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ARTICLE

In Situ Biofilm Collection: Implications for the Management of Historic Submerged Aircraft Wrecks

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Abstract

The impact of microbiologically influenced corrosion on underwater archaeological sites has spurred recent advancements in research examining the link between microorganisms and historic preservation. Although the microbiomes of steel shipwreck sites have been the subject of DNA sequencing studies and other interdisciplinary investigations, aluminum submerged aircraft wrecks, a prominent symbol of World War II, have yet to be the focus of similar research. This article represents the initial attempt to fill this void by describing a biofilm collection method used to obtain samples for DNA sequencing from World War II aircraft sites off Hawaii. Rather than relying on proxies for microbial growth on wrecks or on destructive sampling, the focus is on a methodology that is productive but minimally intrusive. The protocols resulted in the successful collection of in situ biofilm samples from four submerged aircraft wrecks. The methodology was found to be affordable, time efficient, and reproducible, thus feasible for archaeological site management. The development of viable in situ collection methods for biofilm should aid efforts to empirically assess the relevancy of microbiologically influenced corrosion to submerged aircraft while enabling longitudinal studies of microorganisms that potentially affect site preservation.

Resumen

El impacto de la corrosión microbiológica en contextos arqueológicos sumergidos ha fomentado estudios orientados en la relación entre los microorganismos y la conservación del patrimonio. Mientras que los microbiomas propios de los barcos de vapor de acero han sido el principal interés de los análisis sobre secuencias de ADN, además de otros estudios multidisciplinarios, los restos sumergidos de las aeronaves compuestas de aluminio, símbolo de la Segunda Guerra Mundial, han sido dejados de lado en estas investigaciones. Este artículo pretende representar el primer esfuerzo para reducir esta brecha a partir de la obtención de muestras de ADN a través de un método de recolección de biopelículas en sitios de las aeronaves que han sido fechadas en el periodo histórico antes mencionado y que, en la actualidad, se encuentran sumergidas en Hawái. A diferencia de los análisis comunes que se han centrado en estudios proxys como el crecimiento de microbios en contextos sumergidos o la realización de muestreos destructivos, la utilización del método aquí expuesto resulta productivo, y en especial, mínimamente invasivo. Los protocolos llevados a cabo han permitido el muestreo exitoso de una colección de biopelículas recolectadas en los objetos arqueológicos sumergidos. Asimismo, y debido a su replicabilidad, dicha metodología ha resultado eficaz, lo que ha permitido reducir costos y tiempo, facilitando la gestión de las investigaciones en los sitios arqueológicos. De esta manera, con el desarrollo de este tipo de metodologías de recolección de biopelículas in situ, se espera contribuir a la evaluación de la corrosión microbiológica en aeronaves sumergidas, lo que resultará en la posibilidad de la caracterización de los microorganismos que inciden en la conservación de los contextos.

Keywords: aircraft; Hawaii; microbiologically influenced corrosion; World War II

Las palabras clave: avión; Hawai; corrosión influenciada microbiológicamente; Segunda Guerra Mundial

Within maritime archaeology and cultural resource management, an increasing amount of attention has been paid to determining the impacts that the environment has on underwater historical sites that are deemed to be "heritage at risk" (Miller and Wright 2023). Metallic forms of underwater cultural heritage (UCH) are particularly prone to naturally induced degradation through the corrosion

process (MacLeod 1989). Yet rather than just engaging in reactive site management responses, heritage professionals have invested significantly in the interdisciplinary study of corrosive mechanisms attributed to environmental forces (Bethencourt et al. 2018; MacLeod 2002; Moore 2015). Longitudinally tracking the causes and the rate of change makes possible mitigation measures and other proactive approaches to this preservation threat.

A growing body of knowledge on the ways in which the biology associated with submerged cultural sites contributes to ongoing corrosion has emerged from an interest in environmental site formations (Melchers 2014; Moore 2015). Nested within the burgeoning field of "wreck ecology," the study of microbiologically influenced corrosion (MIC) focuses on assessing the damage inflicted on heritage resources, identifying the associated microbes, and elucidating information on specific corrosion mechanisms (Little and Lee 2014; Paxton et al. 2024). This has often been achieved through collaboration and the use of analytical techniques from outside traditional archaeological practices; namely, next-generation DNA sequencing (Mugge, Brock, et al. 2019; Price et al. 2021; Van Landuyt et al. 2022). Using both the collection of on-site samples and mesocosm experiments, the results paint a complex picture of ways in which microorganisms contribute to corrosion.

Much of the MIC pursuits in maritime archaeology have been devoted to steel shipwreck sites, with dissemination of this research not only providing site-specific data but also resulting in the exchange of methodologies (Little et al. 2019; Paxton et al. 2024). The same cannot be said about aluminum aircraft wreck sites, which constitute a globally abundant form of UCH. Considerations of MIC have yet to be applied to submerged aircraft wrecks (SAWs), leaving a gap in understanding the microbiomes associated with this site type and the necessary methodological approaches. This article addresses the latter deficiency by exploring the fundamental issue of microbial sampling.

I describe here a collection study, with sampling efforts targeting microbial biofilms, the primary form of SAW biofouling. Four World War II–era SAWs off the coast of Hawaii were chosen as study sites. This article's methodological focus is on various sampling implements, step-by-step collection protocols, and downstream considerations related to sample preparation for DNA sequencing. It begins with a brief explanation of MIC in UCH contexts and the relevant biochemical processes that make MIC a potential issue for SAWs. Attention is paid to a lack of nondestructive in situ sampling methods, highlighting the utility of the current study. I conclude with a discussion regarding the efficacy of the proposed collection method and, ultimately, how microbial data gathered over time are intended to support the management of historic SAWs.

Microbiologically Influenced Corrosion

MIC is not associated with a single corrosion mechanism but instead refers to the ability of microbes to alter electrochemical conditions and facilitate increased corrosion through their presence and metabolic activities (Little and Lee 2007:1). Research into the intersection of microbial colonization and UCH site corrosion has resulted in a range of interpretations, from fears regarding the "biological extraction" of wreck metals (Church et al. 2007:206; Cullimore et al. 2001:126) to site impacts correlating with contaminant exposure (such as an oil spill) and the subsequent change in the microbes present (Mugge, Lee, et al. 2019; Mugge et al. 2021; Salerno et al. 2018). In some cases, the occurrence of MIC has been empirically refuted (Little et al. 2019; Salazar and Little 2017), whereas other studies have identified taxonomic associations with corrosion products and biocorroders (Price et al. 2021; Sánchez-Porro et al. 2010; Usher et al. 2014).

However, the collection methods used for in situ sampling are frequently destructive. Most often, corrosion and marine concretion samples are transported to laboratories for microscopic analyses (Albahri et al. 2019) and the extraction of microbial genetic material for DNA sequencing. In two recent examples, Price and others (2021) collected loose shipwreck debris and took drilled core samples from a World War II–era landing craft wreck, whereas Van Lan Landuyt and colleagues (2022) recovered two pieces of wreckage from the V-1302 *John Mahn* (1942). The authors of both studies obtained microbial samples by scraping and swabbing the shipwreck fragments in a laboratory setting. Alternatively, in their study of long-term corrosion rates associated with twentieth-century shipwrecks, De Baere and others (2019:11) articulated the point that the in situ preservation ethos, combined with

legal restrictions and international agreements (for example, the 2001 UNESCO Convention on the Protection of Underwater Cultural Heritage), can make it "morally and legally impossible to collect pieces of shipwrecks." The authors, however, noted that "destructive sampling was needed . . . [to] obtain a clear view on the possible influence of MIC" (De Baere et al. 2019:11). Thus, they opted to recover an unprovenanced piece of historic anchor chain as a substitute for this analysis. Similarly, test coupons and other biofilm recruitment arrays have been placed on-site, serving as a proxy for the actual wreck substrate (Cullimore and Johnston 2008; Mugge, Brock, et al. 2019).

The lack of nondestructive in situ sampling methodologies can raise questions of accuracy and ethics, which are further compounded by the inherently dynamic nature of bacterial communities in marine environments. Whether because of anticipated patterns of microbial succession, unpredictable impacts of climate change, or stochastic events, the bacteria colonizing UCH sites are likely to fluctuate, both taxonomically and functionally (i.e., gene expression), after initial sampling efforts (Dang and Lovell 2016; Sauer et al. 2022). Determining whether these changes correlate with increased MIC requires that heritage managers have access to baseline observations of microorganism populations and comparative datasets. The desire for repeated sampling places even more of a premium on the development of sound microbiological collection techniques that yield relevant genetic information while not inflicting further damage on the heritage resource.

MIC of Aluminum

In addition to refining collection techniques, the study of MIC in archaeological contexts can also be improved on by expanding to include SAWs. Despite their relatively modern origins, aircraft wrecks, particularly those related to World War II, are an increasingly important part of submerged cultural resource inventories throughout the world's oceans (Whitehead 2023). The historical preservation of these sites is justified primarily by their cultural and socioeconomic significance, which have been discussed elsewhere (Bush 2021; Edney and Boyd 2021; Fix 2011) and remain outside the scope of a methods-focused article. The emphasis here is on the biochemical processes that justify MIC as a potential preservation threat to SAWs, the investigation of which requires a clearly defined sampling methodology.

For aluminum aircraft wrecks, MIC research should focus on determining the taxonomic composition and functional structure of the microbial communities colonizing the submerged metallic surfaces. This bacterial attachment most often takes form of mucilaginous biofilms, which begin with colonization by aluminum-tolerant taxa (Sancy et al. 2015). As the initial colonizers proliferate, they produce adhesives known as extracellular polymeric substances, which give biofilms their slimy texture and promotes the attachment of additional microorganisms (Dobretsov 2010). These exopolymers can also trap metal ions, creating cathodic sites that further accelerate the corrosion process because aluminum preferentially corrodes (Azeredo and Oliveira 2000).

Previous investigations of aluminum alloys, including duralumin (Al2024; McNamara et al. 2005; Rajasekar and Ting 2010), the primary construction material used in World War II aircraft, have revealed several possible MIC mechanisms (Salvarezza et al. 1983). The first is the disrupting effect that microbial colonization and activity can have on the protective aluminum oxide film that forms on submerged surfaces (Jaume et al. 2022; Li et al. 2017). Left alone, this passivating layer is largely responsible for the anticorrosive properties that lead to aluminum's frequent use in marine engineering (Kaufman 2000:96–117). The breakdown of this barrier by the presence of microbial biofilm and associated metabolic activities enables attacks from chloride ions on the original aluminum (Dexter 2003; Nelson et al. 2017). The increased chloride concentration can then instigate the growth of corrosion pits (MacLeod 1983). Alternatively, oxygen gradients can form within the biofilm layer as microbes in the outer sections metabolize dissolved oxygen from seawater (Melchers and Jeffrey 2008). The deoxygenated environment can prompt increased metal dissolution through redox reactions associated with oxygen concentration cells (Hamilton 2003; Van Loosdrecht et al. 2002).

The varying oxygen concentrations also promote the growth of anaerobic microbes. Specific groups of oxygen-intolerant microbes—namely, sulfate-reducing bacteria (SRB)—have been linked to the MIC of aluminum through the production of corrosive metabolites (Enning and Garrelfs 2014; Hamilton 1985).

A recent review of MIC literature by Amendola and Acharjee (2022:2) revealed that SRB were "responsible for almost half of all MIC related cases." Rather than referring to a specific class or other taxonomic ranking, SRB are a functional group comprising numerous taxa that possess the ability to respire using sulphate as an electron acceptor (Muyzer and Stams 2008). Within marine biofilm systems, SRB metabolism uses the dissolved sulfate in seawater and organic waste products from nearby aerobic microbes (electron donor; Videla 2000). The resulting metabolites, such as hydrogen sulfide, can react with the underlying aluminum substrate, ultimately concluding in increased metal dissolution and the buildup of corrosion products like aluminum hydroxide (Guan et al. 2017; Liu et al. 2014; Nelson et al. 2017). Clades of marine fungi have also been linked to the MIC of aluminum through similar metabolite production (He et al. 2022; Zhang et al. 2022).

Thus, although multiple MIC pathways exist, the underlying cause and effect of MIC remain the same: it is the ability of microorganisms to alter a substrate's microenvironmental conditions, including oxygen and sulfur content, that leads to an increased corrosion rate. The metabolic activities responsible are often enabled by the communal-living strategies of biofilm constituents (Brown et al. 2023; Little et al. 2008). It is important to note that MIC is not a linear process because marine biofilms are subject to cycles of buildup and dispersal, which drastically affect quantity and composition (Salta et al. 2013). The ever-changing nature of marine biofilms therefore highlights the need for effective microbial collection methods that can yield the most accurate understanding of a wreck's current microbiome.

Research Objectives

Before any taxonomic data can be analyzed in terms of relevance to understanding MIC processes, the efficacy of the sampling procedure should be assessed to ensure that sufficient genetic material can be collected for DNA sequencing. For biofilms from SAWs specifically, the earlier mentioned lack of in situ studies created a need to devise a collection methodology for this sample type. I attempted to create such a methodology using the sampling protocols described later, which I evaluated by comparing sample DNA concentrations and sequencing success. Yet, unlike previous in situ microbial collections in UCH contexts, the current study needed to be minimally invasive, given the stipulations put forth by the relevant site management agency (US Naval History and Heritage Command [NHHC]).

Two World War II SAWs off the coast of Maui (Curtiss SB2C-1C Helldiver and Grumman F6F-3 Hellcat) and two off the coast of Oʻahu (Republic P-47 Thunderbolt and Vought F4U Corsair) were chosen for biofilm sampling (Figure 1). Hawaii served as the ideal testing ground for this methodology given the large number of SAWs (41 confirmed locations) and the interests of stakeholders—recreational divers and military enthusiasts—in these sites. Sites were selected not only because of their physical accessibility but also to ensure that the data collected could eventually be used in the management of these culturally significant forms of World War II heritage. Each SAW has been described in previous reports (Bush 2023; National Oceanic Atmospheric Administration [NOAA] 2011; Petrey et al. 2008). A research team of archaeologists and biologists from East Carolina University carried out fieldwork in Māʻalaea Bay, Maui, April 21–28, 2021, and a local nonprofit organization, Naval Exploration and Research Divers (NERD), assisted collections in Waimānalo Bay, Oʻahu, August 7–15, 2021 (Figure 2). Financial support was provided by a National Center for Preservation Technology and Training Grant from the National Park Service.

Methodology

Sample Collection

Questions regarding the collection of marine biofilm samples for DNA sequencing served as the impetus for a study by Pochon and others (2015), which compared four sampling implements—syringe, swab, tape, and sponge—used to obtain microbial material from glass collection plates. After consultation with Dr. Xavier Pochon from the Cawthron Institute in New Zealand and Dr. Gail Ashton of the Smithsonian Environmental Research Center, who had performed similar biofouling studies (e.g., Clark et al. 2019), I decided that this project would assess the feasibility of using syringes to

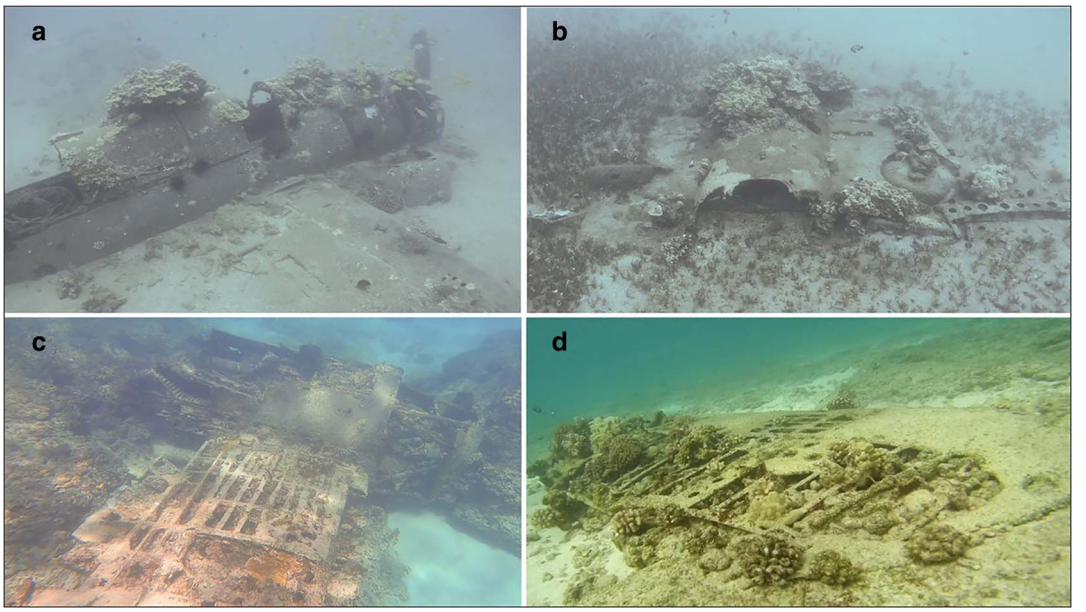

Figure 1. Four study sites: (a) Curtiss SB2C-1C Helldiver (Maui), (b) Grumman F6F-3 Hellcat (Maui), (c) Republic P47 Thunderbolt (O'ahu), and (d) Vought F4U Corsair wing (O'ahu). Photos courtesy of East Carolina University/Naval Exploration Research Divers, 2021.

collect SAW biofilm. Consideration was given to the time required, financial costs, and practicalities of working underwater. Syringes seemed preferable to collection tools such as sponges, swabs, and tape because their suction capabilities aid in gathering dislodged biofilm in an aquatic setting. Furthermore, the syringe volume of 50 mL allowed increased biofilm collection, because it was not limited by the surface area of a sponge or swab.

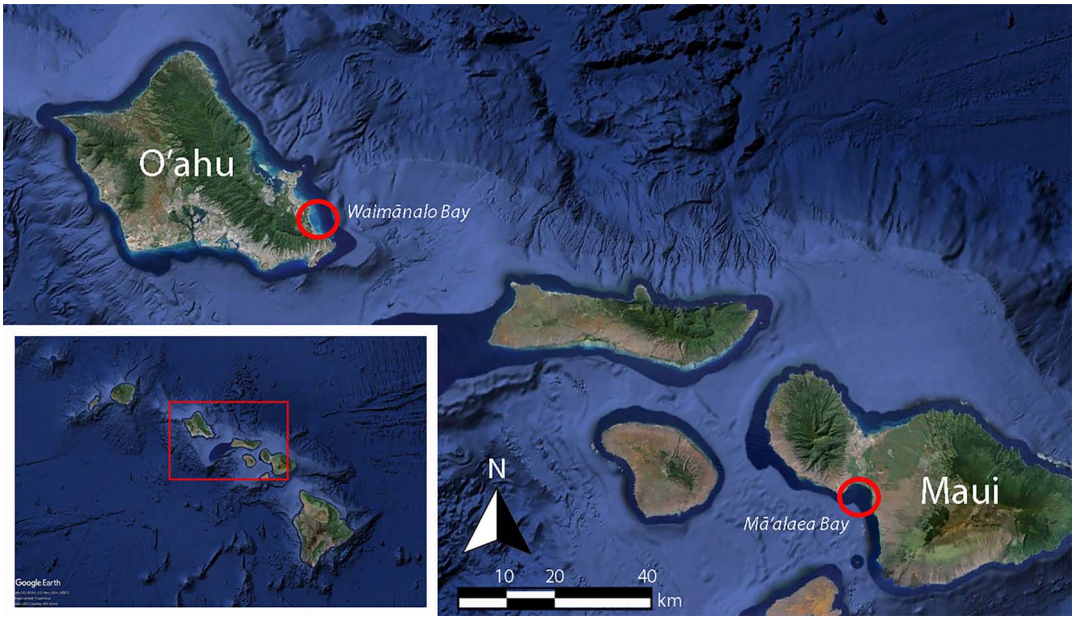

Figure 2. Map of the study area with fieldwork locations off Maui and O'ahu circled. Imagery courtesy of Google Earth.

Before sample collection, all sites except the Corsair were photogrammetrically surveyed using underwater cameras. The resulting imagery was used to make digital models (Agisoft Metashape v1.8), which served as 3D sampling maps (Figure 3). Biofilm sample collection was done by a team of three SCUBA divers, who began by placing a 10×10 cm grid square at the desired location. Sampling locations concentrated on exposed—lacking macrofouling—aluminum that provided sufficient surface area (>100 cm^2). For this initial collection, 10 sampling locations were divided evenly between visibly corroded and noncorroded surfaces (Figure 4). Following pre-disturbance photographs, one diver would dislodge the biofilm with a polypropylene spatula while avoiding damage to the aluminum surface. Simultaneously, another diver would extract a 50 mL syringe fitted with a 1 cm tubing attachment. Capturing as much biofilm material as possible, divers used three syringes per sampling square (Figure 5). Although aluminum is the focus of this study, we were able to do additional biofilm collection at the Hellcat site from two non-aluminum surfaces: a rubber tire and a stainless-steel fitting. Additionally, researchers experimented with collection tools at this site, using WhirlPack Sponge Probes to gather three biofilm samples from aluminum surfaces. This collection method used the same 100 cm^2 sampling grid. On average, five to six biofilm samples were taken on each tank dive, which lasted between 45 and 60 minutes.

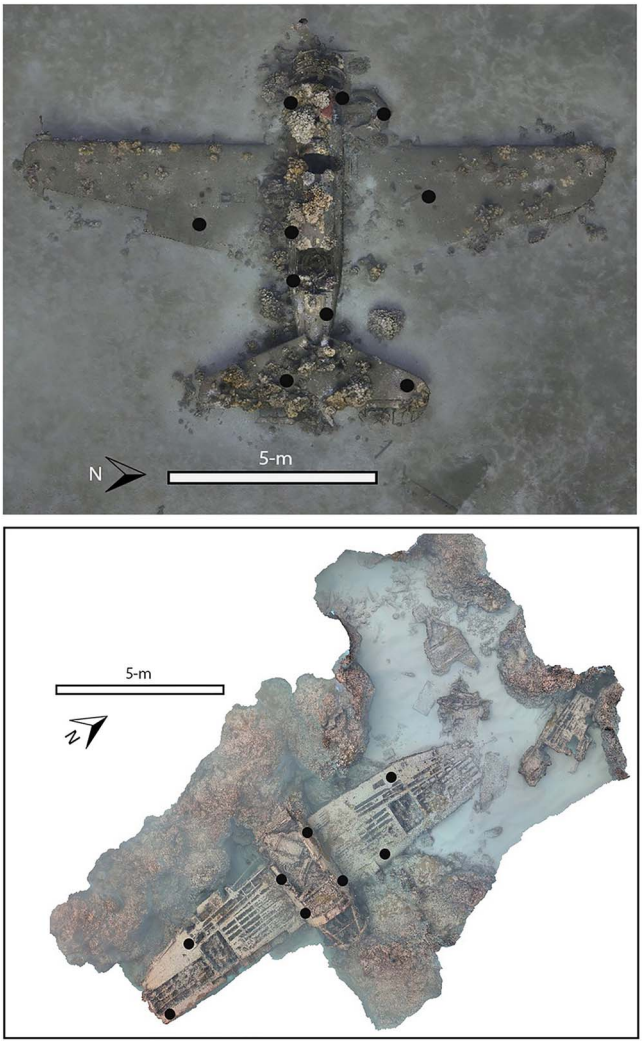

Figure 3. 3D models of the biofilm sampling locations (black dots): *top*, Helldiver (Maui); *bottom*, Thunderbolt (O'ahu) sites. Note an additional three biofilm samples were taken from the Thunderbolt's engine section not pictured. Models created by the author, 2022.

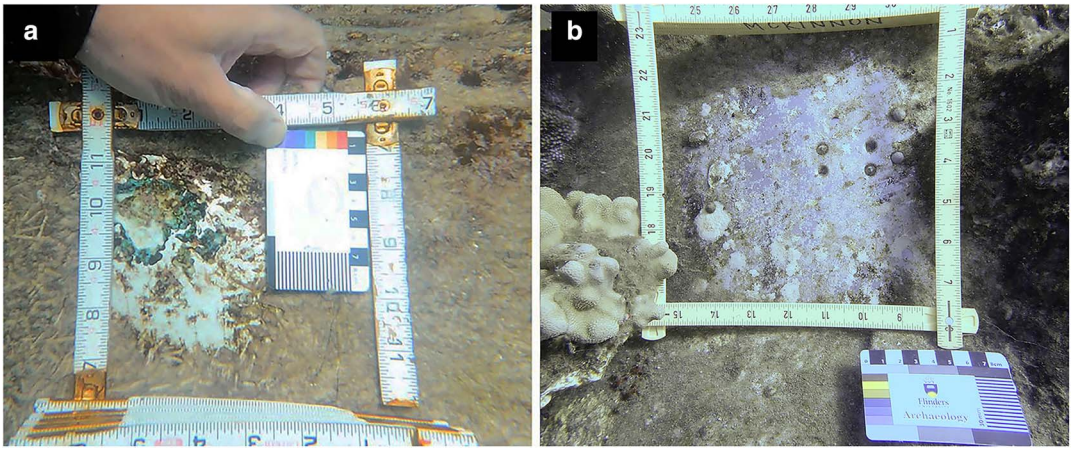

Figure 4. Examples of wreck surfaces samples: (a) blue/green copper corrosion (Thunderbolt site, O'ahu), (b) noncorroded (Helldiver site, Maui). Photos by the author, 2021.

The project team also took sediment and seawater samples from each site. For sediment, divers collected samples in 50 mL falcon tubes from four areas at each site: near a visibly corroded aluminum surface, near a noncorroded aluminum surface, 5 m from the wreck, and 30 m from the wreck. An additional sediment sample was taken from underneath the Helldiver's fuselage and an algae-covered sand patch near the Hellcat. Three 1 L water samples were collected into sterilized plastic containers from above each site. Water samples were then filtered using a 0.2 µm Polyethersulfone filter placed into a plastic holder. The holder was attached to a syringe, so that the water samples could be filtered through in 50 mL intervals. Each filter was then aseptically transferred to a falcon tube. Samples were kept frozen and shipped on ice to East Carolina University's Howell Science Complex, where they were placed in a −20°C freezer until DNA extractions could be performed.

DNA Extractions

All sediment and biofilm samples were processed using a commercial DNA extraction kit (DNeasy PowerSoil Pro). I chose this kit because of its availability and proven track record within our laboratory, but a variety of alternatives exist (Dairawan and Shetty 2020). For biofilm samples, a 100 µL pipette was inserted into the settled biofilm material at the bottom of the falcon tube, minimizing the amount

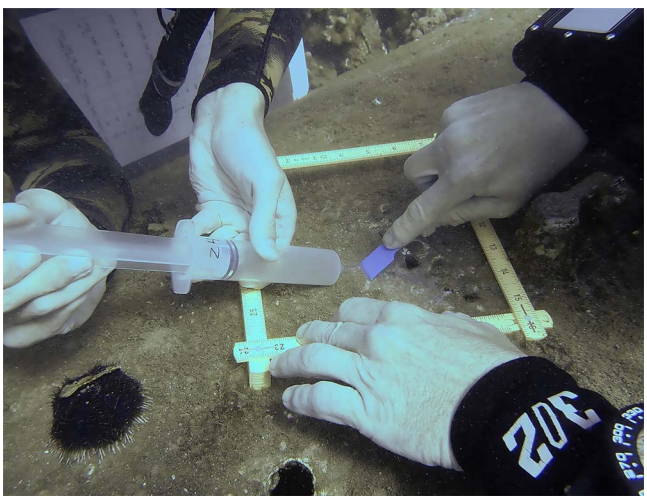

Figure 5. Photo of biofilm sampling method developed during this study. Hellcat site (Maui). Photo courtesy of East Carolina University, 2021.

of seawater placed into the extraction kit's lysis tube. For sediment samples, excess seawater was poured off, and subsamples were loaded into lysis tubes using metal spatulas. Six biofilm samples were reprocessed using a different commercial DNA extraction kit (DNeasy PowerBiofilm) as a way of testing whether one kit was more effective than the other in isolating microbial genetic material from biofilm (Dauphin et al. 2010). A separate extraction kit (DNeasy PowerWater) specifically designed to isolate genetic material from water filters was used for all seawater samples.

To ensure each extracted sample met the minimal yield threshold for sequencing (1 nanogram/microliter [ng/μL]), DNA concentrations were assessed using a Qubit Fluorometer. Ten μL subsamples were shipped to Integrated Microbiome Resource (IMR), where they were sequenced using an Illumina MiSeq. This method produces millions of reads per run on a specified region of DNA, which provides greater coverage from the perspective of community-level statistics (Quail et al. 2012). Sequencing targeted the V4–V5 region of the 16S rRNA gene because its ubiquity in bacteria and diagnostic nature, which make it the most widely studied 16s rRNA region (Schmalenberger et al. 2001).

Results

The results were evaluated in terms of the DNA concentration yields and DNA sequencing success, particularly as they related to comparisons between the biofilm and environmental (seawater and sediment) samples. Although the sequencing data will later be used to understand the effects that wrecks and the surrounding environment have on each other's microbiome, for this study the sediment and seawater samples served as a control. In contrast to the uncertainty regarding whether syringes would obtain adequate genetic material, the sediment and seawater samples were collected using well-established methods. Thus, the resulting data—DNA concentrations, sequencing success, and bacterial sequence counts—can help infer the validity of the biofilm dataset. Statistically similar results across sample types would indicate a successful biofilm collection.

DNA Concentration Yields

A total of 75 samples were collected: 45 biofilm (three by the sponge method), 18 sediment, and 12 water filters. For biofilm and sediment samples, a minimum of 0.25 g (0.4–0.5 g is preferrable) was needed for the two DNA extraction kits. Biofilm weights were of particular interest, because obtaining an adequate amount of material was a concern prior to fieldwork. Each syringe-collected sample, however, produced more than the material threshold. The surplus biofilm was needed, because eight extractions originally failed to yield sufficient DNA. Several sediment samples were also unsuccessful at first, and the extraction process had to be repeated. All water samples passed the concentration check initially. In addition to the third sample obtained with the WhirlPack Sponge, at least 1 ng/μL of DNA was successfully obtained from each sample, including those from the stainless-steel fitting and rubber tire (Table 1). In general, the sponges collected little biofilm material, which precluded repeated extractions. Similarly, DNA concentrations from the other two sponge samples were relatively low. Biofilm samples collected using the syringe method produced an overall mean DNA concentration of 8.25 ng/μL, which was significantly higher ($t = 3.63$; $p = 0.001$) than the mean produced by sediment samples ($n = 18$; mean = 3.03), while being nearly equal with that of seawater samples ($n = 12$; mean = 8.33). The minimum and maximum values of all three sample types compared favorably. DNA yields were not improved by using the PowerBiofilm kit for biofilm samples ($n = 6$; mean = 2.86). Thus, to eliminate any potential biases that could arise from using different kits, the PowerSoil Pro kit was used for final extractions.

DNA Sequencing

Of the samples submitted to IMR, 90.1% (73 of 81) were successfully sequenced, including six that were resubmitted after the first sequencing attempt failed (Table 1). The lone sample that could not be sequenced was a biofilm sample from the Thunderbolt site. Sequencing reads were returned as fastq files, which were then imported into mothur v1.41.3 (https://mothur.org/), an open-source, bioinformatics data-processing software. Following an updated version of protocols by Schloss and others (2013), nucleotide sequences were translated into operational taxonomic units (OTUs), which are used

Table 1. Sample List.

Sample ID #	Location	Type	Material	DNA Yield Range	Average Sequencing Counts
DNeasy PowerSoil Pro Kit					
1–5	Maui: Hellcat	Biofilm	Aluminum, corroded	3.19–19.8	95,986
6–10	Maui: Hellcat	Biofilm	Aluminum, noncorroded	4.2–9.06	50,978
11	Maui: Hellcat	Biofilm	Rubber tire	2.01	4,358
12	Maui: Hellcat	Biofilm	Stainless-steel fitting	1.38	4,297
13–15[a]	Maui: Hellcat	Biofilm (Sponge)	Aluminum, noncorroded	0.07–3.83	6,737
16–20	Maui: Helldiver	Biofilm	Aluminum, corroded	2.45–9.38	15,005
21–25	Maui: Helldiver	Biofilm	Aluminum, noncorroded	2.67–12.5	24,266
26–30	Oʻahu: Corsair	Biofilm	Aluminum, corroded	6.79–27.0	69,190
31–35[b]	Oʻahu: Corsair	Biofilm	Aluminum, noncorroded	1.04–25.8	68,664
36–40	Oʻahu: Thunderbolt	Biofilm	Aluminum, noncorroded	5.9–11.9	89,693
41–45	Oʻahu: Thunderbolt	Biofilm	Aluminum, noncorroded	5.63–10.9	95,611
46–50	Maui: Hellcat	Sediment	Sediment	1.44–12.7	43,083
51–55	Maui: Helldiver	Sediment	Sediment	1.06–5.08	8,766
56–59	Oʻahu: Corsair	Sediment	Sediment	1.38–2.63	33,494
60–63	Oʻahu: Thunderbolt	Sediment	Sediment	1.35–2.3	51,978
Dneasy PowerBiofilm Kit					
1	Maui: Hellcat	Biofilm	Aluminum, corroded	1.3	N/A
7, 9, 10	Maui: Hellcat	Biofilm	Aluminum, noncorroded	2.37–5.01	N/A
11	Maui: Hellcat	Biofilm	Rubber tire	1.84	N/A
12	Maui: Hellcat	Biofilm	Stainless-steel fitting	2.32	N/A
Dneasy PowerWater Kit					
64–66	Maui: Hellcat	Seawater	Seawater	8.11–15.2	11,271
67–69	Maui: Helldiver	Seawater	Seawater	14.2–18.8	11,598
70–72	Oʻahu: Corsair	Seawater	Seawater	2.49–3.47	116,348
73–75	Oʻahu: Thunderbolt	Seawater	Seawater	2.19–4.02	116,313

[a]DNA concentration of Sample 15 below sequencing threshold and could not be reextracted
[b]Sample 31 failed to sequence.

as proxies for traditional taxonomic ranks. Sequences that read at a similarity threshold (97% in this study) for the designated marker gene (16S rRNA) were clustered into a single OTU. The sample OTUs were then identified taxonomically by comparing sample sequences with reference examples (silva v.132 database), which will enable future assessments of community composition. Across the 73 total samples, 3,306,515 bacterial sequences were identified after quality filtering. For each sample type, sequences counts were higher for the Oʻahu samples than the Maui samples. Sequence counts from the Maui biofilm samples were not significantly different from either sediment ($t = -1.11$, $p = 0.277$) or seawater ($t = 1.01$, $p = 0.324$). Conversely, Oʻahu biofilms yielded significantly higher

sequence counts when compared to sediment samples ($t = 2.54$, $p = 0.018$), whereas the opposite was observed when compared with seawater samples ($t = -1.89$, $p = 0.072$). Despite these interisland differences, it appears that biofilm collection was successful.

Discussion

Biofilm Collection Method

This study succeeded in creating a sampling method that produced sufficient microbial genetic material from marine biofilms on SAWs, as inferred by comparisons with sediment and seawater samples. Biofilm sampling was uncertain, given its limited concentration (1 mm thick layer) on wreck surfaces and the absence of studies explicitly examining the microbial ecology of SAWs. Yet, sufficient sample weights, combined with comparable DNA yields and sequencing success across all sample types, indicate that the syringe method of collection can be used for future SAW biofilm studies. Furthermore, this methodology had the additional benefit of being nondestructive: the research team was able to obtain in situ biofilm samples without removing any parts of the aircraft. An inspection of the Thunderbolt site by me 14 months after fieldwork indicated that there were no obvious adverse effects from the initial sampling effort.

In addition to the primary collection method, two additional aspects of the current study warrant further discussion. The first is the attempt to use a different collection device, a WhirlPack sponge. In this study, sponge collection resulted in noticeably less biofilm material and is not recommended for future use. The second methodological insight pertains to DNA extraction kits. For the current study, there were concerns about the ability of the PowerSoil Pro kit to extract sufficient genetic material from biofilm when compared to the PowerBiofilm kit. No appreciable differences in DNA quantity were observed; as such, the PowerSoil Pro kit was ultimately chosen because it will allow for the most appropriate taxonomic comparison between sediment and biofilm samples. This is not to advocate for any specific commercial DNA extraction kit; instead, kit consistency should be an important consideration during the DNA isolation process (Claassen et al. 2013).

Overall, the biofilm collection procedures devised for this study had to account for the practicalities of working underwater and to be amenable to use by SCUBA divers. Biofilm samples were obtained not only in a relatively straightforward process but also economically in terms of time. The project team spent a maximum of two one-hour dives at each site, resulting in the collection of 10–12 biofilm samples per site. The lack of specialized equipment for in situ sampling has additional benefits that make biofilm collection more feasible. The plastic syringes and falcon tubes used can be purchased in packs of 40–50 for less than $100 USD. Other supplies, including the polypropylene spatulas, serological pipettes, and syringe filter (for water sampling), can also be purchased for reasonable amounts.

This methodology is intended to be efficient, minimally invasive, and affordable while still capable of yielding relevant information. The combination of these attributes should make in situ biofilm sampling a viable option for site management agencies. Ultimately, the collection method is meant to support research related to both a baseline understanding of SAW microbiomes and how they may shift in response to natural and anthropogenically induced change, without compromising the heritage resources. The sampling protocols have been shared with the NOAA, National Park Service, Hawaii State Historic Preservation Division, and NHHC. The last of these agencies has already expressed explicit interest in follow-up biofilm collections in hopes of better understanding the microbial communities associated with the UCH resources it manages (NHHC 2024).

Relevance to Microbiologically Influenced Corrosion

Although the current study has documented an effective means of sampling biofilm from SAWs, it is the potential of the taxonomic data that is perhaps most consequential. Translation of the sequenced DNA into identifiable microbial types should enable a more informed assessment of the microbes currently colonizing the SAW, including corrosive metabolite-producing SRB and the co-occurrence of aerobic and anaerobic bacteria, which may be indicative of an oxygen concentration cell (Lenhart et al. 2014). Yet, the taxonomic data, when available, should not be viewed as a definitive characterization nor as evidence of any conclusive links between the marine microbiota and heightened

corrosion (Knisz et al. 2023). Instead, site managers, armed with a baseline understanding of each wreck's microbiome, could perform repeated follow-up biofilm collections and decipher which additional lines of microbiological evidence, such as metagenomic studies of gene profiles, are needed for determining the potential of MIC (Krohn et al. 2021). The interpretation of these results can then be paired with photogrammetric surveys (Yamafune 2024) and electrochemical corrosion assessments (Blackwood 2018) to track potential correlations between increased site degradation and microbial communities.

Such an approach was used to document U-*166*'s corrosion following the Deepwater Horizon oil spill in 2010, where time-series images indicated significant metal loss (Damour et al. 2019). The visual evidence of deterioration was coupled with assessments of the microbes and genes present within biofilms formed on nearby steel recruitment arrays, which exhibited similar metal loss. This suggested a link between the sunken submarine's increased corrosion and microbes likely colonizing its surface in the wake of the oil spill (Mugge, Brock, et al. 2019). For SAWs, the development of a practical and efficient method of collecting in situ biofilm samples without significantly affecting heritage resources makes this a more realistic possibility. From there, site managers can begin to proactively consider mitigation responses. The U-*166* example demonstrates that before the questions of how to inhibit MIC and the degree to which management agencies should invest in mitigation measures can be considered, it is essential to determine "who" may be contributing to MIC and to what extent this phenomenon is manifested.

Yet, the sample collection of biofilms from SAWs need not be reserved for federal agencies, universities, or other well-funded research entities. The use of inexpensive collection materials and a reproducible order of operations presented here can facilitate collaborations with "citizen scientists" (Silvertown 2009). The recreational diving community stands out as a capable and motivated group, given their vested interest in the continued survival of the UCH sites they frequent (Viduka and Edney 2022). Furthermore, recreational divers are often keen observers of site transformations, with insights that can inform scientific interpretations (Viduka 2022). Communication with these divers and other stakeholders can be used to explain not only how to collect biofilm but also why these samples aid site management. This transparency should prevent temporary evidence of past collections serving as an invitation for unwanted contact with SAWs.

In the current study, NERD, a collaborative nonprofit of local archaeologists and recreational divers, proved to be an invaluable research ally. With access to their own watercraft and SCUBA equipment, NERD volunteers were fully capable of repeating the microbiological surveys, if given the requisite collection materials. The possibility of follow-up studies aimed at additional wreck sites and seasonal influences has been discussed with NERD. This kind of knowledge transfer may help local communities develop a stronger sense of stewardship for UCH resources (Viduka 2020). For nonlocal researchers, project costs are greatly reduced by not needing to return for sample collection. Admittedly, the expenses associated with the laboratory analysis, both in terms of consumables (DNA extraction kits) and equipment, likely negate the ability of management agencies and citizen scientists to perform this portion of the project. In such cases, partnerships with academic institutions that have access to the requisite facilities offer a way forward.

Future Directions

Despite the apparent successes of the biofilm collection method and its potential use in furthering our understanding of SAW microbiomes, the research team recognizes the continued challenges inherent in MIC research. Sample areas were chosen based on observed corrosion, but a multitude of influences can stimulate corrosion, many of which are independent from a site's microbiology. The lack of physical indicators unique to MIC and distinct from those associated with abiotic modes of corrosion renders determinations of causation difficult (Little and Lee 2022:182). Furthermore, the metallurgic composition of historical aircraft is far from homogeneous. It not only varied by country and plane type but also by component (for example, frames and skins), which required separate manufacturing techniques, such as extrusion, rolling, and casting and stress resistances (Ouissi et al. 2019). Thus, different variations of aluminum alloys were used, resulting in differential deterioration for certain SAW

components in underwater environments (MacLeod 2006; Richards and Carpenter 2018). This is compounded by the confounding effects that environmental parameters—water temperature, salinity, dissolved oxygen, and pH—and human interference have on the corrosion process (Ezuber et al. 2008; Nişancioğlu 2007). The metallurgic complexity and use of different alloys also likely affect the taxonomic makeup and functional genes of microbial biofilms (Zhai et al. 2022; Zhang, Ma, Duan, et al. 2019; Zhang, Ma, Zhang, et al. 2019), although this has yet to be formally tested for SAWs. Together, these realities may make attributing site damage to MIC and predicting its role in site decay a difficult task.

Yet, despite these challenges and uncertainties, there is reason to invest in the baseline research of SAW microbiomes. Enabled by the nondestructive in situ sampling methodology developed here, follow-up studies can begin to tease out the various influences related to environmental conditions and anthropogenic activities, as well as substrate metallurgy and the presence of chemical residues and treatments. Additionally, the taxonomic baselines established initially will serve as invaluable comparisons for future studies that track changes in a site's ecology. Although outside the scope of this methods-focused article, climate change serves as an obvious motivator of this research, as shifting ocean conditions are expected to affect all forms of UCH (Gregory et al. 2022; Wright 2016). Attempting to predict the exact effect that climate change will have on SAW microbiomes, and in turn, how that will affect site preservation is currently impossible. Nonetheless, there is reason to believe that rising temperatures and increased ocean acidification will alter the makeup and chemistry of marine biofilms (Dobretsov et al. 2019). Thus, collecting the necessary data now, which will allow us later to empirically assess how climate change affects SAW microbiomes, should remain a desirable objective, especially given the low cost and feasibility of the sampling methodology. The development of the current study's protocols, and the greater interest in MIC overall, is intended to be the kind of proactive approach espoused within the concept of "heritage at risk" (Cochran et al. 2023).

Until more conclusive investigations can be conducted into the impact that microorganisms have on the preservation of SAWs, it would be inappropriate to assume any definitive correlation between biofilm and increased correlation. Instead, more possibilities can and should be evaluated using this sampling methodology. For example, in addition to analyzing taxonomic datasets for potential evidence of MIC, SAW biofilm studies should also assess the possibility of microbial growth being protective. Marine biofilms can influence metallic corrosion rates through the ennoblement of surface metals, thus inhibiting galvanic corrosion—a result of more noble metals in contact with less reactive ones (Little et al. 2013; Örnek et al. 2002; Zuo 2007). If this is proven true for SAWs, then microbial biofilm would appear beneficial for site preservation, with its disruption possibly igniting localized corrosion. Questions regarding the potential dual influence of biofilm, as both a source of MIC-linked bacteria and an anticorrosive buffer, underscore the importance of sound collection methods that establish baseline understandings of site microbiomes. Yet, MIC research is not the only use for this collection method. The overall awareness of wreck ecology within the field of archaeology has spurred interest in how a wreck's presence affects the local biology. Recently, a NOAA-funded project used the syringe method of collecting biofilm as a part of a multidisciplinary assessment of SAWs off the island of Saipan (NOAA 2023). The sampling protocols resulted in the successful collection of in situ biofilm samples for eDNA analysis that will be used to track biodiversity and discern the artificial reef role of SAWs.

Conclusion

Currently, the relevance of MIC to SAW management remains preliminary and will require an empirical assessment before more definitive conclusions can be reached. This goal will only be achieved by using effective collection methods for obtaining in situ biofilm samples. In the past, proxies for wreck microbiomes, including test coupons, have yielded valuable insights into the effect MIC has on UCH sites. However, the intricacies of microbial colonization and the microscopic scale in which differences can manifest warrant consideration. This should encourage site managers to acquire as accurate an interpretation of a site's microbiome as possible through preservation-oriented, routine, nondestructive monitoring efforts.

This article presents the first attempt to collect microbial DNA from four SAWs off the coast of Hawaii. The sampling methodology was designed specifically for use by SCUBA divers and was proven to be both cost and time efficient, which further enables citizen science collaborations. The initial results, including sample weights, DNA yields, and sequencing success, highlight the feasibility of the collection and DNA extraction processes. This study is a necessary first step in elucidating information about if and how MIC may be affecting SAWs. Answering these questions in their entirety, however, will be made difficult by the sheer complexity of MIC processes and the multitude of factors, biotic and abiotic, that affect SAW preservation in marine environments.

For this reason, the sampling efforts enabled by this study's protocols should be used in conjunction with additional forms of analyses, including environmental, electrochemical, and photogrammetric studies. Together, these varying lines of evidence can be used to prioritize preservation threats and responses before irreparable damage occurs. This research is especially timely, because a foundational understanding of which microbes are present on SAWs and the environmental factors influencing these communities is warranted. Because changing ocean conditions will undoubtedly alter the seas' microbial distribution, it is imperative that archaeologists consider the possibility that this may further render SAWs as heritage at risk.

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