doi:10.25923/hjf1-zj16

Mote Marine Technical Report Number 2606

December 29, 2022

Ocean Exploration and Research

FY18 FFO Projects

Final Report

I. Overview

1. Grant Number: NA18OAR0110291
2. Amount of funding from OER: $257,442.00
3. Project Title: Development of innovative techniques for exploring novel submarine springs on the Gulf of Mexico Outer Continental Shelf
4. Area of Operation

    Mote Marine Laboratory, Sarasota, FL (**Figure 1**)
    Amberjack Hole: 27.28748, -83.16139
    Green Banana Hole: 27.13695, -83.44023

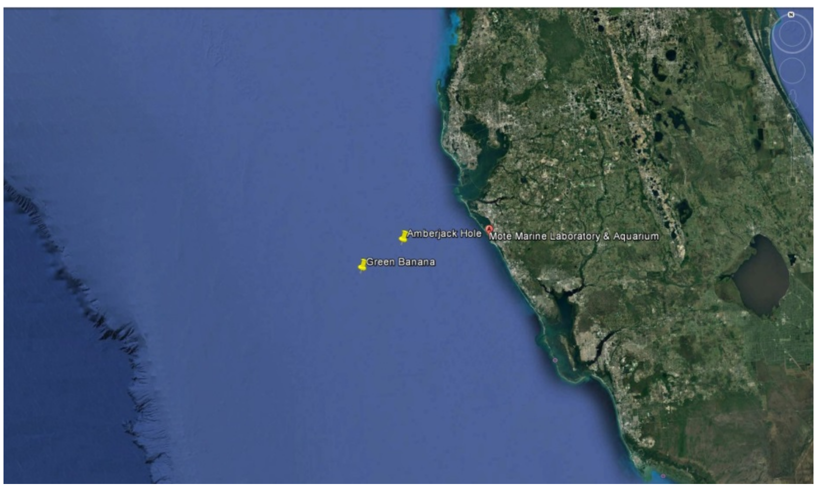

Figure 1. Areas of operation including Mote Marine Laboratory, Amberjack Hole and Green Banana Hole.

5. Principle Investigator

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6. Participating Institutions and personnel

Mote Marine Laboratory: Emily R. Hall, Ph.D., Jim Culter
Florida Atlantic University: Jordan Beckler, Ph.D.
Georgia Institute of Technology: Martial Taillefert, Ph.D.
Montana State University: Frank Stewart, Ph.D.
USGS: Christopher G. Smith, Ph.D.

7. Award Period: From 09/01/2018 to 08/31/2022

8. Period Covered by this Report: From 09/01/2018 to 08/31/2022 (Final Performance Report)

II. Summary:

1. *Abstract*. For this study, the project team assembled an innovative sensor and sampling suite to explore submarine sinkholes and springs (“blueholes”) on the Gulf of Mexico Outer Continental Shelf (OCS). Offshore submerged sinkhole and spring features have received limited scientific study as they frequently exceed normal scuba limits, reaching depths of >130 m, and exhibit openings too small for access with many submersibles. These blueholes host several commercially important and regulated fish species as well as protected species and can be considered ecological hotspots with respect to species composition and diversity. Because of groundwater discharge, organic matter deposition, and circulation regimes, parameters such as temperature, salinity, light, turbidity, circulation, DO, pH, redox, trace metals, carbonate chemistry, and sediment types are heterogeneous and satisfy various biological niches. Fortunately, due to the development of new technology, it is finally possible to overcome the technological limitations and explore the geological, physical, and chemical environments in these karst features, and the resulting resident biological distribution and diversity. The principal objectives were to 1) Repurpose and repackage existing high-tech marine biogeochemical instrumentation to create a benthic lander appropriate for efficient exploration of these difficult-to-access environments; 2) Develop an efficient multi-day logistical plan for safely deploying the platform in conjunction with geochemical, genomic, and macrofauna surveys; 3) Employ this plan in the exploration of two blueholes at depths >100m, and develop a long-term plan for the systematic exploration of other blueholes on the West Florida Shelf and beyond that will ultimately answer important hypothesis-driven questions; and 4) Disseminate exciting data and images through innovative means that will immediately captivate the minds of the public and garner future interest from scientists. Outreach and education tools included videos and photographs, a lesson plan dedicated to blueholes and the technology used to study them for education programs at Mote, and a temporary display in the aquarium that translates and transfers our NOAA Ocean Exploration (OER) research to enhance the level of ocean literacy of the public. There is a fundamental knowledge gap in the level of scientific understanding of these features because of the difficulties in their study. It is necessary to develop baseline measurements of the dynamics and biodiversity of these complex ecosystems, the ability to model and predict the effects of climate change on these systems, and to inform any habitat protection initiatives. Baseline characterizations of poorly known ocean areas, processes, and resources were made, innovative application of existing in situ and time-series data collection platforms enhanced undersea technical capabilities, partnerships were fostered between technology and technical diving experts, audiences were engaged through innovative means, and data will be shared in accordance and compliance with an open data policy and NEPA.

2. *Purpose of Project*. To satisfy several core mission objectives of NOAA and OER, the project team sought to combine an existing autonomous sensor and sampling suite with state-of-the-art geochemical, isotope, and genetic analyses to overcome logistical challenges that have long-hampered exploration of GoM OCS bluehole geology, hydrodynamics, geochemistry, and biology. A formalized plan for the systematic survey of these blueholes was developed to accelerate the pace, scope, and efficiency of the exploration of and discoveries relating to these poorly characterized features. Two blueholes were explored within the proposed scope of work. Overall, baseline

characterizations of poorly known ocean areas, processes, and resources were made (OER Objective 1), innovative application of existing in situ and time-series data collection platforms enhanced undersea technical capabilities (2), partnerships were fostered between technology and technical diving experts, and non-profit, university, and government laboratories (3), audiences were engaged through innovative means (4), and data will be shared in accordance and compliance with an open data policy and NEPA (5,6).

The principal objectives were to 1) Repurpose and repackage existing high-tech marine biogeochemical instrumentation to create a benthic lander appropriate for efficient exploration of these difficult-to-access environments; 2) Develop an efficient multi-day logistical plan for safely deploying the platform in conjunction with geochemical, genomic, and macrofauna surveys; 3) Employ this plan in the exploration of two blueholes at depths >100m, and develop a long-term plan for the systematic exploration of other blueholes on the West Florida Shelf and beyond that will ultimately answer important hypothesis-driven questions relevant to NOAA's mission; 4) Disseminate exciting data and images through innovative means that will immediately captivate the minds of the public and garner future interest from scientists

3. Approach.

a. *Work Performed:* There were four major objectives for this study. The first objective was to upgrade the autonomous benthic lander that was recently developed by the Georgia Institute of Technology. The new lander for this study was miniaturized (3' x 8' base, 3' tall) and ballasted to be only slightly negative with syntactic foam for better maneuverability by diver in and around the blueholes. The foam allows for a depth range as deep as 300 meters. Lander feet were modified to consist of perforated aluminum plates to prevent sinking into the soft, silty sediment. The benthic flux chamber was also redesigned by Florida Atlantic University (FAU) engineers for an improved seal, via installation of a larger hydraulic cylinder and a fulcrum that pushes down on the center of the lid instead of on one side rim. External testing revealed the chamber did not leak air or water for at least 24 hours. The initial plan was for the lander to be deployed to the base of each blue hole for one week instead of < 36 hours; however, given uncertainties of proper functioning during placement in a challenging deep, low visibility, constricted environment, we decided during operational planning that replicate, shorter deployments would allow for more redundancy (i.e. a sealed benthic flux chamber). Depending on the comfort level of the various dive teams, the lander was either free-guided downwards onto the blue hole sediment base or lowered from a cross-line positioned across the hole opening. Once lowered to the hole opening, the lander was carefully positioned by divers so that the benthic flux chamber and micro-profiler were vertically aligned with respect to the sediment/water interface. Four electrodes were juxtaposed on the microprofiler manifold (i.e. at the corners of a 4 x 4 cm square) and operated sequentially with a multiplexer at each incremented depth across the sediment-water interface and downwards into the sediments, thus providing redundant concentration profile measurements to constrain vertical geochemical redox analyte variability.

Voltammetric Hg/Au electrodes were also positioned within a benthic flux chamber that incubated a small parcel of water in contact with sediments for a period of ~24 hrs, with measurements collected at a frequency of ~10 minutes to quantify redox chemical species (i.e. $O_{2(aq)}$, Mn^{2+}, Fe^{2+}, and ΣH_2S). Overlying waters were collected both within the benthic chamber as well as outside (ambient/reference) as a function of time. An in situ benthic lander microcontroller drove two lead-screw systems that triggered 50 mL syringes to collect water samples via spring-loaded mechanisms. The lead-screw was also configured to trigger a mechanical switch to activate a hydraulics systems that closed the benthic chamber lid after an initial settling period immediately after deployment. The chamber contents were gently mixed with a pump. When fully functioning, nine syringe samples were capable of being collected from the chamber during the deployment. Additionally, another nine syringes were configured to sample the ambient waters as an external control, and two electrodes were also positioned external to the chamber on the lander frame. These syringe samples allow determination of the benthic fluxed of species not quantified electrochemically (i.e., DIC, ΣPO_4^{3-}, NH_4^+, SO_4^{2-}, NO_3^-) relative to ambient overlying waters, while also ensure the benthic chamber sealed properly via the injection of an iodide/bromide non-reactive tracer. The lander was also outfitted with a CTD/fluorometer/DO multiprobe sensor as part of an integrated package to monitor bluehole bottom water conditions over time, as well as several other in situ instruments that were tested (i.e. a HydroCycle-P for in situ phosphate measurements outside of the chamber, an Analytical Instrument Systems ISEA-X for redundant electrochemical measurements outside the chamber, a hydrophone, and an oxygen optode for measurements within the chamber).

The second objective was to create a bluehole survey sampling logistical plan. Each exploration campaign required meticulous planning to efficiently deploy the benthic lander, install other sensors, survey macrofauna, and collect samples for genomics and geochemistry. The preliminary logistical plan is considered a specific output of this work that was implemented and refined during this project through the seasonal exploration of two GoM OCS blueholes. The work was performed using Mote's research vessels: a 37' Yellowfin Yacht (R/V William R. Mote; WRM), used for rapid transit to the study sites, support diving operations, and the deployment of small equipment (i.e. CTD/rosette), while the 46' Newton (R/V Eugenie Clark; EC) was used for deployment of the larger benthic lander and collection/processing of water samples. For a single bluehole, each survey was performed in no less than four site visits, with between two and four divers required each day. Technical divers affixed to themselves a CTD data logger to allow passive data collection while in the blueholes. A CTD/rosette was deployed at several locations each day to survey the water column in and around the holes and collect water samples for geochemical and microbiological analyses described below. For more confined blue hole regions, niskin samples were collected by diver. Analyses of water samples included dissolved nitrate-nitrite, ammonia, phosphate, and silica; chlorophyll *a*; dissolved inorganic carbon; total seawater alkalinity; spectrophotometric pH; particulate nitrogen, phosphorus, and carbon; total nitrogen, phosphorus, and carbon. Filtered samples were also voltammetrically screened for redox analyte chemistries, including sulfide, elemental sulfur, sulfide clusters, and thiosulfate (HS^-, S^0, S_x^{2-}, $S_2O_3^{2-}$), dissolved Fe^{2+} and Fe(III) organic complexes, dissolved Mn^{2+}, and aqueous FeS colloids. Samples were then remeasured after acidification and N_2 sparging to dissolve and make labile larger compounds (e.g. colloidal FeS), as well as to remove and correct for the analytical overlap of the confounded S^0 and S_x^{2-} signals. Iron (II) and total acid-labile and reducible Fe (via hydroxylamine-HCl treatment) were finally colorimetrically determined.

The third objective was to perform systematic exploration of known GoM OCS blueholes. The benthic lander upgrades and testing, and the development of the 4-day logistical plan took place between Fall 2018 and Fall 2022. To explore the prospect of aquifer connectivity, tidal effects, and seasonal fluctuations, each hole was surveyed twice: once during the spring (estimated April/May) season, and once during the fall (estimated August/September) seasons. These two sites were selected because of the relative proximity to Mote, simplifying logistics, and because they represent two ends of the spectrum: Amberjack Hole (AJ) is a typical sinkhole configuration and Green Banana (GB) is a suspected spring feature. Both holes have a circular opening. AJ widens ~5m below the rim and assumes an inverted funnel shape, and no internal horizontal tunnels have been observed. In contrast, the walls of GB are hourglass shaped reaching the narrowest point at ~91m, with a series of small ledge microenvironments throughout and reports of horizontal tunnels at the base.

The fourth objective included data dissemination and outreach. Data is in the process of being disseminated via the NOAA National Centers for Environmental Information (NCEI), the Sequence Read Archive, and as a USGS Data Release. Several publications are being prepared with one peer-reviewed article published (Patin et al., 2021) and one in review (Culter et al., in review). The bluehole exploration described conjures images such as “diving to the abyss” and deploying technology analogous to “Lunar” or “Martian” landers to obtain samples of life living in these odd and poorly understood environments. Several previously documented videos are available on Youtube by easily querying the bluehole name. A blog was created by PI Hall that documented preparation and exploration campaigns. There were multiple interviews with each PI. Captivating images were acquired by professional photographer/videographer during the dives, and have been made available to all participating institutions, media, and NOAA. Divers were equipped with Go-Pro cameras to record macrofauna surveys and general logistics. Multimedia was incorporated as features on Mote, Georgia Tech, and Harbor Branch/FAU web-sites, many social media outlets (Facebook, Twitter, quarterly Mote newsletters, and the weekly Mote podcast “Two Sea Fans”). This project was also featured as a PBS Our Changing Seas Episode (Season 12, Episode 1, https://www.pbs.org/video/floridas-blue-holes-oases-in-the-sea-zjxbhl/). Beyond the exciting visuals produced, discussions of these karst features are generally very surprising to the public because the GoM OCS of the West Florida Shelf is often perceived as relatively featureless. Especially interesting was the potential for land to sea connectivity and relationships to the drinking-water aquifer.

All PIs engaged in talks and discussions with the general public throughout this project (e.g. Mote’s “Coffee with a Scientist”). All PIs also hosted undergraduate interns (24 total) who were assigned to assist with analyses or data compilation associated with this proposed research. Art students at Ringling College of Art and Design were asked to create renditions of these blueholes as part of a project called “The Art of Marine Science”.

b. *Project Organization*: PI Hall (Mote) provided project oversight and management, conducted water quality and carbonate system analyses, and lead the last three scientific dive operations. PI Hall also provided expertise within the outreach section of this proposal by acting as the outreach liaison between the public and Ringling College of Art and Design. Co-PI Beckler (FAU/Harbor Branch) participated in research cruises, participated in the benthic lander development and deployment, and coordinated the geochemical analyses of sediment cores. Co-PI Culter (Mote) lead the initial scientific diving field operations, instrument deployment, and assisted with sample collection. Co-PI Taillefert (Georgia Tech) participated in research cruises, coordinated and provided instrumentation for the benthic lander deployments, prepared the equipment for each deployment, and analyzed the electrochemical data provided by each deployment. Co-PI Stewart (Montana) provided equipment, supplies, and personnel for DNA sample collection, sequenced microbial community DNA for taxonomic and metagenomic analysis, and conducted statistical analysis of the sequence data. Collaborator Smith (USGS) participated in research cruises, oversaw the collection and analysis of naturally-occurring radionuclides used to characterize water masses within, above, and adjacent to the blueholes. Multiple other staff (chemists, biologists, dive team, marine operations team) also supported this work.

c. *Data Organization:* Data was collected by each PI, stored at least as an Excel file, and collated in a shared Google Drive folder for each expedition. Initial steps have been made to store data with NCEI and all data will be uploaded within 6 months of the project end date. Data and metadata formats conform to NOAA OER Data Explorer, OER Digital Atlas repository, and NOAA NCEI specifications and include detailed descriptions of collection and analysis procedures. Field observation and analytical data have been stored in flat ASCII or Excel files, which can be read easily by different software packages. Datasets from every instrument/sensor are stored as archive-ready, open-source, non-proprietary formats. Datasets of scientific logging and first-hand scientific observations have been preserved and converted into archive-ready formats. All post-processed datasets, data products, and reports have been documented with metadata describing the processing steps and quality assurance methods. Sequence Read Archive (SRA) data have been assigned a single BioProject identifier with linked metadata. Radioanalytical data obtained on samples collected and analyzed by the USGS (Smith) are published as a USGS data release, which meets Federal open-data requirements. These data are accessible via the web and are freely available to public through the USGS Publication Warehouse and can be found here: https://doi.org/10.5066/P9CKZT3O. Data will be archived for a minimum of 10 years beyond the end of the project.

Observational data and analytical results are currently being made available with the reporting requirements through the NOAA OER Data Explorer, OER Digital Atlas repository, and NCEI data systems (and will be complete within 6 months from the end date). All sequence data has been made publicly accessible fully abides to the Minimum Information about a Genome Sequence and Metagenomic Sequence standards that have been recently established by the scientific community (MIGS and MIMS, respectively).

4. Findings.

a. *Accomplishments and Findings:* After initial lander development, four different research cruise weeks were planned and completed throughout the project timeline. Two cruise weeks took place at Amberjack Hole and two cruise weeks took place at Green Banana Sink. Prior to each week-long excursion, preliminary trips were made to each hole to place diver down lines, anchor lines, and thermographs within the holes (these preliminary trips were completed at no cost to the project using Mote vessels or volunteer vessels). The lander was transported, assembled, and tested multiple times at FAU/Harbor Branch and prior to each of the four surveys at the Mote New Pass dock to test ballasting and functionality and enable deployment to the base of each blue hole for one week. This included deployment by the R/V Eugenie Clark as well as maneuverability and final ballasting tests. There were 8 total lander deployments over the course of this project (**Table 1**).

Table 1. Summary of blue hole sediment/benthic biogeochemical activities. DNW = Did not work; DNU = Did not use, i.e. equipment was not available, NP = Needs processing.

Meta information				Sediment profiles					Benthic flux chamber time series			Lander ambient (i.e. bottom water conditions)	
Site	Dates	Sample/ Event	Time / Location / Notes Time corresponds to core extrusion, lander reaching seafloor (per sonde depth data), or lander syringe start time	Core pore water profiles	Core solid phase profile	Core pH profile	Core voltammetry profile	Lander in situ voltammetry profile	Syringe-collected wet chemistry	Syringe-collected discrete voltammetry	In situ voltammetry	In situ voltammetry	Sonde / Water quality
AJ	5/14/2019	Lander	12:27, Lander deployed top of debris pile but slid down. May not have sealed.	-	-	-	-	DNW	DNW	DNW	DNW	DNW	✓
AJ	5/14/2019	Core	12:40, near lander	✓	✓	✓	✓	-	-	-	-	-	-
AJ	5/16/2019	Lander	15:10, Added spikes to legs	-	-	-	-	DNW	✓	✓	DNW	DNW	DNW
AJ	9/16/2019	Lander	15:30, 93 m, Syringe discrete voltammetry data needs to be processed*	-	-	-	-	DNW	✓	✓*	DNW	DNW	✓
AJ	9/16/2019	Core	~15:35, Slightly upslope of lander	✓	✓	DNU	✓	-	-	-	-		-
AJ	9/18/2019	Lander	~15:45 Lander stopped working after several hours*	-	-	-	-	DNW	✓* NP	✓* NP	DNW	DNW	DNW
AJ	9/18/2019	Core	~10 m away from 9/16 core	✓	✓	DNU	✓	-	-	-	-	-	-
GB	8/31/2020	Lander	12:22 (lander emplaced), 116 m	-	-	-	-	DNW	DNW	DNW	DNW	-	✓
GB	8/31/2020	Core	12:30, 120 m, next to lander.	✓	✓	✓	✓	-	-	-	-		-
GB	9/3/2020	Lander	12:07 (lander emplaced), 116 m, No nitrate data for benthic chamber*	-	-	-	-	✓	✓*	✓	✓	✓	✓
GB	9/3/2020	Core	Collected just after lander deployment, near lander, Microbial mat evident	✓	✓	✓	✓	-	-	-	-		-
GB	5/4/2022	Lander	12:38 (first syringe collected), 120m	-	-	-	-	✓	✓	✓	✓	✓	DNU
GB	5/4/2022	Core	~15:25	✓	✓	✓	✓	-	-	-	-		-
GB	5/5/2022	Lander	~15:45, 50m, deployed outside of blue hole due to diver availability issues, electrodes could not penetrate substrate*	-	-	-	-	✓*	✓	✓	✓	✓	DNU

Cruise Week 1 (Amberjack Hole, May 12-16, 2019) Summary[1]. The first full week of sampling was completed in the Spring of 2019. All Co-PIs arrived at Mote by Sunday, May 12, 2019 (the lander was delivered to Mote the week before and set up on the R/V Eugenie Clark by Co-PI Beckler and team, prior to other Co-PI arrivals). A preliminary dive team meeting (including volunteer divers) was held on Sunday, May 12, 2019 to describe all dive tasks. Bad weather did not allow the team to depart on Monday, May 13 as planned, so the first field day was moved to Tuesday, May 14. Both research vessels were loaded and departed from the Mote Marine Laboratory (MML) New Pass dock. The R/V William R. Mote reached the site first, allowing a team of rim divers (max depth 35m) to finish line setup at the hole. The R/V Eugenie Clark then arrived and began anchoring maneuvers for positioning over the hole. Technical divers entered the water and prepared for the lander deployment. The rest of the field week went as follows: Day 1 – lander deployment, sediment samples collected (from 106m), thermograph and Onset HOBO conductivity loggers were placed on the debris pile. Day 2 – lander retrieved, water column samples and rim water samples were collected by divers. Day 3 – lab-based work on sediment core samples and water samples, lander maintenance and repositioning. Day 4 – lander deployment, rim biological surveys completed, and lines retrieved. Day 5 – lander retrieved, more water samples collected. The lander control system failed for the first deployment, but the problem was fixed for second deployment, for which all lander systems were successful. Technical dive volunteers assisted with all dive days as in-kind match.

During the May 2019 week-long excursion to Amberjack, 17 water samples were collected for geochemical analytes in the bluehole water column and in three directions from the rim of AJ Hole via divers. Water samples were also subject to laboratory redox analyses for discrimination of various sulfur forms, i.e. hydrogen sulfide, elemental sulfur, and thiosulfate. Two sediment cores were collected at maximum depth (106m). One core was subject to Hg/Au voltammetric microprofiling and afterwards was sectioned in coarse 5 cm increments, and frozen for archival. The other core was immediately sectioned in 0.5 – 5 cm depth increments (x20), pore waters extracted via centrifugation, filtered (200 nm), and subject to numerous analyses of both pore waters and the solid phase. A deep-water pump collected six water samples from the Amberjack Hole site and three from a nearby control site. These samples were processed and analyzed for radium-223, 224, and 226 (Vargas et al, 2022). Similarly, a rosette water sampler was deployed to collect water for radon-222 analyses at AJ Hole (6 depths) and the surface control site (2 depths) (Vargas et al., 2022). The rim of AJ Hole was video surveyed for biological diversity. Water samples were also collected at the following depths for microbial community surveys: surface (~1 m), 7.6 m, 15.2 m, 22.9 m, 30 m, 33 m, 46 m, 60 m, 85 m, and 106 m.

The sediment core(s) profiles indicated that anaerobic respiration was extremely intense, and a lack of dissolved O_2 in the pore waters reflected euxinic blue hole bottom waters. High sulfide and diffusive flux profiles suggested sediments are likely responsible for the sulfide accumulation in bottom waters. In fact, the sulfide/oxygen interface was located approximately 10 meters above the sediments instead of at the sediment-water interface. Sediments were predominantly carbonate in nature, albeit with high organic content, reflecting their source either from the presumed carbonate blue hole walls, or from autochthonous production. Indeed, dissolved nutrients suggest Redfield Ratio-like organic material, suggesting a marine source. Sediment nutrient fluxes are intense, suggesting that the blueholes are supporting a diverse ecosystem at least partially because of their role of being hotspots of organic carbon remineralization.

[1] Summary figures for cruise week 1 are presented in previous reports.

Other significant findings included elevated total ^{223}Ra and ^{222}Rn activities inside the hole relative to the overlying water column and the offsite control (Vargss et al., 2022). The highest activities were found not at lowest sampling point, 85 m total water depth (~55 m inside the hole), but rather at 60 m total water depth (~30 m inside the hole) (Vargas et al., 2022). Chlorophyll *a* was elevated at the rim from approximately 20m down to 40m. Dissolved nitrate-nitrite (DNO_{23}-N) was elevated at 46 and 50m. Dissolved ammonia (DNH_4-N) and dissolved phosphate (DPO_4-P) were also elevated at these depths. DNH_4-N, DPO_4-P, and dissolved silica ($DSiO_3$) were most elevated at max depths (106m). Particulate carbon and nitrogen were most elevated just above the rim at 22m. Particulate phosphorus was most elevated at 46m. Water column profiles display significant redox- and nutricline zones above the hole and within the hole as seen by dissolved oxygen, nutrient, temperature, and other redox data.

The microbial communities of the water column were strongly partitioned by depth. Communities from the deepest water layer (at 86 m and 106 m) were dominated by the Woesearchaeota, which composed approximately 40% of the community. This group is rare and poorly studied, with no cultured isolates and other environmental surveys reporting only 1-2% composition of marine water and sediment communities. This level of representation is therefore unprecedented. Woesearchaeota were also observed in the sediment at maximum levels of ~8%. In the mid-water layer, the ammonia-oxidizing archaeal genus *Nitrosopumilus* composed up to 40% of the communities (at 46 and 60 m), which is similar to other low-oxygen marine systems. Other observed taxa include those potentially suggestive of roles in dissimilatory sulfur cycling, nitrification, and anaeorobic biogeochemical processes (Patin et al, 2020).

Cruise Week 2 (Amberjack Hole, September 15-20, 2019) Summary[2]. A modified lander logistical deployment plan was developed for the Fall 2019 cruise at Amberjack Hole based on the Spring 2019 trip. Physical modifications included the addition of a 12” bolt extending below each lander foot to ensure the lander does not shift down-slope during deployment. In addition, the lander was deployed by divers positioned at the crossline extending across the mouth of the blue hole. This enabled divers to lower the lander directly into place via a downline, with great care to ensure a gentle landing so that the benthic flux chamber and micro-profiler would be aligned perfectly with respect to the sediment-water interface. The lander was delivered to Mote on September 12 to allow for final logistical preparation. The R/V William R. Mote, R/V Eugenie Clark, and the privately-owned vessel, Goldens Rule were used for lander deployment, water and sediment sampling, and biological surveys. Prior to the fall expedition, two on site visits to Amberjack Hole were completed to re-install anchor points on both sides of the bluehole rim as well as submerged buoys.

During the September 2019 week-long excursion to Amberjack, all Co-PIs arrived at Mote by Sunday, September 15, 2019 (the lander was delivered to Mote the week before and set up on the R/V Eugenie Clark by Co-PI Beckler and team, prior to other Co-PI arrivals. A preliminary dive team meeting (including volunteer divers) was held on Sunday, September 15, 2019 to describe all dive tasks. The first field day was Monday, September 16, 2019. Both research vessels (R/V William R. Mote and R/V Eugenie Clark) were loaded and departed from the MML New Pass dock. The vessel, Goldens Rule was loaded with the technical diver team and departed at the same time. This was the only day Goldens Rule was used due to a failed engine. The R/V William R. Mote reached the site first, allowing a team of rim divers (max depth 35m) to finish line setup at the hole. Then the R/V Eugenie Clark arrived and began anchoring maneuvers for positioning over the hole. Technical divers entered the water and prepared for the

[2] Summary figures for cruise week 2 are presented in previous reports.

lander deployment. Unfortunately, the transmission on the R/V Eugenie Clark malfunctioned on the trip back to port and was deemed unusable for most of the week. The rest of the field week went as follows: Day 1 – lander deployment, sediment cores collected (from 106m), thermograph and Onset HOBO conductivity loggers were placed on the debris pile. Day 2 – lander retrieved (with the R/V Eugenie Clark on a single engine), water column samples and rim water samples were collected by divers. Two sawfish were recorded at the bottom of AJ Hole that day and reported to the Florida Fish and Wildlife Research Institute (FWRI) and NOAA. Day 3 – sediment core obtained, water samples collected via handheld niskins, and lines retrieved. A team of endangered species recovery experts joined the expedition for recovery of one of the sawfish. Technical dive volunteers assisted with all dive days as in-kind match. Only one successful lander deployment was completed due to the loss of the transmission on the R/V Eugenie Clark.

The lander deployment was mostly successful, with all systems functional. The lander failed due to a software issue approximately 80% through the deployment, resulting in shortened depth profiles and time series data. Duplicate sediment cores were collected from the debris pile during the Fall 2019. One core was subject to Hg/Au voltammetric microprofiling and afterwards was sectioned in coarse 5 cm increments, and frozen. The other core was immediately sectioned in 0.5 – 5 cm depth increments (x20), pore waters extracted via centrifugation, filtered (200 nm), and subject to many analyses of both pore waters and the solid phase. Water samples were collected via hand-lowered niskin bottles at the surface, 10m, 20m, 30m, 40m, 50m, 60m, 70m, 80m, 85m, 90m, and 96m and were immediately filtered or preserved for analyses. Biological surveys were completed with the use of survey tape and GoPros. Radioanalytical (radium and radon) samples were collected similar to the May cruise.

Based on the findings from the first sampling trip, water was collected and filtered for microbial and molecular samples from directly above and within the hole at the surface (~1 m), 10 m, 20 m, 30 m, 40 m, 50 m, 60 m, 70 m, 80 m, 85 m, 90 m, and 95 m (1 L each). The sediment core sections were also preserved for sequencing. Sample preservation and processing was performed as in the May expedition. All samples sequenced successfully with the number of quality-filtered reads ranging from 10,000 to over 100,000 per sample. Taxonomic analyses and ecological statistics were performed using common bioinformatic protocols.

Based on the amplicon sequencing data, four samples were chosen for shotgun metagenomic sequencing: two each from May and September samplings, one 60 m and one from the deepest sampling (106 m from May, 95 m for September). Sequencing was performed on two MiSeq runs and generated over 13 Gbp of data, which were assembled and used to bin high-quality draft genomes of 16 distinct microbial taxa. Assembly and binning was performed using the most cutting-edge bioinformatic algorithms to maximize recovery of biologically relevant taxonomic and functional data. Functional profiling was performed on individual assemblies as well as on genome bins to link biogeochemical activity with taxonomy.

For the Fall 2019 Amberjack Hole "wet season" survey, overall redox and nutrient results were in line with those from Spring 2019 "dry season" survey, suggesting that Amberjack Hole is relatively stable with respect to redox and nutrient regimes; however these measurements only represent "snapshots" of the blue hole conditions. For example, in situ electrochemical profiling revealed sediments were again dominated by sulfate reduction, with $MnCO_3$ mineral dissolution likely below a certain depth based on the accumulation of Mn^{2+} below this depth. Sediments were again acidic (pH < 6), suggestive of carbonate dissolution. Simultaneously, lander syringe samples obtained from the benthic chamber increased in PO_4^{3-} concentrations over the ~18 hours of deployment relative to control samples obtained outside of the chamber, suggesting that sediments are at least partially responsible for the elevated phosphate within the bottom water.

Sediment core profiles confirmed results from May 2019 that demonstrate anaerobic respiration was again extremely intense, and a lack of dissolved O_2 in the pore waters reflecting the euxinic blue hole bottom waters. While both dissolved sulfide and Mn^{2+} were present in this core as well as in the in situ voltammetric profile, the results from the ex situ core could possibly be artifactually more compressed due to the core separation techniques, as evidenced by Mn^{2+} increasing in concentration immediately at the surface.

Compared to sediment core results from May 2019, the September core also showed intense DIC production with depth, although concentrations are more elevated. Interestingly a maxima in PO_4^{3-} concentrations at the surface suggests that there may be a pulsed summertime delivery of organic matter that is locally remineralized, in turn increasing the flux of PO_4^{3-} back to the bottom waters.

With respect to water column, chlorophyll *a* was elevated at the rim from approximately 20m down to 40m. Dissolved nitrate-nitrite (DNO_{23}-N) was elevated from 50m to 85m. Dissolved ammonia (DNH_4-N) and dissolved phosphate (DPO_4-P) were most elevated at the bottom just above the debris pile (80m and deeper). Water column profiles show significant zones above the hole and within the hole as seen by nutrients, dissolved oxygen and temperature data. Samples were also collected to determine if any *Karenia brevis* was present near or around AJ hole, since this is an area where *K. brevis* blooms are often present this time of year. No *K. brevis* was present, but *K. asterichroma* was present near the rim of the hole and just above the rim.

Water column redox chemical measurements illustrated several vertically-distributed dissolved oxygen fluctuations ultimately terminating in a sulfidic bottom water column enriched in in iron and nutrients. The detection of thiosulfate ($S_2O_3^{2-}$) and elemental sulfur at the sulfide/O_2 interface could provide an ideal niche for chemolithotrophic microbes. Overall, Amberjack Hole redox chemical data provides additional support to the emerging theme that blueholes are supporting a diverse ecosystem with intense stratification caused by of their role of being hotspots of organic carbon remineralization.

As was seen in May, the microbial communities of the blue hole water column were strongly partitioned by depth. Communities from the deepest water layer were consistently dominated by the Woesearchaeota, which composed up to 60% of the community. This group is rare and poorly studied, with no cultured isolates and other environmental surveys reporting only 1-2% composition of marine water and sediment communities. This level of representation is therefore unprecedented. Woesearchaeota were also observed in the sediment at maximum levels of ~8%. In the mid-water layer, the ammonia-oxidizing archaeal genus *Nitrosopumilus* composed up to 40% of the communities, which is similar to other low-oxygen marine systems. Other observed taxa include those potentially suggestive of roles in dissimilatory sulfur cycling, nitrification, and anaerobic biogeochemical processes.

Metagenomes produced sixteen high-quality draft genome bins (seven from the mid-water layer at 60 m and nine from the deepest water layer, at 95 and 106 m). Taxonomic analysis showed these genomes represent the following taxa: Woesearchaeota (2), Bacteroidia (2), Thioglobaceae, *Nitrosopumilus* sp. (2), Patescibacteria, Proteobacteria (3), Microtrichales, Marinisomatota, Thermoplasmata, and Verrucomicrobia. Most of these are poorly characterized taxa suggesting blue holes are a rich source of novel biodiversity. The two Woesearchaeal genomes in particular are a valuable addition to the sparse body of knowledge on this cryptic group of microbes.

Moreover, two nearly-identical Woesearchaeal bins were recovered from the deepest water layer from the May and September metagenomes, suggesting there are persistent and stable Woesearchaeal populations at the bottom of the blue hole.

While results from May and September 2019 display remarkably similar biogeochemistries, and the confining nature of a submarine sinkhole would seem to suggest a static water column since the currents of overlying water are mild and do not penetrate into the sink, long term hydrodynamic monitoring demonstrates a more dynamic system not captured by discrete surveys. Temperature for depths of 47, 49, and 53 meters was quite dynamic from November 2018 to September 2019, illustrating a distinct seasonal trend together with much shorter-term fluctuations. The temperatures for the 78- and 104-meter depths were much more stable but still illustrated seasonal changes. An anomalous event occurred at a depth of 78 meters from January 21 – 25, 2019 with a sudden spike of 2.43˚C (16.67 to 19.10˚C) after which the temperature dropped back down to 17.62˚C (February 2) and stabilized at ~18.81˚C with a slow seasonal increase through time. Interestingly the temperature increase at 78 meters coincides with the steep temperature drop minima for the shallower depths. This indicates a possible winter overturn of the sink's water column which at least temporarily forces warmer surface water to the deeper layers of the sink. Temperature then becomes a primary forcing factor which coupled to groundwater flow may allow for significant groundwater exchange with overlying oceanic water.

Cruise Week 3 (Green Banana, August 31 - September 5, 2020) Summary[3]. For the third expedition (first trip to Green Banana), the lander was delivered to Mote mid-August 2020 to allow for final logistical preparation prior to the fall expedition. The R/V William R. Mote and the R/V Eugenie Clark were used for lander deployment, water and sediment sampling, and biological surveys.

During the Fall 2020 week-long excursion to Green Banana all Co-PIs arrived at Mote by Sunday, August 30, 2020 (the lander was delivered to Mote the week before and set up on the R/V Eugenie Clark by Co-PI Beckler and team, prior to other Co-PI arrivals). A preliminary dive team meeting (including volunteer divers) was held virtually with the Mote Dive Safety Officer (Greg Byrd) to describe all dive tasks, dive teams and safety rules. All dives during this mission were considered technical dives (depths>46m, trimix or closed circuit and decompression dives). The first field day was Monday, August 31, 2020. Both research vessels (R/V William R. Mote and R/V Eugenie Clark) were loaded and departed from the MML New Pass dock. The R/V William R. Mote reached the site first, allowing a team of rim technical divers (max depth 46m) to finish line setup at the hole, collect rim water samples via diver handheld niskins, and deliver samples to science team aboard the R/V Eugenie Clark. The R/V Eugenie Clark then began anchoring maneuvers for positioning over the hole. The deep team of technical divers entered the water and prepared for lander deployment. The rest of the field week went as follows: Day 1 (August 31, 2020)– lander deployment, rim water samples, sediment cores collected, and thermograph and Onset HOBO conductivity loggers placed on the debris pile. Day 2 (September 1, 2020) – water column samples collected via rosette and handheld niskins, biological surveys, and lander retrieved. Day 3 (September 2, 2020) – R/V William R. Mote only; due to the larger depth of GB relative to AJ Hole, radium was collected passively using dive bags containing manganese fibers (for radio-isotopes, Vargas et al., 2022). The bags were attached at specific depths along a mooring at Green Banana as well as offsite; and underwater filming was carried out. Day 4 – R/V Eugenie Clark only, lander deployment, sediment samples, moorings with radio-isotope bags retrieved, and some replicate deep-water samples collected. Day 5 – R/V Eugenie

[3] Summary figures for cruise week 3 are presented in previous reports.

Clark only, lander recovery, diver down line recovery. Technical dive volunteers assisted with all dive days as in-kind match. Due to a software issue, data collection for the first lander deployment was limited to the Sonde and hydrophones, while all systems were operational for the second deployment.

Duplicate sediment cores were collected from the debris pile on two separate days and processed as described previously. New for this trip, the microprofiled core was subsequently sectioned in anticipation of analyses of plastic compounds. Rim water samples were collected via divers with hand-held niskin bottles at the hole rim, 9.2m, and 18.3m away from hole in three directions. Water samples were also collected via rosette at surface, 10m, 20m, 30m, 40m, 50m, 60m, 70m, 80m, 90m, 100m, 110m, and 120m. All water samples were immediately filtered or preserved as with previous expeditions.

Samples for microscopy and analysis of microbial DNA/RNA were collected along a vertical profile through the Green Banana water column. Microbial biomass was filtered from water samples (n=48 total; 1 L each) taken from the surface (~1 m), above the hole (10 m, 20 m, 30 m, 40 m, 50 m), and within the hole (60 m, 70 m, 80 m, 90 m, 100 m, 110m, and 120m). Sections of sediment cores (6 sections of one 6-cm core) were also preserved for microbiome analysis. Sample preservation and processing was performed as in the 2019 expeditions. Biological surveys were completed with the use of survey tape and GoPros.

In contrast to Amberjack Hole, the lander data from Green Banana revealed in detail an apparently intense diel cycle over two, separate ~24 hr periods. Suddenly within minutes on these two separate nights (~8:30 pm), the base of the blue hole water column immediately displays relatively low salinity, high sulfide, and high FDOM/chl, persisting until the following morning. High frequency fluctuations suggested a transient phenomenon consistent with some type of pore water or aquifer-driven flow, but only during a discrete nighttime period. Electrochemical data showcased the multi-analyte sensitivity by revealing hydrogen sulfide and oxidized sulfide intermediates and an interplay with dissolved oxygen. Nutrients accumulated in the benthic chamber at higher rates during this evening flow.

During the day time discrete sampling, nutrient and carbonate chemistry results showed elevated dissolved $NO_{2,3}^{-}$N with depth starting around 60m depth (just under the rim). Dissolved SiO_2^{3-} was also elevated with depth. Chlorophyll concentrations were low, however a few different species of *Karenia* spp. were present above the hole and throughout the hole (*K. brevis, K. asterichroma*). Dissolved hydrogen sulfide was never detected in Green Banana during the day. The water column was hypoxic, but during the day did not depress below ~13%. Thiosulfate was detected at intermediate depths, providing evidence of active oxygenation of the water column. We suspect that this thiosulfate detected during the daytime is residual from the night time inputs.

<u>Cruise Week 4 (Green Banana, May 2 – 6, 2022)</u>. The fourth expedition cruise was completed Spring 2022. The benthic lander was deployed twice once on 5/4/22 and once on 5/5/22, although the second deployment was conducted outside of the hole adjacent to the opening due to diver unavailability. For the first time, the lander was “flipped” in the field to allow more efficient redeploy not requiring a return to Mote for reconfiguration. While the primary lander mechanisms functioned in their entirety during both deployments, Co-PI Beckler/Taillefert did not attach the same water quality sonde (YSI EXO2) to the lander for ambient water quality monitoring due to its recent theft during a different project. While PI Hall’s team did affix a sonde (Hydrolab) to the lander as a backup, the sonde flooded after several hours during the 5/4/22 deployment, so time series hydrodynamic monitoring results were not obtained for the full diel cycle. Preliminary from the time series lander deployment from 5/4/22 are presented in **Figure 2**. Even without the hydrodynamic data, we suspect that diel cycling/night time advective

inputs were not intense based on the stability of electrochemical dissolved oxygen measurements during the nighttime period, as well as the relative stability of the nutrient data. Not shown are discrete electrochemical speciation analyses conducted in the laboratory from the lander-collected syringe samples. Anecdotally, we did not detect any hydrogen sulfide in these samples (data is not yet fully processed), unlike in September 2020. **Figure 3** demonstrates an in situ electrochemical profile obtained by the lander microprofiling system. While sediments are mostly sulfidic, concentrations are significantly lower than during the September 2020 survey.

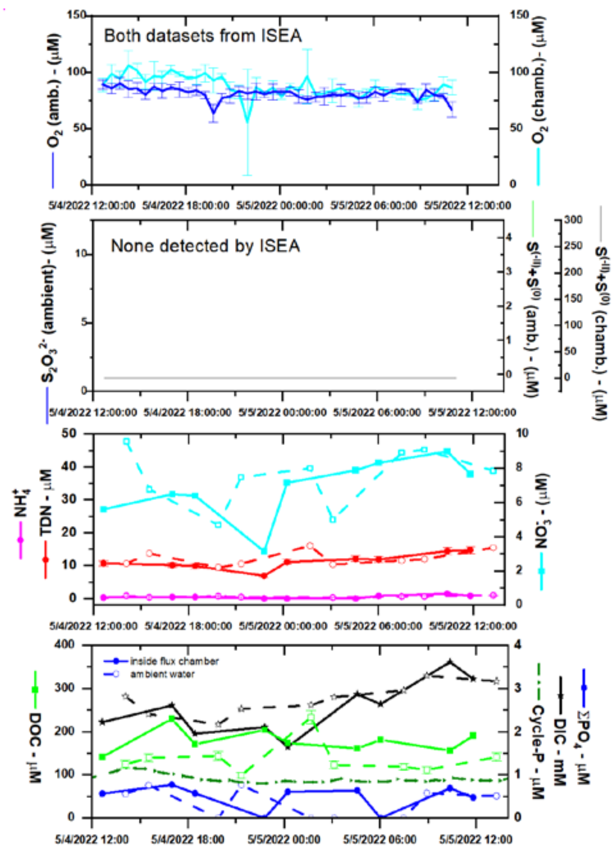

Figure 2. Time series data collected during the lander deployment beginning 5/4/22 from Green Banana. Results demonstrate that dissolved oxygen was surprisingly not consumed to a great extent in the benthic flux chamber ("chamb."), and bottom waters ("amb." = ambient) remained oxygenated overnight. In contrast to results from Amberjack Hole, nitrogen fluxes are dominated by nitrate (not ammonium), while phosphate fluxes remained low.

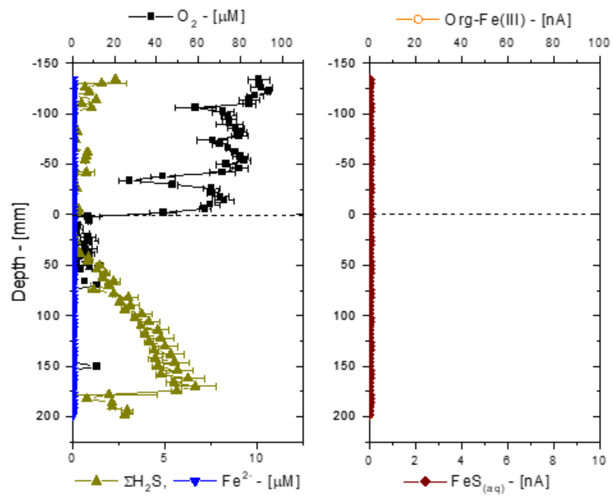

Figure 3. In situ electrochemical profiles collected as collected via the lander microprofiling system deployed beginning 5/4/22 demonstrate a gap between dissolved oxygen and hydrogen sulfide at the sediment-water interface, suggesting sulfide consumption by benthic microorganisms.

Results from the sediment core are presented in **Figure 4**. The sediments are consistent with results from September 2020 with respect to the redox and nutrient geochemistry. However, the electrochemical profile obtained from the sediment core illustrates significantly more hydrogen sulfide than in the in situ electrochemical profile (**Figure 3**). This suggests either a) sediment core collection and transport to the laboratory (i.e. a period of several hours during transport on a small boat) creates significant artifacts; or b) the lander deployment and sediment core collection locations were spatially different. These differences highlight the need for continued advancement and use of in situ monitoring technologies for bluehole survey.

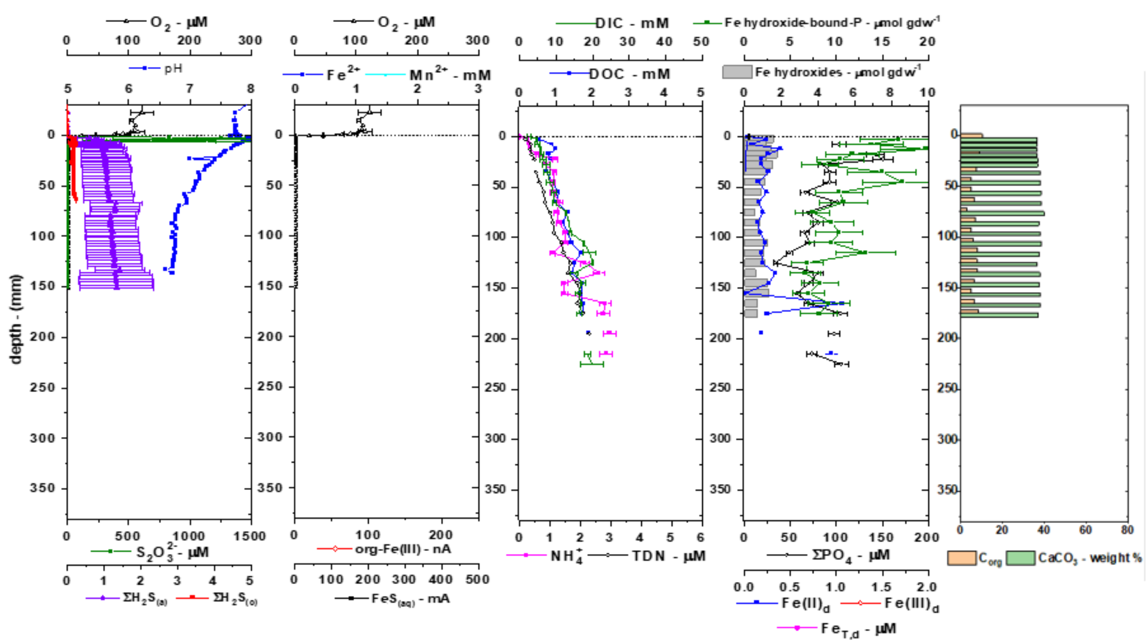

Figure 4. Sediment core profiles from Green Banana, sampled 5/4/22. The sediments are intensely anaerobic and sulfidic, although a zone of thiosulfate is detected at the sediment-water interface.

Nutrient fluxes to the water column are intense – particularly ammonium, but phosphate is apparently incorporated into minerals deeper in sediments.

All field survey and sample collection processes were similar to the Fall 2020 Green Banana excursion. A suite of samples was collected from the blue hole every ~10m from the surface to the bottom of the hole, and biological surveys were again completed. Mooring lines used to passively sample radium were lost during this field effort and the three samples that were returned were not analyzed.

Temperature, oxygen and pH had significant drops between 60 and 70m which was lower than the rim of the hole (**Figure 5**). Salinity remained constant (indicating a well-mixed system). All dissolved nutrients (ammonia, nitrate-nitrite and phosphate) were elevated in the hole from 60m down with nitrate-nitrite the most elevated. Particulate carbon showed elevated concentrations at 30, 60, 90, and 120m, yet did not coincide with any other chemical features (**Figure 6**). Interestingly, chlorophyll *a* was most elevated around between 70 and 100m (**Figure 6**). Carbonate chemistry patterns were similar to previous visits at both Amberjack and Green Banana (**Figure 7**). Collectively, these results comprise the Green Banana "dry season" survey. While more sample and data analyses is pending, results so far are not suggestive of diel cycling, in contrast to the "wet season" Green Banana survey.

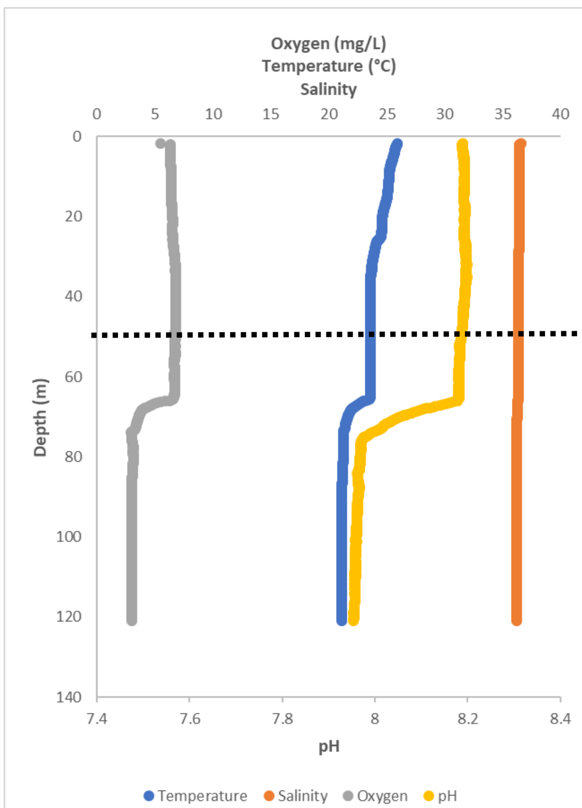

Figure 6. CTD data from the Spring 2022 deployment in Green Banana. The dashed line indicates the rim of the hole.

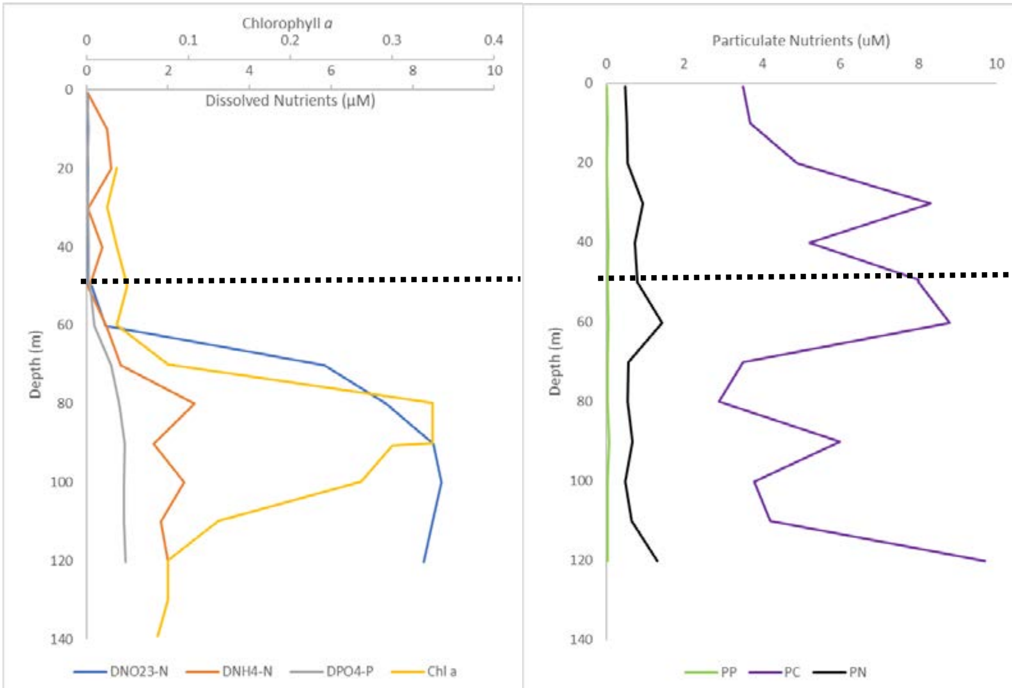

Figure 6. Dissolved and particulate nutrients and chlorophyll *a* data from the Spring 2022 deployment in Green Banana. The dashed line indicates the rim of the hole.

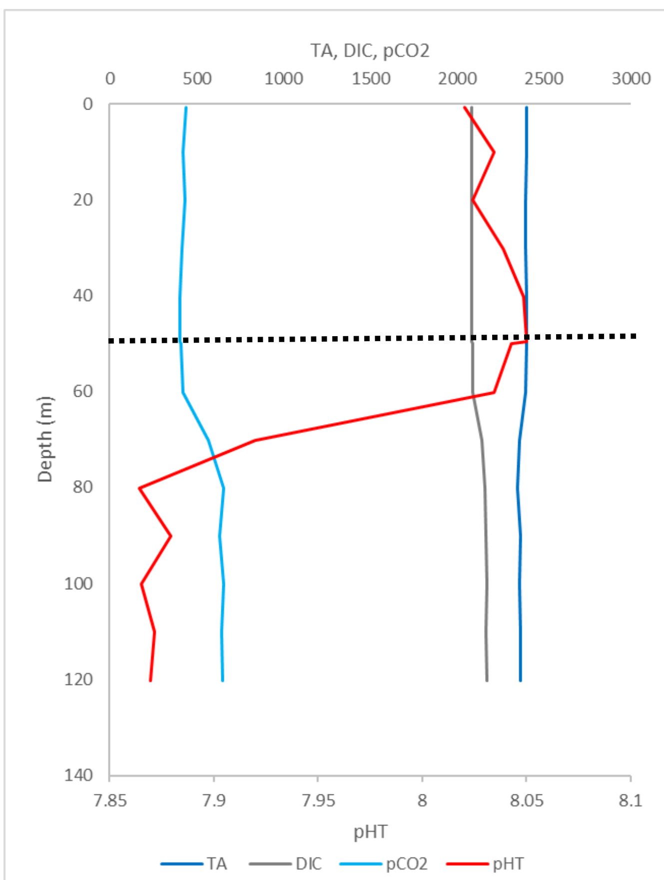

Figure 7. Carbonate chemistry data from the Spring 2022 deployment in Green Banana. The

dashed line indicates the rim of the hole.

For microbial/molecular results, there was a significant difference in alpha diversity concerning OTU richness between samples taken above the bluehole vs. the top of the bluehole (q = 0.003202) and samples taken above the blue hole vs the bottom of the bluehole (q = 0.003202). There was a significant difference in alpha diversity concerning the Shannon diversity (richness and evenness) between samples taken above the blue hole vs inside the blue hole, samples taken above the blue hole and the blue hole surface (interface), and the hole surface and inside the bluehole (q = 0.031106). There was a significant difference in beta diversity concerning the Shannon diversity (richness and evenness) between samples taken above the blue hole vs inside the top of the blue hole (q = 0.00200), between samples taken above the blue hole vs inside the bottom of the bluehole (q = 0.00200), samples taken above the blue hole and the blue hole surface (interface) (q = 0.0030), the hole surface and top of hole (q = .0020), bottom of hole and top of hole (q = 0.0072). There was only a significant difference in beta diversity (Bray Curtis) between the samples collected at the surface of the bluehole and the samples collected at 90 meters (p = 0.032) and 110 meters (p = 0.030).

When comparing Green Banana to Amberjack microbial/molecular results, SAR11 and Cyanobacteria showed a higher relative frequency at shallower depths as expected, yet Woesearcheota do not appear to be enriched at deeper depths which was unexpected. Nitrosopumilus appears to be enriched at deeper and middle depths (ammonia oxidizer) which was somewhat expected (**Figure 8**).

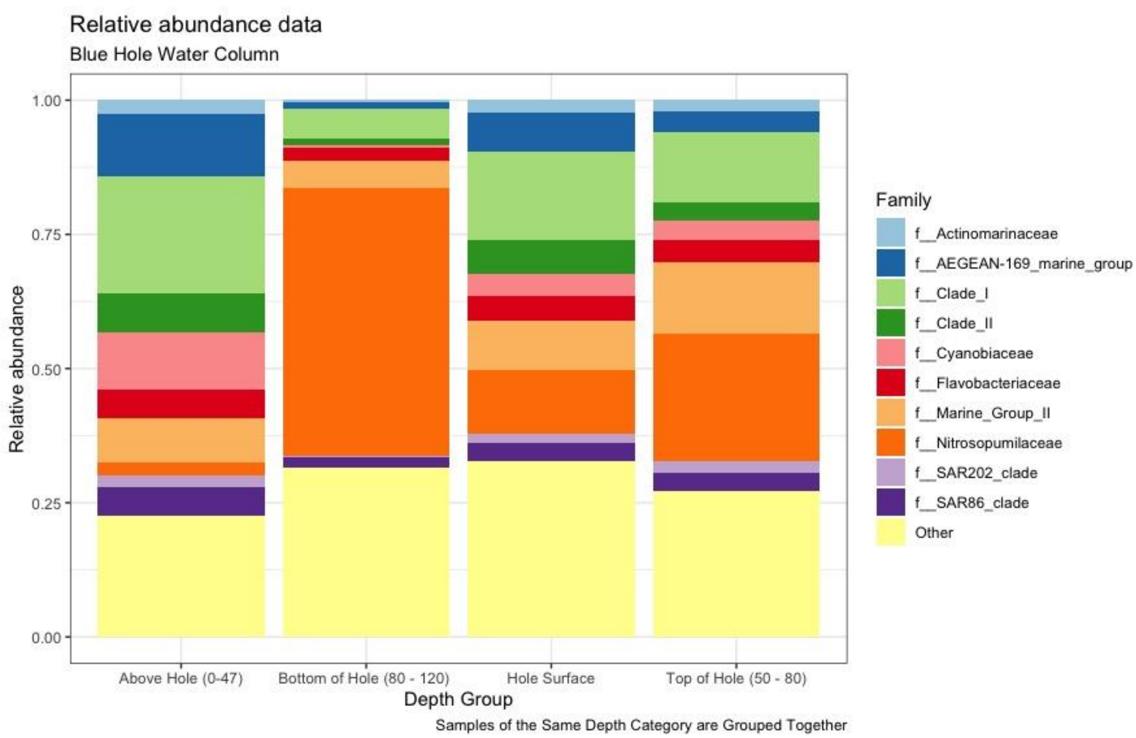

Figure 8. Relative abundance of taxonomic groups with depth for Green Banana, Spring 2022.

b. *Inventory of Activities*: 24 student interns have been working on the project, learning technical skills, instrument skills, sampling techniques and overall project

assistance. Three participants (current AAUS scientific divers) were also trained as technical divers with one diver reaching closed circuit training certification.
Week 1 Amberjack May 2019: There were three days of preliminary dives (eight dives total) by a team of PI Hall, Co-PI Culter, and volunteer divers at Amberjack Hole to survey the first lander station. Six thermographs were placed on a vertical line recording at 10-minute intervals. The first week long lander deployment, water and sediment sampling, and instrument placement occurred. The lander was deployed and retrieved 2 times. There were a total of 7 rim dives (6 rim divers, max depth = 35m). There were a total of 3 technical dives (5 technical divers, max depth = 106m). There were a total of 4 rosette/CTD deployments, and a number of handheld niskin deployments. Two rim dives complete the perimeter of AJ Hole for biological diversity surveys.
Week 2 Amberjack September 2019: There were two days of preliminary dives (four dives total) by a team of PI Hall, Co-PI Culter, and volunteer divers at Amberjack Hole to survey the lander station. Concrete weights were placed for the purpose of rigging diver safety lines with a surface buoy allowing for controlled descent /ascent as well as a bottom reference location. Six thermographs were placed on a vertical line recording at 10-minute intervals. The second primary, week long (Fall 2019) lander deployment, water and sediment sampling, and instrument placement routines were also conducted. The lander was deployed and retrieved once. A total of four sediment cores were collected on two separate days. There were a total of 4 rim dives (6 rim divers, max depth = 35m). There were a total of 3 technical dives (5 technical divers, max depth = 106m). There were a total of 24 handheld niskin deployments. Two rim dives complete the perimeter of AJ Hole for biological diversity surveys.
Week 3 Green Banana August/September 2020: The fourth expedition cruise plans were initiated including the development of a 4-day logistical plan based on experience from the first three expeditions. Six thermographs were placed on a vertical line recording at 10-minute intervals. The third cruise (first cruise to Green Banana) was completed the week of August 31-September 4, 2020 and included lander deployment, water and sediment sampling, instrument placement, and biological surveys. The lander was deployed and retrieved twice. A total of four sediment cores were collected on two separate days. There were a total of 5 rim dives (5 divers, max depth=46m) and 5 deep dives (9 divers, max depth=130m). There were a total of 16 handheld niskin deployments and 10 rosette deployments. One rim dive (three teams of divers) completed the perimeter of Green Banana for biological survey diversity.
Week 4 Green Banana: Prior to the final expedition, two CTDs were placed at the rim and on the bottom (~120m) from May 6, 2021 to June 11, 2021. The fourth expedition cruise plans were initiated including the development of a 4-day logistical plan based on experience from the first three expeditions. Five thermographs were placed at the rim (~49m), ~59m, ~74m, ~94m, and ~119m on a vertical line recording at 10-minute intervals (these thermographs have not been retrieved yet). Two trips to Green Banana occurred (on matching funds): 1. Deploy diver down lines and two CTDs, 2. Recover the diver down lines, the CTDs, and the thermographs. There was a total of 2 rim dives (4 divers, max depth=46m). The benthic lander was deployed twice successfully (once at the bottom of the hole and once at the rim of the hole) for >24 hours each deployment. Thermographs were deployed from the rim down to the bottom of the hole and will remain there for a year. Diver down lines were deployed for the extent of the project. There was a total of three deep dives (>400’ depth) and three rim dives (~200’ depth max) by two teams. All dives and deployments occurred at the Green Banana site (27.13695, -83.44023).

c. *Inventories of Samples*:

Week 1 Amberjack May 2019: No samples were collected during the initial dives. During the week-long excursion, 17 water samples were collected for nutrients and carbonate chemistry in the water column and in three directions from the rim of AJ Hole via divers. Two sediment cores were collected at maximum depth (106m). A deep-water pump collected samples for radium analyses at 6 depths with three other depths of surface control samples (Vargas et al., 2022). Niskin bottles were deployed to collect water for radon analyses at six depths with three other depths of surface control samples (Vargas et al. 2022). Four videos of biological transect data was collected around the rim of the hole.
Week 2 Amberjack September 2019: No samples were collected during the preliminary dives prior to the fall 2019 expedition. During the week-long excursion, 22 water samples were collected for nutrients, carbonate, and redox chemistry in the water column and in three directions from the rim of Amberjack Hole via divers. These include blue hole water column depth profile at 10 m increments. The solid-phase and pore waters were collected from a total of four cores. A deep-water pump collected samples for radium analyses at 6 depths with three other depths of surface control samples. Niskin bottles were deployed to collect water for radon analyses at 6 depths with three other depths of surface control samples. Four videos of biological transect data was collected around the rim of the hole.
Week 3 Green Banana August/September 2020: During the week-long excursion, 28 water samples were collected for nutrients, carbonate, microbial, and redox chemistry in the water column and in three directions from the rim of Green Banana via divers. These include blue hole water column depth profile at 10 m increments. Four benthic boundary layer time series samples (ambient water) were collected via the benthic lander syringes, as well as nine benthic flux chamber samples. Solid-phase and pore waters were collected from a total of four cores. Twelve dive bags containing manganese fibers (for radio-isotopes) were deployed for radio-isotope analyses at 12 depths with 5 other depths of surface control samples. Four videos of biological transect data was collected around the rim of the hole.
Week 4 Green Banana May 2022: Water samples collected = 23 (are partially analyzed for chlorophyll *a*, NOx, PO4, NH4, SiO3, PN, PC, PP, pHT, DIC, TA, DOC, TN, Fe(II), Fe(T), sulfur speciation, microbial community, and molecular analyses). Sediment core samples collected (n= 4) were analyzed for Fe2+, Mn2+, Fe(III), FeS, H2S, Fe(II), Fe(T), Fe(OH)2, DOC, TDN, PO4, DIC, microbial community, and molecular analyses. Radium bags were deployed from the surface to the bottom of the hole = 7 bags, and at a control site = 3 bags, but were deemed damaged/lost upon retrieval. CTD casts were completed daily from the surface to the bottom of the hole and at the control site. Hydrolabs were strapped to divers (rim and deep) to get contours of salinity, temperature, DO, and pH on each dive. The molecular dataset consisted of 45 water column samples with 16s rRNA gene amplicon sequencing data spanning 0m – 120m (as well as two controls from the extractions). Prior to bioinformatic analysis, 4 samples GBH-02, GBH-02, GBH-04, and GBH-4 were removed due to ambiguous labeling reducing the sample size to 41 samples. 16S rRNA gene amplicon sequencing from 41 samples yielded 11,893 total features with 225.0 – 155,468.0 reads per sample after filtering for quality and trimming 30 base pairs off each end.

d. *Resulting Publications*: One peer-reviewed publication has been produced: Patin, N.V., Dietrich, Z.A., Stancil, A., Quinan, M., Beckler, J.S., Hall, E.R., Culter, J., Smith, C.G., Taillefert, M. and Stewart, F.J., 2021. Gulf of Mexico blue hole harbors high levels of novel microbial lineages. The ISME Journal, 15(8): 2206-2232.

A second peer-reviewed publication has been submitted: Culter, J, Hall, E.R., Byrd, G., Cole, C. In review. Diving techniques for exploration of submarine karst features of the West Florida continental shelf. Proceedings of the American Academy of Underwater Sciences.

A summary of the project was also presented in an article in New Frontiers in Ocean Exploration (Raineault and Flanders, 2020).

Multiple conference abstracts have been published with coinciding talks at scientific conferences: One abstract was accepted and presented as a poster presentation at the OceanVisions2019 meeting in Atlanta, Georgia:

> Beckler, J.S., J. Culter, E. Hall, A. Stancil, M. Taillefert, F. Stewart, and C. Smith. 2019. Blue holes as hotspots of ocean deoxygenation and acidification. Protecting Ocean Health: Ocean Acidification and Hypoxia Session, OceanVisions2019 Summit, Atlanta GA (April 2nd, 2019)

Two abstracts were accepted as presentations at the Ocean Sciences Meeting 2020 in San Diego, CA (February 2020):

> Stancil, A., Taillefert, M., Culter, J., Hall, E., Putin, N., Stewart, F., Smith, C., Quinan, M., and Beckler, J. 2020. Blue hole biogeochemistry in the greater Gulf of Mexico context. ASLO Conference, San Diego, CA.
>
> Patin N.V., Dietrich Z., Hall E., Beckler J.S., Taillefert M., Culter J., Stewart F.J. 2020.Woesearchaeal metagenome-assembled genome informs its role in blue hole water column and sediments. ASLO Conference, San Diego, CA.

PI Hall and Co-PI Culter presented this work at the Advanced Diver Conference in Englewood, Florida on October 10-11, 2019. http://www.englewoodeventcenter.com/admconference.html

More than 20 presentations have been made to the public by PIs Hall and Culter. This included a number of Mote Podcasts (Two Sea Fans) as well as other podcasts on Blue Hole Research. One presentation was made to the public by Co-PI Beckler for the FAU Harbor Branch Ocean Science Lecture Series.

Blue Holes were also included as part of Sylvia Earl's Mission Hope declaration of the Gulf of Mexico as a Hope Site.

The PBS Changing Seas Program featuring blue holes was released to the public: https://www.changingseas.tv/season-12/1201/ and all PIs participated in a live Q and A with the release.

Multiple media releases were posted, and a number of news articles were published based on media releases including: BBC, National Geographic, VICE news, ABC, CBS, CNN, Late Night Show.

A blog specific to this project was hosted at Mote Marine Laboratory (mote.org/deepthoughts).

PI-Hall and co-PI Culter gave guest lectures/presentations to the Ringling

College of Art and Design “Ecology of Water” class from 2020-2021 to initiate outreach projects with the art students in the spring semester on the blue hole. A document describing the project goals was presented and agreed upon with the course instructor (Ms. Anamari Boyes). Students submitted mini-proposals halfway through the semester and were approved by Ms. Boyes and Dr. Hall. Final projects were completed and presented to PIs Hall and Culter. Students were provided the opportunity to “donate” their projects to Mote Marine to be used in an exhibit or website. Some of the final projects included paintings, posters, t-shirt designs, game designs, coloring book, comic book, and a children’s book.

e. *Location/Status of Data Archive and Sample Storage/Plan for Public Access*: All data from this project are retained as hard copies at Mote Marine Laboaroaty and includes field logs, sample receipt forms, sorting and identification logs, work orders or benchsheets, identification data sheets, chromatograms, charts, automated printouts, printouts of code for applications programs, records of formulas contained in spreadsheet calculations, and tabulations of QC checks (as required by the Mote Marine Laboratory Quality Assurance Manual (MML, 2022). Data are stored in chronological laboratory files or project specific files as detailed above. Hard copy of data pertinent to laboratory operations (standards and reagent preparation, field meter calibrations, sample container cleaning and kit preparation records) are also retained. All field observation and analytical data have also been stored as Excel or PDF files on the Mote Marine Laboratory Chemical and Physical Ecology Program storage drive.

Datasets from every instrument/sensor (all participating institutions) are stored as archive-ready, open-source, non-proprietary formats. Datasets of scientific logging and first-hand scientific observations have been preserved and converted into archive-ready formats. All post-processed datasets, data products, and reports have been documented with metadata describing the processing steps and quality assurance methods. Sequence Read Archive (SRA) data have been assigned a single BioProject identifier with linked metadata. Radioanalytical data obtained on samples collected and analyzed by the USGS (Smith) are published as a USGS data release, which meets Federal open-data requirements. These data are accessible via the web and are freely available to public through the USGS Publication Warehouse and can be found here: https://doi.org/10.5066/P9CKZT3O.

Observational data and analytical results are currently being made available with the reporting requirements through the NCEI data systems (and will be complete within 6 months from the end date). All sequence data has been made publicly accessible fully abides to the Minimum Information about a Genome Sequence and Metagenomic Sequence standards that have been recently established by the scientific community (MIGS and MIMS, respectively). Data will be archived for a minimum of 10 years beyond the end of the project.

f. *Major Changes/Adjustments to previously submitted documents*: There have been no major changes/adjustments to previously submitted documents.

III. Evaluation:

1. Accomplishments – Explain special problems, differences between scheduled and accomplished work. The third cruise (first cruise to Green Banana) was scheduled for May 2020, but due to COVID-19 and required quarantine and stay in place orders

from the governor of Florida, the cruise had to be postponed to September 2020 (which was fortuitously consistent with previous Amberjack Hole we season month). A no-cost extension was applied for and granted.

The final mission to Green Banana was scheduled for the week of May 10-14, 2021 with a backup week of May 17-21, 2021. The benthic lander frame (an integral piece of equipment required for this mission) was shipped to Mote Marine Laboratory (Sarasota, FL) from Georgia Tech (Atlanta, GA) via UPS Freight. It was due to arrive at Mote April 28, but never arrived. We did postpone the mission to the week of June 7, 2021 with the assumption that the lander would be found, or that Georgia Tech could either modify or build a new lander. Unfortunately, neither option was able to occur in time for the June 7 mission. The lander was never located by UPS, and based on parts availability, a new lander was not able to be constructed in time. The PI team agreed that a no-cost extension should be requested to postpone the final spring season trip to Green Banana to Spring 2022 to allow for the planned "dry season" survey. The no-cost extension was granted to September 2022 and the survey proceeded as planned in May 2022.

2. Expenditures:
   a. *Describe original planned expenditures.* Planned expenditures included salary and fringe for all PIs and supporting staff; supplies (e.g. chemical consumables, thermographs, isotope supplies, gas for divers, sample containers, cameras, lights, lines, dive gear, and buoys); contract and subaward funds to Co-PIs (Georgia Institute of Technology and FAU/Harbor Branch); boat use fees, and indirect charges.
   b. *Describe actual expenditures.* There were no major deviations in planned expenditures from the rewarded funding. Actual expenditures included salary and fringe for all PIs and supporting staff; supplies (e.g. chemical consumables, thermographs, isotope supplies, gas for divers, sample containers, cameras, lights, lines, dive gear, and buoys); contract and subaward funds to Co-PIs (Georgia Institute of Technology and FAU/Harbor Branch); boat use fees, and indirect charges. There were expenditures from matching funds that were larger than anticipated (volunteer diver costs and additional boat time for preliminary trips to each hole).
   c. *Include a final budget table with a column of original planned expenditures and a column of actual grant expenditures*

BUDGET TABLE: $257,442

	Overall Funds	**Actual Expenditures**	**Balance Remaining**
Personnel	$38,490	$37,593.94	$896.06
Fringe Benefits	$13,760	$13,439.84	$320.16
Travel	$1,000	$166.77	$833.23
Equipment	$0	$0	$0.00
Supplies	$24,326	$24,071.12	$254.88
Contractual	$60,702	$59,346.10	$1,355.90
Construction	$0	$0	$0.00
Other	$79,981	$84,553.99	($4,572.99)
Indirect Charges	$39,182	$38,270.25	$911.75
TOTALS	**$257,442**	**$257,442.00**	**$0.00**

   d. *Explain special problems, differences between planned and actual expenditures.* There were only slight differences in salary and fringe (<2% variation from total cost) likely due to differences in salaries of staff throughout the project from original planned project). The largest differences were in travel and other due to changes in gas prices throughout the project (and remained <2% variation from total cost).

3. Next Steps:
   a. *Planned or expected reports (professional papers, presentations, etc.):* Several journal articles are pending preparation, including a project-wide synthesis of blue hole activities. More specific journal articles will also focus on the sediment biogeochemistry and influence on the blue hole redox/nutrient budget, and another focusing on the disparate diel cycling at the two blue holes.
   b. *Brief description of need for additional work, if any (next project phase, new research questions, unaccomplished work, etc.):* Additional work will include data uploads to NCEI and publications. The project team has goals to write proposals to continue this work on other holes (there are currently more than 25 of these holes documented throughout the Gulf of Mexico). New questions that have resulted from this work include: "Are these holes initiation sites for Red Tide (e.g. *Karenia* spp)?" and "Are more of these holes connected to the mainland?".

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