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Quantification of heavy metals and other inorganic contaminants on the productivity of microalgae

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Abstract:	Increasing demand for renewable fuels has researchers investigating the feasibility of alternative feedstocks, such as microalgae. Inherent advantages include high potential yield, use of non-arable land and integration with waste streams. The nutrient requirements of a large-scale microalgae production system will require the coupling of cultivation systems with industrial waste resources, such as carbon dioxide from flue gas and nutrients from wastewater. Inorganic contaminants present in these wastes can potentially lead to bioaccumulation in microalgal biomass negatively impact productivity and limiting end use. This study focuses on the experimental evaluation of the impact and the fate of 14 inorganic contaminants (As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn) on Nannochloropsis salina growth. Microalgae were cultivated in photobioreactors illuminated at 984 µmol m-2 s-1 and maintained at pH 7 in a growth media polluted with inorganic contaminants at levels expected based on the composition found in commercial coal flue gas systems. Contaminants present in the biomass and the medium at the end of a 7 day growth period were analytically quantified through cold vapor atomic absorption spectrometry for Hg and through inductively coupled plasma mass spectrometry for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Se, Sn, V and Zn. Results show N. salina is a sensitive strain to the multi-metal environment with a statistical decrease in biomass yield with the introduction of these contaminants. The techniques presented here are adequate for quantifying algal growth and determining the fate of inorganic contaminants.
Author Comments:	
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Question	Response
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January 29, 2014

Ethan Prather
Associate Editor
Journal of Visualized Experiments

Dear Ethan,

It is my honor to re-submit the article “Quantification of inorganic contaminants and their impacts on microalgae productivity” for consideration for publication in the Journal of Visualized Experiments.

We have revised the manuscript based on the reviewer comments and made every effort to ensure the manuscript is ready for publication. Attached is a detailed response to the reviewer comments.

The authors of this work and their contributions are: Katerine Napan, Derek Hess and Brian McNeil were involved in experiment set-up, protocol development, data collection and analysis; and Jason C. Quinn (Principal Investigator) involved in experimental development and analysis. Please feel free to contact me with any questions or concerns.

Sincerely,

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TITLE:
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SHORT ABSTRACT:
Integration of microalgal cultivation with industrial flue gas will ultimately introduce heavy metals and other inorganic compounds into the growth media. This study presents a procedure used to determine the end fate and impact of heavy metals and inorganic contaminants on the growth of *Nannochloropsis salina* grown in photobioreactors.

LONG ABSTRACT:

Increasing demand for renewable fuels has researchers investigating the feasibility of alternative feedstocks, such as microalgae. Inherent advantages include high potential yield, use of non-arable land and integration with waste streams. The nutrient requirements of a large-scale microalgae production system will require the coupling of cultivation systems with industrial waste resources, such as carbon dioxide from flue gas and nutrients from wastewater. Inorganic contaminants present in these wastes can potentially lead to bioaccumulation in microalgal biomass negatively impact productivity and limiting end use. This study focuses on the experimental evaluation of the impact and the fate of 14 inorganic contaminants (As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn) on *Nannochloropsis salina* growth. Microalgae were cultivated in photobioreactors illuminated at 984 µmol m^{-2} s^{-1} and maintained at pH 7 in a growth media polluted with inorganic contaminants at levels expected based on the composition found in commercial coal flue gas systems. Contaminants present in the biomass and the medium at the end of a 7 day growth period were analytically quantified through cold vapor atomic absorption spectrometry for Hg and through inductively coupled plasma mass spectrometry for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Se, Sn, V and Zn. Results show *N. salina* is a sensitive strain to the multi-metal environment with a statistical decrease in biomass yield with the introduction of these contaminants. The techniques presented here are adequate for quantifying algal growth and determining the fate of inorganic contaminants.

INTRODUCTION:

Compared to traditional terrestrial crops microalgae have been shown to achieve higher biomass and lipid yields due to inherent higher solar conversion efficiencies[1,2]. Cultivation of microalgae at high productivity rates requires the supply of various nutrients including an external carbon source. It is expected that large-scale growth facilities will be integrated with industrial waste streams such as industrial flue gas in order to minimize production costs and at the same time provide environmental remediation. Industrial waste carbon is typically in the form of gaseous carbon dioxide and can contain contaminants that have the potential to negatively impact microalgae production. 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. Some of these elements can be nutrients at appropriate concentrations, however at higher concentrations they can produce cell dysfunction and even death[3].

The integration of microalgae with industrial flue gas has the potential to directly introduce inorganic contaminants into growth media. Coal based flue gas has a variety of inorganic elements (*e.g.* As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn) at various concentrations some of which, in low concentration, represent nutrients for microalgae growth. Inorganic contaminants have a high affinity to bind to microalgae and further be sorbed internally through nutrient transporters. Some inorganic contaminants (*i.e.* Co, Cu, Zn and Mn) are nutrients that form part of enzymes involved in photosynthesis, respiration and other functions[3,4]. However, in excess metals and metalloids can be toxic. Other elements, such as Pb,

Cd, Sn, Sb, Se, As and Hg, are not known to support cell function in any concentration and represent non-nutrient metals which could negatively impact culture growth[3,5,6]. The presence of any of these contaminants has the potential to produce negative effects on microalgae cell function. Furthermore, the interaction of multiple metals with microalgae complicates growth dynamics and has the potential to impact growth.

Large-scale economics have been directly linked to the productivity of the cultivation system[7-19]. Moreover, medium recycle in the microalgae growth system for either open raceway ponds (ORP) or photobioreactors (PBR) is critical as it represents 99.9 and 99.4% of the mass, respectively[20]. The presence of inorganic contaminants in the media could ultimately limit microalgae productivity and the recycling of media due to contaminant build up. This study experimentally determined the impact of 14 inorganic contaminants (As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn), at concentrations expected from the integration of microalgae cultivation systems with coal derived flue gas, on the productivity of *N. salina* grown in airlift PBRs. The contaminants used in this study have been shown to not only be present in coal-based flue gas but municipal waste-based flue gas, biosolids-based flue gas, municipal wastewater, produced water, impaired groundwater and seawater[21-23]. The concentrations used in this study are based on what would be expected if microalgae growth systems were integrated with a coal based CO_2 source with an uptake efficiency demonstrated in commercial PBR systems[20]. Detailed calculations supporting the concentrations of the heavy metals and inorganic contaminants are presented in Napan et al.[24] Analytical techniques were used to understand the distribution of the majority of the metals in the biomass, media and environment. The methods presented enabled the assessment of productivity potential of microalgae under inorganic contaminant stress and quantification of their end fate.

PROTOCOL:

1. Growth system

1.1. Build the following microalgae experimental growth system (Figure 1).

[Place Figure 1 here]

1.1.1. Acquire twelve airlift PBRs consisting of glass tube reactors 4.5 cm in diameter and 80 cm in height with a cultivation capacity of 1.1 L with silicone lids. Acquire pre-cut glass capillary tubes (5 mm external diameter and 1 mm internal diameter) of 10 cm (3 per PBR) and 85 cm (1 per PBR) in length.

1.1.2. Freeze silicone lids in a -80 °C freezer. Lubricate a drill bit with glycerol and while lids are frozen drill 3 holes to host the vent, sampling and gas delivery capillary tubes, and 1 hole of 17 mm diameter to host a pH probe.

1.1.3. Insert the 3 capillary tubes in place with the longest tube extending 2 cm from the bottom of the PBR. In the other capillary tube add a silicon tube with a capillary tube attached

to the other end extending to a desired sampling point. Cover the hole for the pH meter with a silicone stopper size 21D.

1.1.4. Humidify ambient air by bubbling it through water and deliver the humidified air to the air distribution header. Pass the gas through a 0.2 µm filter and deliver it to the algal suspension through the longest glass delivery capillary tube.

1.1.5. Deliver compressed CO_2 into the humidified air stream in order to maintain a neutral pH of 7.0 ± 0.1 in the culture suspension. Control the rate of CO_2 delivery with an automated CO_2 dispensary system (pH controller) that opens a magnetic solenoid when the algal culture reaches pH 7.1 and closes at pH 6.9.

1.1.6. Provide light using 24 T5 fluorescent lamps that result in an average illumination of 984 µmol m^{-2} s^{-1} similar to peak outdoor conditions.

1.1.7. Immerse the PBRs in a water bath in order to maintain a constant temperature of approximately 25 °C. Control the temperature of the system by using a recirculating chiller and an automated heating recirculating water bath unit control.

1.1.8. Monitor temperature and pH in real-time and record with a data logger.

1.1.9. Ensure that all components of the microalgae growth system are properly working, especially before harvesting microalgae inoculum or preparing inorganic contaminants as they cannot be preserved.

2. Lab ware preparation

2.1) Wash volumetric flasks, PBRs, carboys and any containers, with soap and tap water. Rinse with deionized water (DW).

2.2) Acid rinse the lab ware in order to eliminate any traces of inorganic contaminants. This can be done by one of two ways:

2.2.1. Soak lab ware overnight in 10% trace metal grade nitric acid (CAUTION: Do not breath fumes, concentrated nitric acid can produce severe burning and toxic fumes, work in a fume hood using nitrile gloves, goggles and lab coat).

2.2.2. Soak lab ware for 15 min in 50% trace metal grade nitric acid.

2.3) Rinse the lab ware with DW thoroughly at least 3 times making sure all acid is removed. It is critical that PBRs are thoroughly rinsed, especially the sampling tubes and the capillary tubes. Failure to do this will produce acidification of the medium and possible inhibition of growth. Test the pH of the rinse water to verify all acid has been removed.

2.4) Sterilize PBRs, containers and flasks by autoclaving them at 120 °C and standard atmospheric pressure for at least 30 min.

3. *N. salina* medium preparation

3.1) Preparation of solution A: Partially fill a 1 L volumetric flask with DW. Insert a magnetic stir bar and add the chemicals shown in Table 1 one after the other. Ensure that each ingredient dissolves before the addition of the next constituent. Remove the magnet and fill the flask to the 1 L volume mark.

[Place Table 1 here]

3.2) Preparation of vitamin solution: In three separate volumetric flasks add the vitamins as shown in Table 2. Filter each vitamin solution through a sterile 0.2 µm syringe filter to a sterile container. Preserve vitamins at -4 °C in the dark.

[Place Table 2 here]

3.3) Partially fill a 20 L autoclavable container with DW and insert a magnetic stir bar. Place the container on top of a magnetic stirrer plate and add the chemicals shown in Table 3 (except the vitamins), adding them one after the other and after each fully dissolves. Fill the container to reach 20 L.

[Place Table 3 here]

3.4) Sterilize the medium by autoclaving for 30 min at 120 °C and atmospheric pressure. Let the medium cool down to room temperature.

3.5) Place the container on a magnetic stirrer plate. Add the vitamins prepared in step 3.2 and let the medium mix thoroughly.

4. Inorganic contaminants stock preparation

4.1) Partially fill the volumetric flasks indicated in Table 4 with DW and add the individual salt listed. Fill with DW to the required final volume and mix thoroughly. Do not preserve these stocks as some elements adsorb to flask walls

CAUTION: Several inorganic contaminants used in this protocol are carcinogenic, teratogenic and mutagenic, wear a face mask, gloves and lab coat when handling salts.

[Place Table 4 here]

4.2) Sterilize the inorganic contaminant stocks by passing the solution through a sterile 0.2 µm syringe filter and collect the filtrate in a sterile tube.

5. *N. salina* inoculum production

5.1) In a 500 mL Erlenmeyer flask add 200 mL of medium prepared in step 3 and then add 3 g of agar. Cover the flask with aluminum foil and autoclave for 20 minutes at 120 °C. Pour the solution into sterile petri-dishes and let it cool until it solidifies. This should be completed be a sterile hood or at least near a flame in a clean environment to reduce risk of contamination.

5.2) Streak *N. salina* cells in sterile petri-dishes prepared in step 5.1 using a sterile seeding loop. Place the petri-dish cultures on a table illuminated with T12 lights maintained at room temperature. Let microalgae grow until colonies are visible.

5.3) Transfer colonies to sterile baffled Erlenmeyer flasks containing 200 mL of nutrient rich medium prepared in step 3 and keep them on an illuminated shaker table (1000 RPM). Let the culture grow until medium becomes green.

5.4) Transfer the microalgae to a 1.1 L sterile PBR. Place the PBR in an inoculum water bath illuminated at 200 µmol m^{-2} s^{-1} with T8 fluorescent lights and maintained at 23 °C by a recirculating chiller and an automated heating recirculating water bath control. Adjust the air and CO_2 rotometers to 2.5 L min^{-1} and 25 cc min^{-1}, respectively.

5.5) After a week of growth split biomass into new 1 .1 L PBRs containing new medium and let it grow until a total of at least 28 g of dry weight biomass are obtained between the two reactors which can be determined through optical density.

5.6) Harvest the inoculum biomass by centrifugation at 2054 × g for 15 min at 10 °C using sterile centrifuge bottles and sterile techniques to avoid contamination. Dispose of the supernatant and continue cell concentration as needed.

5.7) Once all biomass is centrifuged, re-suspend the cells in 300 mL of fresh sterile medium.

5.8) Dilute 0.1 mL of microalgal culture in 3 mL of DW and then dilute 0.1 mL of this new solution in 3 mL of DW. Ensure the sample is thoroughly mixed. Measure the optical density (OD) of the microalgae concentrate at 750 nm (OD_{750}) immediately using a spectrophotometer.

5.9) Use equation (1) to determine the amount of biomass in the concentrate.

Note: Equation (1) was obtained from the linear regression between OD_{750} versus total suspended solids (TSS in g L^{-1}) for *N. Salina* (R^2=0.9995). Equation 1 was developed for the spectrophotometer model in the Materials Table, generate a new calibration if using another spectrophotometer model.

5.9.1) Using equation (2) calculate the volume of microalgae concentrate (V in L) needed to obtain a 4 g L^{-1} culture density in a PBR of 1.1 L volume (PBR_v in L).

$$TSS = OD_{750} \times 0.2962 + 0.0667 \quad (1)$$

$$V = TSS \times PBR_v \quad (2)$$

5.10) Using sterile techniques, add the volume of microalgae concentrate found in the step 5.9 to an autoclaved PBR to reach an initial culture density of 4 g L^{-1}. Fill PBR with medium to 1.1 L. Repeat this step until 6 PBRs are inoculated. Place the PBRs in the inoculum water bath.

5.11) Let the microalgae in the PBRs grow for 8 days and then harvest the biomass (by repeating steps 5.6 to 5.7). Repeat step 5.8 to calculate the initial inoculum volume for an initial culture density of 1 g L^{-1}.

6. Experimental reactors

6.1) Using sterile techniques add approximately 1 L of medium prepared in step 3 to each of the 12 acid-rinsed sterile PBRs. Place the PBRs in the water bath of the experimental growth system. Turn sparge air on at 1.5 L min^{-1}.

6.2) Sterilize a calibrated pH meter by cleaning it with 70% ethanol. Measure the pH of the medium in the PBR and ensure pH is approximately 7.0; if not, repeat step 2 to remove acid leached from the acid rinsing step.

6.3) Calibrate each pH controller using buffer pH 7, disinfect the probes using ethanol (70%) and then insert them in the PBRs lids.

6.4) To each PBR (except the control PBRs) add 1 mL of each of the sterile inorganic contaminants stocks prepared in step 4. Let the contaminants thoroughly mix in the PBR. The final concentration of the inorganic contaminants in the PBRs are shown in the last column in Table 4, and are the estimated maximum concentrations expected from a coal-fired power plant integration.

6.5) Add 14 mL of sterile DW to the control PBRs.

6.6) Add the concentrated microalgae inoculum obtained in step 5.11 to the experimental PBRs in order to obtain an initial culture density of 1 g L^{-1}. Let biomass mix thoroughly.

6.7) Turn high light intensity lights (of 984 µmol m^{-2} s^{-1}) and pH controllers on and adjust CO_2 to 30 cc min^{-1}. Increase the CO_2 flow to 50 cc min^{-1} from day 3 afterwards. Initial low CO_2 flow rate is critical in order to avoid large changes in pH due to delays in gas/liquid transfer and pH measurement.

6.8) Measure OD_{750} and take samples as needed. Make sure to mark the water level after sampling. (CAUTION: some inorganic contaminants in the PBR are carcinogenic, teratogenic and

mutagenic; use gloves and capped containers when handling samples).

6.9) Add sterile DW daily to the PBRs in order to compensate for losses due to evaporation.

6.10) After 7 days of growth, harvest the biomass by centrifugation at 9936 × g and preserve both, biomass and supernatant medium, at -80 °C.

6.11) Freeze dry the biomass at 0.1 mbar and -50 °C overnight. Powder the biomass (use a spatula to powder biomass inside the centrifuge tube). Preserve freeze dried biomass at -80 °C.

7. Microwave assisted digestion of samples

The digestion of the biomass samples is required as a pre-processing step for ICP-MS analysis.

Note: These steps use a closed vessel microwave digestion system with controlled pressure relief. (CAUTION: High pressures develop during acid digestion, inspect the physical integrity of the digestion vessels and shields, and reshape the microwave digestion vessel lids before every use).

7.1. Wash Teflon microwave digestion vessels with soap and water, rinse with DW and let vessels air dry. To remove trace metal contamination in the vessels digest acid as described in the following steps.

7.2. Reshape the microwave digestion vessel lids and close the vials tightly.

7.3. Add 10 mL of nitric acid to each.

7.4. Introduce the vessel in the safety shield. Ensure that no biomass, water or any reagents are left on the walls of the safety shield or in the outer walls of the digestion vessels in order to avoid damage to the safety shield. Cap the safety shield with the safety valve making sure the spring in the vial is flush. Locate the shield on the rotor with the cap vents pointing outward in the outer row and inwards in the inner row.

7.5. On vessel number one, insert the ceramic thermowell and the temperature sensor. This thermometer monitors the actual internal temperature in the vial and serves as the controlling parameter to execute the digestion program. Ensure that vial number one contains the same sample and reagent amounts as the other vials.

7.6. Input the digestion parameters shown in Table 5 and start digestion. When the program has finished, air cool the vials until they reach room temperature.

[Place Table 5 here]

7.7. Inside a fume hood, insert the pressure relief tool on the shield cap with the cap vents point away from you. Once pressure is released open the cap (CAUTION: Always open digested

vials inside fume hood since biomass digestion using acid produces toxic fumes).

7.8. Dispose of the acid. Rinse the Teflon vessels with DW 3 times. Let vials air dry.

7.9. To digest biomass, add 50 mg of freeze dried biomass to microwave digestion vessels. For quality control (QC) prepare the following vials: in two different vials add either 5 mL of Level 7 ICPMS or 5 mL of Level 7 Hg CVAAS standard prepared in steps 9.1 and 10.1 (the digested solution from this vial is called the laboratory fortified blank (LFB)), leave another vial empty (the digested solution from this vial is called the laboratory reagent blank (LRB)).

7.10. To digest medium, add 10 mL supernatant medium to dry acid rinsed microwave digestion vessels. For quality control (QC) prepare the following vials: In two different vials add 5 mL of Level 7 ICPMS or CVAAS metal standard prepared in step 9.1 and 10.1 (the digested solution from this vial is called the LFB), to another vial add 10 mL of DW (the digested solution from this vial is called the LRB).

7.11. Reshape the microwave digestion vessel lids and close the vials tightly.

7.12. Add 7 mL of concentrated trace metal grade nitric acid and 3 mL hydrogen peroxide to each vial. Homogenize the contents by gently swirling the solution. Digest the contents of the vials by repeating steps 7.4 to 7.7 (use the microwave digestion parameters for sample digestion in Table 5).

7.13. Add digested sample to a 25 mL volumetric flask, rinsing the vessels with DW for increased recovery. Fill the volumetric flask with DW to the mark.

7.14. Transfer digested samples to a capped container. Preserve samples at 4 °C until analysis can be completed. For this study analysis is done the same day for Hg and within three days for the other elements.

8. Quality control (QC) samples

Note: Analyze QC samples in order to assure reliability of the results from experimental samples.

8.1. Partially fill an acid rinsed 1 L volumetric flask with DW. Add 280 mL of concentrated trace metal grade nitric acid and mix thoroughly (this solution is also called the blank solution) (CAUTION: always add acid to water, never add water to acid as the exothermic reaction can be violent). Let solution cool to room temperature.

8.2. In addition to QC samples prepared in steps 7.9 and 7.10, prepare the following QC samples.

8.2.1. For the continuing calibration verification (CCV): Fill a polystyrene tube with calibration

standard (for preparation see step 9.2 and 10.1). Put the Hg standard solution on the CVAAS rack and the ICPMS standard solution in the ICPMS autosampler.

8.2.2. For the continuing calibration blank (CCB): Fill two polystyrene tubes (16 mL) with the blank (solution prepared in step 8.1). Place one sample in the CVAAS rack and the other sample in the ICPMS autosampler.

8.2.3. For the laboratory-fortified matrix (LFM): Randomly choose 1 sample of every 12 samples for each type of sample (i.e. biomass or medium) and use it to prepare a LFM. For ICPMS, add 0.5 mL of ICPMS standard Level 7 and 3 mL of digested experimental sample (from either biomass or medium) to a polystyrene tube.

8.2.4. Mix contents and place the vials on the ICPMS autosampler. For CVAAS, add 2 mL Hg standard Level 7 and 6 mL of digested experimental sample (from either biomass or medium) to a polystyrene tube. Mix contents and place vials on the CVAAS rack.

8.2.5. For the duplicate samples: Randomly choose 1 sample of every 12 samples for each type of matrix (e.g. biomass, medium, LFM or any diluted matrix) and duplicate the vial. Place the repeated vials in the ICPMS autosampler or the CVAAS rack.

8.2.6. For the duplicate samples: Randomly choose 1 sample of every 12 samples for each type of matrix (e.g. biomass, medium, LFM or any diluted matrix) and duplicate the vial. Place the repeated vials in the ICPMS autosampler or the CVAAS rack.

8.3. Define the data quality criteria for the study. For the present study duplicate the quality criteria established by Eaton, Clesceri, Rice and Greenberg [25]. The parameters established for the QC are: percent difference (%D) for CCV within ± 10%[25] (with exception of Pb and Sb, see discussion), LFB percent recovery (%R) within ± 70-130%[25], LFM percent recovery (%R) within 75-125%[25], and relative percent difference (RPD) within ± 20%[25], and a continuing calibration blank (CCB) below method reporting limit (MRL)[25]. See calculation equations in step 9.7.

9. Quantification by Inductively Coupled Plasma Mass Spectrometry (ICPMS)

9.1. On the day of analysis, transfer approximately 5 mL of digested sample to polystyrene tubes and place them in the ICPMS autosampler. Add approximately 15 mL of digested samples to polystyrene tubes and place them in the CVAAS rack.

9.2. The same day of analysis prepare the calibration standards. Add purchased ICPMS standard solution and refill with blank (solution prepared in step 8.1) as described in Table 6 (see standard solution description in Material Table) to acid-rinsed volumetric flasks.

[Place Table 6 here]

9.3. Remove the cones from the ICPMS and sonicate them for 1 min in DW. Dry the cones

and put them back in the instrument.

9.4. Turn on the water chiller, gasses (Ar, H_2, He), the ICPMS, plug lines to internal standard, and fill auto-sampler rinse containers (DW, 10% nitric acid, 1% nitric acid + 0.5% hydrochloric acid).

9.5. Open the Masshunter Workstation software and turn on the plasma, tune the ICPMS and load the method set to parameters in Table 7.

[Place Table 7 here]

9.6. Place calibration standard, QC samples and experimental samples in the autosampler. In the ICPMS software add the analysis sequence and analyze samples. Aspirate the sample inside the instrument to the plasma where the elements are ionized. Then a vacuum withdraws the ions to a counter. The ions will separate depending on their atomic weight from the lightest to the heaviest.

CAUTION: Collect ICPMS waste in hazardous containment and handle appropriately for disposal.

9.7. Ensure that the correlation coefficient (R) value for the calibration curve for each metal or metalloid is greater than 0.995[24].

9.8. During sample analysis, calculate %R, %D and RPD as described in equations 3 to 6 [26] and compare the results to the project data quality criteria in 8.3.

9.8.1. Calculate percent recovery (%R) to determine losses/gaining from the laboratory fortified blank (LFB) and matrix interference from laboratory-fortified matrix (LFM).

$$\%R = \frac{LFB\ result - LRB\ result}{known\ LFB\ added\ concentration} \times 100\% \qquad (3)$$

$$\%R = \frac{LFM\ sample\ result - sample\ result}{known\ LFM\ added\ concentration} \times 100\% \qquad (4)$$

9.8.2. Calculate percent difference (%D) to determine instrument performance changes with time when running CCV samples.

$$\%D = \frac{CCV\ true\ value - \text{CCV}\ found\ value}{CCV\ true\ value} \times 100\% \qquad (5)$$

9.8.3. Calculate relative percent difference (RPD) to determine changes in method precision with time when running experimental samples.

$$RPD = \frac{sample\ result - duplicate\ result}{(sample\ result + duplicate\ result)/_2} \times 100\% \qquad (6)$$

9.9. To reduce matrix interference (%R out of acceptable range), dilute the samples for poor %R to a ratio 1:3 (sample:DW).

10. Hg quantification by Cold Vapor Atomic Absorption Spectrophotometer (CVAAS)

10.1. Prepare calibration standards the same day of analysis. Dilute purchased Hg standard by adding 1 mL of purchased Hg standard solution to a 100 mL volumetric flask and fill with the solution prepared in step 8.1.

10.1.1. Add 2.5 mL of this solution into a 100 mL volumetric flask and fill with the solution prepared in step 8.1 (this new solution is Level 7 Hg standard). Add diluted Level 7 Hg standard to volumetric flasks and fill with blank (solution prepared in step 8.1.) as described in Table 8 (see purchased Hg standard solution description in Material Table).

[Place Table 8 here]

10.2. Open the Ar gas and air valve, turn on the Atomic Absorption Spectrophotometer and the Flow Injection Atomic Spectroscopy (FIAS). Open the CVAAS Winlab software, turn on the Hg lamp and let it warm up until the software's energy parameter reaches 79. Load the program for Hg analysis with the parameters in Table 9. Adjust the light path in the instrument to give the maximum transmittance.

[Place Table 9 here]

10.3. Plug the line to the carrier solution made of 3% trace metal grade hydrochloric acid.

10.4. Plug the line to the reducing agent solution made of 10% stannous chloride (suitable for Hg analysis) in 3% trace metal grade hydrochloric acid. Prepare this solution the same day of analysis as it is prone to atmospheric oxidation (CAUTION: Stannous chloride is very hazardous, use protective wear when working with it. Collect CVAAS waste in hazardous containment and properly dispose).

10.5. Place the Hg standards, QC samples and experimental samples in the CVAAS rack and input the sequence in the CVAAS Winlab software. Run standards and generate the calibration equation.

10.6. Run QC samples and experimental samples. The CVAAS draws approximately 5 mL of sample into the instrument, reduces the Hg present in the sample to elemental Hg (Hg^0) gas and purges the gas from solution with a carrier gas (Ar) in a closed system. The Hg vapor passes through a cell in the Hg lamp light path. A detector determines the light absorbed at 253.7 nm and correlates it to concentration. (CAUTION: Hg vapor is toxic, ensure instrument exhaust hood is in place).

10.7. Calculate %R, %D and RPD in step 9.7 during analysis and compare the results to the

project data quality criteria.

REPRESENTATIVE RESULTS:

Biomass yields

Production of *N. salina* in the PBR system used in this study grew from 1 g L^{-1} to 8.5 ± 0.19 g L^{-1} (N=12) for control reactors and 4.0 ± 0.3 g L^{-1} (N=12) for the multi-metal contaminated in 7 days. The experiments produced repeatable data across triplicate reactors and multiple batches. Figure 2 (A) shows the average culture density with very small standard error based on sampling from three independent PBRs. To ensure this result was not an isolated result, three more batches were grown with similar results. The combined results for all four batches are shown in Figure 2 (B). Although biological variability exists, this study shows that there is a consistent negative impact of inorganic contaminants to *N. salina* production. The biomass yields in the contaminant exposed PBRs were statistically different to the control PBRs from day 2 onwards (ANOVA, $p < 0.05$).

[*Place* **Figure 2** *here*]

Quality Control assessment of inorganic contaminant quantification

Twelve of the fourteen elements analyzed were fully recoverable after digestion as shown by the LFB%R with %R near 100%, indicating no losses, no gains and no cross-contamination of analytes during digestion (**Table 10**). During quantitative analysis of samples %D and RPD were monitored through all analysis and the average of the results are shown in **Table 10**. As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, V and Zn passed the %D and RPD, however %D for Pb and Sb gradually dropped during analysis. The %D for these elements are improved after cone cleaning, however, constant cone cleaning is impractical, and therefore the data quality objectives for Pb and Sb were lowered. CCB for all the analytes were also below the MRL. Matrix effects were assessed by analyzing LFM samples and obtaining the %R. While Co, Hg, V and Sb passed the QC data criteria, it was not passed by As, Cd, Cr, Cu, Mn, Ni, Pb and Zn when digested biomass samples were analyzed, resulting in %R below the QC objectives. Matrix dilution in DW to a ratio of 1:3 (solute:solvent) resulted in %R that passed data quality criteria. Matrix effects were also observed during the analysis of the digested supernatant and were addressed by the same dilution ratio (**Table 10**) making sure the dilution did not compromise the detection limit of the instrument. Issues with the detection of Se and Sn were observed based on unstable readings and a contamination issue, respectively. The unstable readings for Se are attributed to salts in the matrix [27]. The Sn contamination was traced back to an acid used in the digestion step.

[Place **Table 10** here]

Inorganic contaminant concentrations

Heavy metal and inorganic contaminants were found in both biomass and supernatant medium. The concentrations found in the biomass for the 12 elements analyzed are shown in Figure 3. Concentrations in the biomass harvested from triplicate PBRs (N=3) in batch #1 shows a very small standard error (Figure 3 (A)). Combining data from triplicate PBRs from 4 batches

consistently shows that inorganic contaminants are present in the biomass (N=12). The concentrations found in the supernatant medium are shown in Figure 4. Results show triplicate PBRs (N=3) for batch #1 also have small standard error (Figure 4 (A)) and show that most contaminants preferentially were located in the biomass leading to very low concentrations in the supernatant with several sample concentrations close to the MRL of the instrument. Results from all four batches are presented in Figure 4 (B).

[*Place* **Figure 3** *here*]

[*Place* **Figure 4** *here*]

Figure 1. Microalgae growth system. (A) air rotometer, (B) CO_2 rotometer, (C) pH controller with solenoid, (D) data logger, (E) in-line air filters, (F) air distribution header, (G) fluorescent light bank, (H) pH meters, (I) cooling system, (J) water bath, (K) thermocouple wire, (L) air lift photobioreactor, (M) heater, (N) walk-in fume hood, (O) vent, (P) air delivery capillary tube, (Q) air filters, (R) sampling tube, (S) PBR silicone lid, and (T) pH well in silicone lid.

Figure 2. Culture concentration over the cultivation period for contaminated and control PBRs. (A) Culture density in batch #1, results from N=3 PBRs. (B) Culture density in 4 batches, results from N=12 PBRs. Empty circles represent contaminated biomass, filled circles represent the control.

Figure 3. Concentration of inorganic contaminants in biomass. (A) Concentration in batch #1, results from N=1 PBR for Zn and N=3 PBRs for all the other analytes, (B) Concentration from 4 batches, results from N=4 PBRs for Zn and N=12 PBRs for all the other analytes. Mean concentrations are represented by black filled circles, individual data points are represented by grey filled circles. Error bars represent ± one standard error from the mean.

Figure 4. Concentration of inorganic contaminants in supernatant. (A) Concentration in batch #1, results from N=3 PBRs, (B) Concentration from 4 batches, results from N=12 PBRs. Mean concentrations are represented by black filled circles, individual data points are represented by grey filled circles. Error bars represent ± one standard error from the mean.

Table 1. Solution A recipe. Quantities are amounts needed in the preparation of 1 L of concentrated solution.

Table 2. Vitamin solution recipe. Quantities are amounts needed for the preparation of concentrated solution.

Table 3. *N. salina* medium recipe. Quantities are amounts needed in the preparation of 20 L of nutrient-rich medium.

Table 4. Concentrated inorganic contaminants stock preparation. Addition of 1 mL of this concentrated stock to the 1.1 L PBR medium produces the final concentration shown in the last

column.

Table 5. Parameters used in the microwave digestion program.

Table 6. Concentration of calibration standards. Levels 1 to 7.

Table 7 ICPMS operating conditions.

Table 8. Concentration of Hg calibration standard. Levels 1 to 6.

Table 9. CVAAS operating conditions.

Table 10. Summary of the results of quality control samples. R: correlation coefficient, %D: percent difference, %R: percent recovery, RPD: relative percent difference, dilution ratio refers to solute:solvent ratio.

DISCUSSION:

Saline microalgae *N. salina* can be successfully grown in the designed growth system with repeatable results and high biomass yields. Airlift mixing allowed for a well-mixed suspended culture with minimal settling or biofouling over the 7 day growth periods. The minimal light variability across the fluorescent light bank is also shown to not produce noticeable differences in growth.

The study shows heavy metal contaminated media at concentrations representative of integration with coal flue gas negatively impacts biomass growth. Repeatability in the study highlights the impact the multi-metal system has on productivity. Various steps in the process have the potential to negatively impact growth and contaminate the system requiring diligent experimental preparation. Determination of the pH of the medium before starting the experiment is a QC step that allows for verification that the medium is not acidified (*e.g.* resulting from improper PBR rinsing after acid soaking). Acidified medium will affect algal growth 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. 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. Other steps in the process have the potential to introduce unaccounted for metals highlighting the need for the use of proper grade solvents and chemicals. Proper QC through the process can effectively identify the introduction of heavy metal contaminants.

Results show introduced contaminants are distributed between the biomass (Figure 3), media (Figure 4) and environment. Inorganic contaminants found in harvested *N. salina* suggests that this microalgae will incorporate several of the inorganic contaminants present in flue gas. This assimilation can be a result of adsorption onto cell walls due to charged binding sites, absorption inside the cell due to metabolic activity, and precipitation of complexes formed with elements present in the medium[28]. Visually the reactors with inorganic contaminants after a

couple of days appeared yellow in color compared to the dark green of the control reactors. Contaminated harvested biomass was not visually different from the contaminant-free biomass after pellet formation after harvesting by centrifugation. The visual color difference before harvest is attributed to a lower density biomass and stressed microalgae. 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 a necessity for economic viability. The limitation would be dictated by the tolerance to heavy metal contaminants which will be species specific. 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. 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 organic contaminants such as polychlorinated dibenzo dioxins and dibenzo furans. 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.

The methodology presented allowed for the study of inorganic contaminants with repeatable results for microalgae. Some inorganic contaminants used in this experiment are traditionally found in growth systems at low concentrations, but the others do not have a known function in the cell. As a result the multi-element mixture of As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn at the concentration shown in Table 4 inhibited growth. Quantifying the amount of contaminants in the biomass can prove challenging in multi-metal systems. Often, samples with high contents of organics and salts can produce matrix interferences, polyatomic interferences, physical interferences and salt build up in cones that eventually leads to inaccurate readings and loss of analytical accuracy[29,30]. Quality control samples run together with the experimental samples helped to determine the accuracy and precision of the readings. Measurement of the analytes using the protocols developed for this study has shown to be accurate and precise producing acceptable recoveries that are within the acceptable performance for this type of study[25,29]. Digestion of samples by microwave oven was showed to be effective for *N. salina* as digested samples were clear with no presence of cell debris or immiscible portions. The matrix used in this experiments (algal biomass and artificial seawater) produced matrix interferences that were overcome by matrix dilution. However, higher biomass sample sizes than the ones used in this experiment could lead to matrix interferences, and therefore QC should be analyzed for each specific scenario.

DISCLOSURES:
The authors declare that they have no competing financial interests.

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REFERENCES:

1 Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M. & Posewitz, M. C. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Curr Opin Biotechnol.* **19** (3), 235-240, doi: 10.1016/j.copbio.2008.05.007 (2008).

2 Moody, J. W., McGinty, C. M. & Quinn, J. C. Global evaluation of biofuel potential from microalgae. *Proceedings of the National Academy of Sciences*. **111** (23), 8691-8696, doi: 10.1073/pnas.1321652111 (2014).

3 Pinto, E. *et al.* Heavy metal-induced oxidative stress in algae. *J Phycol.* **39** (6), 1008-1018, doi: 10.1111/j.0022-3646.2003.02-193.x (2003).

4 Gupta, A. & Lutsenko, S. Evolution of copper transporting ATPases in eukaryotic organisms. *Curr Genomics.* **13** (2), 124-133, doi: 10.2174/138920212799860661 (2012).

5 Perales-Vela, H. V., Peña-Castro, J. M. & Cañizares-Villanueva, R. O. Heavy metal detoxification in eukaryotic microalgae. *Chemosphere.* **64** (1), 1-10, doi: 10.1016/j.chemosphere.2005.11.024 (2006).

6 Sandau, E., Sandau, P. & Pulz, O. Heavy metal sorption by microalgae. *Acta Biotechnol.* **16** (4), 227-235, doi: 10.1002/abio.370160402 (1996).

7 Amer, L., Adhikari, B. & Pellegrino, J. Technoeconomic analysis of five microalgae-to-biofuels processes of varying complexity. *Bioresour Technol.* **102** (20), 9350-9359, doi: http://dx.doi.org/10.1016/j.biortech.2011.08.010 (2011).

8 Benemann, J. R., Goebel, R. P., Weissman, J. C. & Augenstein, D. C. Microalgae as a source of liquid fuels. *Final Technical Report, US Department of Energy, Office of Research*, DOE/ER/30014-TR (1982).

9 Benemann, J. R. & Oswald, W. J. Systems and economic analysis of microalgae ponds for conversion of CO_2 to biomass. Final report. Report No. DOE/PC/93204--T5; Other: ON: DE97052880; TRN: TRN: 97:004328 (1996).

10 Chisti, Y. Biodiesel from microalgae. *Biotechnol Adv.* **25** (3), 294-306, doi: 10.1016/j.biotechadv.2007.02.001 (2007).

11 Davis, R., Aden, A. & Pienkos, P. T. Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy.* **88** (10), 3524-3531, doi: 10.1016/j.apenergy.2011.04.018 (2011).

12 Jones, S. *et al.* Process design and economics for the conversion of algal biomass to hydrocarbons: whole algae hydrothermal liquefaction and upgrading. *U.S. Department of Energy Bioenergy Technologies Office* (2014).

13 Lundquist, T. J., Woertz, I. C., Quinn, N. W. T. & Benemann, J. R. A realistic technology and engineering assessment of algae biofuel production. Energy Biosciences Institute, Berkeley, CA (2010).

14 Nagarajan, S., Chou, S. K., Cao, S., Wu, C. & Zhou, Z. An updated comprehensive techno-economic analysis of algae biodiesel. *Bioresour Technol.* **145**, 150-156, doi: http://dx.doi.org/10.1016/j.biortech.2012.11.108 (2013).

15 Pienkos, P. T. & Darzins, A. The promise and challenges of microalgal-derived biofuels. *Biofuels Bioproducts & Biorefining-Biofpr.* **3**, 431-440, doi: 10.1002/bbb.159 (2009).

16 Richardson, J. W., Johnson, M. D. & Outlaw, J. L. Economic comparison of open pond

raceways to photo bio-reactors for profitable production of algae for transportation fuels in the Southwest. *Algal Research.* **1**(1), 93-100, doi: 10.1016/j.algal.2012.04.001 (2012).
17 Rogers, J. N. *et al.* A critical analysis of paddlewheel-driven raceway ponds for algal biofuel production at commercial scales. *Algal Research.* **4**, 76-88, doi: http://dx.doi.org/10.1016/j.algal.2013.11.007 (2014).
18 Sun, A. *et al.* Comparative cost analysis of algal oil production for biofuels. *Energy.* **36** (8), 5169-5179, doi: 10.1016/j.energy.2011.06.020 (2011).
19 Thilakaratne, R., Wright, M. M. & Brown, R. C. A techno-economic analysis of microalgae remnant catalytic pyrolysis and upgrading to fuels. *Fuel.* **128**, 104-112, doi: http://dx.doi.org/10.1016/j.fuel.2014.02.077 (2014).
20 Quinn, J. C. *et al. Nannochloropsis* production metrics in a scalable outdoor photobioreactor for commercial applications. *Bioresour Technol.* **117**, 164-171, doi: http://dx.doi.org/10.1016/j.biortech.2012.04.073 (2012).
21 Borkenstein, C., Knoblechner, J., Frühwirth, H. & Schagerl, M. Cultivation of *Chlorella emersoni*i with flue gas derived from a cement plant. *J Appl Phycol.* **23** (1), 131-135, doi: 10.1007/s10811-010-9551-5 (2010).
22 Douskova, I. *et al.* Simultaneous flue gas bioremediation and reduction of microalgal biomass production costs. *Appl Microbiol Biotechnol.* **82** (1), 179-185, doi: 10.1007/s00253-008-1811-9 (2009).
23 Israel, A., Gavrieli, J., Glazer, A. & Friedlander, M. Utilization of flue gas from a power plant for tank cultivation of the red seaweed *Gracilaria cornea*. *Aquaculture.* **249** (1-4), 311-316, doi: 10.1016/j.aquaculture.2005.04.058(2005).
24 Napan, K., Teng, L., Quinn, J.C., Wood, B*., Impact of Heavy Metals from Flue Gas Integration with Microalgae Production*, Algal Research, (in press)
25 Eaton, A. D., Clesceri, L. S., Rice, E. W. & Greenberg, A. E. 3125B. Inductively coupled plasma/mass spectrometry (ICP/MS) method. *Standard methods for the examination of water and wastewater* (2005).
26 Eaton, A. D., Clesceri, L. S., Rice, E. W. & Greenberg, A. E. *Standard methods for the examination of water and wastewater*. APHA-AWWA-WEF (2005).
27 Smith, M. and Compton, J. S. *Matrix effects in the ICP-MS analysis of selenium in saline water samples*. Proceedings of the 2004 Water Institute of Southern Africa Biennial Conference. Cape Town, South Africa (2004).
28 Mehta, S. K. & Gaur, J. P. Use of algae for removing heavy metal ions from wastewater: progress and prospects. *Crit Rev Biotechnol* **25** (3), 113-152, doi: 10.1080/07388550500248571 (2005).
29 EPA, U. Method: 200.8: Determination of trace elements in waters and wastes by inductively coupled plasma - mass spectrometry (1994).
30 Eaton, A. D., Clesceri, L. S., Rice, E. W. & Greenberg, A. E. 3120B. Inductively coupled plasma (ICP) method. *Standard methods for the examination of water and wastewater* (2005).

Figure

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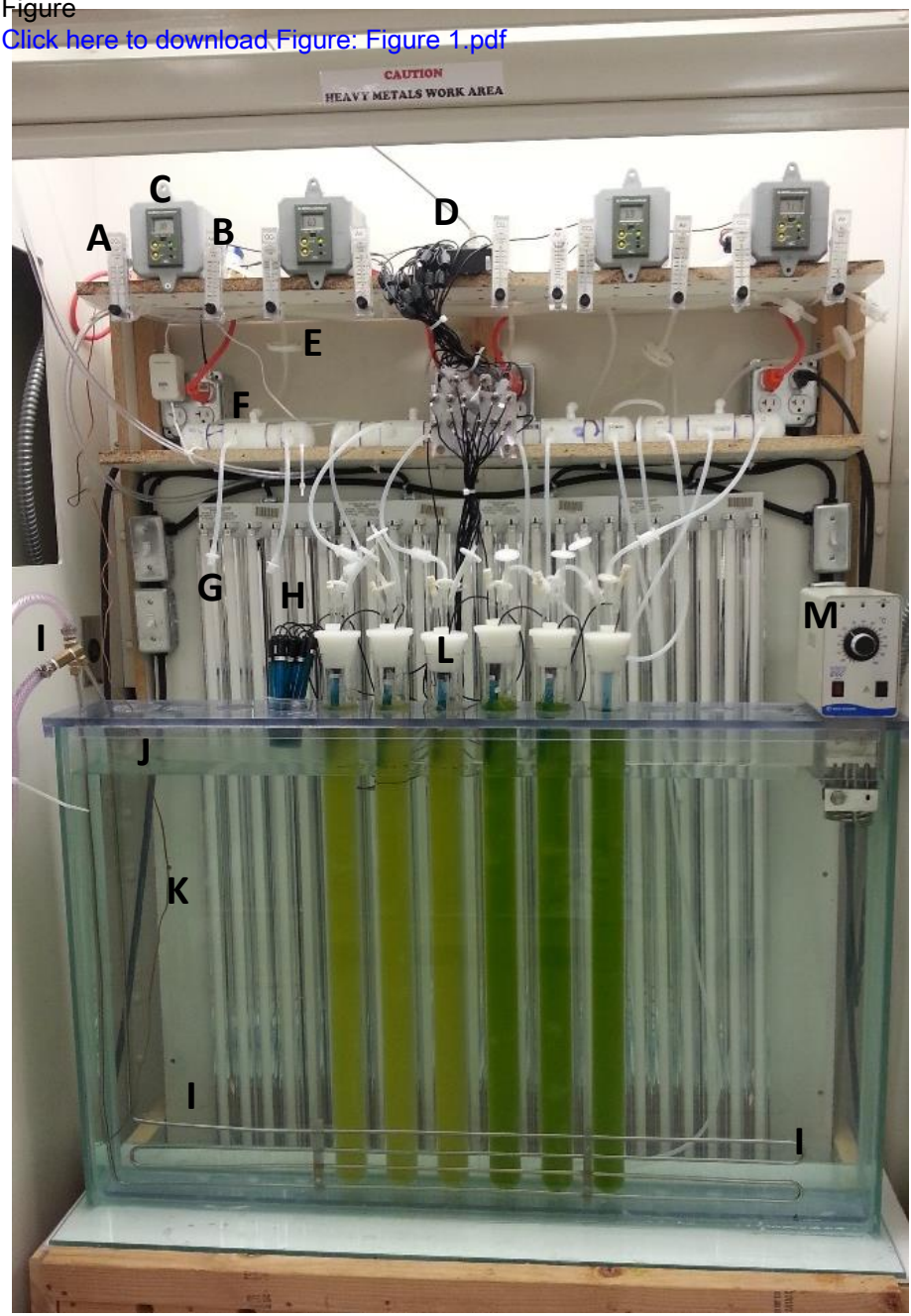

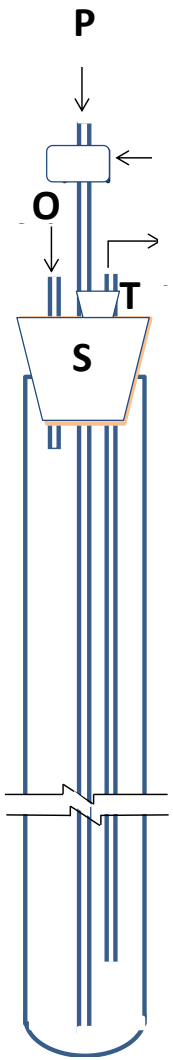

Figure

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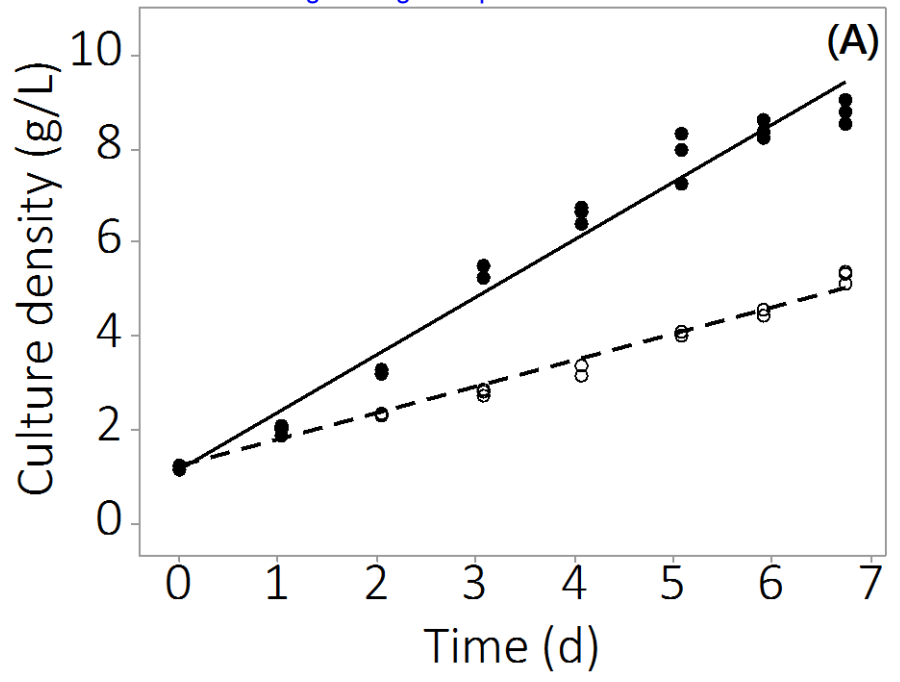

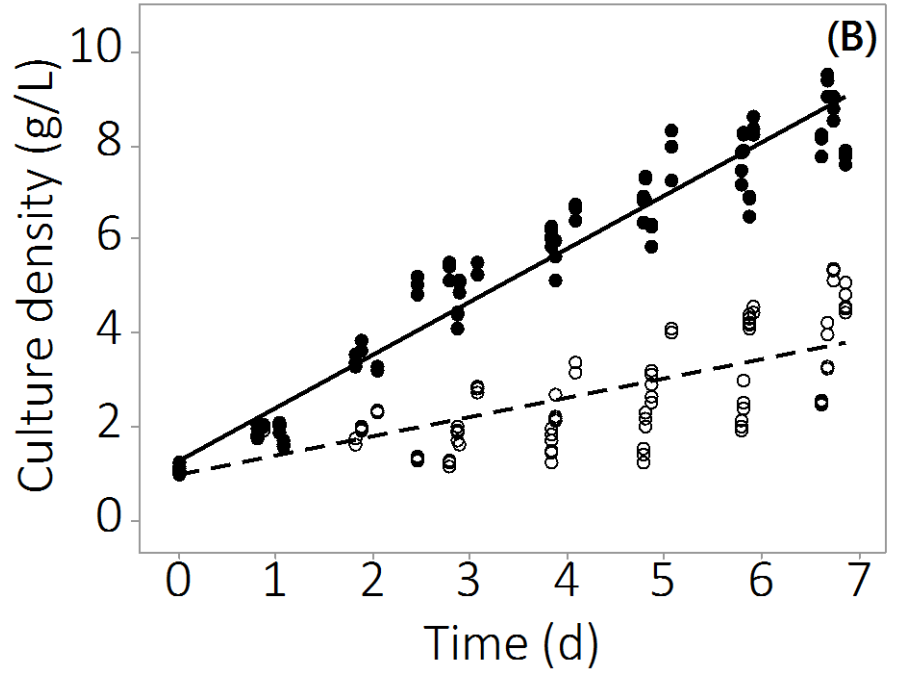

Figure

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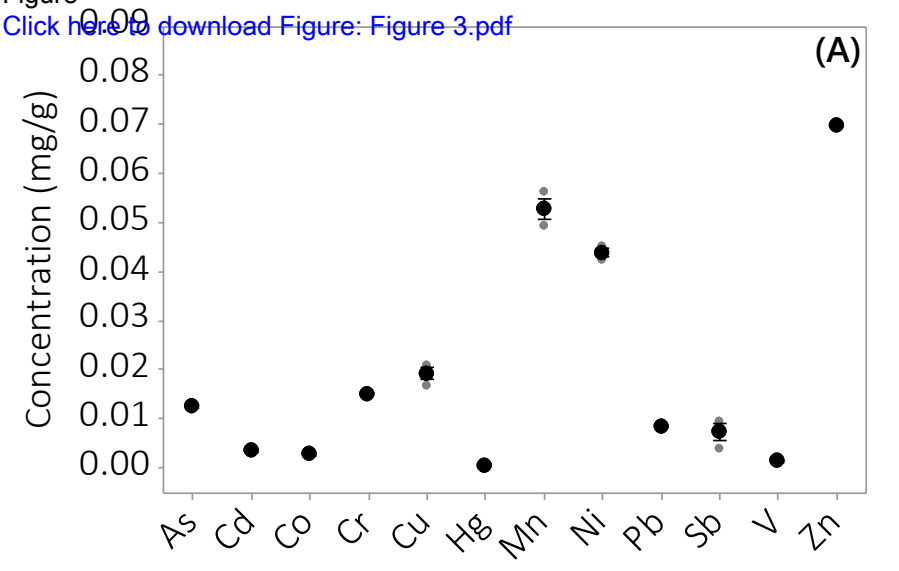

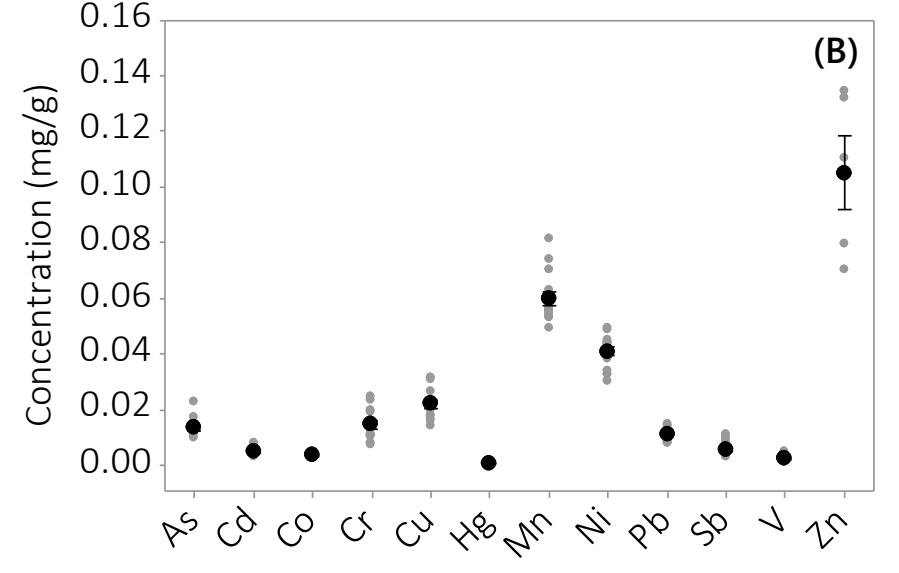

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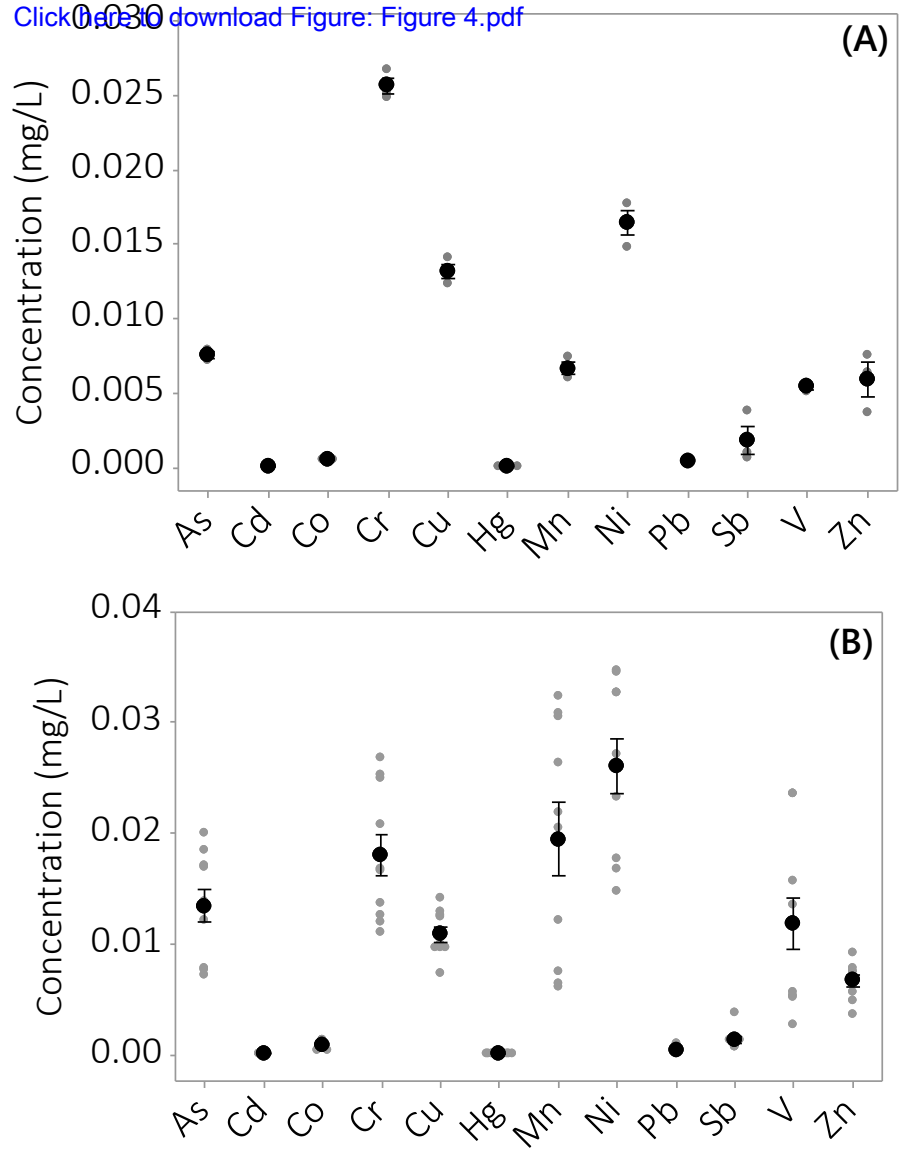

Table

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Component	Amount to add (g)	Final concentration (g/L)
H_3BO_3	0.900	0.900
$Na_2MoO_4 \cdot 2H_2O$	0.012	0.012
$MnCl_2 \cdot 4H_2O$	0.300	0.300
$ZnSO_4 \cdot 7H_2O$	0.060	0.060
$CuSO_4 \cdot 5H_2O$	0.020	0.020

Table
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Vitamins	Amount (mg)	Final volume (mL)	Final vitamin concentration (mg/L)
Biotin	12.22	500	24.43
Vitamin B12	13.50	100	135.00
Thiamine hydrochloride	977.63	500	1955.27

Table
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Component	Amount to add to medium	Unit
NaCl	350.00	g
$CaCl_2 \cdot 2H_2O$	3.00	g
KCl	9.60	g
$Na_2SiO_3 \cdot 9H_2O$	1.14	g
$MgSO_4 \cdot 7H_2O$	29.60	g
KNO_3	20.40	g
KH_2PO_4	1.36	g
Ammonium ferric citrate	0.10	g
Solution A	20.00	mL
Biotin solution*	818.00	µL
Vitamin B12 solution*	296.20	µL
Thiamine hydrochloride solution*	521.60	µL

* Add to cooled autoclaved media

Table
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Analyte	Salt source	Volume of stock to prepare (L)	Salt to add to the flask (mg salt)	Analyte concentration added to the culture (mg analyte/L)
As	$NaAsO_2$	0.1	14.8	7.74E-02
Cd	$CdCl_2$	0.5	13.5	1.50E-02
Co	$CoCl_2.6H_2O$	0.5	34.7	1.56E-02
Cr	$Na_2Cr_2O_7{\cdot}2H_2O$	0.1	40.6	1.29E-01
Cu	$CuCl_2.2H_2O$	0.1	38.3	1.30E-01
Hg	$HgCl_2$	1.0	14.6	9.80E-03
Mn	$MnCl_2.4H_2O$	0.1	58.8	1.49E-01
Ni	$NiCl_2.6H_2O$	0.1	112.0	2.51E-01
Pb	$PbCl_2$	0.5	39.9	5.41E-02
Sb	Sb_2O_3	0.5	26.7	4.06E-02
Se	Na_2SeO_3	0.5	11.8	9.80E-03
Sn	$SnCl_2.2H_2O$	0.5	3.9	3.76E-03
V	V_2O_5	0.1	22.2	1.13E-01
Zn	$ZnCl_2$	0.1	99.9	4.36E-01

Table 5
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Step	Vials rinsing			Sample digestion	
	Temperature (°C)	Time (minutes)	Max. power (W)	Temperature (°C)	Time (minutes)
1	Room temperature to 190	25	1000	Room temperature to 180	15
2	190	10	1000	180	15
Exhaust	-	20	-	-	20

Max. power (W)
1000
1000
-

Table 6
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Parameter	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Purchased standard to be added (mL)	-	-	-	-	-	-
Level 7 to be added (mL)	0.0	1.0	2.5	5.0	20.0	25.0
Final volume* (mL)	-	50.0	50.0	50.0	100.0	50.0
Final concentration (µg/L)						
^{75}As	0.0	2.0	5.0	10.0	20.0	50.0
^{111}Cd	0.0	1.0	2.5	5.0	10.0	25.0
^{59}Co	0.0	10.0	25.0	50.0	100.0	250.0
^{52}Cr	0.0	2.0	5.0	10.0	20.0	50.0
^{63}Cu	0.0	5.0	12.5	25.0	50.0	125.0
^{55}Mn	0.0	3.0	7.5	15.0	30.0	75.0
^{60}Ni	0.0	8.0	20.0	40.0	80.0	200.0
^{208}Pb	0.0	1.0	2.5	5.0	10.0	25.0
^{121}Sb	0.0	12.0	30.0	60.0	120.0	300.0
^{51}V	0.0	10.0	25.0	50.0	100.0	250.0
^{66}Zn	0.0	4.0	10.0	20.0	40.0	100.0

* Achieve this volume by adding the solution prepared in step 8.1

Level 7
10.0
-
100.0
100.0
50.0
500.0
100.0
250.0
150.0
400.0
50.0
600.0
500.0
200.0

Table 7
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Parameters	Values
Internal standards	^{72}Ge, ^{115}In
Rf power	1500 W
Plasma gas flow rate	14.98
Nebulizer gas flow rate	1.1 L/min (carrier and dilution gas combined - 0.6 + 0.5 L/min)
Sampling cone	Nickel for x lens
Skimmer cone	Nickel
Sample uptake rate	0.3 rps
Nebulizer pump	0.1 rps
S/C temperature	2°C
Scanning condition	Dwell time 1 s, number of replicate 3
H_2 gas flow	N/A
He gas flow	4.3 ml/min

Table 8
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Parameter	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
L7 Hg standard to be added (mL)	0	1	2.5	5	20	25
Final volume* (mL)	-	50	50	50	100	50
Final concentration (µg/L)	0	0.5	1.25	2.5	5	12.5

* Achieve this volume by adding the solution prepared in step 8.1

Table 9
Click here to download Table: Table 9.xlsx

Parameters	Values
Carrier gas	Argon, 100 mL/min
Lamp	Hg electrodeless discharge lamp, setup at 185 mA
Wavelength	253.7 nm
Slit	0.7 nm
Cell temperature	100 °C
Sample volume	500 µL
Carrier	3% HCl, 9.23 mL/min
Reductant	10% $SnCl_2$, 5.31 mL/min
Measurement	Peak height
Read replicates	3

Table
Click here to download Table: Table 10.xlsx

Analyte	R	CCV	LFB	LFM for biomass samples			LFM for supernatant samples		
		%D	%R	Dilution ratio	%R	RPD	Dilution ratio	%R	RPD
QC limits [25]	**0.9950**	**±10**	**70-130**	**-**	**75-125**	**±20**	**-**	**75-125**	**±20**
As	0.9998	1.8	101.0	1:3	100.4	5.2	1:3	92.5	-0.5
Cd	1.0000	1.4	102.6	1:3	103.5	4.6	None	92.3	0.6
Co	0.9997	1.7	98.8	None	95.2	-1.4	None	96.5	-1.5
Cr	0.9999	1.5	99.8	1:3	96.5	1.8	1:3	90.1	-0.8
Cu	0.9999	2.9	98.2	1:3	101.4	4.8	1:3	94.4	-0.5
Hg	0.9983	-1.7	103.0	None	98.7	1.5	None	98.0	0.3
Mn	0.9998	2.9	97.6	1:3	83.2	1.8	1:3	95.4	-1.7
Ni	0.9999	0.5	103.5	1:3	98.5	2.1	None	93.3	-0.9
V	0.9998	2.5	97.2	None	95.5	-1.5	None	101.2	-1.9
Pb	0.9998	12.6	105.2	1:3	88.9	0.0	None	93.5	-0.5
Sb	0.9998	1.1	105.7	None	101.8	-9.6	None	90.8	-1.2
Zn	0.9997	5.2	120.8	1:3	90.7	1.4	None	89.2	-1.9

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: JoVE materials - Table 11.xlsx

Name of Material/ Equipment	Company	Catalog Number
	Chemicals	
Sodium chloride	Fisher Scientific	S271-3
Calcium chloride dihydrate	Fisher Scientific	C79-500
Potassium chloride	Fisher Scientific	P217-500
Sodium meta silicate nonahydrate	Fisher Scientific	S408-500
Magnesium sulfate heptahydrate	Fisher Scientific	M63-500
Potassium nitrate	EMD Chemical	PX1520-5
Potassium phosphate monobasic	Fisher Scientific	P285-500
Ammonium ferric citrate	Fisher Scientific	I72-500
Boric acid	Fisher Scientific	A73-500
Sodium molybdate, dihydrate	EMD Chemical	SX0650-2
Manganese chloride tetrahydrate	Fisher Scientific	M87-500
Zinc sulfate heptahydrate	Fisher Scientific	Z68-500
Cupric sulfate pentahydrate	Fisher Scientific	C489-500
Biotin	Acros Organics	230090010
Thiamine	Acros Organics	148990100
Vitamin B12	Acros Organics	405920010
Copper (II) chloride dihydrate	Sigma-Aldrich	221783-100G
Lead (II) chloride	Sigma-Aldrich	268690-250G
Sodium dichromate dihydrate	Sigma-Aldrich	398063-100G
Cobalt (II) chloride hexahydrate	Sigma-Aldrich	255599-100G
Nickel (II) chloride hexahydrate	Sigma-Aldrich	223387-500G
Sodium (meta) arsenite	Sigma-Aldrich	71287
Cadmium chloride	Sigma-Aldrich	202908-10G
Mercury (II) chloride	Sigma-Aldrich	215465-100G
Tin (II) chloride dihydrate	Fisher Scientific	T142-500
Manganese chloride tetrahydrate	Fisher Scientific	M87-500
Vanadium (V) oxide	Acros Organics	206422500
Carbon dioxide	Air Liquide	I2301S-1
Hydrogen peroxide	H325-500	Fisher Scientific
ICP-MS standard	ICP-MS-6020	High Purity Standards
Mercury standard	CGHG1-1	Inorganic Ventures
Argon	Air Liquide	
Helium	Air Liquide	
Hydrogen	Air Liquide	
Nitric acid	Fisher Scientific	A509-P212
Hydrochloric acid	Fisher Scientific	A508-P212
	Equipment	

Scientific prevacuum sterilizer	Steris	31626A
Centrifuge	Thermo Fisher	46910
Spectrophotometer	Shimadzu	1867
pH controller	Hanna	BL981411
Rotometer, X5	Dwyer	RMA-151-SSV
Rotometer, X5	Dwyer	RMA-26-SSV
Water bath circulator	Fisher Scientific	13-873-45A
Compact chiller	VWR	13270-120
Freeze dryer	Labconco	7752020
Stir plate	Fisher Scientific	11-100-49S
pH lab electrode	Phidgets Inc	3550
Inductively coupled plasma mass spectrometer	Agilent Technologies	7700 Series ICP-MS
FIAS 100	Perkin Elmer Instruments	B0506520
Atomic absorption spectrometer	Perkin Elmer Instruments	AAnalyst 800
Cell heater (quartz)	Perkin Elmer Instruments	B3120397
Microwave	Milestone	
Microwave rotor	Milestone	
	Materials	
0.2 micron syringe filter	Whatman	6713-0425
0.2 micron syringe filter	Whatman	6713-1650
0.45 micron syringe filter	Thermo Fisher	F2500-3
Polystyrene tubes	Evergreen	222-2094-050
Octogonal magnetic stir bars	Fisher scientific	14-513-60

Comments/Description
Irritant, Dangerous to the Environment
Toxic, Dangerous to the Environment
Oxidizing, Highly Toxic, Dangerous to the Environment
Toxic, Dangerous to the Environment
Toxic, Dangerous to the Environment
Toxic, Dangerous to the Environment
Highly Toxic, Dangerous to the Environment
Toxic, Dangerous to the Environment
Corrosive. Suitable for Hg analysis. Very hazardous.
Dangerous to the Environment
Compressed
30% in water
1000±6 µg/mL in 5% nitric acid
Compressed
Compressed, ultra high purity
Compressed, ultra high purity
67-70% nitric acid, trace metal grade. Caution: manipulate under fume hood.
35% hydrochloric acid, trace metal grade. Caution: manipulate under fume hood.

SV-120
RC-6 Plus
UV-1800
X4
T31Y
T35Y
Attached to autosampler CETAC ASX-520
Programmable, maximum power 1200 W
Rotor with 24 75 mL Teflon vessels for closed-vessel microwave assisted digestion.

17x100 mm w/cap, 16 mL, polysteryne
Magnets encased in PTFE fluoropolymer

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Author(s): Napan, Katerine; Hess, Derek; McNeil, Brian and Quin, Jason C.

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CORRESPONDING AUTHOR:

Name: Jason C. Quinn

Department: Mechanical & Aerospace Engineering

Institution: Utah State University

Article Title: Quantification of inorganic contaminants and their impact on microalgae productivity

Signature: Jason Quinn Date: 11/4/14

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Rebuttal Comments
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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.