Contamination Levels in Biomass and Spent Media from Algal Cultivation System Contaminated with Heavy Metals

Katerine Napan^a, Karthik Kumarasamy^b, Jason C. Quinn^{a*} and Byard Wood^a

^aDepartment of Mechanical & Aerospace Engineering, Utah State University, Logan, Utah 84321,
United States

^bDepartment of Civil & Environmental Engineering, Utah State University, Logan, Utah 84321,
United States

*Corresponding author: Ph. (435) 797-0341. Address: 4130 Old Main Hill, Logan, UT, United
States. Email: jason.quinn@usu.edu

Abstract

Sustainability assessments have shown that integrating CO₂ from power plants with microalgae production systems can reduce costs and greenhouse gas emissions. However, coal fired flue gases contain contaminants (heavy metals) that could have a deleterious effect on products derived from such systems. To address this concern, photobioreactors (PBRs) were designed to test the hypothesis that metals in a microalgae cultivation system at concentrations derived from flue gas will limit end uses of the biomass and spent medium. *Scenedesmus obliquus* were grown in PBRs spiked with 10 metals found in such flue gases at concentrations representing typical (1X) and high (5X and 10X) metal loading scenarios. Results show that contamination levels can be modulated by managing the harvesting time and by leaching, with EDTA being effective at early growth stage (approximately $\leq$ day 6) and acidified methanol effective afterwards. Although metals limit biomass and medium uses, some uses fall within regulatory limits (e.g., 1X spent medium is suitable for irrigation, and 1X biomass is suitable for bio-fertilizer and select animal feed uses). Results showed

that uses of biomass and medium under higher loading scenarios from such integration will restrict end uses of both biomass and media.

Keywords

Algal bioproducts; heavy metals; microalgae; contaminant regulation; leaching; flue gas

Graphical Abstract

1. Introduction

In response to increased global energy consumption and concerns over the effects of greenhouse gases, several new regulations for reducing carbon emission from industrial point sources are being implemented in several countries. One of the carbon trapping technologies being explored is the integration of photosynthetic organisms such as microalgae with point source carbon dioxide (CO₂) emissions [1]. Microalgae utilize solar energy 10 times more efficiently than terrestrial plants resulting in high biomass and lipid yields [2]; but they require a concentrated

carbon source such as industrial flue gas, with the integration of the two systems being mutually beneficial. However, the utilization of flue gas from various fuels including coal may concurrently introduce contaminants [3, 4] (such as the heavy metals listed in Table B1 in the SI) into the growth system as shown by previous studies [5, 6]. Previous research shows that cell density, biodiesel yields, and metal distribution are affected when microalgae is grown in a multi-metal system at concentration level estimated to come from flue gas with most metals accumulating in the biomass[7].

Techno-economic evaluations of large-scale microalgae production systems often assume a seamless integration of the cultivation system with a low cost CO₂ source such as flue gas producing industries. These studies assume that spent medium can be re-used and the biomass harvested from such systems can be used for animal feed, human food source, bioenergy feedstock, etc. [8-13], without considering any metal contamination. However, the buildup of contaminants within the biomass and spent medium has the potential to limit the end use of microalgae based products and byproducts due to adverse effects of heavy metals on human/animal health and the environment [1]. Depending on the level of heavy metal contamination, the biomass and the medium may not be adequate for the uses proposed in such techno-economic evaluations, or may need to be treated prior to their use or release to the environment.

The information about the extent of heavy metal contamination in the biomass and medium for a microalgae system using flue gas is limited in the current literature. Wide variability in the initial metal concentration and the metals loading rates is expected for a cultivation system using flue gas. This variability originates from several intrinsically variable factors such as: the variable metal concentration in the fuel supply, which varies across coal types and regions [14]; the variable removal efficiency of air pollution control devices (APCD) [15]; the variable trace elements in the water used (e.g. seawater, agricultural runoff, recycled algal spent media, municipal and industrial wastewater); among others. To address this complexity, this study takes a conservative approach by

maximizing the contact time through the use of a batch cultivation system (instead of a fed batch approach) and by evaluating three scenarios (named 1X, 5X and 10X) intended to capture the wide range of possible intermediate concentrations in the complex metal loading scenarios that could occur in a field cultivation system where recycling of the medium is expected. Primary assumptions for determining the baseline concentration are based on the integration of microalgae cultivation systems with coal based flue gas (see detailed assumptions and calculation in [7]). Higher heavy metal exposure doses of 5X and 10X were 5 times and 10 times the 1X concentration, respectively.

Thus, this study addresses the hypothesis that heavy metals present in a microalgae cultivation system will limit potential end uses of products, co-products and by-products. The key objectives of this study are: 1) to determine the metal concentration in the biomass and medium that corresponds to three metals loading scenarios expected to be introduced from coal-based flue gas, 2) to identify management practices (harvesting time, growth dilution and leaching) as means to modulate the contamination levels in biomass, and 3) to identify potential uses and limitations for the contaminated algal biomass and medium based on current regulatory standards. This study aims to provide the algae community with preliminary feasibility information about how different levels of heavy metals could affect algae-based products and how some strategies (such as harvesting time, growth dilution and the use of a leachant) can help with the compliance and planning of potential uses. Moreover, the authors recognize the complexity of this issue and from the beginning attempted to design a research program that addresses the stated hypothesis while providing a path forward for a more comprehensive research program to support the development of microalgal biofuels and bioproducts.

2. Materials and Methods

Microalgae were cultivated in vertical tube photobioreactors (PBRs) (Figure A1 in SI) over a 24 day period under various heavy metal concentrations (1X, 5X, and 10X) with the baseline

concentration (1X) intended to be a conservative estimate of contamination levels from the integration of a growth system with coal flue gas (calculations detailed in [7]). Increasing concentrations (5X, 10X) were used to understand impacts of higher metal loading scenarios. Over the course of the growth period, heavy metals were quantified in the biomass and medium, and the results were used to evaluate the limitations of the use of the biomass and spent medium. The effectiveness of solvents typically used in various industries were evaluated for metal removal.

2.1 Growth system.

The nutritive medium used was composed of NaNO_3 (1000 mg L⁻¹), K_2HPO_4 (200 mg L⁻¹), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (49.1 mg L⁻¹), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (25.1 mg L⁻¹), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (21.5 mg L⁻¹), H_3BO_3 (11.4 mg L⁻¹), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.597 mg L⁻¹), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.086 mg L⁻¹), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.058 mg L⁻¹), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.041 mg L⁻¹) and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.029 mg L⁻¹). This mixture was autoclaved at 121°C. To avoid iron precipitation with medium components during autoclaving, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ solution were separately autoclaved and were added to the medium prior to inoculation to reach concentrations of $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (12 mg L⁻¹) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (4.5 mg L⁻¹) in the final medium. The initial pH was adjusted to 7.0 by HCl addition. *Scenedesmus obliquus*, donated by Arizona Public Service (APS), was chosen for this study because of its ability to thrive well in high CO₂ concentrations [16-18] and due to APS's experience with this algal strain. APS developed test bed experiments and evaluated them using microalgae to sequester CO₂ from the flue gases from power plants with different fuel source: Cholla plant is coal fired and Red Hawk uses natural gas. *S. obliquus* thrived in both situations.

For the experiment *S. obliquus* was grown axenically in petri dishes in order to maintain strain purity. Colonies of microalgae from petri dishes were harvested and grown in 3 L polystyrene spinning bioreactors (Corning®) with nutritive medium for 7 days until cultures reached a density of approximately 2.5 g L⁻¹ (dry weight basis). Light was supplied by cool fluorescent lamps (24/7) and pH was maintained at 7.0 by CO₂ injection. Biomass was harvested by centrifugation (3900

RPM for 5 minutes) and washed twice with fresh medium in order to eliminate metal chelators excreted by microalgae (adapted from Bates et al. [19]). Prior to microalgae cultivation, the PBRs were acid rinsed overnight using 10% HNO₃ and then were rinsed thoroughly with deionized water. The PBRs were autoclaved at 120 °C for 30 minutes and filled with sterile medium without EDTA in order to reduce complexation with metals.

Heavy metals were prepared as individual liquid stocks at 1000X concentrations. The stocks were sterilized by filtration through sterile 0.2 µm sterile syringe filter (instead of autoclaving) in order to avoid changes in the oxidation state. The day of seeding, sterile medium was added to the bioreactor. Air delivery system was turned on, followed by adding heavy metals to the bioreactors with concentrations 1X, 5X or 10X, with 1X composed of As (0.078 mg L⁻¹), Cd (0.015 mg L⁻¹), Co (0.016 mg L⁻¹), Cr (0.13 mg L⁻¹), Cu (0.131 mg L⁻¹), Hg (0.01 mg L⁻¹), Pb (0.054 mg L⁻¹), Ni (0.25 mg L⁻¹), Se (0.01 mg L⁻¹) and Zn (0.44 mg L⁻¹). The growth system was operated in batch mode and allowed the determination of contamination levels of microalgae under the longest time of exposure and the highest initial metal concentration expected; therefore giving insights of maximum contamination levels. A real flue gas fed system is expected to have variable initial metal concentration ranges depending on coal source and a variable exposure time to metals, resulting from the incremental flue gas feeding rates directly proportional to cell multiplication. As a consequence, potentially variable intracellular uptake (driver of metabolic-induced toxicity) could be expected, thus impacting the final contamination levels in biomass and spent medium.

Washed algal biomass was added to each PBR to reach a final density of 0.8 g L⁻¹. The final volume of 1.1 L was achieved by dropwise addition of medium. The mixing of the algal cells in the PBR and the maintenance of pH at 7.0 was accomplished by sparging the PBR with CO₂ enriched air through a vertical glass capillary tube that extended down to 1 cm from the bottom of the PBR. CO₂ delivery was monitored daily and adjusted to maintain the pH at 7. Biomass content was determined as total suspended solids (TSS) through dry mass and optical density measurements.

2.2 *Heavy metals sampling*

Two analytical methods were used to determine metal concentrations. The first method was used to detect As, Cd, Co, Cr, Cu, Ni, Pb, Se and Zn. A total of 12 mL of unfiltered sample (algal suspension) was aspirated from a PBR of which 5 ml was used for analyzing the heavy metal concentration in the algal suspension. The remaining 7 ml was centrifuged at 7500 RPM for 3 minutes and the supernatant was analyzed for heavy metal concentration and is reported as heavy metal concentration in the medium. The wet biomass pellet was then evaluated for desorption of heavy metals by EDTA and is reported as the EDTA non-removable fraction as described below. The total metals in the harvested biomass were determined through the difference between the total metals in the aspired unfiltered sample and the metals in the supernatant. Unfiltered samples, supernatant samples and microalgae pellet samples were transferred into borosilicate test tubes and digested with HNO₃ at 105°C in a heating block until biomass was no longer visible. Digested samples were transferred into volumetric flasks with the volume adjusted to 5 mL (1X experiments) or 10 mL (5X and 10X experiments) using deionized water type I. Samples were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICPMS Agilent 7500 Series). ICPMS metal standards and quality control (QC) samples were prepared as detailed in Napan et al. [20] the night before or the same day of the analysis using concentrated analytical grade stocks (ICPMS 6020, High Purity Standards, USA) [20].

The second method used was the Atomic Absorption Spectrometry to detect Hg. A total of 30 mL of unfiltered sample was aspirated from a PBR of which 10 ml was used for analyzing Hg concentration in the algal suspension. The remaining 20 ml was centrifuged at 7500 RPM for 3 minutes and the supernatant was analyzed for Hg concentration and is reported as Hg concentration in the medium. The pellet was analyzed for Hg and is reported as Hg concentration in the harvested biomass, no sequential EDTA extraction was performed on the pellet due to detection sensitivity of the instrument and sample volume availability. Samples were digested following US EPA 7470A

and US EPA 7471A standard methods for mercury analysis in liquid and semisolid waste. Hg concentration was measured by cold vapor atomic absorption procedure using an Atomic Absorption Spectrometer (AAS, PerkinElmer Analyst 800). Hg standard (CGHG1 1, Inorganic Ventures, USA), QC samples and SnCl₂ solution were prepared the same day of analysis using concentrated stocks standards and chemicals of analytical grade.

2.3 *Heavy metal desorption from fresh biomass using EDTA solution*

Metal chelator EDTA is commonly used to remove heavy metals from biomass as it chelates cell surface-bound cationic metals [21] and also removes anionic metals [22]. In this study the impact of washing the cells with EDTA to remove heavy metals (cationic and anionic metals) was evaluated over the course of the 24 day growing period. The evaluation of surface bound metals was performed through the washing of the biomass with EDTA [21]. The algal cell pellets were re-suspended in 0.1 M EDTA containing 0.08% v/v NaCl solution (to avoid lysis of cells due to hypotonic effect) at pH 7 for 10 minutes. The sample was then centrifuged at 7500 RPM for 3 minutes to remove cationic metals (Cd, Co, Cu, Ni, Hg, Pb, and Zn) that were surface bound. The non-cationic heavy metals (As, Se, and Cr) present in the algal cell pellets were also measured and are reported. The washed pellet was analyzed for heavy metals and is reported as the EDTA non-removable fraction. The sequential extraction method described above is adapted from the operationally defined approach described by [19].

2.4 *Heavy metal desorption with solvents from lyophilized biomass*

The pellets harvested on day 24 were placed in a freezer at -80°C, then lyophilized and powdered. Approximately 20 mg of dry biomass was soaked with 15 mL of various leachants for 60 minutes at room temperature under constant mixing in a tilting shaker (adapted from [23]). The leachants used were deionized water, 0.1 M EDTA, methanol, hexane or a solution of methanol with 5% H₂SO₄ [24]. After 60 minutes, the suspension was centrifuged and the supernatant was discarded. The washed biomass pellet was lyophilized and analyzed for heavy metals content. The

unwashed biomass (control) was also analyzed in order to understand the effectiveness of each solvent in heavy metal removal.

3. Results and Discussion

3.1 Heavy metal concentration in biomass and medium

3.1.1 Time series of contamination levels in IX experiments

A time series of the heavy metal concentration in the medium is presented in Figure 1, with results presented in mg of metals per liter of spent medium. The time series for the medium show that Cd, Cr, Pb, Se and Zn were removed from the medium within the first week of growth, unlike Co, Ni, Cu and As that were removed at a slower rate.

Figure 1. Heavy metal concentration in the medium for 1X experiment. Horizontal lines represent the maximum allowable regulatory limits for the use of this medium in selected applications. Due to the lack of standards, the following maximum limits are not presented: Co, Hg and Pb for Human Health; Co for Drinking water and Aquatic life. Data points are averages from three replicates and error bars indicate $\pm$ one standard deviation. Some error bars are not visible due to overlap with markers that represent data points. Secondary Y axis (same units as primary Y axis) in the right side of the plots (for Cu, Pb, Se and Zn) has a different scale to show the maximum limits.

The time series of concentration of heavy metals in the biomass, differentiated as: the concentration of heavy metals in the harvested biomass (solid line) and as EDTA non-removable metal fraction (the fraction that remains in the biomass after rinsing with EDTA, dashed line) are shown in Figure 2. The concentration of heavy metals in the harvested biomass initially rose to a peak concentration for all heavy metals (except Ni) followed by a drop in concentration. The rise of heavy metal concentration in the biomass is a result of sorption of metals to the cell wall which is a fast process while the uptake of metals into algal intracellular compartments is slower [25]. The drop in metal concentration is a result of cell multiplication, also called growth dilution [26]. For Ni, it was observed that higher peak concentrations in the harvested biomass were reached after several days, suggesting that the production of new cells did not result in growth dilution due to the slow rate of Ni removal from the medium.

These results indicate that heavy metals in fresh biomass are initially lightly sorbed onto the cell wall surface as EDTA is shown to remove a large fraction of heavy metals from the biomass during the first 6 days of growth as shown by the more pronounced difference between the solid line and the dashed line in Figure 2. Towards the end of the growth experiment the effectiveness of EDTA to remove metals diminished with the exception of Co and Ni. This reduced removal capacity by EDTA towards the end of the growth could be attributed to the possible relocation of heavy metals inside the algal cell and/or formation of stronger bonds with the cell wall [21].

Figure 2. Time accumulation of heavy metal concentrations in biomass for 1X experiment. Results are presented for the concentration found in harvested biomass and in the biomass after EDTA rinsing (EDTA non removable). The difference between these two lines represents the EDTA removable fraction. Data points are averages from three replicates and error bars indicate $\pm$ one standard deviation. Some error bars are not visible due to overlap with markers that represent data points.

3.1.2 Ending contamination level in 1X, 5X and 10X experiments

235 *Spent medium*

236 Contamination levels were determined for the end of the 24-day growth period for the
237 medium and biomass. Figure 1 and Figure 3 shows the concentration levels of the heavy metals in
238 the medium (mg L^{-1}) prior to and after cultivation of *S. obliquus* for three initial heavy metal
239 concentrations (1X, 5X and 10X). Results show that higher amounts of As, Cd, Co, Cr, Cu, Hg, Ni,
240 Pb, Se, and Zn were removed from the medium with an increase in initial dose, indicating that the
241 mechanisms responsible for heavy metal removal were not saturated. Although 5X and 10X
242 experiments produced less biomass due to metal toxicity (growth results presented in Napan et al.
243 [7]), the metals removal were not dramatically impacted. The majority of the metals from the 1X,
244 5X, and 10X growth systems were removed from the medium with the exception of As. From these
245 results, it can be inferred that the microalgal system may be able to remove most of the heavy
246 metals even at increased concentrations that could occur as a result of higher metal inputs (e.g.,
247 from flue gas containing higher metal concentrations, recycling of contaminated medium, or
248 increased levels of CO_2 added to the growth system).

Figure 3. Heavy metal concentration in the medium after harvest (24 days growth period) for 1X, 5X and 10X experiments. Horizontal lines represent the maximum allowable regulatory limits for the use of this medium in specific applications. Due to the lack of standards, the following maximum limits are not presented in this figure: Co, Hg and Pb for Human Health; Co for Drinking water and Aquatic life. Data points are averages from three replicates and error bars indicate $\pm$ one standard deviation. Secondary Y axis (same units as primary Y axis) in the right side of the plot except P which has a different scale to show the maximum limits.

A direct comparison of metal concentrations from various end use regulatory limits contrasted with spent medium metal contamination is shown in Figure 3. The terminal extent of spent media contamination expressed as percentage of the standards can be found in the SI. Results show that all heavy metal concentrations for the 1X experimentation were significantly lower than the Food and Agriculture Organization of the United Nations (FAO) [27] recommended maximum limits for crop irrigation after the first couple of days of growth, with ending concentration well below the standard. The spent media exceeded the Criterion Continuous Concentration (CCC) from US EPA aquatic life criterion for long term exposure in freshwater [28], exceeded the US EPA human health criterion focused on protecting people that drink untreated surface water or eat fish, shellfish and wildlife grown in such contaminated water [28], and exceeded the US EPA drinking water standard [29].

The final concentrations for the 5X and 10X scenarios exceeded all of the standards for irrigation, human contact, human consumption, and aquatic life. However, considerations for mixing lengths and dilution of contaminants contained in the National Pollutant Discharge Elimination System (NPDES) permit [30] for some water bodies may allow the spent medium from this study to satisfy US EPA criteria.

Figure 4. Heavy metal concentration in the harvested biomass after a 24-day study period for 1X, 5X and 10X experiments compared to regulated limits shown as horizontal lines. Metal concentrations are presented in two portions: EDTA non-removable and EDTA removable with the sum being the concentration in the harvested biomass. Due to the lack of standards, the following maximum limits are not presented in this figure: Hg (total) for Cattle; Co, Cr and Hg (total) for Fish feed; Hg (total) for poultry; Co and Cr for Biofertilizer. Data points are averages from three replicates and error bars indicate $\pm$ one standard deviation. Secondary Y axis (same units as primary

Y axis) in the right side of the plots. As, Cr and Cu have a different scale to show the maximum limits.

Biomass

The bioaccumulation of heavy metals by *S. obliquus* at the end of the growing cycle (day 24 for this study) for the three concentrations (1X, 5X and 10X) tested is presented in Figure 4. Results show that higher initial heavy metal doses results in higher heavy metal contaminant concentrations in the biomass for most elements (with exception of As). For 1X, the heavy metals associated with the biomass were shown to be strongly bound to the biomass as washing with a metal chelator, EDTA, showed minimal impact on metal removal. Larger doses (5X and 10X) of As, Cd, Cr, Ni, Pb, Se and Zn resulted in larger removable fraction by EDTA washing.

Microalgal biomass has the potential to be a replacement feedstock for use in a variety of industries ranging from food to commodities; however, heavy metal contamination has the potential to limit the end use. Contamination limits specific to microalgal biomass for use as ingredient in human food, animal feed, or bio-products are limited in the literature, primarily due to the infancy of the industry. For comparison purposes, several guidelines and recommended limits were revised (see SI) for trace metals in several products in other industries. A few of the standards included microalgae, and were directly compared with the concentrations in the harvested biomass from 1X, 5X and 10X experiments (Figure 4). The extent of biomass contamination expressed as percentage of the standards can be found in the SI.

From the comparison, it can be concluded that biomass produced exposed to 1X concentrations were above the National Research Council (NRC) [31] fish food recommendations, were under the NRC's MTL (maximum tolerable limit) for cattle feed, were under the recommended poultry MTL (conclusions about Hg is inconclusive due to lack of Hg speciation data) and were below the Association of American Feed Control Officials (AAFCO) [32] recommended MTL. Biomass from 5X and 10X were well above the standards for all animal feeds.

Since, microalgal biomass is added only as a supplement or as a protein replacement to animal feed, it will only form a fraction of the whole feed. Blending of the contaminated biomass with other excipients may reduce the final contaminant concentration. It is recommended that MTLs are established for each use.

Results from the 1X, 5X and 10X experiments were above the Standard 173 NSF International for raw materials to be used as dietary supplements for humans [33]. It is unlikely that biomass grown using flue gas or any other waste stream (e.g., wastewater, produced water, etc.) will be used for human consumption based on the potential of the introduction of various other toxins present in these wastes that are known carcinogens (e.g., dioxins, furans and polycyclic aromatic hydrocarbons (PAHs)) [9, 31].

Heavy metals in biomass from 1X experimentation were below the concentrations recommended by Association of American Plant Food Control Officials (AAPFCO) [34]. Hence, it can be applied as fertilizer or soil amendment. Biomass from 5X and 10X experiments exceeded the AAPFCO standard, thus it cannot be land applied. Landfilling and co-incineration of the non-conforming biomass could be management options as long as the 40 CFR Part 258 (Criteria for municipal solid waste landfills) and the Clean Air Act stipulations are met [35]. Like biosolids, extended land application of the contaminated microalgal biomass could increase metal concentrations in soil, groundwater and surface water [36], hence alternative processing to reduce metals associated with the biomass is advantageous.

Microalgal biomass produced from the 1X experimentation was below the recommendations for use in packaging based on the US Model Toxics in Packaging Legislation [37] and the European Parliament and Council Directive 94/62/EC [38]. Results from this study show 100% of the harvested biomass can be used to produce paper and plastic containers without exceeding the standard. The guidelines for paper in contact with moist and fatty food of the European Paper and Board Food Packing Chain [39] was exceeded. For biodegradable plastic,

contamination in 1X biomass were several times under the ceiling concentration of the American Society for Testing and Materials ASTM D6400 standard [40]. Currently, microalgae are only being incorporated in small quantities during plastic and paper production to limit compromising product characteristics [41], thus the levels of heavy metals in the final product are expected to be well below the recommended standards. Biomass from 5X and 10X experiments exceeded the paper and plastic standards.

A primary research effort in microalgae is in the production of high lipid content biomass that could be used as a biofuel feedstock, however no MTL for heavy metals in feedstock or fuel was found (see SI). Heavy metals present in biodiesel can come from vegetable oils and could be carried over to the purified biodiesel [42]. Metals in fuel act as pro-oxidants that negatively impact fuel properties and shelf life [42, 43] and have negative health effects after exhaust inhalation [44]. Further testing is recommended to understand the transfer and impact of heavy metals from biomass to fuel.

3.2 Strategies to modulate the level of heavy metal contamination in biomass

Depending on the objective of an industry, the levels of heavy metal contamination may need to be modulated. For example, a farm focused on bioremediation of heavy metals will need to remove larger amounts of metal in a short period of time. In contrast, the lowest possible contamination with high yields is desirable for algal biomass intended for human/animal consumption. These modulations can be obtained through management of microalgae culture and process-based practices:

3.2.1 Microalgae culture management practices: harvesting time and growth dilution

Heavy metal bioaccumulation by microalgae is driven by time dependent adsorption and internalization processes [25], therefore the final contamination level of the biomass is expected to be directly impacted by the cultivation time. Figure 1 and Figure 2 suggest that any changes in harvesting time or culture conditions that affect algal growth (e.g., type of bioreactor, light intensity,

nutrient levels, algae specie, etc.) will directly affect the final level of heavy metal contamination in the biomass and medium. In the 1X experiments, metals were constantly internalized by the cell during the cultivation period leading to larger fractions of metals in the biomass. Due to growth dilution, the contamination level per unit of biomass is reduced, however, at this point EDTA alone will not be able to remove the heavy metals. The effect of different growth rates on metal contamination is also shown by Larsson and Lindblom [5], where the biomass from *Chlorella vulgaris* grown in open ponds (with lower growth rates) using flue gas from a municipal wastewater treatment plant resulted in higher concentration of heavy metals per unit of biomass than its counterpart grown in column PBRs which supported higher growth rates [5]. Therefore, to obtain a cleaner biomass, a bioreactor may be more effective than an open pond, as bioreactors have a higher biomass yield.

The growth system in batch mode tested in this study allowed the determination of contamination levels of microalgae under the longest time of exposure and the highest initial metal concentration expected in the microalgae growth system tested; therefore giving insights of maximum contamination levels. A real flue gas fed system is expected to have variable initial metal concentration ranges depending on coal source and a variable exposure time to metals, resulting from the incremental flue gas feeding rates directly proportional to cell multiplication. As a consequence, potentially variable intracellular uptake (driver of metabolic-induced toxicity) could be expected, thus impacting the final contamination levels in biomass and spent medium. It is recommended that future studies use fed-batch and chemostat approaches to evaluate the impact that different flue gas feeding strategies have on the final metal contamination levels of harvested biomass and spent medium.

3.2.2 Process-based management practice: Removal of heavy metals with solvents

The potential to remove heavy metals from the biomass was evaluated using a variety of solvents. EDTA solution was used for fresh biomass; hexane (H), deionized water (W), EDTA (E),

methanol (M) or acidified methanol (Ma) were used for lyophilized biomass (harvested after 24 days of growth). The dashed lines in Figure 2 show that EDTA can clean up the fresh biomass from heavy metals only if rinsing of biomass is performed during the first few days of growth. For a microalgal growth system focused on bioremediation of heavy metals from water, a short time of cultivation (below 6 days) would be more desirable in order to clean-up the biomass using EDTA (as shown in Figure 2), thus reducing the level of contamination in the harvested biomass, while still removing large amounts of metals. This type of management practice could be used in systems where algae is being used for bioremediation of metals in water, for which the main objective is the maximum removal of contaminants rather than biomass yields. For a cultivation system focused on biomass production, a bioreactor system with higher specific productivity (therefore shorter cultivation time) may allow for some removal using EDTA; however, more research is needed in this area to understand how the rate of metal internalization (changes in EDTA-non-removable fraction) is affected by increased specific growth rate.

Heavy metal concentrations in the terminal harvested biomass after rinsing with H, W, EDTA, M and Ma are shown in Figure 5. Hexane removed between 4 and 12% of heavy metals. Deionized water was somewhat effective at removing As and Cr while it had minimal effect on the other metals. EDTA was effective at removing Cd, Co, Cu, Cr, Ni, Pb and Zn. The most effective solvent for the removal of heavy metals was acidified methanol containing 5% sulfuric acid. This solvent removed greater than 90% of As, Cd, Cu, Ni, and Zn and removed more than half of Co, Cr and Pb. The resulting biomass was grey in color indicating that the solvent denatured a significant portion of the chlorophyll. The increased removal of heavy metals observed with acidified methanol can be attributed to the ability of sulfuric acid (a strong oxidizer) to digest cellular components [45]. Methanol alone was not very effective at removing the majority of the heavy metals with the exception of the removal of 37% of the As. This result could indicate the presence of organic As

species bound to the biomass. Foster et al. [45] reports that methanol has an affinity to remove organic As from cells, while inorganic As species are poorly removed.

Figure 5. Heavy metals concentration in lyophilized biomass (after 24 days growth from the 1X growth study) after solvent rinsing compared to regulated limits shown as horizontal lines. Control (C) before solvent rinsing, hexane (H), methanol (M), acidified methanol (M-a), deionized water

(W) and EDTA (E). Due to lack of a standard, the following maximum limits are not presented in this figure: Hg for Cattle; Co, Cr and Hg for Fish feed; Co and Cr for Biofertilizer and Compostable plastic; As, Co, Cu, Ni, Se or Zn for plastic/paper packing. Secondary and tertiary Y axes (same units as primary Y axis) in the right side of the plots have different scales to show the maximum limits.

The different degrees of removal by these solvents imply that the type of solvent used during downstream process of biomass will affect the contamination levels of microalgae derived products. In the case of biodiesel production from contaminated microalgae it can be argued that the choice of solvents (e.g., methanol in supercritical methanol transesterification vs. acidified methanol in *in situ* acid catalyzed transesterification) will produce a biomass remnant and a liquid phase (containing the crude biodiesel) with different degrees of heavy metal contamination. Further research under actual production working conditions would be needed to determine the final levels of heavy metal contamination in any microalgae derived products from contaminated algal biomass.

As shown above, management practices can play a key role on heavy metal contamination levels. It is recommended that the algae farming industry considers the impacts of the presence of heavy metals in the growth system when developing Best Management Practices for the industry.

4. Conclusions

In summary, this study shows that the presence of heavy metals in the growth system effects the contamination levels in the biomass and medium and therefore their potential end uses. The final level of heavy metals in biomass and medium strongly depends on the time of harvest and the growth characteristics of the cultivation system. Management practices such as management of cultivation time and desorption using solvents can help to modulate contamination levels. Although heavy metals present in flue gas contaminates biomass and medium, there are uses that fall within regulations/recommendations. For *S. obliquus* grown for 24 days at the baseline heavy metal concentration (1X), the spent medium was suitable for irrigation, but not for disposal into a natural water system. Metal concentrations in the biomass did not meet standards for direct human and fish consumption, but were below recommendations for select animal feed, biofertilizer, and filler for

paper and plastic. This research strongly suggests that heavy metal contamination from the integration of industrial flue gas will restrict the end uses of the biomass and media, therefore their potential impacts should be considered in techno-economic and life cycle analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://www.sciencedirect.com>.

Acknowledgements

The authors would like to acknowledge funding support from the Department of Energy (grant DE EE0003114), National Science Foundation (award number 1335550), Utah Science and Technology Initiative for Economic Development (Biofuels A16114), and Arizona Public Service (SPO 090448). The authors also thank professors Dr. Sridhar Viamajala, Mark Riley, Joan McLean, Ronald Sims, Raymond Hobbs, Sam Coutore and Sally Sun for their inputs, and Katie Glaittli, Hamud Abdullahi and Hao Chieh Hsieh for their help during data collection.

References

- [1] US DOE. National algal biofuels technology roadmap, in: U.S.D.o. Energy (Ed.), U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Biomass Program, 2010.
- [2] T.M. Mata, A.A. Martins, N.S. Caetano. Microalgae for biodiesel production and other applications: a review, *Renew. Sust. Energ. Rev.* 14 (2010) 217-232.
- [3] S. Nagarajan, S.K. Chou, S. Cao, C. Wu, Z. Zhou. An updated comprehensive techno-economic analysis of algae biodiesel, *Bioresource Technology* 145 (2013) 150-156.
- [4] J.N. Rogers, J.N. Rosenberg, B.J. Guzman, V.H. Oh, L.E. Mimbela, A. Ghassemi, M.J. Betenbaugh, G.A. Oyler, M.D. Donohue. A critical analysis of paddlewheel-driven raceway ponds for algal biofuel production at commercial scales, *Algal Res.* 4 (2014) 76-88.

- [5] M. Larsson, J. Lindblom. Algal flue gas sequestration and wastewater treatment: an industrial experiment, Industrial Engineering and Management, KTH Royal Institute of Technology, Stockholm, Sweden, 2011.
- [6] C. Borkenstein, J. Knobelechner, H. Frühwirth, M. Schagerl. Cultivation of *Chlorella emersonii* with flue gas derived from a cement plant, J. Appl. Phycol. 23 (2010) 131-135.
- [7] K. Napan, L. Teng, J.C. Quinn, B.D. Wood. Impact of heavy metals from flue gas integration with microalgae production, Algal Res. 8 (2015) 83-88.
- [8] ANL, NREL, PNNL. Renewable Diesel from Algal Lipids: An Integrated Baseline for Cost, Emissions, and Resource Potential from a Harmonized Model, US Department of Energy Biomass Program, June 2012.
- [9] L. Laurens, K. Cooksey, J. Sears, R.A. Cattolico, M. Edwards, S. Howell, A. Neblett, R. McCormick, P. Pienkos, G. Clapper, P. Ahlm. The Industrial Algae Measurements 6.0, ABO's Technical Standard Committee, 2014.
- [10] J.L. Manganaro, A. Lawal, B. Goodall. Techno-economics of microalgae production and conversion to refinery-ready oil with co-product credits, Biofuels, Bioproducts and Biorefining 9 (2015) 760-777.
- [11] C.M. Beal, L.N. Gerber, D.L. Sills, M.E. Huntley, S.C. Machesky, M.J. Walsh, J.W. Tester, I. Archibald, J. Granados, C.H. Greene. Algal biofuel production for fuels and feed in a 100-ha facility: A comprehensive techno-economic analysis and life cycle assessment, Algal Res. 10 (2015) 266-279.
- [12] L. Zhu. Biorefinery as a promising approach to promote microalgae industry: An innovative framework, Renew. Sust. Energ. Rev. 41 (2015) 1376-1384.
- [13] M.S. Chauton, K.I. Reitan, N.H. Norsker, R. Tveterås, H.T. Kleivdal. A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: Research challenges and possibilities, Aquaculture 436 (2015) 95-103.
- [14] CIBO. Report to the U.S. Environmental Protection Agency on fossil fuel combustion byproducts from fluidized bed boilers, Burke, VA., 1997, pp. 311.

489 [15] H.Z. Tian, C.Y. Zhu, J.J. Gao, K. Cheng, J.M. Hao, K. Wang, S.B. Hua, Y. Wang, J.R. Zhou.
 490 Quantitative assessment of atmospheric emissions of toxic heavy metals from anthropogenic
 491 sources in China: historical trend, spatial distribution, uncertainties, and control policies, Atmos.
 492 Chem. Phys. 15 (2015) 10127-10147.

493 [16] D. Tang, W. Han, P. Li, X. Miao, J. Zhong. CO₂ biofixation and fatty acid composition of
 494 *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO₂ levels, Bioresour.
 495 Technol. 102 (2011) 3071-3076.

496 [17] C. Yoo, S.-Y. Jun, J.-Y. Lee, C.-Y. Ahn, H.-M. Oh. Selection of microalgae for lipid
 497 production under high levels carbon dioxide, Bioresour. Technol. 101 (2010) S71-S74.

498 [18] R. Hobbs, X. Sun, S. Coutore. Arizona Public Service biosequestration program, private
 499 communication, Phoenix, Arizona, 2009, 2015.

500 [19] S. Bates, A. Tessier, P. Campbell, J. Buffle. Zinc adsorption and transport by *Chlamydomonas*
 501 *varuiabilis* and *Scenedesmus subspicatus* (chlorophyceae) grown in semicontinuous culture, J.
 502 Phycol. 18 (1982) 521-529.

503 [20] K. Napan, D. Hess, B. McNeil, J.C. Quinn. Quantification of Heavy Metals and Other
 504 Inorganic Contaminants on the Productivity of Microalgae, Journal of Visualized Experiments
 505 (2015) e52936.

506 [21] K. Knauer, R. Behra, L. Sigg. Adsorption and uptake of copper by the green alga *Scenedesmus*
 507 *subspicatus* (chlorophyta), J. Phycol. 33 (1997) 596-601.

508 [22] W. Amorim, A. Hayashi, P. Pimentel, M. Da Silva. A study of the process of desorption of
 509 hexavalent chromium, Braz. J. Chem. Eng. 20 (2003) 283-289.

510 [23] E. Grima, A. Medina, A. Giménez, J. Sánchez Pérez, F. Camacho, J. García Sánchez.
 511 Comparison between extraction of lipids and fatty acids from microalgal biomass, J. Am. Oil Chem.
 512 Soc. 71 (1994) 955-959.

513 [24] P.D. Patil, V.G. Gude, A. Mannarswamy, S. Deng, P. Cooke, S. Munson-McGee, I. Rhodes, P.
 514 Lammers, N. Nirmalakhandan. Optimization of direct conversion of wet algae to biodiesel under
 515 supercritical methanol conditions, Bioresour. Technol. 102 (2011) 118-122.

516 [25] S.K. Mehta, J.P. Gaur. Use of algae for removing heavy metal ions from wastewater: progress
 517 and prospects, Crit. Rev. Biotechnol. 25 (2005) 113-152.

518 [26] W.R. Hill, I.L. Larsen. Growth dilution of metals in microalgal biofilms, Environ. Sci.
 519 Technol. 39 (2005) 1513-1518.

520 [27] R.S. Ayers, D.W. Westcot. Water quality for agriculture, Food and Agriculture Organization of
 521 the United Nations, Rome, 1994.

522 [28] US EPA. National recommended water quality criteria United States Environmental Protection
 523 Agency, <http://water.epa.gov/scitech/swguidance/standards/criteria/current/index.cfm>, accessed
 524 June 2015.

525 [29] US EPA. Drinking water contaminants, United States Environmental Protection Agency,,
 526 <http://water.epa.gov/drink/contaminants/index.cfm#List>, accessed January 2015.

527 [30] US EPA. National pollutant discharge elimination system (NPDES) permit writers' manual,
 528 United States Environmental Protection Agency, Office of Wastewater Management, Washington,
 529 D.C., 2010.

530 [31] National Research Council. Mineral tolerance of animals , second ed., National Academies
 531 Press, Washington, D.C., 2005.

532 [32] FAO. Animal feeding and food safety, FAO Food and nutrition paper 69, Food and
 533 Agriculture Organization of the United Nations, Rome, Italy, 1998.

534 [33] NSF International. Dietary supplement-Standard 173, metal contaminant acceptance levels,
 535 [http://standards.nsf.org/apps/group_public/download.php/15297/DS-2007-](http://standards.nsf.org/apps/group_public/download.php/15297/DS-2007-5%20Supplementary%20-%202003%20NSF%20DS%20Metal%20Contaminant%20Acceptance%20Levels.pdf)
 536 [5%20Supplementary%20-](http://standards.nsf.org/apps/group_public/download.php/15297/DS-2007-5%20Supplementary%20-%202003%20NSF%20DS%20Metal%20Contaminant%20Acceptance%20Levels.pdf)
 537 [%202003 NSF%20DS%20Metal%20Contaminant%20Acceptance%20Levels.pdf](http://standards.nsf.org/apps/group_public/download.php/15297/DS-2007-5%20Supplementary%20-%202003%20NSF%20DS%20Metal%20Contaminant%20Acceptance%20Levels.pdf), 2003, accessed
 538 June 2015, pp. 22.

539 [34] Association of American Plant Food Control Officials AAPFCO. AAPFCO's statement of
 540 uniform interpretation and policy (SUIP) #25: the heavy metal rule, Association of American Plant
 541 Food Control Officials, <http://www.aapfco.org/rules.html>, accessed December 2014.

542 [35] US EPA. 40 CFR part 503, United States Environmental Protection Agency, Washington,
 543 D.C., 1993.

544 [36] M.J. McFarland, K. Kumarsamy, R.B. Brobst, A. Hais, M.D. Schmitz. Groundwater quality
 545 protection at biosolids land application sites, *Water Res.* 46 (2012) 5963-5969.

546 [37] Toxics in Packaging Clearinghouse. Model Toxics in Packaging Legislation,
 547 <http://toxicsinpackaging.org/model-legislation/model/>, 2012, accessed June 2015.

548 [38] European Parliament and Council of the European Union. Directive 94/62/EC Packaging and
 549 packaging waste, <http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31994L0062>,
 550 1994, accessed July 2015.

551 [39] European Paper and Board Food Packaging Chain. Industry guideline for the compliance of
 552 paper and board materials and articles for food contact,
 553 <http://www.cepi.org/system/files/public/documents/publications/foodcontact/2012/Industry%20guideline-updated2012final.pdf>, 2012, accessed June 2015.

554

555 [40] G. Kale. Biodegradation of commercially available biodegradable packages in real and
 556 simulated composting conditions, School of Packaging, Michigan State University, East Lansing,
 557 Michigan, 2007.

558 [41] T. Otsuki, F. Zhang, H. Kabeya, T. Hirotsu. Synthesis and tensile properties of a novel
 559 composite of Chlorella and polyethylene, *J. Appl. Polym. Sci.* 92 (2004) 812-816.

560 [42] O. Igbum, K. Asemave, C. Nwadinigwe, N. Akaasah. Heavy metal analysis of blends of
 561 methyl esters obtained from four virgin tropical seed oils, *International J. Sci. Res.* 2 (2013) 463-
 562 467.

563 [43] S. Jain, M.P. Sharma. Correlation development for effect of metal contaminants on the
 564 oxidation stability of *Jatropha curcas* biodiesel, *Fuel* 90 (2011) 2045-2050.

565 [44] Y. Wang, C. Arellanes, S.E. Paulson. Hydrogen peroxide associated with ambient fine-mode,
 566 diesel, and biodiesel aerosol particles in southern California, *Aerosol Sci. Tech.* 46 (2012) 394-402.

567 [45] S. Foster, W. Maher, F. Krikowa, S. Apte. A microwave-assisted sequential extraction of water
 568 and dilute acid soluble arsenic species from marine plant and animal tissues, *Talanta* 71 (2007) 537-
 569 549.