***July 19, 2016***

To: Rogerio Valle
Academic Editor for PeerJ

**Re: Response to editorial review for manuscript: *Characterization of algae from urban stormwater and wastewater systems as candidates for biofuel feedstock* (#2016:05:10974:0:1:REVIEW)**

Dear Dr. Valle,

I wish to thank you and the reviewers for your helpful comments regarding our manuscript entitled *Characterization of algae from urban stormwater and wastewater systems as candidates for biofuel feedstock.*Care has been taken to address all comments, for which responses are itemized below. Please review manuscript changes in “Track Changes” mode for line-numbering continuity.

***Reviewer 1***

***Basic reporting***

*…For clarity, the discussion section should be divided in items e.g.*

*-Diversity of algal isolates*

*-Growth characteristics and tolerance to copper*

*-Potential for biodiesel production*

These recommended subheadings have been added to the discussion.

***Experimental design***

*1. The antibiotics treatment approach to reduce bacteria loads is a valid one for the reasons given by the authors (it reduces interference of bacteria in the FAME profile), although bacteria contamination is normally a minor problem during the exponential growth phase of algal cultures. I wonder how much this antibiotics treatmente influenced growth rates and FAME profiles of the strains used in the experiments. If some of the strains were so susceptible to the antibiotics cocktail that they were removed from the experiments, other strains could have their growth rates negatively affected as well.*

We can see how this passage is not very clear. Antibiotics were only used initially to obtain axenic cultures. During preliminary work to obtain axenic cultures, antibiotics were applied via serial dilution, and colonies from the lowest concentration with no bacteria present were used. As such, no antibiotics were used in any subsequent experiments. Additional phrasing has been added to Lines 113-122 to improve clarity.

 *Also, large-scale production of microalgal biomass will not be axenic thus the FAME profile is likely to change under this conditions. Antibiotics susceptibility could be the reason for e.g. low growth rates of some strains (like Scenedesmus sp. Sp21.12 and Chlorella sp. Sp21.20) This should be acknowledged in the discussion.*

As previously mentioned, no tested isolates in this study were inhibited by antibiotics, a study criterion that has been added to the methods (Line 118). Standard axenic protocols were followed in this initial study to rule out bacterial contributions to the FAME profile. In previous work, we compared axenic and non-axenic FAME profiles so have cited that paper on line 113.

*2.Can you assure you achieved clonal algal cultures? The plate spreading technique is a widespread, straightforward way to isolate fast growing algae, but it does not necessarily produce clonal strains (i.e. a culture originated from one cell or coenobium). This should be taken into account since the authors acknowledged (based on references) that strain variability does exist.*

To clarify, it is never mentioned in the manuscript that strains are clonal, but rather unialgal. It is also described in the manuscript that water samples were serially diluted prior to spread plating on media with agar. Well-isolated colonies were pegged and grown in flasks and then observed microscopically to confirm they were a single strain. This is a protocol provided by Andersen and Kawachi (2005) for isolating algae, a book used by many research labs as a resource for algal isolation. Additional text has been added to clarify that single colony isolation occurred and that single strains were confirmed. Since most strains were sequenced, molecular analyses confirmed that these were unialgal cultures (i.e., there was a single 18S rRNA sequence for each culture).

***Validity of the findings***

*Experiments are scientifically sound. Results are of good quality and relevant to the field of biofuels. Conclusions are supported by the results. DNA sequences of microalgal strains used in this work should be made available in a public database and accession numbers must be provided as supplementary material.*

The reviewer may not have realized, but this information is already required by PeerJ, thus this information is included in the Supplementary Information on the PeerJ website.

***Comments for the Author***

*In the title and throughout the text change algae for microalgae, as the ms deals specifically with these types of microorganisms. Suggestion for the title: Screening microalgae isolated from urban storm- and wastewater systems as feedstock for biofuel. Abstract should me more informative giving e.g. growth rates ranges, maxima, etc. for selected strains that the authors found to be suitable for biofuel feedstock.*

All of the reviewer suggestions have been incorporated into the manuscript, including the consistent use of “microalgae”, new title, and additional details to the abstract.

*line 17: replace "were tolerant of" with "were tolerant to"*

After consulting various grammar authorities, “tolerance of” appears to be more acceptable than “tolerance to”. However, we will leave this to the editor to decide.

*line 35 replace could with can*

Done.

*lines 51-54. These statements need references.*

A reference has been added for the first statement. The second statement is our own idea. It has been reworded to make this more obvious.

*line 70 Replace "Strain Collection and Isolation" for "Sample collection and strain isolation".*

Done.

*line 83. Why was the nitrogen concentration of BG11 medium reduced so much? If it was to bring it close to wastewater N concentrations (as stated in the following sentence) then the typical average or range of N concentration in the storm and wastewater ponds sample in the study should be shown.*

More details were added, including a reference to a paper that reports nitrogen and phosphorus concentrations from the stormwater ponds used in this study.

*line 79. Replace grown in for transferred to*

Done.

*line 86. Add reference for f2 medium. Could you please supply nutrient concentration of surveyed ponds as supplementary material? Would be helpful to compare with nutrient concentration of the media used for isolation.*

The f2 medium reference has been added. Nutrient data has been previously published and the citation (Vincent and Kirkwood 2014) has been included several times throughout the manuscript.

*line 131 replace "to prime PCR reactions at a concentration" with "to prime PCR at a concentration"*

Done.

*line 189. species name should not be in capitals. Please check this throughout the text.*

Fixed.

*line 215. sp. after genus name should not be in italics. Please check this throughout the text.*

Fixed.

*line 246-248. This belongs to M&M.*

It is already mentioned in the M&M that 34 axenic isolates were targeted for FAME analysis. This sentence has been modified as a reminder to readers that not all isolates were assessed for fatty acid composition.

*line 251. Replace "does a good job of preserving" with preserved.*

Done.

*lines 257-259 This belongs to the M&M section.*

Done.

*lines 259-260 This sentence belongs to the discussion*

Since this phrase relates to the previous calculations, it has been moved to the M&M section based on the previous suggestion.

*line 263 Unsaturation should not begin with capitals*

Degree of Unsaturation is a term rather than a phrase. However, the entire term has been uncapitalized to comply with grammar rules for phrases.

*line 296 replace " for biofuel strains" with "for isolation of lipid-rich strains suitable for biodiesel production"*

Done.

*lines 301-302 This statement needs a reference.*

Added.

*Figure 3 could be moved to supplementary material. Add species names for the isolates at the tip of the branches.*

Figure 3 has been moved to supplementary material, but there are no branches in this figure to add species names.

*Table 3 and Figure 5 show basically the same information. I suggest removing Figure 5.*

Figure 5 has been removed.

***Reviewer 2 (Anonymous)***

***Basic reporting***

*My major concerns with this study are related to the taxonomic part and incongruencies between the phylogenetic analysis and the fatty acid analysis.*

*1/ The authors have omitted the % similarity from table 1. A similarity >90% is indicated in the legend, but the authors need to be more specific because 10% variation may mean a lot. Different species may have 99% similarity based on the 18S fragment sequence. Two strains having 91-93% similarity could easily belong to different genera…add % similaity to closest match in Table 1.*

We agree with the reviewer and have added the actual percentages to the table.

*2/The inconsistency between 18S tree and data in table 2 needs to be explained. For instance, Isolates 12.07 and 12.21 are identical in the 18S tree but differ significantly in the Table 3 parameters. Idem 1.43 and 21.23, 21.20 and 23.13. There are more such cases.*

We agree that further elaboration and discussion is required here, but we would like to point out that Fig. 3 (formerly Fig. 4) was included to, in part, addresses this issue. The reviewer does not refer to this figure, so it is unclear if they are aware of this. With respect to the reviewer’s point about the discrepancies between the 18S tree and Table 2, this does warrant further clarification, which has been added to lines 332-341 in the Discussion section.

*3/I did not find WW8 and WW28 in the Table 2, though they are listed in 18S phylogenetic tree. The authors will need to revise the whole set of isolates and make sure the same set appears in all tables and figures.*

A challenging aspect of this study was that not all isolates could be assessed for all characterization procedures, thus we recognize that this may create some confusion for the reader. To address this, we took great care to provide details throughout the methods section on why certain isolates were omitted from certain analyses. In this instance, WW8 and WW28 were not axenic, so were not included in the FAME analyses. This is why they are not included in Table 2, although the title has been amended to remind readers that only axenic strains were included in the FAME analyses.

*Line 288-313 are not convincing; need rewriting focusing on the main findings of the manuscript. Overal discussion needs to be more focused on the results of the present study.*

We are not sure what exactly is not convincing about this first section of the Discussion. Our isolates were all chlorophyte taxa, which are commonly used in biofuel studies. Does the reviewer not agree that our sampling locations were worthwhile sites for isolating biofuel algae? With respect to the discussion needing to be more focused on our results, this is a difficult comment to address since no concrete suggestions were offered. The Results section is where one focuses only on the results of the study, whereas the Discussion offers the opportunity to interpret the results within the larger context of the field. Having reviewed the discussion again, we think that it is presented as a good balance between the results of this study and how they relate to the broader literature. Since these comments do not relate to the technical competency of the manuscript, and Reviewer 1 did not raise any concerns about the Discussion, we have elected to leave it as is, and defer to the editor to offer suggestions.

***Additional***

*Monoraphidium sp. Sp17.38 and Selenastrum sp. Sp14.35 are not in the tree (fig 1). Why? Make sure you get the right taxonomic assignment for these two as they are apparently the most interesting for further investigations.*

Unfortunately, we were unable to amplify sufficient DNA for strains *Monoraphidium* sp. Sp17.38 and *Selenastrum* sp Sp14.35 with the NS1/18L and NS1/ITS2 primers typically used for algae. It is not that uncommon for some algal taxa to not be easily sequenced, for various reasons. We took a conservative approach with phylogenetic tree construction and only used complete NS1 region sequences greater than 400 base pairs (bp), which was stated in the manuscript lines 155-156. Any sequencing information we do have for these taxa are provided in Table 1.

*Please Do not use sp. In italic*

Fixed.

***Experimental design***

*Authors need to revise their methodology in order to confirm inconsistencies between FAME and 18S analysis.*

We believe all of the concerns raised by Reviewer 2 regarding inconsistencies between FAME and 18S analysis have been addressed. Please see responses to these Reviewer 2 concerns above.

I hope these amendments have improved the manuscript sufficiently for publication in PeerJ. Thank you and the reviewers again for such insightful and helpful comments.

Yours sincerely,



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