The American University in Cairo

School of Sciences and Engineering

Microbial and Chemical Profiles of Sediments isolated from *ATLANTIS II and DISCOVERY Deep* Brine Pools in the Red Sea.

A Thesis Submitted to

The Biotechnology Graduate Program

in partial fulfillment of the requirements for

the degree of Master of Science in Biotechnology

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Bachelor of Science in Pharmacy - Ain Shams University

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Spring 2011
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DEDICATION

This thesis is dedicated to my parents,

*my beloved mother:* Mrs Fayza Asa’d Abdel Sayyed

Without her unconditional love, dedication, support, guidance and wisdom, I would not have the goals I have to strive and be the best I can to fulfill my dreams.

*Mum, you are truly missed!*

*my dad:* Eng. Guirquis Rasmy Marsis

Your marvelous endless support and encouragement in every step of this hard road had always pushed me for forward success and a better me.

*I am honored to have you as my parents.*
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ABSTRACT

Microbial and Chemical Profiles of Sediments isolated from *ATLANTIS II* and *DISCOVERY Deep* Brine Pools in the Red Sea.

Sediments lying beneath two brine pools in the Red Sea; *Atlantis II* deep and *Discovery* deep were assessed chemically, geologically and phylogenetically. Understanding the extreme nature of these environments is essential in the microbial diversity as a potential mean for identifying new genes and new bacterial traits for biotechnological applications. Screening for bacteria suggested a high diverse community on the class level using 16S rDNA –DGGE technique. Assessment of 91 amplified DGGE clones had revealed only 45 clones to be of good sequence quality. The sequenced clones fell into nine major lineages belonging to *Bacteria* domain; α, β, and γ subdivisions of *Proteobacteria*, *Cytophaga–Flavobacterium–Bacteroides group* (CFB), *Clostridia*, *Bacilli* and *Cyanobacteria*. Along with the phylogenetic assessment, a complete chemical profile of all the metals in the periodic table had been generated revealing a highly contaminated heavy metal environment. Thirty three metals got detected among which are; nickel, iron, chromium, aluminum, manganese, arsenic and zirconium. A Carbon-Hydrogen-Nitrogen-Sulphur (CHNS) profile for both deeps was also generated. The geological era determination of both deeps was provided with electron microscope pictures revealing foraminifers belonging to the Tertiary period (65 – 1.8 mya) which is the age of mammals. In this study, a link between microbial profile and chemical one is being provided. The microbial profile suggested that more investigations need to be done on this extreme environment due to the huge information that could be revealed. The chemical profile provided important information about high heavy metal contents that are toxic to any living organism such as nickel, chromium, arsenic, iron, copper, titanium, aluminum and antimony. More investigations need to be done in the future in order to provide more information about the organisms inhabiting such an extreme environment.
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1. **Introduction:**

The study of extremophiles had been the new trend in research where living organisms inhabiting environments with extreme stress conditions such as temperature, salinity, pressure, dryness and contaminants; such as heavy metals and radioactive compounds were extensively studied. Scientists had applied variety of techniques in order to study such microorganisms. Although in the past many culturing techniques were applied to study these microorganisms, yet little information was yielded due to failure in laboratory culturing which is mainly attributed to the difficulty in mimicking such extreme conditions that are needed to enable their growth. Therefore, the emergence of metagenomics as a new science had enabled scientists to study such extremophiles. DNA sequencing techniques are very well established where DNA after being isolated from samples is then extensively studied. Information generated from genetic material is applied in many fields. Some scientists applied their studies in the field of pharmaceuticals where novel antibiotics and antiviral agents got discovered. Others applied it in the field of bioremediation where bacteria that tolerated heavy metal and toxic contaminants are being genetically modified to be used in cleaning the environment. Metaproteomics is another field where scientists applied protein engineering on proteins extracted from micro-organisms in order to enhance the protein’s specificity and performance.

In this study an extreme environment is being investigated; two brine pools in the Red Sea; *Atlantis II* deep and *Discovery* deeps. Brine pools are large areas of brine water lying in the ocean/sea basin. The salinity of the brine pool could reach up to five times
that of the surrounding water. Sediments of brine pools typically deposit metals such as sulfides of zinc, copper, iron with significant amounts of silver, gold, cobalt along with many other elements of high economic value. *Atlantis II* deep is suspected to have its own geothermal activity, whereas *Discovery* deep is thought to be a discharge of the former deep. The temperature of *Atlantis II* deep was recorded by the expedition to be 70° C while *Discovery* deep was around 45° C. *Atlantis II* deep and *Discovery* deep sediments taken at about 2,200 meters deep are being assessed chemically, geologically and phylogenetically on the molecular level.
2. **Literature Review:**

Metagenomics\(^1\) is a new emerging science where microbial communities inhabiting environmental samples are being studied without using culturing techniques\(^2\). Samples studied could encounter some stress conditions, hence culturing conditions are not easily mimicked in the laboratory\(^3,4\). Therefore metagenomics; as a new science had overcome such difficulties. The microbial research before metagenomics was a limited one regarding techniques, however a huge area of research had been unlocked for more exploration after metagenomics due to the ease of applied techniques and huge information being yielded as a consequence. Metagenomics had created a link between environmental stresses, metabolic cooperation in mixed species biofilms and assigning novel energy processes to marine bacteria\(^5\). Metagenomics is a sequencing based tool, where DNA of desired community inhabiting a sample is isolated and studied\(^3\). The isolated DNA can serve as the meeting point for many researchers who can study it for many purposes. The first purpose is incorporating DNA information into phylogenetic studies to assess the biodiversity of a specific community; this had been illustrated in many studies. In 2009, a study in Puertollano, Ciudad Real, Spain was done on Polycyclic Aromatic Hydrocarbon (PAH) contaminated area. It was found that the degradation of naphthalene, phenanthrene and anthracene was complete within 18 days of cultivation where the toxicity was highly reduced. The diversity of the bacterial community showed bacteria belonging to *Gamma-Proteobacteria* and uncultured *Stenotrophomonas* ribotypes as PAH degraders\(^6\). Another study was done to disease suppressive soils, by Elsas *et al.* where this spectacular environment showed extensive microbial diversity of both eubacteria and archaeabacteria. The disease suppressive soil
was found to have *Streptomyces* bacteria of antagonistic and antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa* and other mentioned bacteria. The second purpose of metagenomics studies was found to be useful in assessing functional genomics of the studied community. Functional genomics is mainly associated with the study of genetic material that has functional activity either within the microorganism itself or upon the surrounding environment. Functional genomics could include histones associated in DNA folding processes and all the way till antimicrobial agents secreted by the microorganism. Due to the extreme conditions marine environments prevail such as salinity, temperature, pressure and nutrients, marine biochemical compounds had been thought to have unique biochemical activity. Marine chemodiversity had been a huge area for researchers to study natural drug products. Violacein which is a broad spectrum antimicrobial agent isolated from *Chromobacterium violaceum*, a metagenomics isolated bacterium. A third purpose is the study of metaproteomics and metatranscriptomics. This is done through recovering mRNA and proteins from the studied environment. The first metatranscriptome study revealed transcripts encoding for both archaeal and bacterial genes involved in carbon, sulphur and nitrogen cycling. 

One of the purposes of this study is the application of metagenomics in studying the biodiversity of the bacterial community inhabiting sediments isolated from brine pools (*Atlantis II* and *Discovery* deeps) in the Red Sea at a depth starting from 2,200 m using phylogenetic analysis. Since metagenomics as mentioned before had enabled many environments to be studied, deep marine environments constructed the major challenge for many researchers. Many studies had paid special attention to marine environments
especially those of unique nature in order to be explored and investigated. In 1999, a
study had been done on cold marine sediments from Hornsund off the coast of
Spitsbergen, Arctic Ocean. The biodiversity results revealed that *Delta-Proteobacteria*
was the most abundant group (36.8%), yet the sediments were being inhabited by other
groups such as *Gamma-Proteobacteria* (18%), *Cytophaga and Flavobacteria* but no*Cyanobacteria* was detected. Bacteria belonging to phylum *Cytophaga* are known for
their ability to associate and glide on surfaces and to degrade a wide variety of polymeric
substances. Also, in 1999, another study had been done on marine sediments isolated
from Sagami and Tokyo bays in Japan at a depth of 17 m. Those bays represent industrial
and domestic waste areas discharged from an enormous human population. The diversity
of the bacterial community showed *Gamma, Delta, and Epsilon-Proteobacteria* along
with gram positive bacteria. In 2008, sediment samples were collected from Arctic
Ocean at water depth of 1,209 m. The study showed that the deepest sample was being
mainly inhabited with *Chloroflexi* clones, Phyla *Bacteroidetes, Gamma, Beta-
Proteobacteria* and *Fermicutes*. Another unique marine environment had been studied,
was deep sea hydrothermal vents of the east Pacific rise and at the Guaymas basin. In this
study, two novel *Epsilon-Proteobacteria* were being isolated and studied. *Epsilon-Proteobacteria* are known to inhabit chimneys and are closely associated with
invertebrate hosts. The strains studied were found to have fumarate growth properties and
use elemental sulphur, hydrogen and carbon dioxide for growth under anaerobic
conditions. Sediments from Eagle and Blakely harbors heavily contaminated with coal-
tar creosote had also been metagenomically studied. Creosote is composed of polycyclic
aromatic hydrocarbons (PAHs). Twenty two clones were being studied, indicating Alpha, Delta and Gamma-Proteobacteria, gram positive bacteria and Clostridia\(^{15}\).

The biodiversity of a microbial community could be assessed using 16S rDNA sequences. 16S RNA molecule is a small RNA subunit that contributes in the formation of 30S RNA molecule in prokaryotes\(^{16}\). The secondary structure of 16S RNA is folded on itself forming loops and helices through Watson-Crick base pairing\(^{17,18}\). The 16S rDNA sequences vary from one organism to another, yet there are some regions that are conserved whereas others are variable. The conserved regions are slowly evolving sequences whereas the variable regions are the rapidly evolving ones inferring historical and evolutionary relationships\(^{19}\). Hence, these variable regions are the ones of significant importance in phylogenetic studies\(^{20}\). These variable regions are noted v1 to v9 as shown in figure 1, yet v4 is absent from prokaryotes but present in eukaryotes\(^{18}\). In this study v3 region was the hyper-variable region that got analyzed. V3 region; according to Escherichia coli sequence numbering, starts from position 341 and ends at 926, thus a total of 585 bp\(^{19}\). Primers used in this study were according to Muyzer et al\(^{21}\), where amplicons generated are of about 193 bp from position 341 to 534 (Fig 2); the highly variable part in v3 region\(^{21}\). In order to study 16S rDNA sequences, a variety of techniques were applied such as pyrosequencing, 16S plasmid/ clone library, temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE)\(^{22}\). Each one of the previously mentioned techniques had been applied in many studies, DGGE is the one used in this study. DGGE is a rapid, reproducible and inexpensive technique which is used to assess the biodiversity and complexity of
microbial communities. DGGE is based on genetic fingerprinting concept where a pattern of DNA fragments is generated on a gel. The gel has denaturing characteristics gained from mixing urea and formamide as DNA denaturants, whereas DNA fragments are obtained as a PCR product using GC clamp primers. The GC clamp is a GC rich domain (30-50 nucleotides); that is attached to the 5’ prime end of the primer and hence gets attached to the DNA fragment while PCR amplification. GC clamp functions as a high melting domain preventing the DNA fragments from complete dissociation. The genetic fingerprinting of the DGGE gel is generated when double stranded DNA molecules, same in length but different in sequence, partially melt and start to electrophoretically migrate into the gel that has linear gradient of DNA denaturants. The migration of the melted DNA fragments is stopped once the helical structure of the double stranded DNA is changed into partially melted form. Each melted DNA fragment differ from the others in their migrated position according to one base difference, this is mainly because the melting point for each DNA fragment to reach the partially melted form differ according to their sequence. Thus, different sequences will differ in the position they migrated into the gel. DGGE of 16S rDNA had been applied to many studies in order to assess the diversity of microbial communities. One of these studies was done on Sanya Mangrove Nature Reserve ecosystems dominant along tropical coastlines of China. Zhang et al. used PCR-DGGE technique where he used v3 region primers; 338f- 518r. The phylogenetic analysis of seventeen DGGE bands showed Proteobacteria, Bacteroidetes, Gemmatimonadetes, Actinobacteria and Firmicutes to be predominant in the studied samples. The isolated DGGE bands showed nearest neighbors inhabiting similar environments such as mangrove soil, tropical oligotrophic lakes, salt marsh
sediments, high temperature volcanic environments and oil polluted marine microbial sediments. Also, v3 region had been investigated with paleolithic paintings and the surrounding walls of two Spanish caves (Llonin and La Garma) as a sort of investigating the deterioration of paintings due to microbial activity. Samples had been isolated from many areas in the caves, some from red and black paints, rocks, and microbial colonies. The primers used were 341f/ GC clamp- 518r. Forty six clones had been isolated from DGGE and sequencing revealed Proteobacteria (α, β, γ and δ subdivisions), Nitrospira (nitrite oxidizer) group, Cytophaga/ Flexibacter/ Bacteroidetes phyla, members of the Acidobacterium division, Firmicutes and Actinobacteria. The Acidobacteria was found to contribute significantly in the deterioration process of the red and black paintings due to its sulphur-ammonia activity. Actinobacteria, was also found to destroy paintings by excretion of organic and inorganic metabolites. A study on deep sea sediments isolated from South China Sea at a depth of 2,965 m was done by Cao et al. In his study, he investigated v3 region by applying DGGE technique in order to assess the biodiversity of the studied community. The DNA got isolated, PCRed and underwent DGGE technique followed by sequencing and phylogenetic analysis. The Phylogenetic analysis of the study showed a divergent community of Cytophaga/ Flexibacter/ Bacteroidetes phyla (CFB group), Flavobacteria, Delta, Alpha, and Gamma-Proteobacteria. Another study in 2006 was done on four marine sponge isolated from South China Sea as well. The study aimed for bacterial communities associated with marine sponges using 16S rDNA sequences via DGGE technique. Jiang et al. isolated DNA from the four types of sponges after grinding, then the DNA got PCRed using the same primers for v3 region as that used in this study; 341f (GC clamp)- 534r along with other primers also for v3 region;
341f-518r. The phylogenetic analysis of the study revealed four main groups of bacteria; Gamma, Alpha, and Beta-Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria\textsuperscript{27}. In 2009, a study used the same primers as those used in this study; 341f (GC clamp)-534r, was done using DGGE technique for 16S rDNA. The study aimed for assessing the biodiversity of marine bacterio-plankton. Water samples in this study were being collected from both surface and bottom depths in Kongsfjorden, Spitsbergen. The DGGE was successful in isolating 36 pure individual bands. The phylogenetic data of the surface water in this study showed Alpha, Gamma, and Epsilon-Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes and Cyanobacteria, whereas, the bottom water revealed Alpha, Beta and Gamma-Proteobacteria, Bacteroidetes, Actinobacteria and Verrucomicrobia but no Cyanobacteria\textsuperscript{28}. Qu et al. in 2008 used also the same primers as those used in this study, where sediments from the eutrophic Guanting Reservoir in China at a depth of 11 m. The phylogenetic analysis also revealed Proteobacteria as a major group of the studied community\textsuperscript{29}.

In this study, phylogenetic analysis will be done in order to assess the microbial biodiversity of two brine pools in the Red Sea; Atlantis II and Discovery deeps. These brine pools are two of many others that exist in the Red Sea. In 2001, seven deeps of the Red Sea were chemically investigated. The deeps were; Suakin, Port Soudan, Valdivia, Chain, AtlantisII, Thetis and Nereus deeps\textsuperscript{30}. Kebrit deep is another brine pool in the red sea where its sediments in 1999, and its brine water in 2001\textsuperscript{31}, had been metagenomically investigated through 16S rDNA sequences for both bacteria and archeae\textsuperscript{32}. Shaban deep had also been chemically investigated in 2007\textsuperscript{33} and metagenomically investigated in
2008\textsuperscript{34} where a novel lineage of wall-less contractile halophilic bacteria had been found. Among all those brine pools, \textit{Atlantis II} and \textit{Discovery} deep sediments had been extensively chemically and geologically investigated yet had never been metagenomically assessed. So, an additional purpose of this study is linking the biodiversity of the studied community to the environmental stress conditions such as high temperature, salinity, heavy metal and hydrothermal activity. Previous studies for both deeps had revealed some important information related to their origin. In 1965, A.R. Miller and his colleagues from Woods Hole Oceanographic Institution (WHOI) were able to investigate both \textit{Atlantis II} and \textit{Discovery} deeps\textsuperscript{35}. In their study, they were able to investigate the chemical composition, geological and hydrographic structure of both brine deeps. Their findings indicated that brine pools’ sediments were rich in iron deposits as well as other metals; such as silver and manganese, unlike non-brine deeps that were only carbonate rich. Another finding the study showed was the high temperature the brine pools recorded; \textit{Atlantis II} deep at 2,123 m depth was 55.9\textdegree{}C and \textit{Discovery} deep at 2,044 m depth was 44.7\textdegree{}C. The latest finding suggested that these brine pools have their own source of heat to compensate for the heat lost to the surrounding water\textsuperscript{35}. Another study was made by WHOI crew in 1967\textsuperscript{36} where John M. Hunt and his colleagues were able to study both \textit{Atlantis II} and \textit{Discovery} deeps. Their findings concerning temperature and geological aspects did not differ from previous studies. Temperature of \textit{Discovery} deep was recorded to reach 44.7\textdegree{}C while \textit{Atlantis II} deep reached 56\textdegree{}C. Nonetheless, their study did include two novel findings from previous studies. The first finding concerned the temperature of the sediment cores below each deep; where \textit{Atlantis II} deep was found to increase in temperature by 1\textdegree{}C every 16 m depth in sediment core, whereas \textit{Discovery}
deep’s temperature gradient was less pronounced and decreased with increasing the sediment depth. Such a finding suggested that Atlantis II deep has its own geothermal event. The second novel finding of this study is related to microbiological studies done to water columns above the brine pool and the brine water itself. Hunt et al. was able to find anaerobic bacteria living in water column above the brine pool whereas his study to the brine water itself suggested that the brine water was sterile. In March 1971, Ross et al. from WHOI visited the brine area in the Red Sea. According to his findings; concerning the increase in Atlantis II deep’s temperature and slight increase in Discovery deep’s, his study suggested that Atlantis II deep has its own geothermal activity whereas Discovery deep gained its temperature as an overflow from Atlantis II deep. In the same year, Brewer and his colleagues also from WHOI, investigated the Red Sea brine area as well. The study suggested that Discovery deep is an overspill from Atlantis II deep. The hypothesis was based upon the temperature profile obtained that showed no increase in Discovery deep’s temperature whereas Atlantis II deep had risen 2.7°C in 51 months.

Since then Atlantis II deep had been considered of more value than Discovery deep, where many researchers had focused on studying only Atlantis II deep rather than Discovery deep. In 1987, Simoneit; a petroleum researcher, and his colleagues studied the organic matter composition of Atlantis II deep. The aim of such a study was to prove whether Atlantis II deep was petroleum rich or not. Although the researchers were unable to prove that Atlantis II deep was petroleum rich, yet their findings were interesting. The sediments showed low levels of organic carbon content and considerable levels of the following; n-alkanes, isoprenoid alkanes, alkylcyclohexanes, tri-terpenoids, steroid hydrocarbons, and kerogen. Kerogen; a mixture of organic matter that when subjected
to certain stress conditions such as high temperature and hydrothermal activity could lead
to petroleum existence, was found in average amounts. Anshutz et.al also, paid a
special interest in Atlantis II deep, when in 1995 he was able to generate a complete
chemical profile of the deep. The Mineralogy included a wide range of metals such as
iron, manganese, aluminum, zinc, copper, lead, and zirconium among others. Later on
in 2000, he repeated the previous analysis to trace changes in metalliferous composition
of the deep over time. Anschutz, also, was able to study strontium isotope in Atlantis II
deep in specific. Studying the origin of Atlantis II deep and how it evolved as a
hydrothermal system was the main aim of the strontium study. Nonetheless, he
managed to study silica behavior as an indication for some geochemical dynamics in
Atlantis II deep. In 1996, Anschutz continued studying Atlantis II deep; where he
compared the hydrographic data since 1966 till 1992 as an estimation of the flow rate
and temperature of the hydrothermal fluid supplied into the deep.

Although Atlantis II deep was paid much attention as shown above, yet Discovery
deep was also studied by several researchers among other brine pools such as Kebrit and
Shaaban deeps. In 1995, Discovery deep was studied by Monnin where other brines
were included such as Atlantis II, Valdivia and Suakin deeps. Monnin studied the
anhydrite saturation index which indicated that a continuous overspill from Atlantis II
deep into Discovery deep took place over years. In 1998, changes in the physical and
chemical structures for the last 14 to 23 years of Discovery deep were compared to those
of Atlantis II deep. The study showed that strong changes occurred in Atlantis II deep
more than those in Discovery deep.
Until 2009, both Atlantis II and Discovery deeps had not been studied from the microbiological point of view, yet they had been extensively studied from the chemical, geological and physical point of view. In 2010, a crew from both Hong Kong and Saudi Arabia were able to study the water column overlying both deeps where new archaeal and bacterial species were found. Nonetheless, both brine sediments are not yet investigated from the microbiological point of view.
3. **Materials and Methods:**

3.1. **Sampling:**

Sediment Core was taken on October 2008 on the board of R/V Oceanus from Woods Hole Oceanographic Institution by AUC crew; Dr Rania Siam and Mr Ahmed Shibl. The sediment cores were taken from *Atlantis II* (South West/ SW basin) and *Discovery* deeps. The location of *Atlantis II* (the deepest part of the pool) was (21°20.365’N, 38°05.055’E), water depth of 2,170 m where 2.67 m depth were recovered as sediment cores. Whereas the location of *Discovery* (21°16.646’N, 38°03.153’E), water depth of 2,180 m where 2.80 m depth were recovered as sediment cores (fig 3). The sediment cores were divided into six samples for each site. The Sediment level starting from the sea bed was noted as Zero. *Discovery* deep: *Spill From top sample (SFT)*; represent the uppermost layer of the sediment, *0-47 cm sample*; represent the first 47 cm core, and the same applies to *47-94 cm sample, 94-141 cm sample, 141-188 cm sample* and *188-235 cm sample*. *Atlantis II* deep/ 50 gms were collected from HKUST core from brine SW basin: *0-45 cm, 45-90 cm, 90-135 cm, 135-180 cm, 180-225 cm and 225-267 cm samples*. The samples were then refrigerated at -20°C at the American University in Cairo; Biology facility for further analysis.

3.2. **Chemical Analysis:**

3.2.1. **Sample Preparation:**

About 2 grams from each sample had been washed from sodium chloride residues using distilled water and filter paper of 40 mm pore size. Residual sodium chloride was tested using silver nitrate rendering no white precipitate. After washing the samples from
sodium chloride, filters carrying the sediment particles were placed on a petri dish in an oven at 80°C until complete dryness.

3.2.2. Metal analysis:

Metal analysis was done using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). ICP-OES is an analytical technique used for detection of metals and some non-metals such as phosphorus and silicon. It is based upon emission spectrometry where inductively coupled plasma cell is used to excite atoms and ions and hence the metals under investigation will emit electromagnetic waves of a certain wavelength characteristic to each one. Moreover, the intensity of the wave indicates the concentration of the detected element. Multiple standards for each element of different concentrations are being also measured so that a calibration curve is made. Samples are being suspended in solutions; HNO₃/H₂O₂ for organometallics, inorganic metals and trace elements, H₂SO₄/HClO₄ for phosphorus, organometallics and trace elements in organic compounds, HNO₃/HCl/H₂O₂ for inorganic metals and trace elements. Blank solution is also prepared with the samples. (See supplementary data 1)

3.2.3. CHNS analysis:

Total carbon, hydrogen, nitrogen and sulphur (CHNS) content in the sample are being detected using Carlo-Erba 1108 gas chromatography. (See supplementary data 2)
3.3. Geological Analysis:

About 0.5gms from chemical prepared samples, are placed in the electron microscope at the Youssef Jameel Science and Technology Research Center (STRC) at the AUC and viewed under different magnifications.

3.4. Molecular Analysis:

3.4.1. Sodium Phosphate Protocol:

For re-suspension and collection of intact cells from sediments and subsequent DNA isolation to avoid adsorption of extracted DNA, a modified protocol after Pote et al 2010 is used. Add to 7 gm sediment, 35 ml freshly prepared 2% Na Phosphate buffer (1 gm Na$_2$(PO$_3$)$_6$ + 1.75 gm NaCl + to 50 ml PCR-graded DNA-free water such as Ambion and sterile filter the solution prior to use). Agitate at room temperature (preferably at 4°C) for 1 hour. Release the cells in suspension via sonication for 5 min at 80 Watt. Centrifuge at 4°C for 15 min at 750xg. If the suspension is still turbid, place the tubes overnight on ice in a refrigerator to settle fine sediment particles. Next day, transfer supernatant from the o/n settled samples via careful pipetting into a clean corning tube. Note: this step is required for the upper two sediment intervals of Atlantis. We found that the fine particles in these layers still adsorb DNA. This o/n incubation is most likely not required for the other settings and for the deeper Atlantis samples. We are not 100% sure if bacterial cells can also settle o/n at 1x gravity so prevent this step if the supernatant after the centrifugation step is just colored (pigments) but not turbid. Collect the cells by sterile filtration on a 0.2 μm pore size Sterivex filter
(Millipore). Extraction of DNA from filters using Amicon filters and Phenol/Chloroform extraction procedure; shown as below.

3.4.2. DAPI staining:

After bacterial cell isolation on 3, 0.8 and 0.2 µm filter, DAPI staining was done to about 1/6 of the filter and visualized under fluorescent microscope. Samples to which DAPI staining was done are three samples; two samples from *Discovery* deep; *spill from top* and 0-47 cm samples and one *control* sample isolated from sea water.

3.4.3. DNA extraction using Phenol/Chloroform Protocol:

Add 5 ml extraction buffer in a 15 ml corning tube (*Extraction Buffer*: In a 50 ml corning tube, Add 5 ml Tris HCl (100mM) + 30 ml EDTA (250 mM) + PCR H2O to 50 ml. Take from this prepared mixture 10 ml in a new 15 ml corning tube + 1 ml 5M NaCl + 1 ml SDS 10% + Filter with cells). Then add about 500ul zirconium beads. Add filter with cells and vortex for about a minute to remove all the cells from the filter and bring them into the solution. Incubate for about 5 min at 50°C in the water bath and then freeze at –80°C for at least 30 minutes. Thaw the tube at 50°C until liquid and vortex for about a minute and freeze at -80°C for at least 30 minutes. Repeat the freeze/thaw step 3 times. Centrifuge the tube with the filter for 5 min at 3000 rpm. Transfer the extraction buffer with the lysed cells into a new 15 ml corning tube. Add 2.5 ml Phenol chloroform/Isoamyl alcohol and vortex for 30 seconds. Centrifuge at 3000 rpm for 5 minutes. Load 4 ml of the extract in a 15 ml Amicon filter unit and centrifuge for 10
minutes at 3000 rpm. Discard the flow-through and load the rest of the extract. Wash the pellet with 500 ul 1XTE for 5 minutes. Transfer the extract into a 1.5 ml tube.

3.4.4. Cleaning up procedures of extracted DNA

Cleaning extracted DNA from inhibitors was done using “Powerclean™ DNA Clean-Up Kit - Mo Bio Laboratories, Inc.” Catalog Number: 12877-50. Use kit as the instruction manual demonstrates.

3.4.5. DGGE sample preparation:

Cleaned samples are amplified twice using Q-PCR. Primers used in both amplifications have GC clamp for V3 region and are shown in Table 1. The first PCR conditions was: initial melting at 95°C for 1 mins/ 1 cycle, melting at 94°C for 40 secs/ 60 cycles, annealing at 57°C for 40 secs/ 60 cycles, extension at 72°C for 40 secs/ 60 cycles, photo 80°C for 20 secs/ 60 cycles and 4°C for ∞. The first Q-PCR is stopped at cycle 39, and then the samples are re-amplified using the same primers as those of the first Q-PCR with exact the same conditions, yet the Q-PCR is stopped at 9-12 cycles. Q-PCR kit used is picomax™. The Q-PCR mixture for one reaction is as follows: PCR water 19.7 ul, 10x picomax buffer 3 ul, dNTP’s (2.5mM/each dNTP) 3 ul, MgCl2 (25mM) 1.2 ul, Ambion BSA (50ug) 0.6 ul, SyBr green 0.6 ul, Picomax polymerase (2.5 U/ul) 0.6 ul, GC Bac 341f 0.15 ul, Bac 534r 0.15 ul, DNA template 1 ul, final volume 30 ul/ Reaction. Negative controls for each Q-PCR were used.

The double amplified DNA is then run onto a DGGE gel of U (Ureum) and F (deionized formamide) concentration 70% and 20% UF for bacterial primers (See
DGGE is then left to run for 16 hours at 60°C. The gel is then visualized using SYBR gold stain and bands are then cut from the gel and put in TE buffer at 4°C to elute over night and then transferred to -20°C freezer to be stored. The eluted DNA is then amplified by thermocycler with no GC clamp primers and then sequenced using ABI sequencer.

3.4.6. Cell count Q-PCR:

The cell count Q-PCR conditions were: initial melting 98°C for 2 mins/ 1 cycle, melting 98°C for 5 secs/ 60 cycles, annealing 57°C for 15 secs/ 60 cycles, 80°C for 20 secs/ 60 cycles and 4°C for ∞. The Q-PCR is stopped at cycle 45. Q-PCR kit used is SsoFast EvaGreen® Supermix, Bio-Rad Laboratories, Inc.; the Q-PCR mixture for one reaction is as follows: PCR water 8.6 ul, Evagreen supermix 10 ul, Primers used are Bac 341f, 0.2 ul, Bac 534r, 0.2 ul, DNA template 1 ul, final volume 20 ul/ Reaction. Negative controls for each Q-PCR were used. A Q-PCR standard was also amplified in order to perform cell count (See supplementary data 4).

Table 1: Displaying Primers used in Q-PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Strain</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bac 341f</td>
<td>16S rDNA</td>
<td>5’-[GC-clamp]5’-CCT ACG GGA GGC AGC AG-3’</td>
<td>Bacteria, Muyzer et al., (1993)</td>
</tr>
<tr>
<td>Bac 534r</td>
<td>16S rDNA</td>
<td>5’-ATT ACC GCG GCT GCT GG-3’</td>
<td>Bacteria, Amann et al., (1992)</td>
</tr>
</tbody>
</table>

Sequence of 40 bp GC-clamp: (5’-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG)

supplementary data 3).
3.5. Data Analysis:

3.5.1. Sequence trimming:

Sequence trimming was done using “Sequencher” program where low quality ends and primers got trimmed and then contigs are generated from forward and reverse sequence reads.

3.5.2. Blasting:

Blast was done on AUC biotechnology 4GB server, where database used was SSU ref Silva database (SSU r104), bacteria only. The blast line command used was → blastall –p blastn –d “./db/silvaRef” –i input.fasta –o outputname.txt –e 1e-5 –v 50 –b 50.

3.5.3. Clustering using CD-Hits 97%:

After blast, sequences are counted and redundant sequences are removed. Then, clustering of the blast hits alone without the Red Sea/ RS DGGE band sequences was done using CD-Hits online tool. Criterion used was clustering for sequences of 97% similarity.

3.5.4. Alignment using MAFFT

For the clustered sequences combined with the wanted RS DGGE bands either Atlantis II deep, or Discovery deep or both deeps’ multiple alignment was done using online MAFFT (Multiple Alignment using Fast Fourier trasnform) tool using L-INS-i option (similar to that of T-Coffee but faster).

3.5.5. Refinement of alignment:

Jalview program was used in refinement of the alignment. Then realignment using MAFFT online was performed with the same criteria as above.
3.5.6. Phylogenetic tree using PhyML.

Phylogenetic tree was done using PhyML\textsuperscript{54} program version “PhyML v3.0\_360-500M”\textsuperscript{55}. Criteria used were, sequential option, random initial trees used are 3, tree topology are NNI and SPRs, model used is HKY85 and number of taxa= 395.

3.5.7. Display of the tree using iTOL.

The tree generated from PhyML program was displayed using iTOL/interactive tree of life online display tool\textsuperscript{56}.
4. **Results:**

4.1. *Atlantis II Deep*

4.1.1. Chemical Analysis:

4.1.1.1. Metal Analysis:

About 33 metal elements have been detected in each of the six levels of the *Atlantis II* deep. Each varies from one level to another in its detected concentration. Metals and non-metals have been detected and chemical profile includes all the following elements: silver, arsenic, barium, cadmium, cerium, copper, chromium, lithium, lutetium, molybdenum, sodium, niobium, nickel, lead, praseodymium, scandium, silicon, strontium, terbium, vanadium, yttrium, ytterbium, zirconium, aluminum, calcium, iron, potassium, magnesium, manganese, phosphorus, titanium, and zinc.

In table 2, metals detected in *Atlantis II* deep are in ppm and percent units. The quantity of some metal detected either in ppm or percent unit in *Atlantis II* deep were found much more higher in comparison with those of *Discovery* deep (table 3). Silver had been found in *Atlantis II* deep (table 2) of very high quantities reaching more than 60 ppm whereas in *Discovery* deep (table 3) did not reach the 10 ppm. Also, molybdenum in the middle levels: 45-90cm and 90-135cm, recorded levels above the 140 ppm, whereas in *Discovery* deep the maximum level detected was 15 ppm. Phosphorus also ranged minimum level of 185 and exceeded the 800 ppm yet in *Discovery* deep some levels were undetected/0.13 ppm and only 0-47 cm and 47-94 cm were around 800 ppm. Lead was detected in *Atlantis II* deep above the 700 ppm in the 90-135cm level, whereas *Discovery* deep did not even reach the 30 ppm in any of its levels. Praseodymium in levels 45-90 cm
and 90-135 cm in *Atlantis II* deep recorded the highest of value 28, 26ppm respectively, while in *Discovery* deep the highest amount detected was 6 ppm in the spill from top level. Aluminum showed interesting amounts where in *Atlantis II* at 0-45, 45-90, 90-135, and 180-225cm did not really differ from *Discovery* deep levels which did not exceed the 1%, yet only 135-180 and 225-265 cm; last level in *Atlantis II* deep, showed massive amounts of aluminum of 672% and 855% respectively. Another element showed same behavior is manganese where the middle levels of *Atlantis II* deep; 90-135, 135-180, and 180-225 cm showed high amounts; of 793%, 341%, and 780%, while in *Discovery* deep and the rest levels of *Atlantis II* deep ranged from 1% to 3%. The last element that *Atlantis II* deep had higher amounts than *Discovery* deep is iron, where in the latter deep it did not reach the 10% whereas in the former deep it recorded up to 35.35%.

In each level there were certain metals that got detected in a high concentration when compared to the rest of levels of *Atlantis II* deep. In level 0-45 cm, chromium and potassium were detected in a high level when compared to other levels of *Atlantis II* deep, where chromium reached 6 ppm and potassium reached 832 ppm. Level 45-90 cm, lithium was undetectable while molybdenum, praseodymium, ytterbium and iron recorded to be the highest in this level (Fig 5) whereas calcium was the lowest of them all (table 2). In level 90-135 cm, arsenic, lutetium, manganese, niobium, phosphorus, lead, scandium, silicon, terbium, titanium, vanadium and zirconium were the highest in this level (Fig 6 A,B) while lithium recorded the lowest (table 2). Level 135-180 cm showed interesting findings as it showed the largest number detected as the lowest among all the *Atlantis II* level such as arsenic, cadmium, chromium, niobium, nickel, lead, scandium, silicon, titanium, vanadium, zirconium, iron and magnesium (Fig
6 A, B) and only barium and calcium were the highest amount (Fig 7), but lutetium, praseodymium, and terbium were undetected at all (table 2). Level 180-225 cm showed silver, cadmium, copper, magnesium, sodium and zinc as the highest detected metals (Fig 8) while barium and strontium were the lowest and lithium was undetected (table 2). In level 225-267 cm, aluminum, lithium, nickel, and strontium levels were the highest (Fig 9), whereas molybdenum, manganese and zinc were the lowest (table 2) and carbon was undetected (Fig 10).

4.1.1.2. CHNS analysis:

Carbon, Hydrogen, Nitrogen and Sulphur profiles were also detected in all the six levels of Atlantis II deep; as shown in figure 10 and table 4. Nitrogen was detected but it was in trace amounts less than 0.1 % found, whereas hydrogen in both 135-180 cm and 225-267 cm levels only were untraceable. Carbon was detected in all levels except the deepest one; 225-267 cm level, whereas sulphur was highly detected in all the six Atlantis II levels. Level 90-135 cm constructs the highest CHNS profile when compared to the other levels of Atlantis II deep (Fig 10).

4.1.2. Geological Analysis:

The electron microscope (EM) pictures of Atlantis II deep showed total different features than those in Discovery deep. Most of the layers did not show any planktic features as that of Discovery deep. The first and second levels (0-90 cm) (Fig 11, 12) of Atlantis II deep showed what might be predicted as authentic carbonate which could be generated by bacteria. The third level (90-135 cm) (Fig 13) shows a planktic
foraminifer of genus *Globigerinoides*. The fourth level (135-180 cm) (Fig 14) shows needles which are suspected to be aragonite. However the deepest two levels (180-267 cm) (Fig 15) show image which are predicted to be bacterial communities inhabiting the sediments.

The difference in the contents of *Atlantis II* deep’s features and *Discovery* deep’s could be related to either dissolution or dilution. It is most probably dilution; i.e. fast growth of the other materials in compared to coccoliths’ calcification.

4.1.3. Molecular Analysis:

DGGE gel showed bands that are different in both position and intensity from mixed controls. Total bands isolated and sequenced from *Atlantis II* deep were 34 yet only 19 bands were of good quality and length after assembly and trimming (Fig 16). Each level had different bands showing good diversity of the environment. Level 0-45 cm showed good diversity where 4 bands were fully investigated, 45-90 cm showed 6 bands, 90-135 cm only 1 band was of good quality and intensity, 135-180 cm level showed 5 bands, 180-225 cm showed only 1 band and 225-267 cm level showed 2 bands that were fully investigated. According to NCBI top blast hits, the bands showed bacteria isolated from anaerobic, mineral or plant associated habitats, hydrothermal vents and marine environments.

4.1.4. Phylogenetic Analysis:

The phylogenetic tree indicated a diverse community on the class level. The tree composed of *Bacilli*, cyanobacteria, *Alpha-Proteobacteria*, *Gamma-
Proteobacteria and Beta-Proteobacteria. The majority of the sequences were Gamma-Proteobacteria and Beta-Proteobacteria respectively (Fig 17). The nodes and clusters against which the sequences were compared were 332 bacterial sequences yet 151 of which are uncultured bacteria classified only on the class level according to NCBI database (See supplementary data 5). The v3 region, used in the analysis is so small that it would not allow accurate species assignments to the DGGE clone, that’s why the tree is being presented to show the class level of the presented sequences. Hence all the bacteria included in the tree as a database to which the DGGE clones were compared are being downloaded from SILVA database in order to show classification till reach the class level according to SILVA, RDP and greengenes databases but not according to NCBI database as the latter showed a lot of unclassified sequences that were found to be classified in the former databases. For Bacilli only 24 sequences were classified on the class level from NCBI database yet the rest were classified on the class level in the other databases. Cyanobacteria had only 1 sequence classified on the class level yet the rest are fully classified in the mentioned databases. Same applies to Alpha-Proteobacteria where only 33 sequences are NCBI classified, Gamma-Proteobacteria had only 17 sequences classified on NCBI database while Beta-Proteobacteria had 26 sequence classified on the class level yet the rest were rendered unclassified on NCBI database which was not the case with the other databases. Level 0-45cm showed RS 54, 59, and 60 to belong to Gamma-Proteobacteria group, while RS 61 belonged to Beta-Proteobacteria group. Level 45-90cm showed RS 64 and 65 to belong to Beta-Proteobacteria, while RS 67 and 68 belonged to Gamma-Proteobacteria and RS 69 was among Alpha-Proteobacteria group. Level 90-135 cm showed RS 71 to belong to bacillus group. Level 135-189 cm
showed RS 73, 77 and 78 to belong to *Gamma-Proteobacteria* group, and RS 76 and 79 belonged to *Beta-Proteobacteria* group. Level 180-225 cm showed RS 82 to belong to cyanobacteria group. Level 225-267 cm showed RS 83 as a *Gamma-Proteobacteria* and RS 86 as a beta-proteobacteria.

A table showing each sequence of the DGGE bands and its nearest neighbor associated with some of the clustered sequences and the habitat from where these sequences were isolated is indicated (table 5). The table showed environments that are marine in nature, rich in iron, volcanic and thermophilic ones, heavy metal contaminants, and deep sea sediments which support our findings.

### 4.2. *Discovery Deep*:

4.2.1. Chemical Analysis:

4.2.1.1. Metal Analysis:

About 33 metal elements have been detected in each of the six levels of *Discovery* deep. Each varies from one level to another in its concentration detected. Metals and non metals had been detected and chemical profile include all the following elements; silver, arsenic, barium, cadmium, cerium, copper, chromium, lithium, lutetium, molybdenum, sodium, niobium, nickel, lead, praseodymium, scandium, silicon, strontium, terbium, vanadium, yttrium, ytterbium, zirconium, aluminum, calcium, iron, potassium, magnesium, manganese, phosphorus, titanium and zinc.

In table 3 metals are detected in *Discovery* deep in both ppm and percent units. The quantity of some metal detected either in ppm or percent unit in *Discovery*
deep were found to be much more higher in comparison with those of *Atlantis II* deep (table 2). *Discovery* deep was found to have higher concentration of cerium, chromium, lithium, niobium, strontium, vanadium, zirconium and titanium than that of *Atlantis II* deep. Cerium concentrations reached 21 ppm in the deepest level of *Discovery* deep whereas in *Atlantis II* deep was less the 1 ppm in all levels. Chromium concentration reached up to 66 ppm in 188-235 cm level of *Discovery* deep whereas in *Atlantis II* deep it did not reach 10 ppm in any of its levels. Lithium was also highly detected in *Discovery* deep having concentration up to 115 ppm in 141-188 cm level while *Atlantis II* deep was in very low amounts. Niobium, which is used in stainless steel alloys for nuclear reactors, jets and missiles, had concentration that ranged from 83 to 273 ppm in *Discovery* deep whereas in *Atlantis II* deep, it ranged from 1 to 30 ppm (table 2). Strontium levels ranged from 437 ppm till reaching 670 ppm yet in *Atlantis II* deep it recorded 291 ppm as its highest reading. Vanadium also in *Discovery* deep was found at high ranges reaching the 118 ppm whereas *Atlantis II* deep recorded 84 ppm as its highest read. Zirconium in *Discovery* deep was found to range from 10 to 38 ppm while in *Atlantis II* deep was 6-24 ppm. Although titanium in the first and last levels of the *Discovery* deep was undetectable, yet in the middle layers in was found in huge amounts till reaching 915 ppm, while in *Atlantis II* deep it was detectable in all its level but in low amounts ranging from 11-116 ppm. Calcium and magnesium also were recorded in higher percentages in *Discovery* deep when compared to *Atlantis II* deep.

For every element detected, a comparison had been made between the six levels of the *Discovery* deep. Some interesting findings were found as a consequence where spill from top level (the first level) never recorded to have any low amount of any
of the metals detected when compared to the other levels of Discovery deep, yet it was found to have the highest amount of arsenic, barium, cadmium, copper, lutetium, molybdenum, sodium, nickel, lead, praseodymium, scandium, silicon, terbium, ytterbium, vanadium, iron, magnesium, manganese and zinc (Fig 18 A, B, C). On the other hand, level 94-141cm, never recorded to have any high amount of any of the metals detected when compared to the other levels of Discovery deep except for zinc (Fig 21); i.e. it was found to have the lowest amount of Barium, cadmium, cerium, chromium, lithium, nickel, niobium, lead, scandium, yttrium, vanadium, zirconium, aluminum, iron, potassium and magnesium (Fig 18 A, B, C and table 3). Level 0-47 had silver, terbium and titanium as its highest metal detected when compared to other levels of Discovery deep (Fig 19). Level 47-94cm was found to have lead as its highest metal whereas strontium and calcium as its lowest regarding other levels (table 3). Level 141-188cm, had cadmium, lithium, strontium and calcium as the highest (Fig 22) whereas the lowest in arsenic, molybdenum and arsenic (table 3). The deepest level in Discovery deep; 188-235cm, recorded the highest in cerium, chromium, niobium, yttrium, zirconium, aluminum and potassium (Fig 23), while the lowest in silver, copper and praseodymium (table 3).

4.2.1.2. CHNS analysis:

Carbon, Hydrogen, Nitrogen and Sulphur profiles were also detected in all the six levels of Discovery deep; as shown in table 4 and figure 24. Unlike Atlantis II deep, Nitrogen was detected in higher amounts in Discovery deep. The Sulphur content is
much more less than that found in *Atlantis II* deep. As the sulphur content in *Discovery* deep does not exceed the 3% yet in *Atlantis II* deep it reached up to 35%.

4.2.2. Geological Analysis:

The electron microscope (EM) pictures of *Discovery* deep samples showed many features, some showed magnificent details of coccoliths, foraminifers indicating geological eras, whereas others showed some vertebrate debris.

Figure 25 shows the EM images of Dicovery deep level 0-47cm, where the blue drawings indicate *Gephyrocapsa* (modern Neogene era “24-1.8 mya” coccolith) with its characteristic bridge. The angle of the bridge gives an indication of the temperature of the surrounding environment.

Figure 26 shows EM images of level 47-94cm, where the violet part is a reticulofenestrid; genus *Reticulofenestra* yet the species is unidentified due to partial coverage of details with authigenic carbonates. The violet rods/ tubes are *Gladiolithus* coccolith whose basal end is a two part oval shape whereas the other end is a triangular shaped tip, six rods can form a long hollow tube with a crown-like serrated end and the other end of the tube has a small basal plate. The green shaped painting is broken bits of *Emiliania huxleyi* (EHUX) coccolithophore. The orange part is a beautiful example of a multi-rayed discoaster, which suggests Eocene geological era “54-38 mya”.

Figure 27 shows EM images of Level 94-141cm, where the orange painting which looks like a snail is a benthic foraminifer; phylum *Foraminiferida*. It normally exists in deep sea and oceans but is not useful in age determination (late
Cretaceous-Paleogene; early-middle Eocene; Oligocene-early Miocene; middle Miocene-recent). The yellow parts are parts broken from pteropod indicating aragonite saturation. The turquoise spherical shapes are **Globigerinoides sacculifer**, which are dominant in the sample as they are tropical species and can deal with the Red Sea high salinity. Yet the taxonomic features of all the *Globigerinoides* in the samples are difficult to identify them on the species level due to difficult angles they are presented at and hence taxonomic features are hard to be revealed. The round green sphere is *Orbulina Universa* which is a typical subtropical to tropical species. The sample shows a unique heavy calcification of all the features it presented.

Figure 28 shows EM images of level 144-188 cm, where a blend of the previous three levels features are presented; *Gephyrocapsa, Gladiolithus* coccolith, EHUX, benthic foraminifer, *Globigerinoides sacculifer*, *Orbulina Universa*, pteropod and mult-rayed dicoaster. Also, the calcification layering growth at the pore of the foram in picture F is presented.

Figure 29 shows EM images of the deepest level of *Discovery* deep 188-235cm. In this level new features appeared along with a blend from previous levels’ features. The new features are bone and vertebrate debris and some new forms of foraminifers where the taxonomic features are hard to be known due to dense sample presentation.
4.2.3. Molecular Analysis:

DGGE gel showed bands that are different in both position and intensity from mixed controls. Total bands isolated and sequenced from *Discovery* deep were 53 yet only 26 bands were of good quality and length after assembly and trimming (Fig 30). Each level had different bands showing good diversity of the environment. Level SFT/spill from top showed good diversity where 9 bands were fully investigated, 0-47 cm showed 4 bands, 47-94 cm 4 bands were of good quality and intensity, 94-141 cm level showed 4 bands, 141-188 cm showed only 3 bands and 188-235 cm level showed 2 bands that were fully investigated. According to NCBI top blast hits, bands showed bacteria isolated from anaerobic, mineral or plant and coral reef associated habitats, hydrothermal vents, thermophilic and marine environments.

4.2.4. Phylogenetic Analysis:

Phylogenetic tree indicated a diverse community on the class level. The tree composed of *Bacilli*, cyanobacteria, *Alpha-Proteobacteria*, *Gamma-Proteobacteria*, *Beta-Proteobacteria*, *Clostridia*, and CFB group (Cytophaga, Flavobacteria, Bacteriodetes, Sphingobacteria). The majority of the sequences were *Beta-Proteobacteria* and *Gamma-Proteobacteria* (Fig 31). The nodes and clusters against which the sequences were compared were 332 bacterial sequences yet 151 of which are uncultured bacteria classified only on the class level (See supplementary data 6). A table showing each sequence of the DGGE bands and its nearest neighbor associated with some of the clustered sequences and the habitat from where these sequences were isolated is indicated in (table 6). The table shows environments that are marine in nature, coral reefs
associated, rich in iron, thermophilic with heavy metal contaminants, and deep sea sediments which support our findings. The v3 region, used in the analysis is so small that it would not allow accurate species assignments to the DGGE clone, that’s why the tree is being presented to show the class level of the presented sequences. Hence all the bacteria included in the tree as a database to which the DGGE clones were compared are being downloaded from SILVA database in order to show classification till reach the class level according to SILVA, RDP and greengenes databases but not according to NCBI database as the latter showed huge unclassified sequences that were found to be classified in the former databases. For Bacilli, only 24 sequences were classified on the class level from NCBI database yet the rest were classified on the class level in the other databases. Cyanobacteria had only 1 sequence classified on the class level yet the rest are fully classified in the mentioned databases. Same applies to Alpha-Proteobacteria where only 33 are NCBI classified, Gamma-Proteobacteria had only 17 sequences classified on NCBI database while Beta-Proteobacteria had 26 sequence classified on the class level, CFB group (Cytophaga, Flavobacteria, Bacteriodetes and Sphingobacteria) had only 65 sequences classified on the class level, Clostridia had only 14 sequences, yet the rest were rendered unclassified on NCBI database which was not the case with the other databases.

Level SFT showed 9 bands where RS 37 belonged to the CFB group while 39 and 42 bands belonged to Beta-Proteobacteria whereas 40 and 41 bands are Bacillus group, band 43 is a Gamma-Proteobacteria, band 45 is Clostridia and both 46 and 47 bands are Alpha-Proteobacteria. Level 0-47cm showed that RS 28, 31, and 32 bands are Gamma-Proteobacteria while RS 29 is a CFB group. Level 47-94cm showed that RS 17,
21, and 27 are CFB group whereas RS 18 is a *Beta-Proteobacteria*. Level 94-141cm showed that RS 6 is a *Gamma-Proteobacteria*, RS 7 and 10 are *Beta-Proteobacteria* while RS 12 is an *Alpha-Proteobacteria*. Level 141-188cm showed that RS 48 is a *Gamma-Proteobacteria*, RS 49 is a *Beta-Proteobacteria* and RS 51 is an *Alpha-Proteobacteria*. Level 188-235cm showed RS 52 to belong to *Bacillus* group and RS 53 is a cyanobacteria.

4.3. Q-PCR and DAPI staining:

4.3.1. Cell count Q-PCR:

Figure 32 shows a standard curve for all samples of both *Atlantis II* and *Discovery* deeps together. All samples show a low cell count of around 70 cells.

4.3.2. DAPI staining:

DAPI staining was done for qualitative purposes. It was done after the isolation procedure of intact bacterial cells and their filtration in order to confirm whether the isolation procedure is a success or a failure. Figures 33 shows a *Discovery* deep sample 0-47 cm mixed with normal sea water on 3, 0.8 and 0.2 um filters. The figure shows two stained items of different sizes; one big and one very small. Thus, DAPI staining indicates a success of the isolation procedure.
5. Discussion:

In 1967, John M. Hunt was the first one to investigate the microbial communities living in the brine area. He was able to find anaerobic bacteria living in water column above the brine pools whereas his study to the brine water itself suggested that the brine water was sterile. After the emerging of the new science of metagenomics and sequencing technologies, it had become easier to investigate microbial communities that are hard to be cultured in the lab similar to the brine pools’ bacterial communities. The study of both Atlantis II and Discovery deeps’ sediments is made much easier and more informative when metagenomics and sequencing technologies are applied to it. Using DNA isolation and sequencing techniques had made it possible to investigate any microbial communities inhabiting such harsh area, as shown in the results section. DGGE results had shown diverse communities not only in Discovery deep sediments but also in Atlantis II sediments that is suspected to have its own geothermal activity and high heavy metal concentrations.

Phylogenetic analysis on the class level had shown high microbial diversity of the studied bacterial community (table 7). Atlantis II deep; level 0-45 cm when compared to Discovery deep; levels spill from top and 0-47 cm, showed a less divergent community. Two classes were predominant in Atlantis II deep; Gamma and Beta-Proteobacteria, whereas Discovery deep showed a more divergent community of six classes; Alpha, Beta, and Gamma-Proteobacteria, Bacilli, Clostridia as well as CFB group. Level 45-90 cm in Atlantis II deep showed three classes of bacteria; Alpha, Beta, and Gamma-Proteobacteria, while Discovery deep; level 47-94 cm showed Beta-Proteobacteria and
bacteria belonging to CFB group. For *Atlantis II* 90-135 cm level only one class of bacteria; *Bacillus*, was being assessed whereas *Discovery* deep 94-141 cm showed 3 classes of bacteria; *Alpha*, *Beta*, and *Gamma-Proteobacteria*. The next level is 135-189 cm in *Atlantis II* deep and 141-188 cm in *Discovery* deep where the former level showed bacteria of *Beta*, and *Gamma-Proteobacteria* classes, yet the later level showed bacteria of *Alpha*, *Beta*, and *Gamma-Proteobacteria* classes. The last level in *Discovery* deep 188-235 cm and the corresponding level in *Atlantis II* deep; 189-225 cm both showed *Cyanobacteria* for the first and only time. For *Atlantis II* deep level 225-267 cm did not have its correspondence in *Discovery* deep, showing bacteria belonging to *Beta* and *Gamma-Proteobacteria*.

Molecular Analysis had shown that the position of some DGGE bands from *Atlantis II* and *Discovery* deeps are at the same place which indicates similarity either on the sequence level or evolutionary level. For example, bands number 28, 48 from *Discovery* deep and 54, 73 and 83 from *Atlantis II* deep; as shown in figure 34, have the same position, whereas in phylogenetic tree display combining both deeps, they also shared same evolutionary origin (Fig 35). This suggests similarity on the sequence level and when the previously mentioned sequences aligned with MAFFT online tool, a 100% similarity in the sequences had been shown as in figure 36. The five previously mentioned sequences indicated a nearest neighbor; as shown in tables 3 and 4 where *Pseudomonas sp*. class *Gamma-Proteobacteria* inhabiting surfaces of historic Scottish monuments, as well as uncultured bacteria isolated from subsurface water of the Kalahari Shield, South Africa.
Applying the same theory as above, DGGE bands number 37 (spill from top level) and 29 (0-47 cm level) of *Discovery* deep have the same position on the gel (Fig 34), have 100% identity as shown with MAFFFT alignment tool (Fig 37) and share the same origin as displayed in the phylogenetic tree in figure 35, indicating uncultured bacteria of phylum bacteriodetes of CFB group; a group that is widely distributed in marine environments. The habitat indicated contaminated sediments with nitrates and heavy metals such as nickel, aluminum, barium, chromium, mercury, copper, arsenic and iron (tables 5, 6). These findings coincide with the chemical profile of the indicated *Discovery* deep levels; spill from top and 0-47 cm which is among the highest concentrations of nickel, aluminum, barium, copper, arsenic and iron (Fig 18 A, 18 B, 18 C, 19). It is worth mentioning that bands of similar position on the DGGE gel were shared in *Discovery* deep 47-94 cm level, and in *Atlantis II* deep 0-45 cm, 135-180 cm, 180-225 cm levels, but were of low sequence quality after trimming via Sequencher™ (Fig 34).

More bands sharing the same DGGE position are 65, 76 and 86 in *Atlantis II* deep (Fig 34). Yet in the phylogenetic tree, it was found that bands number 65 and 76 share the same origin, yet band 86 (RC= reverse complement) doesn’t (Fig 17). This is explained with the MAFFFT alignment tool (Fig 38) that shows that band 86 had an additional part at the beginning of the sequence not shared by the other two sequences whereas it had a missing part at its end but present in the other two sequences, leading to having the same melting behavior and gel position yet differ in evolutionary origin (Fig 17). The phylogenetic information of bands number; 65, 76 and 86, show uncultured bacteria, class *Beta-Proteobacteria*. The habitat from which these bands are isolated differ; bands
65 and 76 show habitat of contaminated sediments with nitrates and heavy metals such as barium, copper, and iron (table 5, 6). These findings coincide with chemical profile of those levels where at level 45-90 cm and 135-180 cm of Atlantis II deep have the highest amount of barium, copper, and iron (figures 5, 7, 8). However, band 86 show habitats of water biofilms receiving chlorine or monochloramine residual as well as contaminated sediments with nitrates and heavy metals such as nickel and aluminum, which coincides with the chemical profiles’ findings of the level of Atlantis II deep; 225-267cm; shown in figure 9, indicating the highest contents of nickel and aluminum of all levels.

Following the previous rule, bands 61 and 79 in Atlantis II deep levels 0-45 cm and 135-180 cm consecutively have the same position on DGGE gel (figure 37), and share the same origin in phylogenetic tree (figure 16) indicating Burkholderiaceae bacterium class Beta-Proteobacteria isolated from volcanic deposits. Another pair are bands number 64 (Atlantis II; 45-90 cm level) and 39 (Discovery; SFT) that share both the DGGE position and phylogenetic origin (Fig 37, 38) indicating Beta-Proteobacteria that is metal and antibiotic resistant, iron, coal and chromium rich habitat. Also, bands 67 (Atlantis II; 45-90 cm level) and 77 (Atlantis II; 135-180 cm level) are found of same phylogenetic origin with same DGGE position (Fig 16, 17) indicating Gamma-Proteobacteria isolated from anoxic habitat rich in arsenite, sulphur and dolomite associated environment. Additional three bands follow this rule are bands from Atlantis II deep (Fig 16, 17) number 60 (0-45 cm), 68 (45-90 cm), 78 (135-180 cm) indicating Gamma-Proteobacteria isolated from Mariana trench in Japan at depth 11,000m as well as gold rich environment. The last pair following such a rule are two bands from both
deeps, 43 (Discovery; spill from top level) and 59 (Atlantis II; 0-45 cm) indicating *Gamma-Proteobacteria* inhabiting dolomite rocks and lakes (Fig 34, 35).

Exceptions of this rule are bands 31 and 17 from Discovery deep (Fig 30), that share the same DGGE position yet have different phylogenetic origin (Fig 31) where band 31 belongs to *Gamma-Proteobacteria* whereas band 17 belongs to CFB group. The MAFFT alignment (Fig 39) shows multiple bases different in each sequence that may lead to same melting behavior but different composition and this is one of the drawbacks of DGGE technique as mentioned by Andreas Felske; 2007. The difference in phylogenetic origin also could be attributed to sequence quality and trimming accuracy of both sequences, yielding inaccurate bases.

Chemical profile of metallic deposits as well as diversity profile of the microbial communities inhabiting both deeps had revealed a prospective potential for further research in the fields of heavy metal resistance, bioremediation and bio-indicators. *Atlantis II* deep had indicated high concentrations of silver, cadmium, molybdenum, lead, praseodymium, aluminum, iron, manganese, and arsenic, whereas; *Discovery* deep had showed high concentrations of cerium, chromium, lithium, niobium, strontium, vanadium, zirconium and titanium. Moreover, in both deeps the following metals did exist in high concentrations: copper, zinc, nickel, silicon and barium. This indicates the extreme nature of such habitats and the exquisite nature of the genetic capabilities the organisms inhabiting such environment could have. The toxic effect of heavy metals on bacterial cells varies according to the kind of heavy metal that exists in the environment, its concentration and the mechanism of heavy metal resistance the bacterial cell could
encounter. Mercury, cadmium and silver constructs the most lethal metals in the smallest concentration to bacterial cells, yet needed in higher concentrations come next zinc, nickel and copper\textsuperscript{58}. There is no general mechanism for heavy metal resistance\textsuperscript{59}. Some bacterial cells are resistant to arsenic and antimony through efflux pump which operates through \textit{ars}\textsuperscript{59}. Cyanobacterium \textit{Synechocystis} PCC6808\textsuperscript{59} as well as \textit{Alcaligenes eutrophus} were found to be resistant to cadmium, zinc and cobalt through a homologous protein to \textit{czcABC} complex, this is significant due to the presence of cyanobacteria\textsuperscript{59} in both \textit{Atlantis II} deep 189-225 cm level and \textit{Discovery} deep 188-235 cm level (table 7) whereas, \textit{Alcaligenes} sp.\textsuperscript{60} (table 5) showed a hit to RS 66 band in \textit{Atlantis II} deep 45-90 cm level. Silver also represented a major toxic compound to bacterial cells where samples isolated from sediments in Thames River were found to be tolerant to silver nitrate and sequencing revealed \textit{Pseudomonas} sp.\textsuperscript{61}. This is significant due to the predominance of \textit{Pseudomonas} sp. representing the nearest neighbors of many bacteria inhabited the studied sample (table 5 and 6). The Bacterial response to vanadium; which is also a toxic metal detected in \textit{Discovery} deep in considerable high amounts, is not widely investigated\textsuperscript{62}. Nickel is also found in both deeps where RS 65, 76 and 54 in \textit{Atlantis II} deep show hits from environments contaminated with nickel, aluminum, barium, chromium, mercury, copper, arsenic, and iron, literature discussed bacterial cells investigated to be resistant to nickel through biosorption\textsuperscript{63}. 

CHNS profiles indicated high levels of sulphur in \textit{Atlantis II} deep reaching up to 35\% whereas \textit{Discovery} deep reached 2.99\% (table 4). This sulphur content in \textit{Atlantis II} deep supports the idea that this deep in specific has its own geothermal activity.
*Discovery* deep on the other hand had high levels of carbon and nitrogen compared to *Atlantis II* deep, where nitrogen in *Atlantis II* deep was less than 0.1%.

Finally, the geological results of both deeps showed interesting findings. *Atlantis II* deep showed completely different contents than that of *Discovery* deep. *Discovery* deep was full of planktic contents suggesting both Neogene (24-1.8 mya) and Eocene (54-38 mya) geological eras which both belong to Tertiary period (65 – 1.8 mya) which is the age of mammals. While; *Atlantis II* deep showed authentic carbonate, aragonite needles and few planktic foraminifers which suggests either dissolution or dilution due to the fast growth of the other materials in compared to coccoliths’ calcification. This also might explain the high contents of calcium in *Discovery* deep compared to *Atlantis II* deep due to the high amount of calcified coccoliths present in *Discovery* deep.
6. Conclusion:

Bacterial communities in environmental samples such as soil, groundwater, and sediments are known to be extremely complex. *Atlantis II* and *Discovery* deeps are known for their exquisite nature and composition either on the chemical level or the geological level. Previous researches never revealed any information about microbial communities inhabiting these two brine pools, yet cell count and DAPI staining of samples isolated from these two areas provided information in this area of research. Despite of the low cell count done using Q-PCR, the study had proved the survival of intact bacterial cells after sampling and freezing where DNA was studied. Phylogenetic analysis of surviving bacterial cells indicated a high diversity of the microbial community inhabiting both deeps. The molecular analysis had shown the diversity of the community on the class level. Bacteria belonging to *Alpha*, *Beta*, and *Gamma-Proteobacteria*, *Bacillus* and *Clostridia* classes as well as phyla *Cytophaga*, *Flavobacteria* and *Bacteriodetes* (CFB group) had proved how diverse the studied community is. That’s why a DGGE technique is not enough to provide a complete assessment of the community living in such harsh conditions as that of our sample. DGGE is a simple technique that gives an overview about the complexity of the studied community. That is mainly because of two reasons; first, the excised band that gets sequenced represents a few nucleotides of the v3 targeted amplified region of the DNA; i.e not the whole 16S rDNA sequence; hence some information is still missing. Second, the sequence obtained by sequencing technique sometimes is not of high quality, this is mainly attributed to
band multiplicity, i.e. one band could contain multiple sequences having the same melting behavior and hence confuse the sequence analysis.

Another reason why this study gives a general preliminary overview of the bacterial community inhabiting this environment is the technique of DNA isolation. DNA isolation technique used, studied only bacteria that was able to survive, i.e. intact bacterial cells that was able to survive sampling, storing and shipment conditions, where these bacteria got filtered and then underwent DNA isolation (intracellular DNA) as shown in the materials and methods section. The intracellular DNA isolation technique of this study was done as a result of failure to isolate extracellular DNA applying many techniques. The first technique used was a DNA extraction protocol from sediments adopted from Brady et al, 2007; Fierer et al., 2007 and Mesbah et al., 2007. Also, Mobio Powersoil kit™ was not successful in isolating any of the extracellular DNA at a detectable concentration. Another technique was applied for extracellular DNA isolation was using ELUTRAP™ electro-elution system which also did not yield any detectable DNA. The last unsuccessful attempt was direct gel electrophoresis for the sediment sample at 120 volts for 60 minutes for 2% agarose gel and further elution of the isolated DNA in TE buffer. The unsuccessfulness of the previous techniques was suggested to be due to the high adsorption capacity of the sediment particles that contain high concentration of positively charged metals which binds to the negatively charged DNA. Although many techniques were applied to isolate extracellular DNA from the sediments but intracellular DNA isolation was a success. Q-PCR cell count as well as DAPI staining results showed that few cells were able to survive, indicating a high diversity of the microbial communities inhabiting both deeps.
In conclusion, isolation of both more DNA is needed to be done in further analysis to give an accurate overview of the microbial communities inhabiting such extreme environments; this could be done using freshly isolated samples in order to prevent overcharging of positively charged sediment particles and hence adsorption of DNA to those particles. Moreover, a microbiological cell study needs to be done in order to provide a better overview for the bacterial community. Also, a clone library or pyrotagsequencing technique should be done instead of DGGE technique in order to generate an accurate phylogenetic assessment of high quality 16S rDNA sequences. However, in order to generate an accurate phylogenetic tree a computer hardware requirement of more than 6 GB RAM is needed in order to process a huge database of sequences generated by pyrotagsequencing techniques.
List of abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>WHOI</td>
<td>Woods Hole Oceanographic Institution</td>
</tr>
<tr>
<td>CFB group</td>
<td>Cytophaga–Flavobacterium–Bacteroides group</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>CHNS</td>
<td>Carbon-Hydrogen-Nitrogen-Sulphur profile</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbon</td>
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<tr>
<td>TGGE</td>
<td>Temperature Gradient Gel Electrophoresis</td>
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<tr>
<td>ICP-OES</td>
<td>Inductively Coupled Plasma Optical Emission Spectrometry</td>
</tr>
<tr>
<td>MAFFT</td>
<td>Multiple Alignment using Fast Fourier Transform online tool</td>
</tr>
<tr>
<td>iTOL</td>
<td>Interactive Tree Of Life</td>
</tr>
</tbody>
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Figure 1: Prokaryotic 16S rRNA

The bold lines show the conserved regions of relative sequence consistency. The thin lined areas are the variable areas. V1, V2, V3, V5, V6, V7, V8, V9 are prokaryotic variable regions. V4 is absent in prokaryotes.
Figure 2: A schematic diagram of the amplified studied region of 16S RNA molecule

The figure shows the highly variable area in the v3 region of 16 S rDNA sequence where the primers used in this study amplify v3 region from position 341 till position 534 where a GC-clamp of 40bp is being used attached at the 5’ prime end of the primer. 21

Figure 3: Atlantis II deep and Discovery deep locations

A figure showing the depths profile and location of both brine pools from R/V Oceanus (WHOI/AUC) expedition done in October 2008 obtained from KAUST (King Abdullah University for Science and Technology).
A: Shows the Location map of both the brine pools lying in the middle of Jeddah and Sudanese waters.
B: Shows the exact location and depth of both sampling areas of Atlantis II and Discovery deeps; where the violet arrow indicates the South West/ SW basin of Atlantis II deep at depth of about 2,250 m at latitude of about 21°20’N and Longitude of about 38°04’E. While the pink arrow shows Discovery deep location at depth of about 2,200 m at at latitude of about 21°16’N and Longitude of about 38°03’E.
Figure 4: Atlantis II deep, highest 0-45 cm level sample.

Shows compounds that recorded the highest concentration in the first level of Atlantis II deep where the figure is displayed as a comparison of chromium/ Cr and potassium/ K in the six levels of Atlantis II deep. The arrows indicate the concentration in ppm; Cr was found to be 6 ppm whereas K was found to be 832 ppm.
Figure 5: *Atlantis II* deep, 45-90 cm sample

Shows the metals that had the highest concentration in the second level of *Atlantis II* deep where the figure is displayed as a comparison of molybdenum/ Mo, praseodymium/ Pr, ytterbium/ Tb and iron/ Fe in the six levels of *Atlantis II* deep. The arrows indicate the concentration in ppm for Mo 163 ppm, Pr 28 ppm and Tb 6 ppm. Fe was found to be 35.35%.
Figure 6 A: Atlantis II deep, 90-135 cm sample

Shows the metals that had the highest concentration in the third level of Atlantis II deep where the figure is displayed as a comparison of arsenic/As, lutetium/Lu, manganese/Mn, niobium/Nb, phosphorous/P and lead/Pb in the six levels of Atlantis II deep. The arrows indicate the concentration in ppm for As=230 ppm, Lu=7 ppm, Mn=793 ppm, Nb=30 ppm, P=848 ppm and Pb=724 ppm.
Figure 6 B: Atlantis II deep, 90-135 cm sample

Shows the metals that had the highest concentration in the third level of Atlantis II deep where the figure is displayed as a comparison of scandium/Sc, silicon/Si, titanium/Ti, vanadium/V, ytterbium/Yb and zirconium/Zr in the six levels of Atlantis II deep. The arrows indicate the concentration in ppm for Sc=692 ppm, Si=692 ppm, Ti=161 ppm, V=84 ppm, Yb=3 ppm, Zr=24 ppm.
Figure 7: Atlantis II deep, 135-180 cm sample

Shows the metals that had the highest concentration in the fourth level of Atlantis II deep where the figure is displayed as a comparison of barium/ Ba and calcium/ Ca in the six levels of Atlantis II deep. The arrows indicate the concentration in ppm for Ba where it reached 234 ppm. Ca is detected in percent value which reached 9.35%.
Figure 8: *Atlantis II* deep, 180-225 cm sample

Shows the metals that had the highest concentration in the fifth level of *Atlantis II* deep where the figure is displayed as a comparison of silver/Ag, cadmium/Cd, copper/Cu, magnesium/Mg, sodium/Na and zinc/Zn in the six levels of *Atlantis II* deep. The arrows indicate the concentration in ppm for Ag=64 ppm, Cd=172 ppm, Na=329 ppm. In percent value the following were detected; Cu=0.8%, Mg=0.66% and Zn=9.65%.
Figure 9: Atlantis II deep, 225-267 cm sample

Shows the metals that had the highest concentration in the deepest level of Atlantis II deep where the figure is displayed as a comparison of aluminum/ Al, lithium/ Li, nickel/ Ni and strontium/ Sr in the six levels of Atlantis II deep. The arrows indicate the concentration in ppm for Al=855 ppm, Li=5 ppm, Ni=185 ppm and Sr=291 ppm.
Figure 70: *Atlantis II* deep CHNS comparison among six levels

Shows CHNS (total carbon, hydrogen, nitrogen and sulphur content) detected in percentage in *Atlantis II* deep. Nitrogen was detected but it was in trace amounts less than 0.1 % found whereas hydrogen in both 135-180 cm and 225-267 cm levels only were untraceable. Carbon was detected in all levels except the deepest one; 225-267 cm level, whereas sulphur was highly detected in all the six *Atlantis II* levels. The figure shows that level 90-135 cm constructs the highest CHNS profile when compared to the other levels of *Atlantis II* deep.
Figure 8: Geological analysis of *Atlantis II*; 0-45 cm level

Electron microscope image done to the first level of *Atlantis II* deep; 0-45 cm;
A: Is a 10um magnification of the first level. The image shows no planktic features
B: Is a 2um magnification with no characteristic features.
C: Is a 200nm magnification with small rods/needles are visible.
The image was done using electron microscope in the “Youssef Jameel Science and Technology Research Center” lab at the AUC facility.
Figure 9: Geological analysis of Atlantis II; 45-90 cm level

Electron microscope image done to the second level of Atlantis II deep; 45-90 cm;
A: Is a 10um magnification of the sample with no planktic features are visible, authentic carbonate is suspected to be the visible part.
B: Needles are visible of unknown source due to dense sample.
The image was done using electron microscope in the “Youssef Jameel Science and Technology Research Center” lab at the AUC facility.
Figure 10: Geological analysis of Atlantis II; 90-135 cm level

Electron microscope image done to the third level of Atlantis II deep; 90-135 cm;
A: The spherical shape is a planktic foraminifer genus *Globigerinoides*.
B: Shows broken bits of the foraminifer.
C: Another type of foraminifer but due to incomplete features, it remains unidentified taxonomically.
The image was done using electron microscope in the “Youssef Jameel Science and Technology Research Center” lab at the AUC facility.
Figure 11: Geological analysis of *Atlantis II*; 135-180 cm level

Electron microscope image done to the fourth level of *Atlantis II* deep; 135-180 cm; The orange arrows are pointing at argonate needles stacked together. No planktic features were detected. The image was done using electron microscope in the “Youssef Jameel Science and Technology Research Center” lab at the AUC facility.
Figure 12: Geological analysis of *Atlantis II*; 180-225 and 225-267 cm levels

Electron microscope image done to the deepest two levels of *Atlantis II* deep; 180-225 cm and 225-267 cm;
The spherical ball-like shaped images indicate bacterial communities inhabiting these samples.
The image was done using electron microscope in the "Youssef Jameel Science and Technology Research Center" lab at the AUC facility.
Molecular Analysis: DGGE fingerprints of Atlantic II deep bacterial community amplified by universal 16srRNA primers. The total bands isolated and sequenced from Atlantic II deep were 34. Only 19 bands were of good quality and length after assembly and trimming using Sequencher™. The coloring pattern of bands indicates the best hits’ habitat obtained from blast against nr/nt database on NCBI website.
Figure 14: Phylogenetic Analysis of *Atlantis II* bacterial community

Showing tree done using PhyML program, where the green lines and boxes indicate the 19 *Atlantis II* DGGE bands. Sequence trimming was done using "Sequencher" program. Then blast against SSU ref Silva database. Clustering of blast hits was done using CD-Hits 97%. Alignment using MAFFT (multiple alignment using fast fourier transform online tool, L-INS option). Refinement of alignment was done using Jalview program. Phylogenetic tree was generated using PhyML program where 3 parsimony trees were done to generate the final tree. See supplementary data 5 in order to reveal more information on the class level.
Figure 15 A: *Discovery* deep, SFT sample

Shows the metals that had the highest concentration in the uppermost level of *Discovery* deep where the figure is displayed as a comparison of arsenic/ As, barium/ Ba, cadmium/ Cd, copper/ Cu, lutetium/ Lu and molybdenum/ Mo in the six levels of *Discovery* deep. The arrows indicate the concentration in ppm for As=56 ppm, Ba=142 ppm, Cd=3 ppm, Cu=182 ppm, Lu=2 ppm and Mo=15 ppm.
Figure 18 B: *Discovery deep, SFT sample*

Shows the metals that had the highest concentration in the uppermost level of *Discovery deep* where the figure is displayed as a comparison of sodium/ Na, nickel/ Ni, lead/ Pb, praseodymium/ Pr, scandium/ Sc and silicon/ Si in the six levels of *Discovery deep*. The arrows indicate the concentration in ppm for Na=760 ppm, Ni=51 ppm, Pb=29 ppm, Pr=6 ppm, Sc=5 ppm and Si=526 ppm.
Figure 18 C: Discovery deep, SFT sample

Shows the metals that had the highest concentration in the uppermost level of Discovery deep where the figure is displayed as a comparison of terbium/ Tb, ytterbium/ Yb, vanadium/ V, iron/ Fe, magnesium/ Mg, manganese/ Mn and zinc/ Zn in the six levels of Discovery deep. The arrows indicate the concentration in ppm for Tb=2 ppm, Yb=2 ppm, V=2.75 ppm. In percent concentration, the following were detected; Fe=9.58%, Mg=2.75%, Mn=3.93% and Zn=0.15%.
Figure 19: Discovery deep, 0-47 cm sample

Shows the metals that had the highest concentration in the 0-47 cm level of Discovery deep where the figure is displayed as a comparison of silver/Ag, terbium/Tb and titanium/Ti in the six levels of Discovery deep. The arrows indicate the concentration in ppm for Ag=9 ppm, Tb=2 ppm and Ti=915 ppm.
Figure 20: *Discovery deep, 47-94 cm sample*

Shows the metals that had the highest concentration in the third level of *Discovery deep* where the figure is displayed as a comparison of lead/ \( \text{Pb} \) and terbium/ \( \text{Tb} \) in the six levels of *Discovery deep*. The arrows indicate the concentration in ppm for Pb=29 ppm and Tb=2 ppm.
Figure 16: *Discovery deep, 94-141 cm sample*

Shows the metals that had the highest concentration in the fourth level of *Discovery deep* where the figure is displayed as a comparison of zinc/ Zn in the six levels of *Discovery deep*. The arrow indicates the concentration in percent value for Zn which reached 0.15%.
Figure 17: *Discovery* deep, 141-188 cm sample

Shows the metals that had the highest concentration in the fifth level of *Discovery* deep where the figure is displayed as a comparison of cadmium/ Cd, lithium/ Li, strontium/ Sr and calcium/ Ca in the six levels of *Discovery* deep. The arrows indicate the concentration in ppm for Cd=3 ppm, Li=115 ppm and Sr=670 ppm. In percent value Ca reached 10.61%.
Figure 18: *Discovery* deep, 188-235 cm sample

Shows the metals that had the highest concentration in the deepest level of *Discovery* deep where the figure is displayed as a comparison of cerium/ Ce, chromium= Cr, niobium/ Nb, yttrium/ Y, zirconium/ Zr, aluminum/ Al and potassium/K in the six levels of *Discovery* deep. The arrows indicate the concentration in ppm for Ce=21 ppm, Cr=66 ppm, Nb=273 ppm and Y= 1.92. In percent value Al reached 1.92% while K reached 0.61%.
Carbon, Hydrogen, Nitrogen and Sulphur profiles were also detected in all the six levels of *Discovery* deep; Unlike *Atlantis II* deep, Nitrogen was detected in higher amounts in *Discovery* deep. The Sulphur content is much more less than that found in *Atlantis II* deep. As the sulphur content in *Discovery* deep does not exceed the 3% yet in *Atlantis II* deep it reached up to 35%.
Figure 20: Geological analysis of Discovery deep sediments level 0-47cm

EM image showing; the blue part is a *Gephyrocapsa* and the pink rods/ tubes
Figure 21: Geological analysis of Discovery deep sediments level 47-94cm

The overall image is at 10um magnification showing multiple features and enlarged at 1 um to display the details of the colored parts.
Figure 22: Geological analysis of *Discovery* deep sediments level 94-141cm
Figure 28: Geological analysis of Discovery deep sediments level 141-188cm

F: shows calcification layering growth at the pore of the foram
Figure 29: Geological analysis of *Discovery* deep sediments level 188-235cm
Figure 23: DGGE fingerprints of Discovery Deep brine pool bacterial community

Shows DGGE bands of Discovery deep, where DGGE gel shows bands that are different in both position and intensity from mixed controls. Total bands isolated and sequenced from Discovery deep were 53. Only 26 bands were of good quality and length after assembly and trimming using SequencherTM. The coloring pattern of bands indicates the best hits’ habitat obtained from blast against nr/nt database on NCBI website.
Figure 24: Phylogenetic Analysis of Discovery Deep Bacterial Community:

Showing tree done using PhyML program, where the red lines and boxes indicate the 26 Discovery DGGE bands. Sequence trimming was done using “Sequencher” program. Then blast against SSU ref Silva database. Clustering of blast hits was done using CD-Hits 97%. Alignment using MAFFT (multiple alignment using fast fourier transform online tool, L-INS option). Refinement of alignment was done using Jalview program. Phylogenetic tree was generated using PhyML program where 3 parsimony trees were done to generate the final tree. See supplementary data 6 in order to reveal more information on the class level.
Figure 25: The cell count Q-PCR

Q-PCR conditions were: initial melting 98°C for 2 mins/1 cycle, melting 98°C for 5 secs/ 60 cycles, annealing 57°C for 15 secs/ 60 cycles, 80°C for 20 secs/ 60 cycles and 40°C for ∞. The Q-PCR is stopped at cycle 45. Q-PCR kit used is EvagreenTM; the Q-PCR mixture for one reaction is as follows: PCR water 8.6 ul, Evagreen supermix 10 ul, Primers used are Bac 341f, 0.2 ul, Bac 534r, 0.2 ul, DNA template 1 ul, final volume 20 ul/Reaction. Negative controls for each Q-PCR were used. A Q-PCR standard was also amplified in order to perform cell count.

Figure 26: DAPI stain of mixed sea water + sample

DAPI stain of 0.2 um filter of both Discovery 0-47 cm sediment and filtrate of water above sediments and normal sea water cells. The figure shows two stained items of different sizes; one big and one very small. Thus, DAPI staining indicates a success of the isolation procedure.
Figure 27: DGGE fingerprint of both Discovery and Atlantis II deeps
Figure 28: Phylogenetic Analysis of both *Atlantis II* deep and *Discovery* deep

Showing tree done using PhyML program, where the red lines and boxes indicate the 26 *Discovery* DGGE bands and the green lines and boxes indicate *Atlantis II* bands. Sequence trimming was done using “Sequencher” program. Then blast against SSU ref Silva database. Clustering of blast hits was done using CD-Hits 97%. Alignment using MAFFT (multiple alignment using fast fourier transform online tool, L-INS option). Refinement of alignment was done using Jalview program. Phylogenetic tree was generated using PhyML program where 3 parsimony trees were done to generate the final tree. See supplementary data 7 in order to reveal more information on the class level.
Figure 29: MAFFT alignment displayed by BioEdit program of sequences 28, 48, 54, 73 and 83 showing 100% similarity.

Figure 37: MAFFT alignment displayed by BioEdit program of sequences 29 and 37 showing 100% similarity.
Figure 30: MAFFT alignment displayed by BioEdit program of sequences 65, 76 and 86.

Red arrows indicate missing additional parts in band 86 and a missing part at its end.

Figure 39: MAFFT alignment displayed by BioEdit program of sequences 17 and 31.
Table 2: Chemical analysis of *Atlantis II* brine sediments/ Metal compounds

*Atlantis II* Metal compounds, where arrows indicate metals that are higher in concentration than *Discovery* deep.

|          | Ag  | Yb  | As  | Ba  | Zr  | Cd  | Ce  | Cr  | K   | Li  | Lu  | Mo  | Na  | Nb  | Ni  | P   | Pb  | Pr  | Sc  | Si  | Sr  | Tb  | Ti  | V   | Unit |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 0-45 cm  | 50  | 2   | 115 | 137 | 8   | 82  | <1  | 6   | 832 | 2   | 3   | 65  | 207 | 17  | 13  | <1  | 112 | 13  | <1  | 255 | 188 | 3   | 119 | 55  | ppm  |
| 45-90 cm | 49  | 3   | 159 | 94  | 11  | 60  | <1  | 5   | <1  | 7   | 163 | 150 | 12  | 17  | 712 | 480 | 28  | <1  | 590 | 61  | 6   | 66  | 71  | ppm  |
| 90-135 cm| 56  | 3   | 230 | 122 | 24  | 90  | <1  | 6   | 1   | 7   | 145 | 181 | 30  | 36  | 848 | 724 | 26  | <1  | 692 | 80  | 5   | 161 | 84  | ppm  |
| 135-180 cm| 10 | <1 | 8   | 234 | 1   | 17  | <1  | 1   | 198 | <1  | 36  | 202 | 1   | 4   | 219 | 37  | <1  | <1  | 45  | 281 | <1  | 11  | 8   | ppm  |
| 180-225 cm| 64 | 2   | 36  | 64  | 11  | 172 | <1  | 5   | 717 | <1  | 4   | 41  | 329 | 12  | 36  | 797 | 396 | 13  | <1  | 390 | 45  | 4   | 70  | 76  | ppm  |
| 225-267 cm| 16 | <1 | 15  | 135 | 6   | 27  | <1  | 2   | 5   | 1   | 18  | 278 | 2   | 185 | 43  | 2   | <1  | 83  | 291 | 1   | 13  | 11  | ppm  |

<table>
<thead>
<tr>
<th></th>
<th>Al</th>
<th>Ca</th>
<th>Cu</th>
<th>Fe</th>
<th>Mg</th>
<th>Mn</th>
<th>Zn</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-45 cm</td>
<td>0.35</td>
<td>1.17</td>
<td>0.43</td>
<td>20.59</td>
<td>0.37</td>
<td>1.41</td>
<td>2.77</td>
<td>%</td>
</tr>
<tr>
<td>45-90 cm</td>
<td>0.34</td>
<td>0.47</td>
<td>0.42</td>
<td>35.35</td>
<td>0.33</td>
<td>0.36</td>
<td>1.16</td>
<td>%</td>
</tr>
<tr>
<td>90-135 cm</td>
<td>0.54</td>
<td>0.94</td>
<td>0.53</td>
<td>34.58</td>
<td>0.41</td>
<td>793</td>
<td>3.1</td>
<td>%</td>
</tr>
<tr>
<td>135-180 cm</td>
<td>672</td>
<td>9.35</td>
<td>0.55</td>
<td>2.26</td>
<td>0.24</td>
<td>341</td>
<td>0.76</td>
<td>%</td>
</tr>
<tr>
<td>180-225 cm</td>
<td>0.5</td>
<td>0.59</td>
<td>0.8</td>
<td>23.28</td>
<td>0.66</td>
<td>780</td>
<td>9.65</td>
<td>%</td>
</tr>
<tr>
<td>225-267 cm</td>
<td>855</td>
<td>8.1</td>
<td>0.19</td>
<td>4.48</td>
<td>0.32</td>
<td>0.23</td>
<td>0.65</td>
<td>%</td>
</tr>
</tbody>
</table>
Table 3: Chemical analysis of *Discovery* brine sediments/Metal compounds

*Discovery* Metal compounds, where arrows indicate metals that are higher in concentration than *Atlantis II* deep.

<table>
<thead>
<tr>
<th></th>
<th>Ag</th>
<th>As</th>
<th>Ba</th>
<th>Cd</th>
<th>Ce</th>
<th>Cr</th>
<th>Cu</th>
<th>Li</th>
<th>Lu</th>
<th>Mo</th>
<th>Nb</th>
<th>Ni</th>
<th>Pb</th>
<th>Pr</th>
<th>Sc</th>
<th>Si</th>
<th>Sr</th>
<th>Tb</th>
<th>V</th>
<th>Y</th>
<th>Yb</th>
<th>Zr</th>
<th>P</th>
<th>Na</th>
<th>Ti</th>
<th>Zn</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>spill from top</td>
<td>8</td>
<td>56</td>
<td>142</td>
<td>3</td>
<td>13</td>
<td>43</td>
<td>182</td>
<td>25</td>
<td>2</td>
<td>15</td>
<td>228</td>
<td>51</td>
<td>29</td>
<td>6</td>
<td>5</td>
<td>526</td>
<td>522</td>
<td>2</td>
<td>118</td>
<td>7</td>
<td>2</td>
<td>31</td>
<td>760</td>
<td>ppm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-47</td>
<td>9</td>
<td>52</td>
<td>113</td>
<td>2</td>
<td>12</td>
<td>36</td>
<td>120</td>
<td>20</td>
<td>1</td>
<td>12</td>
<td>191</td>
<td>44</td>
<td>27</td>
<td>4</td>
<td>4</td>
<td>433</td>
<td>445</td>
<td>2</td>
<td>97</td>
<td>6</td>
<td>1</td>
<td>26</td>
<td>873</td>
<td>572</td>
<td>915</td>
<td>ppm</td>
<td></td>
</tr>
<tr>
<td>47-94</td>
<td>8</td>
<td>55</td>
<td>98</td>
<td>2</td>
<td>9</td>
<td>31</td>
<td>115</td>
<td>17</td>
<td>1</td>
<td>11</td>
<td>154</td>
<td>36</td>
<td>29</td>
<td>4</td>
<td>3</td>
<td>351</td>
<td>437</td>
<td>2</td>
<td>83</td>
<td>5</td>
<td>1</td>
<td>19</td>
<td>743</td>
<td>504</td>
<td>742</td>
<td>713</td>
<td>ppm</td>
</tr>
<tr>
<td>94-141</td>
<td>4</td>
<td>16</td>
<td>23</td>
<td>1</td>
<td>4</td>
<td>14</td>
<td>72</td>
<td>10</td>
<td>&lt;1</td>
<td>4</td>
<td>83</td>
<td>20</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>170</td>
<td>620</td>
<td>1</td>
<td>31</td>
<td>3</td>
<td>&lt;1</td>
<td>10</td>
<td>0.13</td>
<td>404</td>
<td>ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>141-188</td>
<td>4</td>
<td>10</td>
<td>40</td>
<td>3</td>
<td>8</td>
<td>21</td>
<td>86</td>
<td>115</td>
<td>&lt;1</td>
<td>2</td>
<td>126</td>
<td>22</td>
<td>19</td>
<td>1</td>
<td>3</td>
<td>157</td>
<td>670</td>
<td>1</td>
<td>39</td>
<td>5</td>
<td>&lt;1</td>
<td>16</td>
<td>618</td>
<td>ppm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>188-235</td>
<td>2</td>
<td>20</td>
<td>98</td>
<td>2</td>
<td>21</td>
<td>66</td>
<td>55</td>
<td>20</td>
<td>1</td>
<td>4</td>
<td>273</td>
<td>34</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td>315</td>
<td>586</td>
<td>&lt;1</td>
<td>100</td>
<td>10</td>
<td>2</td>
<td>38</td>
<td>436</td>
<td>ppm</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Al</th>
<th>Ca</th>
<th>Fe</th>
<th>K</th>
<th>Mg</th>
<th>Mn</th>
<th>Zn</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spill from top</td>
<td>1.68</td>
<td>8.39</td>
<td>9.58</td>
<td>0.37</td>
<td>2.75</td>
<td>3.93</td>
<td>0.15</td>
<td>%</td>
</tr>
<tr>
<td>0-47</td>
<td>1.35</td>
<td>7.42</td>
<td>7.26</td>
<td>0.29</td>
<td>2.19</td>
<td>3.02</td>
<td>0.12</td>
<td>%</td>
</tr>
<tr>
<td>47-94</td>
<td>1</td>
<td>5.98</td>
<td>6.36</td>
<td>0.22</td>
<td>1.71</td>
<td>2.62</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>94-141</td>
<td>0.57</td>
<td>8.95</td>
<td>2.62</td>
<td>0.18</td>
<td>1.11</td>
<td>0.86</td>
<td>0.15</td>
<td>%</td>
</tr>
<tr>
<td>141-188</td>
<td>0.74</td>
<td>10.61</td>
<td>2.15</td>
<td>0.29</td>
<td>1.3</td>
<td>0.32</td>
<td>0.14</td>
<td>%</td>
</tr>
<tr>
<td>188-235</td>
<td>1.92</td>
<td>7.75</td>
<td>4.95</td>
<td>0.61</td>
<td>1.88</td>
<td>0.25</td>
<td>%</td>
<td></td>
</tr>
</tbody>
</table>
Table 4: CHNS profile of both brine sediments

shows CHNS profiles of Both Atlantis II deep and Discovery deeps, where arrows indicate elements that are higher in the indicated deep than the other.

### Atlantis II deep

<table>
<thead>
<tr>
<th></th>
<th>0-45 cm</th>
<th>45-90 cm</th>
<th>90-135 cm</th>
<th>135-180 cm</th>
<th>180-225 cm</th>
<th>225-267 cm</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.44</td>
<td>0.39</td>
<td>2.55</td>
<td>1.09</td>
<td>0.44</td>
<td>&lt;0.1</td>
<td>% Found</td>
</tr>
<tr>
<td>H</td>
<td>0.72</td>
<td>0.82</td>
<td>5.19</td>
<td>&lt;0.10</td>
<td>0.83</td>
<td>&lt;0.1</td>
<td>% Found</td>
</tr>
<tr>
<td>N</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.1</td>
<td>% Found</td>
</tr>
<tr>
<td>S</td>
<td>5.11</td>
<td>4.19</td>
<td>35.62</td>
<td>23.26</td>
<td>6.85</td>
<td>24.59</td>
<td>% Found</td>
</tr>
</tbody>
</table>

### Discovery deep

<table>
<thead>
<tr>
<th></th>
<th>Spill from top</th>
<th>0-47 cm</th>
<th>47-94</th>
<th>94-141</th>
<th>141-188</th>
<th>188-235</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5.52</td>
<td>5.41</td>
<td>5.28</td>
<td>8.83</td>
<td>8.44</td>
<td>6.36</td>
<td>% Found</td>
</tr>
<tr>
<td>H</td>
<td>1.3</td>
<td>1.24</td>
<td>1.23</td>
<td>0.93</td>
<td>0.93</td>
<td>1.18</td>
<td>% Found</td>
</tr>
<tr>
<td>N</td>
<td>0.55</td>
<td>0.49</td>
<td>0.47</td>
<td>0.48</td>
<td>0.46</td>
<td>0.61</td>
<td>% Found</td>
</tr>
<tr>
<td>S</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.75</td>
<td>1.46</td>
<td>2.99</td>
<td>% Found</td>
</tr>
</tbody>
</table>
Table 5: Habitat of the nearest neighbor of *Atlantis II* bands in the phylogenetic tree and their clustered CD-hits.

<table>
<thead>
<tr>
<th>Name Of Band</th>
<th>Name of Band</th>
<th>Species of nearest neighbor</th>
<th>Source/ Habitat of neighbors in the same node / Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RS 54</strong></td>
<td>DQ465010</td>
<td><em>Pseudomonas sp.</em> DQ54799</td>
<td>Microbiota on surfaces of historic Scottish monuments. Subsurface water of the Kalahari Shield, South Africa.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium clone</td>
<td></td>
</tr>
<tr>
<td><strong>RS 59</strong></td>
<td>AY661995</td>
<td>Uncultured bacterium clone 010B-A05</td>
<td>Microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid-bearing uranium waste.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RS 60</strong></td>
<td>DQ49280</td>
<td><em>Pseudomonas sp.</em> D87345</td>
<td>Chitinase-Producing Bacteria Isolated from Arctic Sea Water.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unculturable Mariana eubacterium no. 1 DQ0488739</td>
<td>Sediment of the 11,000-m deep Mariana Trench. Communities associated with fracture-derived groundwater in a deep gold mine of South Africa.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td><strong>RS 61</strong></td>
<td>DQ490292</td>
<td><em>Burkholderia cætaceae bacterium</em> KVD-1959-09</td>
<td>Heterotrophic isolates in respective clone libraries from recent Hawaiian volcanic deposits (&lt;300 yr old)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belongs to the same cluster as AY698512 (nearest neighbor) 09% similarity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RS 64</strong></td>
<td>AL46053</td>
<td><em>Ralstonia solanacearum</em> GM11000</td>
<td>Bacteria resistant to metals and antibiotics in freshwater microcosms. Soil of tropical forests. Water from deep wells. pH gradient-induced by Fe(III)-reducing microorganisms in coal mining-associated lake sediments.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belonging to the same cluster: DQ04967</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A856118</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A856112</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A4713401</td>
<td></td>
</tr>
<tr>
<td><strong>RS 65</strong></td>
<td>DQ409099</td>
<td>Uncultured bacterium clone 661185</td>
<td>Contaminated sediments with nickel, aluminum, barium, chromium, mercury, copper, arsenic, and iron.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RS 66</strong></td>
<td>A8234299</td>
<td><em>Alcaligenes sp.</em> TS-MOSK-3</td>
<td>Isolation of triazines degrading-bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Belonging to the same cluster: DQ237948</td>
<td></td>
</tr>
<tr>
<td><strong>RS 67</strong></td>
<td>A8257640</td>
<td>Unculturable gamma proteobacterium EU708509</td>
<td>Endolithic microbial community in dolomite rock in the central Alps. Anoxic-arsenate-oxidizing denitrifying bacteria.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unculturable bacterium A821078</td>
<td>Sulfur-based autotrophic bacteria.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas taiwanensis</em></td>
<td></td>
</tr>
<tr>
<td><strong>RS 68</strong></td>
<td>DQ49280</td>
<td><em>Pseudomonas sp.</em> D87345</td>
<td>Chitinase-Producing Bacteria Isolated from Arctic Sea Water.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unculturable Mariana eubacterium no. 1 DQ0488739</td>
<td>Sediment of the 11,000-m deep Mariana Trench. Communities associated with fracture-derived groundwater in a deep gold mine of South Africa.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td><strong>RS 69</strong></td>
<td>E868273</td>
<td>Uncultured bacterium clone</td>
<td>Activated sludge from a 70 degrees C ultrasound-enhanced anaerobic reactor for treating carbazole-containing wastewater.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Cont. Table 5; showing the nearest neighbor of *Atlantis II* bands in phylogenetic tree and their clustered CD-hits where the associated habitat is indicated.

<table>
<thead>
<tr>
<th>Name Of Atlantis II Deep Level</th>
<th>Name Of Band</th>
<th>Species of nearest neighbor</th>
<th>Source/ Habitat of neighbors in the same node / Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>90-135 cm</td>
<td>RS 71</td>
<td>A8879076 <em>Anoxybacillus rupiensis</em></td>
<td>Thermophilic bacterium isolated from Rupi basin</td>
</tr>
<tr>
<td>135-180 cm</td>
<td>RS 73</td>
<td>DQ465010 <em>Pseudomonas sp.</em> DQ354709 Uncultured bacterium clone</td>
<td>Microbiota on surfaces of historic Scottish monuments Subsurface water of the Kalahari Shield, South Africa</td>
</tr>
<tr>
<td></td>
<td>RS 76</td>
<td>DQ404909 Uncultured bacterium clone 661185</td>
<td>Contaminated sediments with nickel, aluminum, barium, chromium, mercury, copper, arsenic and iron.</td>
</tr>
<tr>
<td></td>
<td>RS 77</td>
<td>AB257640 Uncultured gamma proteobacterium EU708509 Uncultured bacterium AB210278 <em>Pseudoxanthomonas taiwanensis</em></td>
<td>Endolithic microbial community in dolomite rock in the central Alps Anoxic-arsenite-oxidizing denitrifying bacteria Sulfur-based autotrophic bacteria</td>
</tr>
<tr>
<td></td>
<td>RS 78</td>
<td>DQ492807 <em>Pseudomonas sp.</em> D87345 Unculturable Mariana embacterium no. 1 DQ088739 Uncultured bacterium</td>
<td>Chitinase-Producing Bacteria Isolated from Arctic Sea Water. Sediment of the 11,000-m deep Mariana Trench. Communities associated with fracture-derived groundwater in a deep gold mine of South Africa.</td>
</tr>
<tr>
<td></td>
<td>RS 79</td>
<td>DQ490292 <em>Burkholderiaceae bacterium KVD-1959-99</em> Belongs to the same cluster as AY568512 (nearest neighbor) of 99% similarity</td>
<td>Heterotrophic isolates in respective clone libraries from recent Hawaiian volcanic deposits (&lt;300 yr old)</td>
</tr>
<tr>
<td>180-225 cm</td>
<td>RS 82</td>
<td>AF468339 Uncultured bacterium clone ARKCry-93</td>
<td>Arctic sea ice cryoconite</td>
</tr>
<tr>
<td>225-267 cm</td>
<td>RS 83</td>
<td>DQ465010 <em>Pseudomonas sp.</em> DQ354709 Uncultured bacterium clone</td>
<td>Microbiota on surfaces of historic Scottish monuments Subsurface water of the Kalahari Shield, South Africa</td>
</tr>
<tr>
<td></td>
<td>RS 86</td>
<td>A957031 DQ404909</td>
<td>Water biofilms receiving chlorine or monochloramine residual Contaminated sediments with nickel, aluminum, barium, chromium, mercury, copper, arsenic and iron.</td>
</tr>
</tbody>
</table>
Table 6: Habitat of the nearest neighbor of *Discovery* deep bands in the phylogenetic tree and their clustered CD-hits.

<table>
<thead>
<tr>
<th>Name Of Discovery Deep Level</th>
<th>Name of Band</th>
<th>Species of nearest neighbor</th>
<th>Source/ Habitat of neighbors in the same node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spill from top/SFT</td>
<td>RS 37</td>
<td><em>DQ4047041</em> Unguncultured bacterium clone 655917 16S ribosomal RNA gene, partial sequence.</td>
<td>Nitrate and heavy metal contaminated soils</td>
</tr>
<tr>
<td></td>
<td>RS 39</td>
<td><em>AM936569</em> Uncultured <em>Dersia</em> sp. <em>DQ264620</em> Uncultured bacterium clone BANW682</td>
<td>Hydrocarbon-contaminated soil Subsurface groundwater during polylactate stimulated chromate bioremediation.</td>
</tr>
<tr>
<td></td>
<td>RS 40</td>
<td><em>AY397773</em> <em>Brevibacillus</em> sp. R-7152 16S ribosomal RNA gene, partial sequence.</td>
<td>Highly heat-resistant spores of <em>Bacillus</em> sporothermodurans in ultra high-temperature environments with high fatty acid contents.</td>
</tr>
<tr>
<td></td>
<td>RS 41</td>
<td><em>AM934691</em> <em>Bacillus</em> sp. BF98 partial 16S rRNA gene, strain BF98.</td>
<td>Cultured aerobic heterotrophic bacteria in rivulet water and tufa biofilm.</td>
</tr>
<tr>
<td></td>
<td>RS 42</td>
<td><em>CP090378</em> <em>Burkholderia cenocepacia</em> <em>HM042678</em> <em>Burkholderia cenocepacia</em></td>
<td>Soil bacterium. Bacteria associated with solubilization of rock phosphate.</td>
</tr>
<tr>
<td></td>
<td>RS 43</td>
<td><em>AB25640</em> Uncultured gamma proteobacterium. <em>AY345536</em> Uncultured bacterium clone LWS-P3</td>
<td>Endolithic microbial community in dolomite rock in the central Alps.</td>
</tr>
<tr>
<td></td>
<td>RS 45</td>
<td><em>EU636630</em> Uncultured bacterium clone <em>Porites</em> C32cID06 16S ribosomal RNA gene, partial sequence. / <em>Peptoniphilus</em> sp.</td>
<td>Associated with <em>Porites</em> sp. coral.</td>
</tr>
<tr>
<td></td>
<td>RS 46</td>
<td><em>AY177358</em> Phenanthrene-degrading bacterium 35 16S ribosomal RNA gene, partial sequence.</td>
<td>Long-term exposure to phenanthrene in a soil column system.</td>
</tr>
<tr>
<td></td>
<td>RS 47</td>
<td><em>AF358002</em> Uncultured bacterium clone LO13.10 16S ribosomal RNA gene, partial sequence.</td>
<td>Functionally active methanotroph population in a peat soil microcosm.</td>
</tr>
<tr>
<td>0-17 cm</td>
<td>RS 28</td>
<td><em>DQ465010</em> <em>Pseudomonas</em> sp VTTE-052911</td>
<td>Aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments</td>
</tr>
<tr>
<td></td>
<td>RS 29</td>
<td><em>DQ4047041</em> Uncultured bacterium clone 655917 16S ribosomal RNA gene, partial sequence.</td>
<td>Nitrate and heavy metal contaminated soils</td>
</tr>
<tr>
<td></td>
<td>RS 31</td>
<td><em>DQ234143</em> Uncultured Enterobacteriaceae bacterium clone D9059.</td>
<td>Danshuei river estuary of northern Taiwan.</td>
</tr>
<tr>
<td></td>
<td>RS 32</td>
<td><em>AB25640</em> Uncultured gamma proteobacterium. <em>AY345536</em> Uncultured bacterium clone LWS-P3</td>
<td>Endolithic microbial community in dolomite rock in the central Alps.</td>
</tr>
</tbody>
</table>

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Table 6 Cont.:

Table showing the nearest neighbor of *Discovery* deep bands in phylogenetic tree and their clustered CD-hits where the associated habitat is indicated.

<table>
<thead>
<tr>
<th>Name Of Discovery Deep Level</th>
<th>Name of Band</th>
<th>Species of nearest neighbor</th>
<th>Source/ Habitat of neighbors in the same node</th>
</tr>
</thead>
<tbody>
<tr>
<td>47-94 cm</td>
<td>RS 17</td>
<td>DO499465 <em>Sphingobacteriaceae bacterium KVD-nuk-11</em></td>
<td>Volcanic deposits</td>
</tr>
<tr>
<td></td>
<td>RS 18</td>
<td>CP9000884 <em>Deftia acidovorans SPH-1</em> AB076856 <em>Diaphorobacter nitroreducens</em></td>
<td>Sewage soil.</td>
</tr>
<tr>
<td></td>
<td>RS 21</td>
<td>AB015264 Uncultured <em>Cytophaga sp.</em></td>
<td>Deepest cold-seep area of the Japan Trench</td>
</tr>
<tr>
<td></td>
<td>RS 27</td>
<td>AF314434 Uncultured bacterium PHOS-HE79/ unclassified <em>Bacteroidetes</em></td>
<td>Characterisation of the microbial 16S rDNA diversity of an aerobic phosphorus-removal ecosystem and monitoring of its transition to nitrate respiration</td>
</tr>
<tr>
<td>94-141 cm</td>
<td>RS 6</td>
<td>DO351913 Uncultured bacterium clone JH-WH03</td>
<td>Soil iron-manganese bacteria.</td>
</tr>
<tr>
<td></td>
<td>RS 7</td>
<td>AY625146 Uncultured bacterium clone L-2</td>
<td>Perchloroethylene-contaminated ground water. Also, phenol-digesting bacteria.</td>
</tr>
<tr>
<td></td>
<td>RS 10</td>
<td>AF358001 Uncultured bacterium clone LO13 1 DO499310 Uncultured bacterium clone CV71 AJ581620 Uncultured beta proteobacterium</td>
<td>Functionally active methanotroph population in a peat soil. Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. Heavy metal contaminated environments.</td>
</tr>
<tr>
<td></td>
<td>RS 12</td>
<td>BA000012 <em>Mesorhizobium loti</em> MAFF303099</td>
<td>Nitrogen-fixing symbiotic bacterium, <em>Mesorhizobium loti</em> / soil associated</td>
</tr>
<tr>
<td>141-188 cm</td>
<td>RS 48</td>
<td>DO465010 <em>Pseudomonas sp. VTTE-052911</em></td>
<td>Aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments.</td>
</tr>
<tr>
<td></td>
<td>RS 49</td>
<td>DO248270 Uncultured soil bacterium</td>
<td>Carbon tetrachloride contaminated soil</td>
</tr>
<tr>
<td></td>
<td>RS 51</td>
<td>AY293404 Uncultured alpha proteobacterium clone</td>
<td>Travertine Terraces at Yellowstone Hot Springs</td>
</tr>
<tr>
<td>188-235 cm</td>
<td>RS 52</td>
<td>AY289498 AY604055 AY739687 <em>Bacillus sp.</em></td>
<td>Predominant in Soil. also found in dolomite aquifer 896 meters below the surface. also microbial communities inhabiting petroleum contaminated tropical soils.</td>
</tr>
<tr>
<td></td>
<td>RS 53</td>
<td>AJ292689 Uncultured enubacterium WD215</td>
<td>Bacterial living in polychlorinated biphenyl-polluted soil</td>
</tr>
<tr>
<td>Level: 0–45 cm</td>
<td>Level: Spill from top</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-Proteobacteria</td>
<td>Gamma-Proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-Proteobacteria</td>
<td>Beta-proteobacteria</td>
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<td></td>
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<tr>
<td></td>
<td>Alpha-Proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacillus</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Clostridia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFB group</td>
<td></td>
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<tr>
<td>Level: 45–90 cm</td>
<td>Level: 0–47 cm</td>
<td></td>
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<tr>
<td>Beta-proteobacteria</td>
<td>Gamma-Proteobacteria</td>
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<td></td>
</tr>
<tr>
<td>Gamma-Proteobacteria</td>
<td>CFB group</td>
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<tr>
<td>Alpha-Proteobacteria</td>
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<tr>
<td>Level: 90–135 cm</td>
<td>Level: 47–94 cm</td>
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<tr>
<td>Bacillus</td>
<td>Beta-proteobacteria</td>
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</tr>
<tr>
<td></td>
<td>CFB group</td>
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</tr>
<tr>
<td>Level: 135–189 cm</td>
<td>Level: 94–141 cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-Proteobacteria</td>
<td>Beta-proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>Gamma-Proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alpha-Proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level: 189–225 cm</td>
<td>Level: 141–188 cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>Beta-proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gamma-Proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alpha-Proteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level: 225–267 cm</td>
<td>Level: 188–235 cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-Proteobacteria</td>
<td>Cyanobacteria</td>
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</tr>
<tr>
<td>Beta-proteobacteria</td>
<td>Bacillus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References


6. FAU MM, Gonzalez NF, FAU BL, Sanz RF, Simarro RF, Sanchez I FAU - Sanz, Jose,L., Sanz JL. Isolation and genetic identification of PAH degrading bacteria from a microbial consortium. Biodegradation JID - 9100834 0112.


51. CD-HIT package can perform various jobs like clustering a protein database, clustering a DNA/RNA database, comparing two databases (protein or DNA/RNA), and generating protein families. More information is available at CD-HIT homepage. [Internet]. Available from: http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit-est.

52. MAFFT version 6 [Internet]. Available from: http://mafft.cbrc.jp/alignment/server/.


