Reviewer comments is presented in BLUE
Author responses are presented in PURPLE

Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52936_R1_120214.docx) is located in your Editorial Manager account. Please download the .docx file and use this updated version for any future revisions.
All revision to the manuscript have been made on the 52936_R1_120214.docx.

Changes made by the Science Editor:

1. There have been edits made to the manuscript.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript has been thoroughly reviewed for spelling and grammar,

2. What are the volumes of the polystyrene tubes used in step 8?
The manuscript has been updated in step 8 to include the volumes, specifically they are 16 mL tubes.

3. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, *i.e.*, the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Currently there are just under four pages of highlighted protocol text. We suggest removing section 9 from the highlighting as this is difficult to film and the visual aspect won't add much to this material.
Section 9 has been removed and the highlighted text for filming has been reduced to 2.5 pages. Section 10 has also been removed based on the same rationale. The filming will consist of following the preparation of medium and inoculation of the system and the preparation of samples for metals analysis.

Reviewers' comments:

Reviewer #1:
This manuscript presents a procedure used to determine the end fate and impact of 14 metal ions on the growth of Nannochloropsis salina grown in a simulated environment, which could potentially be employed for the study of microalgal cultivation with industrial flue gases. The topic definitely fits within the scope of the journal. However, the authors failed to address the complexity issues associated with actual flue gases, and the correlation between the metal levels

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with the microalgae production is likely to be overgeneralized. The composition of flue gases from different combustion facilities varies a lot, and depends on the type of fuel and the combustion conditions, e.g. the air ratio value. Generally, flue gases consist of high concentrations of the combustion products water and carbon dioxide, as well as oxides of sulphur and nitrogen, fine dust, trace heavy elements, and super-toxics such as dioxins.
The authors agree with the observations made by the reviewer. The introduction has been updated with a statement that highlights this work is intended to be representative of the integration with a typical coal plant and focuses only on heavy metals. The following text has been added to the introduction: "Specifically, flue gas derived from coal will have a variety of contaminants including but not limited to combustion products water and carbon dioxide, as well as oxides of sulphur and nitrogen, fine dust, organic contaminants such as dioxins and furans, and inorganic contaminants such as heavy metals. The impact of the majority of these contaminants including inorganics with some of them known as heavy metals on microalgae productivity have not been explored."

Specifically, below are several major concerns I have:
1. It is suggested that the title be changed to "Quantification of heavy metals and their impacts on microalgae productivity in a simulated environment"
The title has been changed per the suggestion of the reviewer. The authors point out that not all of the contaminants in the study are heavy metals. The new title proposed is "Quantification of heavy metals and other inorganic contaminants on the productivity of microalgae"

2. The protocol is designed for studies being conducted in a simulated algal growth environment, i.e., pH-controlled glass tube reactors spiked with known amount of sterilized metal ion solutions. The authors should elaborate how this method can be adapted to be used in actual industrial flue gases, which are more complex. For example, whether the pH needs to be monitored, and should the samples be sterilized? Critical steps should be added to address these issues.
The discussion section has been updated to address the reviewers comment. A core results is the need to better understand the impacts of integrating waste streams with microalgae cultivation. Previous TEA and LCA work has assumed a seamless integration which based on the results of this study might not be the case. Utilization of flue gas could negatively impact growth and the potential uses of the produced biomass as illustrated with the results from this study. Further, the results from this study can generally be applied to assessing impacts of coal based flue gas integration and the potential for algae to be used for environmental remediation. Both of these points have been added to the discussion section. The following text has been added: "The results of this study highlight the need to better understand the potential negative impacts on integrating microalgae growth systems with waste carbon sources, specifically coal based flue gas. Previous TEA and LCA assessments have assumed a seamless integration without considering the impacts of contaminants such as heavy metals and inorganic contaminants on productivity. In general the results from this work highlight the impact of a multi-metal system on productivity and can be used to understand the potentials of microalgae to bioremediate contaminants. " In terms of pH regulation, algal systems will be monitored for pH as this dictates the rate as which carbon is added to the system. For a commercial system it is likely that one system will be monitored with the assumption that a bank of growth systems will mimic the monitored system. The authors agree with the reviewer that sterilizing the system is not

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necessary and is limited to the need for accurate metal detection. To address this point the discussion has been edited. The following text has been added/edited: "Determination of the pH of the medium before starting the experiment is QC step that allows for verification that the medium is not acidified (e.g. resulting from improper PBR rinsing after acid soaking) will affect algal physiology and change nutrient bioavailability (e.g. changes in inorganic carbon speciation and metals speciation) thus impacting the interactions between algal binding sites, nutrients and metals, and consequently growth. The meticulous preparation of the laboratory equipment for these studies was required such that an accurate mass balance of the introduced metals can be performed. "

3. The study only focuses on 14 metal ions and their potential impacts on the microalgae growth, and does not discuss how the majority of the components in the actual flue gases would affect the outcome. While the steps listed in the procedure would lead to the described outcome, it is very questionable if the same conclusion be made with microalgae sampled cultured with real flue gases.
The authors agree that integration with an existing flue gas system would be ideal. The study presented is intended to evaluate one aspect, heavy metals, on microalgae productivity. It is expected that other contaminants in flue gas will further negatively impact productivity. The discussion has been expanded to point out there are other constituents in flue gas that could further complicate integration of growth systems with flue gas. Specifically the following text has been added: "The results from this study highlight the needs to understand the productivity implications of other contaminants expected to be present in flue gas such as oxides of sulphur and nitrogen, fine dust, and super-toxics such as dioxins."

Reviewer #2:
The paper describes the evaluation of the growth of the saline microalgae Nannochloropsis salina in PBR's with various inorganic contaminants that are often present in flue gas. Although this method is necessarily novel, a clear and comprehensive description of the method is important for future studies as flue gas could be a potential economical source of CO2 for algal cultivation. The authors have done an excellent job of providing detailed information of the procedures used and have made it feasible for the reader to repeat the results and implement the method in his or her own research.
A few minor points were found that could easily be changed and make the paper ready for publication.

- Abstract, line 59: change to: …a statistical decrease in biomass yield with increasing concentration of these contaminants.
The following change was made based on the reviewer's comment, "with the introduction of these contaminants" The work presented tests one metal concentration compared to a control and felt the requested change would indicate a variety of concentrations were tested.
- Line 96: …at concentrations expected…
The change has been made.
- Line 118: change to: -80 C freezer
The change has been made.
- Line 124: to a desired sampling point.

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The change has been made.
- Line 168: indicate the pressure. Was it at a standard 20 psi?
The pressure has been indicated, specifically atmospheric.
- Line 192: indicate the pressure. Was it at a standard 20 psi?
The pressure has been indicated, specifically atmospheric.
- Line 215-216: indicate that this is best to be performed in a sterile hood or at least nearby a flame in a cleaned environment to reduce the risk of contamination.
The change has been made.
- Line 232: is the 28 g per liter or per PBR. Please indicate a total volume.
The total volume has been indicated.
- Line 238: it is assumed that the resuspending happens in fresh, sterile media?
A clarification has been made to the manuscript.
- Line 364: to a capped container
The change has been made.
- Line 448-449: please indicate what LFB and LFM stand for, or possibly include a list of abbreviations (which would actually be useful for the entire paper)
A list of abbreviation has been generated with each abbreviation defined at the first occurrence. Further the definitions of these abbreviations are included at this instance.
- Line 612: add: minimal settling or biofouling during the 7-day growth experiments. (If this was tested for longer please indicate appropriately. Settling and biofouling often happens after longer growth periods so this in itself is not surprising).
The comment has been added to the discussion.
- Line 614 and line 624: add: heavy metal contaminated media (should indicate what type of contamination, to distinguish from organic or bacterial, etc.)
The clarification comment has been added.
- Line 635-638: Please indicate in the discussion the ratio of how much of the added inorganics were accumulated in the biomass versus how much was left over in the supernatants. If a potential limitation of scaling is an issue (as indicated by the authors) it would be good to list how much would be carried over in the super. This is obviously different for the various inorganics, so possibly the reader needs a reference to a table that shows the numbers.
The requested information is presented in figure 4 of the original manuscript. A reference to figure 4 has been made in the discussion as requested by the reviewer. Specifically the following text has been edited/added: "Contaminants not removed in the biomass have the potential to accumulate in the media as illustrated in figure 4. Accumulation in the media represents a potential to limit scale as media recycling represents an economic necessity for economic viability. The limitation would be dictated by the tolerance to heavy metals contaminants which will be species specific."

Reviewer #3:
Manuscript Summary:
The manuscript describes an apparatus and method for growing microalgae in the presence of inorganic ions and then quantifying the amount of the inorganic contaminants in both biomass and growth media.
The detail in the manuscript is almost sufficient - what is missing for me is the suppliers used.

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This is important because drilling the holes for the pH probe, for example, may depend on which pH probe is used and from where it is sourced.
Suppliers of the various pieces of equipment were provided in the original submission documentation. Specifically the excel file with the list of equipment contains all information required for procuring the materials used in this work.
The instructions are generally quite clear and easy to follow. It is a little unclear in places, but reading the entire way through clarified those points, which is realistically what someone would do if they were trying to follow this protocol.
I have a very few suggestions as to where the communication can be improved, but this is well written, technically complete (except for supplier details on hardware) and easy to follow.

Major Concerns:
N/A

Minor Concerns:
1) Lines 69-74 are a condensed version of the paragraph starting on line 76. The easiest way I can see to address this is to simply remove these lines.
The text the first paragraph has been edited based on another reviewers comment. The authors have introduced other concepts in this area of text that differentiates it from the following paragraph.

2) I am not an expert in microalgae production, but in section one in the instructions to build the system, the supplier of the components would be useful for people who would like to actually do this.
Supplier information has been added.

3) In the biomass section, it would be useful to clarify what 'contaminated' is given that a dozen or so inorganic metals are examined here I am not clear which are used for this data.
Clarification has been added to this section, specifically that contaminated refers to the multi-metal contamination.

Reviewer #4:
Major Concerns:
1. From abstract, "Microalgae were cultivated..... in a growth media polluted with inorganic contaminants at levels expected based on commercial flue gas integration." In addition, from the text, the researchers varied the concentrations of inorganic matter into 7 levels. The importance of these levels need to be explained clearly in the content.
Clarification text has been added to the abstract and to the introduction to detail the specific contamination levels. The levels are based on what would be expected from the integration of microalgae production systems with coal flue gas. Flue gas has different concentrations of the various metals and the assumed metals concentration is based on the expected CO_2 requirements.

2. As the title focus on the impacts of inorganic contaminants on microalgae productivity, readers may expect to see the results on the influence of each element or some most relevant elements in flue gas on the microalgae growth; maybe at least the impacts from different levels

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of these contaminants concentration.
The title has been changed based due to a reviewer comment. The authors understand the point by the reviewer but feel the reader will quickly understand the scope of the work by reading the abstract.

3. line 626: the distribution/mass balance of each inorganic matter among biomass, media and environment should be presented.
The requested information is presented in figures 3 and 4. A reference to these figures has been added at this point in the manuscript.

line 619: Which evidences/references show the effect on algal physiology?
The reviewer makes a good point and the language has been changed. The updated manuscripts states the importance of pH on growth not algal physiology.

4. There are many definitions and abbreviations available in the manuscript. Thus, it needs to be clarified additionally their meaning or importance, why to do that, and what they for. That will make readers follow the content clearly.
A list of abbreviations has been added to the manuscript. Each abbreviation is fully defined on the first occurrence.

Minor Concerns:
line 58: There are no Se and Sn in the list of elements which were analyzed by ICPMS.
The list has been updated. Discussion has been added to the text as results for Se and Sn had detection due to matrix interference and contamination issues, respectively. The issues related to Se detection are related to matrix interference. This effect is supported with Smith et al. 2004.
line 98: All studied inorganic matter is related to the flue gas. So it'll be great to provide some information on the concentration of each matter in flue gases.
More information on the concentrations in flue gas have been referenced and discussed in the text. Detailed information regarding the concentrations of heavy metals is presented in Napan et al. (in press).
line 114: ....with silicone lids.
The change has been made.
line 163: 2.3) How to make sure that all acid is removed?
Removal of the acid is ensured through multiple rinses and testing the pH. More text has been added to clarify this point.
line 168: 2.4) Should say 'sterilize PBRs, containers and flasks.....' ? as in the 3.2) and 5.3) you mentioned about sterilized container and flask.
The manuscript has been updated based on the comment.
line 170: You might mention in the text that the method in 3.1) for 'solution A' preparation and the method in 3.2) for ' vitamin solution'.
The suggested change has been integrated into the manuscript.
line 290: You mentioned in the abstract that you used light density at 984 μmol m-2 s-1 (line 53) and the same value in line 136 but in line 227, the value of light density is changed to 200 μmol m-2 s-1 and in line 290, there are no value indicated there. What is the point of these different light intensities at each section ?

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Inoculum is cultivated at a lower light intensity. Clarification on light intensities has been added to the methods.

line 232: How to indicate that a total of biomass already reaches 28 g ? and the 28 g biomass on dry or wet weight?

The biomass is estimated through optical density and the 28 g is referring to dry weight biomass. Clarification has been added to the manuscript.

line 313-340: It'll be good to give a reason that why we need to do the acid rinsed microwave digestion vessel at 7.1-7.8.

Per the request of the reviewer justification for this step has been added at line 313 of the original manuscript. The digestion is required as a pre-processing step for ICP-MS analysis to remove any residual contamination from previous testing.

line 368-370: This one should move to 'Quantification section'.

The change has been made.

line 386: .....standard L5.... It should be identified what is 'L5'.

L5 has been removed and the standards are identified with reference to protocol steps in the manuscript.

line 424: Do you mean.....(Ar, H_2, He)....

The subscript 2 has been added to the H.

line 631-635: The different of color between the inorganic contaminated ones and control one might be from the variation of pigments in algae cells due to the contaminants ?

The authors speculate the differences in pigments are due to stress from the contaminants. A statement to this effect has not been included as it is not a definitive result.

Table 3: The unit of vitamin solution should be μL.

The unit of volume should be written as 'L'(capital letter). That might reduces the confusion between the unit as 'L' and normal 'l' in the text.

Liters has been abbreviated with a capital L per the request of the reviewer.