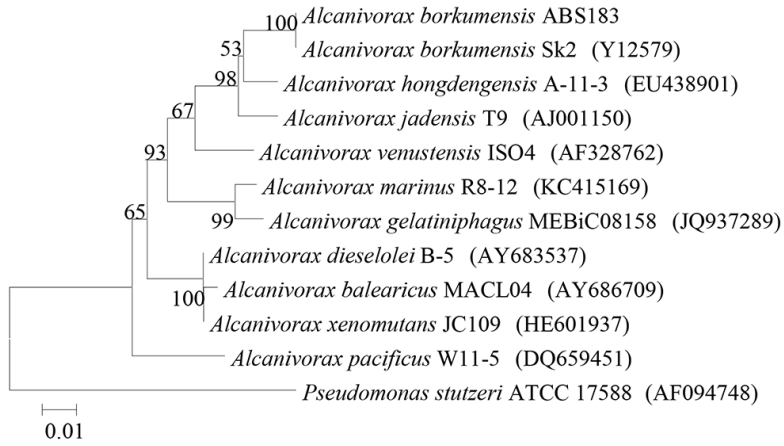

Figure 6. Schematic model of metabolism and cross-membrane transporters

The model was predicted based on the genes in the draft genome of *Alcanivorax borkumensis* ABS183.

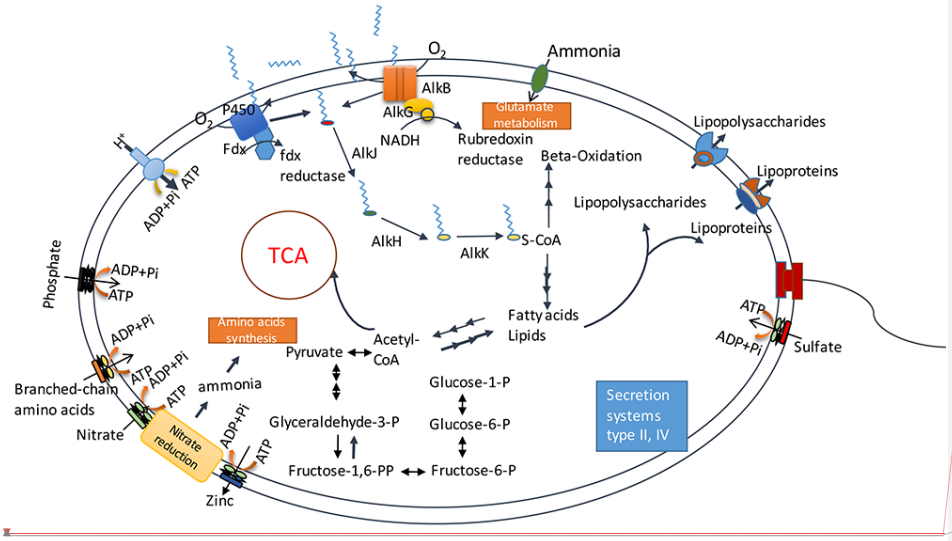

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Figure 7. Fluorescence *in situ* hybridization (FISH) of *Alcanivorax* sp. ABS183 embedded in anhydrite crystals.

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), 16S rRNA probes (E) and the *alkB* probe (F), respectively, and overlaid (G). Using a sample filtered through a 0.22-µm membrane, a dividing *Alcanivorax* sp. ABS183 cell was labeled using the same method and probes (H-J). The microscopic fields shown in H-J are merged in Fig. 7K.