Critical Review – Deep-sea shipwrecks represent island-like ecosystems for marine microbiomes

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Melanie Damour is a Marine Archaeologist from the Florida State University (United States) specializing in, and having extensive experience in, shipwreck and underwater site research (FSU, 2021). She is the Vice President of Submerged Archaeological Conservancy International (SACI, 2021), a non-profit organisation focusing on the preservation and protection of submerged archaeological resources (SACI, 2021), and the Environmental Studies Coordinator for the Bureau of Ocean Energy Management's Gulf of Mexico Region office of the Department of Interior, US (FSU, 2021). In 2014, her and her team received funding for a research project (GOM-SCHEMA: "Gulf of Mexico Shipwreck Corrosion, Hydrocarbon Exposure, Microbiology, and Archaeology"), and subsequently an excellence award from the National Oceanographic Partnership Program for the work conducted for this project. The project sought to understand the impact of the *Deepwater Horizon* blowout on historic shipwreck-associated sediment microbiomes in the northern Gulf of Mexico (Hamdan *et* al., 2018). One outcome of this project is the paper "Deep-sea shipwrecks represent island-like ecosystems for marine microbiomes" (Hamdan *et al*., 2021). In this essay I will review this paper, which focused on the analyses of microbial diversity at various distances around a shipwreck to study how built environments impact microbial biodiversity in benthic environments.

In this paper, the authors propose that artificial, anthropogenic features shape biogeographic patterns of microorganisms, and that, in this particular case, sunken shipwrecks in the deep ocean function as islands that create a hospitable habitat for benthic microorganisms. The authors state that this is of interest to marine research as previous research has only investigated how naturally occurring deposits influence biogeographic patterns of benthic microorganisms. They claim that their research is unique as technology has only recently been developed that allows sampling from deep-sea benthic sediments to undertake this kind of research. Therefore, for this research, the authors intend to explore the deep-sea built environment of the *Anona* shipwreck, sunken in 1944 to the depth of 1258 meters below sea level. The intention is to determine the impact this anthropogenic, artificial environment has on biogeographical patterns at a microbiological scale. In marine research, often big grants are allocated to multi-year projects that require the collaboration between different research centres and scientists, and result in the publishing of several papers with some data-sharing between these papers (e.g., MARBLES funded by Horizon2020; "The race for new space" funded by Royal Society Te Apārangi). The research conducted for this paper was part of a larger BOEM grant, also resulting in the publishing of at least seven other papers (some listed here). This smaller paper is a case study of one specific shipwreck, the "*Anona*", which is a large steel-hulled ship that sank in 1944 in the Gulf of Mexico, was discovered in 1995, then geophysically surveyed in 2002, and since then had been sampled extensively. Over the course of the research grant, the authors take various sediment samples from different orientations, distances, and depths from the shipwreck and analyse their differences in microbial community composition using 16S rRNA sequencing, bioinformatic and statistical analyses. The results showed that microbiome diversity and richness increase the closer the sample is taken to the shipwreck, with a diversity and richness extinction plot suggesting an “island-effect” on microbes. They argue that this is further supported by the findings that, for samples further away from the shipwreck, sediment depth was a larger factor in determining microbial richness and diversity. The results also showed a microbial “halo” around 150m from the shipwreck, where the core taxa of the identified microbiome dropped off, illustrating a transition zone between shipwreck and surrounding environment. The authors suggest that this is only a first “glimpse” of a shipwreck functioning as an island-like ecosystem, and that the abundance of other shipwrecks in the ocean can provide further indication on how built environments determine biogeographical patterns in the deep-sea benthos. Overall, the authors claim that this case study provides new information on how benthic microbiomes are shaped by the arrival of unnatural material on the seafloor.

The objective of this study is to determine whether shipwrecks function as an island-like system for benthic microbiomes. The authors claim that they accomplished this objective and were able to confirm that shipwrecks do, in fact, function as island-like systems for benthic microbiomes. I disagree with this claim. The authors cite the “Island Theory of Biogeography” by Macarthur & Wilson (1967) and argue that the book describes that species richness and diversity in island-like systems are determined by the size of the island, its isolation from a mainland or island providing a source of taxa. Whilst this may be true and is stated in this book, the authors do not measure this with the molecular and statistical techniques they use. They do not compare the microbial diversity and composition to any of the parameters outlined in their argument, i.e., a differently-sized island-like system, or a differently-distanced island-like system. Thus, not providing a point of investigation for the foundation of their claims. The above-mentioned book also describes that the features outlined above dictate extinction and immigration rates of the inhabiting taxa, as also outlined by the authors. Even though the authors undertake their expeditions over the course of 4.5 years, they do not take this into consideration when evaluating the data, and even go so far as to ignore the fact that the samples from various locations were all taken at different, random, timepoints during the 4.5 years. Thus, they do not measure any extinction or immigration rates, and they evidently do not consider time as a variable, despite the book specifically addressing this “dynamic”, time-dependent biome as part of island-like systems. The authors hypothesise that built structures, such as shipwrecks, decrease the isolation of microbiomes on the seabed. The authors do not explicitly state whether this hypothesis was confirmed. However, they discuss that their results show a “halo” of microbes around the shipwreck, the presence of a core microbiome around the shipwreck, and that the benthic microbiome extends around 200m into the surrounding environment. They state that this is an interesting finding but requires more context once other shipwrecks have been investigated where the data can be put together and analysed. I do agree that this is potentially an interesting finding, and I have some thoughts as to why this would be interesting, however I believe that it is the authors role in the discussion to stimulate these thoughts and provide some hypotheses or further suggestions about what could be investigated here. Additionally, I struggled to interpret the hypothesis as it was not further elaborated on in the Introduction or Discussion. I suspect that it intends to state that “smaller” microbiomes (perhaps isolated microbes) are distributed in the ocean and when a built structure arrives on the seabed, these microbiomes adjust to become a united microbiome. If this is the case, I do not agree that the hypothesis is true, or at least, their data does not show that this hypothesis is true. As mentioned above, the data is lacking time as a parameter. When looking at changes in microbiome due to a force entering their environment, it would make sense to consider this parameter. This could be achieved by comparing what the microbiome looked like before this force was able to disrupt the environment, or at least, the microbiome could be studied over a certain amount of time to observe changes in response to the aging of the force.

In the introduction, the authors justify their hypothesis by outlining that microorganisms exhibit biogeographic patterns, i.e. the non-random distribution over time and space, and that, in marine environments, environmental selection and pelagic dispersal have been found to dictate these biogeographic patterns (Hanson *et al*., 2009). This particular review (Hanson *et al*., 2009) found that physical and chemical signals, i.e., habitat features, are what determine the microbial community composition in environmental habitats, and therefore shape microbial biogeographic patterns. The authors state that these factors also play a role in the deep seas as they have been explored in a wide range of natural deep-sea habitats, such as hydrothermal vents, methane seeps, and seamounts (Duchinsky *et al*., 2019; Inagaki *et al*., 2006; Ruff *et al*., 2015). They argue that these findings are based on natural features, and that it is imperative to determine whether these findings are similar in built environments. The authors highlight the “Island Theory of Biogeography” (Macarthur, 1967) which hypothesises that, within island-like systems, the size of the “island” and isolation from the mainland determines the species richness and diversity. The authors argue that the sediment that had occurred from the impact of the shipwreck on the seafloor is within the definition of “island-like system” (Meyer KS, 2017). The rest of the introduction outlines, in excruciating detail, the details of the *Anona* shipwreck, including physical details, how it sunk, and what it carried. Finally, the authors describe that the sinking of the *Anona* created a sediment berm in the vicinity. The introduction overall provided an okay overview of the topic; however, I think there was too much detail provided on the details of the shipwreck as the impact these details have on the microbiome are not actually investigated in the study itself. The authors outline in their introduction observations of the impact physical and chemical factors have on microbiomes, specifically mentioning hydrodynamics, salinity, circulation, and state that microbial composition and habitat features correlate have been found to correlate, yet they fail to measure or address this in the study. This information is arguably interesting, but redundant in context to the study.

The authors describe that they undertook seven expeditions on two different vessels between March 2014 and September 2018, ranging between 9 and 13 months apart, to take sediment samples around the *Anona* shipwreck. Before sample collection, visual surveys were conducted to determine suitable areas for sampling. Three different Remote Operated Vehicles (ROVs) extruded these samples and immediately sectioned the sediments in 2-4cm intervals. The result was a total amount of 138 samples from 23 different locations around the shipwreck, at distances ranging from 2-1000 meters. The samples were then separated into four transects of northwest, southwest, southeast, and northeast. Whilst the authors provide a map of the shipwreck (Fig. 1), there is no map illustrating the sediment locations and transects, and there is only a supplementary table (Table S1) that provides a list of each sample with information about the depth, distance, transect, direction, and date/expedition the sample was taken. However, this table does not include units of measurements for depth and distance, and it seems that this is raw data that was added without considering what value it would add to the paper. It would have been more helpful to include a map to show the transects and sampling locations, complemented by a table that illustrates the total number of samples, and total number of samples per transect, depth and distance. This would allow for an overview of the samples and perhaps allow the reader to follow the description and discussion of the results more easily. Additionally, 138 samples are a small number for a research project over the course of seven expeditions and 4.5 years. Taking samples from all 23 locations at every expedition would have yielded more quantifiable data. Examining the samples in the supplementary table, the data collection dates are unrelated to any of the other parameters of the sample collection. Thus, the samples from different locations cannot reliably be compared to each other without considering the (potentially significant) confounding factor that they have been taken at different times within the 4.5 years. Especially considering that, shortly before the expeditions began, the *Deepwater Horizon* spilt uncountable gallons of oil into the ocean close to the shipwreck site. Additionally, the samples were taken using different ROVs, thus possibly introducing another confounding factor. Perhaps, again, a more valuable approach would have been to sample the same locations, or in close vicinity, repeatedly over the 4.5 years to be able to compare how the microbiome changes (I will discuss this in more detail in the next paragraph). At least, as some sort of “snapshot”, an alternative approach would have been to take samples from all 23 locations at the same time to prevent the time-confound. In the interest of the objective of this study, and considering the information discussed in the introduction, I think it would have been useful to measure the physiochemical parameters of the sediment samples and the surrounding ocean water, such as pH level, salinity, oxygen-, iron-, and carbon concentrations, and finally, the level of degradation of the shipwreck. These parameters would allow us to understand the ecosystem more holistically. It could also allow us to understand how the shipwreck affects this deep-sea environment. Finally, some papers that describe similar studies seem to document the flora on the seabeds in additional to these parameters to determine potential microbiome accumulations or sediment linkages (e.g., Fujiwara *et al*., 2007; Smith *et al*, 2014 at similar depths). I believe this could have benefitted the overall objective also.

For the molecular analyses, the authors extracted the genomic DNA of the sediment samples, amplified the genes with primers specific to the V6-V8 region of bacterial 16S rRNA, and sequenced the results using Illumina. This data was then cleaned up and used to build an ASV feature table. However, this table was not shown and there is no directive provided of where to find it. Turning to the main-output paper of the project, the ASV table also cannot be found as this paper uses OTUs rather than ASVs for taxonomic identification. I think it would have been beneficial to provide this table or at least a bar graph to show the bacterial distributions. However, a figure (Fig. 3) found later in the paper is a bubble plot of the various classes and phyla at each distance category. The ASV table was compared against an rRNA database (SILVA) to identify the taxa within the samples. The methods do not contain an exact description of the sample analysis and instead provide a general overview of the main techniques used, referencing another paper that is also part of the research project for more details (Hamdan *et al*., 2018). However, this paper also refers to earlier papers (Hamdan *et al*., 2013; Comeau *et al*, 2011) instead of providing the detailed description of molecular analyses. Additionally, using V6-V8 16S rRNA to identify microbial communities has been shown be biased towards bacteria depending on which primer is used (Johnson *et al*., 2019). The paper does not state that they consider this bias and attempt to compensate for this, rather, they specifically state that they use primers for bacterial rRNA, and their accompanying figures (Fig. 2-5, Fig. S1) refer to only bacteria and bacterial families and genuses even though the authors continuously refer to the bacterial biome as the “whole” microbiome. Notably, the other paper I have mentioned above seems to be the main output of the full research project. The data in the main-output paper states that primers specific to each, Bacteria and Archaea, were used. Examining the data in the main-output paper it appears that the data from the paper discussed in this critical review overlaps with the main-output paper data, and the authors seem to have made the conscious choice to exclude some parts of the data (specifically Archaea) for this paper. Considering this paper seeks to study marine microbiomes, choosing to only report on bacteria is unlikely going to accurately reflect any kind of microbiome, including marine microbiomes, as microbiomes commonly consist of microorganisms from all domains (e.g. Archaea, Bacteria, Fungi, even protozoans). Additionally, it seems that the author did not find it necessary to undertake any research into the functional profiles of the microbes, for instance metagenomics and/or transcriptomics (ideally both, of course). In combination with the physiochemical parameters suggested in the previous paragraph, this could have enabled us to understand how the microbes interact in the environment, what their main roles are, and how they interact with each other. Furthermore, it would have also been very useful when trying to distinguish between whether the “island-like ecosystem” is due to microbes gathering to degrade an unwelcome compound (i.e., an alloy) in their surroundings, or whether the effect occurs because the microbes (and other organisms) have truly accepted the shipwreck as a hospitable habitat and are integrating it into the environment. Overall, the level of detail of the methods is slightly inconsistent. There is also little detail on the molecular analyses and much detail on the statistical analyses, thus making it difficult to replicate the experiments without considerable effort. Additionally, this further supports the suspicion that the data was taken from the previously referenced paper and subsequently used for further bioinformatic and statistical analyses for this paper.

In the results, the authors present a table (Table 1) with PERMANOVA (multivariate analyses) data which was not described in the methods. The table description states that it was constructed from all the sediment data to look at microbial community differences between depth and distance. The authors stated that the interaction between the two features were statistically not significant, yet they also construct an nMDS from the 16S rRNA data which contradicts this claim. The nMDS found that the less proximate to the ship, the more distinct the microbial groups are based on sediment depth. The authors chose to present a bubble plot (Fig. 3) in lieu of the ASV table or a bar graph to show bacterial abundance, which also shows the samples grouped by distance categories. They do not explain their distance categories which are ranging from just a few meters when next to the shipwreck to growing larger until full 500 meters at the 500-meter mark. I suspect that this may be because the authors expect to see a lesser impact on the surroundings the further away from the shipwreck the sample is taken. However, this should be outlined in the methods or results, and this could potentially introduce confounds (i.e., assuming something to be true may not actually be true), especially considering the contradicting claims in the PERMANOVA and nMDS. One reason for this contradiction could be due to the presence of different flora (or other larger, stationary organisms). As mentioned previously, some other studies chose to take pictures and document the flora of the seabed. This, again, could have helped establish the underlying reasons for this contradiction. Another major issue is that, even though the authors identify some bacteria to below the class level, they only do so in the bubble plot

(Fig.3) as part of the plot, rather than presenting this as a result that can be discussed later. The only comment about the taxa in the plot is to point out that Proteobacteria ("not annotated below the phylum") were significant contributors to the core microbiome at all distances. Overall, the results are not well presented as the authors only shallowly outline their findings, some figures having been inappropriately selected and some contradicting results are not presented in a way that would allow interpretation for the underlying reasons for this.

In the discussion, the authors compare their island-like system to an island-like system of a whale fall, hydrothermal vents, or seeps. However, I want to argue that these three things (and the shipwreck) do not compare to each other. Firstly, hydrothermal vents and seeps are permanent structures. They nurture microbes like extremophiles and provide them with a steady, hospitable environment (Reysenbach *et al., 2006*). One reason why a shipwreck provides a similarly steady environment is because it is made from non-natural matter that is difficult for microbes to degrade (Salerno *et al.*, 2018). Particularly in this case, where the shipwreck is made from steel, an alloy that is made from inorganic iron and carbon, I would expect microbes to remain in the environment for longer as it takes longer to degrade. I would like to argue that it is more likely that this artificial obstruction attracts microbes that need to colonize the area to "clean up" and force microbes whose natural habitat this area is to migrate somewhere else. Secondly, whale fall is the death of an organism that is made from organic material that are naturally degraded by microbes (Smith *et al.*, 2015). Additionally, in the case of whale fall, microbes migrate to the organism to degrade it and then leave to allow naturally occurring microbes to settle back into their habitat (Smith *et al.*, 2015). Thus, the comparison between the shipwreck and the organic compounds of other ecosystems seems inappropriate. Furthermore, the discussion is lacking significant depth and I believe this may be relating to the fact that many confounding factors were not considered and only 16S bacterial rRNA analysed. For instance, the authors extensively discuss the presence of a "microbial halo", which was the elevated microbial diversity found within 170 meters of the shipwreck. They included hypotheses for why the halo exists, for instance they postulate that it is relating to ecotone, which is described as a transition zone that connects spatially and temporally different ecosystems. They alternatively postulate that the halo exists due to either resource depletion or large dispersal around the shipwreck due to the impact when it dropped down. It would likely be possible to narrow down the extensive list of the hypotheses if the authors had taken physiochemical samples or functional profiles of the microbes. Another option could be to discuss the composition of the core microbiome in more detail. Even though it is only identified to the class level, it might explain some of the irregularities in "microbial" (=bacterial) diversity observed at different distance categories (and perhaps accounting for this "halo"). Altogether, this discussion is not clearly addressing or analysing the findings in context of the objective of the study, and is therefore not satisfying.

I realise that, at this stage, I should compare this paper to others that have looked at similar research questions. However, I believe this would be an insult to other studies. This study is so fundamentally flawed that I do not trust the data that was generated. I do not believe that the data has any value as only bacterial 16S rRNA genes were used to "establish" the microbiome, which is, without a question, not what a microbiome is. The authors had the opportunity to provide insight into a very interesting question, but the execution was lacking insight, thought, and scientific rigour as I have (partially) outlined in this critique. It was difficult to find information about the linked papers, the authors, descriptions for methods, and even the reason for why obvious confounds were present, for instance a short explanation why three different ROVs were used or why there was seemingly random temporal sampling. I also found that important, commonly assessed, parameters were not considered, such as physiochemical properties and functional profiling, and claims were made disregarding the possible confounds produced by this lack of data. These parameters would have also contributed to the ability to reach the goal of this research, which was to provide an understanding of a possible “island-effect” on built features. I believe that it is important to understand how built features fit into our ecosystem and how they can affect it. Considering that we are constantly littering the ocean with various inorganic products, large and small, investigating the impact this littering has could allow for meaningful, honest discussions around conservation. The authors continuously highlight the importance of this, yet they fail to satisfyingly answer this overall research question, by, in my opinion, significantly overinterpreting their data and completely disregarding confounds. Lastly, in my opinion, this paper compromises the integrity of scientific research. I believe that we are living in a world where the distrust in scientific research is becoming an increasing state of mind of the public. Particularly in current times, this distrust can become a threat to public health. I believe that we should do everything we can to retain this integrity and sadly, this paper did the opposite.