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DISTRIBUTION OF HEAVY METALS FROM FLUE GAS IN ALGAL BIOREACTOR

Katerine Napan

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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2014

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ABSTRACT

Distribution of Heavy Metal from Flue Gas in Algal Bioreactor

by

Katerine Napan, Doctor of Philosophy

Utah State University, 2014

Co-Major Professors: Byard Wood and Ronald Sims
Department: Biological Engineering

Flue gas from coal-fired power plants is a major source of CO₂ to the atmosphere. Microalgae can use this enriched form of CO₂ as carbon source and in turn the biomass can be used to produce food, feed, fertilizer and biofuels. However, along with CO₂, coal-based flue gas will inevitably introduce heavy metals, which have a high affinity to bind algal cells, could be toxic to the organisms and if transferred to the products could limit their uses. This study seeks to address the distribution and impact of heavy metals present in flue gas on microalgae production systems. To comprehend its effects, algae *Scenedesmus obliquus* was grown in batch reactors in a multimetal system. Ten heavy metals (Cu, Co, Zn, Pb, As, Se, Cr, Hg, Ni and Cd) were selected and were evaluated at four concentrations (1X, 2X, 5X and 10X). Results show that most heavy metals accumulated mainly in biomass and were found in very low concentrations in media. Hg was shown to be lost from the culture, with low amounts present in the biomass. An upper limit for As uptake was observed, suggesting its likelihood to build-up in the system during medium recycle. The As limited bioaccumulation was overcome by addition of sulfur to the algal medium. Heavy metal at 2X, 5X and 10X inhibited both growth and lipid production, while at the reference concentration both biomass and lipids yields were increased. Heavy metal concentrations in the medium and

biomass were time dependent, and at the end of the cultivation most heavy metals in the supernatant solution complied with the recommendations for irrigation water, while biomass was below limits for cattle and poultry feed, fertilizer, plastic and paper. This research shows that bioremediation of CO₂ and heavy metals in combination with energy production can be integrated, which is an environmentally friendly form of biotechnology.

(163 pages)

PUBLIC ABSTRACT

Distribution of Heavy Metal from Flue Gas in Algal Bioreactor

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Katerine Napan, Doctor of Philosophy

Utah State University, 2014

Co-Major Professors: Byard Wood and Ronald Sims
Department: Biological Engineering

Algae are microscopic organisms with a great potential to produce biomass and lipids at productivities several times higher than terrestrial crops. To grow, these organisms consume carbon dioxide (CO₂), a greenhouse gas. This gas, emitted primarily by power plants after coal burning, can be effectively used for algae production, thus resulting in CO₂ remediation and biomass beneficial utilization as feedstuff, industrial filler and biodiesel feedstock. However, since coal is a fuel mined from the earth's crust, it contains heavy metals that are released during coal burning and inevitably enter the algal cultivation system, contaminating the water where algae is grown, the algal biomass and the products derived from such biomass. The distribution of heavy metals from flue gas in algal cultivation systems is unknown, yet necessary to advance this industry. This study focused on quantifying the distribution and effects that ten coal-derived heavy metals (Cu, Co, Zn, Pb, As, Se, Cr, Hg, Ni and Cd) will have on algae strain *Scenedesmus obliquus* and on the potential products derived from this algae.

DEDICATION

I would like to dedicate this doctoral dissertation to my husband, Karthik Kumarasamy, who was my pillar of support during this challenging but rewarding journey. Karthik, I am deeply thankful for your support, which came not only as words of encouragement and technical opinions, but also as those countless silent hours and late nights you awaited for one experiment more to be finished.

On the other hand, I also want to dedicate this work to you, the reader. I wish this study helps you to continue this chain of scientific contributions towards making a better world for all Earth's inhabitants.

Katerine Napan (Katerine Karthik)

ACKNOWLEDGMENTS

I would like to acknowledge Arizona Public Service (APS) and the U.S. Department of Energy (DOE) for the funding support of this research, as part of their vision to progress towards a more sustainable energy.

I am grateful to my major professors, Dr. Wood and Dr. Sims, who guided and advised me, but mainly who taught me to critically evaluate research questions/answers and analyze their broader implications. My deep appreciations to my committee members, Ms. McLean, Dr. Miller and Dr. Stevens, and to professors Dr. Viamajala and Dr. Quinn, because with their exciting classes, their advice and feedback they helped me to grow in knowledge, in a field many times new and challenging for me. I am also very thankful to my colleagues, Mr. Butler, Lihong and Tess, and to all the staff in the Biological Engineering department and Mechanical Engineering department, especially Anne, Jed, Paul and Karen.

Finally, all my gratitude to my husband, Karthik, my parents Pablo and Francisca, my siblings Pablito and Kandy, my nephew baby Hsieh and all my extended family and friends who gave me moral support regardless of the distance that divided us. Thanks to my in-laws, Kumaraswamy, Devaki, Saraswathi, Jack, Mohan and Dinesh, who tacitly helped me to finish this endeavor with their acceptance. My gratitude also goes to Carmen for her support.

To all, my warmest thanks, admiration and appreciation.

Katerine Napan (Katerine Karthik)

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ACRONYMS

AAPFCO: Association of American Plant Food Control Officials

AAS: Atomic Absorption Spectrometry

ACS: American Chemical Society

ASTM: American Society for Testing and Materials

B100: Fuel containing 100% biodiesel

EDTA: Ethylenediaminetetraacetic acid

EIA: Energy Information Administration

EPA: Environmental Protection Agency

FAO: Food and Agriculture Organization of the United Nations

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

MCL: Maximum Contaminant Level

MMT: Million of Metric Tons

MTL: Maximum Tolerable Levels

NPDES: National Pollution Discharge Elimination System

NRC: National Research Council

OD: Optical Density

PBR: Photobioreactor

QC: Quality Control

ROS: Reactive oxygen species

TSS: Total Suspended Solids

Note: Heavy metals in this study are referred by their chemical symbols. The arsenic symbol (As) will only be used whenever there is no chance of confusion with the adverb, conjunction and preposition “as”. Otherwise the full name will be written.

CHAPTER 1

INTRODUCTION

1.1 Background

Feedstock development for the production of renewable biodiesel looks forward to generate a crop that is high in oil content, but that does not compete with food crops. For several years first generation oil crops (*i.e.*, crops for food) have been used for biofuel production, and as of 2012, the main sources of oil for biodiesel production came from conventional terrestrial oil crops such as soybean, canola and corn [1]. The use of these edible oils for biodiesel production has partially resulted in an increase in the prices of these food crops [2-5]. Between 2001 to 2007 the U.S. biofuel demand contributed to a 20 to 25% increase in the price of corn and 7 to 8% for soybean [5]. Microalgae, a third generation feedstock (*i.e.*, from microorganisms using advanced technology), is a very promising feedstock. Compared to the traditional oil-crops, microalgae readily generate biomass at rates of one to two orders of magnitude higher than terrestrial oil crops and have a much higher potential oil productivity (Table 1) [6]. Furthermore, microalgae do not compete for land or water with traditional agriculture, instead they can thrive in non-arable land using municipal wastewater, seawater, produced water, saline water and some types of industrial wastewater [7-10].

Table 1. Oil productivity of several oil-crops

Oil crop	Oil productivity (gallon/acre/year) [6]
Corn	18
Soybean	48
Sunflower	102
Rapeseed	127
Oil Palm	635
Microalgae	5000 - 20 000

Microalgae, henceforth referred to as algae, are photosynthetic microorganisms that convert sunlight, CO₂ and nutrients into biomass. This natural carbon fixing process can be utilized to capture CO₂ from industrial sources to generate algal biomass that can serve as a feedstock for the production of liquid fuels, food, animal feed, fertilizer and as a feedstock for other industries (Figure 1) [10-14]. CO₂ present in industrial flue gas is currently considered a waste of environmental concern. Fast raising temperatures observed from 1971 onwards (with the 1990's being the warmest decade for the past millennium [15, 16]) has been attributed to be a result of the raising atmospheric greenhouse gasses such as CO₂ [17]. Conversion of CO₂ to biomass recycles CO₂ and reduces its impact and at the same time provides energy and food solutions.

The largest emission of CO₂ into the atmosphere come from industries such as coal-fired power plants [18]. According to the Energy Information Administration (EIA), 1458 coal-fired electrical power plants exist in the US [19] and are by far the largest CO₂ producers, accounting for 5637.9 MMT of flue gas which represents 42% of the total anthropogenically-produced CO₂ in the US [18]. Using CO₂ from coal-fired power plants to cultivate algae will aid in carbon recycling and has been widely proposed in the published literature with demonstrated applications [20-24].

Besides CO₂, flue gases from coal-fired power plants also contain heavy metals that when released to the environment have a negative impact on human health due to their carcinogenic, teratogenic and mutagenic effects as wells as adversely affect the environment (Table 2). Several heavy metals derived from coal are toxic pollutants considered by US EPA of high priority for their regulation (Table 2). Heavy metal is a vaguely defined term commonly used to identify some transition metals, metalloids, lanthanides and actinides generally known for their toxicity at low concentrations. One of the definitions for heavy metals is said to refer to chemical elements with a specific gravity at least 5 times that of water that exhibit metallic properties [25], but no consensus exists yet. In this study, we will use this term to refer to As, Cd, Co, Cu, Cr, Hg, Ni, Se, Pb and Zn.

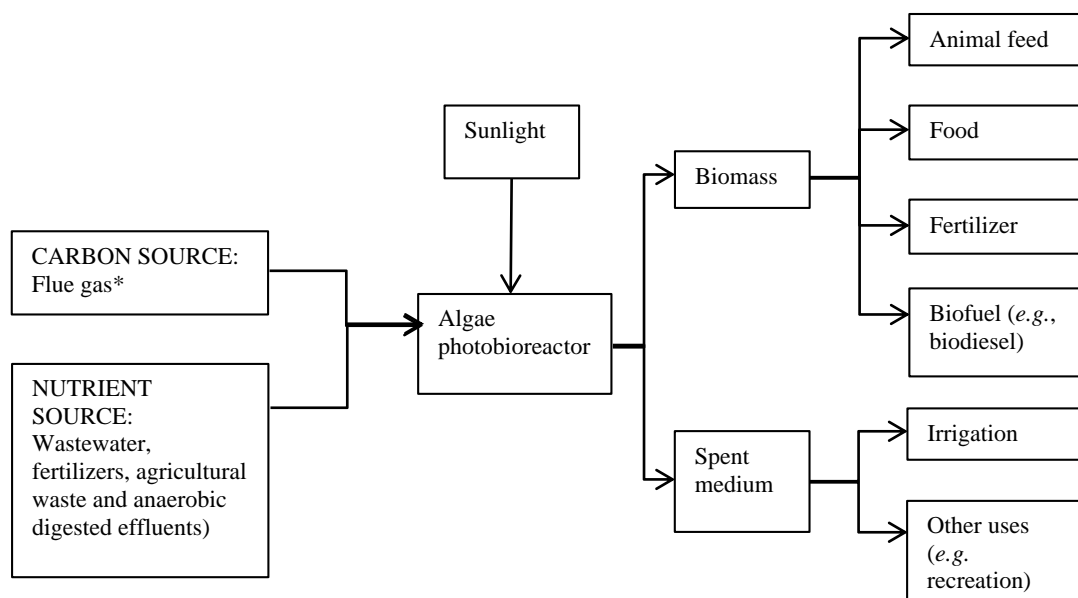


Figure 1. System concept for algae production. (*) Source of heavy metals.

Table 2. Metallic air pollutants from coal-fired electrical generators in the US

Heavy metal	Priority /Non-priority	Percent of total US anthropogenic emission [26-28]	Carcinogen classification
As	Priority	45%	Human carcinogen
Cd	Priority	11%	Probable human carcinogen
Cr(VI)	Priority	27%	Human carcinogen (inhalation route)
Ni	Priority	29%	Human carcinogen (Nickel refinery dust and nickel subsulfide)
Se	Priority	74%	Not classifiable as to human carcinogen
Hg	Priority	48%	Not classifiable as to human carcinogen for elemental Hg, possible human carcinogen for methylated Hg
Pb	Priority	4%	Probable human carcinogen
Co	Non-priority	22%	-

The effect of heavy metals on algal cultures can be positive and/or negative. At high concentrations they can be toxic to algae, affecting photosynthesis and inhibiting growth; however, at low doses some of the heavy metals present in flue gas can serve as micronutrients for algae (*e.g.* Co, Zn and Cu) [23, 24, 29], thus reducing the costly use of fertilizers. The potential negative effects and fate of heavy metals are a cause of concern. Concerns include growth inhibition, restricted use of biomass and liquid medium for biodiesel feedstock, decreased economic value of by-products and increased costs of contaminated solid/liquid waste disposal [22]. These concerns will be further enhanced with medium recycling, leading to heavy metal build up. Currently, published literature about the effects of heavy metals on algae production systems and the use of algal-based feedstock contaminated with heavy metals for energy production does not exist [30]. The funding agencies funded two researchers (a Ph.D. and a master), the specific objectives for the doctoral study are outlined in the following section.

1.2 Research Objectives

The overarching hypothesis for this research is “*Flue gas from coal fired combustion sources contains heavy metals that can be beneficial for the production of algal biomass feedstock for biofuels*”. To test this hypothesis research was carried out to determine if heavy metals from flue gas will impair uses of biomass and medium. Specifically, this study assesses the distribution of 10 heavy metals (As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn) in the bioreactor, quantifies the heavy metals in biomass and effluents, evaluate potential uses from regulatory and beneficial use perspective and discusses the results focusing on the implications of this combined system. Key questions to be answered are:

- What are the sinks for heavy metals in a photobioreactor (PBR) production system, *i.e.*, where do the heavy metals accumulate: biomass or elsewhere?

- What is the capability of algae to uptake heavy metals and what are their bioremediation capabilities?
- Can heavy metals concentration in the algal biomass and medium affect their uses?
- Can bioremediation of As by algae be enhanced by sulfur enrichment?

The hypothesis and experimental design to answer these questions are presented in Appendix A.

1.3 A Guide to the Dissertation

The remainder of this dissertation is divided into four (4) chapters. Chapter 2 will focus on determining the distribution of the heavy metals in the PBR (*i.e.*, the medium, the biomass or elsewhere), the removal of heavy metals from the biomass (internalized or externally bound) and the impact of such heavy metal distribution on algal growth and lipid yield. Chapter 3 presents the heavy metal contamination levels in biomass and medium, identifies the heavy metals that are likely to build-up in the PBR, and explores the use of desorbents and solvents to reduce heavy metal concentration in the biomass. Heavy metal concentration in biomass (before and after rinsing procedure) and medium are compiled and compared against established standards for irrigation, aquatic life, animal feed, fertilizer, plastic filler and paper pulp. Chapter 4 explores a bioremediation treatment in order to reduce heavy metal build-up in the PBR. Finally, Chapter 5 presents conclusions about the viability of producing algae using flue gas.

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CHAPTER 2

HEAVY METAL DISTRIBUTION IN ALGAL PRODUCTION SYSTEMS

Abstract

Integration of algae cultivation with coal-based flue gas is a widely proposed approach to capture and recycle CO₂ from power plants and recover energy through biodiesel production from algal biomass. Besides CO₂, heavy metals (originally present in coal) are introduced to the cultivation system and could impact overall biodiesel production due to the contamination of biomass or medium and due to inhibition of algal growth and lipid accumulation. In this study, green algae *Scenedesmus obliquus* was grown in nutrient rich medium containing 10 heavy metals expected to be introduced from flue gas (As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn). Four concentrations were evaluated, namely, 1X concentration (reference concentration expected from flue gas), 2X, 5X and 10X considering recycle scenarios. Analysis of the distribution of heavy metals indicated that with exception of Hg and As, the biomass was the main sink for all heavy metals. The accumulation of heavy metals by algae inhibited algal growth and lipid yield at 2X, 5X and 10X concentration; however, 1X resulted in higher biomass (12%) and lipid yields (61%) in comparison to the control. The algal suspension was not the main sink for Hg, possibly due to Hg volatilization. Differentiation between cellular bound and internalized heavy metals indicates that the cell surface is the main sink during the early growth period, but gradually the internalized portion become the dominant sink.

2.1 Introduction

Algae production using waste streams such as flue gas is a promising option for flue gas-emitters for complying with air quality regulations and for algae producers who can use it as a carbon source. With the advent of the most recent air quality regulation, coal fired power plants can benefit from the treatment of flue gas with this beneficial use of CO₂, while algae farming can

leverage into current waste streams with nutrient value to lower production costs. However, the flue gas may also carry heavy metals that are introduced to the cultivation system (*i.e.*, photobioreactor-PBR and open ponds) during flue gas injection [1-4].

Heavy metals initially transferred from flue gas to the liquid medium will re-distribute due to several processes such as adsorption, biotransformation, biouptake and complexation with medium components [5]. All these processes happen simultaneously and as a result heavy metals will accumulate in certain compartments such as the medium, reactor walls, the algal cell wall and inside the cell. This re-distribution can be actively changed by algae through their various defense mechanisms that alter the heavy metal trafficking inside and outside the cell in order to reduce cellular damage derived from toxic metals. Some external factors that can also influence heavy metal re-distribution are the cultivation practices such as period of cultivation, biomass productivity, chemistry of the nutritive medium, etc. For instance, for biodiesel production, algae are grown in highly rich nutrient medium in order to obtain high biomass production, and then the cells are stressed by limiting nutrient supply (*e.g.*, N) in order to boost lipid accumulation and in turn boost biodiesel yields [6]. But under nutrient deprivation algae could overexpress nutrient transporters that also internalize nutrient analogs such as toxic heavy metals (*e.g.*, selenate instead of phosphate, Cd instead of Ca, etc.) [7-9]. In a commercial PBR all these complex interactions will occur simultaneously; however, most published literature supporting integration of algae cultivation with flue gas capture does not account for these interactions.

Several studies have been carried out to understand adsorption and toxicity of heavy metals on algae, however they are not fully applicable to the understanding of algae-flue gas integrated systems. For example, studies addressing adsorption of heavy metals by algae are usually performed for short time periods (typically 20 to 300 minutes), at low pH (usually from 1 to 6.5) and at doses that are several times higher than what is expected from flue gas; hence not being representative of commercial algal-biomass production conditions [10-14]. Other studies have

evaluated long term toxicity effects of heavy metals on either growth or lipids, but at concentrations that are higher than the concentrations that would be seen with flue gas [15-20]. Both, short and long term studies reviewed, are either mono-metal or include few elements in multi-metal systems, but are not representative of the various heavy metals that may be introduced from flue gas [12, 21, 22].

Although there are a few studies that report on the use of flue gas to cultivate algae, they have not accounted for the effects of heavy metals. Instead their results represent the combined effects of the cultivation techniques, the effect of several other constituents present in flue gas (*e.g.*, NO_x, SO_x), and the flue gas quality and temperature at the moment of withdrawal (flue gas quality changes with incineration technique, fuel source, level of purification, etc.) [1-4, 23]. Furthermore, a review of the literature highlights the need to better understand the distribution of heavy metals on algal cultivation systems and their impact on biomass and lipid productivity, as described in the National Algal Biofuels Technology Roadmap authored by the US Department of Energy [24]. The aim of this study is to determine the final distribution of 10 heavy metals (As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn) from flue gas in three compartments (aqueous phase, algae-surface-bound and internalized portion) in an algal bioreactor intended for biodiesel production.

2.2 Literature Review

2.2.1 Flue Gas

During the combustion of coal (at temperatures around 1000 to 1600°C) minerals trapped in the coal matrix are released through vaporization, thermal decomposition, fusion and agglomeration [25]. As, Cd, Cu, Pb, Se, Hg, Co, Ni and Zn volatilize in the boiler [25, 26] and part of their vapors is adsorbed by the ash, while the remaining vapors continue in gaseous state until condensation occurs. Larger sized ash particles settle in the bottom of the furnace (also named bottom ash) but ash smaller than 100 µm (also called fly ash) and vapors exit the furnace [27, 28].

During flue gas cooling (about 200°C), most heavy metal vapors pass the dew point and either condense onto the fly ash, associate with chlorides or form aerosols [25, 28-30]. Before flue gas is released to the atmosphere, it passes through pollution control devices such as electrostatic precipitators, cyclone and fabric filters in order to reduce fly ash content [25, 31]. However, sub-micron sized fly ash is not fully captured and escapes even after the flue gas has been treated [25, 29, 32, 33]. These sub-micron sized ash particles are more likely to have higher concentrations of heavy metals due to the preferential re-condensation of vaporized metals onto a larger surface area [27, 28, 30, 32, 33].

Heavy metals in flue gas can form various chemical species, with the formation of volatiles being increased under high chlorine conditions. Due to the high presence of chlorine in US coals, most heavy metals in the flue gas cooling post-combustion atmosphere are likely to react with Cl gas and water vapor, leading to formation of chloride salts [28, 34]. Se, As and Hg are more likely to form vapors even at low temperatures [25, 33], but once they condense they also can react with chlorides. Chloride salts of Se and As are unstable and As and Se are more likely to occur as As_2O_3 and SeO_2 [35]. After reacting with the steam in flue gas, As and Se are expected to form oxyanions arsenite (H_3AsO_3) and selenous acid (H_2SeO_3), respectively, which are common As and Se forms under anoxic conditions [36]. After the cooling process Hg reacts with HCl gas and the primary reaction is $Hg^0 + 2HCl(g) \leftrightarrow HgCl_2 + H_2O$ [26, 35], where only $HgCl_2$ is expected to dissolve in the PBR medium as Hg^0 does not dissolve in water due to its low water solubility ($6 \times 10^{-6} \text{ g} \times 100^{-1} \text{ mL water at } 25^\circ\text{C}$) and high vapor pressure [37]. Moreover, Kelly, Budd and Lefebvre [38] documented the absence of Hg after bubbling $Hg^0_{(g)}$ through a PBR [38].

2.2.2 Algae

Algae are any photosynthetic eukaryotic microscopic organisms that lack leaves and roots [39]. Algae fix CO_2 using the Calvin cycle during photosynthesis and produce sugar phosphate

(glyceride-3-phosphate) which is later converted into glucose and other biomass components. In addition to carbon, algae consume nutrients such as N, P, K, S and essential elements (also called trace elements or microelements) such as Co, Cu, Se and Zn [40] [41]. Algae can also uptake nonessential elements (toxic elements) such as Cd, Hg, As and Pb that do not have a known role in cell growth [41-43].

CO₂ from flue gas can be used as a carbon source in algae cultivation [44]. Enhanced algal growth has been observed by Doucha, Straka and Lívanský [45], Douskova, Doucha, Livansky, Machat, Novak, Umysova, Zachleder and Vitova [3] and Kadam [46] when using flue gas. They hypothesized that either CO₂ or nutrients present in flue gas resulted in enhanced growth [45-47].

Flue gas leaves the stack at about 120 to 200°C and is further cooled down before entering the PBR [3, 48]. After injecting flue gas, heavy metals are transferred to the medium and the biomass [1-3]. Once in the PBR system, heavy metals can redistribute and may undergo chemical and biological transformations. For instance, the interaction of heavy metals with the medium components can form new complexes and precipitates; the redox-sensitive metallic ions can be oxidized or reduced naturally or biologically; the metallic ions can be adsorbed to the algal cell walls and can be internalized by algae.

Once inside the cell heavy metals can cause damage by forming nonfunctional proteins, direct damage of DNA, generation of reactive oxygen species-ROS (singlet oxygen, hydroxyl radical, hydrogen peroxide and superoxide anion) which can damage cell membrane, proteins, lipids and nucleic acid, producing alteration of cell structure [49-51]. Due to metal stress, defense mechanisms are activated and heavy metals can be sequestered by algae and can undergo biologically driven transformations (towards a less toxic form) or they can be excreted [36, 41, 52]. All mentioned interactions are extremely complex. It is not the purpose of this section to provide a comprehensive review of the mechanisms behind these interactions, but rather to describe their

cumulative effect on the distribution of heavy metals and the beneficial and/or negative consequences.

It is hypothesized that heavy metals redistribute to several locations within the PBR after their addition. Literature suggests algae have high affinity for binding heavy metals [5, 11, 53-57]. After this initial interaction, internalization of the heavy metal follows, especially of the ions that have nutrient functions (*e.g.*, Zn, Cu, Co) [58], but also of toxic heavy metals [59]. Eventually both, nutrients and non-nutrient heavy metals, will produce biological responses that will affect metal distribution, algal growth and lipid accumulation. Since algae take several days to weeks to grow and to accumulate lipids (specie dependent), it is hypothesized in this study that heavy metals will redistribute in the PBR; but preferably will be associated with algae, affecting growth and lipid productivity.

2.3 Materials and Methods

Labware and reagents: All glassware, polycarbonate labware and borosilicate PBR were soaked for 12 hours in 10% HNO₃ solution to eliminate any potential contamination. They were rinsed three times with E-pure deionized water (resistivity 17.7megohm·cm) following the soaking process. The reagents used for the preparation of stock solutions and medium were of analytical grade or better. Nitric, sulfuric and hydrochloric acids were purchased from Fisher Scientific and were of trace metal grade. KMnO₄, K₂S₂O₈, NH₂OH, SnCl₂ were purchased from Fisher Scientific and were ACS (American Chemical Society) grade suitable for Hg analysis. Standard solutions for As, Cd, Co, Cr, Cu, Se, Ni, Pb and Zn analysis were purchased from Fisher Scientific. Standard solution for Hg was purchased from Inorganic Ventures. Standard solutions for PO₄³⁻, SO₄²⁻ and NO₃⁻ analysis were purchased from Fluka and Inorganic Ventures.

Strain and medium: Researchers at Arizona Public Service (APS) evaluated different strains and medium compositions appropriate for outdoor cultivation using flue gas. They found

that green algae *Scenedesmus obliquus* was a resilient strain that became dominant over other strains (according to APS scientists [60], personal communication). APS researchers also developed a medium recipe (called APS medium) to suit this strain. *Scenedesmus obliquus* algae and APS medium recipe were the choice for this study and were kindly donated by APS.

Algal cell preparation: Algae *Scenedesmus obliquus* was first cultured in Petri-dishes in order to maintain strain purity. Petri-dish colonies were transferred to 3 L polystyrene spinning PBRs (Corning®) and were grown for 7 days in APS medium under continuous fluorescent light condition at pH 7 until the biomass density reached approximately 2.5 g/L dry weight. Algal cells were harvested by centrifugation at 3900 RPM for 5 minutes and were washed twice with the fresh APS medium in order to eliminate metal chelators contained in the old medium [61]. Washed algal cells were re-suspended and added to the borosilicate PBR.

Medium preparation: APS medium was prepared using NaNO_3 (1000 mg/L), K_2HPO_4 (200 mg/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (49.1 mg/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (25.1 mg/L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (21.5 mg/L), H_3BO_3 (11.4 mg/L), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.597 mg/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.086 mg/L), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.058 mg/L), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.041 mg/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.029 mg/L), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (12 mg/L) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (4.5 mg/L). The medium was autoclaved at 121°C. Mixture of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ + $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ was autoclaved separately in order to minimize iron precipitation [61] and added to the medium afterwards. The pH was adjusted to 7 by HCl addition.

Heavy metals stock solution: Characterization of heavy metals in flue gas is limited. Their ceiling concentrations and bioavailability vary widely as a result of variable fuel source and combustion conditions. Therefore, the selection of heavy metals species and concentrations made for this study considers a conservative case scenario for algae productivity and contamination (details of these calculations can be found in Appendix B). Ten (10) heavy metals (As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn) were selected for this study, with the metal sources and their concentrations shown in Table 3. The heavy metal concentrations are henceforth referred to as 1X,

2X, 5X and 10X concentrations. The 1X concentration represents the highest end concentration that algae are likely to be exposed from flue gas without medium recycling. The 2X, 5X and 10X concentrations were tested to understand the effects of highly contaminated flue gas as well as the higher concentrations resulting from recycling of the medium.

Metal salts in Table 3 were kept in a desiccator overnight [62] and then were weighted and dissolved in E-pure deionized water. Each metal salt was prepared individually to reach a 1000X concentration. In order to avoid a change in the oxidation state, the liquid stocks were sterilized by filtration through sterile 0.2 μm syringe filter instead of autoclaving. Stocks were stored in sterile containers and preserved at 4°C until the following day. Stocks were prepared one day before the experiments.

Borosilicate PBRs: Airlift borosilicate glass tube PBRs of 1.1 L capacity were used in the experiments (Figure 2). The PBRs were built at the USU Chemistry store. A 12-hour photoperiod was used with fluorescent plant lights as the light source. A sterile mixture of CO₂ and air was bubbled into the reactor using a glass capillary tube extended up to 1 cm from the bottom. The completely mixed state in the reactor was achieved by the turbulence created by the raising bubbles. The amount of CO₂ delivered was adjusted to maintain a pH of 7. The PBRs were located inside a walk-in fume hood to prevent any potential release of metal vapors within the laboratory.

Borosilicate PBRs were sterilized by autoclaving at 120°C for 30 minutes and were filled with the APS medium without EDTA to reduce complexation with metals [30]. Aliquots of heavy metal stock were added to the medium to reach the desired concentrations listed in Table 3. Washed algae were added to the PBRs at an initial density of around 0.8 g/L. The algal cells remained in suspension during the entire experiment ensuring homogeneity and no attached growth or surficial foams were observed. Samples were collected at 5 cm from the bottom of the PBR. Growth measurements were taken during the experiment by measuring optical density (OD) at 750 nm, which was correlated with total suspended solids (TSS).

Table 3. Heavy metals concentrations

Heavy metals	Metal source	Concentration ($\mu\text{g metal/L}$)			
		1X	2X	5X	10X
As	NaAsO_2	78	156	390	780
Cd	CdCl_2	15	30	75	150
Co	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	16	32	80	160
Cr	$\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$	130	260	650	1300
Cu	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	131	262	655	1310
Hg	HgCl_2	10	20	50	100
Ni	$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	254	508	1270	2540
Pb	PbCl_2	55	109	273	545
Se	Na_2SeO_3	10	20	50	100
Zn	ZnCl_2	440	880	2200	4400



Figure 2. Airlift tube PBR schematic and set-up

Correction for evaporation: Evaporation of water was observed during the cultivation period. Water lost by evaporation was not compensated to avoid changes to the chemical pseudo-equilibrium reached in the PBR. Instead, evaporation rates were measured and the concentrations reported in this study were adjusted for evaporation.

Determination of biomass concentration: The biomass concentration was estimated using a relationship between measured OD at 750 nm (OD_{750}) and TSS [63]. TSS was determined by using the standard method 2540D [62]. OD_{750} was measured using a Thermo Electron Corporation Genesys 5 spectrophotometer. The TSS in g/L was obtained using the correlation equation $TSS = OD_{750} \times 0.4585 + 0.0116$.

Lipid transesterification and FAME analysis: Lipids in algae were quantified through transesterification of lipids into fatty acid methyl esters (FAME). *In situ* transesterification, a single-step reactive extraction method that combines the sequential extraction followed by transesterification was used. Frozen microalgal pellets from 45 mL samples were freeze-dried and ground into a powder using a mortar and pestle. A subsample of 24 mg of dried algae was transferred into a crimp top gas chromatograph vial containing 0.5 mL acidified methanol (5% H_2SO_4) and was digested for 90 min at 90°C. After digestion the vial was centrifuged and the acidified methanol containing the FAME was transferred into a 5 mL serum bottle containing 4 mL hexane. Complete recovery of FAMEs was achieved by rinsing the biomass with additional 1 mL hexane. The sealed serum bottle was then immersed in a water bath at 90°C for 15 min and cooled down to allow phase separation. The upper phase containing the hexane-FAME was pipetted out and analyzed by gas chromatograph (Agilent Technologies 7890A) using methyl ester standards purchased from Sigma-Aldrich.

Heavy metals sampling: 12 ml of unfiltered sample was aspirated from the PBR of which 5 ml was used for analyzing total heavy metal concentration in the algal suspension (medium containing algae). Additional 30 ml sample was aspirated for Hg analysis from which 10 ml was

used for total Hg quantification in algal suspension. These values are reported as heavy metal concentration in the algal suspension.

The remaining 7 ml algal suspension (or 20 ml for Hg) was centrifuged at 7500 RPM for 3 minutes and the supernatant was analyzed for heavy metal concentration. These values are reported as heavy metal concentration in the medium.

The algal cell pellets left in the centrifuge vials were re-suspended in 0.1 M EDTA containing 0.08% w/w NaCl solution (to avoid lysis of cells due to hypotonic effect) at pH 7 for 10 minutes. They were centrifuged at 7500 RPM for 3 minutes to remove cationic metals (Cd, Co, Cu, Ni, Hg, Pb and Zn) that are surface-bound (EDTA only removes surface bound metals [64]). The washed algal cell pellets were then digested and analyzed with values reported here as internalized metals. The sequential extraction method described above is an operationally defined approach and is shown in Figure 3 [61]. The non-cationic heavy metals (As, Se and Cr) present in the algal cell pellets were also measured and are reported here; however, they are not categorized as internalized metals. The supernatant from EDTA washing was also collected but was not analyzed due to formation of precipitates during analysis; therefore this fraction was obtained as the difference between the heavy metals in suspension, the medium and the EDTA non-removable fraction and is reported as EDTA-removable or surface-bound fraction.

As, Cd, Co, Cr, Cu, Ni, Pb, Se and Zn analysis: Sample digestion was done using HNO₃ digestion in Standard Methods 3030E [62]. The samples (algal suspension, supernatant and algal cell pellets) were transferred to borosilicate test tubes and were digested using HNO₃ at 105°C in a heating block until biomass disappeared. The digested samples were then transferred to volumetric flasks and adjusted to 5 ml by addition of E-pure deionized water. They were preserved in capped containers at 4°C until analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS Agilent 7500 Series). Digested samples were diluted with E-pure deionized water, when needed, while ensuring sample acidity matched the acidity of the ICP-MS calibration standards.

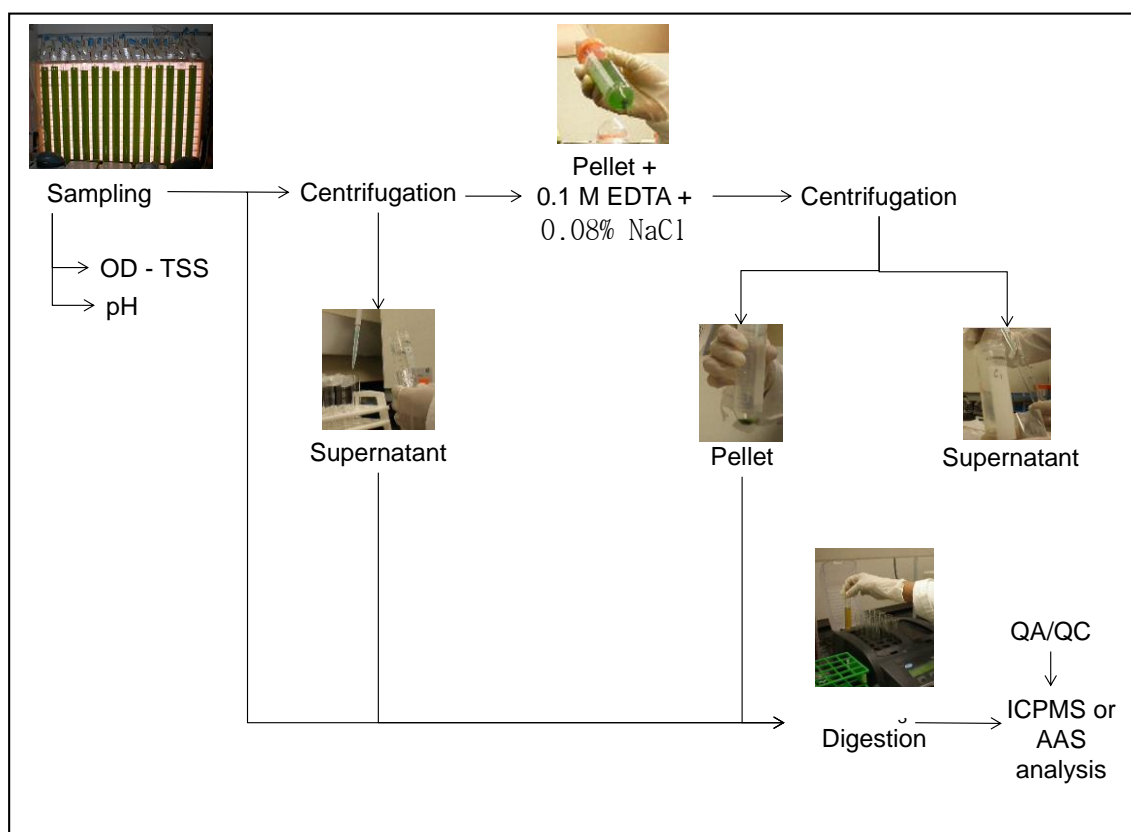


Figure 3. Sequential extraction method for heavy metal analysis

Hg analysis: Sample digestion was done using EPA 7470A and EPA 7471A standard methods. Hg concentration was measured by cold-vapor atomic absorption procedure by Atomic Absorption Spectrometry (AAS, PerkinElmer Analyst 800). Hg standards and SnCl_2 were prepared the same day of the analysis.

Quality Control (QC) samples: It was ensured that correlation coefficients (r) of the heavy metals calibration curve were above the quality criteria (>0.995 [65]). Percent recovery (%R) was monitored during analysis to make sure data were within acceptable recoveries limits (75-125% [65]). Matrix interference (%R outside the acceptable range) was overcome by matrix dilution with acidified deionized water. Overall, calibration curve and percent recoveries (%R) were within acceptable quality control criteria (see Appendix C).

Statistics: Experiments were performed in triplicate PBRs and the data are reported as the mean of the three values. Samples for heavy metals analysis were allowed to be read three times. The standard deviation at each point is represented by error bars that indicate \pm one standard deviation from the mean (n=3). The absence of error bars indicates that they are overlapped within the symbol corresponding to the mean. Data are assumed to be normally, identically and independently distributed (NIID). Comparison of means to find the differences between treatments were done using one-way ANOVA at 95% confidence interval. For multiple comparison of temporal data collected along the growth period, Dunnett's technique was applied using a 95% confidence interval. With these techniques we also identify which pairs of data points (paired by date) are statistically different.

2.4 Results and Discussion

2.4.1 Distribution of Heavy Metals in the Algal PBR

2.4.1.1 Global Distribution of Heavy Metals. Heavy metals added to the medium partitioned to several compartments that in this study were operationally defined based on algae production and potential end use. Of primary interest are the heavy metals that stay in the medium and those that are sorbed by the algae. These are readily measured. Any remaining heavy metals were assumed to be lost to the environment. Thus the compartments are: (i) the medium, (ii) the harvested algal biomass and (iii) the losses from the algal suspension. Figure 4 shows the relative percentages of heavy metal distribution in the three compartments (medium, harvested biomass and losses) from triplicate reactors after 24 days study period for 1X, 5X and 10X (2X was not analyzed), where 100% represents the initial heavy metal concentration added to the algal suspension. Based on Figure 4, it can be observed that biomass serves as the major sink for all metals at the three concentrations tested, except for As and Hg. The main sink for As at 1X was the biomass, but at 5X and 10X it mostly remained in the medium. Hg and Se were consistently lost

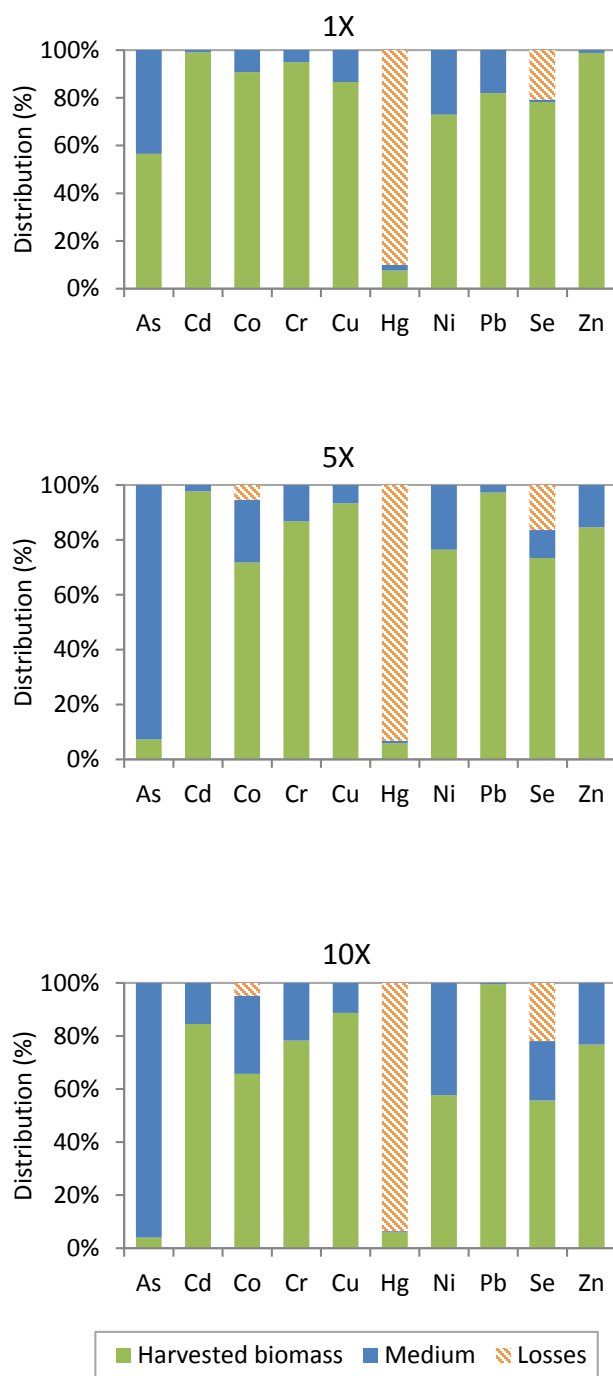


Figure 4. Relative percentages of heavy metals in the medium, harvested biomass and loss after 24 days study period.

from the algal suspension for the three concentrations tested, with Hg being lost in larger quantities. A small percentage of Co was lost from the suspension for 5X and 10X experiments, but was not detected at 1X concentration.

2.4.1.2 Temporal Variation of Heavy Metals in Algal Suspension. Figure 5 shows the temporal variation of heavy metal concentration in the algal suspension during the 24-day study period for PBRs at 1X heavy metal concentration. The concentrations of all heavy metals with the exception of Hg and Se were statistically similar to the initial concentrations added to the PBR (ANOVA, $p < 0.05$). 87% Hg and 27% Se were lost from the algal suspension (only two data points were taken for Hg due to the large sample volume required for the analysis).

Temporal variation of heavy metals in the algal suspension can be attributed to losses of heavy metals to other sinks, for example partitioning from the aqueous phase to the PBR wall (due to sorption) and to the PBR headspace (due to volatilization). The nearly constant concentration observed for As, Cd, Co, Cr, Cu, Ni, Pb and Zn (Figure 5) suggests that these elements were not lost. Adsorption of these elements to glass and silicone walls can also occur but in this experiment they seemed to be negligible. It has been reported that Pb and Cr could form volatile methylated compounds in anaerobic reducing environments [66], but such conditions are not expected to occur in the algal PBR. However, the conditions present in the algal PBR could be favorable for the volatilization of Se and Hg by biotransformation, thus representing an important loss pathway.

Se volatilization by algae is reported in the literature for various algal strains including *Scenedesmus* sp. [67]. Freshwater algae *Chlorella*, *Ankistrodesmus* and *Selenastrum*, exposed to either selenite or selenate, convert Se to lesser toxic and volatile forms such as dimethylselenide (DMSe), dimethyldiselenide (DMDS_e) and trimethylselenonium (TMSe) [68-72]. The entire mechanism for Se volatilization by algae is unknown, but published literature reviewed suggests that after selenite is transferred into the cell, it is converted to selenocysteine (SeCys) and selenomethionine (SeMet) that are precursors to the production of volatile Se forms (Figure 6)

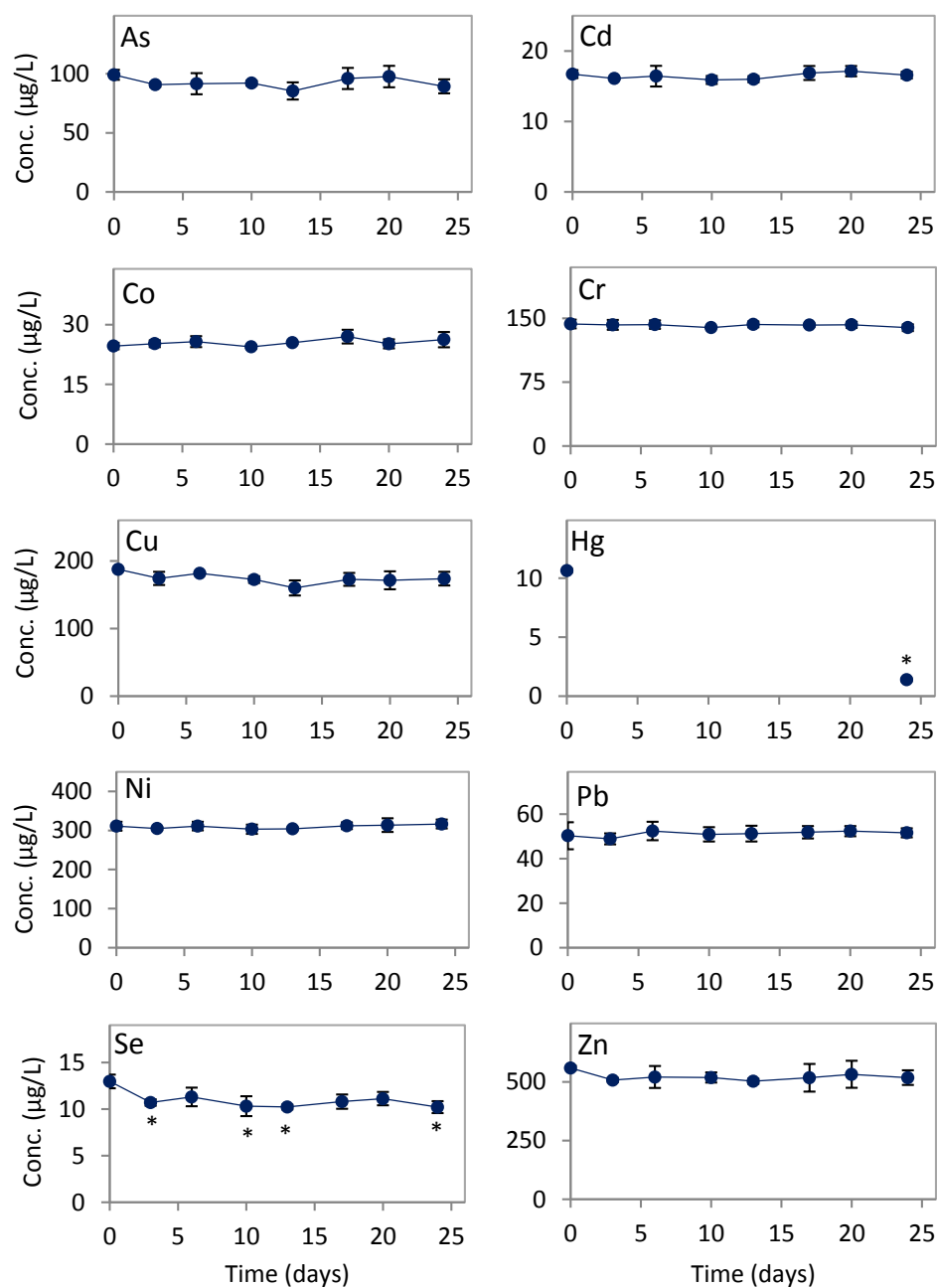


Figure 5. Temporal variation of heavy metal concentration in the algal suspension during 24-day study period for 1X experiment. 100% represents the concentration added to the algal suspension on day 0. Data points shown are the average of three replicates and error bars indicate \pm one standard deviation. Asterisks (*) indicate statistically significant differences ($p < 0.05$) from initial concentration using Dunnett's test.

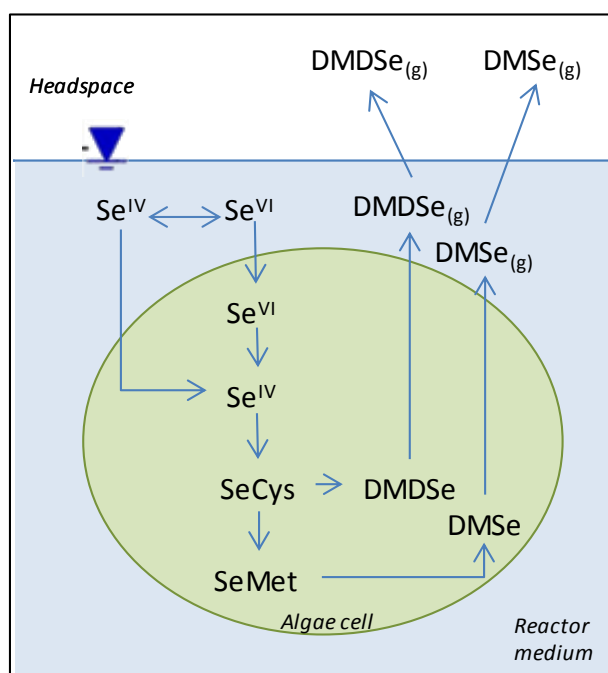


Figure 6. Schematic representation of Se trafficking by algae. Selenomethionine (SeMet), selenocysteine (SeCys), Dimethyldiselenide (DMDSe), dimethylselenide (DMSe).

[68, 70, 72-74]. The Se-methylated forms produced inside the cell diffuse through the cell wall to the surrounding liquid medium and is then it is lost from the liquid medium by outgassing of volatile methylated Se due to its high vapor pressure [75]. Dunnett's test results points to a statistically significant difference ($p < 0.05$) at several times for Se during the study (Figure 5) that can be attributed to the volatilization mechanism described above. Furthermore, these results agree with the low rates of Se volatilization reported by Neumann, De Souza, Pickering and Terry [68] for green algae. Additionally, inorganic Se can volatilize as H_2Se under reducing conditions [76], but such conditions are not expected to occur in an algal PBR.

Algae also uptake inorganic Hg, but reduce its toxicity through various detoxification pathways converting it to less harmful forms. Figure 7 depicts three possible detoxification pathways reported for green algae: (i) volatilization through enzymatic reduction, (ii) thiol chelation and (iii) formation of meta-cinnabar crystals (β -HgS) [38, 77, 78]. Many eukaryotic algae

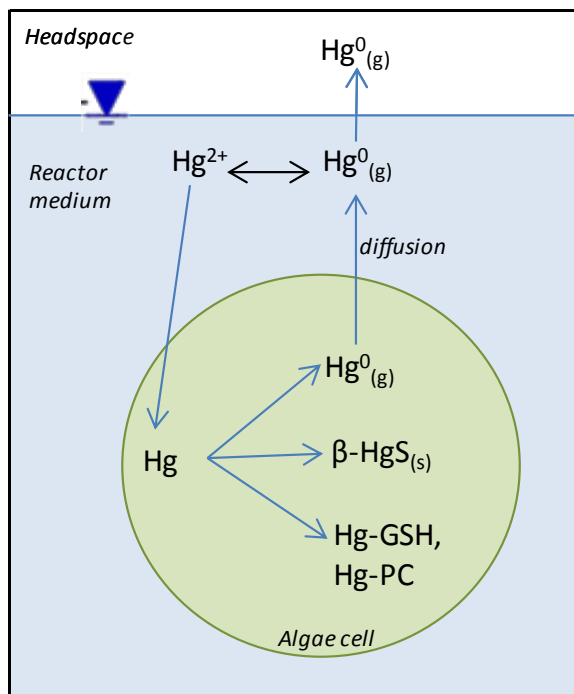


Figure 7. Schematic representation of Hg trafficking by algae. Meta-cinnabar crystals ($\beta\text{-HgS}$), Hg bound to glutathione (Hg-GSH), Hg bound to phytochelatins (Hg-PC).

such as *Scenedesmus*, *Chlorella*, *Dunaliella* and *Selenastrum* have been reported to enzymatically reduce Hg ion (Hg^{2+}) to elemental Hg (Hg^0) in aerobic environment with $\text{Hg}^0_{(g)}$ passively diffusing out of the cell and being lost from aqueous phase by volatilization due to its high vapor pressure [38, 66, 79-84]. Hg^{2+} can also form $\beta\text{-HgS}$ crystals and Hg-bound thiol peptides with glutathione (GSH) and phytochelatins (PC) (Hg-GSH and Hg-PC) within the cell and inhibits further Hg^0 formation [38, 85]. However, the formation of these metal complexes are sulfur-dependent, therefore when the thiol pools are exhausted Hg^0 volatilization dominates [38]. Amongst these three mechanisms, volatilization is a dominant mechanism at sub-lethal concentrations [78] and could be the reason for the losses observed in Figure 5. Another possible mechanism of Hg volatilization is through the formation of organomercuric (CH_3Hg^+) compounds (neurotoxin to humans); however,

until now algae have not been reported to produce CH_3Hg^+ vapors in aerobic conditions like the algal PBR [38].

2.4.1.3 Temporal Variation of Heavy Metals within the Medium and the Harvested Biomass. Figure 8 shows the temporal variation in the distribution of heavy metals in both medium and biomass for PBRs exposed to 1X heavy metal concentration. Differences in uptake were observed for each heavy metal introduced to the PBR, although they were exposed to the same PBR conditions. *Scenedesmus obliquus* exhibited higher affinity towards removing Pb and Cr. From Figure 8 it can be seen that concentrations of Cr and Pb rapidly decreased from the medium to undetectable levels during the first three days, while As, Co and Ni removal was significantly slower and incomplete. Cd, Cu, Se and Zn removal rates were in between these two groups.

Figure 8 also shows the gradual accumulation of Cd, Cr, Co, Cu, Ni and Zn in the biomass, thus indicating that algae have mechanisms to retain them in the biomass and no net release either to the liquid phase or to the headspace was observed. However, a net decrease of Hg, As, Pb and Se from the biomass was observed. Hg, Se and As could have been lost from the cell to the aqueous phase by the diffusion of methylated Se, methylated As, elemental Hg and by excretion of inorganic As. In the case of Pb, however, it was completely removed from the medium on day 6, after which, Pb was released from the harvested biomass into the liquid phase. It is probable that Pb^{2+} associated with the biomass could have been chelated by algal exudates, not present in the initial APS medium, but produced later by algae as a defense mechanism against heavy metal stress [86, 87]. Capelo, Mota and Gonçalves [87] found that *Selenastrum capricornutum* Printz produced high concentrations of an inert exudate in order to complex Pb [87]. This could explain why the Pb that was reintroduced into the aqueous phase was not re-adsorbed or internalized by algae.

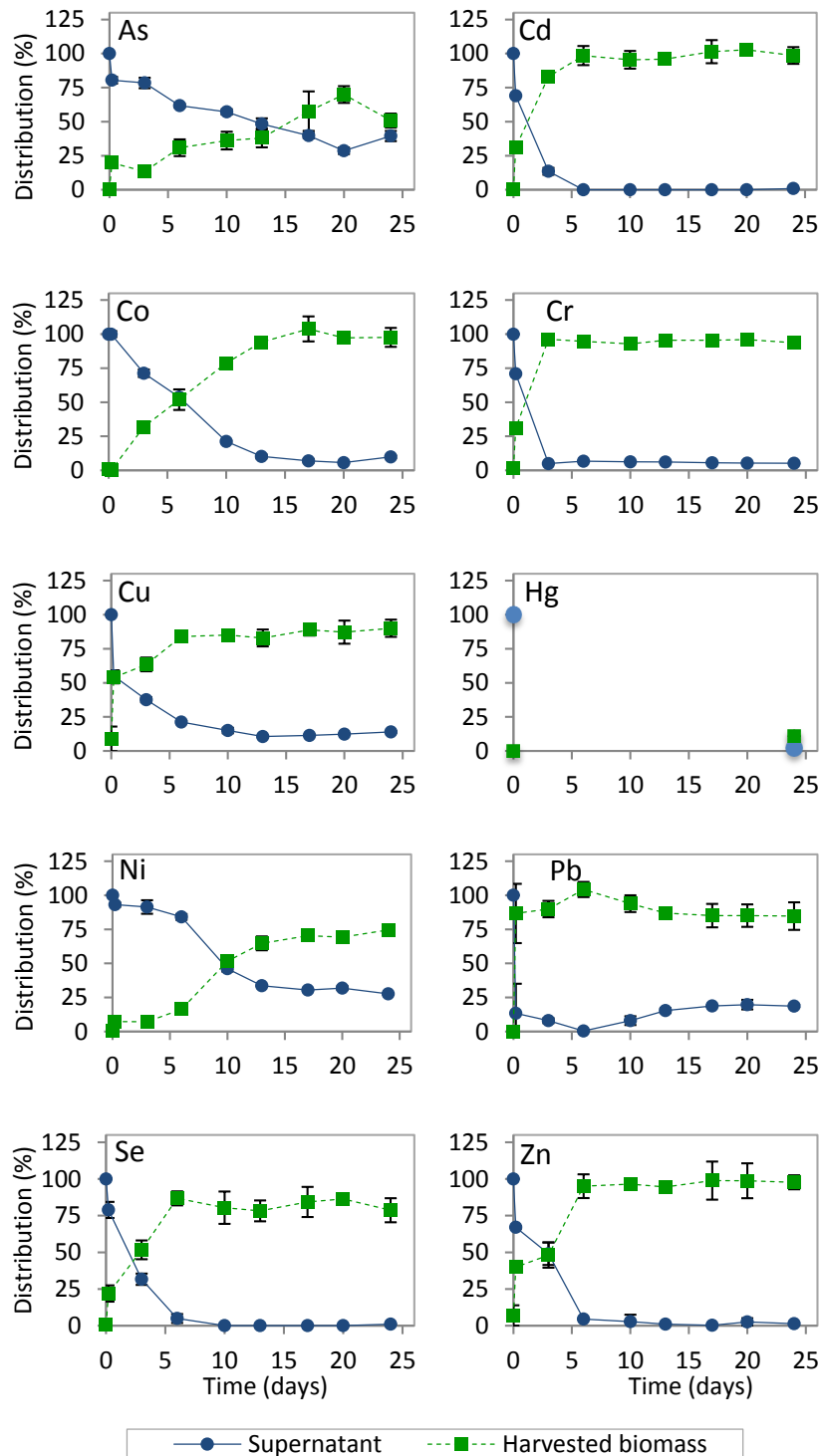


Figure 8. Temporal distribution of heavy metal in the medium and the biomass for 1X experiment. Data points shown are the average of three replicates and error bars indicate \pm one standard deviation.

2.4.1.4 Speciation Modeling and Metal Interactions. Heavy metals can interact with each other and with medium component to form new compounds, thus changing the interaction between algae and metals. Chemical equilibrium models serve in the prediction of such new compounds after a system has reached chemical equilibrium. For the present study, the chemical equilibrium model MINEQL® was used (see speciation in Appendix D). The prediction indicates that besides forming the metal ion specie most heavy metals (except anions As, Se and Cr), have the potential to form new complexes at equilibrium. The complexes predicted are dissolved both, dissolved molecules (charged and uncharged) and solid precipitates. Generally it is considered in that only ionic species could interact with cells; however latest research have shown that larger complexes charged or uncharged can also adsorb to cell walls as a result of their complexation with organic matter or can be taken up inside the cell through more complex uptake channels such as phosphate channels, citrate channel and glycoporins. Formation of metal precipitate is predicted for some heavy metals (Cd, Zn, Cu and Pb), and therefore there is a chance that after centrifugation biomass could contain these precipitates. However, all the chemical species predicted by the software not necessarily are formed as there are several kinetically driven processes (*e.g.* algae uptake and excretion, adsorption) that compete for the same metals during solid nucleation. More studies are needed to further understand the exact nature of the chemical species in the algal system with heavy metals. As far as is concerned in this study, the focus will be put on the quantification of the metal as speciation was out of the scope.

2.4.2 Distribution of Heavy Metals in the Algal Biomass

In the previous section it was shown that harvested biomass was the primary sink for heavy metals introduced from flue gas. The heavy metals associated with the harvested biomass can further be differentiated as: i) fraction removable by EDTA washing and ii) fraction nonremovable by EDTA washing.

2.4.2.1 Cell surface-bound and internalized cationic heavy metals. Figure 9 shows the portion of the heavy metals that was removed by EDTA solution and the portion that was not removed by EDTA solution (100% represents heavy metals initially added to the medium). The EDTA-removable fraction is defined as the fraction of cationic heavy metals (Cd, Co, Cu, Ni, Pb and Zn) that can be desorbed from the algal cell wall [61, 88]. Attachment to the cell surface was an important cationic sink only at the very beginning of the experiment. For example, the samples representing the five hour time period reached the peak (representing highest percent cell surface bound) of 100% for Pb, 41% for Cu, 31% for Zn and 27% for Cd (or Pb>Cu>Zn>Cd>Co>Ni). The peaks occurred much later for Co and Ni and were observed on day 6 and 13 for Co and Ni, respectively. A gradual decrease of this fraction was observed following this measurement and at their lowest points Cd was 0%, Zn was 0%, Cu was 0% and Pb was 25%.

The observed delay for Ni was likely related to competition with other ions. The behavior of Ni can be explained by the S-shaped curve produced by surface-bound Ni (see Ni in Figure 9), with the initial portion of the curve known as a lag period being the result of disadvantageous competition with other chemically analogous cations [89]. This lag period terminated when Zn and Cd were fully removed from the medium (see Cd and Zn in supernatant in Figure 8) and from the cell wall (see surface-bound Cd and Zn in Figure 9). Competition between Ni, Cd and Zn have been reported by Romera, González, Ballester, Blázquez and Muñoz [90] who exposed three algal species to individual and multimetal liquid medium containing either Ni, Zn, Cd, Ni with Zn or Ni with Cd. The multimetal experiments showed less Ni adsorption under the presence of competitors than the individual Ni experiments, thus indicating that adsorption of Ni is in a disadvantageous competition with Cd and Zn [90].

Other researchers have documented similar sorption behavior with regards to Cd, Co, Cu, Ni, Pb and Zn binding to the algal cell wall [5, 55, 91]. The pattern observed for the EDTA-removable fraction was partially analogous to the adsorption affinity reported in the literature for

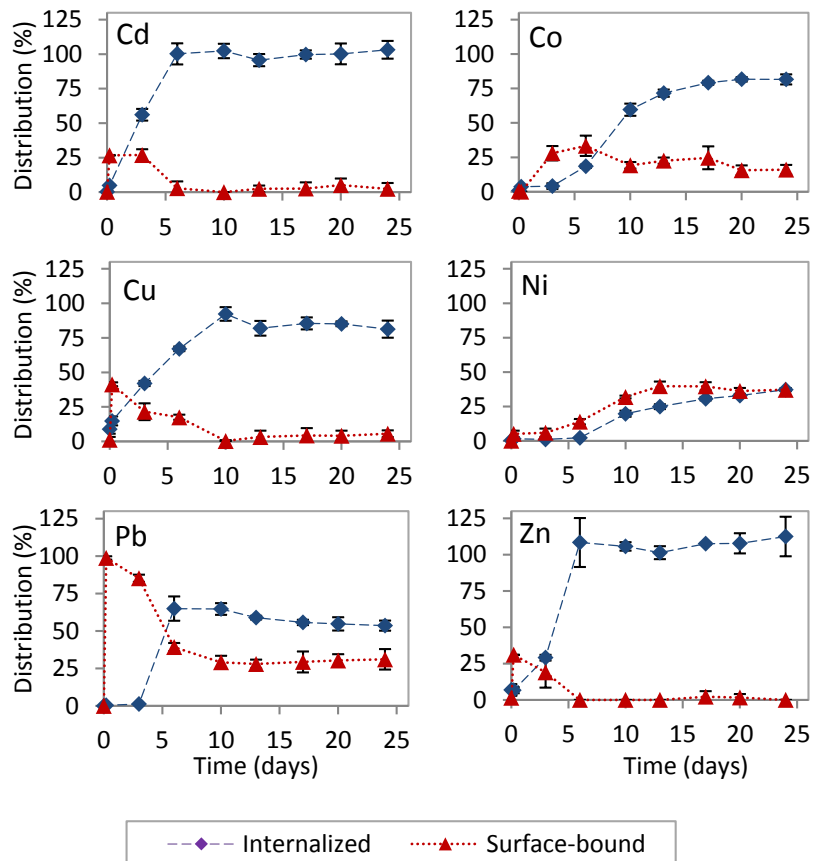


Figure 9. Temporal distribution of surface-bound and internalized cations in harvested biomass for 1X experiment. Data points shown are the average of three replicates and error bars indicate \pm one standard deviation.

algae: $Pb \gg Cu > Hg > Cd \geq Zn > Ni > Co$ [5]. This pattern has been suggested to be in part a consequence of the chemical electronegativity and the element radii size besides heavy metal concentration [5, 11, 53, 92, 93].

This sorption capacity is attributed to algae's surface to volume ratio and to its cell wall chemical composition [5]. Algal cell wall chemical composition varies among species, but generally green algae contains a mixture of functional groups that are involved in adsorption: hydroxyl (-OH), phosphoryl ($-PO_3O_2$), amino ($-NH_3$), carboxyl ($-COOH$) and sulphhydryl (-SH) [5, 53-55, 94]. Each functional group can either lose or gain protons depending on the pH of the

medium [5], thus the final charge of the cell is strongly dependent on the pH of the medium. Typically, the isoelectric point (zero charge) for algae occurs approximately between pH 3 and pH 4 [95, 96], hence in the algal PBR (maintained at neutral pH) algae were negatively charged, thus constituting a good sorbent biomaterial for the cationic heavy metals [5]. The sorptive process may involve several mechanisms occurring at the same time such as adsorption, ion exchange and electrostatic attractions [5, 43]. Regardless of the mechanism involved, it has been observed that the heavy metal sorption to algae is fast [11, 56, 57], which is in agreement with the results presented in this study.

The EDTA-nonremovable fraction is defined as the internalized fraction of cationic heavy metals (Cd, Co, Cu, Ni, Pb and Zn), in other words the fraction that is within the algal cell [61, 88]. The internalized portion was not an important sink for heavy metals in the initial stages of the experiment, but gradually represented the major fraction at the end of the 24-day study period. For example, during the first 5 hours only 5% Cd, 4% Co, 15% Cu, 2% Ni, 0% Pb and 7% Zn were internalized, which is considerably less than the EDTA-removable (surface-bound) fraction. This was followed by a gradual increase of the nonremovable (internalized) fraction and at its highest point Cd was 100%, Co was 82%, Cu was 92%, Ni was 40%, Pb was 65% and Zn was 100%.

This internalization process of heavy metals is facilitated by transporters (embedded in the cell wall) involved in nutrient transport [58]. Figure 10 summarizes some of the heavy metal transporters documented in the literature, with others still unknown [9, 58]. The routes of internalization of nutrients are not nutrient specific and most carriers accept molecules within a wide range of sizes and charges, thus leading to the transport of non-essential metals like Cd, Pb, etc. Thus, toxic heavy metals enter the cell by molecular mimicry of essential metals (due to some similarities in ionic radius and charge), by binding to low molecular weight thiols (*e.g.*, aminoacid transporters) and by endocytosis [41, 59]. Due to sharing of the same transport carriers between several metals (Figure 10), competition for internalization between them is expected. The author

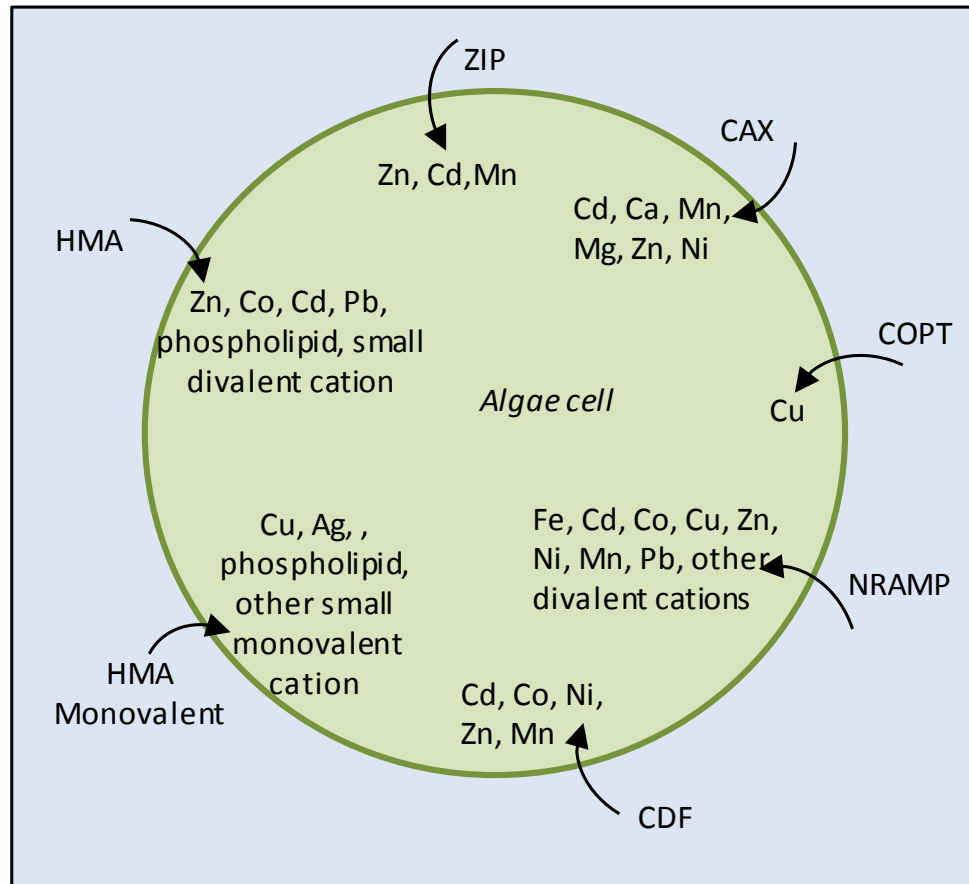


Figure 10. Schematic representation of the mechanisms for heavy metal internalization. Cation Diffusion Facilitators (CDF), Zrt-Irt-like Proteins (ZIP), Cation Exchangers (CAX), Copper Transporters (COPT), Heavy Metal P-type ATPases (HMA), Natural Resistance-Associated Macrophage Proteins (NRAMP) and Multidrug Resistance-associated Protein (MRP) and ABC Transporter of the Mitochondria (ATM) and Heavy Metal Tolerance (HMT) subfamilies of the ATP Binding Cassette (ABC) transporters.

speculates that the unfavorable internalization competition of Ni with Zn and Cd observed in this study could be related to the competition for entry using mutual transporters for the three metals such as the Natural Resistance-Associated Macrophage Proteins (NRAMP) and Cation Diffusion Facilitators (CDF) (Figure 10).

This competition between ions may be dynamic because of the continuous change in the concentration of cell wall-bound ions due to internalization. After internalization of the ions with

highest affinities, it is expected that the freed cell wall binding sites will interact with the least competitive ions. This process probably continues until: the ions in the medium are depleted, until adsorption and uptake equilibrium is reached, or until complexation with organic ligands excreted by algae outcompete the cell wall binding sites.

2.4.2.2 EDTA-removable and EDTA-nonremovable Anionic Heavy Metals. Unlike the cationic heavy metals, the anionic heavy metals, namely, As, Se and Cr, cannot be chelated by EDTA, but they are known to be removed from cells by EDTA solution [97]. The results in this study indicate that the EDTA washing procedure did remove As, Cr and Se along with the cations. Since anions are not known to be chelated by EDTA from the cell walls; As, Cr and Se removed from this procedure are not described in terms of sorbed and internalized portions. Instead the data are reported here to document the occurrence of this removal. The extent of this removal for these three anions is shown in Figure 11 (100% represents heavy metals initially added to the medium). Both, the cationic fraction that was desorbed and the metalloid fraction that was removed, displayed similar trends and peaks. Peak removal rates for As was 18% , Cr was 16% and Se was 24% and occurred at 5 hours for As and at day 3 for Cr and Se. Similar to cations, the washing with EDTA on day 24 resulted in no anion removal. Adsorption of As, Se and Cr to algae have been reported previously in the literature [92, 98-101], but the mechanisms are not well understood. It is believed that the amino group ($-NH_3$, pK_a between 8.8 and 10.3 [102]) carrying a positive charge at pH medium of 7 binds the anionic heavy metals (As, Cr and Se) [53, 100].

2.4.2.3 Mathematical Modeling of the Heavy Metal Distribution. Khummongkol-Ting model [103], developed specifically for algae systems under growth conditions and under multimetal systems at pH 7, was used in this study. This model was found to only fit data for Cr and Cd but poorly predicted the other metals (see results in Appendix E). In fact, researchers from laboratories other than the developer lab have not reported a successful use of this equation.

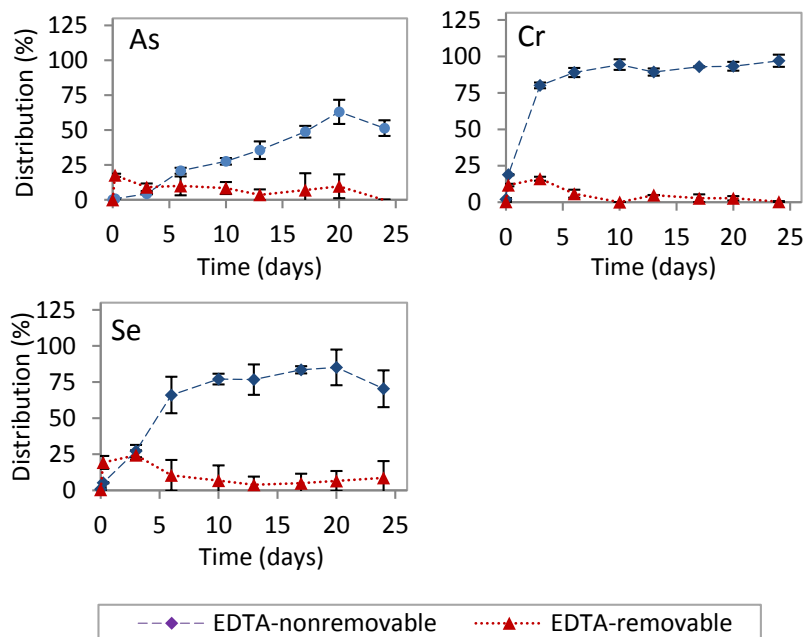


Figure 11. Temporal distribution of EDTA-removable and EDTA-nonremovable anions in harvested biomass for 1X experiment. Data points shown are the average of three replicates and error bars indicate \pm one standard deviation.

Several other kinetic and equilibrium (assuming pseudo equilibrium) models were used in the attempt to describe the experimental data (*e.i.* Langmuir, first order equation [89], reversible first-order kinetic model [89], intra-particle diffusion kinetic model, Fick's mass law, Elovich equation and Lagergren pseudo-second order kinetic model) with poor fitting for most of them. The only model that best fit the data was the empirical Lagergren pseudo-second order kinetic model (equation 1) when q_t represented the heavy metals contained in the whole biomass (internalized + adsorbed) (plot of $\frac{t}{Q}$ vs t should yield a straight line for best fit) (Figure 12).

$$\frac{t}{q_t} = \frac{1}{k} q_{eq}^2 + \frac{t}{q_{eq}} \quad (1)$$

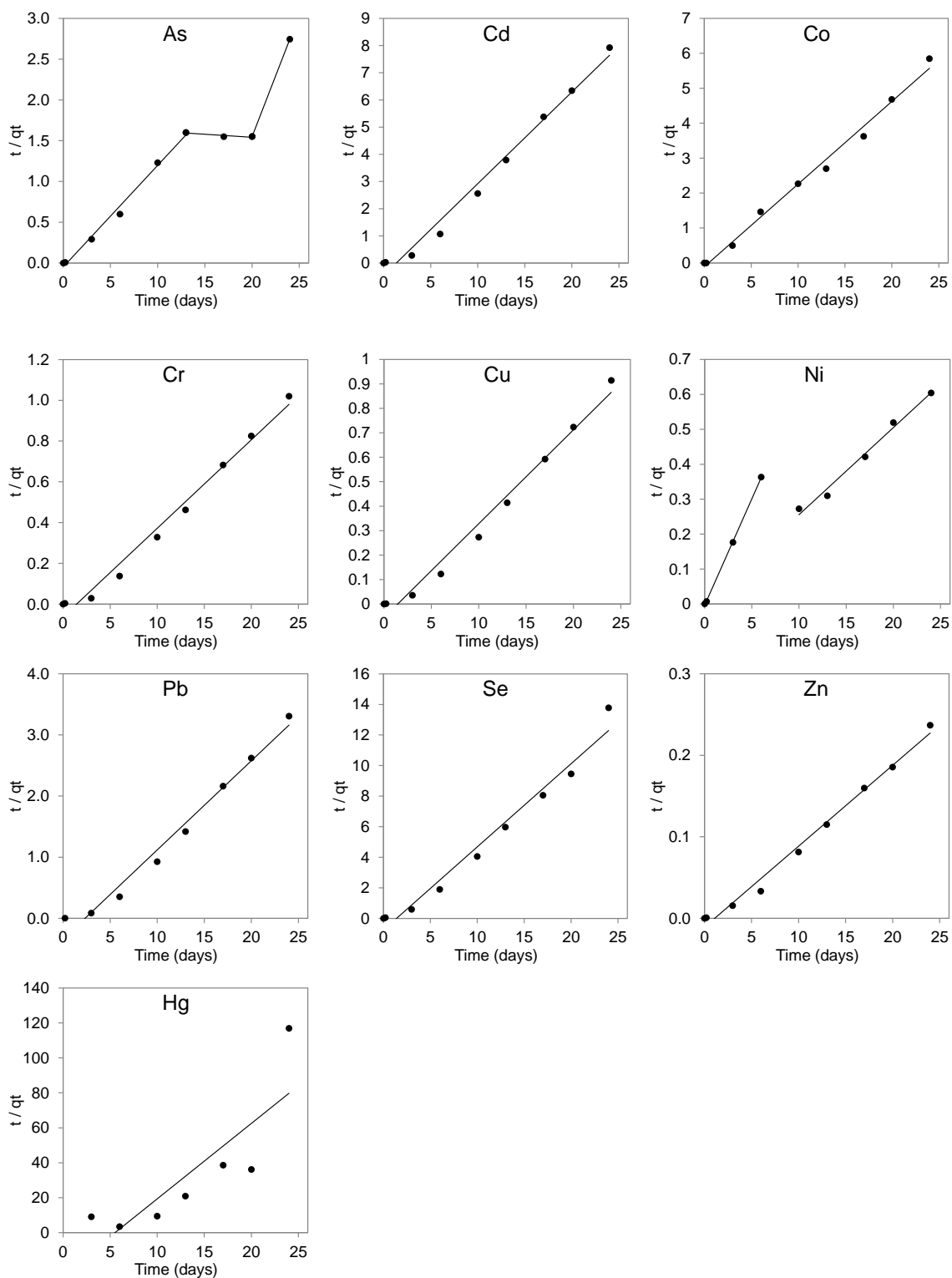


Figure 12. Linearized empirical pseudo-second order kinetic model for 1X experiment. Data points represent experimental data from 1X experiment.

where

q_t = amount of metal uptake at any time t (mg/kg)

q_{eq} = amount of uptake on algae at equilibrium (mg/kg)

k = rate constant of uptake (kg/mg.h)

t = time (days)

A common observation in water quality data is that the rate expression usually provides good fit for a range of data, as a consequence adjustments of the rate parameters need to be done to fit other ranges of data. Therefore, Ni and As had to be fitted using more than one equation (See As and Ni in Figure 12). The need for more than one equation could be a result of the several processes affecting the distribution of these heavy metals (*i.e.* excretion and disadvantageous ionic competitions) as detailed in sections 2.4.2.1 and 4.4.4, respectively. Hg did not have a good fit possibly due to volatilization (see section 2.4.1.2).

2.4.3 Effects of Heavy Metals on N Uptake, Biomass and Lipid Productivity

2.4.3.1 Change in Nitrogen Removal. Essential and non-essential ions within the cell can be toxic if the intracellular concentrations are not regulated by the cell [9]. These unregulated ions can produce reactive oxygen species (ROS) such as hydroxyl radical (HO \cdot), superoxide radical (O $_2\cdot^-$) or hydrogen peroxide (H $_2$ O $_2$) that cause oxidative damage to internal cell components [50]. To cope with this stress, several mechanisms are activated by the cell (Figure 13): (i) heavy metals excretion to maintain a lower concentration, (ii) oxidation state change to a less toxic form, (iii) precipitation of insoluble metal complexes, (iv) complexation of metal ions with metabolites (such as metallothioneins, GSH, proline, cysteine and others antioxidants and PCs), (v) vaporization and elimination and (vi) methylation [36, 41, 42, 58, 104]. Specifically, *Scenedesmus obliquus* are known to perform most of these mechanisms [38, 52, 82, 104-108]. These mechanisms need

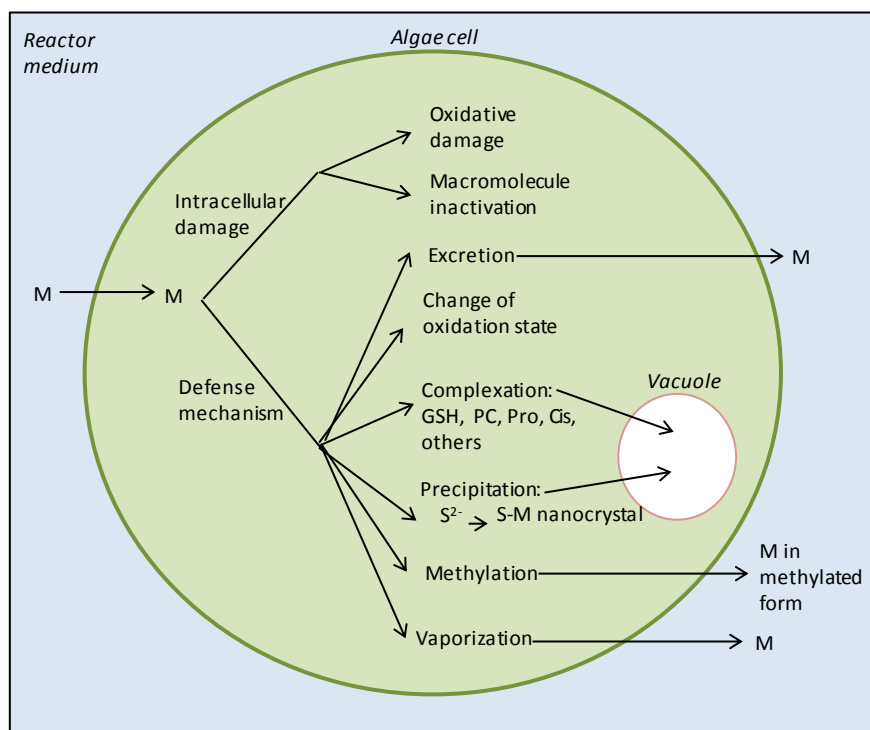


Figure 13. Heavy metal trafficking inside the algal cell. Heavy metal (M), glutathione (GSH), phytochelatin (PC), proline (Pro), cysteine (Cis) and sulfur (S).

nutrients to be performed (*e.g.* N, S), therefore the presence of heavy metals could impact nutrient uptake patterns.

Within the nutrients that form part of antioxidant molecules is N. Higher N consumption is reported in the literature for antioxidant production when algae are exposed to heavy metals [109] and this behavior was observed for *Scenedesmus obliquus* in this study as shown in Figure 14. PBRs exposed to 1X, 2X and 5X heavy metal concentration removed nitrate faster than the control although the control produced considerably higher biomass (5.9 g/L) than in the 2X (4.69 g/L) and 5X (2.62 g/L) experiments (see section 2.4.3.2). Experiments containing 10X were strongly inhibited after day 10, therefore its N consumption decreased. The rate of nitrate uptake (g NO₃⁻/kg algae · day) in Figure 15 shows a peak demand for nitrate occurring between day 6 and 13, with 10X concentration requiring the highest amount, followed by 5X, 2X, 1X and

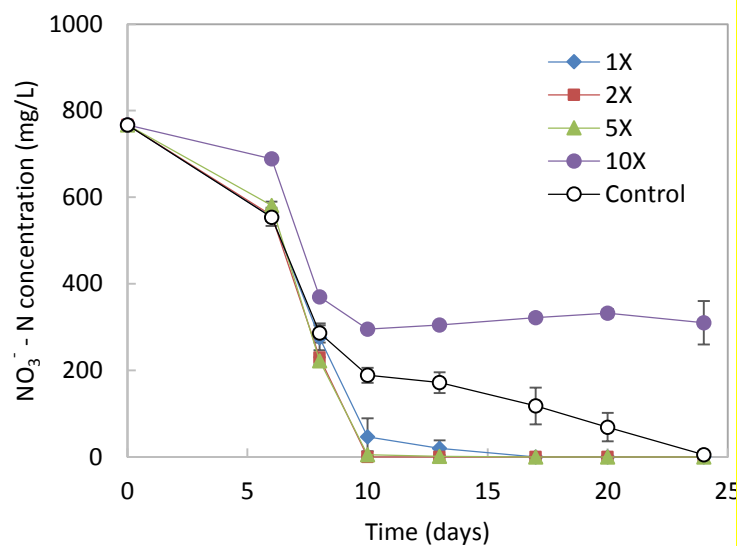


Figure 14. Nitrate concentration in medium . *Scenedesmus obliquus* exposed to 1X, 2X, 5X and 10X heavy metal concentrations for 24 days. Data points are average from three replicates and error bars indicate \pm one standard deviation.

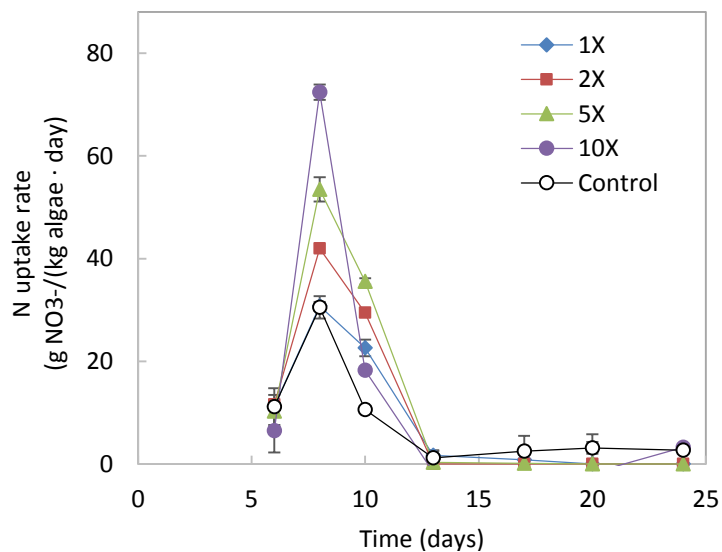


Figure 15. Rate of nitrate uptake *Scenedesmus obliquus* exposed to 1X, 2X, 5X and 10X heavy metal concentrations for 24 days. Data points are average from three replicates and error bars indicate \pm one standard deviation.

ultimately the control. This behavior is consistent with the increased nitrate uptake reported in the literature for green algae *Chlamydomonas reinhardtii* and photosynthetic diatom *Thalassiosira pseudonana* exposed to increasing levels of Cu, Co, Fe and Cd [109, 110].

2.4.3.2 Impact of Distribution of Heavy Metals on Biomass and Lipid Production. Figure 16 and Figure 17 show the effects of the four heavy metal concentrations on growth and lipid production (fatty acid methyl ester, FAME or biodiesel is produced by transesterification of lipids). Toxic effects was evident for PBR exposed to 2X, 5X and 10X affecting cell division and lipids accumulation. However, PBR exposed to 1X showed an increase in both growth and lipid accumulation.

Exposure to heavy metals triggers physiological and biochemical changes in algae that impact cell multiplication and lipid accumulation [15, 111, 112]. The results of this study indicates that over 2X concentration, higher concentration of heavy metals added to the medium produce stronger inhibition, but at 1X concentration the toxicity, if any, was bearable for *Scenedesmus obliquus* (Figure 18). It has been suggested that the extent of damage produced by heavy metals to the cell depends on the amount of heavy metals internalized [9, 113]. This statement agrees with our observations since the stronger inhibition is also directly related to higher cation internalization (Figure 18) (anions were not included as they are not called internalized in this study). From the point of view of biodiesel production, two zones can be identified in Figure 18: Unfavorable zone for production (yields are lower than the control) and favorable zone (yields are higher than the control).

In the favorable zone, oxidative stress could have enhanced biodiesel yields due to ROS-induced lipid accumulation. ROS are free radicals that are natural by-products of aerobic metabolism and are mainly produced in the chloroplast [41, 114]. ROS molecules are very reactive and rapidly attack intracellular biomolecules. This is the reason why the cell produces several ROS scavengers antioxidants (*e.g.*, β -carotene, tocopherols, etc.) [114-116]. It has been suggested that the

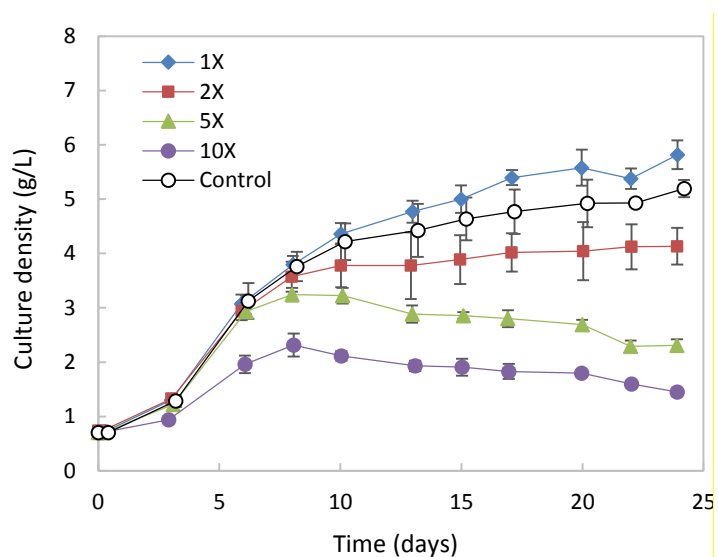


Figure 16. Algal biomass yield. *Scenedesmus obliquus* exposed to 1X, 2X, 5X and 10X heavy metal concentrations for 24 days. Data points are average from three replicates and error bars indicate \pm one standard deviation. Jittered data points to show separate error bars.

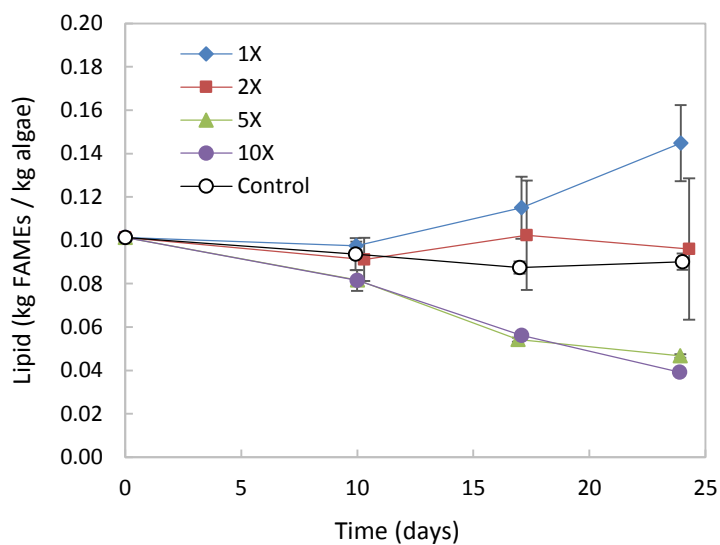


Figure 17. Lipid yield. *Scenedesmus obliquus* exposed to 1X, 2X, 5X and 10X heavy metal concentrations for 24 days. Data points are average from three replicates and error bars indicate \pm one standard deviation. Jittered data points to show separate error bars.

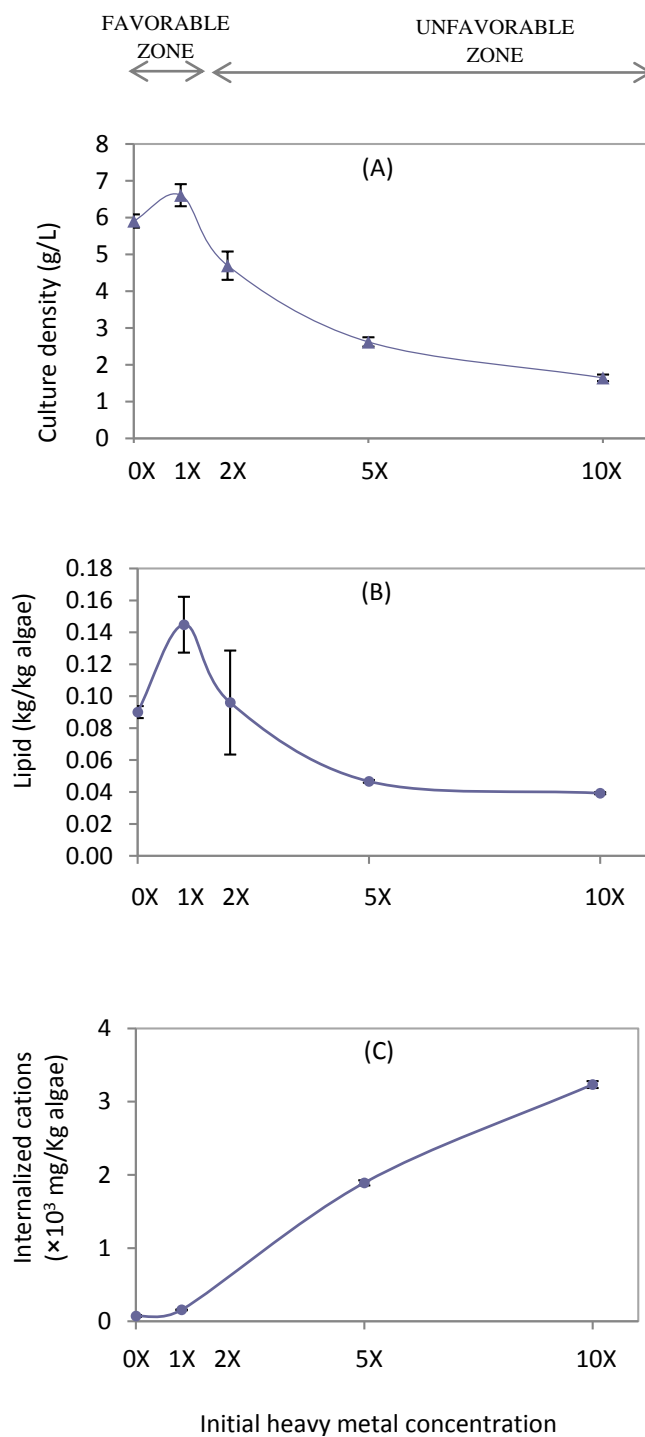


Figure 18. Dose response curve. *Scenedesmus obliquus* exposed to 1X, 2X, 5X and 10X heavy metal concentrations for 24 days. Algal growth (A). Specific lipid yield (B). Internalized cations (C). Data points are average from three replicates and error bars indicate \pm one standard deviation.

production of larger amount of lipids under ROS stress is a protective mechanism, where over-produced cytoplasmic lipid droplets serve as a sink to sequester ROS-scavenger molecules, thus accelerating scavenger molecules biosynthesis [115, 117].

Since heavy metals exacerbates ROS accumulation either through the catalysis of the Fenton reaction by redox-active Co(II), Cr(VI), Cu(II) and Cd(II); or through the consumption of the antioxidant pool by non-redox-active heavy metals such as As(III), As(V), Zn(II) and Pb(II) [9, 50, 116, 118], it can be concluded that heavy metals could directly induce lipid accumulation observed in 1X experiment. Enhanced lipid accumulation in algae due to heavy metal stress has also been observed in other strains such as *Chlorella vulgaris* and *Euglena gracilis* exposed to Cd, Fe, Cu or Zn [112, 119-123].

ROS is also produced by nitrogen starvation, hence the increase of lipids in 1X could have also been the result of the heavy metal-induced nitrogen depletion discussed in section 2.4.3.1. Nitrogen starvation has been demonstrated to enhance lipid accumulation [6, 121, 124] and recently N starvation has been linked to ROS production [118].

In the favorable zone, the increase in biomass could have been a result of cell overprotection. Increase in growth when algae is exposed to very low levels of toxins (as is the case in the favorable zone) has also been related to the algae adaptation to elevated stress levels producing an overcompensation effect generated by the homeostatic regulatory system in the algae that leads to the activation of metabolic and antioxidant production mechanisms to overcome toxicity (also called hormesis) [125]. For example, an increase in the growth for *Selenastrum capricornutum* and *Euglena gracilis* exposed to heavy metals (Zn, Cd or Pb) was documented to be a manifestation of hormesis [120, 126]. Some heavy metals could have enhanced algal growth by acting as nutrient, thus providing an additional nutrient source to the already existing sources in the medium (*e.g.*, Cu is part of cytochrome oxidase and amino oxidase, Cu and Zn are cofactors in enzymes and are essential for mitochondrial and chloroplast functions) [41-43, 58, 63].

Growth inhibition and reduced lipid production observed in the unfavorable zone could be explained as some combination of detrimental effects induced by heavy metal stress (*i.e.*, cell division disruption, inactivation of proteins, disorganization of chloroplast and mitochondrial structures, chloroplast envelop rupture, deterioration of membrane integrity and lysis) [15, 16, 113, 127, 128]. Specifically, strong impairment of the chloroplast (organelle involved in CO₂ fixation) and disturbance of the endoplasmic reticulum (organelle involved in lipid synthesis) are reported to be affected as a result of heavy metal induced stress [16, 120, 128, 129].

If a PBR needs to operate in the unfavorable zone, heavy metal distribution and their bioavailability should be changed. Examples that can help achieve this objective include; addition of competitors that inhibit adsorption and intracellular transport of toxic elements (*e.g.*, Ca, Mg, P) [127, 130], addition of nutrients that increase the generation of antioxidants (*e.g.*, N and S), change in pH which in turn will change the speciation of heavy metals and addition of heavy metal chelators and precipitation/complexation agents such as the ones found in saline waters [131].

2.5 Conclusions

This chapter evaluated the distribution of heavy metals from flue gas in an algal cultivation system and determined the effects that this distribution have over biomass and lipid yields. Some of the key conclusion of this chapter are:

- Heavy metals from flue gas interact with the algal cell and the PBR environment, producing a time-dependent change in the distribution of heavy metals. During the early stage of the study, algal interaction with cationic heavy metals (Cd, Co, Cu, Ni, Pb and Zn, except for Hg) results in the cell wall being the main sink; however, the internal part of the cell is the main sink at later stage.
- EDTA-removable anionic heavy metals (As, Cr and Se) decreased with time, whereas, the nonremovable fraction increased with time.

- Large percentage of Hg and a small percentage of Se were lost from the algal cultivation system at the three concentrations tested.
- Inhibition of Ni sorption and internalization was observed in the initial stage. It was overcome as soon as competitor ions (Cd and Zn) were removed from the medium and the algal cell wall.
- Heavy metals associated with the biomass affected production of biomass and lipid in both favorable and unfavorable ways. Biomass and lipid yield reduction was observed at higher internalized heavy metal concentrations; however, at the lowest internalized concentration the biomass and lipid production were enhanced. It is plausible that the enhancements observed were driven by ROS, whose production is exacerbated by heavy metal stress.

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CHAPTER 3

**POTENTIAL USES OF ALGAL BIOMASS AND MEDIUM CONTAMINATED WITH
HEAVY METALS FROM FLUE GAS**

Abstract

Algae cultivation integrated with coal-based flue gas capture is a promising alliance to obtain revenues from algae-based products while recycling CO₂, a greenhouse gas. However, the feasibility of use of such products can be affected by the heavy metals introduced along with combustion gasses. This chapter determines the contamination levels in medium and biomass grown in photobioreactors exposed to 10 heavy metals from coal-based flue gas at a base concentration (1X) likely to come from flue gas and at 5 fold (5X) and 10 fold (10X) to assess for medium enrichment with heavy metals resulting from recycling of the medium after each harvest. *Scenedesmus obliquus* showed large capacity for removal of heavy metals introduced from flue gas with exception of As, which has high likelihood to remain in the medium and accumulate in the recycled medium. After algae production exposed to 1X concentration, the medium complied with irrigation water recommendations; but did not comply with EPA aquatic life and human health national recommended criteria (without considering dilution and mixing lengths). The biomass harvested contained heavy metals levels that exceeded direct human consumption and fishfood standards, but were under poultry and cattle feedstuff, compostable plastic, paper and biofertilizer standards. Removal of heavy metals from the biomass was evaluated using deionized water, EDTA, hexane, methanol and acidified methanol, common solvents that are used by the food and biodiesel industries. The implication of using heavy metal contaminated biomass as biofuel feedstock is further discussed.

3.1 Introduction

In response to the needs for lessening global climate change, several new regulations (*i.e.*, subsidies, carbon tax and carbon trading) for abating carbon emission from industrial point sources are being put in place in several countries [1, 2]. This has created a new carbon market that allows several CO₂ carbon trapping technologies to compete, being one of them the photosynthetic capture of CO₂ [3]. Photosynthesis-based technologies use sun light to reduce inorganic C present in CO₂ to organic carbon conforming the biomass. Between the several photosynthetic-based options (*i.e.*, forestation, ocean fertilization and photosynthetic algae cultivation), algae can fix CO₂ using solar energy 10 times more efficiently than terrestrial plants thus leading to higher biomass productivity [3].

In algae, the organic carbon represents between 36 to 58% of the biomass [4] and the lipids can constitute up to 75% of the dry biomass [5, 6]. Consequently algae have an oil areal productivity of 136 900 L/ha·year, which is considerably larger than other important oil-crops such as palm (5366 L/ha·year), canola (974 L/ha·year), soybean (636 L/ha·year) and corn (172 L/ha·year) [5]. This high productivity, together with the advantages of a year round cultivation, use of non-agricultural land, high solar energy capture efficiency, integration with wastewater treatment, use of seawater, produced water, saline water and some types of industrial wastewater, give algae a competitive advantage over other terrestrial crops [5, 7-14]. Consequently, algae have gained importance as a promising feedstock for the production of renewable biodiesel and as foodstuff due to their protein, antioxidant and essential fatty acid content [8]. All these advantages make algae a doubly attractive option because it can provide CO₂ pollution remediation and biomass utilization.

Techno economic analyses for the scale up of algae production often assumes integration with flue gas-producing industries, mainly power plants [15-17]. But besides CO₂, flue gas also introduces heavy metals in the growth system. The unforeseen introduction of heavy metals could

be detrimental to the end use of the biomass and medium, having the potential to limit algal-based products and by-products usage due to concerns about heavy metals adverse effects on human health and the environment in the form of carcinogenic, teratogenic and mutagenic effects [18, 19]. Depending on the level of contamination it could be necessary to treat the contaminated meal and water prior to their discharge to the environment.

Often, studies neglect to include the impact of heavy metals in their assessments. Therefore published literature reviewed describing the levels of biomass contamination due to coal-based flue gas is lacking. The literature available for other fuel-based flue gasses besides coal show that flue gas introduction in ponds and photobioreactors delivers a wide range of heavy metal concentrations into the growth system [20-23]. The objective of this chapter is to determine if the heavy metals transferred from coal-based flue gas will limit algal biomass and medium commercial uses. The cultivation conditions chosen resemble potential scale-up conditions and the levels of heavy metal concentrations used conservatively assumes the highest transference expected (see Appendix B for calculations). This study also preliminarily screens if EDTA and solvents commonly used in the food and biodiesel industry (*i.e.*, methanol, acidified methanol and hexane) remove heavy metals from biomass; thus lessening their contamination level.

3.2 Literature Review

Some uses of the spent medium and algal biomass after harvesting are described in the following sections.

3.2.1 Algal Medium

Depending on the specie, the liquid medium to grow algae can range from freshwater, brackish water, saline water to ocean seawater. The medium must contain macronutrients (N, P, K, S), micronutrients (*e.g.*, Mg, Fe, Co, Zn, etc.) and inorganic carbon (H_2CO_3) that are needed by algae in order to grow. These nutrients are partially present in these types of waters, but addition

of fertilizers is almost always required in order to provide the adequate level of nutrients for maximum biomass productivity. Under equilibrium conditions, small amounts of CO₂ exist in water, causing a rise in pH after its consumption by algae. Therefore, enriched sources of CO₂ (*e.g.*, pure CO₂ gas or flue gas containing between 10-20% CO₂ v/v) needs to be bubbled through the medium in order to replenish the lost carbon [3]. The medium remaining after biomass harvesting is expected to be low in nutrients and can be disposed to the environment or can serve again as medium after nutrient enrichment.

At laboratory scale, the medium left after algae harvesting is small, but on a commercial scale it can be significant [18]. In fact, vast quantities of water will be required for commercial scale production of algae. If the productivities reported in Chapter II are used, the smallest economical biodiesel plant (5 million gallons of biodiesel/year capacity [24]) is projected to discharge 14.3 million gallons of spent medium per day. This volume is equivalent to the current influents in the Logan, UT wastewater treatment plant. As reported in [25], Yang et al. estimated that in a commercial biodiesel plant 3018 kg water/kg biodiesel of water footprint would be discharged after algae harvest; therefore a 5 million gallons of biodiesel/year capacity plant could potentially discharge around 38 million gallons water per day, about 3 times the current influents in the Logan, UT wastewater treatment plant. With such large volumes, it is expected that this water must be, for water conservation and economic reasons, recycled after harvesting, to either grow more algae or to be used in activities such as crop irrigation, aquaculture or for recreational purposes [26].

3.2.2 Algal Biomass

3.2.2.1 *Algae for the Food and the Animal Feed Industry.* Whole algae and lipid extracted algae (LEA) can be used as food and feed. Below, several uses widely proposed in techno-economic analysis are described.

Human: For human consumption whole algal biomass is sold mostly as pills or capsules, but not as whole biomass because it is not appealing to the consumer. This refusal towards eating algae is because of the consistency of fresh algae (slimy when wet and hard when dry), the slight fishy smell and the palatability (bitter taste with forage-like flavor) [8, 27, 28].

Fish: Whole dry or wet algae are used as food for fish, bivalves, mollusks and shrimp, or supplement in their foodstuff with improved results [8]. For instance, salmon and trout obtain the characteristic red color desired in the fish meat when fed with algae [27]. Nile tilapia fed with up to 50% dry weight algae *Chlorella* spp. and *Scenedesmus* spp. showed increase in body weight and protein content [29]. Algae remnants after biodiesel production can also be used as protein feedstuff. For example, whole algae *Tetraselmis* and *Nanofrustulum* and their lipid extracted biomass (obtained after hexane extraction method) were fed as protein replacement to Atlantic salmon (up to 10%), common carp (up to 40%) and white leg shrimp (up to 40%) without showing statistical difference with the control without algae [30].

Poultry: Algae are fed to poultry as a partial protein replacement and as a supplement to improve skin color and egg yolk quality [8, 27, 31]. Laying hens fed with algae produced eggs with reduced cholesterol (10%), 2.4 fold higher carotenoid content and increased linoleic and arachidonic acid levels (24 and 29%, respectively) [32]. Birds and chicken fed with a suspension containing *Chlorella* spp. increased their weight by 10 - 25% [33]. Algae can also supplement Se in chicken's diet. Se is a nutrient that helps feathering, improves cellular integrity of the meat, thus reducing water loss during handling, storage and cooking (indicator of a poor meat quality) [34]. Se is commonly supplemented in poultry diet by addition of inorganic selenite, selenate and Se-enriched yeast; however Se-enriched algae is a good replacement for these traditional sources and have a better bioavailability than the inorganic forms used [35, 36].

Cattle: Algae-fed cattle have shown positive results on cattle weight gain and milk production. Chowdhury, Huque, Khatum and Quamrun [37] fed *Chlorella* and *Scenedesmus* algal

suspensions to cattle for 120 days and observed higher increase in the daily weight gain in the algae-fed cattle than in the controls fed with sesame seed oil cake [37]. Cows fed with algae *Schizochytrium* sp. showed increase in essential fatty acid in milk (linoleic acid, docosahexaenoic acid-DHA and transvaccenic acid) [38, 39]. Kulpys, Paulauskas, Pilipavicius and Stankevicius [40] fed 200 g *Arthospira (Spirulina) platensis* to lactation cows daily for 90 days in addition to their normal diet. This author observed an 8 - 11% increase in body weight and 21% increase in milk production [40]. Currently the New Mexico State University and Texas A&M University have ongoing studies using LEA as feed for animal. As of now, they have reported that 60% of LEA in feed can be used without altering palatability [41-43].

3.2.2.2 *Algae as Biofertilizer*. Incorporation of organic matter (e.g., crop residues, leaves, roots, manure, compost, etc.) into soils is a common practice to improve water holding capacity and soil structure. Aquatic green algae (e.g., *Chlamydomonas*, *Clorococcum*, *Chlorella*, *Neochlorosis*, *Scenedesmus* and others) can also be used as soil conditioner or biofertilizer as they attach to soil walls and continue growing [44-46]. Once in the soil, a gelatinous capsule (proteoglycans) protects the organism from desiccation, heat and mechanical damage, and is responsible for improving soil characteristics [44]. For example, *Chlamydomonas* applied for 3 years to Quincy loamy sand parcels improved wet and dry aggregates stability, therefore the soil can better resist tillage, wind erosion, raindrop impact and water erosion [45]. Also, because of the higher soil porosity created by the stable aggregates, the soil can have better water infiltration and root aeration, hence improving soil agricultural potential. In another experiment, *Chlamydomonas Mexicana* was applied in Chihuahua desert and as a result the production of potatoes and cotton was increased by 5-15% while water usage was reduced by 35-40% [44].

Not only live algae, but also post-extracted algal residue can be used as biofertilizer because after oil extraction most of the nutrients and proteins are still present in the remaining biomass. For instance, Andrews [46] tested the potential plant available N supplementation from

feather meal, urea and LEA (hexane oil extraction method) in the production of sweet corn. This author found no statistical differences between these three treatments and concluded that algal biofertilizer is as effective in delivering N as the other two traditional N sources [46].

3.2.2.3 Algae for the Paper Industry. The paper industry uses wood and wastepaper to produce the pulp for paper production. Chipped wood is processed in a pulp mill, where this material is digested in acid solution, washed and bleached. Then, the wood pulp and wastepaper are mixed with water in order to produce a homogeneous slurry. For most paper types, fillers such as calcium carbonate and clays are added to give opacity and density (except for tissue paper) and also dyes and optical brighteners can be added to improve the paper appearance. The fiber slurry, at around 1% solids at this stage, then enters the paper machine where the paper sheet is formed, pressed, dewatered, coated, dried (to 96% solids) and wound to form the parent roll [47].

Algae can be added to the slurry to replace fibers or fillers with positive results. Ververis, Georghiou, Danielidis, Hatzinikolaou, Santas, Santas and Corleti [48] used an acid-digested mixed community of algae (*Chlorella*, *Scenedesmus*, cyanobacteria, diatoms and macro-algae) and produced tissue paper with enhanced breaking length, bursting stress and tearing resistance, possibly improved by the natural proteins present in algal biomass (*e.g.*, chitin) [48]. Hon-Nami and Kunito [49] used *Tetraselmis* sp. to produce handmade paper, which showed improved paper density, smoothness and good ink absorptivity. Shannon, Shi, Pelky, Besaw and Bernd [50] from Kimberly-Clark Corp. used dried *Spirulina* powder to partially replace eucalyptus in the fiber slurry. The author tested 6%, 12% and 18% replacement of total sheet and found that the sheet physical properties (bulk and specific absorption capacity) were not negatively impacted by algae addition [47, 50].

3.2.2.4 Algae for the Plastic Industry. Biopolymers such as starch, cellulose and proteins can be converted into biodegradable plastic (*e.g.*, polylactide, biodegradable acetyl cellulose and thermoplastic starch) and non-biodegradable plastic (*e.g.*, polyethylene, nylon 11 and non-

biodegradable acetyl cellulose) [51-53]. Bio-based plastics can be used to manufacture cutlery, toys, bottles, containers, straws, plant pots, drinking cups, phone casing, car interiors, plastic pipes, etc. They have the advantage to be environmentally friendly because they come from renewable sources (unlike fossil-fuel derived plastics). The bio-based biodegradable plastic helps to reduce landfill usage because it is degraded by enzymes and eventually gets converted into CO₂ and H₂O [53]. The bio-based non-biodegradable plastic allows semi-permanent fixation of CO₂ trapped in the biomass, thus retarding CO₂ emission [53-55].

Currently, mostly potato, corn, wheat, soybean, rice, canola and, at lesser rate, algae are being used to produce bio-based plastics. Algae have advantages over the other crops because algae produce lesser impact on food sources and does not need pre-treatment because of their small size [54-58]. Moreover, the undesirable green color of algae can be removed by chlorophyll bleaching (*e.g.*, chlorine-based and enzyme-based method) [56].

During algae-based plastic production several additives (*i.e.*, flexibilizers, plasticizers, colorants, biodegradable polyesters or non-biodegradable polyesters) are added besides algae [53, 54]. For example, Zhang, Kabeya, Kitagawa, Hirotsu, Yamashita and Otsuki [58] used *Chlorella* and PVC and found algae to be a suitable filler because it decreased the density of the resultant plastic (which reduces freight weight). The tensile strengths were suitable for rigid PVC (when algae was below 50%) and for plasticized PVC (when algae was below 20%) [58]. Zeller, Hunt, Jones and Sharma [54] produced plastics made of 100% algae (*Chlorella* or *Spirulina*) or made of a mixture of algae, polyethylene and glycerol blends. This author found that the formulation of 50% polyethylene, 37.5% *Spirulina* and 12.5% glycerol produced homogeneous blending and good mechanical properties [54]. Shi, Wideman and Wang [55] used up to 80% dry algae (*Nannochloropsis* and *Spirulina*), native cornstarch, hydroxypropylated corn starch, with non-biodegradable polymer (polylefin) to produce plastic films, fibers and injection molded articles. This author mentions that this blend is suitable for production of containers, building materials,

automobile parts, electrical apparatus, etc. and suggest this plastic serves for CO₂ capture [55, 56]. 40% *Chlorella* was used by Otsuki, Zhang, Kabeya and Hirotsu [57] to produce a plate and a disk-like moldings thermoplastic polyethylene [57]. Currently, Cereplast and Soley Biotechnology Institute commercializes algae-based plastic using LEA remnants from algal-biofuels and nutritional industries [59, 60]. For Cereplast's products, LEA currently replaces petro-based resins to up to 20% in their Biopropylene 109D® and to up to 51% in Biopropylene A150D® [60, 61]. Cereplast expects to replace 100% petro-based resins within five years [60, 61].

3.2.2.5 Algal as Biodiesel Feedstock. Biodiesel consists of a mixture of fatty acid methyl esters (FAME) that are produced by transesterification of algal lipids with alcohol. Figure 19 illustrates a transesterification reaction between an ester (algal triglyceride) and methanol (least expensive alcohol) to form a correspondent fatty acid methyl ester [14, 18, 62, 63]. This production technology can use either the lipids extracted from algae or can use the whole algal biomass (also known as *in-situ* transesterification) which is more desirable because it reduces the individual step for cell wall breakage [14, 18, 63]. Two conversion technologies for *in-situ* transesterification of algal oils will be discussed in this study: (i) Acid-catalyzed *in-situ* transesterification and (ii) supercritical methanol transesterification.

Acid-catalyzed *in-situ* transesterification: The acid-catalyzed *in-situ* transesterification uses sulfuric acid as catalyst. Whole freeze-dried algae, methanol and sulfuric acid are added to a reaction vessel and then heat is added for the transesterification to occur. Once the reaction vessel cools down, an organic solvent such as hexane or chloroform is added in order to recover the FAME from the polar alcohol phase. After recovery the crude biodiesel is purified [14, 18, 64, 65].

Supercritical methanol transesterification: At supercritical conditions (above critical point of 512.6 K and 8.09 MPa) methanol liquid and gas properties become identical [66]. This critical fluid has higher diffusion coefficients and lower viscosity than liquid methanol, therefore is able to diffuse better into a solid matrix and co-exist with oil, forming a single phase [63, 66, 67]. During

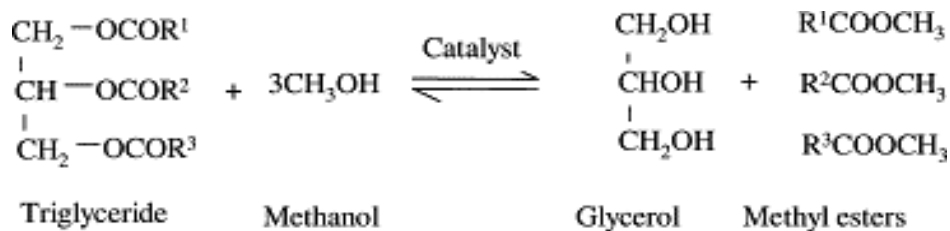


Figure 19. Overall reaction of biodiesel production by transesterification [12]

supercritical methanol transesterification, methanol simultaneously extracts the oils from the algal cell and convert them into biodiesel without the addition of acid catalyst [18, 66, 68]. Once the reaction vessel reaches room temperature, methanol becomes immiscible in biodiesel and can easily be separated [69].

3.3 Materials and Methods

Biomass production: *Scenedesmus obliquus* donated by APS was grown axenically on petri dishes in order to maintain strain purity. Colonies of algae from petri dishes were grown in 3 L polystyrene spinning bioreactors (Corning®) for 7 days until it reaches approximately 2.5 g/L (dry biomass per liter of culture). Light was supplied by cool fluorescent lamps (24/7) and pH was maintained at 7 by CO₂ injection. Biomass was harvested by centrifugation at (3900 RPM) for five minutes and washed twice with fresh medium in order to eliminate metal chelators excreted by algae to the old media (adapted from Bates, Tessier, Campbell and Buffle [70]). Washed algal biomass was re-suspended and added to airlift tube photobioreactors for cultivation with heavy metals.

Experimental photobioreactor (PBR) set-up: Airlift tube PBR were acid-rinsed overnight using 10% HNO₃ and then were rinsed thoroughly with deionized water. Then, the PBRs were autoclaved at 120°C for 30 minutes and were filled with APS medium without EDTA in order to reduce complexation with metals [71]. Heavy metal stock aliquots were conveniently added into

the medium in order to achieve the concentrations in Table 3 in Chapter III (for multimetal experiments) or to achieve 1.56 mg As/L (for individual 20X As experiment). After 5 hours of equilibration washed algal biomass was added to the bioreactors at an initial density of 0.8 g/L [70]. Growth measurements were taken during the course of the experiment by taking optical density (OD), which correlates with total suspended solids (TSS). pH was monitored frequently during the first day and daily until the end of the experiment. The calculation of the heavy metals concentration used in this study can be found in Appendix B.

Lipid transesterification and FAME analysis: Lipids in algae were quantified through transesterification of lipids into fatty acid methyl esters (FAME). *In situ* transesterification, a single-step reactive extraction method that combines the sequential extraction followed by transesterification was used. Frozen microalgal pellets from 45 mL samples were freeze-dried and ground into a powder using a mortar and pestle. A subsample of 24 mg of dried algae was transferred into a crimp top gas chromatograph vial containing 0.5 mL acidified methanol (5% H₂SO₄) and was digested for 90 min at 90°C. After digestion the vial was centrifuged and the acidified methanol containing the FAME was transferred into a 5 mL serum bottle containing 4 mL hexane. Complete recovery of FAMEs was achieved by rinsing the biomass with additional 1 mL hexane. The sealed serum bottle was then immersed in a water bath at 90°C for 15 min and cooled down to allow phase separation. The upper phase containing the hexane-FAME was pipetted out and analyzed by gas chromatograph (Agilent Technologies 7890A) using methyl ester standards purchased from Sigma-Aldrich.

Heavy metals sampling: 12 ml of unfiltered sample was aspirated from the PBR of which 5 ml was used for analyzing total heavy metal concentration in the algal suspension for all heavy metals (except Hg, where 30 ml sample was aspirated and 10 ml was used for analysis) and are reported as heavy metal concentration in the algal suspension. The remaining 7 ml (or 20 ml for Hg) was centrifuged at 7500 RPM for 3 minutes and the supernatant was analyzed for heavy metal

concentration and are reported as heavy metal concentration in the medium. The algal cell pellets were re-suspended in 0.1 M EDTA containing 0.08% w/w NaCl solution (to avoid lysis of cells due to hypotonic effect) at pH 7 for 10 minutes and centrifuged at 7500 RPM for 3 minutes to remove cationic metals (Cd, Co, Cu, Ni, Hg, Pb and Zn) that are surface-bound (EDTA only removes surface bound metals [72]). Intracellular heavy metal concentration was then analyzed from the washed algal cell pellet and are reported as internalized metals. The sequential extraction method described above is an operationally defined approach [70] and is shown in Figure 3. The non-cationic heavy metals (As, Se and Cr) present in the algal cell pellets were also measured and are reported here; however, they are not categorized as internalized metals. The supernatant from EDTA washing was also collected but was not analyzed due to formation of precipitates during analysis; therefore this fraction was obtained as the difference between the heavy metals in suspension, the medium and the EDTA non-removable fraction and is reported as EDTA-removable or surface-bound fraction.

Heavy metal desorption from fresh biomass using EDTA solution: Algae were harvested by centrifugation at 7500 RPM for 3 minutes. Then the pellet was washed with 0.1 M EDTA containing 0.08% w/w NaCl solution at pH 7 for 10 minutes. After 10 minutes, the sample was centrifuged at 7500 RPM for 3 minutes and the pellet was collected for analysis [70].

Heavy metal desorption from dead freeze dried biomass: The pellets harvested on day 24th were placed in a freezer (-80°C), freeze-dried (0.1 mBar at -50 °C overnight) and powdered. Approximately 20 mg of dry biomass was soaked with 15 mL of leachant solution for 60 minutes at room temperature under constant mixing in a shaker. The leachants used were deionized water, 0.1 M EDTA, methanol [73], hexane [74] or a solution of methanol with 5% H₂SO₄ [65]. After this process, the biomass was centrifuged, the supernatant was discarded and the biomass was rinsed with clean leachant (twice) in order to eliminate remaining leachate. The pellets were freeze-dried, digested and analyzed for 9 heavy metals (Hg was not analyzed due to large sample size

requirements). The unwashed biomass was also analyzed in order to obtain the percent removal of each solvent.

Total As, Cd, Co, Cr, Cu, Ni, Pb, Se and Zn analysis: Supernatant samples, algae pellets and algal suspension samples were collected in borosilicate test tubes and digested with HNO₃ at 105°C in a heating block, until biomass disappeared. Digested sample were transferred into a volumetric flask and adjusted to 5 or 10 mL using deionized water. Analysis was done by Inductively Coupled Plasma Mass Spectrometry (ICP-MS Agilent 7500 Series). Heavy metal standards were prepared the night before or the same day of the analysis using concentrated stocks of analytical grade.

Total Hg analysis: Supernatant, fresh biomass and algal suspension samples were digested following Standard Methods 3030E [75], EPA 7470A [76] and EPA 7471A [77] methods. Hg concentration was measured by cold-vapor atomic absorption procedure using Atomic Absorption Spectrometer (AA, PerkinElmer Analyst 800). Hg standards and SnCl₂ solution were prepared the same day of analysis using concentrated stocks standards and chemicals of analytical grade.

3.4 Results and Discussions

3.4.1 Heavy Metal Removal Capacity from the Medium

Figure 20 shows the concentration (in mg/L) of heavy metals before and after cultivation of *Scenedesmus obliquus* for three heavy metal concentrations (1X, 5X and 10X). In these figures it can be observed that larger amounts of Cd, Co, Cr, Cu, Ni, Pb, Se, Zn and Hg (except As) were removed by algae at increasing initial doses, indicating that the heavy metal binding sites in *Scenedesmus obliquus* were not saturated at 1X and 5X. Although 5X and 10X produced less biomass due to metal toxicity (Figure 16), that did not seem to be an impediment for a higher removal capacity. The percent removal for these nine metals (Figure 21) were over 62% even at the higher concentration. With this removal capacity, buildup of these nine elements in the recycled

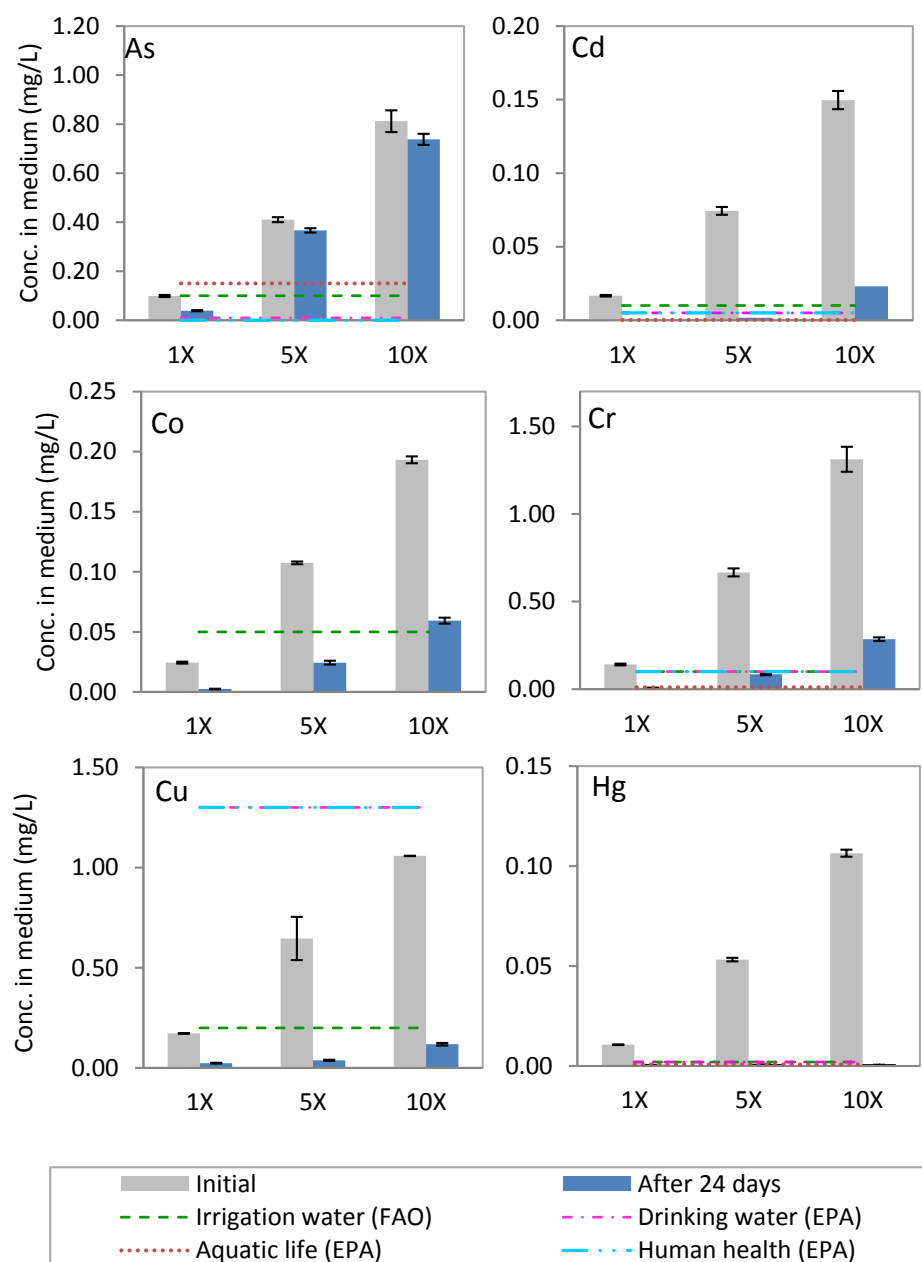


Figure 20. Heavy metal concentration in the medium after 24-day study period for 1X, 5X and 10X experiments. Broken lines represent the maximum allowable regulatory limits for the use of this medium in other activities. Data points are average from three replicates and error bars indicate \pm one standard deviation.

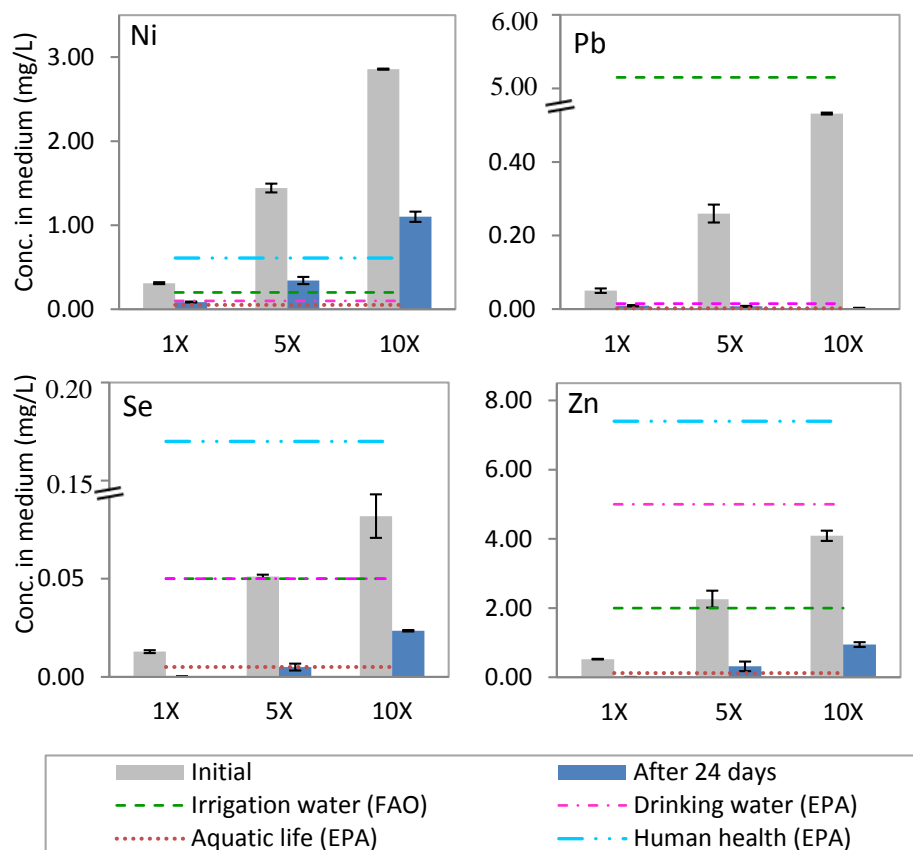


Figure 20 (continued). Heavy metal concentration in the medium after 24-day study period for 1X, 5X and 10X experiments. Broken lines represent the maximum allowable regulatory limits for the use of this medium in other activities. Data points are average from three replicates and error bars indicate \pm one standard deviation.

medium when using flue gas is less likely, even under the worst cultivation scenario of reduced biomass yields.

Contrary to these nine heavy metals, the amounts of As removed by algae did not increase with increasing initial dose (Figure 20), instead it was observed that percent As removal decreased (Figure 21). The concentration of As accumulated in the algal biomass was not statistically significant (ANOVA, $p < 0.05$) and reached in average 13.9 ± 6.1 mg/kg dry weight. This saturation for As bioaccumulation in *Scenedesmus obliquus* can affect the quality of the recycled medium,

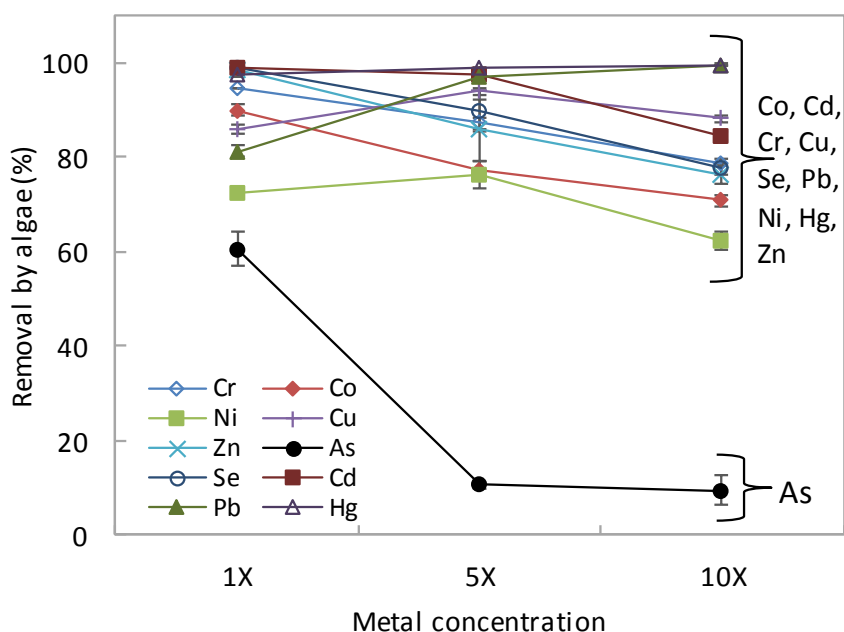


Figure 21. Percent removal of heavy metals by *Scenedesmus obliquus* for 1X, 5X and 10X experiments. Data points are average from three replicates and error bars indicate \pm one standard deviation.

especially because in a commercial algae setting the medium is expected to be reused for about 20 times before being discarded (personal communication [26]).

Limited bioremediation of As has been reported in the literature and suggest that algal defense mechanisms excretes As from the biomass towards the medium [78-81]. Moreover, the chances of As build-up in the recycled medium increase owing to the lack of As volatilization mechanisms by *Scenedesmus obliquus* [79, 81-85]. It is very likely that As can become a silent contaminant in this type of production system, especially because As does not inhibit biomass or lipid yields, not even at 1.56 mg As/L (20X the heavy metal reference concentration) (Figure 22 and Figure 23).

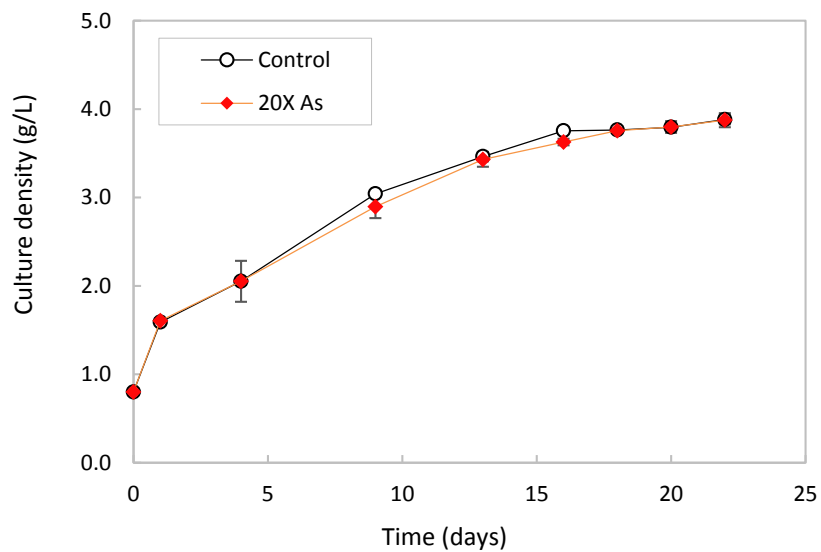


Figure 22. Growth of *Scenedesmus obliquus* under 20X As concentration. Data points are average from two replicates and error bars indicate \pm one standard deviation.

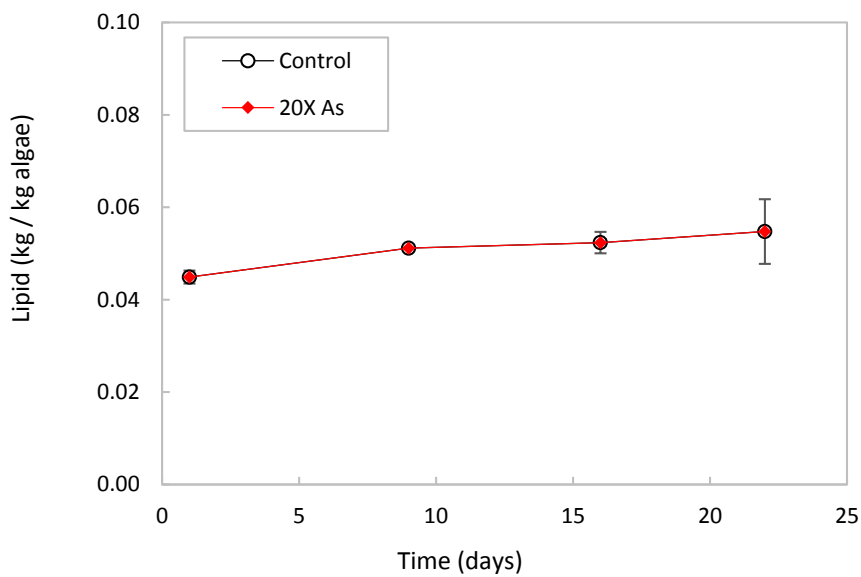


Figure 23. Lipid yield from *Scenedesmus obliquus* grown under 20 X As concentration. Data points are average from two replicates and error bars indicate \pm one standard deviation.

3.4.2 Effects of Cultivation Time on the Level of Medium Contamination

Because heavy metal bioaccumulation by algae is driven by time-dependent adsorption and internalization processes, the final cleanness of the medium depends on the cultivation time. For *Scenedesmus obliquus* grown using 1X heavy metal concentration, the length of the cultivation affected the quality of the medium as shown in Figure 24. Heavy metal Cd, Cr, Cu, Pb, Se and Zn were removed very fast and would not be a problem for medium quality since it is unlikely that harvesting occurs early during the growth. On the contrary Co, Ni, Cu and As were removed slower and could generate concern if the biomass is harvested earlier than the 24 days (for this study).

3.4.3 Potential Uses of the Spent Medium

3.4.3.1 Irrigation Water. If the spent medium is going to be used for irrigation or is going to be discharged in another water body, the concentrations of heavy metals need to comply with maximum contaminant regulations and recommendations. In this regard,

Figure 20 and Figure 24 compare the heavy metals concentrations found in the medium against the ceiling heavy metals concentration for irrigation water recommended by Ayers and Westcot [86] from the Food and Agriculture Organization of the United Nations (FAO) (see Appendix F) [86]. From this comparison it can be observed that for the 1X experiment all heavy metals concentrations in the medium were significantly lower than the recommended maximum limits for crop irrigation (Figure 20 and Figure 24). Final concentrations achieved in 5X and 10X PBR experiments exceeded the FAO recommendations (Figure 24).

3.4.3.2 Aquatic Life. Water discharged from any point source (any discernible, confined and discrete conveyance) into another water body typically requires a National Pollution Discharge Elimination System (NPDES) permit, with exception of return flow from irrigation agriculture. This permit is issued by government authorities who establish effluent limitations on quantity, discharge rate and concentration of pollutants at the point of discharge in order to meet the water

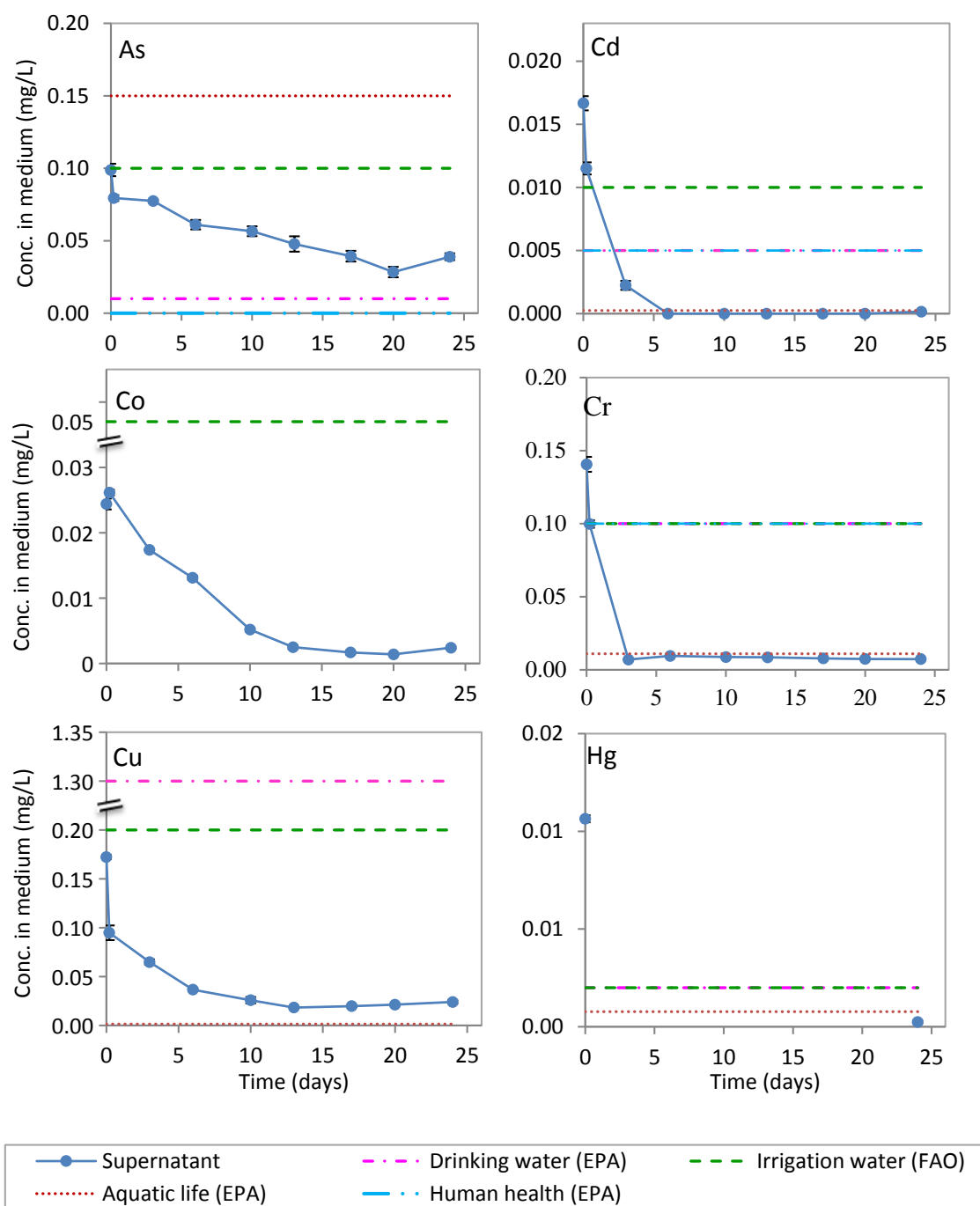


Figure 24. Temporality of heavy metal concentration in the medium for 1X experiment. Broken lines represent the maximum allowable regulatory limits for the use of this medium in other activities. Data points are average from three replicates and error bars indicate \pm one standard deviation.

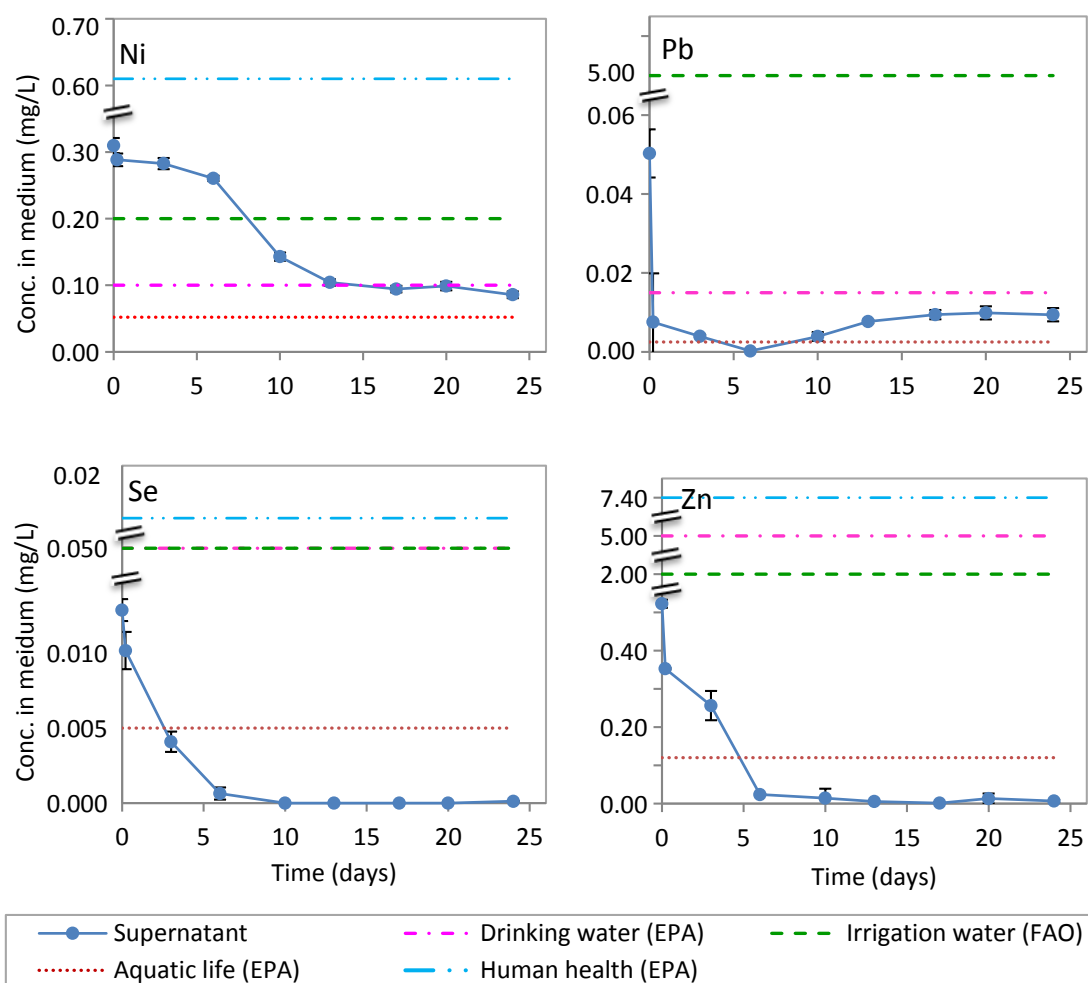


Figure 24 (continued). Heavy metal concentration in the medium for 1X experiment. Broken lines represent the maximum allowable regulatory limits for the use of this medium in other activities. Data points are average from three replicates and error bars indicate \pm one standard deviation

quality criteria for the receiving water. The water quality criteria are maximum limits of contaminants set to protect the ecological system, its organisms (*e.g.*, plankton, fish, shellfish, wildlife and plant life), aesthetics and recreation. The nationally recommended numeric criteria for aquatic life and human health is divided in Criterion Continuous Concentration (CCC) and Criterion Maximum Concentration (CMC) (see Appendix F). The CMC is the estimate of the highest concentration of the pollutant in surface water to which aquatic organisms can be exposed briefly without unacceptable effects, while the CCC (the most protective of both) is the

concentration to which aquatic organism can be exposed indefinitely without unacceptable effects. These criteria need to be met at the point of discharge whenever the water quality standard of the receiving water body does not allow considerations of dilution or mixing zones [87].

Figure 20 and Figure 24 compare the concentrations of heavy metals in the medium versus the CCC EPA aquatic life criterion for long term exposure in freshwater [88]. From these figures it can be observed that the concentrations of As, Cd, Cr, Hg and Se in 1X experiment were lower than the CCC while concentrations of Cu, Ni, Pb and Zn were higher (Cu was 16.6 times, Ni was 1.7 times and Pb was 3.8 times higher than the standard). This implies that after harvesting, the medium is not adequate for aquatic life that develops in a natural ecosystem. However, additional considerations of dilution and mixing lengths during NPDES calculation may allow the medium to be discharged in a water body without affecting aquatic life [87]. For 5X and 10X only Hg was below the standard, while all other heavy metals exceeded it.

3.4.3.3 Human Health. The EPA human health criterion (see Appendix F) establishes limits for contaminant concentration in water bodies in order to protect people that drink untreated surface water or eat fish, shellfish and wildlife grown in such contaminated water. With this criterion it is ensured that such consumption will not produce long-term risk to human health [88]. In this regard,

Figure 20 and Figure 24 compare the concentrations of heavy metals in the medium versus the EPA human health criterion. From Figure 24 it can be observed that the concentrations of Cd, Cr, Cu, Ni and Se for 1X experiment were lower than the standard, while only concentrations of As were 2162 times higher than the standard. For 5X only As exceeded the standard, For 10X, Ni, Cr, Cd and As exceeded. Overall, the spent medium exceeded US EPA standards for its consumption (for example during recreational activities such as swimming) and consumption of aquatic food grown in it. However, when the medium is discharged into another water body, the considerations of dilution and mixing lengths in the NPDES may allow compliance with the human health criterion.

In addition to these two standards, Figure 20 and Figure 24 also compare the heavy metal concentration in the medium to that of EPA drinking water standard [89]. In this regard, it can be observed that for 1X experiment Cd, Cr, Cu, Hg, Ni, Pb, Se and Zn were lower than the maximum contaminant levels (MCL) for drinking water standards. Only As was 3 times higher than the MCL for As (0.01 mg/L) enforced from 2006 onwards. The author does not imply that, with exception of As, this water is suitable for drinking purposes; but is confident that this comparison helps the reader to be aware of the level of danger that the heavy metals in the spent medium represent.

3.4.4 Heavy Metals Bioaccumulation in Biomass

Figure 25 shows the bioaccumulation of heavy metals by *Scenedesmus obliquus* at the end of the growing cycle (day 24 for this study) for three concentrations (1X, 5X and 10X). In this figure the concentration of heavy metals are presented in two portions: the EDTA-removable and the EDTA-nonremovable portion, except for Hg that shows the sum of both. This figure shows that high initial heavy metal doses lead to higher biomass contamination for most elements (with exception of As). For 1X, the heavy metals associated with the biomass were shown to be strongly bond to the biomass as washing with metal chelator, EDTA showed minimal impact on metal removal. Increases in the initial dose (5X and 10X) resulted in larger removable fraction. This suggests that higher input of heavy metals to the culture system (for example due to incomplete flue gas purification, use of already contaminated medium, increase in flue gas delivery rate, etc.) will directly impact the biomass quality in terms of contamination with toxic metals. In addition, the level of accumulation of metals in *S. obliquus* was comparable to the levels observed by other authors [20-22]. Hyperaccumulation of heavy metals was not observed in this strain (Appendix G).

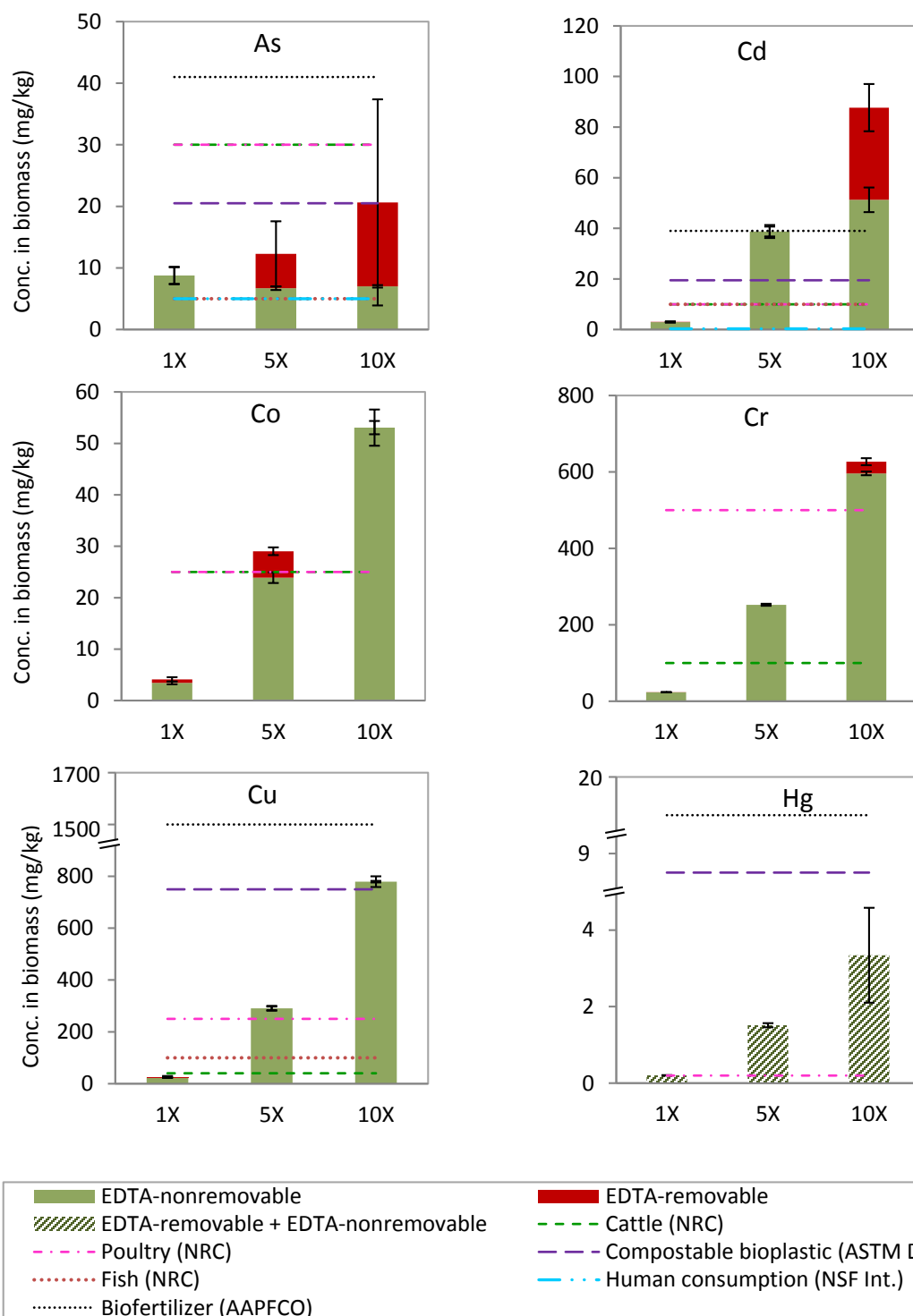


Figure 25. Heavy metal concentration in the harvested biomass after 24 days study period for 1X, 5X and 10X experiments. Data points are average from three replicates and error bars indicate \pm one standard deviation.

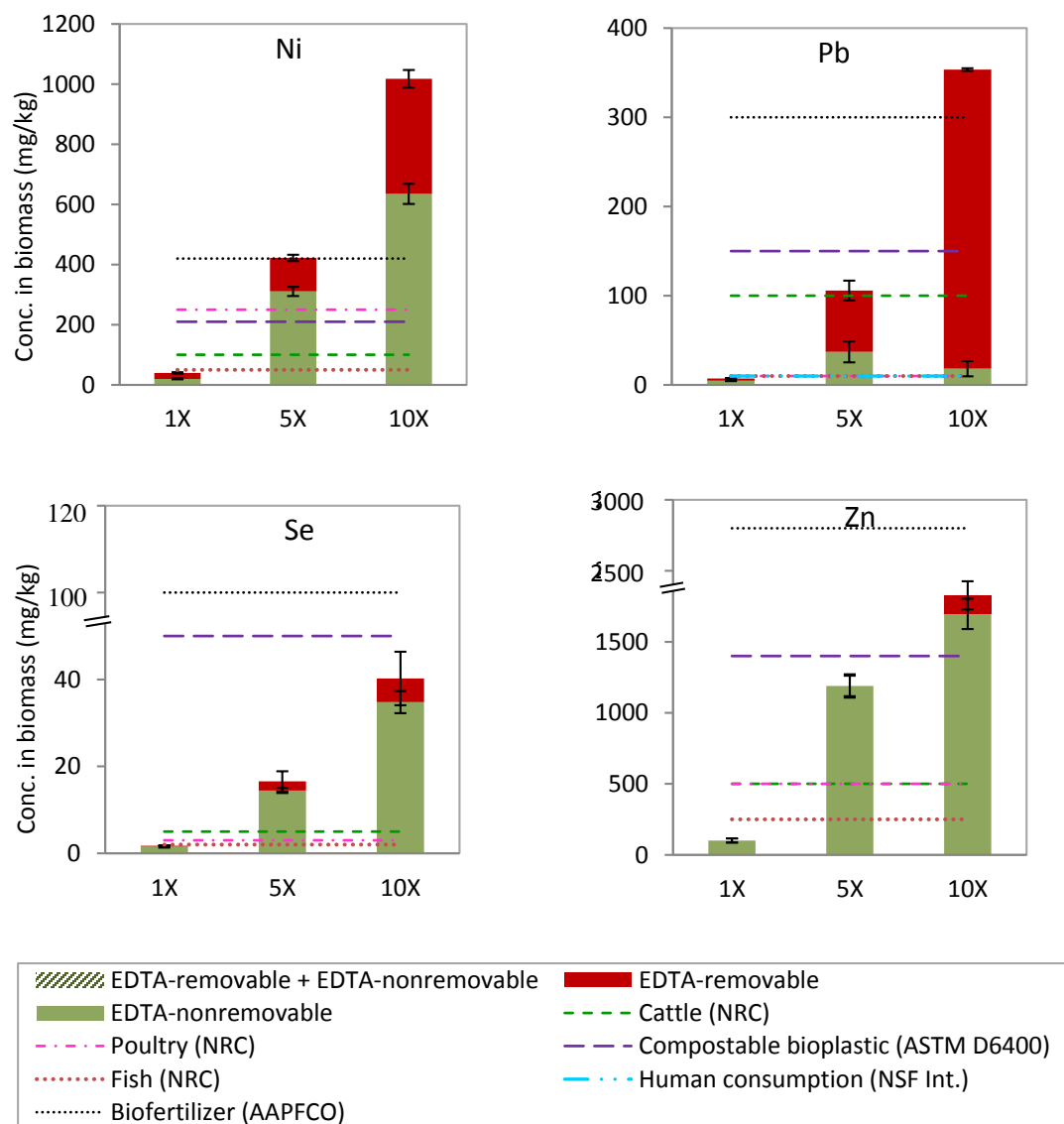


Figure 25 (continued). Heavy metal concentration in the biomass after 24 days study period for 1X, 5X and 10X experiments. Data points are average from three replicates and error bars indicate \pm one standard deviation.

3.4.5 Effect of Cultivation Time on the Level of Biomass Contamination

Figure 26 shows the temporal changes in heavy metals concentration in the biomass in mg/kg dry weight with the solid line representing the heavy metal concentration in the whole biomass harvested by centrifugation. In this figure the concentration of heavy metals initially rose

to a peak concentration (except Ni) and was followed by a drop in concentration. That drop is a result of cell multiplication, also called growth dilution and has been previously observed in algae and diatom [90-92]. For Ni, it was observed that higher peak concentrations were reached after several days, suggesting that the production of new cells did not produce growth dilution, but rather Ni enrichment. This possibly occurs due to presence of Ni in the medium even at the time the experiment was ended (see Figure 24 for Ni), therefore, constant replenishment of Ni to the new cell walls was ensured.

This result suggests that any changes in culture conditions that affect algal growth (*e.g.*, type of cultivation, light intensity, nutrient levels, cultivation period, etc.) will directly affect the final level of heavy metal contamination. In fact, the selection between open pond and PBR can become critical when using flue gas. In average, a PBR can reach up to 8 g/L biomass concentration while a raceway open pond can reach anywhere from 0.3 up to 1 g/L [93], hence the dilution of the initial peak concentration observed would not be as effective in a raceway as in a PBR. Such is the case of *Chlorella vulgaris* grown using flue gas (biogas and syngas combustion) from a municipal wastewater treatment plant where the biomass from open ponds presented higher concentration of heavy metals (68.18 mg Cr/kg, 88.33 mg Cu/kg and 43.33 mg Ni/kg) than its counterpart grown in columns PBR (18.18 mg Cr/kg, 61.18 mg Cu/kg and 40.91 mg Ni/kg) [20].

3.4.6 Effect of Cultivation Time on the Level of Heavy Metal Removal from Fresh Biomass

The leaching of heavy metals from biomass looks towards producing a cleaner biomass. The broken lines in Figure 26 represent the concentration of heavy metals achieved after rinsing the fresh wet biomass with EDTA solution. The different level of cleanliness achieved at different times indicates that the length of cultivation is an important factor affecting biomass clean-up, especially during the first days of growth. It can be observed in this figure that EDTA removed

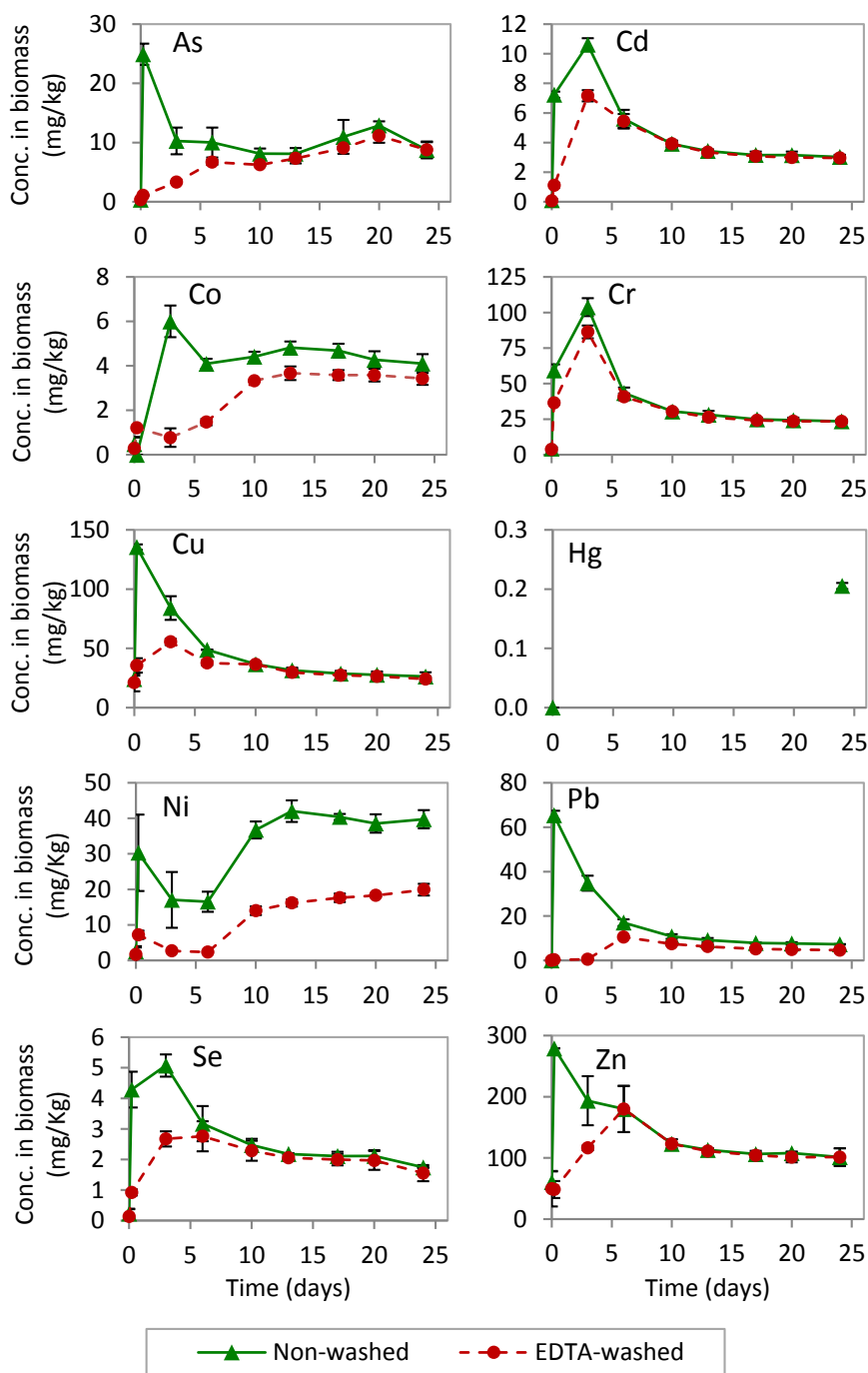


Figure 26. Temporality of heavy metal concentrations in biomass for 1X experiment. Lines represent the concentration found in fresh harvested biomass before (non-washed) and after EDTA-rinsing (EDTA-washed). The area between this two lines represents the EDTA-removable fraction. Data points are average from three replicates and error bars indicate \pm one standard deviation.

heavy metals from the biomass during the first 6 days of growth (differences between the solid line and the broken line are more pronounced) (except for Co and Ni). For cations this difference could be understood as the heavy metals dislodged by EDTA from the cell surface. For a cultivation system focused on biomass production, harvesting at this early stage is unlikely to occur due to the low cell concentrations achieved. By the time the biomass reaches desirable cell concentration, EDTA was not able to remove heavy metals anymore (with exception of Ni), probably because they relocated inside the cell, stronger bonds have been formed between the heavy metals and the cell wall or they had precipitated [72].

3.4.7 Heavy Metal Removal from Freeze-Dried Biomass

Heavy metal concentrations in solvent-rinsed freeze-dried biomass from 1X experiments are shown in Figure 27. The solvents used were hexane (H), methanol (M), acidified-methanol (M-a), deionized water (W) and EDTA (E). Hexane removed between 4 to 12% of heavy metals from biomass. Deionized water was somewhat effective at removing Cr and As. EDTA was effective at removing Cd, Co, Cr, Cu, Ni, Pb and Zn. But the largest removal of most heavy metals was obtained with acidified methanol containing 5% sulfuric acid. Acidified methanol removed 91% As, 98% Cd, 71% Co, 61% Cr, 92% Cu, 96% Ni, 53% Pb, 23% Se and 94% Zn and also denaturated great portion of chlorophyll leaving behind a gray biomass. Methanol alone was not very efficient in removing heavy metals (only removed between 1 to 10%), except for As for which 37% was removed. This affinity towards As species is reported in literature but is not well understood in the published literature, nevertheless it is believed that organic forms of As have affinity for methanol, while inorganic As species are poorly removed [94, 95]. On the contrary, the high removals of As observed with acidified methanol are attributable to the ability of acid solutions to liberate more inorganic As from the matrix. Also, sulfuric acid in the acidified methanol can act as a strong oxidizer capable of digesting cellular components thus liberating internalized metals [94].

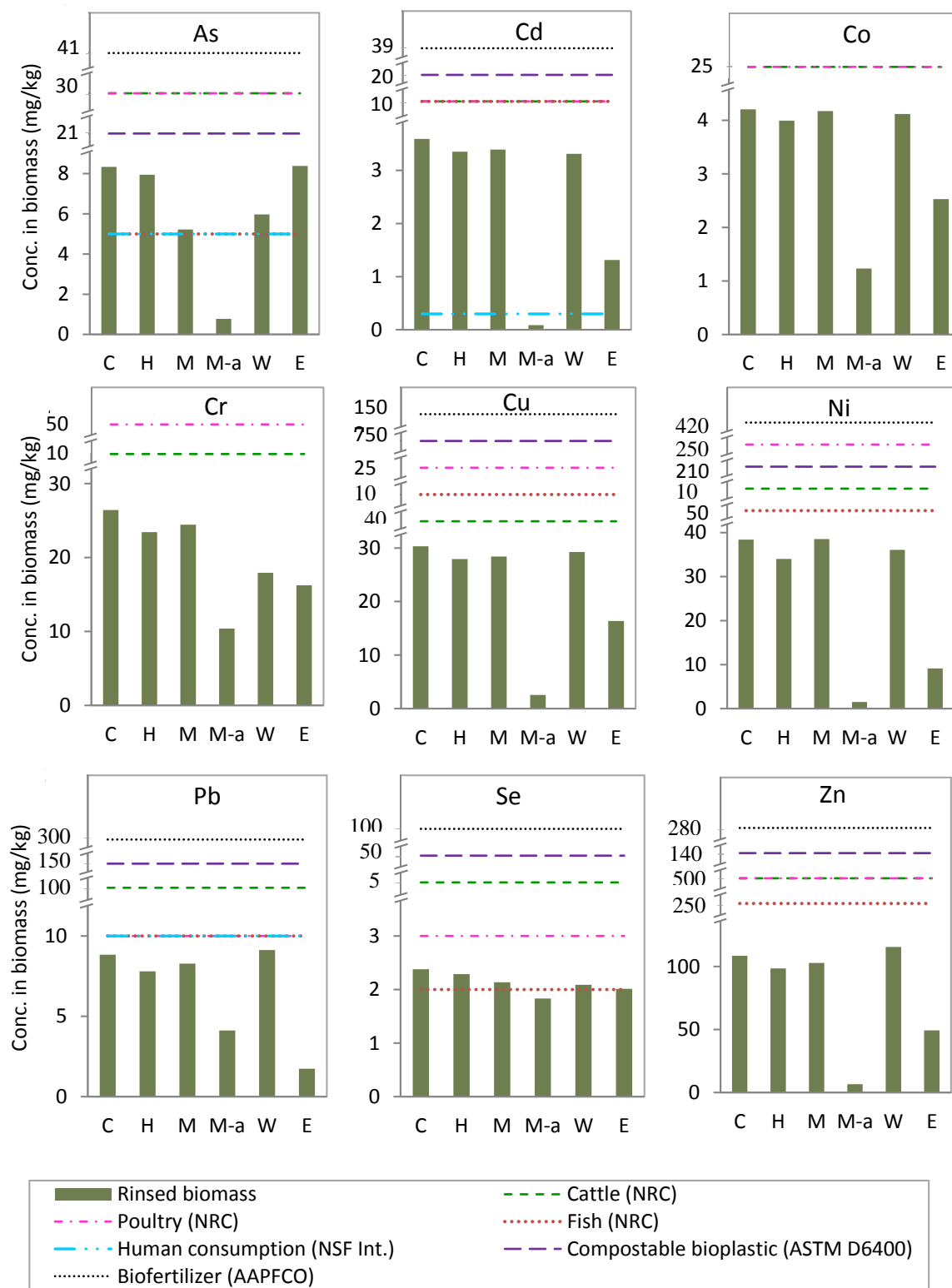


Figure 27. Heavy metal concentration in freeze-dried biomass after solvent rinsing. Control (C), hexane (H), methanol (M), acidified methanol (M-a), Deionized water (W) and EDTA (E).

The different degree of removal produced by these different solvents implies that the type of process chosen for biodiesel production (*e.g.*, *in-situ* acid catalyzed transesterification that uses methanol and acid vs. supercritical methanol that uses methanol without acid) will produce a biomass remnant and a liquid phase (containing the crude biodiesel) with different degrees of heavy-metal contamination. Consequently, level of heavy metal contamination of the products and by-products during biodiesel production could be affected. Further research under actual biodiesel-production working conditions is needed in order to determine the final levels of heavy metal contamination.

3.4.8 Potential Uses of Algal Biomass

Figure 26 and Figure 27 compare the concentrations of heavy metals in the algal biomass against ceiling concentrations established for human food, animal feed (fish, cattle and poultry), biofertilizer, thermoplastic filler and paper filler (common practical uses proposed for algae biomass in the literature). Each type of use is discussed below as well as the challenges that could arise from using algal biomass contaminated with heavy metals for biodiesel production.

3.4.8.1 Use for Human Food. Because heavy metal concentrations in algae are variable (depending on the specie, medium, time of exposure, physiological algal stage, etc.) no official standard regulates their concentration in the biomass [96]. Therefore, for comparison this study uses the Standard 173 NSF International [97] for raw materials to be used as dietary supplements for humans: As (5 mg/kg), Cd (0.3 mg/kg) and Pb (10 mg/kg). From this comparison, for the 1X experiment it was found that As was 1.7 times and Cd was 12 times higher than the NSF International standard, thus the harvested biomass was not suitable for direct human consumption. Heavy metal concentrations after hexane and methanol rinsing were also higher than this standard. In contrast, biomass rinsed with acidified methanol lowered the As and Cd concentrations to acceptable levels. However, 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 because of the fear to the various other toxins also present in these streams (*e.g.*, dioxins, furans and polycyclic aromatic hydrocarbon-PAH) [98-100].

3.4.8.2 Use for Feedstuff. There are no official standards or limits that regulates the content of heavy metals in algae used as animal feed [96, 101]. To the author's knowledge the only regulation is the 21CFR 73.185 concerning heavy metals concentration in *Haematococcus pluvialis* meal as a source of red color additive (astaxanthin) for fish food. However, this regulation is not applicable to this study because the culturing conditions for astaxanthin production are out of the scope. However, there are some guidelines and recommended limits for some trace metals in animal feed and supplements that are discussed below.

Fish: The National Research Council (NRC) of the National Academy of Science provides maximum tolerable levels (MTL) of minerals in animal feed (*e.g.*, fish, poultry and cattle). These MTL recommendations are concentrations that will not affect animal health (when fed for a period of time) or human health (after the consumption of the animal) [97, 102, 103]. NRC's MTL for fish feed are As (5 mg/kg), Cd (10 mg/kg), Cu (100 mg/kg), Ni (50 mg/kg), Pb (10 mg/kg), Se (2 mg/kg) and Zn (250 mg/kg) [102]. From the comparison it was found that As and Se exceeded the NRC standard by 1.7 times and 1.2 times, respectively, while all other heavy metals were lower than the standard (note that NSF International does not have standards for Co, Cr and Hg). This implies that a diet containing 100% whole algal biomass from 1X experiment may not be adequate for fish feedstuff; but diets that incorporate algae as a partial replacement may not exceed the standard. Only removal by acidified methanol reduced the As and Se concentration to acceptable levels for fish foodstuff. In contrast biomass from 5X and 10X were well above the standard.

Cattle: NRC's MTL for cattle are As (30 mg/kg), Cd (10 mg/kg), Co (25 mg/kg), Cr (100 mg/kg), Cu (40 mg/kg), Ni (100 mg/kg), Pb (100 mg/kg), Se (5 mg/kg) and Zn (500 mg/kg) [102]. The concentration in algae from 1X experiments were under the standard (As was 3.6 times, Cd

was 2.8 times, Co was 5.9 times, Cu was 1.3 times, Cr was 3.8 times, Ni was 2.6 times, Pb was 11.3 times, Se was 2.1 times and Zn was 4.6 times lower). Therefore, feed composed of 100% not-rinsed and rinsed algal biomass from 1X experiment would not exceed the allowable limits. Actually, algae biomass can only be partially added to cattle feedstuff because it is usually limited by its ash content, salt content and protein content [41, 42]; therefore it is expected that the heavy metal concentration in the final feed using this biomass would be below NRC standards. In contrast biomass from 5X and 10X were well above the standard.

Poultry: Heavy metals MTL recommended by NRC for poultry feed are As (30 mg/kg), Cd (10 mg/kg), Co (25 mg/kg), Cr (500 mg/kg), Cu (250 mg/kg), Hg (0.2 mg/kg), Ni (250 mg/kg), Pb (10 mg/kg), Se (3 mg/kg) and Zn (500 mg/kg) [102]. All heavy metals from biomass from 1X experiment were under the recommended concentrations (As was 3.6 times, Cd was 2.8 times, Co was 5.9 times, Cu was 8.2 times, Cr was 78.9 times, Ni was 6.5 times, Pb was 1.1 times, Se was 1.3 times and Zn was 4.6 times lower). Since algae biomass is added only as a supplement or as a protein replacement to poultry feed, it will only form a fraction of the whole feed. Biomass from 5X and 10X PBRs exceeded the standard.

3.4.8.3 Use for Biofertilizer. There is not an official standard that regulate the concentration of heavy metals in fertilizers, however there are recommendations given by the Association of American Plant Food Control Officials (AAPFCO). AAPFCO recommends that heavy metals in all compost products (manure, manipulated manure) and any other fertilizer making nutrients claims should be below the USEPA part 503 that determines the ceiling concentrations for biosolids (composted materials from sewage sludge). Those concentrations are 41 mg/kg As, 39 mg/kg Cd, 1500 mg/kg Cu, 17 mg/kg Hg, 420 mg/kg Ni, 300 mg/kg Pb, 100 mg/kg Se and 2800 mg/kg Zn [104, 105].

All heavy metal concentrations in algal biomass from 1X PBR were several times below the ceiling concentration recommended by AAPFCO (As was 4.8 times, Cd was 11.1 times, Cu

was 49.5 times, Ni was 10.9 times, Pb was 34 times, Se was 42 times and Zn was 25.8 times lower). This means that whole algae grown using flue gas can be applied as fertilizer or soil amendment without exceeding regulatory concentrations. This practice however is unlikely to happen prior to the recovery of valuable products from algae.

Cd, Pb and Ni concentrations in biomass from 5X and 10X exceeded the AAPFCO standard, suggesting that this biomass cannot be land applied. Landfilling and co-incineration of this biomass could be management options as long as the 40CFR 258 and the Clean Air Act are met [105].

3.4.8.4 Use for Plastic and Paper. The regulations applicable for plastic and paper are a function of the intended use. For packing material, the US Toxic in Packaging Prevention Act states that the sum of the concentrations of Hg, Pb, Cd and Cr(VI) should not exceed 100 mg/kg by weight. If the paper or board is going to be in contact with moist or fatty food, the European Industry Guidelines Paper and Board Materials and Articles for Food Contact recommends a maximum concentration of Hg (0.3 mg/kg), Pb (3 mg/kg) and Cd (0.5 mg/kg) [106].

Rinsed and not-rinsed biomass were below the recommendations in the Toxic in Packaging Prevention Act. The sum of Hg, Pb, Cd and Cr was 2.6 times lower than the mandated ceiling concentration of 100 mg/kg. This means that 100% of the harvested biomass can be used to produce paper and plastic containers without exceeding the standard. Non-rinsed biomass exceeded the guidelines for paper in contact with moist and fatty food, even when only 30% was assumed as filler. Only when 30% of acidified-methanol-rinsed biomass was considered, the standard was met.

Some industries produce environmentally friendly biodegradable plastic, which can be made using algae. For biodegradable plastic, the American Society for Testing and Materials - ASTM D6400 standard recommends concentrations of heavy metals in the material to be lower than 50% of the amount listed in the 40CFR 503.13, Table 3 [107]. All the heavy metal concentrations in the biomass were several times under the ceiling concentration in this standard

(As was 2.4 times, Cd was 5.6 times, Cu was 24.7 times, Ni was 5.5 times, Pb was 17 times, Se was 21 times and Zn was 12.9 times lower) (notice that Cr and Co do not have maximum concentration established). This means that 100% rinsed and not-rinsed biomass can be used to produce compostable plastic materials without getting near to the maximum heavy metal concentration limits. Moreover, as now only partial incorporation (up to 51%) of algal biomass has been used effectively during plastic production without compromising its characteristics [57, 58, 60, 61], thus the levels of heavy metals in the final product are expected to be well below the recommended standards. Also, using the lipid extracted biomass resulting from *in-situ* acid-transesterification biodiesel production have the advantage of having lower content of chlorophyll, therefore aiding on the removal of this undesired pigment for paper and plastic production. Biomass from 5X and 10X experiments exceeded the ASTM D6400 standard.

3.4.8.5 Use for Biodiesel Feedstock. Transfer of heavy metals from contaminated biomass to biodiesel could present challenges to biodiesel storage because metals such as Pb, Ni, Zn, Co and Cu, present in flue gas, are pro-oxidants that accelerate the oxidation of biodiesel as demonstrated by a reduced induction period in the Rancimat test; therefore resulting in a low quality biodiesel with shorter shelf life [108-115]. Furthermore, the ignition of heavy metals contained in diesel has been linked to negative health effects, such as lung cancer, cardiopulmonary diseases and asthma from inhalation and/or ingestion of emission particulate matter [116, 117].

Currently, there are no limits set for the concentration of heavy metals in biomass intended to be used for biodiesel production. In the biodiesel community, it is widely assumed that most metals will remain in the lipid extracted remnants or in the liquid polar phase after transesterification, but not in the biodiesel [18]. However, it has been suggested in the literature that heavy metals present in biodiesel can come from vegetable oil [118-121] and can be carried over to the purified biodiesel [121]. Such is the case of the Cu, Cd, Zn and Fe found in the unblended biodiesel (B100) produced from 4 degummed oils sources (seeds of *Canarium schweinfurthii*, *Hura*

crepitans, *Telfaria occidentalis* and *Cucumeropsis manii*) by base-catalyzed transesterification, water washing (distilled water) and heat drying [121].

Currently there is no published literature that allows for the determination of heavy metal transfer from biomass to biodiesel, but for Ca and Mg (metals with similar valence number to several heavy metals in this study) 44-66% was transferred from crude palm oil biodiesel to refined biodiesel when ceramic membrane separation and water washing purification was used [122]. For sake of comparison several assumed transfer coefficients were evaluated in order to gain insights about what could be the level of contamination in unblended biodiesel (B100) produced with the algal biomass from IX experiment. In our comparison (see Appendix H), when 5% transfer coefficient was used, heavy metals such as Cr, Ni, Zn, Cd, Pb and Cu exceeded the concentrations in diesel fuel reported by Wang [117].

The oxidative damage to biodiesel produced by heavy metals can be suppressed by addition of natural or artificial antioxidants (*e.g.*, citric acid, phosphoric acid, amino acids, butylated hydroxytoluene, butylated hydroxyanisole, tertiary-butylhydroquinone, propyl gallate, etc.), which is a common practice used to prolong biodiesel shelf life [108-110, 113, 123]. The disadvantage of this approach is that the addition of metal chelators will affect the final biodiesel price and will supply a product containing heavy metals that will be emitted from vehicle exhausts. In order to be environmentally acceptable as a replacement for diesel, biodiesel must contain the same or lesser metallic pollutants than its counterpart diesel. As now, there is little published literature in this area. Given the important impact of heavy metals to biodiesel, the amount of metals in the final biofuel product and by-products must be quantified in future research for accurate assessment of the full impact of the integration of flue gas with algae cultivation.

3.5 Conclusions

Algal cultivation systems using flue gas as carbon source allow for various heavy metals

to accumulate in the biomass and medium, thus affecting their quality in a temporal fashion. Although heavy metals from flue gas contaminate biomass and medium, there are some uses for which the maximum levels of heavy metals regulations and recommendations are not exceeded. For *Scenedesmus obliquus* grown for 24 days in APS medium at 1X heavy metals concentration:

- The level of cleanliness of the medium and the biomass are time dependent and are directly affected by growth dilution.
- Removal of heavy metals from the biomass is also time dependent, with the larger removal occurring only early in the cultivation period.
- The spent medium was found to be suitable for irrigation uses.
- Disposal of spent medium into a natural stream will require that the water quality standards for the water body allow consideration for dilutions and mixing zones before discharge.
- If medium is recycled for further algae production, As build up in the medium is likely to occur mainly because *Scenedesmus obliquus* does not have high capacity to uptake As. Since As is not toxic for *Scenedesmus obliquus* even at 20X, this metal can build up without jeopardizing algae production; but it can negatively impact operative costs due to As treatment before effluent discharge.
- The algal biomass did not meet the standards for direct human consumption due to the elevated concentration of heavy metals; but those concentrations were below the standards recommended for cattle feed, poultry feed, biofertilizer and fillers for the paper and plastic industry. Levels of As and Se were above the standards for fish food. Final heavy metal concentrations in animal feed can be lowered below the standards if algae form only a fraction of the total feed.
- Due to lack of standards for biodiesel feedstock, it is not possible to determine if this biomass can be appropriate for biodiesel production.

- Removal of heavy metals from freeze dried biomass by hexane or methanol was not significant. Only acidified methanol containing 5% sulfuric acid significantly removed heavy metals.

Note of caution: In addition to heavy metals, flue gas delivers hundreds other pollutants that have not been addressed in this study. The level of knowledge of our society about the final effects of pollutant and their further biotransformations is still very limited and thus they should be further evaluated before their introduction into the food chain as animal feed or human food.

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CHAPTER 4

ENHANCING ARSENIC BIOREMEDIATION IN AN ALGAL PRODUCTION SYSTEM

Abstract

Algae cultivation using coal-based flue gas is likely to result in As build up in the recycled medium. Since As is a contaminant attributed to health issues such as cancer, it would be ideal to remediate it within the same cultivation system before disposing it to the environment. Since green algae *Scenedesmus obliquus* have a limited capacity to bioaccumulate As in their biomass, this study explores the use of sulfur (S) to enhance As retention in biomass, therefore increasing As bioremediation efficiencies. Experimental results point to an increase in S uptake that correlates with increased As removal from the medium. An increase in S in the medium (from 19.2 to 58 mg S-SO₄²⁻/L) enhanced the removal of As from 15% to 61%, respectively. The results indicate that the algae cultivation system could be used to remediate As upon S addition.

4.1 Introduction

Our society has a growing demand for liquid transportation fuel as economies grow. In the last few years liquid fuel supply has been shortened due to tensions in petroleum-producing nations, resulting in a increase in the prices. It is in this scenario that biofuels from algae step-up as an alternative to petroleum-based diesel. One of the advantages of algae is the adaptability of this organism to thrive in cultivation systems using waste streams such as flue gas, even when it introduces toxic heavy metals [1, 2]. Algae have been shown to have a large uptake capacity for most heavy metals transferred from flue gas, with the exception of As, which is expected to build up once the medium is recycled for further growing cycles.

Arsenic is a human carcinogen shown to cause liver, lung, kidney and bladder cancer [3, 4]. Natural As is very mobile, difficult and costly to remediate, consequently it has become a worldwide problem affecting several countries (*e.g.*, Bangladesh, India, China, USA, Peru,

Argentina and many others) [5]. In order to reduce As contamination in the environment, algal production facilities should be able to treat any As-contaminated medium at the production facility. A low-cost alternative for As remediation is the biological accumulation of As within the biomass [4], which can then be removed during the biodiesel production process.

For this bioremediation approach to work, bioaccumulation of As by *Scenedesmus obliquus* has to be increased. Published literature suggests that addition of sulfur (S) to the medium could play a role in increasing As bioaccumulation [3, 6, 7]. The hypothesis for the research described in this chapter is that, by increasing sulfate in the medium, As bioremediation by *Scenedesmus obliquus* would be enhanced.

4.2 Literature Review

4.2.1 Occurrence of As in Algal Photobioreactor

Arsenic occurs naturally in surface water, groundwater and soil. Arsenic is especially high in coal (0.3 - 35000 mg/kg) and is released from coal matrix during incineration forming gaseous arsenic oxides in the flue gas environment [5, 8, 9]. Although flue gas from coal-fired plants go through purification systems (*e.g.*, electrostatic precipitators, wet scrubbers, mechanical collectors and fabric bag-house filters); submicron fly ashes, aerosols and gas containing As escape to the atmosphere [9]. It has been reported that As penetration through electrostatic precipitators and wet scrubbers can vary between 2.5 - 11.5% [9]. Airborne As then settles at rates of 1 - 1000 $\mu\text{g}/\text{m}^2$ year causing contamination of soil and water bodies [5].

While integrating algae production and CO₂ capture, flue gas is bubbled through the liquid medium in order to transfer CO₂ to serve as a carbon source for algal growth [10, 11]. During the course of this gas exchange, As in flue gas is also transferred to the medium and then to the biomass, as documented in algal systems supplemented with flue gas from municipal waste and coal incineration [2, 12].

Arsenic originating from flue gas is likely to form As(III) in water [5]. Transformation of As(III) to As(V) is possible but the kinetics of this oxidation is slow [5, 13]. For this experiment As(III) was the As specie added to the PBRs.

4.2.2 Sulfur in Algal Photobioreactor

S is an essential macronutrient that plays a structural and catalytic or electrochemical functions in the cell. For green algae, S is taken up by the cell as sulfate anion (SO_4^{2-}), it is incorporated into the sulfur-based amino acid cysteine (Cys) that is further synthesized into methionine, GSH, metalloenzymes and proteins [14-17]. The thiol in Cys forms disulfide bonds to maintain protein structure, while the thiol (SH) in Cys and GSH perform redox cycles to protect the cell against oxidative stress [17]. GSH is formed by glutamate, cysteine and glycine (Figure 28) where the soluble thiol (SH) of the cysteine molecule binds to toxic free metals and metalloids [14, 18, 19]. When cells are expose to stressors such as heavy metals, these S-based molecules are overproduced, therefore causing an increase in the S assimilation pathway [14, 17].

4.2.3 As Uptake by Algae

Algae are unicellular aquatic organisms that consume nutrients, CO_2 (as a carbon source) and light (as energy source) [20, 21]. Nutrients dissolved in the medium are removed by algae

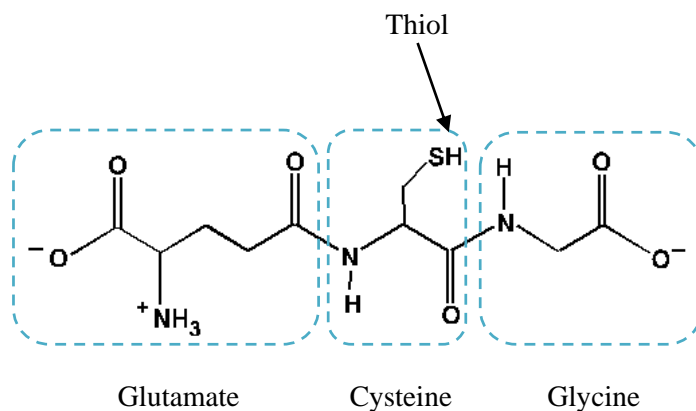


Figure 28. Glutathione (GSH) molecule

through transporters embedded on their cell wall and through carriers that mobilize the nutrients into the cell [20]. These transporters and carriers are not always specific and they also deliver toxic elements such As [22-24]. It has been suggested that arsenate As(V) is transported through phosphate transporters and arsenite As(III) is transported through glycerol and hexose transporters [25, 26].

Inside the cell As can result in oxidative stress and the inactivation of critical molecules. For example, As(V) compete with phosphate in functions such as ATP formation, while As(III) reacts with sulfhydryl groups of enzymes and proteins [26]. To reduce cellular damage from this interaction, free As is regulated by either excretion, methylation, reduction/oxidation and chelation (Figure 29). Excretion of As(III) and As(V) allows the cell to eliminate these toxic ions without any change [18, 27, 28].

Biomethylation is another mechanism used by algae to reduce the toxic effect of As. Methylation of As is not fully understood [29], but it is commonly accepted that it is only biologically driven and follows the Challenger's mechanism (Figure 30). Once the As is methylated, it passively diffuses out of the cell [13, 27] and can be re-uptake for further methylation. The extent of methylation in algae does not produce volatile As forms due to algae's inability to further reduce methylated compounds [29-34].

Another defense mechanism against As is the induction of glutathione (GSH, a reservoir of nonprotein thiol) and phytochelatins (PC, a GSH-based peptide) [18, 22, 35, 36] that binds As ions making them no longer available to interact with cellular components [14, 18, 19]. The new complexes formed between As and GSH or PC are then sequestered in the vacuole [7, 18, 37]. Sequestration of As in the vacuole can be enhanced by the overexpression of these chelators [25].

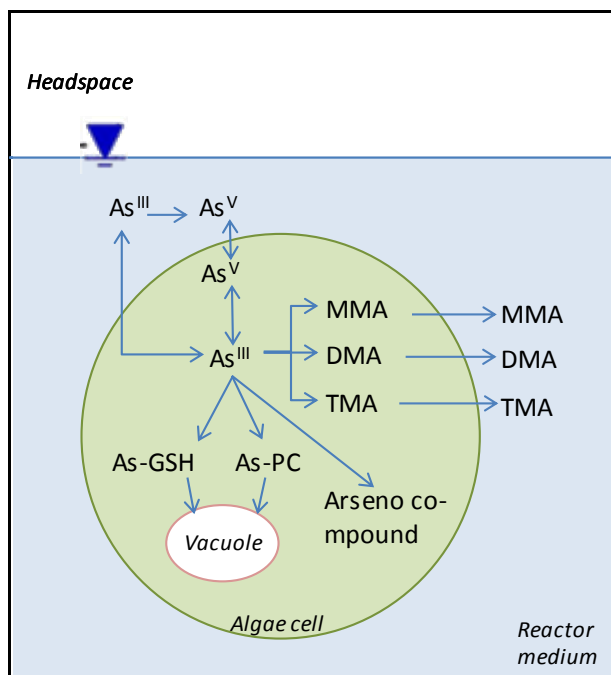


Figure 29. Schematic representation of As trafficking by algae. Monomethyl arsenic (MMA), dimethyl arsenic (DMA), trimethyl arsenic (TMA), As bound to glutathione (As-GSH), As bound to phytochelatin (As-PC).

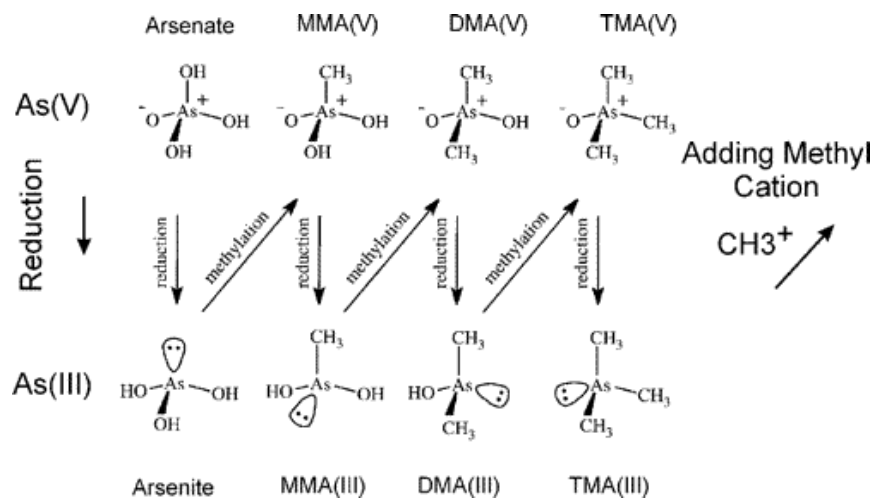


Figure 30. Challenger mechanism for As methylation [33]

For instance, Yamaoka, Takimura, Fuse and Murakami [6] demonstrated that the addition of GSH in the culture medium increased the intracellular accumulation of As in *Dunaliella salina* by eight times [6].

In fact, Srivastava and D'Souza [7] demonstrated that the addition of sulfate in the medium enhanced both the intracellular formation of As(III)-GSH and its sequestration in vacuoles in *Hydrilla verticillata* [7]. Improving As sequestration in algae by addition of S had not been reported previously in the literature. It is the aim of this study to evaluate if S can increase As bioremediation performed by *Scenedesmus obliquus*.

S is an element that can change As redox. In the presence of sulfur, As can form As precipitates such as As_2S_3 and AsS . However, these complexes are only expected at substantially reduced conditions that are not optimal for algal growth, thus are not expected in a PBR [13].

4.3 Materials and Methods

Algae strain and growth medium: *Scenedesmus obliquus* donated by Arizona Public Service (APS) was grown axenically in solid agar APS medium. Colonies were transferred to 3L polystyrene spinning bioreactors (Corning®) for 7 days under continuous light and at pH 7. The biomass was harvested by centrifugation at 3900 RPM and rinsed with S-free APS medium. Experiments were performed in airlift borosilicate glass tube PBR. The bioreactors were initially filled with EDTA-free APS medium without S. S-free APS medium was prepared using $NaNO_3$ (1000 mg/L), K_2HPO_4 (200 mg/L), $CaCl_2 \cdot 2H_2O$ (25.1 mg/L), $MgCl_2$ (18.97 mg/L), H_3BO_3 (11.4 mg/L), $MnCl_2 \cdot 4H_2O$ (0.597 mg/L), $ZnCl_2$ (0.041 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (0.058 mg/L), $CuCl_2 \cdot 2H_2O$ (0.041 mg/L), $CoCl_2 \cdot 6H_2O$ (0.029 mg/L). S stock was prepared the same day using Na_2SO_4 and was autoclaved at 121°C. S stock was added to the PBR to obtain four (4) different concentrations: 19.2 mg $S-SO_4^{2-}/L$ (control or one fold-S), 38 mg $S-SO_4^{2-}/L$ (two fold-S), 58 mg $S-SO_4^{2-}/L$ (trifold-S) and 77 mg $S-SO_4^{2-}/L$ (fourfold-S); where the control is the normal concentration

contained in APS medium. Concentrated As stock (1000X) was also prepared the same day from NaAsO₂, filtered through sterile 0.2 µm syringe filter and added to the reactors to reach 0.39 mg-As/L (5X concentration in previous chapters). Control experiments using the various sulfate concentrations in the absence of As were also carried out in parallel.

Growth monitoring: OD₇₅₀ was measured using the Thermo Electron Corporation Genesys 5 spectrophotometer and then transformed to TSS using the equation $TSS = OD_{750} \times 0.4585 + 0.0116$ developed during preliminary studies. Samples were centrifuged at 7500 RPM for 3 minutes and supernatant was analyzed for As, PO₄³⁻ and SO₄²⁻

Macronutrients analysis: Samples were filtered through a 0.45 µm syringe filter and then placed in an IC cartridge for analysis. PO₄³⁻ and SO₄²⁻ were analyzed using DIONEX ICS-1000 ion chromatograph with self-regeneration system, carbonate/bicarbonate eluent, AS12A anion-exchange column. Standards were purchased from Fluka.

As analysis: Total As concentration in medium was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS Agilent 7500 Series). Samples were digested with HNO₃ at 105°C (Standard Methods 3030E [38]) and adjusted to 10 mL with deionized water. Standards were purchased from Fisher Scientific and the diluted standards were prepared the same day of the analysis.

4.4 Results

4.4.1 Scenedesmus obliquus Growth Under Various S Concentrations

Figure 31 shows the biomass concentrations in the PBRs exposed to four different S concentrations, namely 19.2 mg S-SO₄²⁻/L (control or one fold-S), 38 mg S-SO₄²⁻/L (two fold-S), 58 mg S-SO₄²⁻/L (trifold-S) and 77 mg S-SO₄²⁻/L (fourfold-S). From this figure it can be seen that As-free PBRs followed closely to their corresponding As-treated counterparts, indicating that As at a concentration of 0.39 mg/L did not produce any effects (increment or inhibition) in growth. It.

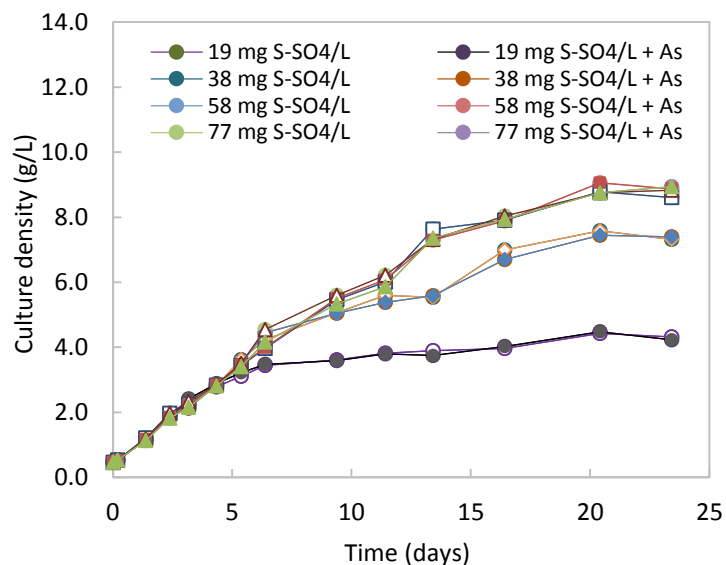


Figure 31. Effect of sulfur in *Scenedesmus obliquus* growth. 19.2 mg S-SO₄²⁻/L is the baseline containing the normal S concentration in APS medium. Empty symbols represent the As-free PBRs, filled symbols represent As-added PBRs.

can also be observed that higher S concentration resulted in higher biomass yields, but reached its maximum at threefold-S concentration

4.4.2 Sulfate in the Medium

Sulfate concentration in the medium is presented in Figure 32. Figure 32A shows the changes in S concentration in mg/L along the growth, while Figure 32B shows S concentration in the medium normalized by biomass yield. Both figures show that until day three S was consumed by algae at similar rates in As-free and As-treated PBRs, after which an accelerated S uptake was observed in As-treated PBRs containing threefold-S and fourfold-S. However, it has been suggested that no intracellular storage compound for S exist in algal cells (unlike other nutrients such as phosphorus), thus continual uptake is possibly to provide a supply of S to the cell [39].

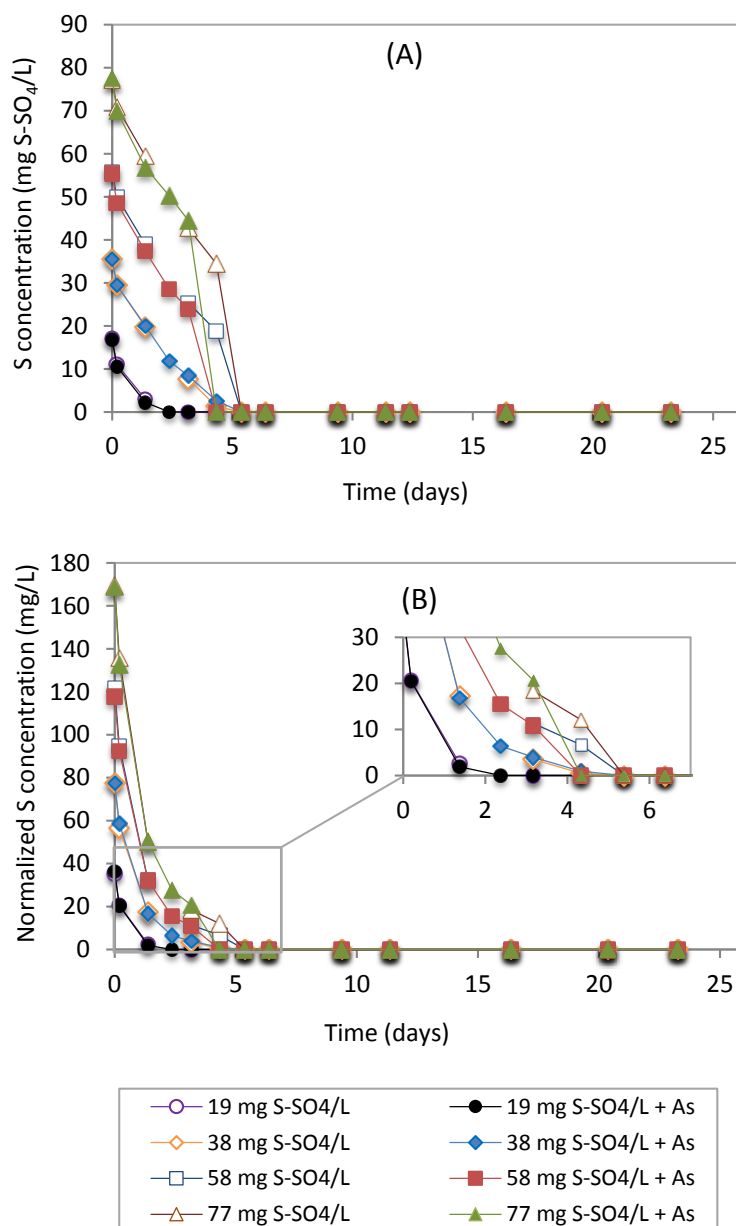


Figure 32. Sulfate concentration in medium after exposure to 0.39 mg As/L. 19.2 mg S-SO₄²⁻/L is the baseline containing the normal S concentration in APS medium. S concentration in mg/L (A) and S concentration normalized by biomass dry weight (B). Empty symbols represent the As-free PBRs. Filled symbols represent As-added PBR.

Increase in S uptake due to metal stress had been observed before by Mera, Torres and Abalde [40], [41, 42]. Literature suggests that under high demands of S-based metabolites resulting from heavy metal stress, genes encoding S transporters and S activation enzymes are induced. As a result the sulfate transporters are up-regulated leading to an increased activity of S uptake [14, 17, 41, 42]. This suggests that the higher S uptake observed in As-treated PBRs could have been the result of high S-based metabolite demand induced by As. It is well known that heavy metals, including As, produce oxidative stress in *Scenedesmus* sp., resulting in overproduction of antioxidants (*e.g.*, metallothionein, PC and GSH) that subsequently accelerates the rate of S uptake [14, 17-19, 27, 35].

4.4.3 Phosphate in the Medium

Phosphate concentration in the medium is shown in Figure 33. The concentration expressed in mg/L is shown in Figure 33A and the specific phosphate concentration normalized by growth is shown in Figure 33B. It can be seen in both figures that until day three phosphate was being depleted from the medium in all PBRs. But on day four, phosphate was reintroduced to the medium in As-treated PBRs and a day after was also reintroduced in As-free PBRs. Interestingly, the phosphate excretion to the medium happened the same day sulfate was totally removed from the medium. Also, it can be observed that larger S uptake resulted in larger P excretions, suggesting that they are related.

To the author's knowledge, it is the first time that excretion of phosphate as a result of uptake of sulfate has been reported in literature. This behavior could be explained by a cellular regulation to maintain intracellular electroneutrality. Nutrient uptake imply intracellular transport of unequal quantities of anions and cations. To avoid charge imbalance, the cells regulate their cation-anion balance by excretion of H^+ or OH^- [43]. However, green algae *Scenedesmus* can also excrete anions such as phosphates [44], thus it can be suggested that the excretion of phosphate

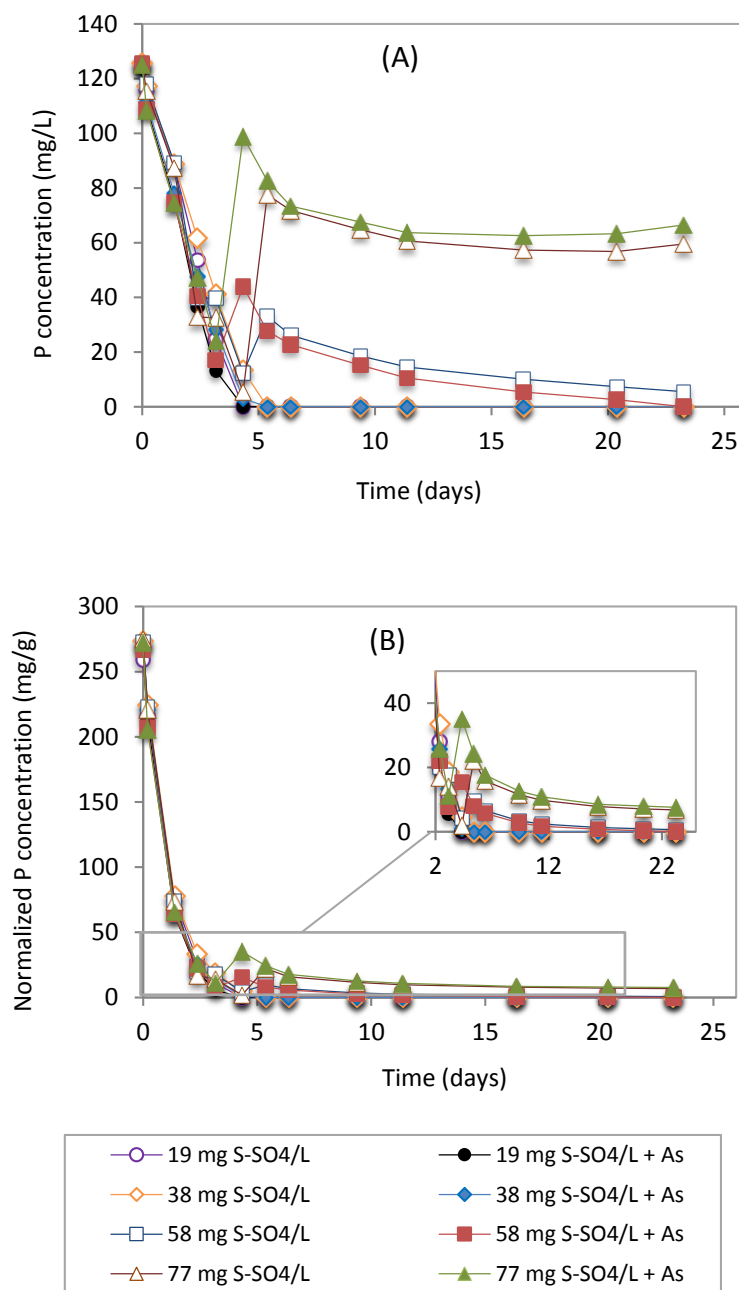


Figure 33. Removal of phosphate from medium. Reduction of phosphate concentration in medium after exposure to 0.39 mg As/L (A) and normalized phosphate concentration by biomass dry weight (B) 19.2 mg S-SO₄²⁻/L is the baseline containing the normal S concentration in APS medium. Unfilled symbols represent the As-free PBRs. Filled symbols represent As-added PBR.

observed in this study could have happened in order to reduce the negative intracellular charge augmented due to sulfate and phosphate uptake. Normally, algae does luxury P uptake and maintains excess P in polyphosphate bodies inside the cell [39], therefore the excess phosphate was expendable.

4.4.4 Bioremediation of As by *Scenedesmus obliquus*

4.4.4.1 Removal of As from the Medium. Figure 34 shows the concentration of As in the medium for all the PBRs tested. Figure 34A shows As concentration expressed in mg/L while Figure 34B shows the concentration in the medium normalized by biomass yield. Both figures demonstrate that supplementation of S at threefold-S and fourfold-S resulted in As removal (60% - 61%) higher than the control (15%). In contrast, treatments of onefold-S and twofold-S produced As removal only during the first 9 days, but thereafter they reintroduced As to the medium.

Results normalized by biomass yield (Figure 34B) show that biomass concentration did impact As removal. Increase in the number of cells is expected to provide more As transporters that can lead to higher internalization, however it can also amplify the response of the defense mechanism. For example, if the cell performs excretion of As as a defense mechanism, then a more effective As elimination is expected due to the higher number of cells performing As efflux. This must be the case for the twofold-S treatment that produced higher biomass and higher As excretion than the control (excretion occurred from day 9 onwards).

4.4.4.2 Modeling of As Bioremediation. Since algal cultivation system used in this study does not lose As (see Figure 5), arsenic content in the biomass can be estimated by $(C_0 - C_t)/B_t$, where C_0 is the initial As concentration, C_t is the As concentration at time t and B_t is the biomass concentration at time t. The results were fitted the empirical Lagergren pseudo-second order kinetic model (equation 2) and is shown in Figure 35 and Table 4.

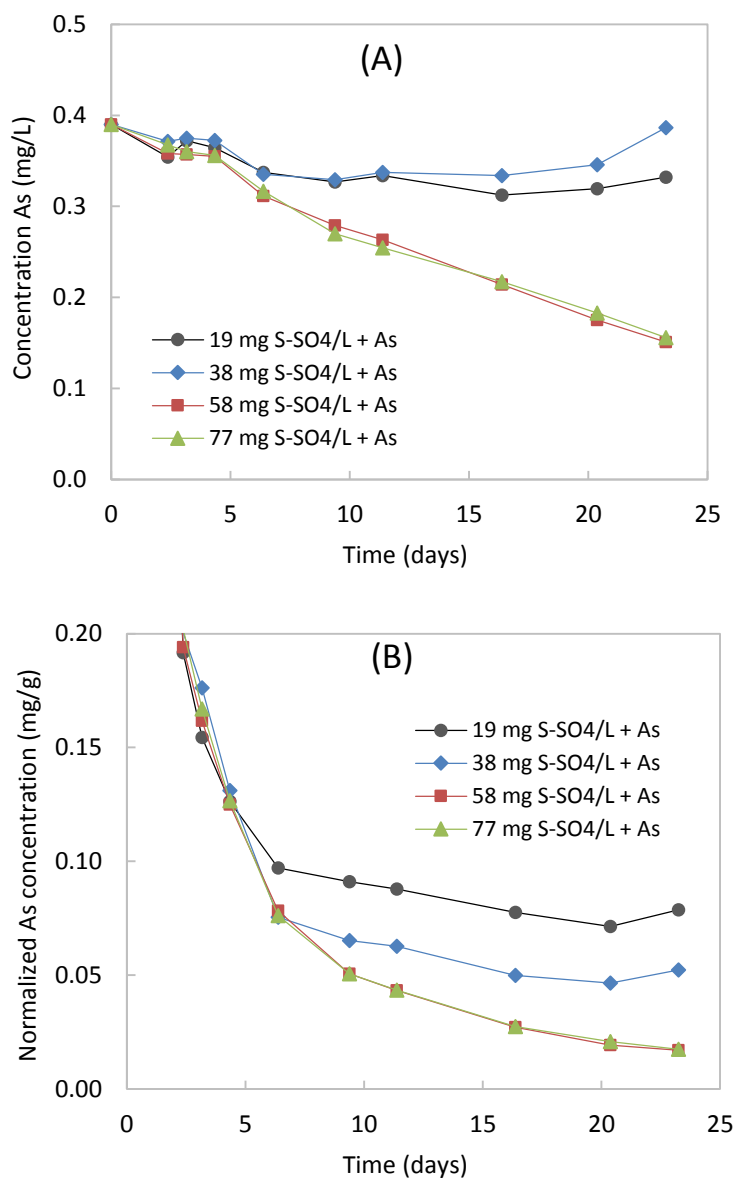


Figure 34. As concentration in medium. Concentration of As in mg/L (A) and As concentration in the medium normalized by biomass dry weight (B). 19.2 mg S-SO₄²⁻/L is the baseline containing the normal S concentration in APS medium.

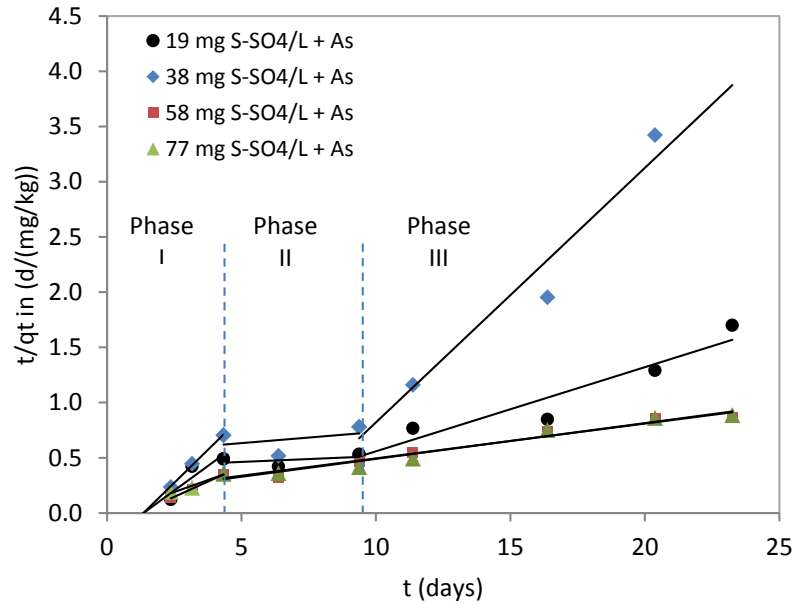


Figure 35. Empirical Lagergren pseudo-second order kinetic fit. Symbols from treatment 58 and 77 mg S-SO₄/L +As overlap.

$$\frac{t}{q_t} = \frac{1}{k} q_{eq}^2 + \frac{t}{q_{eq}} \quad (2)$$

where

q_t = amount of metal uptake on algae cell surface at any time t (mg/kg)

q_{eq} = amount of uptake on algae at equilibrium (mg/kg)

k = rate constant of uptake (kg/mg.h)

t = time (days)

Phase I and II show a similar behavior for all four experiments until day 9, after which the slope of trend lines change drastically for onefold-S and twofold-S experiments, but continue unchanged for threefold-S and fourfold-S experiments (phase III). The trend line for threefold-S and fourfold-S experiments in region III seems to be a continuation of phase II with a positive k (Table 4). That indicates that accumulation of As in biomass (q_t) increases with time. On the

Table 4. Biouptake kinetic model rate constants for phase III

Treatment	k	q_{eq} (mg/kg)	R^2
Control (onefold-S)	-159.85	9.0	0.89
Twofold-S	-2.88	2.8	0.93
Threefold-S	4507.00	30.0	0.96
Fourfold-S	4603.00	29.1	0.96

contrary, onefold-S and twofold-S experiments have a negative k (Table 4), indicating that biomass losses As with time, possibly due to excretion.

4.4.4.3 Mechanism Leading the Boost of As Bioremediation. Based on a review of the published literature, the following mechanism is believed to produce the improved As bioremediation observed in the threefold-S and fourfold-S treatments. A schematic of this mechanism is shown in Figure 36.

Upon As uptake by algae, As is converted rapidly to As(III) [45]. As(III) can follow three pathways namely: i) excretion, ii) methylation or iii) chelation. Due to its higher reactivity, As(III) preferentially will undergo chelation by GSH or GSH-based antioxidants (*e.g.*, PC) as long as these antioxidants are available in the cell. As(III) has a high tendency to react rapidly with thiol groups in these antioxidants [19, 27, 31, 35, 46]. These complexes, however, are not stable at the cytoplasmic neutral pH but are stable under the acidic conditions prevalent in the vacuole [26]. Therefore, As-GSH and As-PC complexes are transferred inside the vacuoles with the aid of Mg-ATP energized ABC transporters [7, 25, 27, 47-52]. If antioxidants are not available, secondary options such as methylation and excretion of As(III,V) get activated.

With the addition of threefold-S and fourfold-S concentration in the medium, the medium provided the S needed to encourage *Scenedesmus obliquus* to perform the chelation (and later storage) of As intracellularly instead of performing the other two excretory pathways (methylation and excretion of inorganic As). Conversely, the onefold-S and twofold-S concentration in the

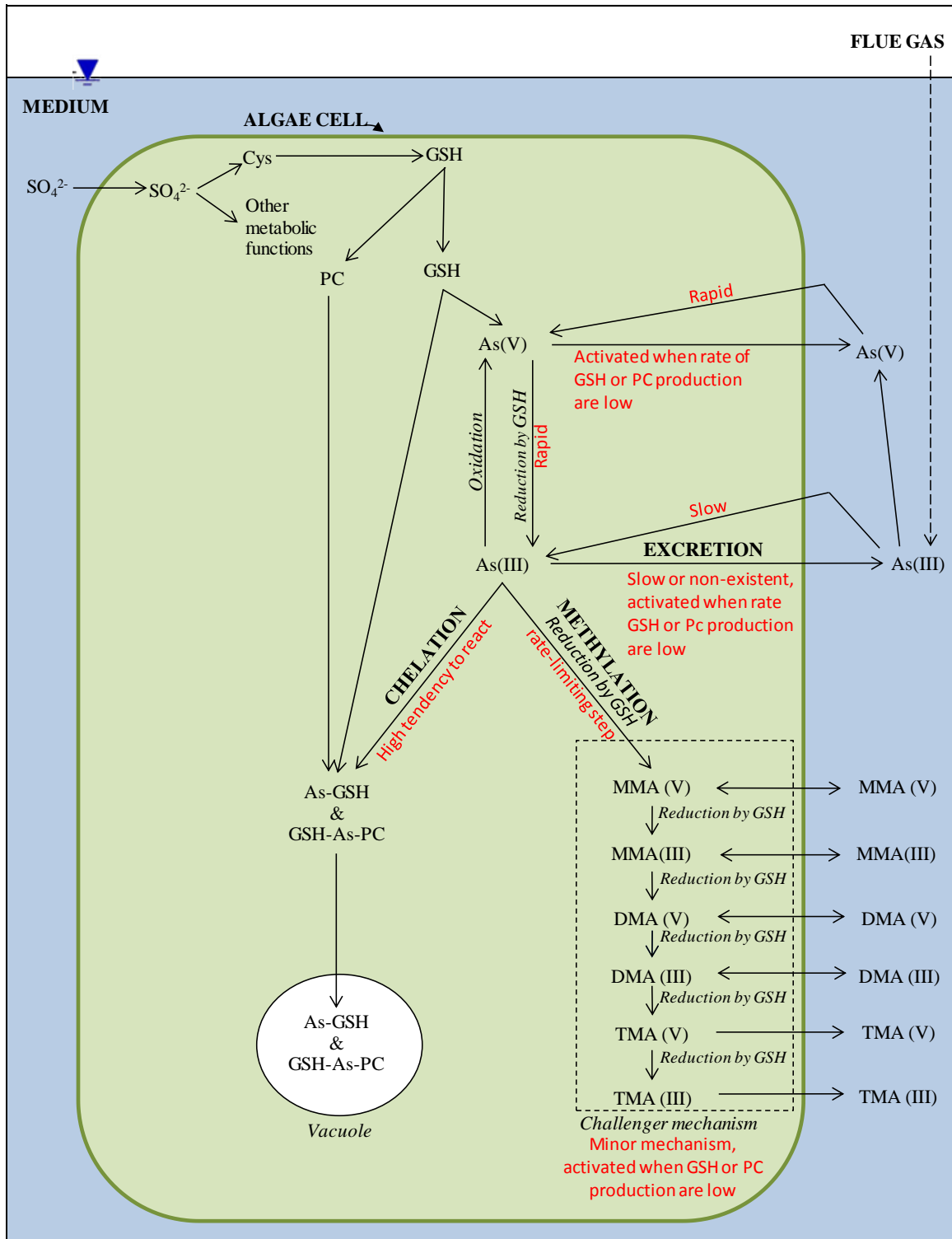


Figure 36. Schematic representation of proposed mechanism enhancing As bioremediation. Glutathione (GSH), phytochelating (PC), As bound to glutathione (As-GSH), As bound to glutathione and phytochelating (GSH-As-PC), monomethyl arsenic (MMA), dimethyl arsenic (DMA), trimethyl arsenic (TMA).

medium were probably insufficient to continue chelation after day 9, leading to excretion after the cell ran out of S-based antioxidants. Further studies of the speciation of As in the medium, the intracellular As compartmentalization and the levels of GSH and PC are needed in order to elucidate the actual mechanisms driving the enhancement of As bioremediation in an integrated carbon capture-algal cultivation facility.

4.5 Conclusions

Algal cultivation systems contaminated with As could be a problem for the production facility. Bioremediation using algae is a promising economical option; however, it faces the challenge that As removal by *Scenedesmus obliquus* is inefficient under the normal cultivation conditions using APS medium. This study tested the hypothesis that S can improve retention of As in the cell and found:

- Increase of S in the medium from 19 to 58 mg S-SO₄²⁻/L improved the removal of As from 15% to 61%. Literature suggests that improved As retention in the biomass could be the result of As chelation by S-based antioxidant molecules.
- Addition of 19.2 to 38 mg S-SO₄²⁻/L produced limited As removal until day 9 and afterwards As was reincorporated in the medium leading to a bioremediation of 15% and 1%, respectively. Literature suggests that poor As retention in the biomass could be the result of inorganic As excretion and As methylation defense mechanisms, usually activated after depletion of S-based antioxidant molecules.

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CHAPTER 5

CONCLUSIONS AND RECOMMENDED FUTURE RESEARCH

5.1 Conclusions

This study aimed to identify the effects that heavy metals from flue gas have on algae used as a feedstock for biofuels. After testing 4 different heavy metal concentrations (1X, 2X, 5X and 10X) in a multimetal system containing 10 heavy metals (As, Cd, Co, Cr, Cu, Hg, Ni, Se, Pb and Zn), the following conclusions can be reached:

- Integration of algal cultivation with carbon capture from flue gas will have to deal with heavy metals redistributed in the biomass, the medium and possibly the by-products.
- On average 87% Hg and 21% Se were lost from the microalgal suspension at 1X, 93% Hg and 16% Se at 5X and 94% Hg and 22% Se at 10X.
- Heavy metals were mostly within the algal biomass. Cd, Pb, Cu, Co, Zn and Cr, were rapidly removed by algae, while the removal of Ni was slower probably due to low affinity with the cell binding sites and due to competition with other cations. Regardless of the mechanisms, it was demonstrated that between 50 to 100% of the heavy metals studied were sorbed in the biomass.
- Heavy metals did affect biomass growth, lipid accumulation and nitrogen uptake in algae. At low concentration (1X) algae responded to the stress with higher growth and larger lipid accumulation, while at higher concentration (2X, 5X and 10X) growth and lipids were inhibited. This behavior is believed to occur as result of heavy metal induced oxidative stress.
- *Scenedesmus obliquus* had a large capacity for sorption of 9 of the 10 heavy metals studied, even at low biomass content. As removal by algae was limited, thus it would probably build up in the recycled medium.
- Bioremediation of As from the medium can be performed by algae, provided that additional sulfur is added to the nutrient medium. It is suggested that the presence of sulfur in the cell induced

algae to perform As complexation instead of As excretion or methylation. More studies are needed to determine the mechanism.

- The concentration of heavy metals in the algal biomass did exceed standards for fish food, human food and paper fillers that are in contact with food. However, the concentrations were below maximum standards for cattle and poultry feedstuff, paper filler, plastic fillers and as biofertilizers.
- The medium produced after harvesting contained low concentration of heavy metals that comply with recommendations for irrigation water. However, these concentrations were above US EPA's aquatic life and human health criteria. Consideration of dilution and mixing lengths may be required before discharging the medium to a water body.

The cultivation of algae using waste streams from other industries are activities that are aligned. Optimal systems can arise if regional alliances between waste producers and algae farms are established. In the big picture, the success of this system can enhance economic growth and energy independence while being environmentally sound.

5.2 Recommended Future Research

- Future studies need to focus on the understanding of the mechanism underlying the biomass and lipid gains and determine if metal stress played a role.
- Study of the effect of individual heavy metals should be carried out in order to understand if the effects observed in growth and lipids is the result of a specific metals or metalloid. This can also serve for the purpose of determining which element is more offensive and should be removed from flue gas to improve productivity.
- The experiments in this study should be repeated using other strain known for their higher oil productivity.
- Heavy metals can alter the final lipid profiles in several organisms. Further determination of fatty acid profile of cells under heavy metals stress can indicate if the saturation or unsaturation

of the lipids or the length chain changes. This can be useful to understand if the lipids are still useful for biofuel applications and if the nutritional character of algae oil are maintained even under heavy metal stress.

- Determine if heavy metals are transferred from biomass to oil and to biodiesel under different extraction and transesterification techniques.
- Other organic and inorganic contaminants present in flue gas should be studied before using contaminated biomass for animal feed.

APPENDICES

Appendix A. Experimental Design

Table A.1. Experimental design

Research objectives	Hypothesis	Experimental design
<p>What are the sinks for heavy metals in a photobioreactor (PBR) production system, <i>i.e.</i>, where do the heavy metals accumulate: biomass or elsewhere?</p>	<p>Concentration of heavy metals in the spiked biomass are greater than the control</p>	<p>Cultures are spiked with 1X, 2X, 5X and 10X heavy metals concentration. Biomass concentration and heavy metal concentration are monitored. Biomass and lipid concentrations from heavy metals contaminated PBRs are compared to biomass and concentrations from metal-free PBRs and tested for statistical differences.</p>
<p>What is the capability of algae to uptake heavy metals and what are their bioremediation capabilities?</p>		
<p>Can heavy metals concentration in the algal biomass and medium affect their uses?</p>	<p>Concentration in biomass and medium are greater than maximum limits stipulated in the regulations</p>	<p>Biomass grown using heavy metals are exposed to several leachant. Biomass grown in free-metal medium is compared to biomass grown with heavy metals and biomass leached with solvents. The concentrations achieved are compared against regulatory standards. Heavy metal concentration in medium is also compared against regulatory standards. Heavy metal concentrations greater than the regulatory standard indicate that the biomass or medium cannot be used in the evaluated activity.</p>
<p>Can bioremediation of As by algae be enhanced by sulfur enrichment?</p>	<p>Addition of sulfur to the medium will reduce As concentration in the medium</p>	<p>Three different S concentrations are tested in a system containing As and a control free of As. Concentration at the end of the experiment are compared and tested for statistical differences.</p>

Appendix B. Determination of Heavy Metal Concentrations

Heavy metal concentration data for uncaptured fly ash is lacking, for this study such concentrations are estimated based on literature using the assumptions, equations and input data summarized in Table B.1 with the final heavy metal concentrations partitioned to the PBR presented in Table B.2. Conservative assumptions were made in order to conservatively evaluate the impact of heavy metals in microalgae cultivation systems integrated with coal based flue gas. The primary assumptions include, the PBR runs at typical CO₂ capture efficiency for an algal growth system [1, 2], heavy metal contamination is derived from uncaptured fly ash, concentration of heavy metals in uncaptured fly ash are equivalent to concentrations in captured fly ash, and heavy metals in the fly ash are fully transferred into the growth medium. Detailed calculations are presented below (equations 1 to 6) with the nomenclature and assumptions presented in Table B.1:

Determination of mass of fly ash per liter of flue gas CO₂ (Ash_{L_gas})

$$Ash_{coal} = (Coal \times Ash_{f_coal}) \times Fly_ash_{f_ash} \quad (1)$$

$$V_{CO_2} = \frac{C_{f_coal} \times Coal \times \left(\frac{MW_{CO_2}}{MW_C}\right)}{\delta_{CO_2}} \quad (2)$$

$$Ash_{L_gas} = \left(1 - \frac{Eff_{fil}}{100}\right) \times \frac{Ash_{coal}}{V_{CO_2}} \quad (3)$$

Determination of the volume of flue gas CO₂ delivered per 1 L culture in PBR ($CO_2\ delivered$)

$$C_{L_culture} = C_f \times X \quad (4)$$

$$CO_{2\ delivered} = \frac{\left(\frac{C_{L_culture} \times \frac{MW_{CO_2}}{MW_C}}{\delta_{CO_2}} \right)}{\frac{Eff_{CO_2}}{100}} \quad (5)$$

Determination of heavy metal concentration (mg/L) in the PBR (M_{PBR})

$$HM_{PBR} = \frac{HM_{fly_ash}}{1000} \times CO_{2\ delivered} \times Ash_{L_gas} \quad (6)$$

Table B.1. Summary of parameters

Parameter	Abbreviation	Value	Unit	Reference
Fraction of carbon in coal	C_{f_coal}	0.6	-	[3]
Fraction of total ash in coal	Ash_{f_coal}	0.22	-	[4, 5]
Fraction of fly ash in total ash	$Fly_ash_{f_ash}$	0.8	-	[6]
Carbon dioxide molecular weight	MW_{CO_2}	44	g/mol	-
Carbon atomic weight	MW_C	12	g/mol	-
Volume of flue gas CO ₂ delivered per 1 L culture in PBR	$CO_2\ delivered$	-	L/L	-
Carbon dioxide density (1 atm, 25°C)	δ_{CO_2}	1.8	g/L	-
Mass of coal	$Coal$	1	g	-
CO ₂ volume in coal	V_{CO_2}	-	L	-
Mass of fly ash	Ash_{coal}	-	g	-
Mass of fly ash per liter of flue gas CO ₂	Ash_{L_gas}	-	g/L	-
Carbon contained in microalgal biomass in 1 L of culture	$C_{L_culture}$	-	g/L	-
Air pollution system filtration efficiency	Eff_{fil}	99	%	[7]
Carbon fraction in biomass	C_f	0.5	-	[8]
CO ₂ capture efficiency	Eff_{CO_2}	4	%	[1, 2, 9]
Algal biomass yield	X	5.3	g/L	-
Heavy metal concentration in fly ash	HM_{fly_ash}	-	mg/kg	-
Heavy metal concentration in PBR	HM_{PBR}	-	mg/L	-

Table B.2. Concentration of heavy metals in fly ash and in PBR

Element	Heavy metal concentration in fly ash (mg metal /kg) [10]	1X heavy metal concentration in PBR *	
		(mg metal/L)	(μ M)
As	391	0.078	1.04
Cd	76	0.015	0.13
Co	79	0.016	0.27
Cr	651	0.13	2.50
Cu	655	0.13	2.06
Hg	49.5	0.010	0.05
Pb	273	0.054	0.26
Ni	1270	0.25	4.33
Se	49.5	0.010	0.13
Zn	2200	0.44	6.73

References

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Appendix C. QA/QC for the Heavy Metals Evaluated

Table C.1. Quality criteria for the study

Parameter	Symbol	Quality criteria [1]
Blank	-	<method detection limit
Percent recovery for laboratory fortified matrix	%R	75-125%
Correlation coefficient	r	>0.995

Table C.1. Summary of the percent recovery

Analyte	r	%R for different matrix		
		Supernatant	Biomass	Total
As	0.999	99.5	103.5	96.5
Cd	0.999	98.6	102.9	103.2
Co	0.999	96.1	104.7	97.3
Cr	0.999	98.4	101.3	98.5
Cu	0.999	99.1	102.3	99.0
Hg	0.995	103.1	115.6	119.0
Se	0.998	87.9	101.8	101.5
Ni	0.999	99.2	101.5	98.5
Pb	0.999	104.4	98.4	100.1
Zn	0.996	84.4	100.9	98.3

References

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Appendix D. Chemical Speciation of Heavy Metals

Table D.1. Metal chemical speciation using MINEQL

Component speciation	Major precipitates	Major dissolved species	Component speciation	Major precipitates	Major dissolved species
As	0.0%	100.0%	Ni	0.0%	100.0%
H ₃ AsO ₃		99.5%	Ni ²⁺		88.6%
Others		0.5%	NiHCO ₃ ⁺		4.7%
Cd	61.5%	38.5%	NiNO ₃ ⁺		2.6%
Cd ²⁺		33.7%	NiSO ₄ (aq)		3.1%
CdCl ⁺		1.9%	Others		1.0%
CdNO ₃ ⁺		1.3%	Se	0.0%	100.0%
CdSO ₄ (aq)		1.4%	HSeO ₃ ⁻		96.20%
CdMoO ₄	61.5%		SeO ₃ ⁻²		3.80%
Others		0.2%	Zn	100.0%	0.0%
Co	0.0%	100.0%	Zn ₃ (PO ₄) ₂	81.4%	
Co(2+)		67.2%	Others	18.6%	
CoHCO ₃ ⁺		2.3%	Hg	0.0%	100.0%
CoHPO ₄ (aq)		26.4%	Hg(OH) ₂		31.5%
CoNO ₃ ⁺		1.3%	HgClOH (aq)		51.3%
CoSO ₄ (aq)		2.4%	HgCl ₂ (aq)		16.9%
Others		0.4%	Others		0.3%
Cr	0.0%	100.0%	Cu	55.0%	45.0%
CrO ₄ ²⁻		71.9%	Cu ²⁺		17.1%
HCrO ₄ ⁻		23.3%	CuOH ⁺		5.5%
NaCrO ₄ ⁻		4.2%	CuCO ₃ (aq)		20.5%
Others		0.6%	Cu ₃ (PO ₄) ₂	55.0%	
Pb	100.0%	0.0%	Others		1.9%
Pyromorphite (Pb ₅ (PO ₄) ₃ Cl)	100.0%				

Appendix E. Modeling the Distribution of Heavy Metals

Khummongkol-Ting model description

As previously reported by Khummongkol, Canterford and Fryer [1], adsorption based models under predicts heavy metals concentration in algae when exposed for long term. In other words Khummongkol, Canterford and Fryer [1] reported the importance of considering intracellular accumulation, which is also documented by Sloof, Viragh and Van Der Veer [2]. Hence, model for this study was chosen to describe processes which included adsorption, internalization and algal growth. Variables considered in the modeling effort include time, algal biomass, metal bound to algal surface, metal internalized and metal in the liquid phase. The mechanistic model that describes metal uptake by algae with growth is described by Khummongkol-Ting [1-3] and is used here to evaluate the experimental data in this study.

The growth data was divided into two phases with the initial phase used to describe the exponential growth and the later phase for the linear growth. Metals data corresponding to those periods were then modeled as either exponential or linear growth rates. The biphasic growth observed in this work is consistent with other studies and is reported by other workers. Parameters for Khummongkol-Ting's model are estimated based as described below:

Estimation of K: Initial uptake data were measured at $t = 5\text{h}$ and was observed to be not equal to zero. Hence, approach used by Ting, Lawson and Prince [3] was modified to account for the metal uptake at $t=5\text{h}$ to estimate K. In other words C_2 was assumed not equal to zero as shown in Equation 1. K value was estimated based on a single point using the estimation procedures given by the authors.

$$K = \frac{X_5 m}{A - m - X_5 C_2} \quad (1)$$

Estimation of μ : The 1st phase of growth was well described by an exponential growth equation (equation 2). Figure E.1 shows the regression analysis performed to estimate the exponential growth rate μ which was found to be 0.2356 /d.

$$x = X_o * e^{\mu * t} \quad (2)$$

where

X_o = Initial algae concentration (mg/L)

t = time (h)

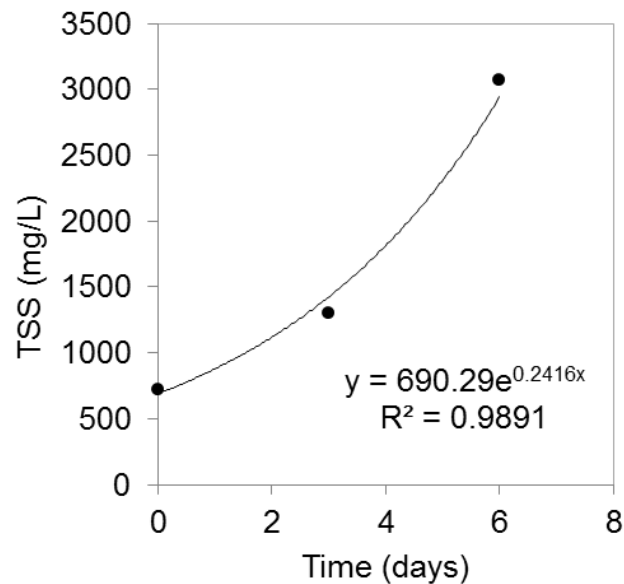


Figure E.1. Regression analysis to estimate the exponential growth rate μ . Filled symbols indicate experimental data; solid line is fit considering exponential growth.

Estimation of L: The 2nd phase of growth was described well using a linear growth model. L, the linear growth rate was estimated as the slope of the line from day 8 to day 24. A linear regression was performed using Excel and the value of L was estimated to be 113.01 mg dry wt/L.h.

Estimation of R_1 and R_2 : These parameters are estimated using multiple regression using the Excel's Data analysis add-in with the zero intercept checked. The equation used to solve is given in Equation 3.

$$\frac{d\alpha}{dt} = \beta R_1 - \alpha R_1 R_2 \quad (3)$$

where α and β are defined as shown in Equations 4 and 5

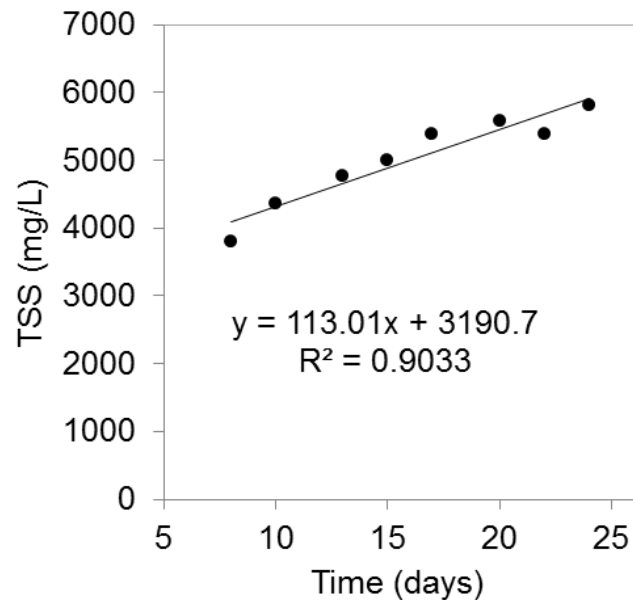


Figure E.2. Regression analysis to estimate the linear growth rate L. Filled symbols indicate experimental data; solid line is fit considering linear growth.

$$\alpha = \frac{KA - m(K + x)}{K} \quad (4)$$

$$\beta = \frac{xm}{K} \quad (5)$$

where

m = Equilibrium concentration of adsorbate (mg/L)

Using the estimated values, parameters equations 6 and 7 are solved for the exponential growth phase and equations 8 and 9 for the linear growth phase. Ode's given in equations 7 and 9 ([3]) are solved numerically using Matlab's ode45 routine (see example below). The heavy metals concentration in biomass predicted by this model are shown in Figure E.3.

$$S_2 = \frac{R_1}{\mu} \quad (6)$$

$$\frac{d(x[C_2])}{dx} + S_2 \left(\frac{1}{K + x} + \frac{R_2}{x} \right) x[C_2] = \frac{S_2 A}{K + x} \quad (7)$$

$$S_1 = \frac{R_1}{L}$$

$$\frac{d(x[C_2])}{dx} + S_1 \left(\frac{1}{K + x} + R_2 \right) x[C_2] = \frac{S_1 A x}{K + x}$$

Example of Matlab routine used to solve Ting's model:

```
% the following m file is used to solve the ode
% to estimate the value of z, which is defined as x(C2)
% this file is used to model the As uptake during the exponential growth phase

function rk = f_exp_As(x,z)      % Function declaration
mu = 0.2356;                    % mu: Exponential growth rate (day-1)
A = 0.099096667;               % A: total metal concentration in the reactor (mg/L)
K = 3043.644169;               % K: adsorption constant (mg cell dry wt/L)
R1 = 5.378481219;              % R1: carrier rate constant (unitless)
R2 = -2.704999328;             % R2: ratio of chemical reaction rate constants (unitless)
S2 = R1/mu;

rk = S2*A/(K+x)-S2*(1/(K+x)+R2/x)*z; % Equation 27 from Ting et. al, 1989
```

Command window

```
>> x_range = (721.35 3073.8);
>> mu = 0.2356;
>> X0 = 705.2130812;
>> z_initial = 0.000779767;
>> (x,z)=ode45(@f_exp_As,x_range,z_initial);
>> C2 = z./x;
>> t=(log(x/X0))/mu;
>> plot(t,C2);
```

References

- [1] D. Khummongkol, G.S. Canterford, C. Fryer. Accumulation of heavy metals in unicellular algae, *Biotechnol. Bioeng.* 24 (1982) 2643-2660.
- [2] J. Sloof, A. Viragh, B. Van Der Veer. Kinetics of cadmium uptake by green algae, *Water, Air, and Soil Pollution* 83 (1995) 105-122.
- [3] Y.P. Ting, F. Lawson, I.G. Prince. Uptake of cadmium and zinc by the alga *Chlorella vulgaris*: Part 1. individual ion species, *Biotechnol. Bioeng.* 34 (1989) 990-999.

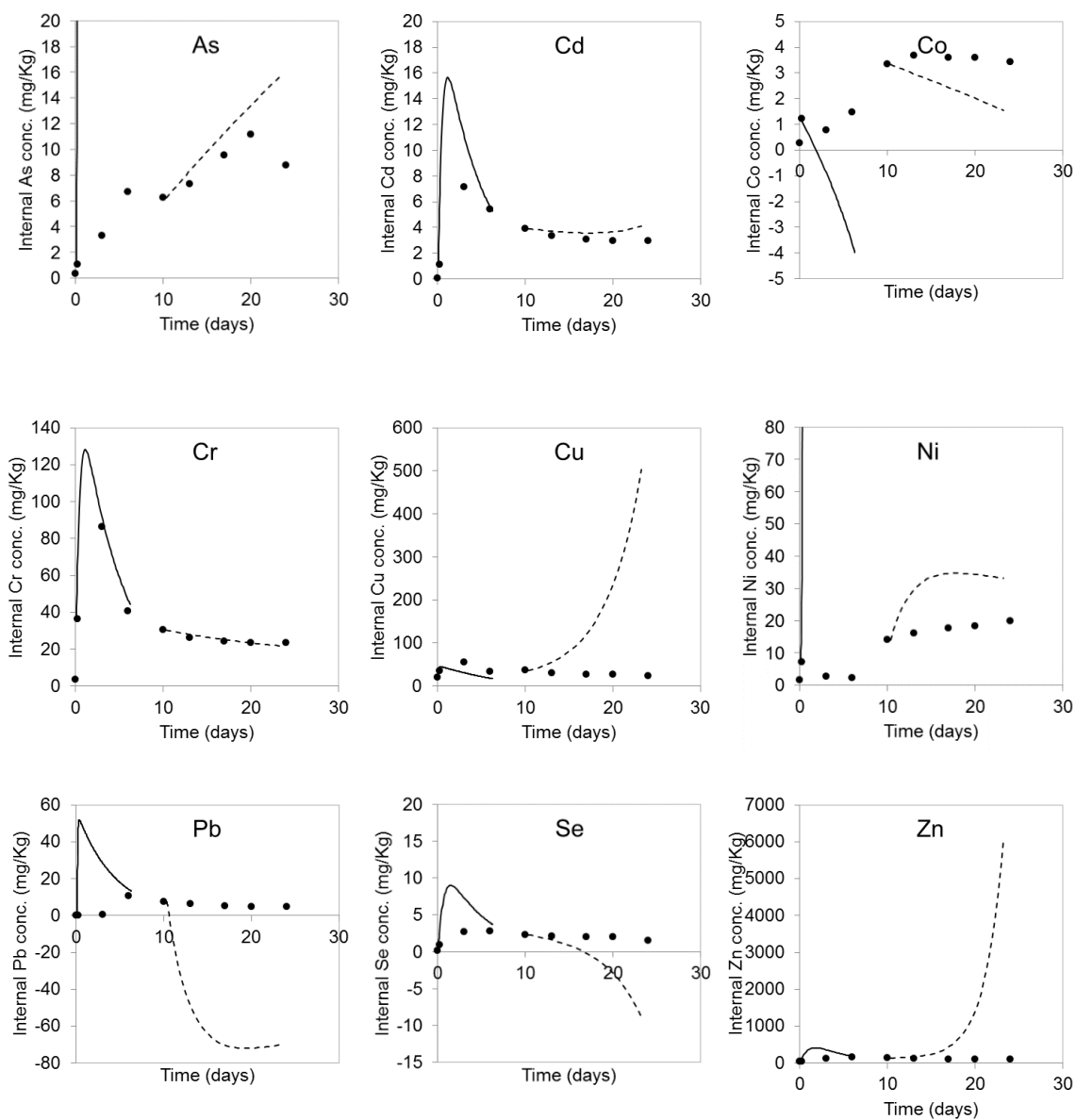


Figure E.3. Predicted heavy metal concentration in biomass due to absorption and uptake. Filled symbols indicate experimental data; solid and dashed lines are fit considering an exponential and linear growth phases respectively. The Y axis ranges are different for each plot.

Appendix F. Regulations Concerning Heavy Metals in Algal Biomass and Medium

Table F.1. Heavy metals standards concerning the medium

Heavy metal	Criteria concentration (ug/L)			
	Human health Consumption of water + organism (EPA) [1]	Drinking (EPA) [2]	Aquatic life CCC (EPA) [1]	Irrigation (FAO) [3]
As	0.018	10.00	150.00	100.00
Cd	Z	5.00	0.25	10.00
Co	NS	NS	NS	50.00
Cu	1300.00	1300.00	1.45	200.00
Cr	Z	100.00	11.00	100.00
Hg	NS	2.00	0.77	2.00
Ni	610.00	100.00	52.00	200.00
Pb	NS	15.00	2.50	5000.00
Se	170.00	50.00	5.00	50.00
Zn	7400.00	5000.00	120.00	200.00

Z = A more stringent maximum level contaminant has been issued by EPA under the Safe Drinking Water Act

NS = No standard established

CCC = Criterion Continuous Concentration (Chronic)

References

[1] United States Environmental Protection Agency. National recommended water quality criteria <http://water.epa.gov/scitech/swguidance/standards/criteria/current/index.cfm>, accessed August 2010.

[2] United States Environmental Protection Agency. Drinking water contaminants, 2013.

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

Table F.2. Heavy metal standards concerning the biomass

Maximum concentration (mg/kg)							
Heavy metal	Human raw dietary supplement (NSF) [2]	Feed			Fertilizer		Plastic/paper [1]
		Cattle (NRC) [3]	Fish (NRC) [3]	Poultry (NRC) [3]	Compostable (ASTM D6400) [4]	Biofertilizer (AAPFCO) [5]	
As	5.0	30.0	5.0	30.0	21.0	41.0	
Cd	0.3	10.0	10.0	10.0	20.0	39.0	
Co	NS	25.0	NS	25.0	NS	NS	
Cu	NS	40.0	100.0	250.0	750.0	1500.0	Sum of Hg, Pb, Cd and Cr(VI)
Cr	NS	100.0	NS	500.0	NS	NS	
Hg	NS	NS	1.0	0.2	9.0	17.0	
Ni	NS	100.0	50.0	250.0	210.0	420.0	< 100.0
Pb	10.0	100.0	10.0	10.0	150.0	300.0	
Se	NS	5.0	2.0	3.0	50.0	100.0	
Zn	NS	500.0	250.0	500.0	1400.0	2800.0	

References

[1] European Paper and Board Food Packaging Chain. Industry guideline for the compliance of paper and board materials and articles for food contact, <http://www.cepi.org/system/files/public/documents/publications/foodcontact/2012/Industry%20guideline-updated2012final.pdf>, 2012, accessed August 2013.

[2] NSF International. Dietary supplement-Standard 173, metal contaminant acceptance levels, http://standards.nsf.org/apps/group_public/download.php/15297/DS-2007-5%20Supplementary%20-%202003_NSF%20DS%20Metal%20Contaminant%20Acceptance%20Levels.pdf, 2003, pp. 22.

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

[5] Association of American Plant Food Control Officials. AAPFCO's statement of uniform interpretation and policy (SUIP) #25: the heavy metal rule, <http://www.aapfco.org/rules.html>, accessed August 2013.

Appendix G. Comparison of Heavy Metal Uptake Removal Capacity by Algae

Table G.1. Comparison of Heavy Metal Uptake Removal Capacity by Algae. Concentration in Harvested Biomass (mg/g).

Heavy metal	This study (mg/g)	Other studies [1]	
		(mg/g)	Strain
Cd	0.088	48.33	<i>Pilayella littoralis</i>
		120.04	<i>S. platensis</i>
		112.40	<i>S. vulgaris</i>
Co	0.08	15.32	<i>Oscillatoria angustissima</i>
		55.40	<i>Pilayella littoralis</i>
		0.01	<i>Spirulina sp.</i>
Cu	0.55	45.97	<i>Ulva reticulata</i>
		66.72	<i>Palmaria palmata</i>
		54.01	<i>Pilayella littoralis</i>
Cr	0.63	45.75	<i>Sargassum sp.</i>
		25.98	<i>Spirulina platensis</i>
		54.60	<i>Padina sp.</i>
Hg	0.003	4.68	<i>Pilayella littoralis</i>
		31.72	<i>Sargassum sp.</i>
		1.40	<i>Spirulina sp.</i>
Ni	1.02	180.83	<i>Sargassum sp.</i>
		30.18	<i>Scenedesmus obliquus</i>
Pb	0.35	349.09	<i>Laminaria japonica</i>
		304.56	<i>Lyngbya taylorii</i>
Zn	1.83	641.28	<i>O. angustissima</i>
		29.42	<i>Pilayella littoralis</i>
		88.96	<i>Sargassum fluitan</i>

Reference

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

Appendix H. Estimation of Heavy Metals in Biodiesel

The estimation of the potential heavy metals concentration transferred from biomass to biodiesel was done using the equation below and considering *Scenedesmus obliquus* with a 14.4% lipid content per dry biomass.

$$C_{md} = \frac{k \cdot C_m \cdot W}{\left(\frac{Eff \cdot P_o \cdot W}{F} \right)}$$

where

C_{md} = Metal concentration in biodiesel in $\frac{\text{mg metal}}{\text{L biodiesel}}$

k = Metal transfer factor from algae to biodiesel (unitless)

C_m = Concentration of metal in biomass in $\frac{\text{mg metal}}{\text{kg algae}}$

W = algae biomass in kg

Eff = oil extraction efficiency (unitless)

P_o = Percent oil content in algae biomass in $\frac{\text{kg unrefined oil}}{\text{kg algae}}$

F = Biofuel conversion factor in $\frac{\text{kg unrefinedoil}}{\text{L biodiesel}}$

Table H.1. Potential heavy metals concentration transferred from biomass to biodiesel

Element	Concentration in algal biomass in this study (mg/kg)	Concentration in diesel fuel (mg/L) [1]	Metal transfer factor		
			k=1% Concentration in diesel fuel (mg/L)	k=5% Concentration in diesel fuel (mg/L)	k=10% Concentration in diesel fuel (mg/L)
Cr	23.54	4.40	1.89	9.43	18.85
Co	4.10	2.04	0.33	1.64	3.29
Ni	39.73	2.61	3.18	15.91	31.82
Cu	26.26	2.78	2.10	10.51	21.03
Zn	101.31	5.63	8.11	40.57	81.13
Cd	3.03	0.53	0.24	1.21	2.42
Pb	7.26	2.04	0.58	2.91	5.81

Reference

- [1] Y.-F. Wang, K.-L. Huang, C.-T. Li, H.-H. Mi, J.-H. Luo, P.-J. Tsai. Emissions of fuel metals content from a diesel vehicle engine, *Atmos. Environ.* 37 (2003) 4637-4643.

VITA

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EDUCATION

Utah State University, Logan, UT

- Doctor of Philosophy in Biological Engineering *January 09-Present*
- Master of Science in Biological and Irrigation Engineering *September 07-December 08*

Ohio State University, Columbus, OH

September 2012

- Entrepreneurship of scientific research, a workshop on REACH for commercialization organized by project CEOS.

Wageningen University-Holland, ONU/CEPAL and IPROGA-Peru, Lima, Peru *January 05*

- Water Law and Indigenous Rights: Anthropology of water rights; Water, gender and power; Local, federal and project water laws; and integrated management of water in a basin.

National Agrarian University, Lima, Peru

- Specialization in Agricultural Business Management. *October 02-April 03*
- Bachelor of Engineering in Agricultural Engineering (**First Place**). *August 97-July 02*

Julio César Tello, Lurín, Peru

- Elementary, middle and high school (**First Place for all 11 years**). *March 86-December 96*

CERTIFICATION

- Laboratory Safety Training *May 10 and Annual refresher*
Chemical hygiene principles, spill prevention, hazardous waste management, fire safety.

EXPERIENCE

RENEWABLE ENERGY AND WATER QUALITY-RELATED EXPERIENCE

Utah State University, UT, USA

Effects of metals from flue gas on microalgae biofuels and co-products: Sustainability and scalability (Funding Agency: National Science Foundation)

[Type text]

Graduate Research Assistant

September 2013 - present

NSF is funding the continuation of my PhD work. This new project will study (i) heavy metals carry-over from biomass to biodiesel, (ii) effects of heavy metals present in the algal remnant on fermentation processes and (iii) life-cycle analysis and scalability of the integrated processes. Currently I am working on:

- Developed the experimental design, standard operating procedures and build photobioreactors with automated CO₂ delivery and datalogging capabilities.
- Teach graduate and undergraduate students.

Project: Effect of heavy metals from flue gas in algae growth, FAME production and metal distribution (PhD); Funding Agency: Arizona Public Service-APS and Department of Energy-DOE

Graduate Research Assistant

January 09 – present

APS and DOE wanted to recycle CO₂ from coal fired power plants' flue gas by growing algae and producing algal biodiesel and other co-products. I studied the interactions between heavy metals, nutrient medium and algae, and recommended practical applications (bioremediation, fuel, feed, fertilizer, paper and plastic uses).

- Performed cultivation of several algal strains (fresh and sea water) in open and close bioreactors, with daily monitoring of growth parameters (nutrient analysis, pH, density by optical density).
- Evaluated fate and distribution of 10 heavy metals (Cd, Co, Cu, Pb, Se, As, Hg, Zn, Cr and Ni), based on the physical interaction with cell wall and bioreactor, the chemical interaction with the nutrients and the biological processes inside the cells.
- Produced biodiesel from algae biomass by acid catalized *in-situ* transesterification.
- Analyzed samples using ICP-MS, IC, GC-MS and AAS.
- Used mathematical models for metal distribution in batch reactor.
- Developed a technique for the bioremediation of Arsenic-contaminated water using algae.
- Evaluated the practical applications for heavy metal contaminated biomass (biodiesel, animal feed, fertilizer, paper and plastic fillers) and for the spent medium (*e.g.* irrigation water, recreation and aquaculture uses).
- Co-authored a proposal for NSF funding (US\$ 300 000) for production of other fuels (*e.g.* methane, acetone, butanol and ethanol) using heavy metal contaminated algal remnants.

Project: Algal-based biofuel production at the Logan Lagoons Wastewater Treatment Plant-WWTP (Funding Agency: City of Logan, UT)

Graduate Research Assistant

July 09 – September 09

The Logan Lagoons WWTP uses microalgae for removing nutrients from wastewater and wants to harvest this microorganism from the effluent to produce biofuel. Utah State University works with the City of Logan to achieve this goal and I collaborated with the Logan Lagoons team in the following activities:

- Developed protocol and performed analysis of chlorophyll content in samples collected from 7 ponds of the Logan Lagoons WWTP.

- Evaluated dissolved air flotation technique for algae harvesting from wastewater under various coagulant settings.

EVALUATION OF PROJECTS-RELATED EXPERIENCE

Ministry of Economy and Finance of Peru, Lima, Peru

General Direction of Public Sector Multi-Year Programming – Office of Emergency Projects:

Technical Evaluator of Emergency projects

September 06 – August 07

The General Direction is in charge of planning and approval of disaster-relief projects across the nation (similar to US FEMA). Some activities performed were:

- Evaluated economic and technical feasibility of disaster-relief projects (e.g. re-seed and crop fertilization, animal feed distribution, food assistance and reconstruction of water treatment plants, water wells, irrigation canals, bridges, roads, river protection, schools, etc.). Feasible emergency projects were recommended to the President’s Council of Ministers for approval of emergency funds (funds up to US \$ 16.9 million).
- Participated in the development of “Methodological Guidelines for the Incorporation of Analysis of Risks in Public Investment Projects” produced in partnership with Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ)
http://www.mef.gob.pe/inv_publica/docs/instrumentos_metod/PautasRiesgos.pdf
- Reviewed and provided technical opinion of new decrees and laws concerning emergency projects proposed by the congress, ministries and other regulatory institutions.

AGRICULTURAL WATER MANAGEMENT-RELATED EXPERIENCE

Utah State University, Utah, USA

Utah Water Research Laboratory:

Project: Seepage evaluations in Cache Valley Irrigation System (MS; Funding Agency: Utah Water Research Laboratory)

Graduate Research Assistant

October 07 – December 08

Cache Valley irrigation system has seepage losses and the canal companies needed to identify the sections with highest seepage rates in order to plan water management and canal lining. Some activities performed were:

- Assessed canal seepage using acoustic Doppler flow meter.
- Evaluated the behavior in 11 canals in Cache Valley Irrigation System and related the observations with soil type, landscape, water flow and local physical characteristics.
- Evaluated the performance of 6 installed Parshall flumes in Tremonton Irrigation System.
- Calibrated, installed and monitored data loggers (WaterLOG® H-500XLTM) and water level sensors (WaterLOG® H-3301, digital shaft encoders with a 12-inch circumference pulley, float

and counterweight) in stilling wells adjacent to Parshall flumes and Broad crested weirs in 2 irrigation canals.

- Evaluated flow meters: mechanical Price-Pygmy with vertical axis, mechanical Ott with horizontal axis, electromagnetic Marsh McBirney and FlowTracker acoustic Doppler velocimeter.

Ministry of Agriculture of Peru, Lima, Peru

Sub-Technical Administration of Lurin and Chilca Sub Irrigation District:

Assistant Engineer

September 04 – August 06

I performed technical evaluations that guided Chief Engineer in the decision making. I was the only technical evaluator and high responsibility and self-organization was a key skill. Some activities performed were:

- Evaluated the technical feasibility study to increase the capacity of Tuctococha dam (3 million m³).
- Technical evaluation of studies for the industrial discharges into water bodies.
- Technical evaluation of studies for the modification of riverian and irrigation infrastructure.
- Provided technical assistance in the irrigation water management and crop scheduling
- Delineated and verified floodplain boundaries at sectors in Chillón, Rimac, Lurin and Chilca rivers.
- Performed field inspection, used tools such as AutoCAD, ArcGIS, HEC-RAS and authored technical reports.

Technical Administration of Mala-Cañete Irrigation District:

Intern

July 03 – September 03

This Administration is responsible for water resources planning. I was in charge of the following activities:

- Discharge measurement in irrigation canals.
- Measured and monitored parcel irrigation and runoff in 7 irrigation company lands.
- Performed field tests (water advance/recession in furrows; soil core sampling, auger sampling, soil density, moisture determination).
- Performed technical evaluation and calibration of infrastructure (e.g., flumes and weirs)

Water Management Authority of Santa-Lacramarca River (Chinecas Project):

Intern

February 01 – March 01

CHINECAS is a hydroelectric and irrigation project that works towards diverting water from El Santa river to irrigate 50000 hectares in arid land. During this internship I was in charge of:

- Trained water users to create update and maintain conveyance infrastructure inventory.
- Performed inventory of irrigation infrastructure and generated maps.

PRESENTATIONS

- Napan, K.; Butler, R.; Sims, R.; Wood, B. and Quinn, J. C. 2014. Environmental impact and effects of heavy metals on microalgae productivity from integration with flue gas. Algae Biomass, Biofuels and Bioproducts organized by ELSEVIER conferences. June 15 - 18, 2014. Santa Fe, NM.
- Napan, K.; Sims, R.; Wood, B. and Quinn, J. C. 2014. Microalgae cultivation in photobioreactors: growth parameter optimization. USU-UNLV Symposium on Biotechnology, Renewable Energy, and Novel Materials. May 5 – 6. Las Vegas, NV.
- Napan, K.; Butler, R.; Wood, B. and Quinn, J. C. 2014. Influence of heavy metals from flue gas integration with algal production on biodiesel making. USU Graduate Research Symposium. April 10, 2014. Logan UT. **Honorable mention** in the Engineering category competition.
- Napan, K. and Quinn, J. C. 2014. Assessment of heavy metal effects in algal-biodiesel production with flue gas integration. SBI conference February 12, 2014. Logan UT.
- Napan, K.; Teng, L. and Wood, B. R. 2013. Distribution of heavy metals from flue gas in algal bioreactor – A bioremediation and biodiesel production focus. 7th Annual ABO Summit 2013. September 30-October 3, 2013. Orlando, FL. **2nd place** in the poster competition.
- Napan, K.; Teng, L.; and Wood, B. Speaker at the **invitation-only** “2012 Biodiesel Technical Workshop” organized by the National Biodiesel Board. October 31, Kansas City.
- Napan, K.; Butler, R.; Teng, L.; Wood, B. and Sims, R. 2012. Biodiesel production in combination with bioremediation of heavy metals from flue gas using microalgae. 6th Annual ABO Summit 2012. September 24-27, 2012. Denver, CO. **2nd place** in the poster competition.
- Napan, K.; Butler, R.; Wood, B. and Sims, R. 2012. Long term algal biomass and FAMEs yield from mono and mixed heavy metals exposed batch reactors. IBE Annual Meeting 2012. March 1-3, 2012. Indianapolis, IN.
- Butler, R.; Napan, K.; Wood, B. and Sims, R. 2011. Effects of heavy metals from flue gas on algal biomass for biodiesel: A focus on growth, lipid accumulation and biomass/water quality. Algae Biomass Summit Meeting. October 25-27, 2011. Minneapolis, MN.
- Napan, K.; Butler, R.; Sims, R.; Wood, B. 2011. Effects of heavy metals from flue gas on alga growth and lipid production and their distribution in the system. Spring meeting & 7th Global Congress on Process safety. The American Institute of chemical Engineers, AIChE. March 13-17, 2011. Chicago, IL.
- Napan, K.; Butler, R; Sims, R. and Wood, B. 2010. **Panel presentation**: Impact of metals from flue gas on algae growth and its distribution in the system. Biomass'10, Renewable power, fuels and chemicals workshop. The Energy & Environmental Research Center, EERC. July 20-21, 2010. Grand Forks, ND.

- Napan, K.; Butler, R; Sims, R.; Viamajala, S. and Wood, B. 2010. Impact of metals from flue gas on algae growth and its distribution in the system. 2010 Annual conference. Institute of Biological engineering, IBE. March 4-6, 2010. Boston, MA.

PUBLICATIONS

- Napan, K.; Teng, L; Quinn, J.C. and Wood, B. 2014. Impact of heavy metals from flue gas integration with microalgae production. *Algal Research*. In-review.
- Napan, K.; Hess, D.; McNeil, B. and Quinn, J.C. Quantification of inorganic contaminants and their impact on microalgae productivity. *Journal of visualized experiments*. In-review.
- Napan, K.M.; Merkley, G.P. and Neale, C.M.U. 2009. Seepage evaluations in Cache Valley irrigation canals. *Proceeding Fifth International Conference on Irrigation and Drainage – USCID*. Salt Lake City, UT.
- Napan, K. 2008. Seepage evaluations in Cache Valley irrigation canals. MS thesis, Utah State University master thesis. Logan, UT.
- Study of pre-feasibility for the installation of a factory of flour and maca pills (*Lepidium Peruvianum Chacón*) for export to American and Japanese market. Undergraduate thesis. La Molina National Agrarian University. Lima, Peru. 2003.

COMPUTER SKILLS

- *Chemical Speciation*: MINEQL
- *Hydraulics*: HEC-RAS
- *Programming*: MATLAB, Visual Basic
- *Statistics*: R, Minitab
- *Optimization Tools*: LINDO
- *Technical Drawing and Mapping*: Autodesk Map, ArcGIS and Surfer
- *Other tools*: MS Office

LEADERSHIP/ SERVICE

- Graduate student representative at Biological and Irrigation Engineering department (2008-2009)
- Design and taught summer school classes for low income teenagers in Peru (2004)
- Student representative in the La Molina National Agrarian university council (year 2001)
- Student representative in the Agriculture Engineering department council (year 2000)

AWARDS

- 2nd place in the 2013 Algae Biomass Summit poster competition
- 2nd place in the 2012 Algae Biomass Summit poster competition
- 2012 CEOS at Ohio State University Award
- 2012 RGS Graduate Student Award
- 2012 Graduate Student Senate Award
- 2010 Graduate Student Senate Award