



RESEARCH ARTICLE

REVISED Laboratory growth of denitrifying water column microbial consortia from deep-sea shipwrecks in the northern Gulf of Mexico [version 3; referees: 2 approved]

Dhanya Haridas ¹, Justin C. Biffinger^{2,3}, Thomas J. Boyd ³, Preston A. Fulmer³, Leila J. Hamdan⁴, Lisa A. Fitzgerald³

¹American Society for Engineering Education, Washington, DC, USA

²Chemistry Department, University of Dayton, Dayton, OH, USA

³Naval Research Laboratory, Washington, DC, USA

⁴University of Southern Mississippi, Hattiesburg, MS, USA

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Abstract

Background: Shipwrecks serve as a rich source for novel microbial populations that have largely remained undiscovered. Low temperatures, lack of sunlight, and the availability of substrates derived from the shipwreck’s hull and cargo may provide an environment in which microbes can develop unique metabolic adaptations.

Methods: To test our hypothesis that shipwrecks could influence the microbial population involved in denitrification when a consortium is grown in the laboratory, we collected samples proximate to two steel shipwrecks in the northern Gulf of Mexico. Then under laboratory conditions, we grew two independent denitrifying microbial consortia. Each consortium was grown by using the BART assay system and analyzed based on growth kinetics, ion chromatography and 16S amplicon sequencing.

Results: Both denitrifying consortia were different from each other based on varied growth profiles, rates of nitrate utilization and 16S amplicon sequencing.

Conclusions: Our observations conclude that the laboratory grown water column microbial consortia from deep-sea shipwrecks in the Gulf of Mexico are able to undergo aggressive denitrification.

Keywords

shipwreck, denitrification, microbe, 16S, Gulf of Mexico

Open Peer Review

Referee Status:

	Invited Referees	
	1	2
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REVISED version 2 published 12 Jun 2018		 report
version 1 published 13 Oct 2017	 report	 report

- Maxim Ru-bin Blum** , Max Planck Institute for Marine Microbiology, Germany
Israel Oceanographic and Limnological Research, Israel
- Dimitri Kalenitchenko**, Université Laval, Canada

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Comments (0)

Corresponding author: Lisa A. Fitzgerald (lisa.fitzgerald@nrl.navy.mil)

Author roles: **Haridas D:** Conceptualization, Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; **Biffinger JC:** Conceptualization, Supervision, Writing – Review & Editing; **Boyd TJ:** Investigation, Writing – Review & Editing; **Fulmer PA:** Resources, Writing – Review & Editing; **Hamdan LJ:** Resources, Writing – Review & Editing; **Fitzgerald LA:** Funding Acquisition, Resources, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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REVISED Amendments from Version 2

Version 3 has a revised Discussion section. The reviewer recommended rewording the statement “Each shipwreck site...” to “The water mass passing by each shipwreck site...” to reflect the fact that the DN consortium identified at each site was obtained from the water sample isolated from a region surrounding the shipwreck and not the shipwreck itself.

See referee reports

Introduction

The biogeochemical process that transforms dissolved inorganic nitrogen to nitrogen gas is known as denitrification (DN). This metabolic pathway impacts the nitrogen (N) cycle by returning elemental N to the atmosphere^{1,2}. It can alternatively be defined as the reduction of more oxidized forms of nitrogen (NO_3^- , NO_2^- , NO and N_2O) to N_2 gas, where it can be linked to the oxidation of iron, sulfur and reduced carbon species³. It is primarily performed by facultative heterotrophic or chemolithoautotrophic bacteria under anoxic or very low-oxygen conditions³, where microorganisms utilize nitrate or nitrite as the terminal electron acceptor⁴. DN, alongside other biogeochemical processes (carbon and sulfur cycles), plays a key role in maintaining the nutrient balance in marine habitats⁵.

In recent years, shipwrecks have been identified as areas from which novel microbial species have been isolated, because of the introduction of foreign material to the area⁶. Thus, they would be an ideal location to discover unique microorganisms and metabolic activity, as these areas are known to be diverse habitats for macroorganisms in the marine environment⁷. The goal of this research was to prospect for novel DN microbial consortia near deep-sea shipwrecks in the Gulf of Mexico, culture the consortia under laboratory conditions and determine their DN activity. In this study, we collected water samples proximal to two steel shipwreck sites located in the northern part of the Gulf of Mexico, and analyzed the denitrifying and culturing potential of the microbial consortia obtained from the two sites.

Methods

Environmental sampling

We obtained water samples ~600 m down current from two steel-hulled shipwrecks investigated as part of the Shipwreck Corrosion, Hydrocarbon Exposure, Microbiology and Archaeology (SCHEMA) study, which addresses the effect of the 2010 *Deepwater Horizon* spill on deep-sea shipwrecks in the northern Gulf of Mexico (<http://www.boem.gov/GOM-SCHEMA/>). Samples were collected onboard the R/V *Pelican* using a CTD-rosette during the PE15-22 expedition in May 2015. The shipwreck *Halo*, is a steel-hulled steam tanker, resting in ~140 m of water, and ~50 miles west of the Mississippi River's Southwest Pass. The double steel-hulled German U-Boat *U-166* shipwreck rests in ~1400 m of water within 10 km of the Macondo wellhead, the epicenter of the 2010 *Deepwater Horizon* spill. The water samples were stored in sterile plastic bottles at 4°C until further use.

Enrichment of denitrifying microbial consortium

The commercially available denitrifying Biological Activity Reaction Test (DN-BART) assay (HACH, Colorado, USA) was used to enrich for DN bacteria. Briefly, the lyophilized media in the DN-BART was solubilized with 15 mL water sample from either the *Halo* or *U-166* shipwreck site. The assay was performed based on manufacturer's instructions, with the exception that the assay was incubated for 30 days instead of the suggested 4 days. The enriched microbial consortium obtained from the DN-BART assay was used as the inoculum to perform the growth curve and Ion Chromatographic (IC) studies.

Growth curve studies

Nunc tubes (Chemglass, NJ, USA) containing 10 mL of modified Indole Nitrite medium (pancreatic digest of casein 20 g/L, disodium phosphate 2 g/L, dextrose 1 g/L, potassium nitrate 1 g/L) were used for all assays. Sterile nitrogen gas was bubbled through the media for 15 min prior to inoculation to de-gas and establish an anaerobic environment. The enriched *Halo* and *U-166* DN consortium derived from the DN-BART assay was used as the inoculum to perform the growth curve studies. Each tube was inoculated with 100 μL of DN-BART consortium. The inoculated Nunc tubes were analyzed for a period of 24 h at 30°C (Excella E25, Fisher Scientific, MA, USA). The optical density of the samples was measured at 600 nm (OD_{600}) every 2 h post-inoculation using the Spectronic 200 Spectrophotometer (Thermo Scientific, PA, USA) over a 24 h period after inoculation. All inoculated samples were done in triplicates.

Ion chromatography studies

The nitrate/nitrite concentrations were tracked by IC using a Dionex IC-3000 IC fitted with an IonPac AS16 column. The mobile phase was 9 mM Na_2CO_3 at 1.0 mL/min and a Dionex 7 anion standard mix was used for calibration before and half way through sample runs. The DN microbial consortium was cultured similarly to the growth curve assay (*i.e.* anaerobically to enable the denitrifying conditions to develop). The *Halo* and *U-166* cultures were sampled (1 mL) using a sterile syringe and needle (BD, NJ, USA) every 2 h over a period of 24 h from the inoculated Nunc tubes. The sample was centrifuged at $12,000 \times g$ for 3 min in a sterile 1.5 mL eppendorf tube (Eppendorf, NY, USA). The supernatant was additionally syringe filtered (0.2 μm filter Syringe filter, SC, USA) and stored in 1 mL IC vials (Thermo Scientific, Pittsburg, PA). All analyses were performed in duplicate or triplicate. Nitrate concentrations were often above the calibration level (100 mg/L) and are annotated as estimated values (*J*). Standard errors for replicate measurements ranged from 0 to 2.26% with an average of 0.31% for the aggregate runs.

16S amplicon sequencing

To determine the DN phylotypes present in the DN consortia, genomic DNA was isolated using DNA isolation solutions I, II and III (bioWORLD, Dublin, OH) from the *Halo* and *U-166* denitrifying microbial consortia after 24 h of growth and 16S amplicon sequencing of the V4 region and bioinformatics analysis

was performed by Seqmatic (Fremont, CA). High throughput NGS was performed using the Illumina MiSeq platform using 2x250bp reads and the FASTQ data was processed using the Qiime pipeline.

Results

Hydrographic conditions of the water samples

The depth, temperature, salinity and dissolved oxygen (DO) of the water samples obtained from the region proximal to the *Halo* and *U-166* shipwreck sites were obtained with the CTD (Table 1). The water sample obtained proximal to the *U-166* shipwreck site had a higher DO content (6.6 mg/L) and lower temperature (4.3°C), as compared to the *Halo* shipwreck site that had 4.1 mg/L DO and 17.7°C. This indicates that both the water samples have varied hydrographic conditions.

Denitrifying microbial consortia

Both water samples were also analyzed for the presence of denitrifying microbial consortia using the commercially available DN-BART assay. Upon performing the assay, it was observed that *Halo* and *U-166* water samples did not produce foam or bubbles around the ball or in the tube after 4 days, the recommended duration for developing a positive reaction. Hence, due to the nature of the unique water samples, the assay was continued for 30 days. Following the 30 days, foam was detected around the ball, providing evidence for potential DN microbial consortia from the *Halo* and *U-166* shipwreck sites. The DN consortia that were enriched using the DN-BART assay were further analyzed for microbial growth, nitrate/nitrite media concentrations and microbial composition over 24 h.

Growth profile of the DN microbial consortium

To determine the growth profile of the DN consortia, the growth curve assay was performed. It was observed that the DN consortia from *Halo* grew ($OD_{600} = 0.980$) much slower than the *U-166* consortia ($OD_{600} = 2.448$) over the 24 h period (Figure 1; Dataset 1). Thus providing the first evidence that both the DN consortia are different from each other. All analyses were performed in triplicate (Figure 1).

Table 1. Hydrographic conditions of water column samples collected proximate to two steel shipwrecks *Halo* and *U-166* in the northern part of Gulf of Mexico. The depth, temperature, salinity and dissolved oxygen (DO) for the water samples collected from *Halo* and *U-166* are listed below.

	<i>Halo</i>	<i>U-166</i>
Depth (m)	141.3	1448.9
Temp (°C)	17.7	4.3
Salinity (PSU)	36.3	35.0
DO (mg/L)	4.1	6.6

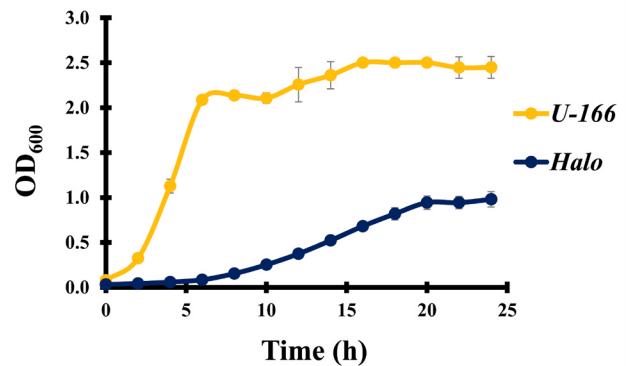


Figure 1. Growth curves from *Halo* and *U-166* denitrifying population. The growth profiles of both DN consortia were analyzed for a period of 24 h at OD_{600} . The *U-166* DN consortia grew at a much faster rate compared to the *Halo* DN consortia.

Nitrate and nitrite ion chromatography analysis

Ion chromatography (IC) studies were performed to identify the denitrifying potential of the isolated microbial consortia. The *Halo* microbial supernatants showed a steady decline in nitrate concentration (734 mg/L to 0.7 mg/L) as microbial growth entered into the logarithmic growth phase. As the nitrate concentration decreased, there was an increase in nitrite concentration from 1.4 mg/L, to a maximum of 130 mg/L and tapered down to 4.3 mg/L at 24 h. The *U-166* microbial consortium rapidly converted nitrate into nitrite, as shown with a decrease in nitrate concentration (730 mg/L to 2.5 mg/L) followed by an increase in nitrite concentration (0 to 240 mg/L), which was later followed by a subsequent decrease in nitrite levels to 2.2 mg/L (Figure 2; Dataset 2).

16S amplicon sequencing

Since the growth curve and IC studies indicated that the DN consortia from *Halo* and *U-166* are mutually exclusive, we wanted to determine the microbial composition of both *Halo* and *U-166* DN consortia using 16S amplicon sequencing. The *Halo* DN consortium primarily consisted of the *Pseudomonas* genus (98.1%), while the *U-166* DN consortium was dominated by the *Citrobacter* genus (72.6%). At the species level, *P. tropicalis* and *P. aeruginosa* for *Halo*, and *C. werkmanii* and *C. freundii* for *U-166* were primarily detected (Figure 3; Dataset 3)), thus indicating that both DN consortia are mutually exclusive.

Dataset 1. Growth curve assay: Growth curve studies were performed over a 24 h period for both the *Halo* and *U-166* DN consortia

<http://dx.doi.org/10.5256/f1000research.12713.d179765>

The optical density of the cultures were measured every 2 h at 600nm.

Dataset 2. Ion chromatography studies: Nitrate and nitrite levels of the DN consortia isolated from *Halo* and *U-166* sites were determined every 2 h for a period of 24 h using ion chromatography

<http://dx.doi.org/10.5256/f1000research.12713.d179766>

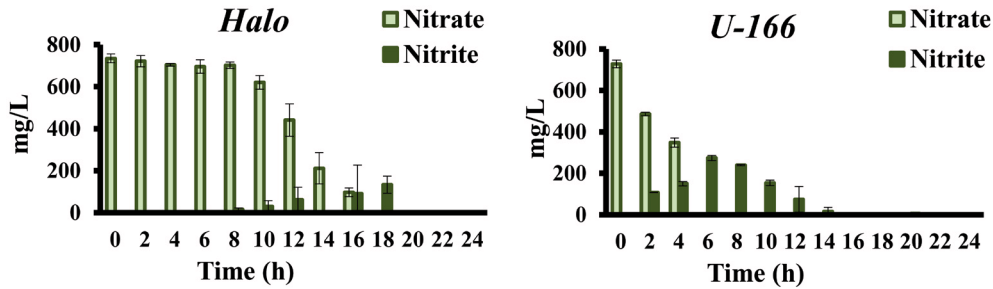


Figure 2. Ion chromatographic studies of the DN microbial consortium isolated from *Halo* and *U-166* shipwreck sites. Samples were collected every 2 h for a period of 24 h and nitrate and nitrite levels were determined. Note: Nitrate values were above the calibration level (100 mg/L) and are thus estimates (but proportional).

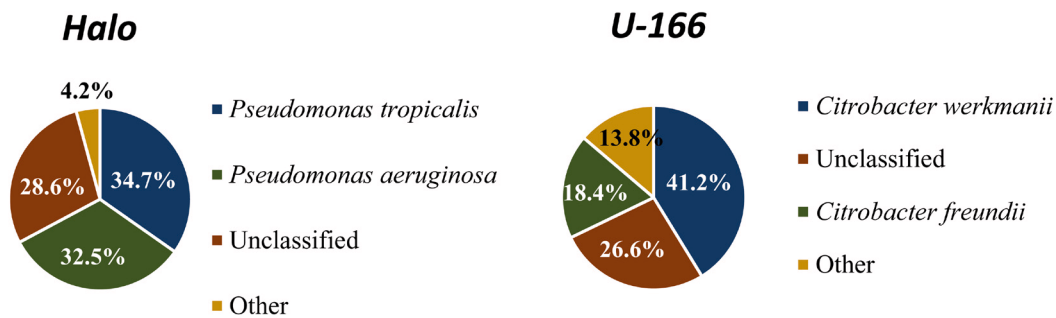


Figure 3. 16S amplicon sequencing on the *Halo* and *U-166* DN consortium after 24 h culturing in Indole Nitrite medium. Genomic DNA was isolated from the *Halo* and the *U-166* DN consortium and the V4 region of the 16S was analyzed.

Dataset 3. 16S amplicon sequencing: Genomic DNA was isolated from DN consortia after 24 h of growth and the V4 region was analyzed using the 16S metagenomics sequencing
<http://dx.doi.org/10.5256/f1000research.12713.d179767>

Discussion

The deep sea, identified with shelf depths greater than 200 m, has been documented to be the largest hypoxic and anoxic environment present on earth⁸. The varied living conditions mentioned earlier induces microbes to adopt unique metabolic adaptations. Hence, the marine dark biosphere has been recognized as a rich resource of unique microbial populations. Apart from the unique microbial life detected in the marine dark biosphere, shipwreck sites located in the deep sea also serve as a rich source of distinct flora and fauna⁶. Using two different shipwrecks at varying depth and material allows for the comparison of the metabolic activity of DN microbial consortia isolated from steel shipwreck sites.

One of the biggest challenges in characterizing new microbes from the deep-sea is the ability to successfully culture them in the laboratory. The initial approach to identifying a DN consortium was to assess growth using the commercially available DN-BART assay. The DN-BART assay provided the necessary nutrients in a modified nitrate medium and the presence of a potential DN microbial consortium from both *Halo* and *U-166* shipwreck sites was confirmed. To further characterize the DN microbial

consortia, the turbidity of the media was monitored and the nitrate/nitrite concentrations were examined every 2 h over a 24 h time period.

The water sample from the *Halo* shipwreck site was able to grow under the conditions set forth in this study, but at a much slower rate when compared to *U-166*. When the *Halo* microbial consortium began its logarithmic growth, there was a steady decline in the nitrate concentration and a subsequent increase in the nitrite concentration in the supernatant. The *U-166* DN consortium also grew and the turbidity of the culture was greater as compared to *Halo* DN consortium. The IC studies further corroborated the decrease in nitrate levels and a concurrent increase in the nitrite concentration during the logarithmic phase of growth. Further, the *U-166* consortia consumed nitrite, most likely as nitrate was completely consumed, at a rate 2 times slower than that observed in the *Halo* DN consortium (30 mg/L compared to 65 mg/L respectively). To determine the DN phylotypes present in the DN consortia, 16S amplicon sequencing was performed, and it was observed that at the species level *P. tropicalis* and *P. aeruginosa* for *Halo* and *C. werkmanii* and *C. freundii* for *U-166* were the most dominant and are known denitrifiers^{9,10}. It was also observed that the *Citrobacter* dominating the *U-166* DN consortia consumed nitrate at a rate that was faster than other industrial microbial consortia containing *Citrobacter* adapted for denitrification¹¹.

This study indicates that *Halo* and *U-166* were good prospecting sites for novel microbial consortia related to

denitrification. The water mass passing by each shipwreck site has a distinct DN consortium which can be grown under laboratory settings. The *U-166* DN microbial consortium performs denitrification at a much faster rate than the *Halo* DN microbial consortium and most known industrial microbial consortia. This elevated DN activity could be the result of local hydrodynamic conditions or the proximity to the shipwreck, but additional studies are needed to identify the exact parameters. In conclusion, both DN consortia isolated from novel prospecting sites (shipwrecks) in the Gulf of Mexico can be cultured in the laboratory and can utilize a DN metabolic pathway for growth.

Data availability

Dataset 1: Growth curve assay: Growth curve studies were performed over a 24 h period for both the *Halo* and *U-166* DN consortia. The optical density of the cultures were measured every 2 h at 600nm. DOI, [10.5256/f1000research.12713.d179765](https://doi.org/10.5256/f1000research.12713.d179765)¹²

Dataset 2: Ion chromatography studies: Nitrate and nitrite levels of the DN consortia isolated from *Halo* and *U-166* sites were determined every 2 h for a period of 24 h using ion chromatography. DOI, [10.5256/f1000research.12713.d179766](https://doi.org/10.5256/f1000research.12713.d179766)¹³

Dataset 3: 16S amplicon sequencing: Genomic DNA was isolated from DN consortia after 24 h of growth and the V4 region was analyzed using the 16S metagenomics sequencing. DOI, [10.5256/f1000research.12713.d179767](https://doi.org/10.5256/f1000research.12713.d179767)¹⁴

Competing interests

No competing interests were disclosed.

Grant information

Funding was provided by the Office of Naval Research (ONR) through the Naval Research Laboratory (PE# 61153N), the Bureau of Ocean Energy Management (BOEM) No. M13PG00020, and the Navy Platform Support Program.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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<http://www.doi.org/10.5256/f1000research.12713.d179766>
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<http://www.doi.org/10.5256/f1000research.12713.d179767>

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Version 3

Referee Report 09 August 2018

doi:[10.5256/f1000research.17320.r36863](https://doi.org/10.5256/f1000research.17320.r36863)



Dimitri Kalenitchenko

Université Laval, Quebec City, QC, Canada

The manuscript version that has been submitted answered all the comments made on previous version.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Referee Report 27 July 2018

doi:[10.5256/f1000research.16633.r34921](https://doi.org/10.5256/f1000research.16633.r34921)



Dimitri Kalenitchenko

Université Laval, Quebec City, QC, Canada

The revised version of "Laboratory growth of denitrifying water column microbial consortia from deep-sea shipwrecks in the northern Gulf of Mexico", allows a better interpretation of the data thanks to the new mat and met section. I still have concern about the fact that the method used by the author can't really test that each shipwreck has a particular DN consortium. I can understand that samples are difficult to obtain but I would recommend to change a little bit the last paragraph to temper your words.

For example instead of saying "Each shipwreck site" say "The Water mass passing by each shipwreck site". It is a small difference but I think a reader that only read the last section of the discussion would think that the shipwreck itself has its own DN consortium. It might be true but it is not what you test for.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 03 Aug 2018

Dhanya Haridas, US Naval Research Laboratory, USA

Dear Reviewer,

Thank you for taking the time and reviewing the paper again. We have included the edit suggested by you in the discussion.

Respectfully,
Dhanya Haridas

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 29 March 2018

doi:10.5256/f1000research.13771.r31968



Dimitri Kalenitchenko

Université Laval, Quebec City, QC, Canada

The paper entitled “Laboratory growth of denitrifying water column microbial consortia from deep-sea shipwrecks in the northern Gulf of Mexico” by Haridas *et al.* explores the potential of steel shipwrecks to host a specialized community with a focus on denitrifying bacteria. Overall the paper is well written and easy to follow, their results are relevant but before suggesting the acceptance of the manuscript, I suggest that authors resubmit a modified version of the manuscript that include a detailed material and method section.

16S amplicon sequencing section :

- Please provide the DNA extraction kit details
- Please provide the name of the sequencing platform and primers
- Please explain the bioinformatics treatment including the taxonomic affiliation used

Denitrifying microbial consortia :

- Why did the authors extend the incubation time up to 30 days, I am wondering if they checked for contamination from external source.

16S amplicon sequencing :

- I am very surprised about the very low diversity they obtained, I agree that they enriched the community in DN bacteria but with only two tubes for two conditions I am not convinced they do not just select for a contaminant or a bacterium in each tube.
- How did the authors obtained these percentages, BLAST ?

Discussion

I think the conclusion that shipwreck are good prospecting area for novel microbial consortia is highly speculative based on these data especially without a reference point before the water mass flow across the wreck.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Jun 2018

Dhanya Haridas, US Naval Research Laboratory, USA

Dear Reviewer,

Thank you for taking the time to review and suggest comments/edits to the manuscript. We have included information about the DNA isolation kit, the sequencing platform on which the 16S metagenomics studies were performed and the bioinformatics pipeline used. We hope the reviewer will find the responses satisfactory.

You expressed your concern on the need for us to extend the incubation time from 4 days to 30 days for the BART assay. We appreciate the reviewer raising this concern and the reason we decided to extend the incubation time was due to the uncharacterized marine environmental samples used and it is known the concentration of viable microbes is less (as determined by a 3H-Leucine incorporation assay) than when grown in a rich medium. Hence extending the incubation time in this study resulted in an increased concentration of the viable microbial culture which helped determine if the assay was positive for microbes which could perform denitrification. You mentioned another concern about the study selecting for a contaminant. The raw data (excel file) containing all the sequencing tags obtained from the 16S metagenomics sequencing has been provided. The authors would like to mention that the microbial population obtained from the BART assay served as the starting inoculum for all the assays mentioned in the paper and therefore any potential aerobic or facultative aerobic bacteria cultured in the BART assay medium would not have propagated in the Nunc tube due to the anaerobic nature of the environment, thus further diminishing the diversity and potential for an aerobic contaminant. The percentages were part of the bioinformatics report submitted to us by Seqmatic. For example, in the Halo sample the total number of reads at the species level was 169,918 of which 59,117 reads were classified as

Pseudomonas tropicalis (By Seqmatic) thus making it 34.72% of the total species population. We hope we addressed all your concerns in this response and thank you once again for reviewing the manuscript.

Respectively,
Dhanya Haridas

Competing Interests: No competing interests were disclosed.

Referee Report 26 March 2018

doi:10.5256/f1000research.13771.r31967



Maxim Ru-bin Blum  1,2

¹ Department of Symbiosis, Max Planck Institute for Marine Microbiology, Bremen, Germany

² Israel Oceanographic and Limnological Research, Haifa, Israel

This study aims to examine the cultivable denitrifying microbial consortia from marine environment near deep-sea shipwrecks in the Gulf of Mexico. The authors hypothesized that the shipwrecks could influence the microbial population involved in denitrification. The methods used in this study, however, have not adequately tested this hypothesis, as no control samples were obtained. On the other hand, the authors were able to report enrichment of two interesting bacterial consortia, one of which appeared to perform denitrification at a much faster rate than that measured for most known industrial microbial consortia, providing an opportunity to study these bacteria in more detail in the future.

If I understood correctly, only a single water sample from each location was used for the denitrifying Biological Activity Reaction Test, allowing to isolate a denitrifying consortium per sampling site. It is likely that these bacteria occur in a patchy manner in the environment, thus further sampling may reveal very different denitrifying consortia. Broadening the sampling effort is needed to test if either environmental factors or presence of shipwrecks affects the diversity of denitrifying bacteria.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Marine Microbiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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