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Exploring the biodiversity of anammox in Atlantis II and Kebrit brine pools' interfaces

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Exploring the Biodiversity of Anammox in Atlantis II and Kebrit Brine Pools' Interfaces

A Thesis Submitted to
The Biology Department

In partial fulfillment of the requirements for
the degree of Master of Science

By
Ibrahim Farouk Farag

Under the supervision of
Dr. Rania Siam
March 2012

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Has been approved by

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DEDICATION

THIS WORK IS DEDICATED TO MY PARENTS, MY WIFE AND MY BELOVED SONS. WITHOUT THIER CONTINUOUS SUPPORT AND ENCOURAGEMENT, I WOULDN'T BE ABLE TO REACH MY AIM.

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ABSTRACT

The Red Sea brine pools are unique environments to assess the biochemical adaptation of marine bacteria. The role of nitrogen in marine biogeochemistry is central and greatly influences the diverse elements including carbon and phosphorus.
Anammox communities play a significant role in the total nitrogen loss especially in deep sea and deep ocean ecosystems. Despite this, the biodiversity of Anammox bacteria have not been previously investigated in any brine pool ecosystems. With the advances of the metagenomics based approaches, the exploration of the yet uncultured microbial communities including the Anammox bacteria has taken different perspectives. Anammox communities are currently analyzed using 16srRNA with some unique functional genes e.g. hydrazine oxidase, hydrazine synthase and Cytochrome cd1-containing nitrite reductase encoding gene nirS.

In this study, we examined the biodiversity of Anammox microbial communities inhabiting two Red sea brine pools' Atlantis II and Kebrit deep interface layers. Comparative and comprehensive analysis of the unique and specific functional gene, hydrazine oxidase was performed. Anammox hydrazine oxidase gene was amplified from DNA isolated from the 0.1 μm serial fractionation of the water samples of Atlantis II interface layer and Kebrit upper interface layer. *hzoA/hzoB* libraries were constructed and a total of 81 and 44 specific clones were identified in the interface layers of Atlantis II deep and Kebrit deep, respectively. The identified sequences matched hydrazine oxidases from uncultured *Planctomycetes*. Alpha and beta diversity analyses were performed using statistical analysis tests and multiple regression analysis was done to assess the level of uniqueness of the Anammox bacteria inhabiting the examined samples using Unifrac. Eight and nine different Anammox related phylotypes were identified in Atlantis II and Kebrit upper interface layers, respectively. *Scalindua* species predominated the sampled interface brine layers. Moreover, the principle component analysis depicted a unique presence of Anammox communities. This study addresses and identifies the unique microbial community in the interface of the Red Sea Brine Pools.

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1.1 Atlantis II Deep and Kebrit Deep Red Sea Brine Pools

1.1.1: - General Description

The Red Sea was formed 3-5 million years ago upon the divergent movement of the Arabian and the African tectonic plates [1], [2]. Recent oceanographic measurements indicated that the Red Sea is an ocean basin formed by splitting apart continents, a phenomenon known as "*ocean in statue nascendi*" [3], [4]. This is evidenced by the current structure of the Red Sea Axial Rift. This Rift expands toward the south and is subdivided into four different regions namely; the Northern Region, the Transitional Region, the Multi-deeps Region and the Rift Valley region (fig 1) [2], [3], [5]. In the past 50 years, more than 25 Red Sea Brine Pools have been discovered and most of them are located in the northern and the central regions of the Red Sea Axial Rift [3]. Among those brine filled pools, Atlantis II is located in the Central Rift around (21 ° 20'), extending 60 km^2 wide and 2200 m deep. It is characterized by the presence of thick, metalliferous deposits underlying its deepest layer [1]. Miller *et al*. discovered this pool using the Atlantis II vessel in 1965 [6]. The Atlantis II brine pool consists of four physically separated layers; the deepest one is the Lower Convective Layer (LCL), which extends from the brine bottom to 2047 m below the sea surface. LCL is over-layered by three stratified UCLs (UCL1, UCL2, and UCL3) exhibiting remarkable differences in salinity and temperature. Above the two brine layers, there is a transitional zone separating that brine pool from the above seawater and extending from 1900 to 2000 m below sea surface; this layer is known as the Interface Layer. Atlantis II brine is known by its hydrothermal activities and this is emphasized by the gradual increases of its temperature over time as reported in the measurements taken from 1965 to 2008 [1], [3], [5].

The second brine pool addressed in this study is Kebrit Deep. It was discovered in 1971 during the *Valdivia* expedition [5]. It is located in the northern region of the Axial Rift at (24 ° 44'N, 36 °17'E) taking an oval shape, which extends for 1 to 2.5 km^2 wide, and ranges from 7m to 1.5 kilometers in depth [4], [5], [7], [8]. The thickness of the brine itself is 84 m with slight acidic pH and no dissolved oxygen[7].

1.1.2 Physicochemical Characteristics

Atlantis II is considered to be the saltiest and the hottest Red Sea brine pool. Its temperature reaches up to 70°C at LCL and its salt concentrations are 25.4% (about 7.5 times higher than the normal seawater) [3], [5]. It is also characterized by the presence of metalliferous deposits due to the high concentrations of heavy metals (iron, zinc, copper, etc.). It was reported that nitrogen and methane are highly abundant in the brine with smaller fractions of carbon dioxide, ethane and hydrogen sulfide [3], [8]. On the other hand, the Kebrit Deep Brine Pool, its name being derived from the Arabic word for sulfur, shows a sharp increase in its salinity from 4% to 26%. It also exhibits an increase in temperature from 21.6° C to 23.4° C. Methane and Hydrogen Sulfide are significantly detected in the Kebrit brine pool with smaller amounts of nitrogen and no dissolved oxygen. The seawater/brine interface is characterized by a high-density gradient that acts as a sieve trapping both organic and inorganic substances[5], [7].

1.1.3 Microbiological Studies: - an Overview

Microbiological analyses of Atlantis II date back to the late 1960s. Culture dependent approaches didn't support the growth on all media tested, which has driven a conclusion that Atlantis II Deep is a very harsh environment that can't support the existence of any forms of life. This conclusion quickly changed after sulfate-reducing bacteria had been successfully isolated by Truper *et al*. 1969 [9]. In 1990, four isolates of *Flexistipes sinusarabici* were isolated, which were characterized later as a new phylum known as *Deferribacteres* [10].

Microbiological studies in Kebrit have then been conducted using contemporary approaches; phylogenetic analyses using primers specific for both bacterial and archaeal 16srRNA have been carried out. Six bacterial and five archaeal unique clones have been obtained. The six bacterial sequences are branched between *Aquificales* and *Thermotogales*. Of the five archaeal clones, three belonged to *Euryarchaeota* group II and III, while the other two clustered in a separate branch [4]. This study has been followed by cultivation-based trials; two rod shaped species belonging to the genus *Halanaerobium* were successfully isolated. Phylogenetic analyses of the isolates

16srRNA sequences revealed that its sequences have been detected in the previous study [4], [7].

With advances in the metagenomics' approaches, an initiative to explore the Red Sea Brine Pools and the water column overlying it has been taking place. 16srDNA profiles of the microbes inhabiting the water column covering the Red Sea Brine Pool, Atlantis II, have been examined at depths of 50m, 200m, and 1500m. The obtained results of the bacterial pyrotags analyses revealed the dominance of *Cyanobacteria* in the surface layers, whereas *Proteobacteria* prevails in the deeper layers. The archaeal pyrotags analyses showed the prevalence of *Euryarchaeota* and *Crenarchaeota* in the upper and the lower layers, respectively[2]. Further investigations were done to determine the metabolic capacities of microbes present in the Atlantis II Brine Pool. The investigations concluded that bacteria inhabiting this brine pool might possess the genetic capability to metabolize hydrothermally produced aromatic compounds[11].

1.2 Nitrogen Cycle: A Marine Perspective

Nitrogen is the most abundant molecule on earth. It is essential for all forms of life as it's an important constituent of most biochemical molecules (e.g. nucleic acids, amino acids, proteins etc.). It exists in different forms ammonium, nitrate, nitrogen oxides, azides and hydrazines. It is also involved in many industrial applications such as the use of nitrates as fertilizers and hydrazines as rocket fuel.

1.2.1 Nitrogen Fixation

Atmospheric nitrogen is the largest reservoir of molecular nitrogen, which is commonly fixed to enter the biological nitrogen cycle through the activities of some groups of bacteria, archaea and fungi. In terrestrial habitats, these microbes establish symbiotic relationships with plants and provide it with the fixed nitrogen forms [12]. In marine habitats, biologically available nitrogen is a crucial factor in the productivity of the ecosystem. The efficiency of nitrogen fixation process in marine ecosystems is controversial [13]. It was perceived that N_2 fixation processes are not abundant in the marine ecosystems and that the major source of fixed nitrogen is the N_2 run-off from the non-marine sources. This notion was based on the hypothesis that most of marine organisms can utilize inorganic nitrogen sources like ammonium, nitrate and nitrite but not fixing N_2 [14]. In fact N_2 fixation in marine habitats is underestimated even after

the discovery of the N_2 fixation capabilities of the ubiquitous cyanobacteria (*Trichodesmium)* [14]. However, after the advances of molecular techniques, Zehr *et al*. 2003 established N_2 fixers profiling approach using a marker gene *nif* H, which is conserved among N_2 fixing bacteria [15], [16]. This approach together with the ^{15}N tracing experiments paved the way to the discovery of key players in the N_2 fixation cycle [17]. It was found that both *Trichodesmium* and other *diazotrophs* are the major suppliers of fixed nitrogen compounds in different marine ecosystems [14], [17].

Determining the spatio-temporal role of marine microbes in the nitrogen fixation process in marine habitats has been a challenging task. N2 fixation processes is also found in close association with nitrogen loss through the activities of denitrifiers and Anammox bacteria [13].

1.2.2 Nitrification

Nitrification is the oxidation of ammonia to nitrite and then to nitrate under aerobic conditions. These reactions are mainly carried out by the activities of two distinct groups of chemolithotrophic bacteria including *Nitrosomonas* (oxidizing ammonia to nitrite) and *Nitrobacter* (oxidizing nitrite to nitrate) [12]. Nitrification reactions are controlled by a set of nitrite oxidoreductases and nitrate monooxygenases that is regulated by the levels of ammonia and oxygen present [13]. *Nitrosomonas* and *Nitrobacter* species belong to Beta and Gamma subclasses of the phylum *Proteobacteria,* respectively [12]. These bacteria exploit the energy produced through the nitrification process as their sole energy source [15]. Furthermore, this process fuels both Anammox bacteria and denitrifiers with the required oxidized nitrogen substrates that act as electron acceptors [18]. Recently, it was discovered that a marine archaeon affiliated with the phylum *Crenarchaeota* could grow chemolithotrophically by converting ammonia to nitrite under aerobic conditions. This archaeon belongs to a group known as Ammonium Oxidizing Archaea (AOA) [19]. Moreover, recent synopsis on the kinetics of the AOA revealed that it might outnumber the Ammonia Oxidizing Bacteria (AOB) at nutrient deprived ecosystems. In photic zones AOA is more light tolerant than AOB [20]. In addition to the autotrophic nitrifiers, a group of heterotrophic nitrifying bacteria and fungi has been also detected[21].

1.2.3 Denitrification

Denitrification is a crucial process in the nitrogen biogeochemical cyclethat was believed – until recently – to be the only way for N_2 production [22]. This concept has been completely upended after the discovery of Anammox and its roles in the nitrogen cycle [23]. The pathway is carried out under limited oxygen conditions and consists of four consecutive steps. In the first step, nitrate is reduced to nitrite and this is mainly attained by the action of nitrate reductases. At this step, this process is split into two distinct pathways, the canonical denitrification and dissimilatory nitrate reduction to ammonia (DNRA). In the former, nitrite is further reduced to nitrite oxide, then to nitric oxide and finally the nitrogen is released back to the atmosphere. Where in the later, nitrate is reduced into ammonia [22], [24]. It is worth noting that *Thioploca and Thiomargarita* are the only genera known to carry out DNRA [25]. The microbial key players in the canonical denitrification process belong to heterotrophic bacteria e.g. *Paracoccus denitrificans*, however it was reported that some autotrophic bacteria are capable of denitrification e.g. *Thiobacillus denitrificans*. Generally, taxonomy doesn't strictly determine denitrification. However, most of these bacteria are ranked within the subdivisions of the phylum *Proteobacteria* [26]. Beyond the bacterial groups, it was also discovered that some autotrophic and heterotrophic archaea possess the necessary machineries to undergo denitrification [25], [26].

1.2.4 Anammox (ANaerobic AMMonium OXidizers)

About two decades ago, Anammox has been determined to be one of the leading causes of nitrogen loss [23]. The anaerobic ammonium oxidation was carried out by a group of uncultured *Planctomycetes* through coupling ammonium - as electron donor - to nitrite - as electrons acceptor - in an energetically favorable reaction to generate di-nitrogen [22], [27]. This reaction takes place in special intracellular compartments called Anammoxosome[28]. It is believed that Anammox communities contribute by more than 50% in eliminating the fixed nitrogen forms from marine water bodies. This reaction releases the required energy to support the chemolithotrophic nature of Anammox bacteria [22]. Genome analyses and metabolic reconstruction experiments on *Kuenenia Sttutgartiensis* revealed the presence of some peculiar intermediates in this pathway such as hydrazine. It also reported that over 200 genes were employed to carry out the catabolic and respiratory functions in the Anammox process [29]. Out of

the 200 genes, nine genes belong to octahaeme cytochrome c hydroxylamine/hydrazine oxidoreductases [29], [30]. These genes encode for functional enzymes that mediate the Anammox pathway.

The presence of Anammox activities in environmental samples was first reported by analyzing marine sediments obtained from the Danish coast using the labeled nitrogen technique [31]. The existence of Anammox bacteria in the suboxic zones of the Black sea was revealed by 16srRNA analyses [32], [33].

So far, five genera belong to Anammox bacteria (the uncultured group of *Planctomycetes* phyla) have been discovered. These genera are *Kuenenia*, *Anammoxoglobus*, *Scalindua*, *Jettenia* and *Brocadia*. It is also worth mentioning that these genera have been isolated from both Anammox enrichment cultures and different environmental samples [34].

1.3 ANAMMOX

1.3.1 Discovery

In 1965, a profound decrease in ammonia levels has been detected in anoxic basins [22]. This observation has pointed out that a possible anaerobic oxidation process may be taking place. However, almost for the rest of the century, heterotrophic denitrifiers were considered to be the only fate of fixed nitrogen loss in marine environments [22]. These previous notions came from the ability of these denitrifiers to respire nitrate or nitrite producing nitric or nitrous oxides, which are further processed to form N_2 gas [26].

In the beginning of 1990s, the missing part of the nitrogen biogeochemical cycle puzzle was solved. In a wastewater treatment plant (Delft, Netherlands), Mulder *et al*. 1995 observed that increasing levels of nitrate and ammonium consumption are accompanied by increasing levels of nitrogen gas [35]. The study also concluded that ammonium is anaerobically oxidized using nitrate or nitrite - as electron acceptor - releasing di-nitrogen gas as a final product. This biological process was given the term “Anammox”. A year later, two publications done by Astrid *et al* discussed the microbiological aspect of the Anammox pathway and formulated the first medium to isolate bacterial species harbouring such unique capabilities [23], [36]. This culture

medium was designed to support the autotrophic machineries of Anammox bacteria by providing it with ammonium and nitrite as the sole electron donor and acceptor, respectively. Carbonate was supplied as the sole carbon source. Furthermore, they found that this process is not only restricted to water treatment plants but also found in marine sediments, water columns close to suboxic regions and in oxygen minimum zones (OMZ) [13], [37]. This paved the way for deeper analyses to explore the unique physiology of the Anammox bacteria.

Using ^{15}N labeled compounds, the Anammox pathway was assembled. It was postulated that hydroxylamine, derived from nitrite, mediates the ammonium oxidation process by acting as an electron acceptor. This results in the formation of hydrazine as an intermediate that is further processed to di nitrogen and a small fraction of nitrite is oxidized into nitrate[38]. (Figure 3)

1.3.2 Anammox Molecular and Cellular Physiology

Genomic data available on Anammox bacteria have been deciphered through the analysis of the 4.2 Mbps genome draft of *Kuenenia Sttutgartiensis* published in 2006. This genome has been assembled from metagenomics sequences retrieved from a complex bioreactor continuously supplemented by artificial wastewater enriched with nitrite, ammonia and bicarbonate [29]. Interestingly, it was found that more than 200 genes were involved in the catabolic and respiratory activities of this Anammox species. Among which, nine hydroxylamine oxidase like genes were reported. Additionally, only two genes commonly involved in denitrification were detected. These versatile catabolic capabilities provided Anammox species with the required energy needed for their growth. Furthermore, the *Kuenenia* genome showed that Anammox bacteria are strictly autotrophic and carbon fixation is accomplished via acetyl-CoA pathway, while the other carbon fixation pathways were absent. Also, it was proved that hydrazine is the source of electrons passing through ferrodoxin to activate both acetyl CoA synthase and CO dehydrogenase. On the other hand, additional enzymes weren't needed to compensate the utilized hydrazines but only to revert electron transport [29].

Apart from Anammox genomic characteristics, Anammox are slow growing bacteria that exhibit long doubling time normally exceeding 10 days. So far,

Anammox species couldn't be obtained in pure cultures. However, it was isolated by enrichment cultures technique. Generally, Anammox bacterial cell is coccoid in shape, lacking peptidoglycan and has a protein-like cell wall [39], [40]. Anammox reactions show activity at wide range of temperatures but only exhibit maximum activity at mesophlic temperature range. Nonetheless, it was also observed to be both psychrophilic (Arctic ice and sediments) and thermophilic (hydrothermal vents and hot springs) [41-44]. Anammox bacteria show maximum activity at pH 8 and tolerate oxygen levels up to 0.5% air saturation; they also demonstrate relatively high affinity toward ammonium and nitrite with affinity constant ≥ 5μM [45], [46].

Anammox reactions take place in a special compartment known as Anammoxosome. A dense staircase like membrane surrounds the anammoxosome. This membrane is formed of a special type of lipid known as ladderane that acts as a barrier to reduce the toxic effects of the Anammox pathway intermediates such as hydrazine and hydroxylamine. The chemical structure of ladderane displays unique arrangements by linking five cyclobutane moieties attached to a glycerol backbone by ester or ether bonds. The second proposed function of this membrane is to provide the Anammox enzymes with larger space to boost its activity and generate ATP by the proton motive force machineries located on its surface [28], [39], [47].

1.3.3 Anammox Phylogeny

Anammox bacteria belong to the uncultured group of the phylum *Planctomycetes*. The position of this phylum on the tree of life has been controversial. Previously, rRNA-based analysis showed that *Planctomycetes* were found at the base of the bacterial phylogenetic tree suggesting that it could be one of the first emerged bacteria [48]. Moreover, The compartmentalized structure as well as the presence of single or double membranes around *Planctomycetes'* genetic materials had driven a conclusion that *Planctomycetes* might be the link between the eukaryal and bacterial domains of life [48]. Recently, *Planctomycetes* phylum has been placed together with the phyla, *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae*, *Poribacteria* and *OP3* in one large superphylum called PVC [49].

However, The genera affiliated to *Planctomycetes* could be divided into two major groups. The first group comprises the heterotrophic cultured representatives and

it includes the genera (*Planctomyces*, *Pirellula*, *Blastopirellula*, *Rhodopirellula*, *Isosphaera*, and *Gemmata*). The second class is known as the extensively diverse autotrophic uncultured group of *Planctomycetes*. This group belongs to the order *Brocadiales* and has been detected in various ecosystems. So far, five Anammox related genera have been identified and these genera are called *Kuenenia*, *Anammoxoglobus*, *Scalindua*, *Jettenia*, and *Brocadia* [34].

1.3.4 Anammox Habitats and Ecosystems

Anammox bacteria are believed to be more widespread than was originally assumed. Generally Anammox bacteria exist in the anaerobic niches at the oxic-suboxic areas located in water column and sediments. However, following its discovery in the wastewater treatment facility in Delft, Anammox could be located in wide arrays of ecosystems [40]. Although, environmental Anammox activities were first reported in the Danish coast but its real existence was verified through the 16srRNA analysis done on the largest anoxic reservoir the Black Sea [31], [32], [33]. Afterwards, it was found in different marine coasts, water column, sea surface, subsurface sediments and estuary ecosystems [50], [51]. It was also detected in fresh water lakes such as Lake Tanganyika in East Africa [52]. Anammox existence is not limited to mesophilic habitats. It was also found in 5-deep sea hydrothermal vents located in Mid-Atlantic ridge and in a hydrothermal vent located in Guayamas basin [43], [50]. Its presence was also observed in California and Nevada hot springs, Arctic ice and Arctic sea sediments [41], [42], [44].

To explore the Anammox-associated communities, a labeled nitrogen analysis based study was performed on Black sea water samples. This revealed that Anammox bacterial communities are more likely to exist in direct or indirect association with aerobic ammonium oxidizers (nitrifying bacteria) in order to supply it with the required nitrate [53].

The Anammox environmental studies done so far showed that Anammox related genera exhibit habitat preferences. This was emphasized by the usual presence of the genera *Kuenenia*, *Anammoxoglobus*, *Jettenia*, and *Brocadia* in fresh water ecosystems. While *Scalindua* family is generally found in marine habitats, its presence in fresh water ecosystems was also detected [34].

1.3.5. Advances in Anammox Molecular Detection Approaches

The first approach used to estimate the contribution of Anammox pathways in the total nitrogen loss was isotope-labeling technique. In this approach, the tested samples were incubated with a mixture of $^{15}NH_4$ and $^{14}NO_2^-$;then the Anammox activities were measured using isotope mass spectrometer [54]. The second approach is based on the quantification of the Anammox biomass by measuring the levels of ladderane lipids that specifically present in Anammoxosome membrane [55].

Since Anammox bacteria couldn't be successfully isolated in pure cultures, the molecular detection approaches have been used to explore its presence in different habitats. Several studies have based their observations on the analyses done using primers specifically targeting Anammox 16srRNA. So far, 16srRNA analyses were able to assess the existence and the diversity of the Anammox communities in different ecosystems [56].

With the increased knowledge about Anammox-related enzymes and its roles in the Anammox pathway, studying Anammox communities using functional gene approaches was implemented. Accordingly, Koltz and Stein recommended hydrazine oxidase *(hzo),* a member of octaheme cytochrome C hydroxylamine oxidoreductase protein family, to be used as a phylomarker. The phylomarker *hzo* would help explore the functional Anammox communities since hydrazine oxidases are only detected and identified in Anammox related species [50], [57]. Additionally, two different recent studies done by Ford *et al*. and Harhangi *et al*. in 2011 based their analyses on two different functional phylogenetic markers. These phylomarkers are the Cytochrome cd1-containing nitrite reductase encoding-gene "*nirS*" and hydrazine synthase "*hzsA*" [58], [59]. The only limitation considered when applying these functional phylomarkers based approaches is that the number of sequences available from Anammox enrichment cultures is so scarce.

Hydrazine oxidases *(hzo)* have been employed in different studies to analyze the Anammox communities dwelling different habitats. The first two studies addressed the Anammox diversity using *hzo* in combination with 16srRNA gene were conducted by (Quan and his colleagues in 2008 and 2009); they addressed the diversity of Anammox bacteria present in Anammox bioreactor plants[60], [61]. In 2010, the

presence of Anammox bacteria in high temperature petroleum reservoirs has been detected using both *hzo* and 16srRNA molecular phylomarkers. The phylomarker *hzo* was also used to detect the Anammox profiles in different marine ecosystems such as Jiaozhou bay sediments, deep-sea hydrothermal vents in Guayamas basin, Montserrat deep sea tephra deposits, Surface sediments from equatorial pacific, subsurface sediment of South China sea and Arabian sea [50], [62-65]. It was also applied as a functional marker to explore Anammox bacteria inhabiting estuary sediments of Mai Po Nature Reserve and Cape Fear River[50], [51]. Furthermore, samples from wastewater bioreactor and North Carolina ground water, were investigated for its Anammox profiles using *hzo*[50].

1.4 Metagenomics and Microbial Diversity

Marine habitats are considered to be the largest ecosystem on earth; it covers approximately 70% of the earth surface area. Marine environments are very diverse in nature ranging from surface sunlit areas that support the growth of photosynthetic microbes to 11,000 m deep ocean areas where microbes with extremophilic characteristics prevail. It also varies greatly in its temperature ranges, pressure, oxygen levels, salt and heavy metal concentrations [66].

Based on the fact that only 0.1% of the microbes are culturable, microbial populations inhabiting any ecosystem including the marine ones were largely underestimated. Recently, with the advances of molecular approaches, sequencing technologies (e.g. Illumnia-Solexa, Roche 454, ABI 3730 XL, etc.) and the emergence of metagenomics field, the microbial populations estimates have been amended and increased noticeably by at least three orders of magnitude[67]. Handelsman and colleagues have first described the term metagenomics in 1998; it defines a cultivation-independent approach to extract all DNA present in a certain niche collectively[68]. This enables the reconstruction of metabolic pathways present in the examined niche through assembling large genomics data and extrapolating its metabolic functions[69]. Additionally, the diversity of the microbial communities dwelling the examined ecosystem is commonly investigated using metagenomics' approaches. These are mainly testing the variations of the 16srRNA as well as other unique functional genes e.g. hydrazine oxidase as a phylomarker of Anammox bacteria[66].

In addition to analyzing the structure of microbial communities, metagenomics can be widely used to screen for novel sequences that encode for enzymes with unique functions. Two strategies are commonly utilized for such purpose; sequence-based and function-based approaches [66]. In the sequence-based approach, the function of the generated sequencing data can be inferred through comparing it to sequences located in different databases. While functional approach is based on the phenotypes or expressed gene products that can be assayed through specific substrates[70]. Different enzymes from marine habitats have been discovered using function-based approaches such as lipases, esterases, chitinases, and cellulases [66].

CHAPTER 2: - MATERIALS AND METHODS

2.1 Sample Collection and DNA Isolation

During the R/V Aegaeo cruise in spring 2010, water samples were taken from two Red sea brine pools Atlantis II deep and Kebrit deep. Atlantis II brine pool water samples were extracted from Interface, UCL and LCL samples. Samples collected from Kebrit deep were classified into Kebrit upper interface layer, Kebrit lower interface layer, and Kebrit brine samples. Microbial cells were fractionated in all samples collected according to its size by subjecting it to series of Millipore Mixed Cellulose Ester filters with pore sizes 3μm, 0.8μm and 0.1μm. Filters were preserved in sucrose buffer at -20 ºC during the expedition and till being transported to AUC genomics labs. Upon reaching the lab, the filters were immediately stored at -80 ºC.

This was followed by DNA isolation from 0.1 μm filters of Atlantis II interface layer and Kebrit upper interface layer by Mr. Amged Ouf following Rusch *et al*. 2007 protocol [71]. The concentration of the isolated DNA was measured using Picogreen assay using Nanodrop™ 3300 Fluorospectrometer.

2.2 Metagenomic Libraries Construction and Protein Based Phylogeny

Approximately 500ng of extracted DNA from each sample was used to construct 454 metagenomics library following GS FLX titanium library protocol. DNA was sheared by nebulization and DNA fragments with size ranges from 400-900 bps were selected by double SPRI method. Subsequently, DNA fragments were amplified using emulsion PCR then underwent through pyrosequencing using (454 life sciences, Branford CT) in the genomic lab, American University in Cairo (AUC). The Red sea metagenomics team at AUC carried out the entire DNA pyrosequencing process.

454 Sequences datasets were analyzed using Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) server cloud. Protein based phylogeny was done for Atlantis II interface and Kebrit upper interface datasets that were analyzed using sequence similarity search tool Blast implemented in the MG-RAST package

against Genebank non-redundant (nr) database considering a minimum E-value cut off score 10^{-5} and minimum identity cut off score 70% [72]. *Planctomycetes*-related hits were assigned to its related species and results from both samples were compared.

2.3 *hzo* Amplification, Library construction and Sequencing

A part of hydrazine oxidase gene (*hzo*) (approximately 600 bp in length) was amplified using a pair of *hzo* specific primers hzoAB4F 5'-TTGARTGTGCATGGTCTAWTGAAAG-3' and hzoAB4R 5' GCTGACCTGACCARTCAGG-3' in a direct PCR approach. PCR reactions were performed following the conditions described by Hirsch *et al*. 2010 [50]. PCR results were examined on 1% agarose gel by electrophoresis, extracted out of the gel and purified using QIA quick gel extraction kit (QIAGEN, Valencia, CA).

The purified PCR products were ligated to pGEM- T EASY vector following the manufacturer procedure (Promega). Then transformed to Top10 *E.coli* electrocompetent cells using Micropulser electroporation device (Bio-Rad, Hercules, CA) according to manufacturer procedure. A total of 96 clones from each sample were grown on LB media to be further subjected to DNA extraction using R.E.A.L prep 96 plasmid extraction kit (QIAGEN, Valencia, CA).

A total 96 clones from each library were sequenced using 96-capillary ABI 3730XL DNA analyzer. Cycle sequencing was performed using M13F primer and Big Dye Terminator Kit. Sequences generated were truncated to remove any vector related sequences and further subjected to quality control assessments by "Codoncode Aligner" (Codoncode Corporation, Dedham, MA) to filter sequences with lengths shorter than 300 bps and/or containing ambiguous nucleotides "N" more than 1%.

2.4 Red Sea Atlantis II and Kebrit Interface hzo Sequence Analyses

2.4.1Alpha and Beta Diversity Analyses

The filtered DNA sequences were aligned together using Clustalw, a multiple sequence alignment (MSA) algorithm[73]. The MSA results obtained were used to generate a distance matrix using dnadist in the PHYLIP package maintaining distance cut-off score 0.03 [50], [74]. The generated matrix was used later to estimate the level of coverage, richness and the evenness of the Anammox communities in the tested

samples. This was achieved by plotting rarefaction curves and calculating alpha diversity non-parametric indices Chao1, Simpson, and Shannon using mothur v.1.18.1 pipeline [75].

To interrogate the distinctions between the structure of Anammox communities inhabiting both Atlantis II and Kebrit interface layers, beta diversity analyses were conducted to estimate the evolutionary distance between both communities using ∫-LIBSHUFF and the number of shared OTUs softwares, which are adopted by mothur v.1.18.1 pipeline [75].

2.4.2 Phylogenetic Tree Based on the Deduced Amino Acid Sequences of *hzoA* / *hzoB*

BlastX(s) of the sequences generated from hydrazine oxidase (*hzo*) libraries of both Atlantis II and Kebrit interface layers were conducted against non-redundant (nr) database using Blastx software installed in our local server maintaining a minimum E-value score 10^{-5}. The best 100 hits were compiled from all sequences matched hydrazine oxidases and the redundant accession numbers were removed.

The isolation source of each accession number present in the hits was detected, and the accessions belong to the same isolation source were clustered using cd-hit server with minimum identity threshold 97%. The deduced amino acid sequences of those clustered hits compiled to construct an *hzo A*/ *hzo B* dataset. The sequences of the hydrazine oxidase libraries were translated using transeq server supported by European Bioinformatics institute (EBI) website. All protein sequences were aligned together using Muscle and then edited using Jalview. The refined alignment was used to construct a maximum likelihood tree using Phyml [76]. The tree was viewed and edited using Interactive Tree Of Life v2 server (iTOL) [77].

2.4.3 Comparative Studies between Anammox Communities Using UniFrac

A comparative analysis study between Anammox communities inhabiting both Atlantis II, and Kebrit interface layers was conducted using phylogenetic based analyses tests adopted by UniFrac web application package [78]. Moreover, The environments representing the isolation sources of the blastx best hits were implemented in this analysis.

In this study, two different statistical values, Unifrac significance test and P-test, were calculated to describe the relation between the examined Anammox communities. Additionally, a multivariate test, principle component analysis (PCoA), was carried out to reveal the effect of the geographical distribution on the structure of Anammox communities under investigation.

CHAPTER 3: - RESULTS

3.1 Metagenomic Analysis of Red Sea Atlantis II and Kebrit interface for Anammox Bacteria

3.1.1 Dataset of Atlantis II Deep and Kebrit Deep Metagenome

The general information describing the 454 metagenomics libraries under study showed that Kebrit upper interface library has a larger dataset (more than 1.5 million reads). These reads include approximately 400000 proteins, which are clustered to 365000 different functional categories. Atlantis II deep interface dataset contains approximately 800,000 reads. Of which, only 50000 encode for identified proteins and further grouped into 47000 different functional groups. The data is summarized in (table 1), as provided by MG-RAST Server Cloud [72].

3.1.2 Bacterial Phyla Abundance in the Interface Layers of Atlantis II Deep and Kebrit Deep

A quantitative analysis of bacterial phyla inhabiting Atlantis II deep and Kebrit deep was done using annotated proteins based phylogeny executed by MG-RAST server. The 454-metagenomics libraries sequencing data of the interface layers were matched to Genebank database using Blast tools implemented in MG-RAST server. The generated best hits results were curated using E-value cut off score 10^{-5}, and minimum identity cut off value 50%. Accordingly, 295 and 4743 hits were assigned to the phylum *planctomycetes* representing 0.26% and 0.92% of the total bacterial communities inhabiting the interface layers of Atlantis II deep and Kebrit deep respectively (Supplementary table 1). Further analyses were done to explore the distribution of *planctomycetes* related hits. Most of the identified *planctomycetes* reads belonged to the known cultured group and few hits matched uncultured species including Anammox. In Atlantis II Interface, *Planctomycetes* community is largely predominated by the cultured species *Pirellula staleyi*, representing 41.5% of all *Planctomycetes* related species. The uncultured *Planctomycetes* group identified was insignificant, since only one read was detected. In Kebrit Upper interface layer, the cultured *Planctomycetes* related hits were approximately equally distributed among five different species and more than 90 reads (~2%) were assigned to species belong to

the uncultured group of *Planctomycetes*. The data is shown in (Supplementary table 2), and was annotated using MG-RAST Server Cloud

3.2 Identification of hzoA/hzoB Gene from both Atlantis II Deep and Kebrit Deep Interfaces

The diversity of Anammox bacterial communities inhabiting the interface layers of Atlantis II deep and Kebrit deep were tested using primers targeting the functional gene hydrazine oxidase (hzoA/hzoB). Positive amplifications in both Atlantis II and Kebrit deep samples were detected. Consequently, amplified fragments with sizes (~ 600bp) were extracted and used to construct a library for each hydrazine oxidase positive samples and sequenced using ABI 3730XL DNA analyzer. The PCR amplification results are shown in (figure 8).

3.3 Computational and Statistical Analyses of Atlantis II and Kebrit Interfaces' Anammox Bacterial Communities

3.3.1 Alpha diversity Analysis of Anammox Bacteria Exist in Atlantis II Interface and Kebrit Upper Interface Ecosystems

Alpha diversity has been analyzed for the interface layers under study using mothur pipeline's α diversity analyses package. Alpha diversity is basically performed to estimate the coverage, the richness and the diversity of the microbial species dwelling the examined samples. In this study, rarefaction curves have been plotted for 81 hydrazine oxidase sequences obtained from Atlantis II interface sequenced clones (figure 9) and 44-hydrazine oxidase sequences recovered from Kebrit upper interface sequenced clones (figure 10). Eight and nine different Anammox phylotypes have been identified within Atlantis and Kebrit analyzed clones, respectively. Both curves have reached asymptote phase and this denotes sufficient coverage of Anammox phylotypes present in both libraries.

Additionally, we applied three non-parametric indices, Chao1, Simpson and Shannon, to conclude Anammox phylotypes richness and diversity. Chao1 used mainly to extrapolate the number of species present in the sample by applying a correction factor to already identified species. Chao1 scores indicated that the estimated number of phylotypes in Atlantis II is almost equal to the number of phylotypes detected by the functional phylomarker approach. For Kebrit Upper Interface, Chao1 score showed that

three Anammox phylotypes were left undetected in the Kebrit sample. Both Simpson and Shannon indices estimate the richness of the sample. The obtained results of both tests implied that the Anammox community in the Kebrit Interface layer is more diverse than the one present in the Atlantis II Interface layer. The overall results of alpha diversity statistical analyses were presented in (table 4).

3.3.2 Beta Diversity Analysis of Anammox Bacteria Inhabit the Examined Layers by Using ∫-LIBSHUFF

∫-LIBSHUFF analysis was performed to gauge the beta diversity among the Anammox communities present in both Atlantis II deep and Kebrit deep interfaces. The results indicated that Anammox community of Atlantis sample is different from those inhabiting the Kebrit sample; dCXY= 0.1401 with P value = <0.0001. Whereas the Anammox species inhabiting Kebrit sample is different from those inhabiting the Atlantis one dCXY= 0.0627 with P value = <0.001. The longest evolutionary distance between both communities is 0.00473.

3.3.3 Phylogenetic Analysis of (hzoA/hzoB) Sequences Retrieved from Atlantis II Deep Interface and Kebrit Deep Upper Interface Libraries

To examine the phylogenetic relationships exhibited between true functional Anammox bacterial communities including ours, an hzoA/hzoB sequences based phylogenetic tree was constructed. A total 81 *hzo* positive clones (eight phylotypes) from Atlantis II deep and 44 *hzo* positive clones (nine phylotypes) from Kebrit deep were evolutionarily compared to other Anammox phylotypes originated from diverse ecosystems using its deduced protein sequences, which show high similarity to previously identified *hzo* (≥ 90% similarity). The obtained sequences were aligned to entries from Genebank nr database using blastx and the first 100 hits for each sequence were retrieved maintaining a 90% minimum identity cut off. Non-redundant blastx matches were clustered according to its isolation sources and the unique OTUs $_{(0.03)}$ within each cluster were identified. All unique OTUs were compiled to construct our *hzoA*/*hzoB* phylogeny dataset. Accordingly, Our sequences were compared to the phylogeny dataset and the relations were displayed in one phylogenetic tree.

The overall results imply that (~60%) of our sequences are closely related to hydrazine oxidases isolated from different marine sources (e.g. deep sea hydrothermal

vent, deep sea tephra deposits, and sea subsurface sediments). Nevertheless, 20% of our sequences are significantly unique and haven't been previously identified in any similar ecosystems.

The resulting tree shows that all *hzo* sequences analyzed including our sequences are grouped into five different clusters (figure 12). Cluster A includes 27 Kebrit *hzo* sequences, these sequences show a close relation to an Anammox phylotype retrieved from deep-sea hydrothermal vent ecosystem. Cluster B contains 24 Atlantis II *hzo* sequences exhibiting close affiliation to a hydrazine oxidase sequence isolated from Candidatus *Kuenenia Stuttgartiensis*. Cluster C includes three Atlantis II *hzo* sequences, closely related to Anammox phylotypes, isolated from sea surface sediments, hydrothermal vent, ground water and coastal estuary sediment ecosystems. Cluster D comprises distinct phylotypes recovered from both Atlantis II and Kebrit interface layers with no close affiliation to any previously recognized *hzo* sequences, to be a putative unique *hzo* cluster. Finally, cluster E includes 51 and two *hzo* sequences retrieved from the interface regions of Atlantis II and Kebrit, respectively. Those sequences are grouped together with previously recognized Anammox phylotypes obtained from various ecosystems including KSU-1 *planctomycetes* enrichment cultures, Anammox bioreactors, ground water, river sediments, coastal estuary sediments and sea surface sediments.

3.3.4 Sequencing Data Analysis Using Blastx

After quality assessment analysis, a total of 81 sequences from Atlantis II deep interface library and 44 sequences from Kebrit deep upper interface library had good (30 Phred-Phrap) qualities. High quality sequences where aligned to entries in Genebank nr database using Blastx similarity search tool keeping E-value cut off score 10^{-5}. Blastx results retrieved were summarized in the following tables (2 and 3).

The overall Blastx results of the tested sequences obtained from both libraries revealed that all the sequences were matching hydrazine oxidases from uncultured *Planctomycetes*. The Atlantis II Interface sequences showed similarity with hydrazine oxidases isolated from marine sources except one short read matched a hydrazine oxidase isolated from Cape Fear River sediment. Out of these 80 sequences, 52 reads matched two hydrazine oxidases retrieved from Jiaozhou Bay sediment and 26 reads

showed similarity to two hydrazine oxidases isolated from Montserrat deep-sea tephra deposits. Only two reads obtained from Atlantis II interface library are similar to hydrazine oxidases isolated from enriched cultures dominated by *Planctomycete* KSU-1. All the Kebrit sequences were matching hydrazine oxidases isolated from marine ecosystems. 34 sequences showed similarity to a hydrazine oxidase isolated from Montserrat deep-sea tephra deposit. Additionally, five sequences were similar to two hydrazine oxidases isolated from Guayamas deep-sea hydrothermal vent and other five reads were similar to two *hzo* sequences isolated from Jiaozhou Bay sediment.

3.4 Comparative Analysis of Anammox Communities

To understand the diversity and the uniqueness of the Anammox communities in Atlantis and Kebrit interface layers, we compared *hzo* deduced amino acid sequences using Unifrac (comparing microbial communities using phylogenetic data) from different environments including Atlantis and Kebrit interfaces [78]. The environments included in the analysis are Atlantis interface layer, Kebrit upper interface layer, Jiaozhou bay sediment, deep-sea hydrothermal vent, deep-sea tephra deposits and upper Cape Fear River sediment. Table 5 discusses the environments examined, abbreviations used in the analysis and the number of *hzo* sequences analyzed from each environment.

The phylogenetic information (phylogenetic tree in Newick format) of all *hzo* sequences under study was processed using Unifrac and the phylogenetic relations were presented in figure 13. The phylogenetic tree depicts the presence of a putative unique cluster composed of 14 Kebrit and six Atlantis II Interface sequences. This cluster doesn't show close relation to any previously identified hydrazine oxidases. The rest of the examined *hzo* Kebrit and Atlantis II interface sequences showed close association to hydrazine oxidases isolated from Jiaozhou Bay sediment, deep-sea tephra deposit, hydrothermal vent sediment and Upper Cape Fear river sediment.

The similarities and differences between the compositions of Anammox communities inhabiting samples under study were analyzed using two different statistical tests, P-test and Unifrac significance test. The result of these statistical analyses indicated possible similarity between the structure of the Anammox

community inhabiting Kebrit Upper Interface with Anammox communities present in Atlantis II Interface and Deep-Sea hydrothermal vent. Moreover, Atlantis II Anammox bacteria showed insignificant differences with the Anammox present in Deep-Sea Hydrothermal vent. The results were presented in tables 6 and 7. The Principal Component Analysis (PCoA) was employed to further separate the environments according to the nature of its Anammox communities and to detect the actual diversity as well as the uniqueness of our samples. The results generated, upon analyzing the samples using two principal coordinates P1 and P2, were illustrated in figure.14. To further support our differential analysis, lineage specific test was done to explore which sequences have the major contribution in distinguishing the investigated samples. The results obtained where presented in table.8.

CHAPTER 4: - DISCUSSION

Marine Anammox bacteria are an underrepresented group in comparison to other phylogroups such as SAR11 (They represent approximately ≤ 1% of the total microbial population in most of the studied habitats and never exceeded 4%) [13], [79]. Based on this fact, studying marine Anammox bacteria may shed the light on the potential diversity of the Red sea brine pools' rare biospheres.

To our knowledge, this is the first study to explore the composition and the phylogenetic diversity of Anammox bacterial communities in the interface layers of Red sea brine pools. This analysis was carried out using a functional biomarker (hydrazine oxidase) gene approach. This gene encodes for an enzyme that belongs to the octaheme cytochrome C hydroxylamine oxidoreductase protein family [30]. This critical enzyme converts hydrazine intermediate in the Anammox pathway to di-nitrogen [50]. To date, hydrazine oxidases were only successfully detected and isolated from different Anammox bacteria e.g. Candidatus "*Scalindua*" and Candidatus "*Kuenenia"*[40]. Accordingly, Koltz and Stein suggested hydrazine oxidases could be used as a functional phylomarker for the Anammox group [57]. Different studies have used this approach to assess the diversity of Anammox communities in surface and subsurface marine sediments [62-64], estuary sediments [50], [51], oil reservoirs [54], river sediments [50], hydrothermal vents [43]and deep-sea tephra deposits [50].

The first glimpse on the results shows that Anammox bacteria are present in both Atlantis II and Kebrit interface layers. Higher levels of diversity and richness were observed in Anammox inhabiting Kebrit upper interface layer than those inhabiting Atlantis II interface layer. This might be due to the presence of oxygen in Atlantis II interface (43 µ moles /L equivalent to 0.5% air saturation) [46] . This exerts oxygen stress over the Anammox communities present suggesting the ability of these Anammox bacteria to tolerate mild aerobic conditions. Furthermore, these findings are supported through the work done by Strous *et al*. 1997 that proved Anammox bacteria could tolerate dissolved oxygen concentrations up to 0.5% [46].

Preliminary protein-based phylogeny analysis was conducted using the MG-RAST server cloud[72]. The analysis showed that 4743 reads representing 0.92% of the total bacterial reads of the Kebrit upper interface metagenomics library were assigned to *Planctomycetes*. Most of the reads belong to the cultured group specially the genera *Blastospirellula*, *Isosphaera, Pirellula* and others that we could not detect by our functional biomarker approach since they lack the unique hydrazine oxidase gene. Only 92 reads were assigned to uncultured *Planctomycetes* that may include our target; the Anammox group. On the other hand, 295 of the Atlantis II metagenomics library representing 0.26% of the total bacterial communities were assigned to *Planctomycetes*. Only one read matched with uncultured *Planctomycetes* group.

The observed discrepancies in the abundance of Anammox bacteria present in both samples could be a result of high sulfur contents and anaerobic conditions that characterize Kebrit upper interface ecosystem. These characteristics stimulate the sulfur reduction metabolic capabilities that are widespread among the cultured *Planctomycetes* group as reported by Elshahed *et al*. 2007 [80]. In addition, the high concentrations of hydrogen sulfide create anaerobic niches favored by the Anammox bacteria. Despite the significant existence of *Planctomycetes* phyla in Kebrit upper interface layer, the presence or absence of Anammox group in either Kebrit or Atlantis II interface layers couldn't be confidently substantiated out of the results obtained from protein based phylogeny analysis. Therefore, a functional biomarker gene approach was employed to gain insight on the Anammox bacterial communities inhabiting both layers.

DNA from both samples was amplified using hzoAB4F and hzoAB4R primers in a direct PCR approach targeting Anammox hydrazine oxidases. The amplified fragments were further processed to construct *hzo* clone libraries from the layers under investigation. A total of 81 clones from Atlantis II deep and 44 clones from Kebrit deep were sequenced. BlastX results showed that 100% of the sequences match hydrazine oxidases of uncultured *Planctomycetes*. In Atlantis II, 80 sequences were found to be similar to hydrazine oxidases isolated from marine sources. Only one short sequence (336bp), which matched a hydrazine oxidase from Upper Cape Fear river sediment, was found. Out of the 80 hydrazine oxidases in Atlantis, 47 sequences are binning to the accession number (ADD16762) isolated from Jiaozhou bay sediment

with identity scores ranging from (94-98%) except a single read shows identity score 87%. Also, five sequences show matches with the accession number (ADD16849) retrieved from Jiaozhou bay sediment with identity values > 98%. Moreover, 26 sequences are binning to the accession numbers (ADO21164) and (ADO21144) recovered from Montserrat deep-sea tephra deposits with identity results >97%. Out of the 81 sequences only two reads matched hydrazine oxidases from Anammox strain *Planctomycete* KSU-1 with identity values 90%. At this point, the structure of Anammox community in Atlantis interface couldn't be fully described due to the diverse nature of Anammox bacteria inhabiting the Jiaozhou bay sediment ecosystem [62].

Kebrit sequences show only similarity with hydrazine oxidases isolated from marine ecosystems with identity scores ≥ 93%. The best hits of more than 75% of the Kebrit match sequences isolated from Montserrat deep-sea Tephra deposits [50]. Five sequences match two different accession numbers retrieved from Guayamas deep-sea hydrothermal vent. Another five sequences match two different accession numbers obtained from Jiaozhou bay sediment ecosystem [50], [62]. Previous studies showed that sequences analyzed from both Montserrat deep-sea tephra deposits and Guayamas deep-sea hydrothermal vents showed intimate relations to *hzo* isolated from *Scalindua* enrichment cultures. Therefore, it is speculated that the majority of Anammox species inhabiting Kebrit upper interface layer are most probably dominated "*Scalindua sp.*"

To assess the diversity of Anammox phylotypes that exist in Atlantis II interface layer and Kebrit upper interface layer, alpha diversity analyses were performed using mothur pipeline. Rarefaction curves were plotted for both samples and indicated good coverage of Anammox phylotypes by attaining the plateau phase in both curves. Reaching this phase means that even if more sequences were added, the number of the phylotypes will remain the same. Additionally, three α-diversity indices were performed; Chao1, Simpson and Shannon. Chao1 is a non-parametric index estimating the approximate number of phylotypes that might be present in the examined samples. For Atlantis II, Chao1 value is 8.3 approximately equal to the actual number of phylotypes that was retrieved from the sample. For Kebrit, the Chao1 value is 12 suggesting that only three phylotypes are missing from our Kebrit dataset. Both Simpson and Shannon indices reflect the diversity and the richness of the tested

sample. Shannon values range between 1 designating low diversity to 5 designating high diversity while Simpson values range between 0 representing high diversity to 1 representing low diversity. The overall alpha diversity indices results imply that Anammox bacteria in Kebrit upper interface ecosystem are richer and more diverse than the one inhabiting the Atlantis II interface.

To estimate the diversity and the evolutionary distances among Anammox communities inhabiting both analyzed samples, beta diversity analyses were done using ∫-LIBSHUFF and shared OTUs test implemented in mothur software. The results showed that Anammox bacteria in Kebrit are significantly different than Atlantis II Anammox bacteria (dCXY= 0.0627). It also indicated that there are no shared OTUs between both examined Anammox groups and the evolutionary distances between the tested phylotypes are > 0.03 (more than 3 amino acids per 100). It could also be inferred that both communities nearly share the same Anammox genera as concluded from the evolutionary distances (< 0.05).

It has been reported in previous studies that the Anammox bacteria *Candidatus Scalindua* species mainly dominate marine water columns, surface and subsurface sediments of the sea. In addition to the presence of *Candidatus* "*Scalindua* sp.", other Anammox bacteria were detected in hydrothermal vents located in the Mid-Atlantic ridge area including *Candidatus* "*Kuenenia* sp.", *Candidatus* "*Brocadia* sp." and *Candidatus* "*Jettenia* sp." It was also concluded that the lower abundance of *Scalindua* species in ecosystems with thermal activities is because *Scalindua sp*. adapts better to ecosystems with lower temperatures [43].

Based on these observations, our interpretations of the phylogenetic analysis data revealed the presence of five different Anammox bacteria clusters in both Atlantis and Kebrit interfaces (figure 12). Cluster A comprises 28 sequences. Of which, more than 60% were recovered from Kebrit. These sequences are phylogenetically related to a single phylotype retrieved from a deep-sea hydrothermal vent located in Guayamas basin. Considering that all the *hzo* sequences isolated from Guayamas basin hydrothermal vents belong to *Candidatus* "*Scalindua*." Therefore, cluster A sequences are more or less related to *Scalindua* species [50]. This is further supported by the fact that *Scalindua* dominate marine habitats with low to moderate temperatures, similar to Kebrit upper interface conditions [40]. The second cluster comprised 24 sequences

representing 30% of Anammox bacteria present in Atlantis II are affiliated to Candidatus "*Kuenenia Sttutgartiensis*." This finding is in agreement with the results found in Mid- Atlantic deep-sea hydrothermal vent [43], as Atlantis II brine pool is characterized by its hydrothermal activity (temperature ≈ 70°C). Cluster C represents three Atlantis II *hzo* sequences clustered with phylotypes isolated from surface sediments of equatorial pacific, Jiaozhou bay sediment, subsurface sea sediments, Montserrat deep-sea tephra deposits and Guayamas basin deep-sea hydrothermal vents. *hzo* sequences recovered from surface sediment of equatorial Pacific [63], deep-sea hydrothermal vents and deep-sea tephra deposits were affiliated to *Candidatus "Scalindua species"* as showed by both 16srRNA and *hzo* based functional analyses [50]. It can be concluded that *hzo* sequences in cluster C most probably belong to Candidatus "*Scalindua species*". Interestingly, cluster D - being composed of 14 Kebrit *hzo* sequences and six Atlantis II *hzo* sequences - didn't show any connection to a previously identified *hzo* sequences. This settles it as a unique *hzo* cluster and hypothesizes that it is a possible signature for Anammox bacteria in brine pools' interface layer ecosystems. It can be also concluded that brine pool ecosystems may exhibit evolutionary forces that might influence the unique diversity of Anammox species. Finally, cluster E seems to be more ubiquitous than formerly mentioned *hzo* clusters. It groups 48 *hzo* sequences isolated from Atlantis II interface layer and two *hzo* sequences from Kebrit upper interface layer with large subset of hydrazine oxidases isolated from different sources including surface sediments from black river, Jiaozhou bay sediment, coastal estuary sediments, Upper Cape Fear river estuary sediment, North Carolina ground water, high temperature oil reservoirs and even from Anammox bioreactors. Different Anammox genera including *Scalindua*, *Jettenia*, *Brocadia* and *Kuenenia* have been identified in ecosystems that grouped in cluster E. This is due to either the nature of the ecosystems themselves that support the presence of these species or the anthropogenic interventions reported at some of these habitats [54], [62]. This coincides with the limited number of *hzo* sequences available in the Genebank database. For instance, there are only two hydrazine oxidase sequences available from *Scalindua* enrichment cultures. Thus, the exact Anammox genus representing this cluster couldn't be accurately identified [63], [64].

To estimate the diversity levels and the degree of uniqueness of the targeted samples, a comparative study between our samples and other ecosystems chosen based

on the isolation sources of the blastx best hits results that retrieved from Genebank nr database was conducted. This study was executed using Unifrac software. Unifrac phylogenetic tree indicates the presence of two *hzo* clades - belonging to Kebrit and Atlantis II - that don't match any other previously isolated *hzo* sequences. This coincides with the results presented by the previously discussed aLRT phylogenetic tree and the lineage specific test as well. Furthermore, the results obtained from another two statistical analysis tests, P-test and Unifrac significance test, indicate that Anammox community inhabiting Kebrit upper interface layer is suggestively close in its structure to those inhabiting both Guayamas basin deep-sea hydrothermal vent and Atlantis II interface ecosystems than other Anammox communities tested. This observation is in agreement with the phylogenetic analysis done, as more than 60% of Kebrit *hzo* sequences matched an Anammox phylotype isolated from Guayamas deep-sea hydrothermal vent and the rest cluster at different positions on the aLRT tree with Atlantis *hzo* sequences. Whereas, Anammox community in Atlantis II interface layer is not significantly different than Guayamas basin deep-sea hydrothermal vent, Kebrit upper interface layer and, at a lower extent, not different than Montserrat deep-sea tephra deposits. These relations are depicted in a Unifrac cluster model of the examined ecosystems and supported by Jacknife confidence values (supplementary figure 1). Finally, the PCoA analysis drew a conclusion that Anammox communities inhabiting either Atlantis II interface layer or Kebrit upper interface layer exhibit a unique geographical distribution pattern. This supports the assumption that the multiple extremophilic nature of these ecosystems might influence the evolution of various Anammox related species differently than other marine habitats.

It is also worth noting that there are three pitfalls that should be highlighted in our study. First, the results of this functional approach should be correlated to the 16srRNA analyses results of both tested samples. This will broaden our perception of the level of the Anammox species diversity present in the examined samples and improve our interpretations of Anammox profiles detected by hydrazine oxidase based approach. In addition, the shortage in the ammonium and nitrogen levels data available on Atlantis II and Kebrit interfaces limited our scope in linking the Anammox diversity based on the fluctuations of nitrogen and ammonia levels. Also, the limited number of hydrazine oxidase sequences available from Anammox enrichment cultures significantly restricts our analyses. This is owing to the presence of only two

hydrazine oxidase sequences from *Candidatus* "*Scalindua* sp.", one from "*Brocadia* sp.", one from "*Jettenia* sp.", two from *Candidatus* "*Kuenenia* sp." and one isolated from *Candidatus* "*Anammoxoglobus*" [64].

In conclusion, the study of the diversity profiles of Anammox communities inhabiting Atlantis II and Kebrit brine pools' interface layers shed the light on the presence of some unique Anammox phylotypes and phylogenetic patterns. These newly discovered phylotypes and phylogenetic patterns differ considerably from Anammox profiles previously described in other marine ecosystems.

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FIGURES

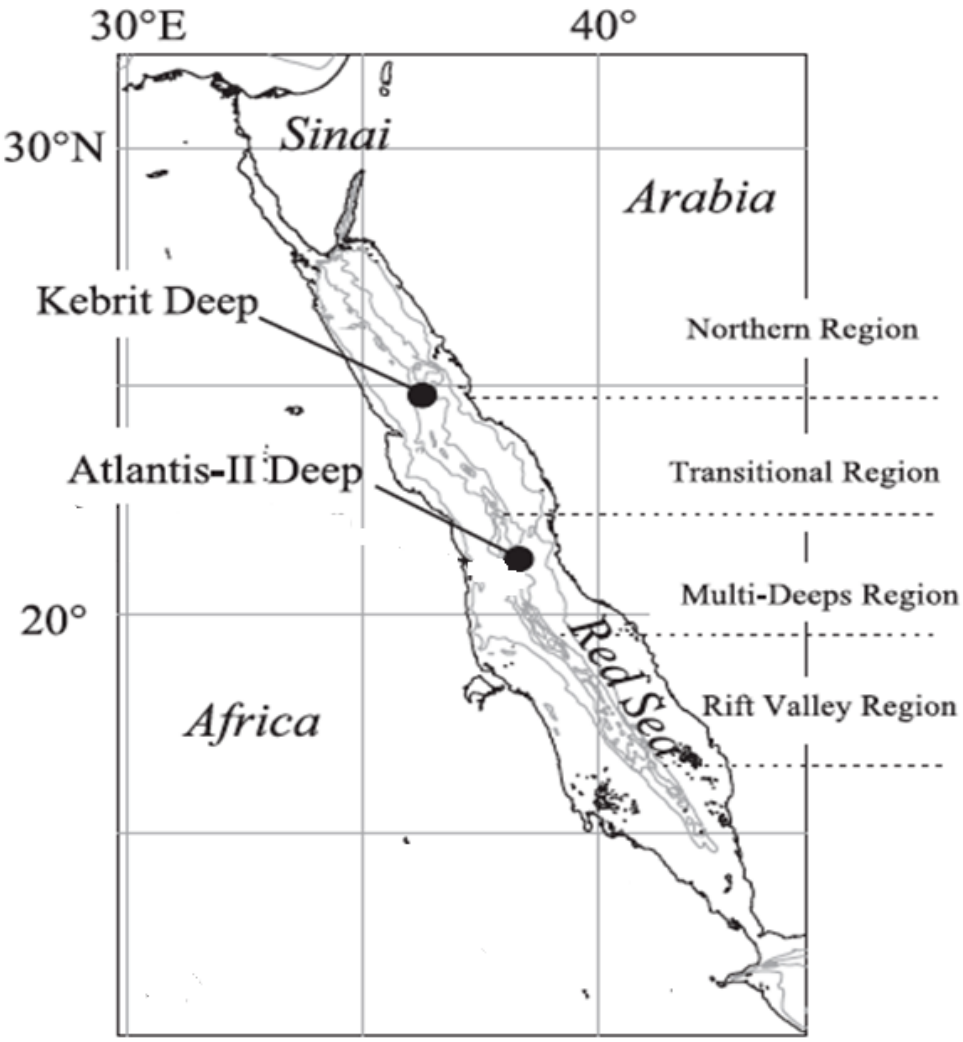
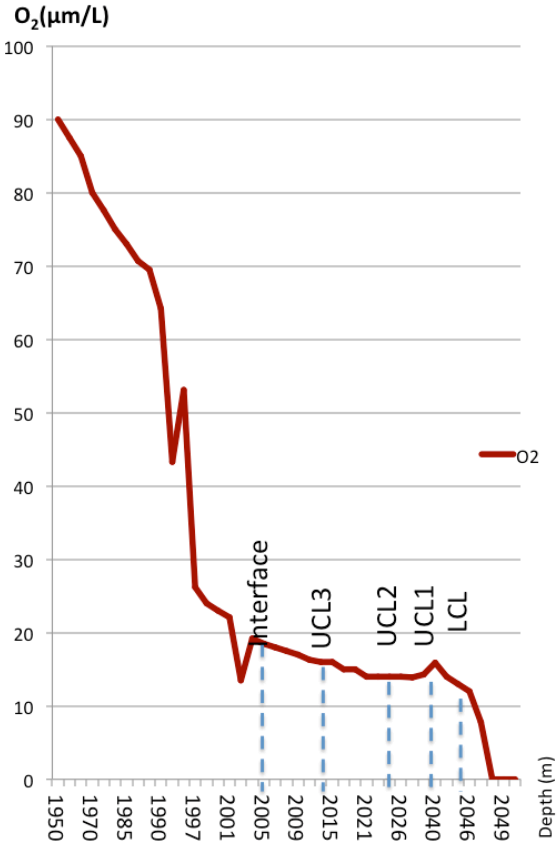
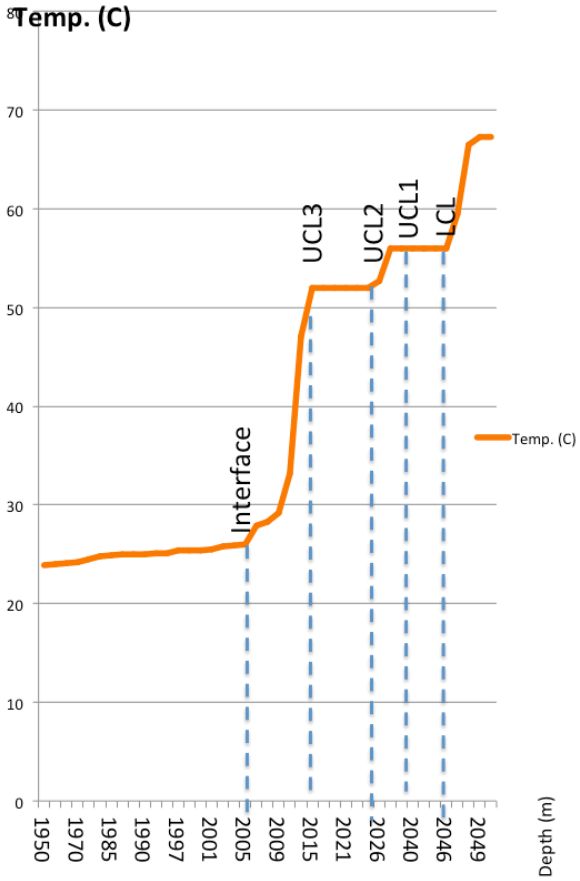


Figure 1 The Location of Red Sea Brine Pools

This figure modified from Schmidt *et al*., 2003; demonstrating the location of two Red sea brine pools, Kebrit Deep and Atlantis II Deep [3].

Atlantis II Deep





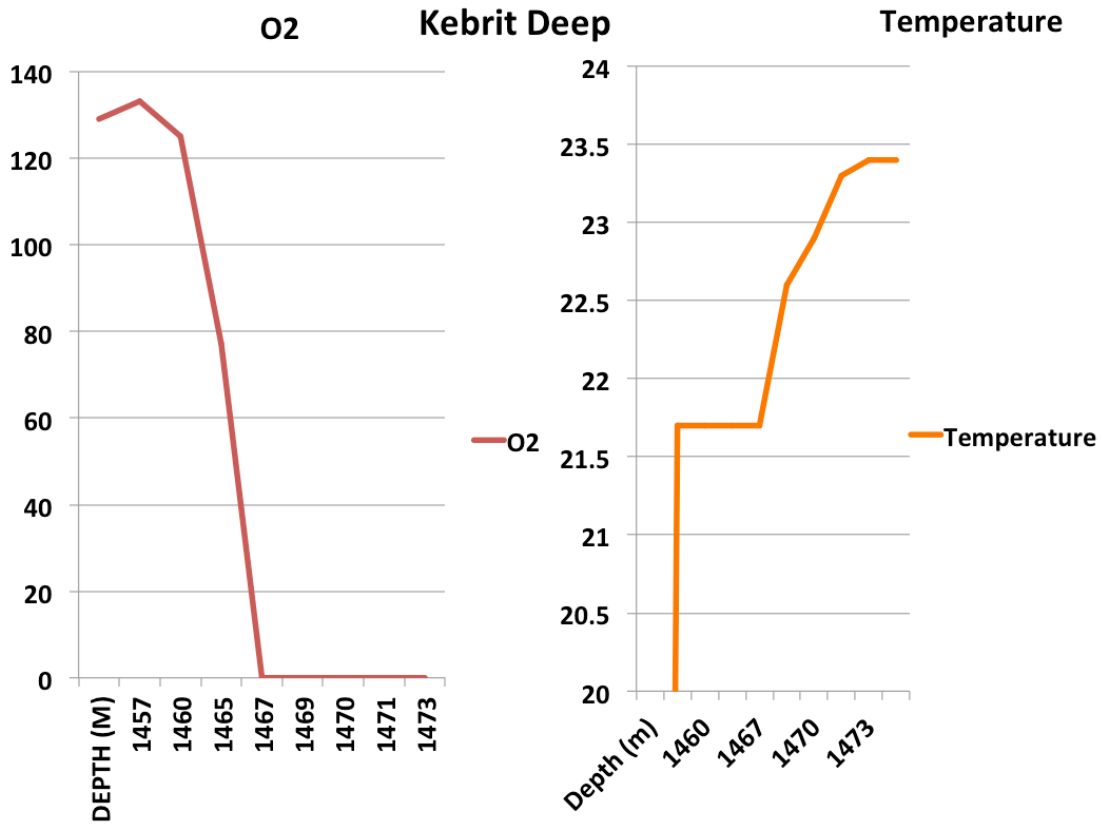


Figure 2 Physicochemical Characteristics of Atlantis II Deep and Kebrit Deep

These graphs show the variations in the oxygen and temperature across different layers of Atlantis II Deep and Kebrit Deep Brine Pools. These graphs were adapted from Schmidt *et al*. 2003 [3]

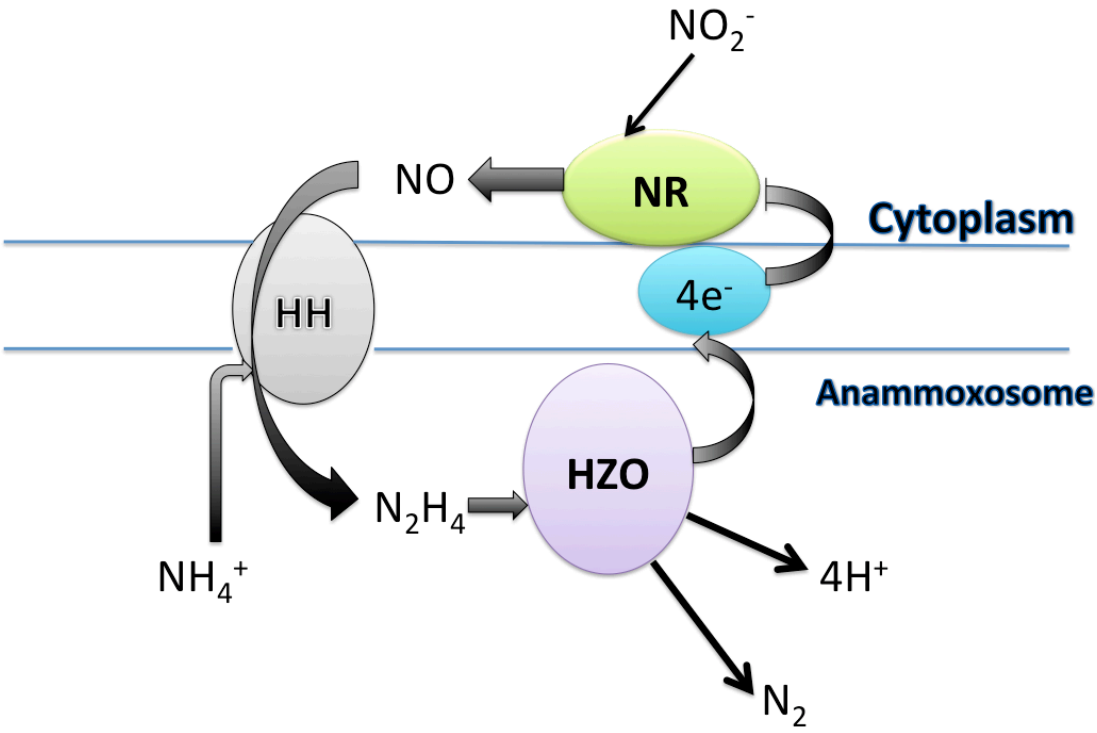


Figure 3 The Anammox Pathway

This illustration depicts the Anammox pathway machinery. Hydrazine hydrolase (HH) produces hydrazine from ammonia and nitric oxide (the product of nitrite reduction via nitrite reductase (NR)). Hydrazine oxidase (HZO) generates di-nitrogen through hydrazine oxidation reaction. This figure is modified from (Strous *et al*. 2006) [29].

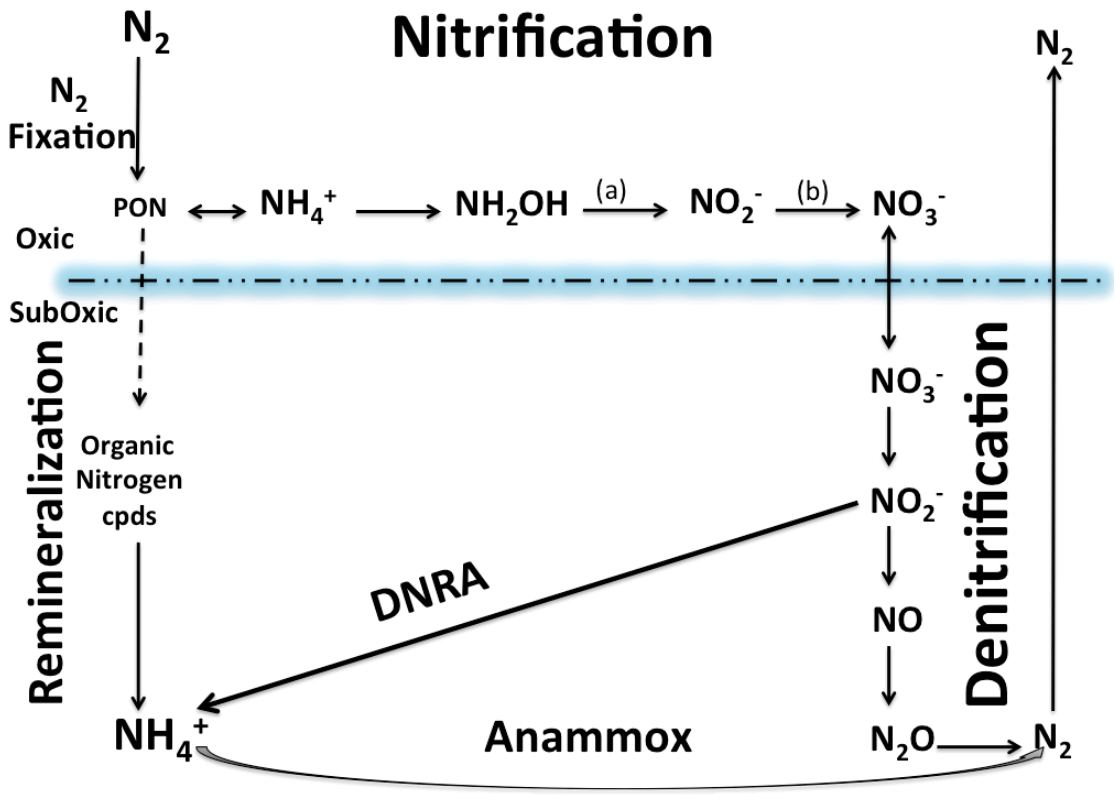


Figure 4 The Major Pathways in the Marine Nitrogen Biogeochemical Cycle

This figure depicts the four major pathways comprising the marine nitrogen biogeochemical cycle. Under oxic conditions nitrogen gas is fixed to particulate organic nitrogen compounds (PON) through nitrogen fixation and ammonia is nitrified in two sequential steps: a) ammonia is oxidized to nitrite through the action of nitrate oxidase possessed by *Nitrosomonas Sp*. b) nitrite is oxidized to nitrate through the action of nitrate monooxygenase possessed by *Nitrobacter Sp*. Accordingly, nitrate seeps from oxic regions to suboxic ones and is reduced to nitrite to be modified either to ammonia by DNRA pathway or to N_2 by denitrification, which is released back to the atmosphere. Ammonia is available in the suboxic niches either through mineralization of organic compounds or DNRA pathway is further processed via Anammox bacteria to generate N_2 gas. This figure is adapted from Francis *et al*. 2007[22].

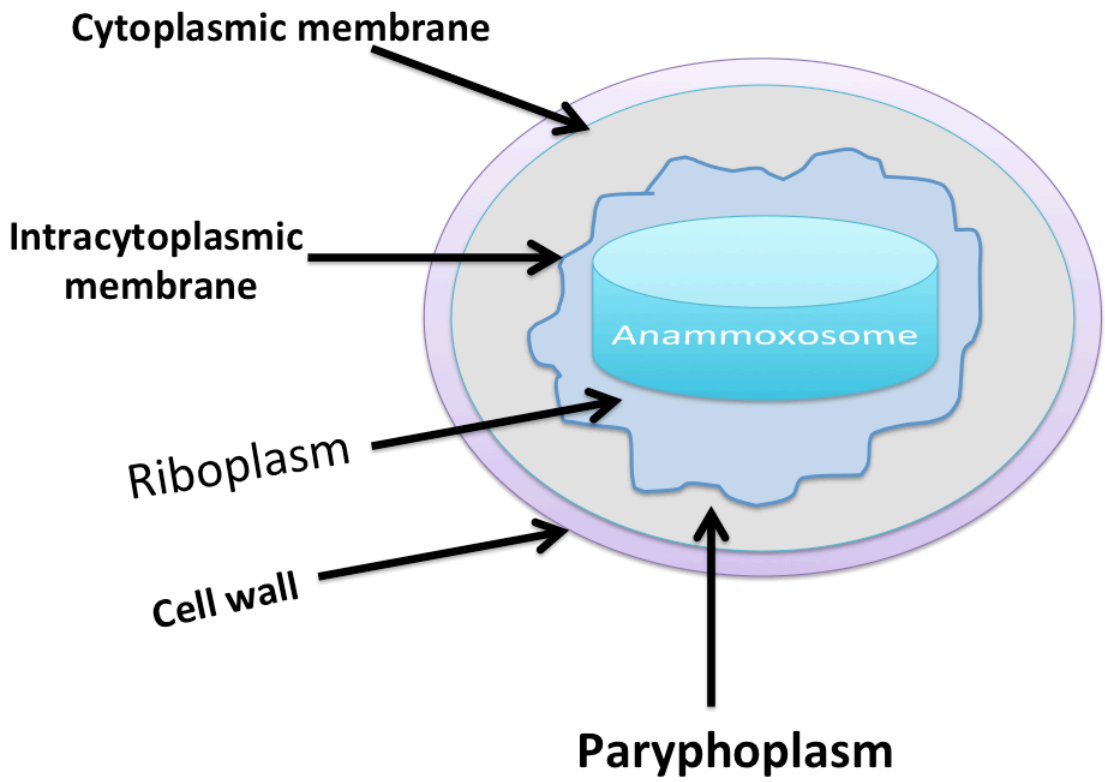


Figure 5 Schematic Representation of the Structure of the Anammox Unique Compartment (Anammoxosome)

The figure depicts the organization of the compartmentalized Anammox cell, modified from Kuenen 2008 [40].

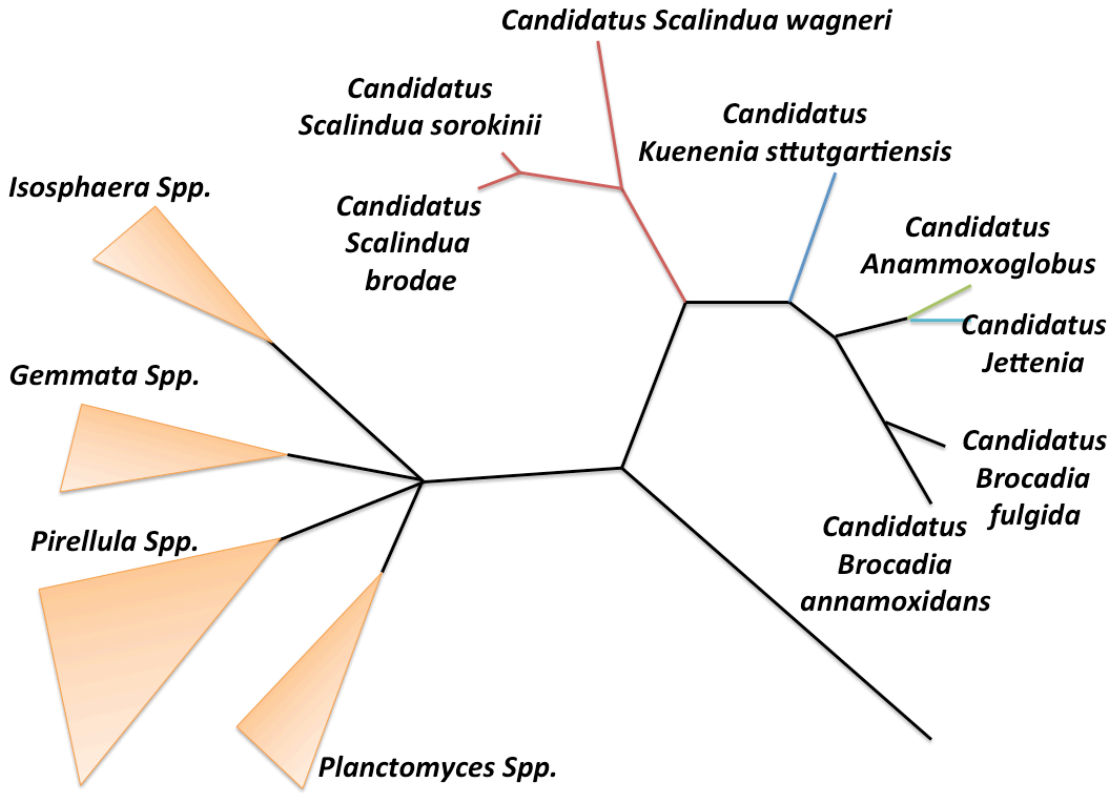


Figure 6 Phylogenetic Representation of the Phylum Planctomycetes

Schematic representation of Planctomycetes phylogenetic tree based on 16srRNA-deduced amino acids depicts the phylogenetic relations between Planctomycetes related species. This tree modified from Kuenen *et al*. 2006 [40]

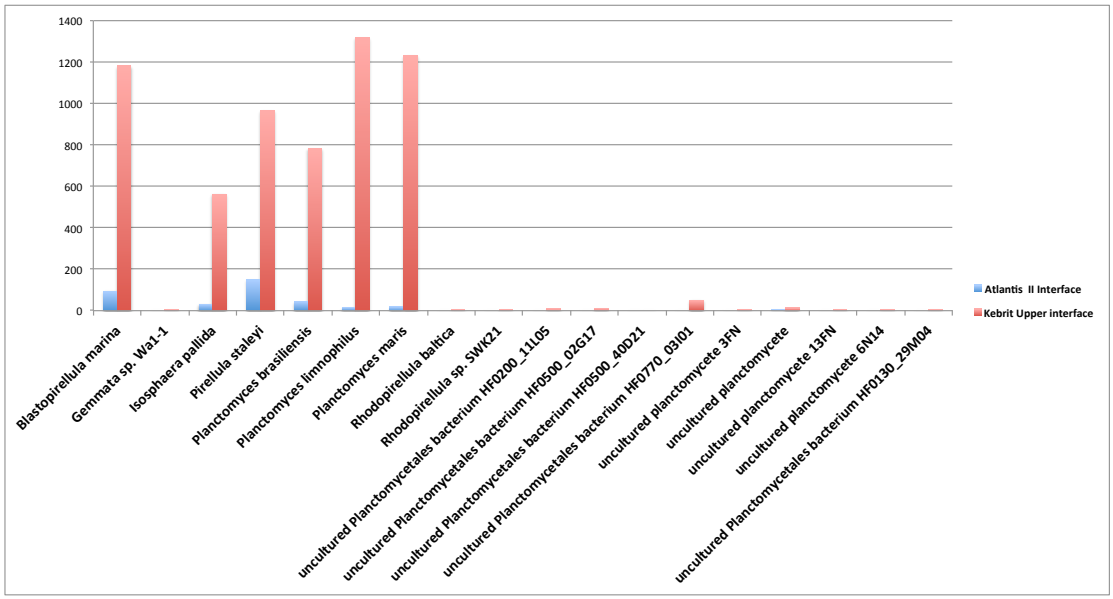


Figure 7 The Overall Distribution of Both Planctomycetes Cultured and Uncultured Species as Identified by MG-RAST Server Cloud

The graph represents the total reads assigned to both cultured and uncultured *planctomycetes* in Atlantis II (blue) and Kebrit upper (red) interface libraries according to MG-RAST Cloud sequence similarity search against GenBank database with a cutoff E-value of 1^{-5}.

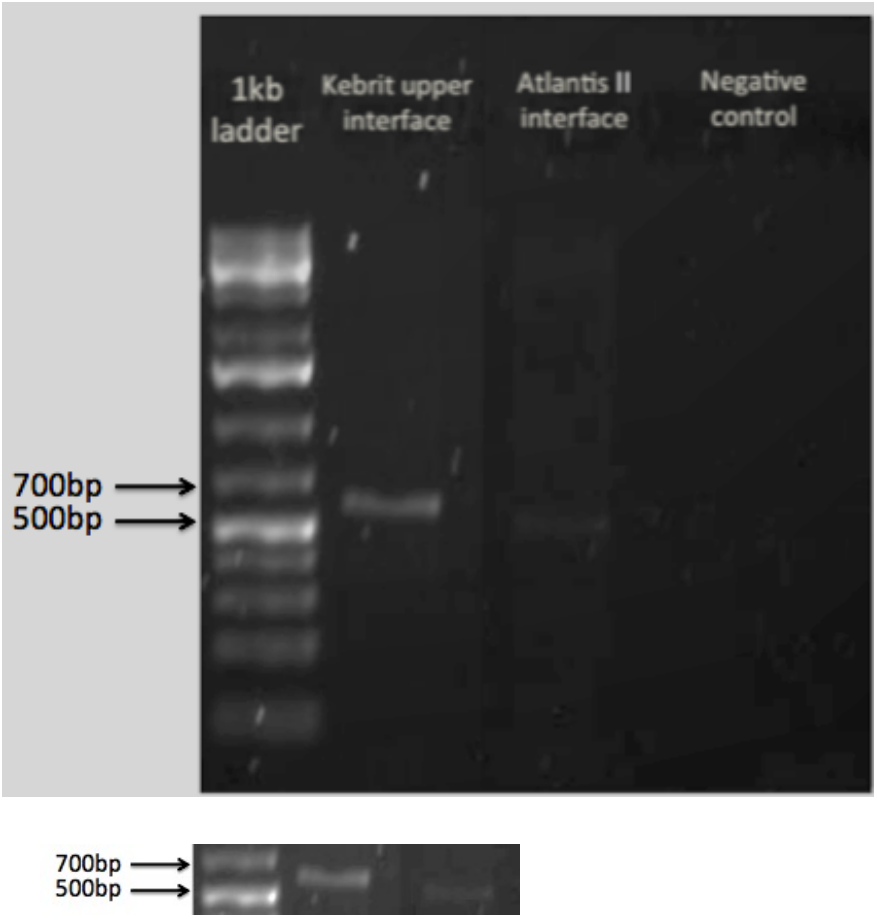


Figure 8 PCR Amplification Results of *hzoA*/*hzoB* from Both Kebrit Upper Interface and Atlantis II Interface Layers

The figure shows PCR amplification results generated using hydrazine oxidase specific primers set hzoAB4F, and hzoAB4R. The products sizes were measured using 1kb ladder (New England Biolabs, Ipswich, MA). Amplifications were observed in Atlantis II deep and Kebrit deep samples, all the amplicons had a length (~ 600bp) as expected.

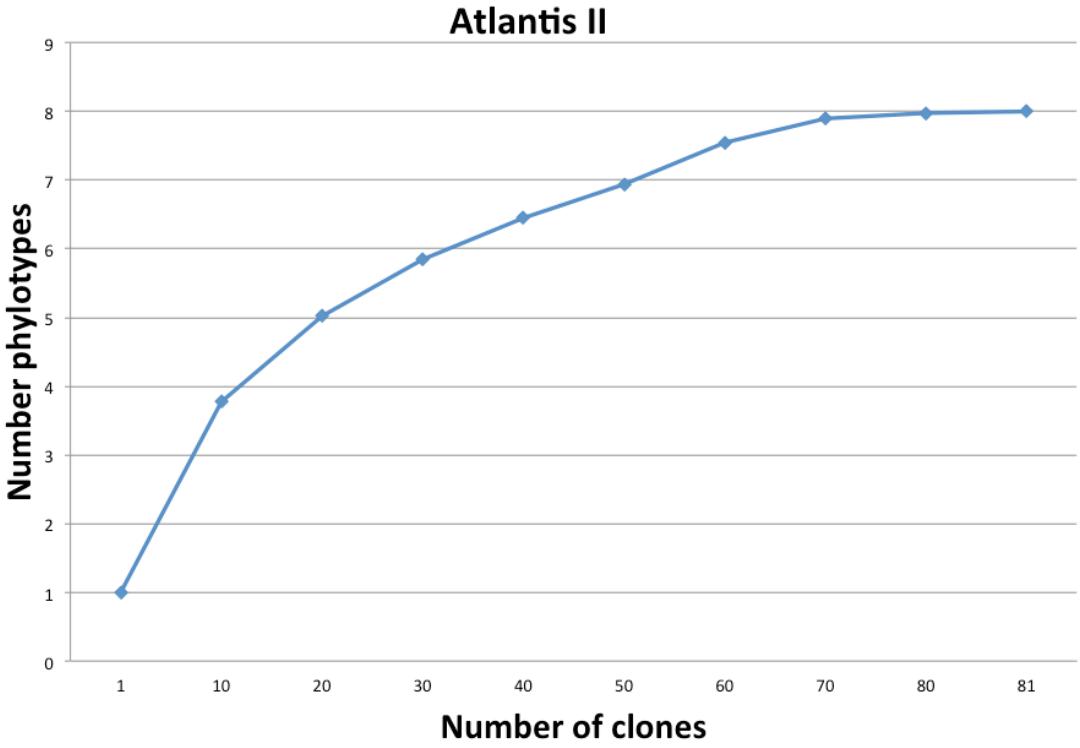


Figure 9 Rarefaction Curve Plotted to Indicate the Coverage Level of Anammox Phylotypes Inhabiting Atlantis II Interface Layer

This graph is plotted to explain the relation between the eight Anammox phylotypes identified from 81 hydrazine oxidase sequences recovered from Atlantis II deep interface library. It also demonstrates a good coverage of Anammox phylotypes present in the layer, which is indicated by the plateau phase reached by the curve.

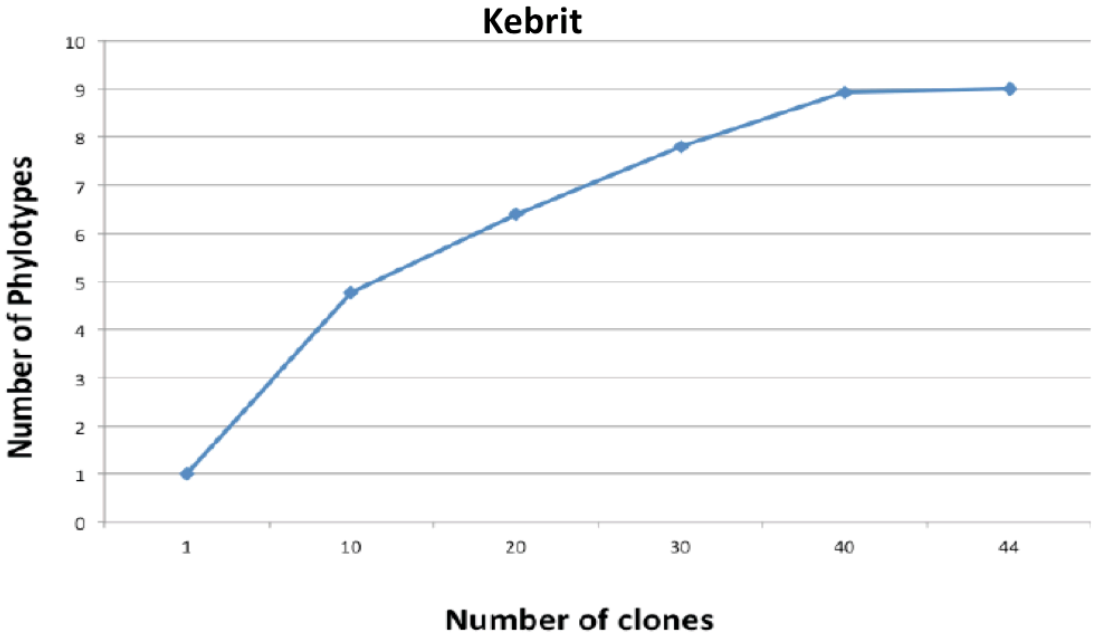


Figure 10 Rarefaction Curve Plotted to Indicate the Coverage Level of Anammox Phylotype Inhabting Kebrit Upper Interface Layer

This graph is plotted to depict the relation between the nine Anammox phylotypes identified based on 44 hydrazine oxidase sequences recovered from Kebrit deep upper interface library. It also demonstrates a good coverage of Anammox phylotypes present in the layer, which is indicated by the plateau phase reached by the curve.

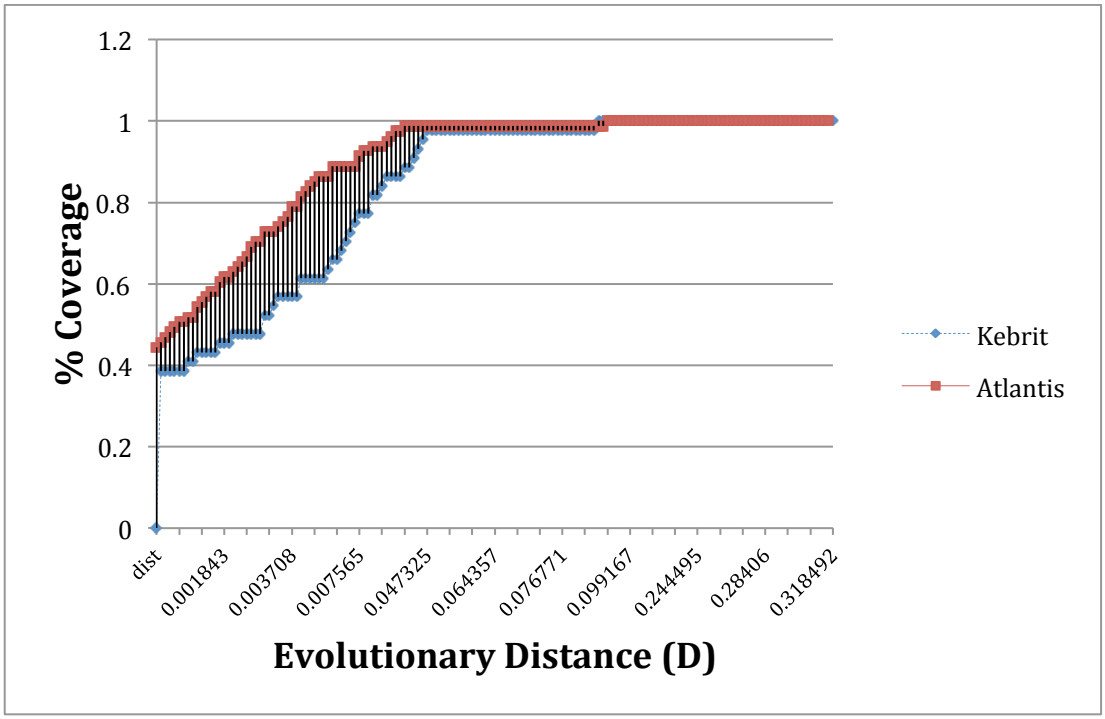


Figure 11 The Evolutionary Distance Between Hzo Sequences Recovered from the Samples Under Study

This graph illustrates the evolutionary distance between the Anammox phylotypes present in the tested samples based on LIBSHUFF analysis of *hzo* sequences. The relation was displayed by plotting the evolutionary distance (D) on the x-axis and the percentage of *hzo* sequences covered (% coverage) on the y-axis.

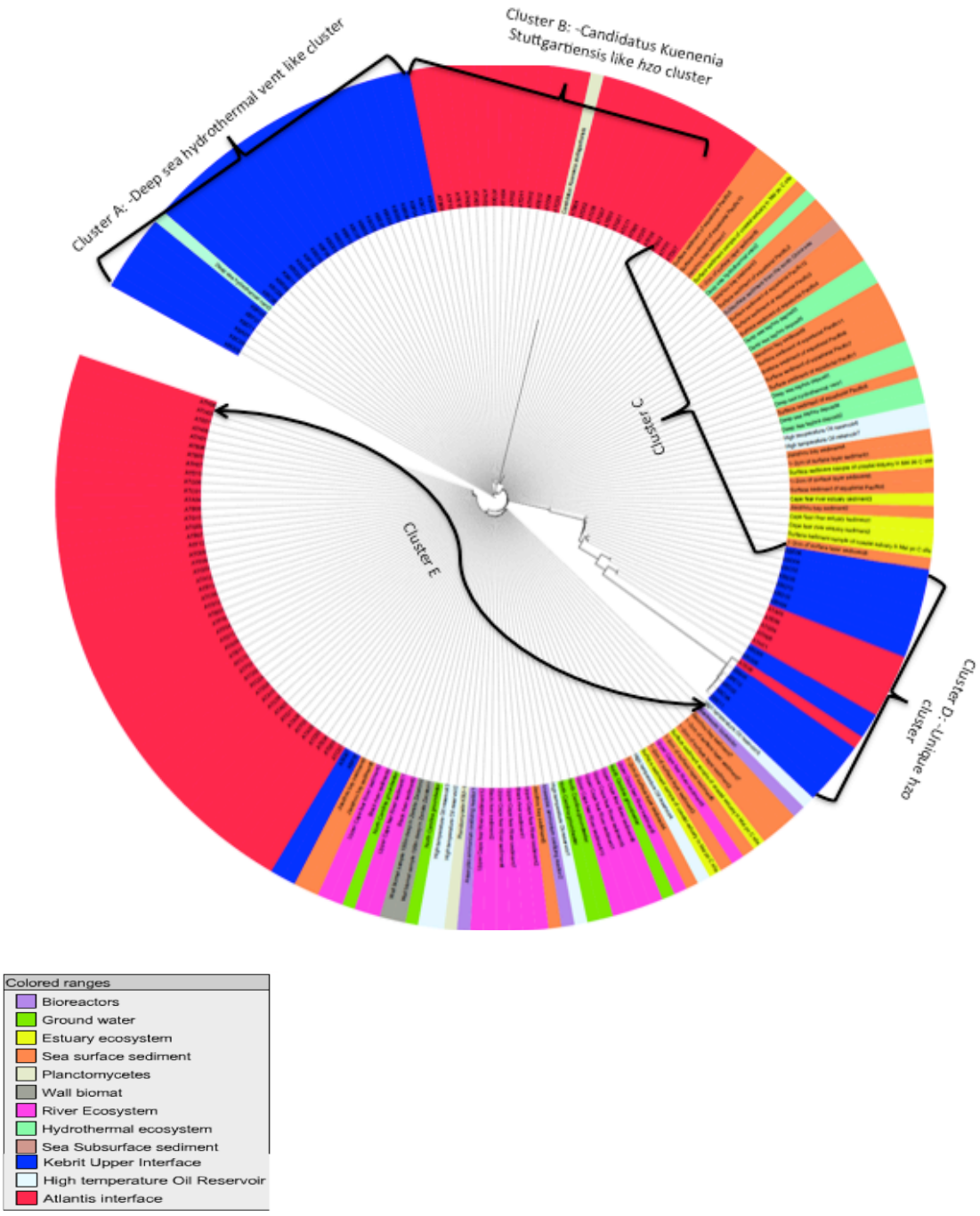


Figure 12 Approximate Likelihood Phylogenetic Tree Based on hzo Sequences Deduced Amino Acids Alignments.

A maximum likelihood tree based on hzo deduced amino acids sequences recovered from the examined layers. Those sequences were evolutionary compared to other *hzo*-based Anammox OTUs obtained from different marine and non-marine ecosystems. The color codes are (purple= Anammox bioreactors, green= ground water, yellow= estuary coastal sediment ecosystem, orange= sea surface sediment, off-white= Anammox enrichment cultures, gray= wall biomat sample, fuchsia= river ecosystem, light green= hydrothermal vents, brown= sea subsurface sediment, blue= Kebrit upper interface layer, white= high temperature oil reservoirs and red= Atlantis interface layer). The tree

confidence values were calculated using approximate Likelihood-Ratio Test (aLRT) and only branches with bootstrap supporting values > 0.5 were presented.

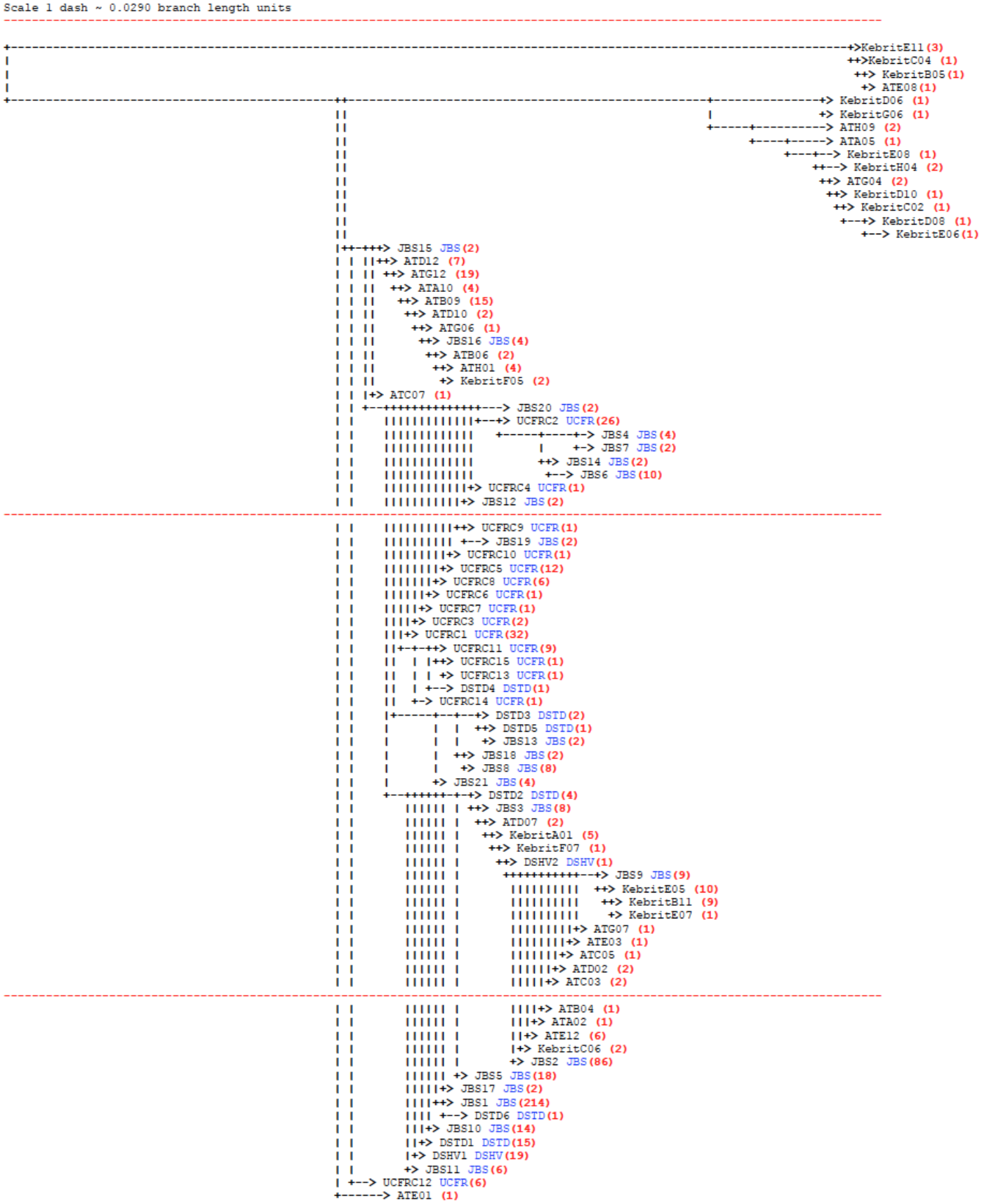

Scale 1 dash ~ 0.0290 branch length units
KebritE11 (3)
KebritC04 (1)
KebritB05 (1)
ATE08 (1)
KebritD06 (1)
KebritG06 (1)
ATH09 (2)
ATA05 (1)
KebritE08 (1)
KebritH04 (2)
ATG04 (2)
KebritD10 (1)
KebritC02 (1)
KebritD08 (1)
KebritE06 (1)
JBS15 JBS (2)
ATD12 (7)
ATG12 (19)
ATA10 (4)
ATB09 (15)
ATD10 (2)
ATG06 (1)
JBS16 JBS (4)
ATB06 (2)
ATH01 (4)
KebritF05 (2)
ATC07 (1)
JBS20 JBS (2)
UCFRC2 UCFR (26)
JBS4 JBS (4)
JBS7 JBS (2)
JBS14 JBS (2)
JBS6 JBS (10)
UCFRC4 UCFR (1)
JBS12 JBS (2)
UCFRC9 UCFR (1)
JBS19 JBS (2)
UCFRC10 UCFR (1)
UCFRC5 UCFR (12)
UCFRC8 UCFR (6)
UCFRC6 UCFR (1)
UCFRC7 UCFR (1)
UCFRC3 UCFR (2)
UCFRC1 UCFR (32)
UCFRC11 UCFR (9)
UCFRC15 UCFR (1)
UCFRC13 UCFR (1)
DSTD4 DSTD (1)
UCFRC14 UCFR (1)
DSTD3 DSTD (2)
DSTD5 DSTD (1)
JBS13 JBS (2)
JBS18 JBS (2)
JBS8 JBS (8)
JBS21 JBS (4)
DSTD2 DSTD (4)
JBS3 JBS (8)
ATD07 (2)
KebritA01 (5)
KebritF07 (1)
DSHV2 DSHV (1)
JBS9 JBS (9)
KebritE05 (10)
KebritB11 (9)
KebritE07 (1)
ATG07 (1)
ATE03 (1)
ATC05 (1)
ATD02 (2)
ATC03 (2)
ATB04 (1)
ATA02 (1)
ATE12 (6)
KebritC06 (2)
JBS2 JBS (86)
JBS5 JBS (18)
JBS17 JBS (2)
JBS1 JBS (214)
DSTD6 DSTD (1)
JBS10 JBS (14)
DSTD1 DSTD (15)
DSHV1 DSHV (19)
JBS11 JBS (6)
UCFRC12 UCFR (6)
ATE01 (1)

Figure. 13 The Phylogenetic Relations between all hzo Sequences Under Study

Unifrac phylogenetic tree illustrates the relations between all the *hzo* sequences analyzed. *hzo* sequences from each ecosystem were clustered together using identity cut off score 97%, equivalent to the cut off normally used to distinguish between different phylotypes. The frequency of the occurrence of each phylotype was provided in parentheses. The codes provided with Atlantis II and Kebrit sequences e.g. KebritE11, represent the location of these sequences in the library plates. Meanwhile, the numbers given with the other environments' sequences denote the cluster number. The scale bar used (0.029) estimates the divergence distance between the tested phylotypes (three different amino acids per 100).

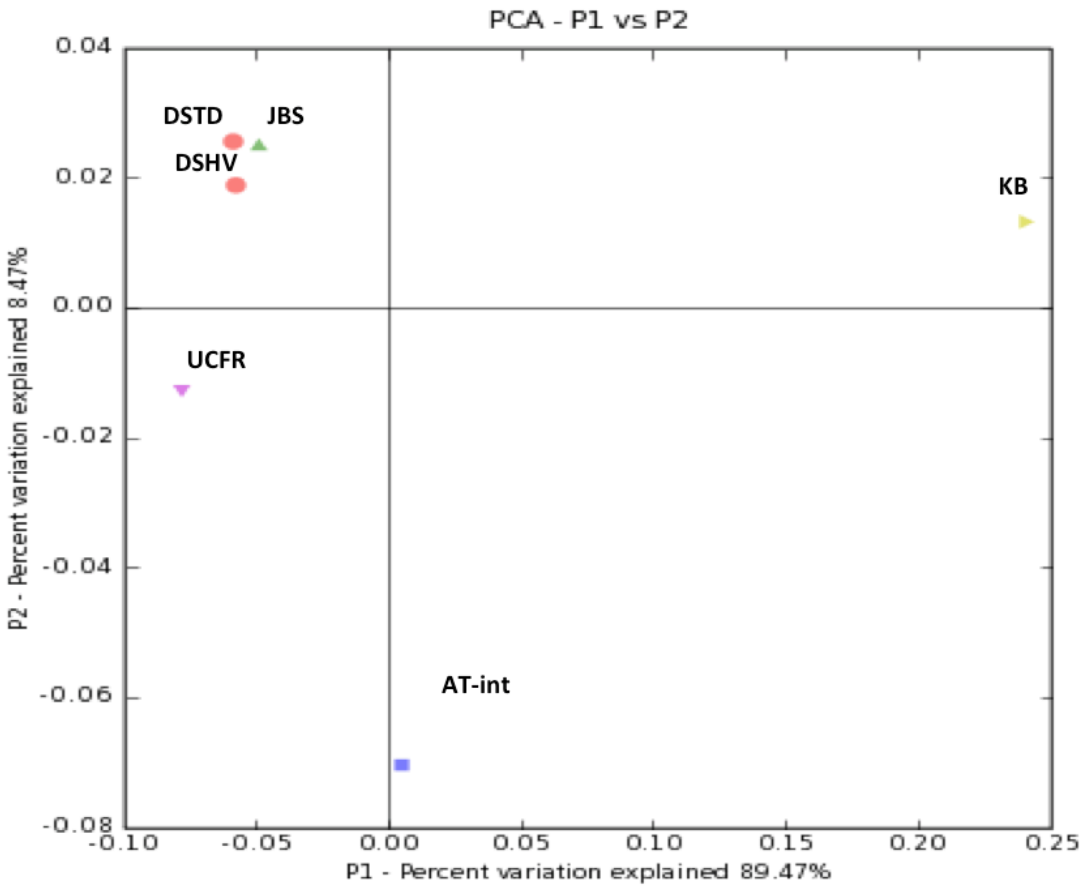


Figure 14 Weighted Unifrac Principal Component Analysis (PCoA) of hzo Sequences

Weighted Unifrac PCoA analysis using the deduced hydrazine oxidase amino acid sequences of the six examined ecosystems. The red circles represent both deep-sea hydrothermal vent and deep-sea tephra deposits samples. The green triangle represents Jiaozhou bay sediment. The purple triangle represents Upper Cape Fear river sediment. The blue square represent Atlantis II interface layer and the yellow triangle represent the Kebrit upper interface layer.

TABLES

Table 1 The Main features of the Metagenomics Libraries Constructed from the Interface Layers of Atlantis II Deep and Kebrit deep

Analysis features	*Atlantis II Interface Deep*	*Kebrit Upper Interface Deep*
Number of 454 runs	1	1
Number of sequences	832,138	1,562,521
Number of Nucleotides	268,756,307 bp	470,950,183 bp
Mean Sequence length	322 ± 120 bp	301 ± 110 bp
Identified Proteins	54,917	407,643
Identified Functional Categories	47,948	365,109

The table presents features of the metagenomics libraries under study. These features include the number of 454 runs, the number of sequences generated, the total number of nucleotides per each library, the mean of sequence length, the number of proteins identified and finally the number of functional groups.

Table 2 BlastX Results of the 81 Sequences Retrieved from Atlantis II Interface Library

Number of Sequences	Best hits Accession Numbers / abundance	Hits Sources	Description
81	(ADD16762) /47	Jiaozhou Bay Sediments	Hydrazine oxidoreductase [uncultured anaerobic ammonium-oxidizing bacterium]
	(ADO21164) /14	Deep sea tephra deposits	Hydrazine oxidase [uncultured planctomycete]
	(ADO21144) /12	Deep sea tephra deposits	Hydrazine oxidase [uncultured planctomycete]
	(ADD16849) /5	Jiaozhou Bay Sediments	Hydrazine oxidoreductase [uncultured anaerobic ammonium-oxidizing bacterium]
	(BAF36964) /2	Planctomycete KSU-1	Hydrazine-oxidizing enzyme [planctomycete KSU-1]
	(ADO21372) /1	Upper cape fear river sediment	Hydrazine oxidase [uncultured planctomycete]

This Table discusses the number of Atlantis II deep interface library sequences aligned to entries in Genebank nr database using blastx similarity search tool with E value cut off score 10^{-5}. The best first matches' accession numbers, with their abundances among the examined sequences, were presented. The table also included the isolation sources where the hits have been recovered and its analysis description as retrieved from the Genebank database.

Table 3 BlastX Results of the 44 Sequences Retrieved from Kebrit Upper Interface Layer

Number of Sequences	Best hits Accession Numbers /abundance	Hits sources	Description
44	(ADO21151) /34	Deep Sea Tephra deposits	Hydrazine oxidase [uncultured planctomycete]
	(ADO21124) /4	Deep Sea Hydrothermal vent sediment	Hydrazine oxidase [uncultured planctomycete]
	(ADD16812) /4	Jiaozhou Bay Sediment	Hydrazine oxidoreductase [uncultured anaerobic ammonium-oxidizing bacterium]
	(ADO 2115)/ 1	Deep sea hydrothermal vent	Hydrazine oxidase [uncultured planctomycete]
	(ADD16823)/ 1	Jiaozhou bay sediment	Hydrazine oxidoreductase [uncultured anaerobic ammonium-oxidizing bacterium]

This Table discusses the number of Kebrit deep upper interface library sequences aligned to entries in Genebank nr database using blastx similarity search tool considering E value cut off score 10^{-5}. The best first matches accession numbers with their abundances among the examined sequences were presented. The table also included the isolation sources where the hits have been isolated and its analysis description as retrieved from the Genebank database.

Table 4 The Number of Sequences, Observed Hzo Based OTUs (0.03), and Alpha Diversity Indices of the Samples Under Study

Sample ID	Number of clones	Number of OTUs (0.03)	Chao1	Shannon	Simpson
Atlantis II -Interface	**81**	**8**	**8.333**	**1.33353**	**0.368827**
Kebrit Upper Interface	**44**	**9**	**12**	**1.719554**	**0.208245**

The table was created using mothur software and presents α diversity results of the examined samples at a cut-off score of 0.03. It shows the number of clones examined in addition to the number of OTUs detected using similarity cut off score 97%. Chao1 was applied to indicate the level of coverage of the phylotypes in each layer. Shannon and Simpson indices were calculated to reflect the richness and the diversity of the samples examined.

Table 5 The Ecosystems Involved in the Comparative Study and Number of Hzo Recovered from Each Environment

Environment Name	*Hzo* Sequences Count
Upper cape fear river sediment (UCFR)	101
Jiaozhou Bay Sediments (JBS)	403
Atlantis II brine deep interface layer (AT)	81
Deep Sea Hydrothermal vent sediment (DSHV)	20
Kebrit brine deep upper interface layer (Kebrit)	44
Deep sea tephra deposits (DSHV)	24
Total Count	671

The table was generated by Unifrac software, which represents all the ecosystems included in the study, its abbreviations as provided in the parentheses, and the total number of hzo sequences analyzed from each sample.

Table 6 The Differences Between the Anammox Communities Existing in the Ecosystems Under Study Using P-test

P-test	AT-int	DSHV	DSTD	JBS	KB	UCFR
AT-int	-	1	0	0	0.02	0
DSHV	1	-	1	1	1	0
DSTD	0	1	-	1	0	0.03
JBS	0	1	1	-	0	0
KB	0.02	1	0	0	-	0
UCFR	0	0	0.03	0	0	-
(< 0.001) Highly significant	(0.001-0.01) Significant	(0.01-0.05) Marginally significant	(0.05-0.1) Suggestive	(> 0.1) Not significant		

Table 7 The Differences Between the Anammox Communities Existing in the Ecosystems Under Study Using Unifrac Significance test

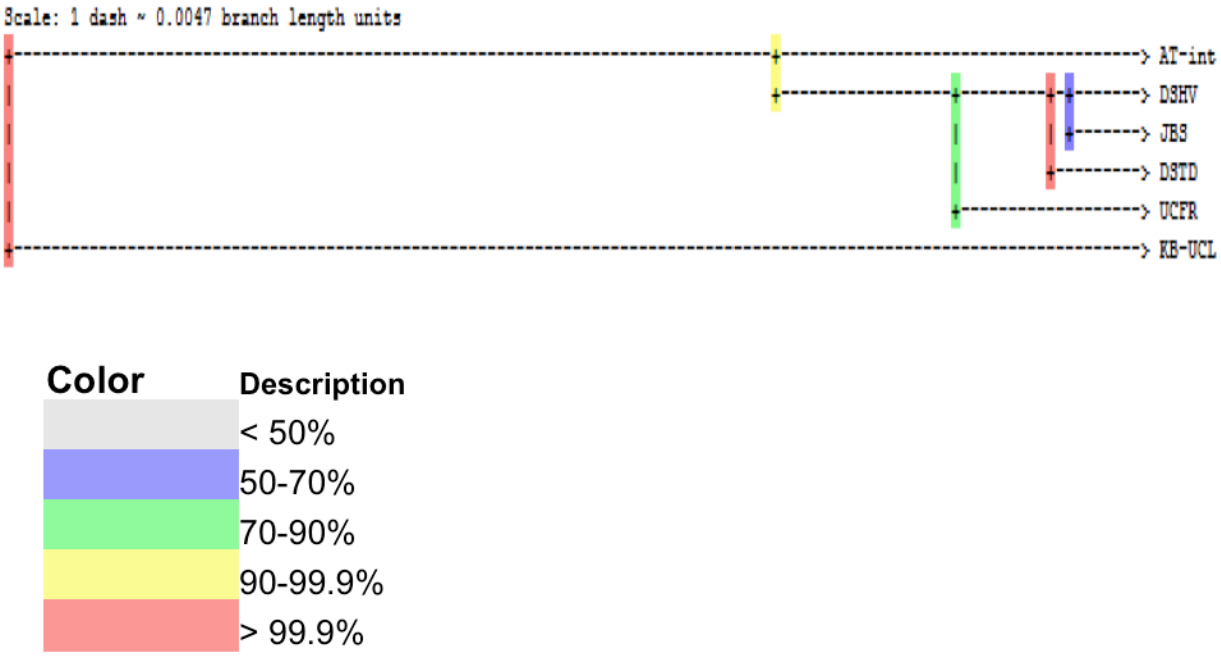
Unifrac Significance test	*AT-int*	*DSHV*	DSTD	JBS	KB	UCFR
AT-int	-	0.77	0.11	0	0.17	0
DSHV	0.77	-	0.65	0.39	0.08	0.34
DSTD	0.11	0.65	-	0.42	0	0.18
JBS	0	0.39	0.42	-	0	0
KB	0.17	0.08	0	0	-	0
UCFR	0	0.34	0.18	0	0	-
< 0.001) Highly significant	(0.001-0.01) Significant	(0.01-0.05) Marginally significant	(0.05-0.1) Suggestive	(> 0.1) Not significant		

Table 8 Lineage Specific Test

Node	P Value	Environment Name	Observed	Expected
node2	0.010624657	AT-int	1	0.706408346
node2	0.010624657	DSHV	0	0.178837556
node2	0.010624657	DSTD	0	0.214605067
node2	0.010624657	JBS	0	3.603576751
node2	0.010624657	KB	5	0.393442623
node2	0.010624657	UCFR	0	0.903129657
node5	0.010624657	AT-int	78	78.29359165
node5	0.010624657	DSHV	20	19.82116244
node5	0.010624657	DSTD	24	23.78539493
node5	0.010624657	JBS	403	399.3964232
node5	0.010624657	KB	39	43.60655738
node5	0.010624657	UCFR	101	100.0968703

The table demonstrates the most unique *hzo* sequences that contribute in the differentiation between Anammox communities analyzed. The output locates the sequences' nodes on the Unifrac generated tree, the P-value of the test, the corresponding environments where the sequences retrieved, the number of sequences observed and the expected number if the samples are uniform in its size.

APPENDIX



Supplementary Figure 1 Unifrac Cluster Model of the Two Red Sea Brine Pools and Other Similar Ecosystems.

The Unifrac cluster model depicts the relations between the Anammox bacterial communities dwelling the brine pool interface layers under study and other ecosystems. The obtained results are supported by Jacknife confidence value

Supplementary table 1 The Number of Atlantis II Interface and Kebrit Upper Interface Layers Related Hits and its Corresponding Percentages, which are Assigned to Different Bacterial Phyla as Identified using MG-RAST Server Cloud.

Kebrit Upper interface	hits: 518180	Atlantis II interface	hits: 113433
Proteobacteria	244408 (47.17%)	Proteobacteria	97508 (85.96%)
Bacteroidetes	43084 (8.31%)	Actinobacteria	4516 (3.98%)
Chlorobi	12560 (2.42%)	Chloroflexi	992 (0.87%)
Acidobacteria	4300 (0.83%)	Chlorobi	599 (0.53%)
Bacteria	5811 (1.12%)	Deinococcus-Thermus	400 (0.35%)
Firmicutes	110679 (21.36%)	Bacteroidetes	1390 (1.23%)
Planctomycetes	4743 (0.92%)	Firmicutes	5142 (4.53%)
Chloroflexi	9185 (1.77%)	Cyanobacteria	959 (0.85%)
Nitrospirae	2725 (0.53%)	Planctomycetes	295 (0.26%)
Verrucomicrobia	4805 (0.93%)	Thermotogae	259 (0.23%)
Cyanobacteria	13764 (2.66%)	Bacteria	214 (0.19%)
Spirochaetes	6643 (1.28%)	Nitrospirae	93 (0.08%)
Candidatus Poribacteria	716 (0.14%)	Acidobacteria	236 (0.21%)
Actinobacteria	30833 (5.95%)	Aquificae	302 (0.27%)
Deferribacteres	1174 (0.23%)	Lentisphaerae	65 (0.06%)
Aquificae	4285 (0.83%)	Dictyoglomi	57 (0.05%)
Lentisphaerae	1086 (0.21%)	Verrucomicrobia	168 (0.15%)
Chrysiogenetes	546 (0.11%)	Deferribacteres	27 (0.02%)
Dictyoglomi	1066 (0.21%)	Elusimicrobia	24 (0.02%)
Thermotogae	4080 (0.79%)	Chrysiogenetes	17 (0.01%)
Deinococcus-Thermus	3123 (0.60%)	Tenericutes	28 (0.02%)
Synergistetes	2224 (0.43%)	Synergistetes	25 (0.02%)
Fibrobacteres	377 (0.07%)	Chlamydiae	31 (0.03%)
Fusobacteria	3274 (0.63%)	Spirochaetes	59 (0.05%)
Chlamydiae	1449 (0.28%)	Fusobacteria	26 (0.02%)
Elusimicrobia	301 (0.06%)	Gemmatimonadetes	1 (0.00%)
Tenericutes	895 (0.17%)		
Gemmatimonadetes	44 (0.01%)		

This table describes the number of hits detected in both Atlantis II interface and Kebrit upper interface layers and its corresponding percentages as annotated using BlastX non-redundant database adopted by MG-RAST server cloud and maintaining at least 50% identity and minimum E-value cut off score 10^{-5}.

Supplementary table 2 The Distribution of Planctomycetes Related Hits Recovered from Atlantis II Interface and Kebrit Upper Interface Layers Among its Different Species.

species	Atlantis II Interface	Percentage2	Kebrit Upper interfa	Percentage3
Blastopirellula marina	95	26.46	1183	19.28
Gemmata sp. Wa1-1	0	0.00	2	0.03
Isosphaera pallida	32	8.91	562	9.16
Pirellula staleyi	149	41.50	964	15.71
Planctomyces brasiliensis	45	12.53	782	12.74
Planctomyces limnophilus	17	4.74	1317	21.46
Planctomyces maris	20	5.57	1232	20.07
Rhodopirellula baltica	0	0.00	2	0.03
Rhodopirellula sp. SWK21	0	0.00	1	0.02
Uncultured Planctomycetales bacterium HF0200_11L05	0	0.00	9	0.15
Uncultured Planctomycetales bacterium HF0500_02G17	0	0.00	10	0.16
Uncultured Planctomycetales bacterium HF0500_40D21	0	0.00		0.00
Uncultured Planctomycetales bacterium HF0770_03I01	0	0.00	51	0.83
Uncultured planctomycete 3FN	0	0.00	3	0.05
Uncultured planctomycete	1	0.28	14	0.23
Uncultured planctomycete 13FN	0	0.00	3	0.05
Uncultured planctomycete 6N14	0	0.00	1	0.02
Uncultured Planctomycetales bacterium HF0130_29M04	0	0.00	1	0.02
	359		6137	

This table demonstrates the number of hits and its corresponding percentages that are allocated for each *Planctomycetes* related species. These assignments were annotated using BlastX non-redundant database adopted by MG-RAST server cloud. Minimum identity cut off score 10^{-5} and identity cut off score 50% were applied.

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