Assessment of Bacterial Activities within Shipwrecks along Iwafe Estuary of New Calabar River in Rivers State

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Authors' contributions

This work was carried out in collaboration among all authors. Author CGDO designed the study, wrote the protocol and first draft of the manuscript. Authors CGDO, EIO and YMT managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Qualitative assessment of bacterial activities within shipwrecks along Iwafe estuary of New Calabar River was studied. The study was aimed at identifying the various bacterial communities present in a shipwreck ecosystem in an estuary located in the Niger Delta. Water, sediment and rusticle samples were collected aseptically from three shipwrecks submerged in the river and were subjected to Biological Activity Reaction Test (BARTS) to investigate the different bacterial activities within the shipwrecks. Three bacterial communities were identified, Heterotrophic Active Bacteria, (HAB), Iron Reducing Bacteria, (IRB) and the Sulphate Reducing Bacteria, (SRB). In less than 24hrs after incubation the HAB test for all the samples showed complete aerobic activity producing a predicted population of 5.4×10^6 Pac/ml for water, 5.4×10^6 Pac/g for sediments and rusticles. The days of delay (dd) revealed the presence of SRB in the sediment and rusticle samples only. Shipwrecks ecosystem in New Calabar Estuary showed bacterial involvement, in corrosion processes even in shallow water ecosystem.

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1. INTRODUCTION

Microorganisms are ubiquitous because they are found in virtually all ecosystems including marineand freshwater, soil, sediment etc., in association with plants, animals and humans. They play vital role in geochemical cycles of minerals in nature, which contributes in the maintenance of environmental stability [1]. For instance, microbes in the ocean play an active role in the dynamics of minerals, detritus biodegradation and nutrient cycling [2].

Microorganisms are found in virtually all sections of the aquatic ecosystem including water column at various depths, the sediment surface and sediments column. Cyanobacteria are heterotrophic aerobic bacteria that play an important role in ocean processes, including the development of stromatolites. Living in colony, the cyanobacteria are autotrophs and produces oxygen during the process of photosynthesis. These organisms are capable of existing in practically any environment accumulating energy from a variety of sources, ranging from organic to inorganic substances. Some bacteria are to utilize organic substances as their source of energy and carbon are known as heterotrophically active bacteria (HAB) or simply heterotrophs or organic busters [3]. They can function under aerobic and anaerobic conditions. Seeing that HAB are major players of degradation of organic their presence becomes crucial in aerobic environment. Other groups of microbes found in the aquatic systems especially in coastal areas include iron oxidizing bacteria (IOB). Iron reducing bacteria, (IRB), and sulphate reducing bacteria (SRB) among others. These different microbial groups are involved in the food chain and transformation of minerals in the ecosystem. Relationally, marine microbes' communities can evolve rapidly in response to environmental shifts and could be used as indicators of environmental change [4].

Global trade involves the transportation of goods between countries including food, machineries, petroleum and petroleum products etc. Ship is the cheapest means of transportation of bulky goods between countries. When a ship becomes damaged beyond repairs, it is sometimes abandoned. Damaged and abandoned ships, often called shipwrecks are common in many coastal towns especially in developing countries, where there are weak or non-existent regulations for the management of shipwrecks.

A shipwreck is the remains of a damaged ship, which are found either beached on the sand or sunken to the bottom of a body of water. Shipwrecks are often not intentionally introduced into the water body; they were once functional sea-going vessels that accidentally became wrecked (as a result of different factors) and often abandoned by their owners due to high cost of removal [5,6].

When a ship is wrecked, accumulation occurs including silt, sand and sediments organic matter with biota. The accumulation of these organic materials on the metal surface results in the formation of artificial reef containing biofilms [7]; which microbially induced corrosion [8]. The wreck also affected by the physiochemical properties of the water and sediments including temperature, depth, current, salinity and pH. Marine creatures such as corals, octopus, mussel and crustaceans begin to colonize and graze on the benthic bacteria and planktons that settled with the silts and sediments on the wreck [2]. Together with the prevalent abiotic factors the organisms cause degradation of the ship. This damage is referred to as "stratification and contamination" [9]. Stratification brings about the loss of aesthetics on the ship's original state, while chemicals change in the water column also affects the shipwrecks [9,10].

Shipwrecks have several environmental impacts including obstruction to marine transportation, risk of marine accidents, colonization by invasive species and causes siltation of navigable channels. Recent studies have shown that shipwrecks cause environmental pollution [11,12,13] and therefore a cause for concern.

In the past, shipwrecks had been thought to be time capsule frozen in time [14,15] whereas microbial corrosion occurs converting the wrecks to red dust in the sediment. Microbial degradation of complex materials, is not done by individual species, but a consortium of organisms [16]. Hence, the need to study the various groups of microbes associated with shipwreck and their possible role in biodeterioration of the wrecks. Investigation of wrecks using the traditional culture methods might exclude the non-culturable population [17]. Hence, in this study of shipwreck at Iwafe Creek, located at the upper reaches of the New Calabar, River, Niger Delta, we used a modified cultural technique based on the biological activity testing to identify the groups of

microbes associated with the wrecks. The Activity Reaction Test (BARTs) approach is a modified culture-dependent method, it does not reveal the individual genus or species in the samples but rather the consortia of bacteria community present and their possible functions.

2. METHODOLOGY

2.1 Sampling Site and Sample Collection

Water samples were collected with sterilized polypropylene bottles. Sediment samples were collected using a Van Veen grab into a sterile polyethene bag. Rusticle samples were collected aseptically by scrapping the disintegrating metals of the ship hull and transferred to polyethylene bags. All the samples were subjected to Biological Activity Reaction Tests (BARTs) immediately after collection.

2.2 Biological Activity Reaction Tests (BART)

BART^{TM} testers were used as designed by Cullimore, [3] to determine the types and the level of bacterial activity present in the samples. The water, sediment and rusticle samples were subjected to BART test, in order to get a semi-quantitative measurement of the number of bacteria in each type of samples. DroyconBART^{TM} kits were used to approximate population of predicted active cells per ml (Pac/ml) which is equivalent to colony-forming units per milliliter (CFU/mL). The following BART testers HAB-BART^{TM}, IRB-BART^{TM} and SRB-BART^{TM} (Figs. 2-4) were used in screening the

Fig. 1. Study locations plotted on Google Earth image
Scale 1:100 000
Sampled points
Key: Point A. Shipwreck 1 as SH-D
Point B. Shipwreck 2 as SH-E
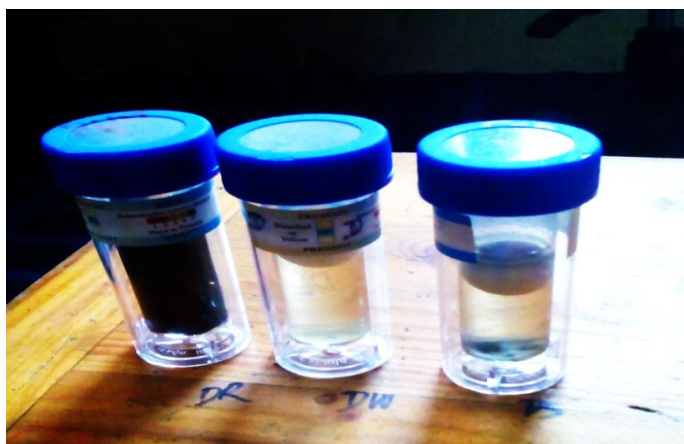*Point C. Shipwreck 3 as SH-F*

Fig. 2. HAB BART tester

Fig. 3. IRB BARTs

activities of heterotrophic aerobic bacteria, iron related bacteria and sulphate reducing bacteria respectively as described by Cullimore, [3,18]. The Pac/ml was determined using the certificate of analysis which accompanied each box of tester.

3. RESULTS

According to Cullimore [3], the BART test was designed such that the results are evaluated based on the number of days needed for the reaction to occur (days of delay, dd) and the strength of the reaction as compared to a BART comparator chart. For all the samples (water, sediment and rusticles) aggressive reactions were observed in less than 24h for total heterotrophic active bacteria, (HAB) (Table 1). Within 7h the total aerobic tests on all the water column had exhibited aerobic activity as shown

by the complete bleaching of methylene blue dye in the test medium. For the sediment and rusticle samples in the three wreck sites the aggressive reactions were observed within 18h. In less than 24 hours of incubation the aerobic test for all the samples showed complete aerobic activity, consequently yielding a predicted population of 5.4×10^6 Pac/ ml for water and 5.4×10^6 Pac/g for sediment and rusticle samples.

Iron related bacteria generated several reactions that occurred within the tester (Table 2). The first reaction was used to determine the time lapse for IRB and also the predicted active cell population triggered off in the phase two reactions. Formation of foam which is a distinguishing characteristics of phase two reaction in IRB tester was seen on all the samples on day 2 except for water sample from shipwreck 1 that generated foam on day 3.

Fig. 4. SRB BARTs

Table 1. Predicted population of heterotrophic aerobic bacteria

Samples	Days to reaction	Predicated heterotrophic active bacteria population (Pac per unit of sample)
SH-1W	< 7 hrs.	5.4×10^6 pac/ml
SH-1S	≤ 18 hrs.	5.4×10^6 pac/g
SH-2W	< 7 hrs.	5.4×10^6 pac/ml
SH-2S	≤ 18 hrs.	5.4×10^6 pac/g
SH-3W	< 7 hrs.	5.4×10^6 pac/ml
SH-3S	≤ 18 hrs.	5.4×10^6 pac/g
SH-1R, SH-2R, SH-3R	≤ 18 hrs.	5.4×10^6 pac/g

KEY: SH-1W = water sample from shipwreck 1, SH-1S= sediment sample from shipwreck 1, SH-1R rusticle sample from shipwreck 1; SH-2W = water sample from shipwreck 2, SH-2S= sediment sample from shipwreck 2, SH-2R rusticle sample from shipwreck 2; SH-3W = water sample from shipwreck 3, SH-3S= sediment sample from shipwreck 3, SH-3R rusticle sample from shipwreck 3,
NOTE; Pac/mL = Predicted active cells per ml, which may be considered equivalent to the traditional colony forming units per ml (cfu/ml)

Foam formation was at its peak on samples SH-1R, SH-2S, SH-2R, and SH-3W while for SH-2W and SH-3R there was moderate occurrence of foam formation and it was faintly noticed for samples SH-1R, SH-1S and SH-3S. Cloudy growth, another phase two reaction was sparsely notice in the BART Tester. The cloudiness of the medium was briefly observed as it gave way to the Phase three reaction. The occurrence of Brown ring, a characteristics reaction of phase three was evident on all the samples, especially samples; SH-2S, SH-2R, SH-3W, SH-2W, SH-3R and SH-3S. The days of reactions for all the samples fell within 2-5 days range of incubation which is indicative of aggressive reactions (Table 3). The black base (BB) reaction observed in which the base of the tester becomes black around the bottom up to 2 to 4mm of the sides of the ball was notice within day 2 and 3. All the phases of the reactions for all samples was completed within 5days. The strength of the reaction showed a high presence of iron related bacteria in the samples. The black base (BB) reaction occurring without days of delay (dd) suggested that the predicted population of iron related bacteria in all the samples is 1.4×10^5 pac/ml except for SH-1W that recorded 3.5×10^4 pac/ml.

SRB in the rusticles showed high aggressive reaction, evident with the formation of Black base BB on day 3 and day 4. The test indicated a high presence of SRB in all the wreck sites (Table 4).

4. DISCUSSION

BART's works on the principle that specific bacteria within a given sample would be able to generate activities or reactions within a time lapse which is proportional to the active population of those bacteria being investigated. Less active populations would take longer time for activities and reactions to be generated. This time lapse is converted to predicted active cells per mL (Pac/mL) using the standard equations according to BARTs Comparator Chart [18].

Aerobic activity was detected in the water column, sediment and rusticles of the three shipwrecks. A very aggressive reaction, with no day of delay, dd was observed, for all the samples collected. The bleaching of methylene blue to colourless in the HAB-tester shows that the activities of heterotrophic active bacteria were aggressive enough as observed on the samples to have 'respired off' the oxygen in the tester. This means that the methylene blue act as an indicator of bacterial activity being blue under respiratory (oxidative) conditions [19,20]. The bleaching of the medium to colourless was indicative of the amount of respiratory function of the heterotrophic bacterial activities in the samples. The bleaching of the methylene blue that was observed from bottom up wards further

Table 2. Reaction patterns of IRB observed in the samples

Samples ID	Phase 2		Phase 3		Phase 4
	CL	FO	BC	BR	BL
SH-1W	_	*	*	*	*
SH-1S	*	*	**	*	**
SH-1R	*	***	*	**	**
SH-2W	-	**	*	***	***
SH-2S	*	***	**	***	*
SH-2R	**	***	**	***	*
SH-3W	*	***	**	***	***
SH-3S	*	*	**	***	**
SH-3R	**	**	***	***	***

*Keys; *** = Highly occurred, ** = Moderately occurred, * =Just occurred,*
CL=Cloudy growth, FO =Foam formation, BC= Clouds, BR=Brown ring, BL = Black.
KEY: SH-1W = water sample from shipwreck 1, SH-1S= sediment sample from shipwreck 1, SH-1R rusticle sample from shipwreck 1; SH-2W = water sample from shipwreck 2, SH-2S= sediment sample from shipwreck 2, SH-2R rusticle sample from shipwreck 2; SH-3W = water sample from shipwreck 3, SH-3S= sediment sample from shipwreck 3, SH-3R rusticle sample from shipwreck 3

Table 3. Predicted population of iron related bacteria

Samples	Days of reaction	Predicated IRB populations
SH-1W, SH-1S, SH-1R	Day 2	$1.4x10^5$pac/mL
SH-2S, SH-2R		
SH-3S, SH-3R		
SH-3W, SH-2W	Day 3	$3.5x10^4$pac/mL

KEY: SH-1W = water sample from shipwreck 1, SH-1S= sediment sample from shipwreck 1, SH-1R rusticle sample from shipwreck 1; SH-2W = water sample from shipwreck 2, SH-2S= sediment sample from shipwreck 2, SH-2R rusticle sample from shipwreck 2; SH-3W = water sample from shipwreck 3, SH-3S= sediment sample from shipwreck 3, SH-3R rusticle sample from shipwreck 3

Table 4. Predicted population of sulphate reducing bacteria, SRB

Samples	Days	Predicated SRB Populations
SH-1R	Day 3	$1.0x10^5$pac/ml
SH-2R, SH-3R	Day 4	$1.8x10^4$pac/mL Aggressive
SH-1S, SH-2S, SH-3S	Day 7	$5.0x10^2$pac/mL Moderately aggressive
SH-1W, SH-2W, SH-3W	Day 9	Negative non aggressive

KEY: SH-1W = water sample from shipwreck 1, SH-1S= sediment sample from shipwreck 1, SH-1R rusticle sample from shipwreck 1; SH-2W = water sample from shipwreck 2, SH-2S= sediment sample from shipwreck 2, SH-2R rusticle sample from shipwreck 2; SH-3W = water sample from shipwreck 3, SH-3S= sediment sample from shipwreck 3, SH-3R rusticle sample from shipwreck 3

substantiate that aerobic bacteria activities predominated in the three samples (Fig. 2). The repository process that led to a reductive state in all the samples occurred in less than 24hrs, which revealed the high populations of heterotrophic aerobic bacteria recorded in the samples and their activities within the samples from the three wreck sites.

Risk analysis for this reaction would primarily relate to aerobically active bacteria. UP reactions are clear signal that the HAB- are dominated by aerobic (oxidative) activities and these bacteria tend to grow prolifically within the three samples possibly forming biofilm on the sediments and rusticles.

Cloudy growth though briefly observed is the first reaction that usually occurs in the IRB-BART tester, its presence does signify that the analyzed sample is oxidative. The observation of a foam ring (FO) which occurred only when the gas bubbles have risen to form a foam ring was at about 75% of the way round the floating BART ball in IRB population.

The phase 2 and 3 reaction patterns were used to determine the dominant iron related bacteria, IRB and their population size [21,3]. The days of delay, and consequently predicted bacteria active cell population for the samples were also determined using phase 2 reaction. The reaction patterns observed were indicative of the nature of iron related bacteria found in the study samples. According to Cullimore [22] the sequence of reaction can reveal potential IRB present in the samples. An observable foam formation (FO) that is, a complete foam ring was formed around the tester ball. FO usually reveals fermentative activities of IRB dominating in the tester with the evolution of gases that formed the bubbles which were locked into the foam layers around the ball. In this study the foam formed did not collapse throughout the incubation period but continued to grow which is indicative of nitrogen gas [23]. FO reaction therefore suggests that the samples are likely to be dominated by iron reducing bacteria. The reaction progressed with Cloudiness, CL which was still observed as a phase 2 respiratory function (Fig. 3). Another significant reaction observed was the phase 3 reaction, the brown cloudy, BC and brown ring, BR reactions. BC reaction indicates possibly a mixed heterotrophic IRB while BR reaction is usually an enabling environment for the sheathed iron oxidizing bacteria such as *Gallionella* and *Crenothrix* [22]. Lastly, the phase four IRB

reaction is the BL (black liquid) which terminates the IRB reactions. The series of reactions observed in all the samples from the three wrecks had so much to reveal about the activities of iron bacteria engaging in the degradation of the shipwrecks. Although the scope of this study did not identify the individual IRB, but is obvious that it was a combination of the iron reducers and the iron oxidizers that are involved in the corrosion of the wrecks.

High aggressive reaction was observed within the first two days of the incubation in almost all the samples with the population size predicted at 1.4×10^5 Pac/ml. The shorter the time lag then, the greater is the activity and the larger the population. These IRB with high aggressiveness with no day of delay, and high population size is indicative of corrosion on the surface of the ship hull which will in turn impact the microflora of the sediments [24,25]. IRB as a complex consortium is probably a mixture of both the stalked and sheathed bacteria.

SRB-BART^{TM} did not show any reaction for the water samples collected from the surroundings of the three shipwrecks. This suggest the absence of SRB in all the water samples from the three shipwrecks. This result agrees with Cullimore, [22] that SRB may not be present in the free-flowing waters over the site of the corrosion. The detection of SRB has always been challenging because they are anaerobic and tend to grow deep within the biomass as a part of the microbial community. Detection of the SRB is therefore made even more difficult because they grow deeper down within the biomass [23]. The rusticles showed a black base, (bb) reaction with2-3 days of delay, which is still indicative of a high aggressive reaction. This was indicative of the high population (1.15×10^5 and 1.8×10^4 Pac/g) of the SRB present in the rusticles. Furthermore, after 6days of delay, the sediments on all the shipwrecks gave a black base reaction (Fig. 4), indicating a moderately aggressiveness. This result explains that the population of SRB in sediment samples were quite low compared to the rusticles. Black base, may be considered to mean that there is a deep-seated anaerobic SRB infestation, that could result to pitting and perforation particularly of the steel materials of the wreck hulls [3,26]. Secondly, bb reaction connotes the reduction of sulfate to hydrogen sulfide which in turn reacts with the ferrous iron present in the rusticle to form black iron sulfide. The corrosion of the shipwreck hulls cannot only be left to chemical corrosion but significantly

observed from our study is also due to a combination of activities by different bacteria groups especially the SRB that generates Hydrogen sulfide (H_2S) that is significant in the initiation of corrosive processes.

The impact of SRB on artificial substrates relates more directly to corrosion generated by the biomass dominated by sulphate reducers. This study showed the presence of microbial groups that might have contributed to the deterioration of shipwrecks as seen through the increase in populations of HAB, IRB and SRB observed on the rusticles.

5. CONCLUSION

The natural breakdown of shipwrecks over time, whether wooden or metal-hulled, is influenced heavily by microorganisms. Various types of microorganisms colonize a shipwreck almost immediately after the vessel comes to rest on the seafloor. Investigation of potentially active bacterial communities eating away the hull of the wrecks cannot be limited to pure culture approach alone. An attempt to culture 'pure' cultures essentially connotes destroying the 'pure' bacterial community and identifying only those that could only be cultured. BARTs tester formulation although a culture-dependent method appeared to be more effective at the detection of potentially active bacterial communities in shipwrecks and other environments experiencing corrosion. The involvement of bacterial communities in shipwreck environment could be revealed through BARTs concept. This study attempted to identify the presence of the bacterial communities such as HAB, IRB and SRB on the wrecks and their possible functions on Shipwrecks.

6. LIMITATION

The limitation of this study is that it was a qualitative investigation, which we hope to present a detailed quantitative analysis which will identify the genus and species of the bacteria involved in corrosion of shipwreck submerged in an estuary habitat using biochemical analysis and culture independent methods.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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