Reviewer #1 comments to authors'rebuttal

Most of my comments were answered with satisfaction. However, I have few changes/comments (that I put in yellow) that the authors have to consider

Manuscript #PeerJ_24978

Dear Editor,
First, we would like to thank the reviewers who carefully read the manuscript and for their valuable comments and suggestions, which have helped us improve this final version.
We have made the modifications suggested made by the two reviewers, and we include below a detailed answer to all their questions, especially to the ones of reviewer 1.
We believe that this modified and improved manuscript is now at a stage that can be accepted for publication in PeerJ.

Our answers are in blue and the modified text in the manuscript is also in blue.

Reviewer #1

General comments

The authors studied the denitrifying bacterium *Marinobacter hydrocarbonoclasticus* strain 617, (1) by describing the gene clusters encoding the 4 denitrification reductases (deduced from the strain ST2 genome) and some of the regulatory denitrification genes, (2) by performing in silico analysis of DNA binding sequence of FNR and NarL, (3) by measuring the transcript levels of the 4 denitrification genes under microoxic culture conditions, (4) by determining that the *nosRZDFYL* gene cluster is in fact a polycystronic operon, (5) by comparing (i) the growth and (ii) the dynamic of nitrate and nitrite (measuring nitrate and nitrite) of strain 617 cultured under microoxic and anoxic conditions, and (iii) the dynamic of the activities of the NO and N2O reductase in whole-cell (measuring enzymes activity) in these cultures. The manuscript is in general well written but suffers of some inconsistencies.

We can start with the letter to the reviewer and editor (which raised some expectations of what I was expected to see in the text)

1- "The denitrification pathway of a marine bacterium *Marinobacter hydrocarbonoclasticus* is characterized for the first time." Not necessary since the same research group did publish data on strain 617 for the enzymatic activities of denitrification enzymes. Besides the genome of *Marinobacter hydrocarbonoclasticus* ST2 is in NCBI since 2012 and with annotations related to the denitrification pathway.

2- "The approaches used go from transcriptomics to the measure of metabolites." Transcriptomics is usually referred to RNA seq. Here qPCR assays were used. One cannot measure metabolites from qPCR. This provide indication of the level of gene expression.

3- "The results support the hypothesis that this organism mitigates the release of nitrous oxide to the atmosphere." This is a big jump here from artificial culture conditions (with sufficient nitrate and carbon source) to open ocean. N2O was not measured in the cultures. The N2O reductase is active yes, but it does not mean that N2O is not released.

Format

The authors did not respect the format for the "Abstract" as defined in the "Author guides", as well as the interlined spaces (should have been 2-line space)

The line spacing was changed to 2-line space and the abstract was modified to comply with the "Author guidelines".

Introduction

- Throughout all the manuscript, "aerobic culture conditions" has to be changed to "oxic culture conditions"; "microaerobic" to "microoxic" and "anaerobic" to "anoxic".

The manuscript was modified according to the reviewer's suggestion. OK

- The section is too detailed for some aspects, and not enough detail in other aspects. For instances, lines 61-62 can be deleted and also lines 63-75 (the whole paragraph), as the concept described in these lines were not a subject of study in this work.

The introduction was modified as suggested by the reviewer. The paragraph was removed partially, and the part that mentioned the co-existence of genes encoding enzymes catalysing the same denitrification step was kept, as it is relevant for the results presented in this manuscript. OK

- In line 77, not all denitrifying bacteria have their denitrification genes clustered in close vicinity, as it seems to be with *M. hydrocarbonoclasticus*. For instance, *Hyphomicrobium denitrificans*.

We acknowledge that not all denitrification genes are clustered together, but in this sentence what it is mentioned is that the genes encoding the enzymes and accessory factors are clustered together and many times organized in polycistronic units. Therefore, the sentence was not modified. OK

- Line 89: Reference to Zumft 1997. Although Zumft 1997 did a very comprehensive review on Denitrification at that time, it is getting a bit old reference and some more recent reviews can be added.

Two other references were added, one review from 2016, and a on denitrification book (of which two of the authors are editors).

One reference is only "2017" and refers in the list of references to an incomplete reference

- Line 90: delete "other families of bacteria, especially" It is true that not many marine bacteria with denitrifying activities are subject of studies. However, some works should be mentioned. For instance, the

marine bacterium *Methylophaga nitratireducenticrescens* JAM1 with the work of Mauffrey et al 2015 (In Frontiers in Microbiol) and Mauffrey et al. 2017 (in PeerJ). Another marine denitrifying bacterium is *Shewanella baltica* that some works were reported.

The sentence has been modified to account the reviewer's comment. OK

- Line 98. Replace "of urge" by "a need". Still in this line, I would delete "as is the case of *Marinobacter hydrocarbonoclasticus*." However, this could be better developed down to why *Marinobacter hydrocarbonoclasticus* should be used as a model to the characterization of the denitrification pathway in marine bacteria.

Text was modified to account for this suggestion. OK

- Line 100. Replace "species" by "spp." Also, replace "are one of" by "among". For the abundance, I looked to the two references. Yoon 2003 described only *Marinobacter litoralis*, and provided no indication on the abundance of *Marinobacter*. Kaye JZ, and Baross JA. 2000 provided some evidence of abundance of halotolerant bacteria in pelagic water and it seems that *Marinobacter* spp. are found in several samples. I would refer as *Marinobacter* spp. been ubiquitous in marine environments.

We agree with the reviewer and modified the manuscript accordingly. OK

- Lines 102-103. Replace "species" by "strains". Also this sentence has to be rewritten. Grimaud 2012 described the genome of *M. hydrocarbonoclasticus* SP17 (Type strain), Ling 2017 the genome of *M. hydrocarbonoclasticus* STW2, and Singer 2011 the genome of *Marinobacter aquaeolei*, not *M. hydrocarbonoclasticus*. Delete "pointing out its biological importance" in line 103 (too trivial).

In fact, *Marinobacter aquaeolei* has been renamed as *Marinobacter hydrocarbonoclasticus* (Int J Syst Evol Microbiol. 2005;55(Pt 3):1349-51), and so the text was modified, explaining the renaming of this species. OK

- Line 109, at the end of the sentence (sources.), please provide at least one reference. This would be a good place to explain why *M. hydrocarbonoclasticus* is a good candidate to study the denitrification pathway in marine environments. Related to *M. hydrocarbonoclasticus* 617, what makes strain 617 different from the two other *M. hydrocarbonoclasticus* strains?

Manuscript was modified accordingly. OK

- Lines 109-110. Delete "Therefore, a detailed analysis of the denitrification pathway in this organism is timely important,"

This part of the sentence was removed as suggested by the reviewer. OK

- Lines 111-112. The authors seem to have done a good amount of works with the enzymatic systems of this denitrifying bacterium. Briefly summarized the results of these studies. The Figure 1 provides no

substantial information that it is not been described from other systems and can be moved in supplemental data.

The authors would still prefer to keep this figure in the main manuscript, as it illustrates the type of enzymes that have been isolated from this organism and the flow of electrons. OK

- Line 117. add "strain 617" after *hydrocarbonoclasticus*

Manuscript was modified accordingly. OK

M&M

- Move (lines 128-132) "Identification of putative gene functions was performed by BLAST search using the web platforms of blastp and blastn suites (NCBI) (Altschul et al. 1997) (statistic data are provided in Supplementary Information Table S1), gene and protein alignments using ClustalOmega (McWilliam et al. 2013), and comparison of gene organization with other denitrifying microorganisms." with the Table S1.

The part of the sentence was transferred to Supplementary Information S1. OK

- Line 143. Starkey 1938. This reference is very old. Some reader may have problem getting this reference. Provide the recipe.

The composition of this solution is presented in the revised version of the manuscript in Supplementary Information S3. OK

- Line 149. Confusing: Described the dimension of the reactor. 2-L: is the work volume of the reactor? Also, what do you mean as 10% volume? Does the reactor have 20 L work volume with 2L medium? Or did you inoculate 200 mL of the final inoculum with 1.8L medium in the reactor?

The text was modified to clarify the working volume of the reactor and the volume used as inoculum. OK

- Line 157: "Cultures were visualized under an optical microscope to confirm its purity." I have strong reservation that observation of the cultures by optical microscope can provide indication that the cultures are contaminated or not.

We can understand the reservations of the reviewer. Nevertheless, this was necessary to be sure that we did not have contamination with microorganisms with different size and morphology. OK

- Line 159. "small aliquots" How the aliquots were sampled and what was the volume? Provide a schematic of the reactor with location(s) of where you took the samples.

The sampling volume is now mentioned in Material and Methods. 4-mL samples were removed from inside the reactor, near a portable flame and into sterile vials, and stored frozen in liquid nitrogen until further use. A scheme of the reactor is provided in Supplementary Information. OK

- Line 164: Cultures in the 100 ml flasks. Are these flasks flushed with N2 gas?

The flasks were flushed with argon before sterilization. The manuscript was modified describing this fact. OK

- Line 173. Again, how the aliquots were sampled and what was the volume?

These aliquots were sampled near the flame through the septum using a sterile filter connected to a syringe and the samples were collected through sterile tubes into sterile vials and stored immediately in liquid nitrogen. This description has been added to the Materials and Methods. A scheme of the reactor is presented in Supplementary Information. OK

- Line 176: What was the volume taken from the reactor for nucleic acid extraction?

The volume used was 0.75 mL and is now mentioned in Materials and Methods. OK

- Line 194. What is this reference "2004"? How can I find this in the list of reference? This is probably taken from instructions provided by Applied Biosystem. This is not a proper reference. Normalization of the transcript levels of the targeted genes was done with the transcript levels of the 16S ribosomal RNA gene determined also by qPCR.

This reference was removed as in fact it is a reference to the Instruction Manual of the equipment used. OK

- In line 202 (and Figure 3), the authors claimed that the expression of the 16S genes remained stable. If it is so, provide all the data in raw data files (not only for one culture but also for the three cultures with the 16S results and the calculation). I have strong reservation of using 16S gene as normalization control. First, there are 3 copies of this gene in the genome, which makes complicate whether these copies are expressed equivalently. Second, 16S rRNA genes are not a housekeeping gene and their expression are strongly influenced by the growth phases, meaning that per cell there are more 16S transcripts during the exponential growth than during the stationary phase, which is not desirable for a housekeeping gene. A real housekeeping gene keeps the same number of transcripts per cell whatever the growth phase. Finally, more than one housekeeping gene have to be used to get reliable results.

The authors have looked carefully to the raw data and analysed the 16S gene expression for the 3 replicates over time. The conclusion is that the expression can, in our opinion, be considered stable during the different growth phases. There are changes but these do not occur at the same time points in the different replicates, and the authors believe that the differences correspond to experimental errors.

The relative expression was calculated divided the expression value of each gene by the median of the 16S gene expression. This value (median of 16D gene expression) is similar either removing the deviant values or considering all the values. The value used was the median including all the values.

You should read this: http://normalisation.gene-quantification.info/ and doi: 10.1007/s10482-015-0524-1. As I mentioned, 16S is not the proper gene for normalization. I will not reject your data because others used this gene before, but please consider using other suitable genes in the future. In the "peerj-24978-

Raw_data_Figure3_Figure5_rev.xlsx" file, add how you calculate values to get the results for the relative transcript levels

- Lines 210-212. Please provide data for the two other replicates in the raw data. Another way to illustrate the results is to have Standard deviation to the time points (i.e. samples taken at 3, 6, 8 h, could be 5.7 h ±SD)

Additional data is now provided. The authors would prefer to keep the representation of the data as it is, so that there is a larger number of points represented in the region that there is larger changes (late exponential phase).

OK

Line 231: defined "RT"

RT stands for reverse transcription.

My comment was to put the definition of RT in the text, not just in the answer

Line 237: "NO-saturated water to a final concentration of 9.6 µM". Provide the source of NO (company). How NO-saturated water was made? How the concentration of NO was measured?

Line 249. N2O-saturated water. How this solution was made? How the concentration of N2O was measured?

The preparation of NO- and N_2O-staurated solution is not explained. The concentration of the solution is determined based on the solubility of the gas in solution and on the percentage of gas in the source used, as it has been described in the literature by many groups working in this subject.

In this case, please provide references where we can see how this work was done for readers not familiar with these protocols

Results

Genomic organization of denitrification genes in *M. hydrocarbonoclasticus*

Lines 257-352. First of all, this subsection described the gene annotations of strain SP17, not strain 617. The authors assumed that both genomes are the same, for the denitrifications genes, which seems to be true, as they have no difficulty to get PCR amplification from primers derived from the strain SP17 genome. However, the authors should acknowledge that differences could occur between their strain and strain SP17. The part of this subsection is a summary of the annotations of strain SP17 that Grimaud reported in 2012 and the annotations are already available in NCBI since then. For these lines, no new information is provided. The authors should only point out the new information not on the NCBI site.

The authors argue that this analysis is still different from the one reported in the NCBI, and have kept the information that is different from the one in NCBI site, moving the other text to Supplementary Information S6, where a figure schematically representing this information was already presented.

OK, but somewhere in the text, you should mentioned that the strain 617, was used for the cultures, you assume that the gene clusters involved in denitrification are present and identical in sequences than strain SP17.

Line 379. The NarL5 and NaL6 sites site in the *narK* gene. This should be mentioned and goes with the probability that this is not a proper binding site. For instance, *narK* gene and *narGHJI* are part of a polycistronic operon in *Methylophaga nitratireducenticrescens*. See Mauffrey et al 2015 (Front in Microb).

The promoter regions of denitrification genes were re-analysed. In the previous version of the manuscript the location of the promoter was given as the distance to the starting codon. In this revised version, the promoter regions were re-analysed to identify the -10 and -35 boxes and then the location of the other transcription binding sites (NarL and FNR/DNR). In the case of the *nar* gene cluster, as there are no -10 / -35 boxes upstream *narG* gene, it is proposed that *narK* together with *narGHJV* are transcribed as a single transcriptional unit, forming an operon. Upstream these genes a FNR and NarL binding sites were identified. Figure 2 was modified according to this analysis.

OK

Line 391 (and this subsection). Are these the results of the 2L-reactor cultures?

This subsection refers to the cells grown in the 2L-bioreactor, as indicated in the title (microoxic growth conditions). Nevertheless, the reference to the 2-L bioreactor was added to the text.

OK

Lines 398-403. Without proper statistical analysis (results from replicate cultures), you cannot draw any conclusion on whether one gene is more expressed than the other genes. Here the only information is relative to the time of higher expression for specific gene.

The authors have data from replicate cultures, in which it is observed the same profile. Therefore, the manuscript was only slightly modified to comply with the reviewer suggestion and opinion.

I will explain myself more clearly. It is recognized that comparison of the transcript levels between 2 different genes is not relevant biologically. To a certain time point, the level of *nosZ* transcripts can be 10 times higher than *cnorB*. But biologically what does it mean? Nothing. In fact, other factors can contribute that this such as the RT-PCR itself: the primers used the PCR, and the sequence amplified are different between genes. There is also posttranslation regulation that could occur that are different from one gene product to another.

You can only compare the transcript levels between time points for the same gene. In figure 3, one can tell that all genes are more expressed in the first 6 hours, than the leveld dropped afterwards, except of

qnorB, where its level remained the same. Do not mention that *nosZ* is more expressed than the others after 1 hr. The conclusion will remain the same.

Figure 3. Use linear scale to show the OD as this is the most usual manner to illustrate growth. Also, because it is difficult to clearly see distinctly the first 10 hours, the growth should be shown (or expand) for the first 20 hours only.

Although disagree with this representation, as it is rather difficult to determine the duration of the growth phases, the Figure was modified accordingly.

We can see at the opposite. The logOD scale masked the fact that growth still occur substantially after 12 hours

Line 420. State why *M. hydrocarbonoclasticus* cultures were performed under microoxic conditions (this is only explained at the end of the manuscript).

The manuscript was modified to account for this suggestion, and not just explain why the two type of growth were performed at the end of the conclusions. OK

Figure 5. Use linear scale to show the OD. Here, the way the two panels is presented, we have to look carefully to notice that under anoxic conditions the OD did not grow more than 0.1 log OD (or 1.2 OD) and under microoxic conditions it reached up to 0.4 log OD or 3.5 OD. This is 3 times the biomass yield. This is a substantial difference that could be explained by the presence of oxygen. Although oxygen was recorded at 0% in the microoxic conditions, that means that aerobic respiration occurred and generated more biomass than under anoxic conditions.

Similarly to Figure 3, Figure 5 was modified according to the reviewer suggestion. The maximum OD is different in two growths, being much lower under, as expected, under anaerobic conditions. As explained before, when the oxygen is registered as being 0%, it only means that it is being completely consumed by the bacteria, while in the anoxic growth it is absent. Thus, under microoxic conditions, the bacteria is using both oxygen and nitrate as electron acceptors and thus there is a higher growth rate. OK

Line 425. What is a dead phase?

This was a misspelling, which was corrected. OK

Line 425. The diauxic growth observed by Hahnke 2014 is observed during the transition of nitrite accumulation after nitrate reduction, to the beginning of nitrite reduction. During this transition, the growth slowed before resuming when nitrite started to be reduced. In figure 5, the log scale for growth is misleading. With the raw data provided, when I was able to draw at linear scale, and it was obvious that for the first 10 hour of culture, the grow rate was faster than the 10 to 50 hours period with a linear growth at slower growth rate. The authors mentioned that the other replicates behaved the same, but this should be presented in the raw data. As mentioned for the comment above for Figure 5, the first period probably illustrates both respirations (oxygen and nitrate) that occurred in the culture and the

second period is the growth with oxygen as the only electron acceptor. A proper control should have be done with strain 617 cultured under oxic conditions (full aeration) with and without nitrate.

We agree with the reviewer on the proper control, but this is not the purpose, as denitrification would not occur under full aeration. Under these conditions nitrate would be consumed but the nitrite would not. The scale used in just a mathematic treatment of the raw data, to be able to identify the two exponential phases, which are easily observed in this representation: in the exponential phase the linear region indicates the presence of an exponential phase. The diauxic phase is observed in different growth of *Paracoccus denitrificans*, in the presence of nitrate, and in the work referenced by Hahnke 2014, the second phase is interpreted as corresponding to the consumption of nitrite. In our study, probably because a lower concentration of nitrate is used, the second exponential occurs after the consumption of nitrite.

The interpretation of the reviewer is the same as ours, as in the first 5h of growth there is oxygen and nitrate that can be used by the microorganism as electron acceptors, while after that nitrate has been consumed and nitrite nearly exhausted, and the rate is also slowed down, because the aeration is slower than before (aeration rate is the same but the agitation has been reduced to 50 rpm). This has been added to the results section.

OK

Line 435. No aeration provided so no substantial growth afterward as nitrate is exhausted.

We agree with the reviewer that since the growth in the sealed flask have no other electron acceptor, and this organism does not ferment the growth ceased after nitrate and nitrite is exhausted. This is mentioned in the results section.

OK

Lines 465-471. The authors measured the NO and N2O reductase activities, but never measured the NO and N2O concentration generated in the cultures. The recording of activities in whole-cell (which I found elegant) only measured the reductase abundance in the cell (as mg protein extract). Therefore, in line 466 referring to "maximum NO reduction rate" should be "maximum NO reductase production by the cells". See also line 540. The same goes with the N2O.

The authors disagree with the suggestion of the reviewer, as the activities reported indirectly indicate the presence of NO and N2O reductases in an active state in the cells, and not the of NO/N2O reductase production by the cells. The production of reductases (presence of polypeptide) would be correctly determined using a western blot, if antibodies would be available for these two enzymes.

These assays report the ability of cells to reduce NO and N2O externally provided, which is an indirect measure of the presence of active NO/N2O reductases in the living cells.

OK

Figure 6. Except for putative motifs found upstream of denitrification gene clusters, none of the regulatory pathway was proven from the results. This remains speculative and replicates what was proven in other

denitrifying bacteria. This can be moved in the supplemental data Table 1. Delete. This is information available in many reviews or books or moved this in supplemental data Table 2. Added the expected length of the PCR products, and indicate which primers were used for qPCR.

Table 1 was moved to Supplementary Information. The list of primers used in the qPCR are listed in Table 2, which include now the expected size of the PCR products.

I am very surprise of the short size of the qPCR products. Just to be sure, the size of these fragments includes the primer sequences. If so, this means that 40 nt out of 60 nt (for instance cnorB) are primers, and 20 nt is synthesized by the polymerase.

Discussion

- First paragraph is a repetition of the Results

- Line 491. Delete Remarkably.

The first paragraph of the discussion section was rewritten, and the work was removed as suggested. OK

- Lines 497-498. As mentioned before, although oxygen was recorded at 0, it does not mean it was absent, but because it was consumed by cells as electron acceptor (respiration).

As discussed before the oxygen levels are not zero, but due to the low aeration rate, it is expected that there are in fact regions of the growth media in which the organisms experience anoxic conditions. Therefore, this is why there is induction of the expression of these genes that are under the control of FNR.

OK

- Line 504-508. Too speculative. As mentioned before, low aeration provided O2 in the medium. A control is missing with qPCR assays done with cultures under anoxic conditions flushed with N2 so no oxygen is provided.

OK