Video Article

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 $984 \mu\text{mol m}^{-2} \text{sec}^{-1}$ 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.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52936/>

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 death³.

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 system⁷⁻¹⁹. 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, respectively²⁰. 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 seawater²¹⁻²³. The concentrations used in this study are based on what would be expected if microalgae growth systems were integrated with a coal based CO₂ source with an uptake efficiency demonstrated in commercial PBR systems²⁰. 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

Figure 1. Microalgae growth system. (A) air rotometer, (B) CO₂ 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. [Please click here to view a larger version of this figure.](#)

1. Build the following microalgae experimental growth system (**Figure 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.
 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.

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.
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.
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.
6. Provide light using 24 T5 fluorescent lamps that result in an average illumination of $984 \mu\text{mol m}^{-2} \text{sec}^{-1}$ similar to peak outdoor conditions.
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.
8. Monitor temperature and pH in real-time and record with a data logger.
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

1. Wash volumetric flasks, PBRs, carboys and any containers, with soap and tap water. Rinse with deionized water (DW).
2. Acid rinse the lab ware in order to eliminate any traces of inorganic contaminants. This can be done by one of two ways:
 1. Soak lab ware O/N 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. Soak lab ware for 15 min in 50% trace metal grade nitric acid.
 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.
 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

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.

Component	Amount to add (g)	Final concentration (g/L)
H_3BO_3	0.900	0.900
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.012	0.012
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.300	0.300
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.060	0.060
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.020	0.020

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

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.

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	1,955.27

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

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.

Component	Amount to add to medium	Unit
NaCl	350.00	g
CaCl ₂ ·2H ₂ O	3.00	g
KCl	9.60	g
Na ₂ SiO ₃ ·9H ₂ O	1.14	g
MgSO ₄ ·7H ₂ O	29.60	g
KNO ₃	20.40	g
KH ₂ PO 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 3: *N. salina* medium recipe. Quantities are amounts needed in the preparation of 20 L of nutrient-rich medium.

4. Sterilize the medium by autoclaving for 30 min at 120 °C and atmospheric pressure. Let the medium cool down to RT.
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

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.

Analyte	Salt source	Volume of stock to prepare (L)	Salt to add to the flask (mg salt)	Analyte concentration added to the culture analyte/L (mg)
As	NaAsO ₂	0.1	14.8	7.74E-02
Cd	CdCl ₂	0.5	13.5	1.50E-02
Co	CoCl ₂ ·6H ₂ O	0.5	34.7	1.56E-02
Cr	Na ₂ Cr ₂ O ₇ ·2H ₂ O	0.1	40.6	1.29E-01
Cu	CuCl ₂ ·2H ₂ O	0.1	38.3	1.30E-01
Hg	HgCl ₂	1.0	14.6	9.80E-03
Mn	MnCl ₂ ·4H ₂ O	0.1	58.8	1.49E-01
Ni	NiCl ₂ ·6H ₂ O	0.1	112.0	2.51E-01
Pb	PbCl ₂	0.5	39.9	5.41E-02
Sb	Sb ₂ O ₃	0.5	26.7	4.06E-02
Se	Na ₂ SeO ₃	0.5	11.8	9.80E-03
Sn	SnCl ₂ ·2H ₂ O	0.5	3.9	3.76E-03
V	V ₂ O ₅	0.1	22.2	1.13E-01
Zn	ZnCl ₂	0.1	99.9	4.36E-01

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.

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

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 min at 120 °C. Pour the solution into sterile petri-dishes and let it cool until it solidifies. This should be completed in a sterile hood or at least near a flame in a clean environment to reduce risk of contamination.
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 RT. Let microalgae grow until colonies are visible.
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 (1,000 RPM). Let the culture grow until medium becomes green.
4. Transfer the microalgae to a 1.1 L sterile PBR. Place the PBR in an inoculum water bath illuminated at 200 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ 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 CO₂ rotometers to 2.5 L min⁻¹ and 25 cc min⁻¹, respectively.
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.
6. Harvest the inoculum biomass by centrifugation at 2,054 × 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.
7. Once all biomass is centrifuged, re-suspend the cells in 300 ml of fresh sterile medium.
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 () immediately using a spectrophotometer.
9. Use equation (1) to determine the amount of biomass in the concentrate.
Note: Equation (1) was obtained from the linear regression between versus total suspended solids (in g/L⁻¹) for *N. Salina* (R²=0.9995). Equation 1 was developed for the spectrophotometer model in the Materials Table, generate a new calibration if using another spectrophotometer model.
 1. Using equation (2) calculate the volume of microalgae concentrate (in L) needed to obtain a 4 g/L⁻¹ culture density in a PBR of 1.1 L volume (in L).

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

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

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.
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

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.
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.
3. Calibrate each pH controller using buffer pH 7, disinfect the probes using ethanol (70%) and then insert them in the PBRs lids.
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.
5. Add 14 ml of sterile DW to the control PBRs.
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.
7. Turn high light intensity lights (of 984 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) and pH controllers on and adjust CO₂ to 30 cc min⁻¹. Increase the CO₂ flow to 50 cc min⁻¹ from day 3 afterwards. Initial low CO₂ flow rate is critical in order to avoid large changes in pH due to delays in gas/liquid transfer and pH measurement.
8. Measure 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).
9. Add sterile DW daily to the PBRs in order to compensate for losses due to evaporation.
10. After 7 days of growth, harvest the biomass by centrifugation at 9,936 × g and preserve both, biomass and supernatant medium, at -80 °C.
11. Freeze dry the biomass at 0.1 mbar and -50 °C O/N. 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).

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.
2. Reshape the microwave digestion vessel lids and close the vials tightly.
3. Add 10 ml of nitric acid to each.
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.
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.
6. Input the digestion parameters shown in **Table 5** and start digestion. When the program has finished, air cool the vials until they reach RT.

Step	Vials rinsing			Sample digestion		
	Temperature (°C)	Time (min)	Max. power (W)	Temperature (°C)	Time (min)	Max. power (W)
1	RT to 190	25	1,000	RT to 180	15	1,000
2	190	10	1,000	180	15	1,000
Exhaust	-	20	-	-	20	-

Table 5: Parameters used in the microwave digestion program.

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).
8. Dispose of the acid. Rinse the Teflon vessels with DW 3 times. Let vials air dry.
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)).
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).
11. Reshape the microwave digestion vessel lids and close the vials tightly.
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).
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.
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.

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 RT.
2. In addition to QC samples prepared in steps 7.9 and 7.10, prepare the following QC samples.
 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.
 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.
 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.
 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.
 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.
 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.
3. Define the data quality criteria for the study. For the present study duplicate the quality criteria established by Eaton, Clesceri, Rice and Greenberg²⁵. The parameters established for the QC are: percent difference (%D) for CCV within $\pm 10\%^{25}$ (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)

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.
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.

Parameter	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
Purchased standard to be added (ml)	-	-	-	-	-	-	10.0
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	100.0
Final concentration (µg/L)							
⁷⁵ As	0.0	2.0	5.0	10.0	20.0	50.0	100.0
¹¹¹ Cd	0.0	1.0	2.5	5.0	10.0	25.0	50.0
⁵⁹ Co	0.0	10.0	25.0	50.0	100.0	250.0	500.0
⁵² Cr	0.0	2.0	5.0	10.0	20.0	50.0	100.0
⁶³ Cu	0.0	5.0	12.5	25.0	50.0	125.0	250.0
⁵⁵ Mn	0.0	3.0	7.5	15.0	30.0	75.0	150.0
⁶⁰ Ni	0.0	8.0	20.0	40.0	80.0	200.0	400.0
²⁰⁸ Pb	0.0	1.0	2.5	5.0	10.0	25.0	50.0
¹²¹ Sb	0.0	12.0	30.0	60.0	120.0	300.0	600.0
⁵¹ V	0.0	10.0	25.0	50.0	100.0	250.0	500.0
⁶⁶ Zn	0.0	4.0	10.0	20.0	40.0	100.0	200.0
* Achieve this volume by adding the solution prepared in step 8.1							

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

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.
4. Turn on the water chiller, gasses (Ar, H₂, 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).
5. Open the Masshunter Workstation software and turn on the plasma, tune the ICPMS and load the method set to parameters in Table 7.

Parameters	Values
Internal standards	^{72}Ge , ^{115}In
Rf power	1,500 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 sec, number of replicate 3
H ₂ gas flow	N/A
He gas flow	4.3 ml/min

Table 7: ICPMS operating conditions.

- 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.
- Ensure that the correlation coefficient (R) value for the calibration curve for each metal or metalloid is greater than 0.995²⁴.
- During sample analysis, calculate %R, %D and RPD as described in equations 3 to 6²⁶ and compare the results to the project data quality criteria in 8.3.
 - Calculate percent recovery (%R) to determine losses/gaining from the laboratory fortified blank (LFB) and matrix interference from laboratory-fortified matrix (LFM).

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

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

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

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

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

$$RPD = \frac{\text{sample result} - \text{duplicate result}}{(\text{sample result} + \text{duplicate result})/2} \times 100\% \quad (6)$$

- 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)

- 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.

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).

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

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.

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 9: CVAAS operating conditions.

3. Plug the line to the carrier solution made of 3% trace metal grade hydrochloric acid.
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).
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.
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 (Hg⁰) 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).
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 2A** 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 2B**. 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).

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 matrix²⁷. The Sn contamination was traced back to an acid used in the digestion step.

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

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.

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 3A**). 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 4A**) 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 4B**.

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 $\pm$ 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 $\pm$ one standard error from the mean.

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 medium²⁸. 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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