Reviewer comments are in black, our responses are in red.

Reviewer #2

General comments:

Clearly a lot of work went into this study, however there is still much work that needs to be done with the paper. Currently the paper reads like a draft that still needs several more rounds of circulation between authors. The introduction and discussion sections need better organization/flow with more specific, relevant background/literature pertaining to sediment microbes and mangroves, and importance of tidal zone (how that might influence microbial communities and mangroves). There are numerous sentences within the results section that belong under either the methods or discussion sections. The discussion lacks focus and synthesis. Overall, this paper needs a significant overhaul. It seems like it would be useful for the authors to clarify specific objectives, if not for the paper, for themselves, to achieve better focus and clarity in conveying this study and its findings. Another major consideration is that the authors should be very careful about what and how they convey findings and conclusions on metabolic pathways and functionality of microbes when only using 16S rRNA amplicon data. Particularly, they should be weary and cautious about using a tool like PICRUST2 to make any major conclusions with respect to microbial functionality. Personally, I think that if you are going to use PICRUST2 as a tool here you should be backing up as much of those findings with literature as possible. For example, you could compare your findings with mangrove metagenomics studies such as those done by Andreote et. al. 2012. Also, it is important to very explicitly state the pitfalls of PICRUST2 analyses (see Sun et. al. 2020, https://link.springer.com/content/pdf/10.1186/s40168-020-00815-y.pdf) and that these are just inferential findings and would need to be confirmed via additional analyses such as transcriptomics or experimental setups.

We thank the reviewer for their time and insightful comments. We have significantly revised our manuscript to address their concerns and are confident that the paper has substantially improved as a result. As per their advice, we have re-written the introduction to clarify the intent of this paper and situate it within the larger body of mangrove metagenomic studies. We have also sought to make clear the limitations of our methodologies. We particularly appreciate the reference to the Sun et al. 2020, which was published the same month we had submitted our manuscript. By incorporating the findings of Sun et al we have highlighted the intrinsic and extrinsic limitations of PICRUSt2 in relation to our data.

Specific Comments:

Abstract

Line 21: The term metagenomic at this point is used solely to describe shotgun or whole metagenomic sequencing, not amplicon sequencing.

We have corrected the text throughout our manuscript to appropriately refer to the method used as 16S rRNA amplicon sequencing.

Line 27: You say “significantly different prokaryotic communities but in Line

This comment appears to be truncated.

Line 31: Change metagenomics to “amplicon” or “16S rRNA”. I don’t think you should include “function in the keywords, as functionality is solely inferred indirectly via amplicon sequencing and Picrust2 analysis. Choose either “Mangrove” or “Pristine Mangrove Forest”.
We agree with the reviewer and have corrected the text.

Introduction

Line 37: Be more specific when you say “large portion”, how much do they constitute?
We have revised this to more specifically describe their size.

Line 42: Instead of talking about studies which look at microbial communities and plant development, include specific background on microbes and mangroves that is relevant to your study.
We agree with the reviewer that this was an overly broad background and we have now condensed the references to focus on those that are most pertinent to our study.

Line 45: You haven’t really established “dependency” of mangrove forest on sediment microbiome at this point. You can expand on how they can be considered dependent or remove this type of wording.
We agree with the reviewer and have added additional text and citations that establish the dependency of mangroves and microbes.

Line 47: I’m not sure what you mean by “single type of sediment.” Since you don’t discuss types of sediment or measure sediment characteristics such as grain size or grain type (silt, sand, etc.) in this paper you should not mention sediment type. I think you might mean that due to the fact that mangroves are highly influenced by tidal flow, which results in variations of “environmental conditions across small spatiotemporal scales,”
The reviewer is correct and we have removed the confusing text.

Line 60: What is the “original area”? Do you have specific information on this?
We have clarified the text to ‘approximate pre-historical area’ and have included the relevant citation.

Line 71: Not sure what you mean by “terrestrial processes.” Do you mean biogeochemical processes?
We have corrected the text to refer to biogeochemical processes.

Line 74: I would say something more like “understand the differences between microhabitats within mangrove systems” instead.
We agree with the reviewer and we have reframed this to reflect the focus of previous work.

Line 75: By “mangrove regions” do you mean different tidal zones? If not, you might want to briefly explain what the different regions of mangroves are.
The reviewer is correct, we had intended this to be synonymous with ‘tidal zones’. We have changed it to be ‘tidal zones’ to avoid confusion.

Line 78: Use something like “We identified taxa which may be driving different utrient cycles between zones.” I should note that you may want to rethink this sentence altogether as you don’t really show that there are different nutrient regimes/cycles between different zones as of now. You could include literature that suggests this or data of your own to support it.
We have rephrased this to emphasize that the taxa have different abundances between sites as well as making substantial inferred contributions to nutrient cycling.

Lines 81 -85: You could circle back to this in the discussion, specifically you could theorize what changes in community structure you might expect based on your findings and the literature in contaminated mangrove sediments.
We appreciate the reviewer’s insight and have followed their suggestions in our revised Discussion section.

Methods

Line 100: What is meant by tidal influence? It would be good to include demarcations for this, i.e. distances, vegetation, etc. Based on Fig.1 it seems like you may have used sediment water content, or time of water coverage.
We agree with the reviewer that the estimation of tidal influence was incompletely explained. We have amended the text to include soil water content, coverage at time of collection, vegetative line and the agreement of a local guide.

Line 104: When you write “disruption of rhizospheres” do you mean “contamination” of rhizospheres associated with vegetation, because you aren’t wanting to include those communities?
Yes, we wanted to avoid a possible interference of the rhizosphere microbiome on our analysis. We have clarified this in the text.

Line 107: Were vegetation densities measured, if so, what was the metric?
Vegetation densities were only qualitatively measured at the time of collection. We now state this in the text.

Line 111: How was organic matter content measured?
Organic matter was measured using the mass loss on ignition (LOI) method. We have amended the text to make this clear.

Line 127: I don't think you need the "ILLUMINACLIP" section, especially if you already have your code published on Github. Additionally, you explain your code in text immediately following.
We agree with the reviewer and have removed this.

Line 131: You mention just QIIME, and QIIME2 in the following steps. QIIME is no longer supported or kept up, so if you used the original QIIME I would recommend using QIIME2 for that step.
We agree with the reviewer and have implemented the addition of QIIME2 for this step. Doing so we have seen an improvement in performance with an average of 2.1% more reads per sample than before. Correlation of abundances at the level of families resulted in a median r^2 = 0.9995 between the two methods.

Line 132 – 141: DADA2 calls ASVs and not OTUs. I have only superficially used QIIME2, as I typically use mothur or DADA2 directly in R, but my understanding is that QIIME2 and DADA2 primarily call ASVs, but gives the option to then cluster those ASVs after they have been called. If this is what you did, you should explain that process briefly. In reading on (Line 138), it would seem that there is either a miscommunication or misunderstanding of the bioinformatic steps with respect to clustering and taxonomic assignment. "Open reference" refers to a clustering method which can be done using Vsearch in QIIME2. It looks like when assigning taxonomy after OTU clustering, Q2 gives you 3 different option, I am thinking you used classify-consensus-vsearch? I would also combine sections 2.4.2 and 2.4.3 in to one section where all bioinformatics workflows in QIIME 2 are discussed together. For reference: https://docs.qiime2.org/2020.8/tutorials/otu-clustering/ and https://docs.qiime2.org/2020.8/tutorials/overview/

We apologize for the confusion. We had initially tested the performance of vsearch and the sklearn methods offered by QIIME2, and identified a degree of dissimilarity between results from the two methods, especially at the species level. Ultimately, we chose to use the sklearn approach based on the work of Bokulich et al. 2018 (https://doi.org/10.1186/s40168-018-0470-z). We have revised the text to reflect the exclusive use of the sklearn method.

Line 149 – 151: What dissimilarity metric did you use for metaMDS, i.e., jaccard, braycurtis etc.? I am wondering why you didn't run something like Canonical Analysis of Principle Coordinates (capscale in Vegan) to investigate correlations between environmental variables with community structure.

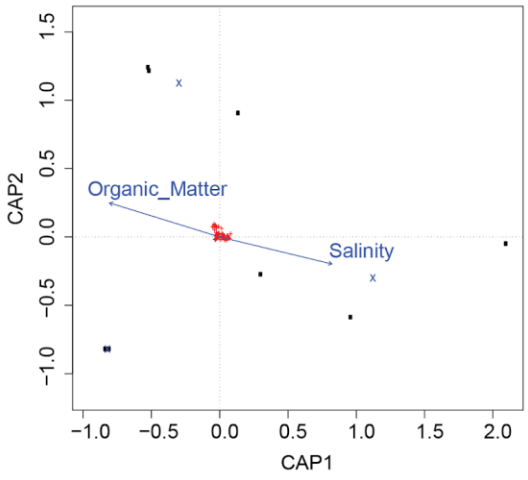
For metaMDS we used the Bray-Curtis metric. This is now stated in the methods section.

To address the reviewers concerns we also ran capscale . The model we started with was a basic linear module wherein all terms are additive and no terms are 'partialled out' (Oksanen, "Vegan: an introduction to ordination", https://cran.r-project.org/web/packages/vegan/vignettes/intro-vegan.pdf). Notably, we did not find this combined model to be statistically significant (Pr > 0.143) (figure below). Partialling out environmental variables resulted in increased model performance (Pr < 0.1 for Organic Matter

and Pr < 0.05 for Salinity). These agree with our own (unconstrained ordination) results using MetaMDS and Envfit.

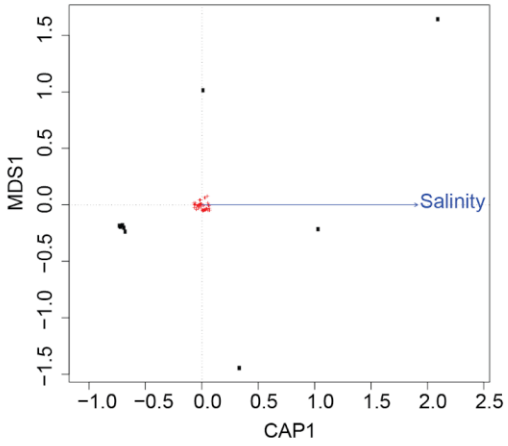
All Variables
(Salinity + Organic_Matter + Temperature + Water_content)

	Df	SumOfSqs	F	Pr(>F)
Model	2	1.3214	1.2026	0.143
Residual	6	3.2964		



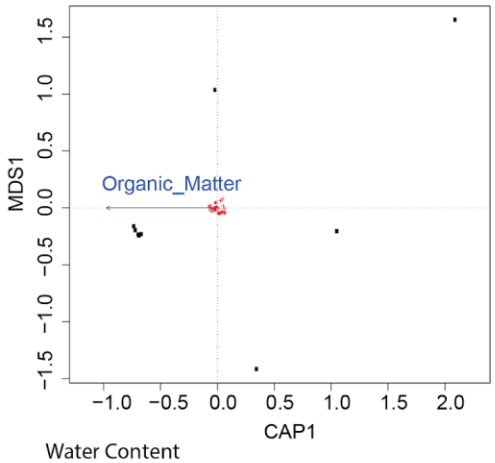
Salinity

	Df	SumOfSqs	F	Pr(>F)
Model	1	0.8307	1.5354	0.038 *
Residual	7	3.7872		



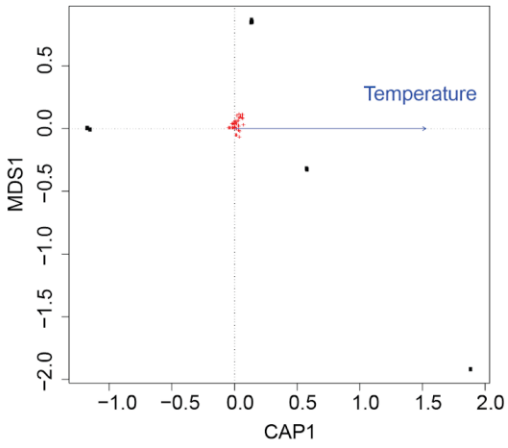
Organic Matter

	Df	SumOfSqs	F	Pr(>F)
Model	1	0.8185	1.508	0.087 .
Residual	7	3.7994		



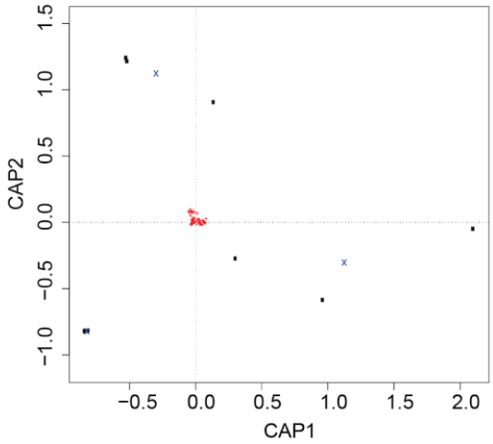
Temperature

	Df	SumOfSqs	F	Pr(>F)
Model	1	0.6613	1.17	0.249
Residual	7	3.9565		



Water Content

	Df	SumOfSqs	F	Pr(>F)
Model	2	1.3214	1.2026	0.136
Residual	6	3.2964		



Line 153: Did you take the limitations in the link provided below into consideration when running these analyses? PiCRUST2 Limitations Link: http://picrust.github.io/picrust/tutorials/quality_control.html

We thank the reviewer for their suggestion and we have now taken these cautions into consideration. Because of the concerns surrounding under-represented taxa we did compare PICRUSt2 performance using the default NSTI cut-off of 2 and of 0.15. We found that the average Spearman *rho* correlation between the two sets was ~0.91, with a standard deviation of 0.01.

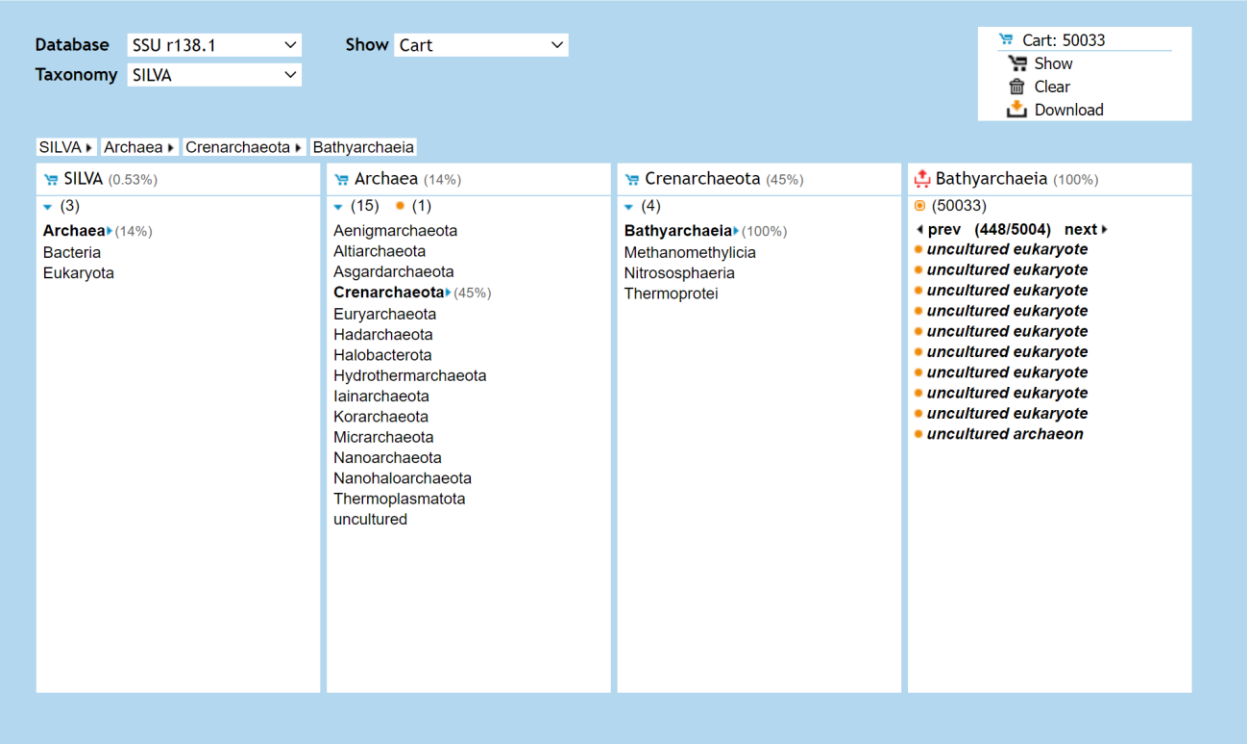
That said, the total ASVs retained using a 0.15 NSTI cutoff was only 12.5% - suggesting that the results (while similar by correlation) were not representative for the majority of taxa present in the sample. We have included this analysis in the Supplemental section.

However, it is important to note that, while the NSTI value of 0.15 is considered an upper bound at the taxonomic level of species (as per the PICRUSt1 manual), it is unclear what the acceptable upper bound would be for the taxonomic level of families, which is what we use in this paper. Indeed, while the median NSTI value for all ASVs in ~0.46 we find that the median minimum NSTI at the level of families is ~0.25.

Line 159: Explicitly explain how you conducted the taxa enrichment analysis. I could not find the reference paper for Spealman et. al. 2020.
We apologize to the reviewer for the confusion. Spealman et al. was a more expansive version of the supplemental information that was deposited in a citable archive upon submission of this paper as per Biogeosciences policy. We have now included this text in the supplement so that it may be more accessible to the reader and provided information on the taxa enrichment analysis in the Methods section.

Results

Line 186: Do you mean uncultured “prokaryote” not “eukaryote”? I don’t believe there is any eukaryotic designations in the 16S SILVA taxonomy reference. You should also qualify further why you felt comfortable assigning an archaeal taxon to the uncultured “eukaryote”.
Unfortunately, the current version of SILVA does have several mislabeled entries for ‘eukaryote’ under the Bathyarchaeia taxa (see below). These are certainly not eukaryotes but mislabeled entries, of which there are several in the Bathyarchaeia taxa. To prevent confusion we have changed the text such that label of “SILVA uncultured eukaryote (SUE)” will instead read “Uncultured Bathyarchaeia”. We have included a short note as to the original mislabelling present in the Silva database.



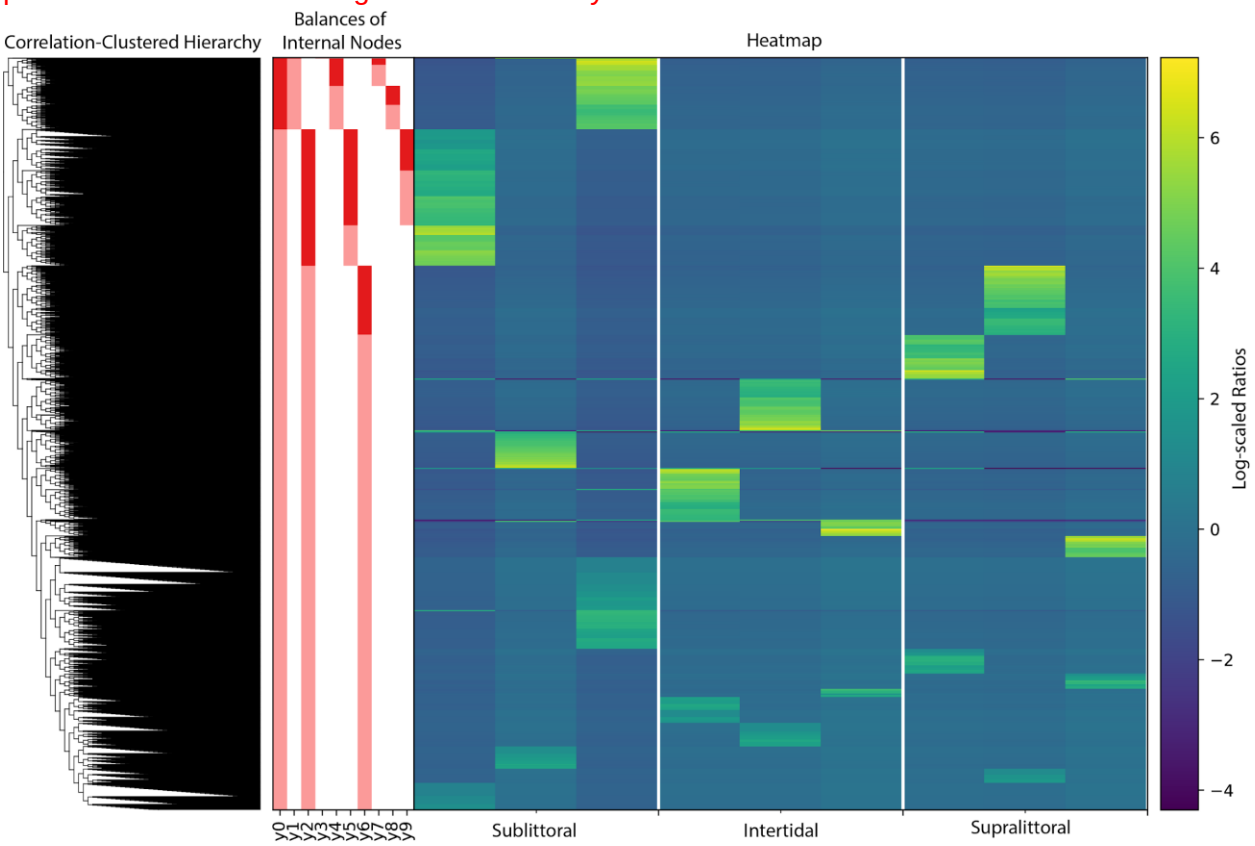
Line 196: Figure 1B colors are difficult to differentiate. Consider adding a pattern to colors which are too close to differentiate. For figure 1B, consider changing the y-axis range to 32 so that we can see more of the other bars.
We agree with the reviewer that individual taxa were difficult to identify and we have corrected the figure.

Line 201, 204, 205: QIIME2 sentences belong in methods
We have moved sentences about QIIME2 to the methods section.

Line 207: P-value for Bray-Curtis is not significant
As per the reviewer comment for Line 211, we have removed this figure

Line 211: Why did you use both distance metrics, i.e., Jaccard and Bray-Curtis? You should choose the one most appropriate to your data and study. Did you try hierarchical clustering to see how data might cluster without apriori considerations like tidal zone?
We originally considered both the Bray-Curtis and Jaccard as they are different measures (quantitative and qualitative, respectfully). In the interests of concision we have removed the Bray-Curtis plot.
To address the reviewer's comment we performed hierarchical clustering (Gneiss, correlation clustering), but found that the balanced clusters only partially describe tidal zones (see below). This is not very surprising given that there is no statistically significant difference in their

pairwise combinations using the Beta-diversity tests.



Line 222: What is your organic matter (OM) metric, is it total OM? I don't think this is the best/clearest way to analyze these data with environmental variables. See methods comment Line 145-151.

As described above we used Loss on Ignition to measure organic matter content. We also evaluated these variables using Vegan's capscale method.

Line 227-229: These sentences belong in the methods section

We have revised the text by moving them to the methods section.

Line 231: What is "elevation" in this context? Do you mean zonation?

The reviewer is correct and we have revised the text to make this clear.

Line 236: What is an "icon" in this context?

We have now specified that the geometric shapes that are used are intended to show which pathways are enriched.

Line 237: What specifically about "carbon metabolism"? As all microbes need a carbon source, this should be explained in a bit more detail.

We agree with the reviewers and have revised our approach. Instead of looking at enrichment of KOs within broad functional categories we now look for enrichment within KEGG modules. This

has allowed us to describe the inferred functional enrichment in a more meaningful way. We have revised the figure, text, and methods to reflect this.

Line 247: In figure 5 it would be good to use different colors for differentiating bacteria and archaea as you are already using green and blue in the figure legend.
We agree with the reviewer that the color palette was confusing and have distinguished these domains in the figure.

Line 252: This sentence belongs in Methods
We have moved it to the methods.

Lines 255 & 256: don't need the "above both" phrase, it is confusing.
We agree with the reviewers and have removed this.

Line 261/262: "Taken together, KO enrichment reinforces the previously observed trend of reduced abundance in the Intertidal site, and greatest abundance at the Sublittoral zone." This sentence seems like it should be in the discussion, especially if the "previous trend" you are referring to is one form the literature. Also, be consistent on whether you are capitalizing the tidal zones or not. I think it is more correct not to capitalize.
We apologize for the confusion, we were referring to the observations of abundances we had described in an earlier section, not previous research. We have clarified the text.
We have revised the text throughout the manuscript to remove capitalization of the tidal zones.

Line 268-276: I may have missed the results in this paragraph, but it seems like all of this belongs in the methods section as it is describing how something was done versus reporting the findings of what was done.
We agree with the reviewer and have removed this paragraph.

Line 270: Where is here? Is it this study or a figure?
This line has been removed.

Lines 277 – 327: I would combine all of the "cycling" sections under one section called "biogeochemical cycling" or something like this. You have several sentences throughout this section that would be more appropriate in the discussion section. Essentially any of the sentences with citations should probably be in the discussion. Examples: Lines 287, 291-293, etc.
We agree with the reviewer and have condensed this section. As the citations were intended to provide additional lines of evidence to support the potential metabolic functional analysis we have moved the most relevant ones to the discussion and included the rest as supplemental information.

Line 328: I appreciate the effort that went into this figure. Personally, I would like to see this figure with less taxa, only ones you specifically mention within the text, so that it is less busy. I am not sure why the # of nodes legend needs to be a tapered triangle. It makes me think I

should be considering both the color and thickness of lines when I'm looking at nodes. I also think you should use a more differentiating way to denote taxa with an associated metabolic role in the literature and taxa with KO greater than 10%. You could use black and white circles, and add a gray circle for those with both if they exist.
We agree that the figure is overly complex and have followed the reviewer's suggestions in making a simpler version with cleaner layout and reduced text.

Discussion

I decided to make an overarching comment here, instead of going line by line, because the discussion needs a lot of work and restructuring. I think one of the best ways to go about fixing the discussion will be to come up with a thesis statement for each paragraph and figure out what points you are trying to convey. This will help you to clarify and re-organize your thoughts. I would like to see in the discussion more synthesis of your findings, such as why you think you might find certain taxa with potential metabolic capabilities enriched in certain tidal zones. It seems to me that you set out to study the sediment microbiome of pristine mangrove environment across 3 tidal zones to serve as a baseline and to characterize differences in taxa and potential biogeochemical cycling that is predominant in those zones. However, neither your introduction or discussion provide enough clear, relevant background or support for your overarching goal. I would have also liked to see some discussion on anaerobic taxa, and where you find more anaerobic taxa with respect to tidal zone. You us the term microhabitat in both the intro and discussion, but it is unclear what this means in the context of your study. You should clearly define what your usage of microhabitat means. Are you talking about microbial habitat, are you taking about spatial scales, millimeters – meters?
In accordance with the reviewer's comments, the discussion section has been restructured in order to provide a better synthesis of our findings with respect to the hypothesis and prior literature. We have also revised the introduction to provide the relevant background for the overarching goals of the study and a definition of the concept of microhabitats that we are using. We believe that these substantive changes have addressed the reviewer's concern and have significantly enhanced the clarity of our manuscript.

Technical Corrections:

Line 57: Latter not "later"
We have corrected the text.

Line 62: I am assuming that the A in APA is referring to Ambiente, but just want to point out you use the English "Environmental" just before APA, so I'm not sure if you should write EPA or use the word Ambiente.
We agree with the reviewer that the original Portuguese is 'Área de Proteção Ambiental' - however, we have tried to faithfully translate this into English as 'Environmental Protection Area' while retaining the un-Anglicized acronym. Similar to how Germany retains the DEU abbreviation. We will amend the text with the full phrase in Portuguese.

Line 117: Use protocol instead of "program"

We have changed the wording to “protocol”.

Line 154: All KEGGs should be capitalized. Line 193: Genera instead of “genus”.

The wording has been corrected throughout the text.

Line 101: I don’t know if superficial is the correct word, I typically see the use of “surficial”.

The text has been corrected.