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Carbon Fixation Pathways From the Marine Dark Biosphere

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14. ABSTRACT

The dark marine biosphere (DMB) is an environment that is permanently separated from light-driven energy production mechanisms. Survival in the DMB is challenging: no light for photocatalytic processes, extremes in temperature, oxygen limitation, scarce sources of energy, and high concentrations of carbon dioxide. Despite these extremes, ~70% of the Earth's microorganisms live in and are adapted to generate cellular energy in the sub-seafloor sediments [1], yet only ~5% of the sea floor has been explored [2]. The DMB also covers more than two-thirds of the Earth's area and is alternatively known as the most isolated CO_2 sink of the Earth [3]. This biosphere has the largest capacity to absorb and convert CO_2 based on not just its surface area alone but also passive and active pumping mechanisms from microbial activity in the sediment and surrounding water. Thus, the microbiome (all microorganisms, their genetic elements and their abiotic interactions) of the DMB must adapt to this changing concentration of CO_2. Therefore, this biosphere is the greatest untapped resource for the discovery of new dark carbon fixation pathways and carbon fixing microorganisms. In addition, how this microbiome fluctuates could provide evidence for how some of the most untouched areas on earth are responding to changing CO_2 levels. Nevertheless, in the last decade, three new carbon fixation enzyme systems have been discovered in the dark biosphere and given the vastness of the uncultured majority, the probability for discovery of more carbon fixation pathways is extremely high. Despite the demonstrated discovery potential of the dark biosphere, a vast majority of bioenergy studies remain focused on harnessing the process and byproducts (e.g. cellulosic biomass) of oxygenic photosynthetic carbon fixation. Photosynthesis is highly evolved and restricted by numerous limiting factors (nutrients, oxidizing conditions, light limitation/inhibition, 2D geometries, and defined enzyme systems). Given the variability and austerity of the dark biosphere, its microbiome and metabolic potential has evolved with more diversity and since sunlight is not required, carbon fixation can occur in a 3D space. This research effort proposed to harvest new genomic and mechanistic information from the dark biosphere, treating it as a new frontier for discovery of energy producing microorganisms from non-photosynthetic, cold, and carbon dioxide-rich environments. The successful outcome of this work will be the discovery, identification, and storage of novel chemolithoautotrophic environmental microbial consortia that will sequester and convert CO_2 and identify key members of the microbial population responsible for the conversion of CO_2 in this dark biosphere.

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EXECUTIVE SUMMARY

The dark marine biosphere (DMB) is an environment that is permanently separated from light-driven energy production mechanisms. Survival in the DMB is challenging: no light for photocatalytic processes, extremes in temperature, oxygen limitation, scarce sources of energy, and high concentrations of carbon dioxide. Despite these extremes, ~70% of the Earth's microorganisms live in and are adapted to generate cellular energy in the sub-seafloor sediments [1], yet only ~5% of the sea floor has been explored [2]. The DMB also covers more than two-thirds of the Earth's area and is alternatively known as the most isolated CO_2 sink of the Earth [3]. This biosphere has the largest capacity to absorb and convert CO_2 based on not just its surface area alone but also passive and active pumping mechanisms from microbial activity in the sediment and surrounding water. Thus, the microbiome (all microorganisms, their genetic elements and their abiotic interactions) of the DMB must adapt to this changing concentration of CO_2. Therefore, this biosphere is the greatest untapped resource for the discovery of new dark carbon fixation pathways and carbon fixing microorganisms. In addition, how this microbiome fluctuates could provide evidence for how some of the most untouched areas on earth are responding to changing CO_2 levels. Nevertheless, in the last decade, three new carbon fixation enzyme systems have been discovered in the dark biosphere and given the vastness of the uncultured majority, the probability for discovery of more carbon fixation pathways is extremely high. Despite the demonstrated discovery potential of the dark biosphere, a vast majority of bioenergy studies remain focused on harnessing the process and byproducts (e.g. cellulosic biomass) of oxygenic photosynthetic carbon fixation. Photosynthesis is highly evolved and restricted by numerous limiting factors (nutrients, oxidizing conditions, light limitation/inhibition, 2D geometries, and defined enzyme systems). Given the variability and austerity of the dark biosphere, its microbiome and metabolic potential has evolved with more diversity and since sunlight is not required, carbon fixation can occur in a 3D space. This research effort proposed to harvest new genomic and mechanistic information from the dark biosphere, treating it as a new frontier for discovery of energy producing microorganisms from non-photosynthetic, cold, and carbon dioxide-rich environments. The successful outcome of this work will be the discovery, identification, and storage of novel chemolithoautotrophic environmental microbial consortia that will sequester and convert CO_2 and identify key members of the microbial population responsible for the conversion of CO_2 in this dark biosphere.

This report presents research conducted by Dr. Lisa Fitzgerald (6115), Dr. Dhanya Haridas (6115), Dr. Thomas Boyd (6114), Dr. Justin Biffinger (former 6115), Dr. Preston Fulmer (former 6115), Dr. Leila Hamdan (former 6114), Dr. Bradley Ringeisen (former 6115), Dr. Yukari Maezato (former ASEE postdoctoral associate), and Dr. Christina Krute (former NRC postdoctoral associate).

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CARBON FIXATION PATHWAYS FROM THE MARINE DARK BIOSPHERE

1. INTRODUCTION

1.1 Objective

The Naval Research Laboratory (NRL) conducted this research to isolate, contour, identify, and categorize novel microbial consortia from the dark marine biosphere (DMB) capable of concentrating and fixing inorganic carbon in seawater between 4 - 25°C. A fundamental understanding of how a microbial population isolated from the DMB uses and adapts to inorganic carbon will lead to the identification of new organisms and pathways for the dark conversion of inorganic carbon. The isolation of consortia and identification of key members associated with the conversion of CO_2 will generate biotechnology systems with unprecedented rates for the fixation of CO_2 and would be a major advancement towards the applicability in "Clean Coal" and prevent technological surprises in this very competitive research space. This program also addresses many transformative areas of biotechnological research such as how biocomplexity coupled with synthetic biology could generate results greater than synthetic biology alone. Results from this fundamental program will lay the foundation for how novel non-photosynthetic microbial consortia are responsible for carbon sequestration and will lead to future programs to isolate the microbes responsible and understand the microbial metabolic pathways utilized for carbon sequestration. Once the microorganisms are identified, these potentially unique dark carbon fixation pathways can be incorporated into transformational synthetic biology applications.

1.2 Motivation

Prokaryotic non-photosynthetic carbon capture technologies are not currently available and the commercial biological carbon captures systems primarily use algae. However, the current negative life cycle analysis of algal systems (energy for compressing air, water reuse, light exposure, and maintenance of algal strains) makes them economically unviable [4]. The challenges for dark microbial systems are less with the major drawbacks including the decreased activity of most microbial species in high CO_2 atmospheres and the production of only two major potential by-products, methane or acetate, from the reduction of CO_2. Given the variability and austerity of the DMB in addition to the temperature of activity (4 -10°C), the microbiome and metabolic potential of the DMB has evolved in a 3D space suitable for scalable biotechnology advances. Unlike current photosynthetic biological systems (*i.e.,* algae) which are hobbled by unfavorable energy balances or water limitation, the DMB can offer highly efficient dark carbon fixation solutions. Additionally, biological systems that can operate in the dark are easier to maintain for longer periods of time and with current advances in synthetic biology, organisms that can fix CO_2 will only increase.

The Calvin-Benson Cycle is the most extensively studied pathway for the fixation of inorganic carbon by prokaryotes. However, several new alternative dark carbon fixation pathways have been discovered (three in the last decade) from prokaryotes in extreme environments like the DMB. Given the vastness of the uncultured majority, the probability for discovering additional carbon fixation pathways from our samples is extremely high which will lead to new targets for the synthetic biology field. Synthetic biology approaches have created solutions for novel biological agents, key enzymatic intermediates, and even fuel production. However, many of these genetic pathways are transposed from the target organisms to an easily mutable model; but this is the inherent limitation to all synthetic biology approaches. Our ability to predict and model pathways better than the organisms themselves is still in its infancy. Thus, the discovery of new unique microbes will lead to transformative biotechnological processes superior to or enhance current system biology approaches and models for carbon fixation.

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A strategy for isolating and culturing members of the dark energy microbiome is applicable to the Power and Energy Naval S&T focus area. These efforts opens a new field of study to a biosphere which is largely unknown. Further discovery and defining of the microbial genes, their functions and their regulation in dark and carbon dioxide rich environments will advance the study of non-photosynthetic carbon fixation, and open opportunities for synthetic biology to engineer high yield dark carbon dioxide fixing microorganisms. Thus, this strategy will yield strategic and monetary benefits for the Navy by characterizing microbial mechanisms to remediate carbon dioxide rich environments while providing a renewable source of energy.

2. APPROACH

This program sought to culture the unculturable microbes from Gulf of Mexico deep sea water and sediment samples and to understand their metabolic function within this dark environment.

2.1 Collection of Dark Marine Biosphere Samples

This study leveraged ship time from NRL platform support during FY14-17 to employ a molecular-, micro- and systems-biology approach for characterizing, expressing and cultivating microorganisms with dark energy capabilities from the Gulf of Mexico. Sediment and water samples were collected from the Northern Gulf of Mexico on board R/V *Pelican* and R/V *Point Sur*. To culture microbes from various shipwreck microbiomes and identify and characterize new microbes having novel applications, field activities were necessary to generate material and environmental samples. This was done in collaboration with a Bureau of Ocean Energy Management (BOEM) program to allow access to shipwreck sites. The BOEM program was funded to comparatively access the *Deepwater Horizon* spill's impact on shipwreck microbiomes and the synergistic effects on those communities and surrounding environment. Figure 1 shows the location of the shipwrecks and how they were impacted by the oil spill and the photos in Fig. 2 show the biological flora that abounds on the shipwrecks. The availability of new samples coupled with recent microbiological and molecular biology breakthroughs enabled our team to uncover new functionally distinctive microorganisms which to date have eluded conventional cultivation and characterization.

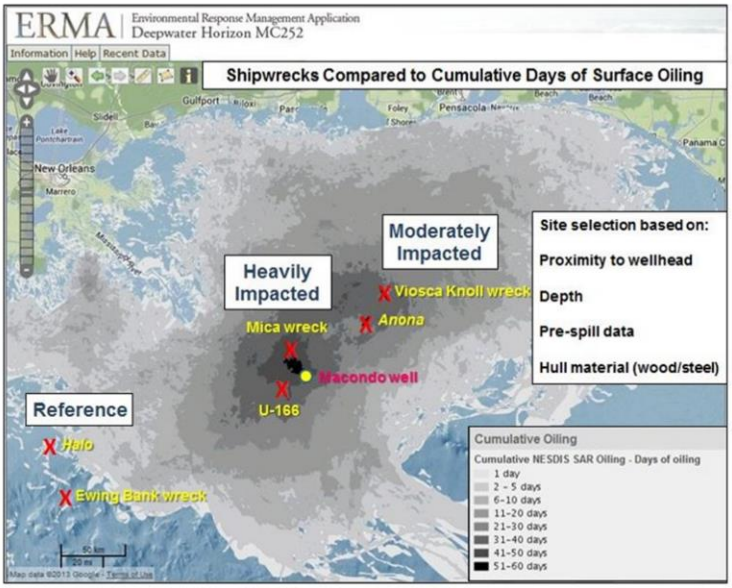

Fig. 1 — Location of sampled shipwrecks in relation to the *Deepwater Horizon* oil spill

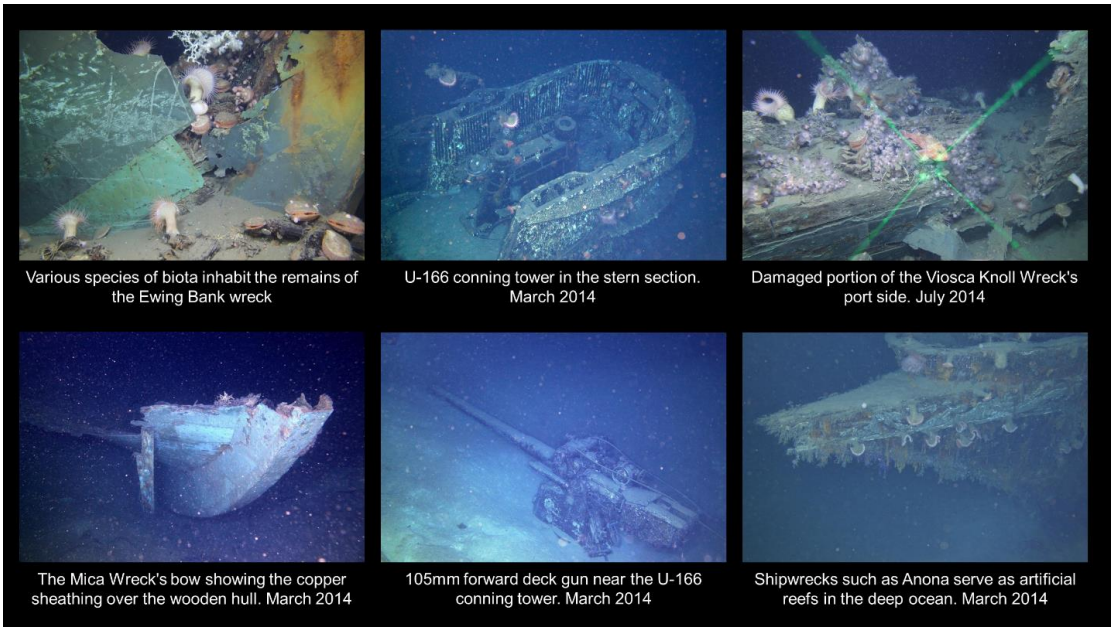

Fig. 2 — Photographs of shipwrecks to show the abundance of biological flora. All photographs by Deep Sea Systems International's Global Explorer ROV and courtesy of BOEM.

2.2 Microbiology and Molecular Biology

2.2.1 Culturing the Unculturable

Several different approaches for culturing were discussed at the beginning of this program and a strategy to compile selective media was determined. High throughput growth experiments were performed in selected media formulations using ammonium and carbonate as the core carbon and nitrogen source to a modified ONR7A medium. The modified medium had a salinity of 35 and was consistent with the salinity of saltwater. Since this medium had many of the common electron acceptors removed from it, the salinity of the base salt formulation was maintained with sodium and potassium chloride salts.

The compilation of electron acceptors & electron donors along with the vitamin solution (ATCC MD-VS, 1% (v/v)) were added to the media in an anaerobic chamber. Several replicate 96 well plates were created using the core salts solutions and the selected variables. The lids of the plates were coated in Triton X-100 using a published procedure to limit the amount of water that condensed on the lid of the plate [5]. Considering the anaerobic nature of these experiments and no viable vendor of anaerobic plates, we had to systematically address issues with the measurement of growth in the Biotek plate reader which was not housed in the anaerobic chamber. Growth was confirmed in these experiments using the change in optical density at 600 nm which is a common indicator of change in turbidity. However, using anaerobic containers (R685025, Thermo Fisher) and low gas diffusion electrical tapes, we were able to maintain anaerobic growth conditions for these experiments for 2 months at both 4°C and 25°C. In addition to growth in modified 96-well plates, samples were prepared in Nunc tubes under anaerobic conditions.

To assist in high-throughput media compilations, a liquid handling system, QIAgility, was used (Fig. 3). Each base medium included the modified ONR7A with the addition of the vitamin mixture. In order to tease out what electron acceptors were used from growth in the cocktail mixture, an array of media containing a combination of the electron acceptors was made.

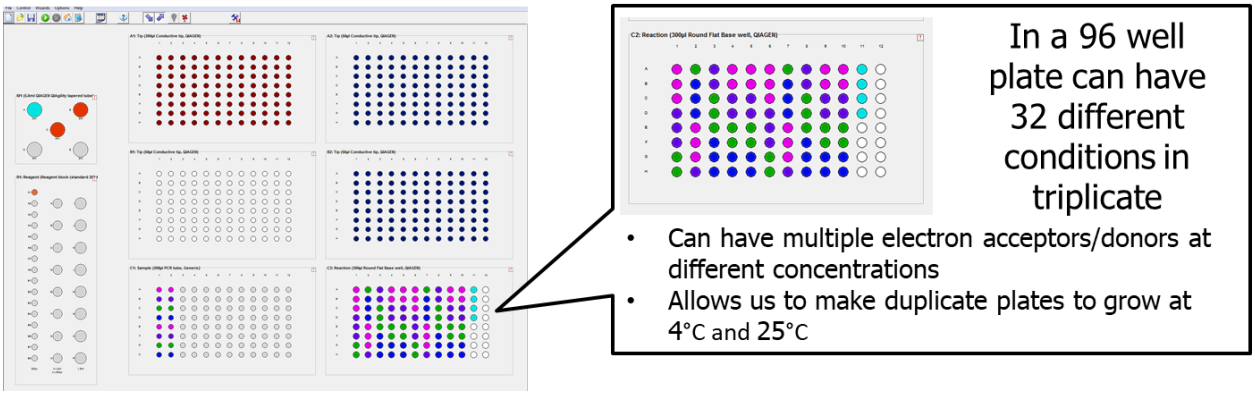

Fig. 3 — QIAgility platform used to assist in making select media

2.2.2 Biological Activity Reaction Tests (BART)

The majority of the microbes in the dark biosphere have been historically 'unculturable' with standard laboratory protocols. One way to address diverse culture conditions is to utilize Biological Activity Reaction Test (BART) assays. These assays are an easy way to culture microbes which might only grow under a particular condition. The 8 assays explored in this study target the growth of different classes of microbes; iron related bacteria, sulfate reducing bacteria, heterotrophic aerobic bacteria, fluorescent pseudomonas, slime forming bacteria, acid producing bacteria, denitrifying bacteria, and nitrifying bacteria. Therefore, these assays were essential to begin to understand the growth conditions of microbes present in water samples collected from shipwreck sites (with higher biodiversity) located in the marine dark biosphere.

2.2.3 Characterizing Denitrifying Bacterial Consortia

The biogeochemical process that transforms dissolved inorganic nitrogen to nitrogen gas is known as denitrification (DN). This metabolic pathway impacts the nitrogen (N) cycle by returning elemental N to the atmosphere [6, 7]. It can alternatively be defined as the reduction of more oxidized forms of nitrogen (NO_3^-, NO_2^-, NO and N_2O) to N_2 gas, where it can be linked to the oxidation of iron, sulfur and reduced carbon species [8]. It is primarily performed by facultative heterotrophic or chemolithoautotrophic bacteria under anoxic or very low-oxygen conditions [8], where microorganisms utilize nitrate or nitrite as the terminal electron acceptor [9]. DN, alongside other biogeochemical processes (carbon and sulfur cycles), plays a key role in maintaining the nutrient balance in marine habitats [10].

In recent years, shipwrecks have been identified as areas from which novel microbial species have been isolated, because of the introduction of foreign material to the area [11]. Thus, they would be an ideal location to discover unique microorganisms and metabolic activity, as these areas are known to be diverse habitats for macroorganisms in the marine environment [12]. The goal of this research was to prospect for novel DN microbial consortia near deep-sea shipwrecks in the Gulf of Mexico, culture the consortia under laboratory conditions and determine their DN activity. In this study, we collected water samples proximal to two steel shipwreck sites located in the northern part of the Gulf of Mexico, and analyzed the denitrifying and culturing potential of the microbial consortia obtained from the two sites.

2.2.4 Sequencing – 16S amplicon, metagenomics and whole genome

For pure isolate microbial identification, DNA was extracted, amplified with 16S rRNA primers and sent to Genewiz (NJ, USA) for sequencing. To determine the phylotypes present in a consortium, genomic DNA was isolated using DNA isolation solutions I, II and III (bioWORLD, Dublin, OH) and 16S amplicon sequencing of the V4 region and bioinformatics analysis was performed either by Seqmatic (Fremont, CA) or Genewiz. High throughput NGS was performed using the Illumina MiSeq platform using 2x250bp reads and the FASTQ data was processed using the Qiime pipeline. For whole genome sequencing, the purified DNA was sent to Seqmatic for sequencing and analysis.

2.3 Geochemistry

2.3.1 Porewater Analysis

Porewater from sediment samples from both the cruises of 2014 were analyzed by ion chromatography (IC) and ICP-OES to quantify major elements. Samples were then run on a Dionex DX-120 ion chromatorgraph equipped with an IonPac AS16 column using 9 mM $NaHCO_3$ buffer eluent with 1.2 mL flow rate. A calibration curve was created using serial dilutions of Dionex 7 anion combined standard (#057590). Limits of detection for this method are < 0.1 mM. For the ICP-OES analysis, porewater samples were diluted 1:10 with 2% (v/v) nitric acid. International Association for the Physical Sciences of Oceans (IAPSO) seawater standards were similarly diluted and used to create a standard curve for calibration. A Jobin-Yvon Ultima C ICP-OES equipped with a SeaSpray nebulizer and baffled concentric spray chamber was used for all analysis. Major elements (Ca, Mg, Sr, Na, and K) were quantified using diluted IAPSO standard. Trace elements (Fe, Si, Mn, Al) were quantified by adding these certified quantities of these elements to obtain a calibration curve. A standard curve was generated for each analyte. Standard errors for replicate measurements ranged from 0 to 21% with an average of ~5% for the aggregate runs (chloride, bromide, nitrate, sulfate). Detection limits for minor elements were ~ 0.5 parts per million (ppm) (Al) and > 0.05 ppm for Fe, Si, and Mn. Instrument drift was corrected by IAPSO standard runs with 1 ppm addition of trace elements. Drift was less than 2% for all elements. All analyses were performed in duplicate.

Porewater dissolved inorganic carbon (DIC) isotopes were measured using a Thermo Delta Plus XP isotope ratio mass spectrometer. A gas chromatography (Thermo Trace) with a 30 m Restek RQ-BOND capillary column (0.32 mm ID; 10 μm film) was used to resolve CO_2 peaks. Gas chromatography conditions were as follows: 40°C isothermal, 1:10 split, 4 mL min^{-1} flow). Pore water samples were acidified with 50% (v/v) H_3PO_4, shaken and the headspace sampled with a 10 μL syringe. Ten total injections were made during the run course alternating between sample injections and known CO_2 standard injections. Isotope values were corrected to the standard and averaged for each core slice. Porewater DIC ranged from -7.18 to 10.6 ‰ with a standard error of ± 0.47 ‰.

2.3.2 Sediment Analysis

Concentration and stable isotope ratios for sediments were determined using a Thermo Flash 2000 coupled through a Thermo Conflo IV interface to a Thermo Delta V Advantage isotope ratio mass spectrometer. TOC/TON samples were treated with 10% HCl and dried to drive off carbonates. All samples were dried at 60°C, homogenized and weighted out (13 mg) in triplicate into silver combustion capsules (TOC/TON) or tin combustion capsules (TC). A concentration calibration curve was created for each run using acetanilide standards. Isotopic calibration curves ($\delta^{13}C$ and $\delta^{15}N$) were created using IAEA C8, and USGS-40 standards as scale anchors. Isotopic values were converted to the per mil scale ($‰_{VPBD}$) using scale anchor standards. Analytical precision for carbon and nitrogen concentrations averaged 3% and 6%, respectively. Isotopic precision was 0.4 $‰_{VPBD}$ and 0.2 $‰_{VPBD}$ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

3. EXPERIMENTS

3.1 Characterization of New Microbes

Growth of new microbes was accomplished through the numerous media compilations created with the use of the QIAgility. Once a 96-well was determined to have microbial growth, the consortium in the well was streaked out on an agar plate to isolate pure colonies. The DNA was extracted from the pure colonies and the 16S rRNA was sequenced to determine if a new microbe was cultivated and isolated in the laboratory. Table 1 is sample from a set that was sent out for sequencing. While some of the 16S rRNA sequences were 99% identical to other microbes in the database based on a BLAST search, some microbes were quite unique. In fact one was only 83% identical.

Table 1 — Example of 16S rRNA sequence analysis of pure microbes

Designation	BLAST Result	Percent Identical
1-001	Photobacterium sp. SS12.11	99
1-002	Photobacterium profundum SS9	99
1-007	Photobacterium sp. strain E703-4	99
2-001	Needs to be repeated	
2-003	Photobacterium profundum SS9	99
3-003	Photobacterium sp. SS13.1	83
3-004	Uncultured gamma proteobacterium clone GC234-4-38	93
2-005	Photobacterium frigidiphilum strain SL13	97
2-006	Photobacterium sp. strain E703-13	99
2-007	Photobacterium frigidiphilum strain SL13	99

Four pure isolates were sent for whole genome sequencing (Table 2). Of the four, three are identified as *Phosobacterium* and one as *Psychrilyobacter.* Each genome has over 3000 genes identified with 33-47% of the genes annotated as hypothetical. These leads way to the potential identification of several new biochemical pathways.

Table 2 — Whole genome sequencing of four pure isolates

16sRNA	Genome Size (~ Mb)	Contigs	Genes	Hypothetical	Repeats	RNAs	CRISPR
97.03% to *Photobacterium profundum* Strain DSJ4	2.2	6556	5918	2192	315	199	124
98.88% to *Photobacterium profundum* Strain DSJ4	2.8	6492	5965	2120	326	201	0
99.02% to *Photobacterium frigidiphilum* strain SL13	3.5	6240	5716	1875	304	220	0
99.37% to *Psychrilyobacter atlanticus* strain HAW-EB21	3	3545	3194	1496	197	82	72

3.2 BART Assays

During the field activities aboard the R/V *Pelican*, samples were collected from the water column near 6 shipwreck sites and in sites related to the DWH spill of 2010; two control shipwreck sites (Ewing Bank and *Halo*), two heavily oil impacted sites (Mica and *U-166*) and two moderately oil impacted sites (*Anona* and Viosca Knoll). The water samples were collected at the middle and near the bottom of the water column. Nine samples collected were used in eight different BART assays (Table 3). These assays determined the presence/absence of culturable iron related bacteria (IRB), sulfate reducing bacteria (SRB), heterotrophic aerobic bacteria (HAB), fluorescent pseudomonas, slime forming bacteria, acid producing bacteria (APB), denitrifying (DN) bacteria and nitrifying bacteria. It is important to note that these tests will only identify viable microbes which are culturable under the conditions provided by each test. All tests were performed according to the BART protocol provided for each assay.

Table 3 — Water samples analyzed with BART assays

Site	Type of site in relation to oil spill	Bottom of Water Column Sample ID	Middle of Water Column Sample ID
Halo	Control	X	
Ewing Bank	Control	X	X
U-166	Heavily Impacted	X	
Mica	Heavily Impacted	X	
Viosca Knoll	Moderately Impacted	X	X
Anona	Moderately Impacted	X	X

After completing the various BART assays (IRB, SRB, HAB, FLOR, SLYM, APB, DN and N) on the water samples obtained from the 6 ship wreck sites located in the Gulf of Mexico various bacterial population groups were identified (Table 4). From these assays it was observed that the water samples did not contain viable fluorescent Pseudomonas, slime forming bacteria or nitrifying bacteria under the conditions outlined from the BART assay protocols. However all the water samples contained iron-related bacteria, heterotrophic aerobic bacteria and acid producing bacteria. Furthermore, all the water samples contained denitrifying bacteria except the water sample from the middle of the water column collected at *Anona*. All the ship wreck sites had one water sample that was positive for sulfate reducing bacteria except for Mica. However, it should be noted that Mica might have viable sulfate reducing bacteria in the middle water column as seen with the Viosca Knoll and *Anona* water samples. As expected, this study indicates that diverse bacterial populations exist at the various ship wreck sites and these previously uncultured bacterial populations isolated from the deep sea can be potentially cultured in laboratory conditions.

Table 4 — Summary of BART assay results

Site	Location	Sample	FLOR	IRB (cfu/ml)	SLYM	SRB	HAB (cfu/ml)	APB (cfu/ml)	DN	N
Halo	Bottom	W001	-	35,000	-	+	50,000	50,000	+	-
Ewing Bank	Bottom	W004	-	2300	-	+	50,000	<100	+	-
	Middle	W005	-	2300	-	-	50,000	<100	+	-
U 166	Bottom	W007	-	2300	-	+	500,000	10,000	+	-
Mica	Bottom	W010	-	9000	-	-	50,000	10,000	+	-
Viosca Knoll	Bottom	W013	-	35,000	-	-	500,000	10,000	+	-
	Middle	W014	-	9000	-	+	500,000	10,000	+	-
Anona	Bottom	W016	-	2300	-	-	50,000	<100	+	-
	Middle	W017	-	2300	-	+	50,000	1000	-	-

Not detected
Presence detected
Low
Moderate
High

FLOR Fluorescent Psuedomonas
IRB Iron Related Bacteria
SLYM Slime forming Bacteria
SRB Sulfate Reducing Bacteria
HAB Heterotrophic Aerobic Bacteria
APB Acid Producing Bacteria
DN Denitrifying Bacteria

3.3 Characterizing Denitrifying Bacterial Consortia

From the DN consortium that grew in the BART assays from U-166 and *Halo*, we investigated the ability of this consortium to grow in a laboratory setting and the ability to perform denitrification [13]. This study indicates that *Halo* and *U-166* were good prospecting sites for novel microbial consortia. Each shipwreck site has a distinct DN consortium which can be grown under laboratory settings. The *U-166* DN microbial consortium performs denitrification at a much faster rate than the *Halo* DN microbial consortium and most known industrial microbial consortia. The DN microbial consortium metabolism may be adapted for a more rapid nitrate turnover due to local hydrodynamic conditions or the proximity to the shipwreck, but additional studies are needed to identify the exact parameters. In conclusion, both DN consortia isolated from novel prospecting sites (shipwrecks) in the Gulf of Mexico can be cultured in the laboratory and can utilize a DN metabolic pathway for growth. The same analysis can be explored with other consortia that grew in the BART assays.

Ion chromatography (IC) studies were performed to identify the denitrifying potential of the isolated microbial consortia. The *Halo* microbial supernatants showed a steady decline in nitrate concentration (734 mg/L to 0.7 mg/L) as microbial growth entered into the logarithmic growth phase. As the nitrate concentration decreased, there was an increase in nitrite concentration from 1.4 mg/L, to a maximum of 130 mg/L and tapered down to 4.3 mg/L at 24 h. The *U-166* microbial consortium rapidly converted nitrate into nitrite, as shown with a decrease in nitrate concentration (730 mg/L to 2.5 mg/L) followed by an increase in nitrite concentration (0 to 240 mg/L), which was later followed by a subsequent decrease in nitrite levels to 2.2 mg/L (Fig. 4).

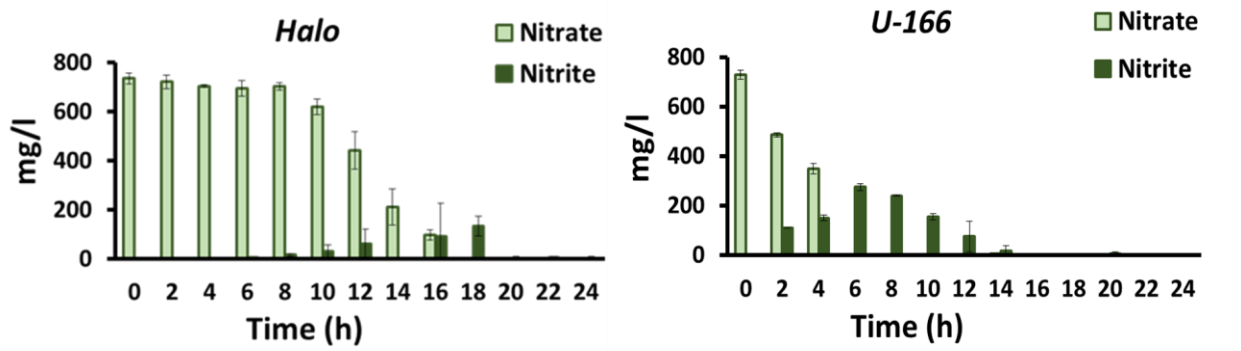

Fig. 4 — Ion chromatographic results of the DN microbial consortium isolated from Halo and *U-166* shipwreck sites

Since the growth curve and IC studies indicated that the DN consortia from *Halo* and *U-166* are mutually exclusive, we wanted to determine the microbial composition of both *Halo* and *U-166* DN consortia using 16S amplicon sequencing. The *Halo* DN consortium primarily consisted of the *Pseudomonas* genus (98.1%), while the *U-166* DN consortium was dominated by the *Citrobacter* genus (72.6%). At the species level, *P. tropicalis* and *P. aeruginosa* for *Halo*, and *C. werkmanii* and *C. freundii* for *U-166* were primarily detected (Fig. 5), thus indicating that both DN consortia are mutually exclusive.

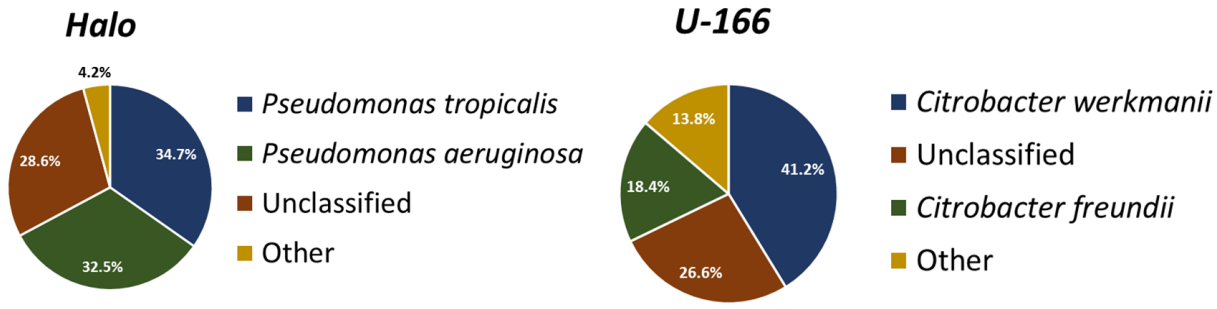

Fig. 5 — 16S amplicon sequencing on the *Halo* and *U*-166 DN consortium after 24 h culturing in indole nitrate medium

3.4 Sediment Organic and Inorganic Carbon and Stable Carbon Isotopes

During the study 166 sediment core slices were analyzed for total nitrogen (TN), total organic carbon (TOC) and total carbon (TC). Additionally, isotopic signatures were determined on core slices ($\delta^{13}C$ and $\delta^{15}N$). Cores were sectioned at sea every 10 cm and stored in whirl-pak bags frozen until processed in the lab. Sediment core material was dried at 40 °C, homogenized and weighed (~5-20 mg) into tin (TC) and silver (TOC) combustion boats. TOC samples were transferred to a desiccator and exposed to acid fumes (concentrated HCl) to liberate any inorganic carbonate material. Each core slice sample was run in triplicate on a Thermo Flash 2000 elemental analyzer coupled to a Thermo Conflo IV and DeltaV isotope ratio mass spectrometer (EA-IRMS). Briefly, a sample is dropped from the autosampler wheel into a combustion tube (Cr_2O_3, Silver Cobaltous oxide, elemental copper) held at 900 °C. Combusted material (CO_2 and N_2) is then separated in-line with a small GC column held at 50 °C. A thermal conductivity sensor (TCD) allows peak area determination after which, ‰ the separated CO_2 and N_2 transfer to the IRMS for isotopic measurement (Fig. 6).

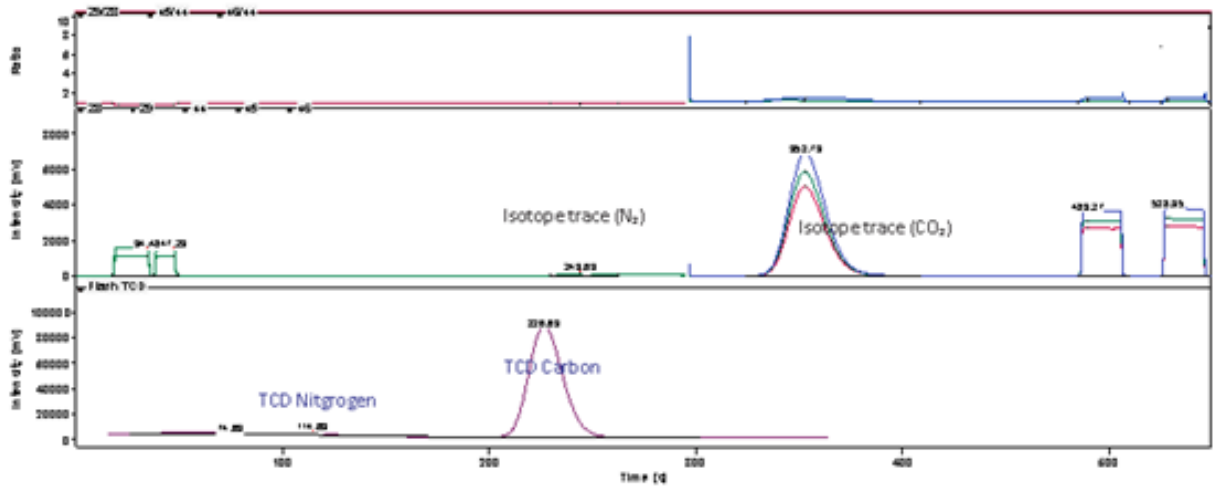

Fig. 6 — Example TC/TN trace from EA-IRMS

TOC, TIC and processed data are used to understand carbon cycling within a sediment horizon (using the core section values as proxy). Visualizing and calculating concentration differences or isotopic enrichments down-core allow screening for conditions conducive to carbon fixation. For example, anthropogenic N inputs (from terrestrial run-off) are generally enriched in ^{15}N relative to natural N sources derived from surface layers in the ocean [14-16]. Sediment reworking under anoxic conditions – creating denitrifying zones within the sediment horizon lead to ^{15}N-depleted sedimentary material moving down-core (Fig. 7). Sediment and water column $\delta^{15}N$ varies considerably by ocean region, but reworking within the environment (*e.g.* oxic to anoxic transition zones) are regions for increased fractionation (*c.f.* [17]).

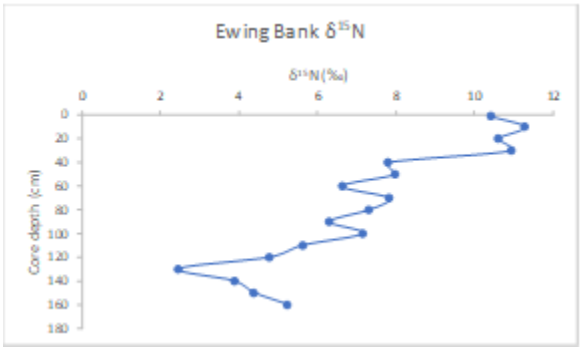

Fig. 7 — $\delta^{15}N$ in a sediment core from Ewing Bank

Shifts $\delta^{13}C$ in also occur in sediment horizons where biogeochemical processes drive fractionation. Organic matter utilization produces CO_2 relatively depleted in ^{13}C leaving residual organic material somewhat ^{13}C enriched. When autotrophic processes occur (CO_2 fixation), lighter CO_2 is enzymatically preferred which creates ^{13}C-depleted sedimentary organic matter. An equilibrium between respiration and fixation occurs below thus producing isotopically-heavier sediment organic matter. Methanotrophy may lead to ^{13}C-depleted organic matter deeper in cores due to highly depleted CH_4 formed deep in sediment horizons (*c.f.* [18]).

These analyses provide information on the source and relative sourcing of depositional material at each site. Additionally, isotopic analyses will assist in identifying substrate sources to microbial populations, carbon assimilation pathways, and the relative contribution of spill related materials to each study location.

4. CONCLUSIONS

Based upon the above data, we have identified methods to culture microbes from the dark marine biosphere. Analysis of the 16S rRNA shows that these microbes have eluted typical metagenomics analysis and a pure culture has not been isolated and deposited into the database. Furthermore, the whole genome sequencing identifies several hypothetical genes which could lead to the discovery of unique biochemical pathways. The ability to grow microbial consortia in defined growth media (*i.e.*, BART assays) has led to the ability to characterize novel consortia from the Gulf of Mexico. In this study we were able to investigate the DN consortia but more importantly we have identified a path forward for future studies of unique environmental samples.

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