THE APPLICATION OF ALGAE FOR THE REMOVAL OF METALS AND NAPHTHENIC ACIDS FROM OIL SANDS TAILINGS POND WATER

by

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

Doctor of Philosophy

in

Environmental Engineering

Civil and Environmental Engineering
University of Alberta

ABSTRACT

Bitumen recovery produces a large volume of TPW, which must be reclaimed using an efficient and cost-effective method. In this thesis, the ability of algae for *in situ* treatment of TPW, with a focus on metal and total acid-extractable organics (TAOs) removal, was assessed.

An indigenous alga, found in cyclone overflow water, was capable of removing metals ^{53}Cr, Mn, Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, As, ^{88}Sr, ^{95}Mo and Ba. Through the amplification of the 23S rRNA gene, the indigenous alga was identified as *Parachlorella kessleri*. Highest metal removal was achieved with the highest nutrient supplements (1.98 mM NO_3^- and 0.20 mM PO_4^{3-}) in Syncrude TPW, and the lowest nutrient supplements (0.24 mM NO_3^- and 0.016 mM PO_4^{3-}) in Albian TPW. This implies that higher concentrations of nutrient supplements do not necessarily improve the metal removal efficiency. FT-IR spectra revealed the presence of amide I, amide II bands, and carboxylic functional groups on the surface of *P. kessleri*; these sites likely contribute to metal removal. However, an acid-base titration showed that the carboxylic group was the only active proton binding site for metal binding. Intracellular bioaccumulation was the dominant mechanism of metal removal, with extracellular bioaccumulation and precipitation playing a smaller role.

For the biodegradation of TAOs, a consortium of indigenous algae-bacteria was employed. Bacteria demonstrated the greatest removal of TAOs with a half-life removal rate of 203 days (first-order kinetics). The TAO removal rate did not

correlate with detoxification of TPW, where most toxicity reduction was observed in samples containing the algae-bacteria consortium. Principal component analysis (PCA) was conducted on the FT-IR spectra. The significant loading wavenumbers, which likely indicated bio-transformed functional groups and bonds in the TAO molecules, were identified as: hydroxyl, carbocyclic and amid groups along with C-H, aryl-H, aryl-OH and N-H bonds. The observations from this research indicate that indigenous algae play an important role in the removal of metals and TAOs from oil sands TPW. However, for future engineering application, more investigates are required to optimize the operating conditions to improve the removal efficiency.

Preface

This thesis is an original work by Hamed Mahdavi under supervision of Dr. Ania C. Ulrich and Dr. Yang Liu. Chapter 3 of this thesis has been published as Mahdavi, H., Ulrich, A.C., Liu, Y., 2012. "Metal removal from oil sands tailings pond water by indigenous micro-alga." *Chemosphere*. 89, 350-354. In addition, a version of Chapter 4 has been published as Mahdavi, H., Liu, Y., and Ulrich, A. C. (2013). "Partitioning and Bioaccumulation of Metals from Oil Sands Process Affected Water in Indigenous *Parachlorella Kessleri", Chemosphere*, 90(6), 1893-1899.

Principal Component Analysis of FT-IR spectra in Chapter 5 (Figure 5-4c and Appendix Q) was kindly conducted by Dr. Vinay Prasad in Department of Chemical and Material Engineering at the University of Alberta.

Dedication

I would like to dedicate this thesis with my deepest love and gratitude to my kind grandmother and grandfather for all their sacrifices they made on my behalf; my sweet heart, Azadeh, for all her affection and love; and my sister, Maryam, for being a tremendous support all my life.

Acknowledgements

I would like to acknowledge and thank my advisors, Drs. Ania C. Ulrich and Yang Liu, for their continuous supervision, advice, and patience throughout my course work and research. Their invaluable guidance and comments allowed me to develop my knowledge in the field of environmental engineering. It was a great pleasure to have the opportunity to work under their supervision. Kind collaboration of Dr. Vinay Prasad for conducting the principal component analysis is appreciated.

I would like to gratefully acknowledge the Helmholtz Alberta Initiative, University of Alberta Teaching and Learning Enhancement Fund, Canadian School of Energy and the Environment, and Natural Sciences and Engineering Research Council of Canada (NSERC) Collaborative Research and Development (CRD) Grant for providing generous funding for my research. I would also like to thank Syncrude Canada Ltd and Albian for providing tailings pond water samples. This research would not have been possible without the technicians in the Civil and Environmental Engineering Department at the University of Alberta, including Elena Dlusskaya, Christine Hereygers, Jela Burkus, Maria Demeter, Steve Gamble, and Judy Yu. Their valuable support, suggestions, and assistance aided the success of my experiments.

I am also thankful to my good friends (too many to list here) for providing their support and friendship.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

APPI	Atmospheric Pressure Photoionization
As	Arsenic
ATS	Patented Algal Turf Scrubber
Ba	Barium
BCF	Bioconcentration Factor
BLM	Biotic Ligand Model
BOD	Biochemical Oxygen Demand
CCME	Canadian Council Of Ministers of The Environment
Cd	Cadmium
CG-MS	Gas Chromatography Mass Spectrometry
Co	Cobalt
COD	Chemical Oxygen Demand
CPCC	Canadian Phycological Culture Centre
Cr	Chromium
Cu	Copper
DCM	Dichloromethane
DGGE	Denaturing Gradient Gel Electrophoresis
DIC	Dissolved Inorganic Carbon
DO	Dissolved oxygen
DOC	Dissolved Organic Carbon
EC50	Effective Concentration 50%
EPA	Environmental Protection Agency

ESI-FT-ICR MS	Ultrahigh Resolution Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
ESI-MS	Electrospray Ionization-Low Resolution Mass Spectrometry
FT-IR	Fourier Transform Infra-Red
GCxGC/MS	Gas Chromatography–by-Gas Chromatography/Mass Spectrometry
HPLC/HRMS	High-Pressure Liquid Chromatography/High-Resolution Mass Spectrometry
HRAP	High-Rate Algae Pond
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
IC20	Inhibitory Concentration 20%
KBr	Potassium Bromide
LC50	Lethal Concentration 50%
LCxLC/MS	Liquid Chromatography-by-Liquid Chromatography/Mass Spectrometry
MCT	Mercuric Cadmium Telluride
MLSB	Mildred Lake Settling Basin
Mn	Manganese
Mo	Molybdenum
NAs	Naphthenic Acids. NAs is a classical term for the total acid organics in the crude oil or bitumen which follows the following formula: $C_nH_{2n+Z}O_2$, where n is the total carbon number and Z represents the hydrogen deficiency indicating a homologous series. Due to a large deviation in molecular structure, there are some organic acids that do not follow the general formula for NAs; therefore some recent publications use the term total acid-extractable organics (TAOs) instead of NAs.
Ni	Nickel
NMR	Nuclear Magnetic Resonance

PAW	Process-Affected Water. PAW is a general term for the wastewater produced by the oil sands industry. Due to toxicity, PAW is impounded in some tailings pond. Tailings pond water (TPW) is the term that is used for the PAW after collection in tailings ponds. The water chemistry of PAW may be different from TPW.
Pb	Lead
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SAGD	Steam-Assisted Gravity Drainage
Sample A-B	A tailings pond water sample for algae and bacterial growth
Sample A-B-N	A tailings pond water sample for algae, bacterial, and *Navicula pelliculosa* growth
Sample B	A tailings pond water sample for bacterial growth
Sample N	A tailings pond water sample for *Navicula pelliculosa* growth
Sr	Strontium
TAN	Total Acid Number
TAOs	Total Acid-Extractable Organics. To find the difference with naphthenic acids (NAs) please see NAs.
TPW	Tailings Pond Water. TPW is process affected water (PAW) which is impounded in tailings ponds.
USEPA	US Environmental Protection Agency
Zn	Zinc

CHAPTER 1: INTRODUCTION

1.1. Oil Sands Industries and Process-Affected Water Production

The third largest oil deposit in the world is located in Alberta, Canada (170.2 billion barrels or 11 percent of total global oil reserves), and 99% of oil deposits occur in the form of oil sands. In 2011, the oil sands industry in Alberta produced more than 1.7 million barrels of crude bitumen per day (www.energy.alberta.ca). Every produced barrel of oil is accompanied by 1.25 m^3 of tailing (sand, fines and water mixture). Presently this process operates under a zero-discharge policy held by the Alberta Environmental Protection and Enhancement Act (1993) (Giesy et al. 2010). Therefore, process-affected water (PAW) is collected in large tailings ponds on site, according to compliance with environmental regulations.

Recycling and reusing PAW reduces reliance on fresh water by 80-85%, however, it still increases the concentration of pollutants in recycled water (Allen 2008). The volume of the tailings pond water (TPW) in Syncrude's Lease 17/22 was around 1 billion m^3 in 2004 (Allen 2008) and in Mildred Lake Settling Basin (MLSB) was more than 200 m^3 in 2007 (Siddique et al. 2007). The total volume of impounded tailings sludge from all oil companies has reached more than 700 million m^3 (Allen 2008). Oil sands companies are responsible for reclaiming the TPW and tailings for release back into the environment via various reclamation techniques (Giesy et al. 2010).

TPW is toxic to aquatic organisms and its toxicity has been documented since the early stage of oil sands development. The toxicity of TPW results from pollutants entering into water during the recovery process of bitumen from oil sands ore. The pollutants in TPW can be categorized into two major groups: inorganic and organic pollutant compounds. Water quality in different tailings ponds differs depending on the quality of the ore, the bitumen recovery method, etc. The main dissolved inorganic compounds in TPW comprise anions such as sulphate, chloride and bicarbonate species and the abundant cation is sodium along with calcium, magnesium and ammonium. High concentrations of calcium and bicarbonate species cause a high total hardness and alkalinity respectively, and the presence of metals of environment concern make TPW an inappropriate

environment for the most organisms. Another group, organic pollutants, mainly consists of unrecovered hydrocarbon (heavy oil) as well as extractable organic acids commonly called naphthenic acids (NAs). NAs are a complex mixture of alkyl-substituted acyclic, monocyclic, and polycyclic carboxylic acids (Misiti et al. 2013). In addition, NAs are considered corrosive surfactants, which are the primary toxicity source in TPW and a threat to aquatic life (Allen 2008).

With oil production expected to increase three-fold over the next decade (Allen 2008), TPW will be a critical issue for oil sands operators. Therefore, finding a feasible and cost-effective method for TPW treatment will become important. Different treatment techniques have been investigated to meet discharge regulations, but it is believed that there is no single reclamation option that is capable of treating TPW to meet the discharge standards in a manner that is technically and economically feasible and viable (Quagraine et al. 2005). Therefore, an integrated reclamation approach is needed. The first step for reclaiming TPW might be a detoxification process that makes it suitable for growing microorganisms and for further biological treatments.

1.2. Bioremediation Process Using Algae and Bacteria

In this research, *in situ* reclamation of TPW is investigated using an algae-bacteria consortium. Due to the vast volume of PAW impounded in the tailings ponds and the growing rate of TPW production, it is essential to have an appropriate treatment method. Conventional treatment methods, used to treat common wastewater, are not feasible for the treatment of TPW because of high operation and maintenance costs, while it is believed that *in-situ* reclamation technology is an efficient and economically feasible method for TPW reclamation.

Algae are photosynthetic organisms that can be categorized into unicellular (microalgae) and multicellular forms. Algae are capable of performing photosynthesis because of chlorophyll pigments. In photosynthesis process, carbon dioxide combines with water in the presence of visible light to produce

oxygen. Algae have been employed for industrial effluent treatment, heavy metal removal and organic pollutant bioremediation (Lourie et al. 2010; Ngo et al. 2009; de-Bashan and Bashan 2010; Semple and Cain 1996; Matsunaga et al. 1999).

Laboratory studies have demonstrated that some algae species, such as green algae and diatoms, exhibit the potential to tolerate and likely degrade model naphthenic acid compounds (4-MCHAA, 4-MCHCA, 3-MCHCA)(Quagraine et al. 2005). Algae and other phototropic organisms are also capable of bioaccumulation, which makes them valuable as treatment tools. Adsorption of dissolved metals by algae cells can lead to a decrease in PAW toxicity. In water contaminated with NAs and ions, some algae divisions, such as *Chlorophyta*, show high tolerance to high concentrations of NAs (Leung et al. 2003). Using algae as a photosynthetic organism to pretreat oil sands water and mitigate toxicity is a new approach that can open a new field of investigation and new hopes for resolving a potential oil sands eco-disaster.

There are certain bioremediation criteria, enumerated by Alexander (1999), that must be considered as a practical means. These criteria might be considered for the reclamation of PAW: (1) microorganisms, which are capable of tolerating the extreme conditions of PAW and can biodegrade toxic organic compounds, must exist in the system; (2) those adapted microorganisms must be able to biodegrade toxic organic compounds at a reasonable rate and decrease toxicity to target levels; (3) microorganisms must not produce other toxic compounds; (4) the site must not contain toxic material or inhibitors which prevent bioremediation; (5) the target pollutants, which in this research are metals of environmental concern and NAs, and must be available to the microorganisms; (6) the conditions at the site or the bioreactor must be appropriate for the microorganisms' growth and activities; (7) the cost for *in situ* bioremediation techniques must be less or no more expensive than other potential technologies.

If even one of the above-mentioned criteria is not met, it can result in a rejection of bioremediation. The term "microorganisms" in the above criteria stands for both algae and bacteria. In this research, these criteria are investigated for *in situ*

reclamation and bioremediation of TPW, using interactions between the algae-bacteria consortium and pollutants.

1.3. Research Objectives

The goal of this investigation is not the perfect and complete treatment of TPW to reach the standards of water discharge. However, that is reclamation and mitigation of toxicity via removal of target pollutants. The target pollutants in this research are metals of environmental concern (inorganic fraction) as well as NAs (organic fraction):

Metals of environmental concern: Algae bioaccumulate dissolved metals via two different mechanisms: intracellular bioaccumulation, which is the uptake of metals into the cell cytoplasm; and extracellular bioaccumulation, which is the adsorption of metals on the surface of algal cells. The objective of this part of the research is to investigate the removal rate of target metals though these two mechanisms.

Naphthenic acids (NAs): The oxygen produced by algae will increase the dissolved oxygen concentration in TPW, and results in the domination of aerobic bacterial species which are able to mineralize, biodegrade or biotransform recalcitrant organic compounds including tailings-associated NAs. However, some algal species have the potential to remove NAs. The objective of this part of the research is to study the application of the algae-bacteria consortium in NAs removal and to determine how algae and bacteria contribute to the biodegradation of NAs.

In this research, water chemistry is monitored during the reclamation process and pollutant removal efficiency is discussed.

1.4. Research Scope

The scope of this research is restricted to the following areas:

Stage I: Sampling and algae isolation: Indigenous algae, which have been adapted to the toxic environment of TPW, must be isolated and identified using molecular biology techniques. Purchasing the isolated algal species from algae collections may be considered for the known species for NAs removal. In the case of purchasing from algae collections, gradual acclimatization stages must be taken to adapt the algal strain to the TPW environment. The purchased algal stains should grow in media with water chemistry similar to that of TPW in terms of the composition of total dissolved solids, cations, and anions.

Stage II: The removal of metals of environmental concern: TPW inoculated with algae is incubated at a certain time and under certain conditions. To study the mechanism of metal removal using algae, the extracellular and intracellular bioaccumulation are monitored over the course of the experiment. Since algal photosynthesis increases the TPW pH, the precipitation of metals is another mechanism of removal that must be considered.

Stage III: The removal of NAs using algae-bacteria consortium: The influence of algal oxygen, the presence of *Navicula pelliculosa* (a diatom), and the contribution of algae and bacteria to the removal of NA are assessed. Toxicity mitigation in TPW is monitored over the course of the experiment, along with the water chemistry. Molecular biology techniques are employed to monitor the bacterial and algal growth.

1.5. Thesis Outline

This thesis consists of six chapters focusing on the reclamation of TPW using the removal of metals of environmental concern as well as the removal of NAs, respectively. A literature review on the mechanism of metal removal using algae along with measurement, toxicity, and biodegradation of NAs are presented in

Chapter 2. Chapter 3 concentrates on the general aspects of removing metals of environmental concern, such as indigenous algae isolation and identification and total metal removal. In Chapter 4, the removal of metals is investigated in detail to elucidate the extracellular and intracellular bioaccumulation mechanisms, along with identifying the chelating agents on the surface of the algal cell wall, which contributes to extracellular bioaccumulation. The general view of the NAs removal and toxicity mitigation associated with the reclamation process is discussed in Chapter 5. Chapter 6 summarizes the conclusions from this thesis, and presents industrial applications for the results.

CHAPTER 2: THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1. Algae and the Treatment of Oil Sands Tailings Pond Water (TPW)

Algae have been used extensively for the removal of nutrients, organic contaminants, heavy metals, and pathogens from various kinds of wastewater. Algae furnish oxygen to heterotrophic aerobic bacteria during the biodegradation of organic pollutants, and they use the carbon dioxide released from bacterial respiration. The production of oxygen via algal photosynthesis can be considered to be a cost effective tool to reduce the expenses related to mechanical surface aeration in treatment ponds (Muñoz et al. 2004). The synergistic relationship of algae and bacteria is not limited to oxygen-carbon dioxide exchange. Algae release extracellular compounds, which may enhance bacterial activity for the biodegradation of organic pollutants (Wolfaardt et al. 1994).

Algae have been successfully utilized to remove nutrients such as phosphate and nitrogen from domestic wastewater. Hazardous contaminants such as heavy metals are efficiently removed from industrial wastewater by algal cells. Algae have also been employed for biological oxygen demand (BOD) removal, pathogen removal, toxicity monitoring, and biogas production (Muñoz and Guieysse 2006).

Generally, microalgae are sensitive to toxicity resulting from contaminants, and consequently are used in toxicity measurement. For example, some hazardous materials such as heavy metals, ammonia and even high pH inhibit the photosynthesis process in algae (Muñoz and Guieysse 2006). Among algae, *Chlorella* species have demonstrated more tolerance to contaminants and toxic conditions. However, *Chlorella* species are still more sensitive than activated sludge microflora; therefore, in the treatment of wastewater using an algae-bacteria consortium, degrading bacteria show more tolerance than their associated algae (Muñoz and Guieysse 2006).

The surface mining procedure for bitumen recovery from oil sands ore produces a large volume of oil sands process-affected water, which is collected and impounded in tailings ponds. Tailings pond water (TPW) contains sand, clay,

unrecovered hydrocarbons, and contaminants such as metals and total acid-extractable organics (TAOs), which are traditionally called naphthenic acids (NAs). Due to the toxicity level and the huge volume of TPW, an efficient, feasible and cost-effective reclamation technique must be employed to prevent a future eco-disaster (Wang et al. 2014). Many reclamation methods, such as the use of adsorption, membranes (micro-, ultra-, and nanofiltration), reverse osmosis, electrodialysis, biological treatment, advanced oxidation, and treatment wetlands, have been evaluated for the treatment of oil tailings pond water (Allen 2008). The large volume of water is one of the main challenges in the treatment of TPW; hence the traditional pump and practice treatment approach (*ex situ* treatment) becomes costly and impossible. However, *in situ* treatment, which is more realistic in this case, eliminates the expenditures associated with transportation of water.

In the biological treatment, the biodegrading microorganisms are traditionally bacteria. However, algae have shown great potential to treat metal-polluted water; and also algae-bacteria consortia have been used in the biodegradation of recalcitrant organic contaminants (Lourie et al. 2010; Ngo et al. 2009; de-Bashan and Bashan 2010; Semple and Cain 1996; Matsunaga et al. 1999).

In this chapter, published reports on the application of algae for the removal of metals, and on the mechanism of metal uptake by algal cells, will be discussed. In addition, the characterization, toxicity and biodegradation of total acid-extractable organics (TAOs) will be studied.

2.2. Removal of Metals Using Algae

2.2.1. Introduction

The surge of industrial development in the last century has increased the environmental problems related to industrial activities. Due to vast application, metals are widely used in many industries; consequently, wastewater from these

industries might be polluted with metals. Accumulation of dangerous metals may adversely affect the ecosystem and host environment. Moreover, some metals are considered as threating pollutants to human health. It is proven that at certain levels, some metals may seriously damage human organs. For instance, cadmium (II), copper (II), and nickel (II) may damage the kidney and liver or cause disorders such as Wilson's disease, dermatitis or chronic asthma (Febrianto et al. 2009). In the United States, the concentration of many metals is regulated by the Environmental Protection Agency (EPA) for the protection of human health or environment. In Canada, the Canadian Council of Ministers of the Environment (CCME) has developed guidelines of acceptable levels of metals for recreational water, agricultural water uses, and general water quality guidelines to protect freshwater and marine aquatic life from both short-term and long-term exposure of many pollutants including metals (www.ccme.ca).

Physical and chemical treatment methods for the removal of metals can be expensive and maintenance intensive. While many plants and microorganisms, which are capable of uptaking and removing metals, can be used for the treatment of metal polluted water. The metal uptake by plants and microorganisms occurs through biosorption and bioaccumulation mechanisms. Biosorption is a passive mechanism that takes place in non-living cells, whereas bioaccumulation is an active, selective and metabolism-dependent mechanism in living cells (Terry and Stone 2002).

Vast research has been successfully conducted on the removal of metals via biosorption (nonliving algae) (Vijayaraghavan et al. 2005; Wang and Chen 2009; Romera et al. 2007; Lourie et al. 2010), whereas despite the many advantages, there is not much literature on the application of bioaccumulation (living algae) for the treatment of metal-contaminated water. One of the main advantages of the bioaccumulation process is the self-regeneration of living cells via cell proliferation. This advantage has made the algal bioaccumulation process an economically cost-effective method, whereas biosorption requires the regeneration of adsorption beds and maintenance. In this chapter, the theory and

conceptual modeling behind the bioaccumulation of metals by living cells are explained, and important parameters influencing bioaccumulation rate are discussed.

2.2.2. Theoretical Background and Modeling

The importance of water chemistry in metal uptake and its bioavailability has been recognized for many years. There are many publications reporting the bioaccumulation of metals and their associated toxicity in various conditions. Several models have been developed based on the theory of the uptake process (Di Toro et al. 2001; Santore et al. 2001; Fernández et al. 2006; Worms et al. 2007; Croisetière et al. 2005). One of the most widely accepted and commonly used models is the Biotic Ligand Model (BLM). In this thesis, this model is utilized to easily explain the complexity of bioaccumulation theory (Di Toro et al. 2001).

According to the model's theory, toxicity and, consequently, metal uptake are not only functions of the total metal concentrations in solution, but are functions of both metal complexation with inorganic and organic ligands as well as metal competition with cations for the site of action. Here, the site of action can be functional groups on the surface of algal cell wall.

The metal complexation plays an important role by reducing metal bioavailability to the site of action. Based on Fig. 2-1, only a fraction of dissolved metals is free and bioavailable to be adsorbed by the algal cells. In the metal removal process using algae, it is important to realize that if the free metal concentration exceeds a certain threshold, it results in the disruption of the cell wall, which leads to the mortality of algal cells (Kaduková and Virčíková 2005).

On the other hand, major cations in the solution, such as sodium, compete with free metal ions for binding with the site of action, and consequently reduce the

metal removal rate. In addition, major cations mitigate the toxicity of metals by reducing their bioavailability.

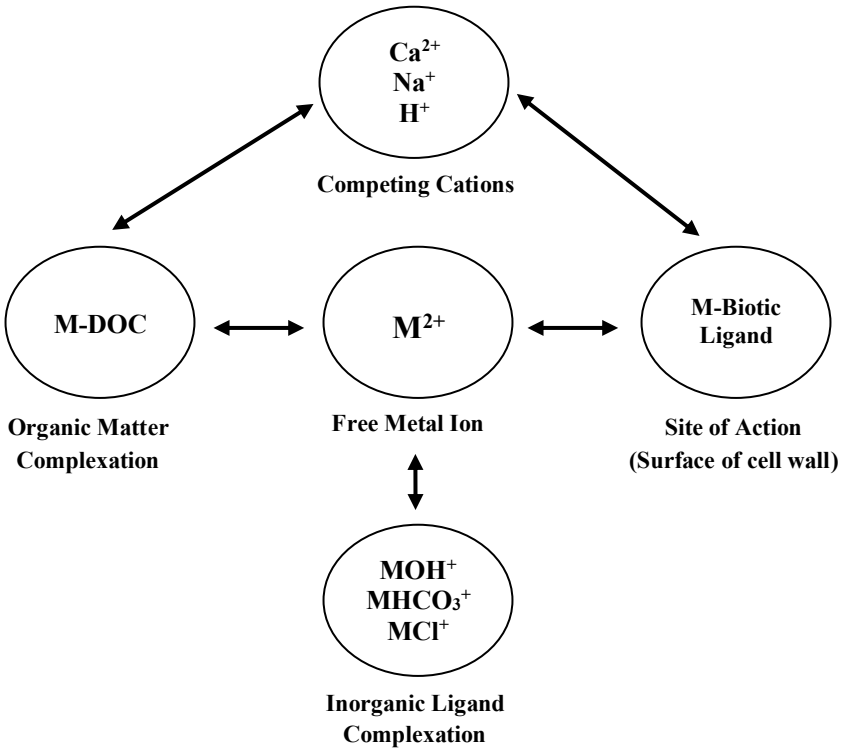


Figure 2-1 The BLM explains the mechanism of bioaccumulation by considering the formation of inorganic and organic ligand-metal complexes (Di Toro et al. 2001). In this model, the mechanism of metal transportation from the cell wall into the protoplasm is not considered.

Theory Equations

The BLM has been developed by the assumption that "interaction between free metal ion and biotic ligand is the same as interaction of metal with organic and inorganic ligands." If [L_b^-] is the biotic ligand (the molar concentration of site of action per weight of biomass), and M_i^{2+} is a divalent metal cation, then the concentration of the metal-biotic ligand complex ($M_i L_b^+$) is calculated as follows:

$$[M_i L_b^+] = K_{M_i L_b} [M_i^{2+}][L_b^-]$$

Where $K_{M_iL_b}$ is the stability constant for the metal-biotic ligand complex. Since the biotic ligand possesses a negative charge, the protonation may occur as follows:

$$[HL_b] = K_{HL_b}[H^+][L_b^-]$$

Where K_{HL_b} is the stability constant and HL_b is a proton biotic ligand complex. A mass balance on the total biotic ligands results in:

$$[L_b]_T = [L_b^-] + [HL_b] + \sum_{i=1}^{N_{M_i}} [M_i L_b^+]$$

Where $[L_b]_T$ is the total site of action density of the biotic ligand, N_{M_i} is the number of metal complexes including competing cations.

The same equations can be developed for organic and inorganic ligands (L_j^-) in the solution:

$$[M_i L_j^+] = K_{M_iL_j}[M_i^{2+}][L_j^-]$$

$$[HL_j] = K_{HL_j}[H^+][L_j^-]$$

$$[L_j]_T = [L_j^-] + [HL_j] + \sum_{i=1}^{N_{M_i}} [M_i L_j^+]$$

Where $K_{M_iL_j}$ and K_{HL_j} are stability constants for metal and proton ligand complexes, respectively. Finally, a mass balance for metal cations will be as follows:

$$[M_i^{2+}]_T = [M_i^{2+}] + [M_i L_b^+] + \sum_{j=1}^{N_{L_i}} [M_i L_j^+]$$

Where N_{L_j} is the number of metal-ligand complexes.

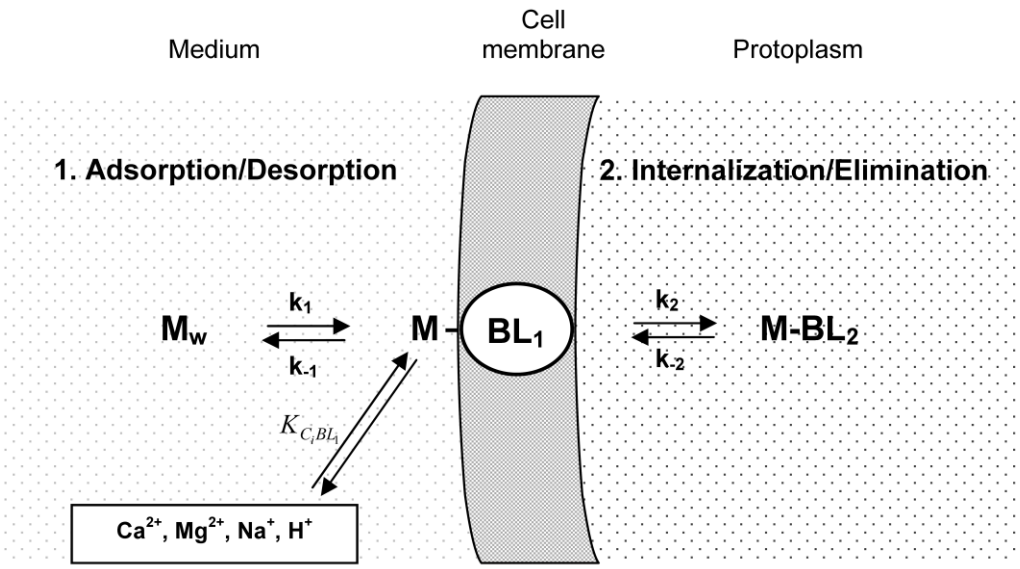


Figure 2-2 The conceptual model of extracellular and intracellular bioaccumulation by Ferreira et al. (2009). Mw, M-BL1 and M-BL2 are dissolved metal ions, and extracellular and intercellular metal ligands, respectively. In this model, the organic and inorganic ligands-metal complex is not considered.

Despite all the comprehensive factors involved in the BLM, this model does not consider the transportation of metals from the cell wall to into the cytoplasm. Ferreira et al. (2009) developed a metal exchange model for moss (Fontinalis antipyretica) which considers the intracellular transportation of metals (see Figure 2-2). This model employs two components involving the bioaccumulation of metals on the cell wall ($M-BL_1$), as well as the bioaccumulation of metals inside the cell cytoplasm ($M-BL_2$). Although the interference of cations with the bioaccumulation of metals was considered in this model, the role of organic ligands and inorganic ligands' complexations with metal was ignored.

Assuming first-order kinetics, the metal mass balances in the medium results in the following equation:

$$\frac{d[M_w]}{dt} = -k_1 . \{BL_1\}_{free} . [M_w] + \frac{m_{bryos}}{V} . k_{-1} . \{MBL_1\}$$

Where $k_1 (g\,mol_{BL_1}\,s^{-1})$ and $k_{-1} (s^{-1})$ are the rate constants for the adsorption and desorption of metals from the cell wall, respectively; $m_{bryos}(g)$ is the dry mass of the algal cell suspended in solution with a volume of (V). $\{BL_1\}_{free}$ and $\{MBL_1\}$ are the concentrations of free and metal-bound sites (functional groups) on the surface of the cell wall ($molg^{-1}$); $[M_w]$ is the concentration of dissolved metal.

With the same assumption (first-order kinetics), two mass balances on the algal cell wall (extracellular bioaccumulation) and protoplasm (intracellular bioaccumulation) are developed as follows:

$$\frac{d\{MBL_1\}}{dt} = \frac{V}{m_{bryos}}.k_1.\{BL_1\}_{free}.[M_w] - k_{-1}.\{MBL_1\} - k_2.\{BL_2\}_{free}.\{MBL_1\} + k_{-2}\{MBL_2\}$$

$$\frac{d\{MBL_2\}}{dt} = k_2.\{BL_2\}_{free}.\{MBL_1\} - k_{-2}.\{MBL_2\}$$

Where $k_2 (g\,mol_{BL_2}\,s^{-1})$ and $k_{-2} (s^{-1})$ are the rate constants for the internalization and elimination of metals from the protoplasm, respectively. $\{BL_2\}_{free}$ and $\{MBL_2\}$ are the concentrations of free and metal-bounded sites (functional groups) in the protoplasm ($molg^{-1}$).

2.2.3. Factors Affecting Metal Bioaccumulation Capacity

There are many interfering factors that can contribute to metals' uptake by algae. Llorente-Mirandes et al. (2010) studied the accumulation of As in algae strains collected from the Western Mediterranean Sea and reported that light intensity, turbidity, temperature, depth, salinity, and nutrient uptake were influencing factors in the bioaccumulation of As in algae. In addition, algae belonging to Chlorophyta (green algae) had a higher As content compared to other phyla, such as Rhodophyta (red algae) and Heterokontophyta (brown algae). Therefore, it can

be concluded that algal strain is another factor contributing to the metal bioaccumulation in algal cell.

pH also influences the metal removal in wastewater treatment ponds. pH increases due to algae growth and photosynthesis, but the rate at which it increases depends on the solution's buffering capacity (Sawyer et al. 2003). Photosynthesis consumes dissolved inorganic carbon (carbonate species) as a source of carbon dioxide. Carbonic anhydrase is an enzyme that plays an important role in algal photosynthesis, by catalyzing the dehydration of bicarbonate (the predominant carbonate species in aquatic systems) to carbon dioxide (Wang et al. 2006). This enzyme-based reaction consumes proton ions from a solution, which increases pH. At pH>9, some metals (such as Cd) may precipitate and are removed from water by alkalinization (Matsunaga et al. 1999).

Metals can also be removed through extracellular and intracellular bioaccumulation by algae. Extracellular bioaccumulation is the adsorption process of metals via binding with functional groups, such as carboxyl and amino groups, on the algal cell wall (Murphy et al. 2007). At a higher pH, more functional groups are deprotonated. Therefore, more free sites will be available for metal binding and, consequently, the bioaccumulation of metals on the cell surface increases (Chojnacka 2007). In addition, nutrients such as nitrogen and especially phosphorous accelerate the algal growth rate, which results in higher cell numbers (or an increase in biomass), which elevates the removal of metals.

It was previously reported that bioaccumulation capacity, which is the weight of metal accumulated per weight of biomass, stays constant during the cultivation period (Zhang et al. 1997). In this theory, it is assumed that each cell possesses a constant metal bioaccumulation capacity, leading to the conclusion that the bioaccumulation of metals is an energy-independent process (Zhang et al. 1997).

The following section focuses on some important factors to the bioaccumulation of metals, as reported in the literature:

Presence of other metals and ions. The interaction of metals in medium and ionic strength may influence the removal efficiency. Peña-Castro et al. (2004) studied the removal of Cr(VI), Cd(II), and Cu(II) in a continuous culture of micro-alga *Scenedesmus incrassatulus* in artificial wastewater. It was found that Cr and Cd interact positively, which increases the percentage of metal removed in the presence of the other. However, no interaction with presence of Cu was detected. In this study, the removal rate for all target metals ranged between 25 and 78%.

The bioaccumulation efficiency varied considerably in a multi-ionic medium in comparison to a single ion medium. In a multi-ionic system, the metal binding capacity is lower than in a single-ion system and consequently the bioaccumulation rate declines (Chojnacka 2007). According to the BLM theory, the competition of cations with metals for functional groups on the cell wall reduces the metal removal rate (Di Toro et al. 2001).

Bioaccumulation capacity. The bioaccumulation capacity of Cd and Cu was studied in *Scenedesmus abundans* by Terry and Stone (2002). The metal bioaccumulation capacity increased in a low algal cell concentration. At the low algal cell concentration, Cd and Cu compete for bioaccumulation. This result implies that at an adequately high concentration of algal cells, the removal rate in a multi-component metal solution is similar to the level of removal in an individual metal solution.

Results from various journal articles suggest that the bioaccumulation capacity depends significantly on the algae stain, media, and metal species. For instance Terry and Stone (2002) reported bioaccumulation capacity as a factor influenced by other parameters, whereas based on the observations of Zhang et al. (1997), bioaccumulation capacity is a constant factor. According to Zhang et al. (1997), the bioaccumulation capacity of uranium for *Scenedesmus obliquus* stayed constant (12.4 mg g^{-1}) over the course of a 15-day incubation period, which indicates that the uranium uptake process is an energy-independent process and the physiological activity does not affect the bioaccumulation of uranium (Zhang et al. 1997).

pH. pH is another important parameter which influences the metal bioaccumulation. Metals can be adsorbed on the cell surface and bind with cation-binding functional groups, such as the carboxyl group (extracellular accumulation). At a lower pH, the functional groups are protonated and thus are not available for the adsorption of metal ions, while at a higher pH, functional groups are deprotonated. Therefore, it is expected that the bioaccumulation rate on the cell surface will increase (Chojnacka 2007).

Organic matter. The presence of dissolved organic matter may alter the bioaccumulation of metals in algal cells. For instance, colloidal organic matter in wastewater effluent affects the metal uptake rate in *Chlorella kessleri*. The high concentration of colloidal organic matters results in a lower intracellular Cd bioaccumulation because of the increase in the complexation of Cd with organic matter (Worms et al. 2010). It has been proven that colloidal organic matter acts as metal-binding ligands and may reduce the bioavailability of metal, because of competition for metal with active sites on the surface of the algal cell wall (Peña-Castro et al. 2004; Di Toro et al. 2001). In contrast with Cd, intracellular Pb content in *C. kessleri* cells was enhanced when exposed to colloidal organic matters (Worms et al. 2010).

Lamelas and Slaveykova (2007) reported the formation of a ternary complex, in which metals have affinity either with the functional groups on the cell wall or organic matters adsorbed to the algal cell surface. Based on the ternary complex theory, the presence of organic matters in the solution and their adsorption on the cell wall may result in an increase in the total bioaccumulation of metals in the algal cell (Lamelas and Slaveykova 2007).

A similar complexity in the bioaccumulation of Cu and Zn in periphyton was reported by Meylan et al. (2004), in which the bioaccumulation of Zn was only a function of the free Zn ion concentration, while the bioaccumulation of Cu was controlled by the free Cu ions as well as the weak Cu-organic complex.

Functional groups on the surface of cell wall. Various functional groups have been reported as contributing to extracellular bioaccumulation as chelating agents. The proton and metal binding capacity of functional groups on the surface of algal cell can be determined by potentiometric titration. The potentiometric titration involves an acid-base titration to determine pKa associated with each functional group as well as its proton and metal binding capacity (Andrade et al. 2005).

Table 2-1 List of functional groups detected by FT-IR spectroscopy and their corresponding stretching frequencies (Murphy et al. 2007)

Wavenumber (cm^{-1})	Corresponding chemical bond in functional groups
3280	Bonded –OH, –NH stretching
2920	Asymmetric stretch of aliphatic chains (–CH)
2854	Symmetric stretch of aliphatic chains (–CH)
1740	C=O stretch of COOH
1630	Asymmetric C=O
1530	Amide II
1450	Symmetric C=O
1371	Asymmetric –SO3 stretching
1237	C–O stretch of COOH
1160	Symmetric –SO3 stretching
1117	C–O (ether)
1033	C–O (alcohol)
817	S=O stretch

Another method to characterize the functional groups on the cell surface is Fourier transform infrared spectroscopy (FT-IR) which has been used in many studies (Han et al. 2007; Murphy et al. 2007; Sheng et al. 2004; Fourest and

Volesky 1996). Murphy et al. (2007) listed the main stretching frequencies reported by various authors and the corresponding chemical bond in functional groups for seaweed (see Table 2-1). Although the FT-IR spectra qualify and quantify the presence of functional groups on the cell wall, they do not clarify the capacity of those groups for metal binding.

2.2.4. Comparison of Dry Cell Biosorption and Living Cell Bioaccumulation

Biosorption, the metal binding ability of dead and dried algal cells, has been studied and compared with bioaccumulation (the metal bioaccumulation capacity of living cells) (Kaduková and Virčíková 2005). It was found that the binding capacity of living cells of *C. kessleri* is significantly less than that of dead cells. In addition, it was found that at very high Cu concentrations, the cells surfaces might be damaged. The damage may partially release the accumulated Cu back into the medium and lead to a loss of cell-binding abilities (Kaduková and Virčíková 2005).

Matsunaga et al. (1999) investigated bioremediation of cadmium-polluted seawater using *Chlorella sp*. After 12 days of cultivation, 67% of removed Cd was intracellularly bioaccumulated and 25% was adsorbed on the cell surface. The maximum Cd adsorption in living cells was estimated to be 37.0 mg Cd (g dry cells)$^{-1}$, whereas it was approximately 91 mg Cd (g dry cells)$^{-1}$ in dead biomass. The maximum adsorption was calculated using the Langmuir sorption model. In addition, the maximum Cd adsorption in living cells has been reported as 19.9 mg Cd (g dry cells)$^{-1}$ for *Tolypothrix tenuis* (cyanobacteria) (Inthorn et al. 1996), 5 mg Cd (g dry cells)$^{-1}$ for *C. vulgaris* (green algae) (Geisweid and Urbach 1983), and 7.9 mg Cd (g dry cells)$^{-1}$ for *C. regularis* (green algae) (Sakaguchi et al. 1979).

2.2.5. Algae-Based Technologies for the Removal of Heavy Metals

The most conventional algae-based technologies for the removal of metals are the high-rate algae pond (HRAP) as well as the patented algal turf scrubber (ATS). The suspended cells of green algae, cyanobacteria or both are employed in these methods for the removal of metals from water (Perales-Vela et al. 2006). The heavy metal removal rate of HRAP and traditional stabilization ponds were examined by Toumi et al. (2000) to determine the efficiency of metal removal using urban polluted water with trace concentrations of Zn, Cu, and Pb. The results indicated that HRAP removed higher rates of heavy metal per volume per day. However, the observed removal rate might be larger than the actual metal bioaccumulation, due to metal precipitation resulting from the high pH, which occurred because of the algal photosynthesis.

ATS systems, employing suspended cyanobacteria and green algae for metal removal, have been used for the removal of heavy metals from polluted underground water as well as the removal of chlorinated and aromatic organic compounds. In addition, a consortium of algae and cyanobacteria was successfully utilized to decrease the prohibitive Mn concentration to an environmentally safe level in acid mine drainage. Bioaccumulation and precipitation were the main mechanisms for the removal of Mn using algae (Phillips et al. 1995).

2.3. Biodegradation and Characterization of Total Acid-Extractable Organics (TAOs), Commonly Called Naphthenic Acids (NAs)

2.3.1. Introduction

The third largest reserve of oil deposits in the world, containing 170.2 billion proven barrels, is located in Alberta, Canada. Almost 99 percent of the oil deposits in Canada are in the form of oil sands, which is a mixture of bitumen, viscous and tar-like super heavy hydrocarbon, with sands and clay

(www.energy.alberta.ca). Currently, there are two methods for recovering bitumen from oil sands ore: surface mining and in-situ (www.energy.alberta.ca). Both methods utilize water for the bitumen extraction process and consequently produce process-affected water (PAW) comprising suspended solids (sand and clays), dissolved organic and inorganic compounds, and unrecovered bitumen. Due to its pollutant content and toxicity, PAW is not released to the environment but is impounded in tailings ponds on-site, according to existing directives and environmental policies.

Similar to conventional crude oil, bitumen naturally contains some organic acids, which are dissolved in water during the process of bitumen recovery. Wide ranges of organic acids with acyclic, cyclic, aliphatic, and aromatic structures are formed during the *in situ* biodegradation of hydrocarbons, by oil-degrading microorganisms, in oil deposits (Scott et al. 2005; Corinne 2010). The term of naphthenic acids (NAs) have collectively been used to define "a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids," with a general formula of $C_nH_{2n+Z}O_2$, where n is the total carbon number and Z represents the hydrogen deficiency indicating a homologous series (Holowenko et al. 2002). NAs with one ring belong to the Z=-2, those with two rings belong to Z=-4 and so on. Distillated NAs from petroleum are commercially available for industrial usage as preservatives, emulsifiers, surfactants, paint dryers in textile and wood industries, and as adhesion promoters in tire manufacturing (Scott et al. 2005). Due to a large deviation in molecular structure, some articles have used the term total acid-extractable organics (TAOs) instead of NAs (Grewer et al. 2010).

There are several concerns regarding the TAO content of bitumen. TAOs are corrosive, have destruction effects on bitumen extraction facilities (Clemente and Fedorak 2005) and react with metals and form a precipitation of metal naphthenates, which blocks pipelines. In addition, the presence of TAOs increases the total acid number (TAN). Petroleum products with a high TAN are categorized as having less commercial value. Therefore, removing TAOs is of great economical interest to the oil industry (Corinne 2010). However, the main

concern regarding TAOs is their acute and chronic toxicity to a number of different organisms in the ecosystem (Leung et al. 2003; Leishman et al. 2013; Scarlett et al. 2012; Scarlett et al. 2013; Rowland et al. 2011).

Toxicity is one of the major concerns regarding TAOs. In 1986, TAOs were first reported as the polar organic carboxylic acids responsible for the toxicity of PAW (MacKinnon and Boerger 1986). TAOs and naphthenate salts have been shown to be acutely and chronically toxic to many organisms including plants, amphibians, zooplankton, phytoplankton, mammals, and bacteria (Corinne 2010). In this section the molecular structure, methods of characterization and fingerprint methods, toxicity, and biodegradation and mineralization are discussed.

2.3.2. Molecular Structure, Characterization and Fingerprint Methods

Due to a deviation in molecular structure, TAOs do not necessarily follow the classical molecular structure for NAs ($C_nH_{2n+Z}O_2$). Han et al. (2009) and Barrow et al. (2010) reported the presence of oxidized NA molecules containing more than two oxygen atoms ($C_nH_{2n+Z}O_x$) in samples from oil sands PAW. The number of oxygen atoms in the molecular structure may range from 2 to 5. Grewer et al. (2010) analyzed the TAO content of samples from various oil sand tailing ponds (MLSB, WIP, pond 9, Demo , Pond 2/3, Pond 5, and Albian), produced water from Steam Assisted Gravity Drainage (SAGD), commercial NAs (Merichem, Acros, and Kodak), rivers (Athabasca, N. Sask, Red Deer, Bow, and S. Sask), and Gregoire Lake, and found that the sum of the number of the oxy-naphthenic acid peaks ($3<X<5$) was greater than classical NAs ($X=2$) in all samples (where X is the number of oxygen atoms in NA molecules). Based on this article, the abundance of classical ($X=2$) and oxidized ($3<X<5$) NAs ranges between 30.5 up to 49% of the total peak. Later, Wang et al. (2013) found that oxidized NAs containing O_3 and O_4 are mainly composed of OH-NAs and $(OH)_2$-NAs.

The presence of nitrogen and sulfur in the TAO content of oil sands PAW and SAGD- produced water makes the molecular structure even more complex

(Grewer et al. 2010). Since the toxicity of PAW is the result of "all organic acid content" and not classic NAs, a broad range of species must be investigated. Headley et al. (2011) marked the TAO compounds that contained species such as O_n where n=1-16, NO_n and N_2O_n where n=1-13, and O_nS and O_nS_2 where n=1-10 in oil sands PAW. Fig. 2-3 illustrates the representative molecular structures for the complex mixture of TAOs.

Toxicity is a fairly molecular structure dependent parameter. Therefore, for studying the environmental impact of oil sands PAW, it is necessary to characterize and fingerprint TAOs using high resolution instruments. Historically, there are diverse classical analytical techniques for the total measurement and characterization of TAOs (Clemente and Fedorak 2005; Headley and McMartin 2004; Headley et al. 2009). The speciation of oxidized TAOs containing nitrogen and sulfur can be done using recently developed high- and medium-resolution mass spectrometry, coupled with multi-dimensional chromatography or ion-mobility separation. In addition, aromatic acids are quickly detected by synchronous fluorescence spectroscopy in the mixture of TAOs (Headley et al. 2013). The speciation of diverse TAOs elucidates the source of pollution, and facilitates PAW reclamation, toxicity studies, and risk assessment. In this section, some recently developed analytical techniques for chemical characterization and species identification of TAOs are discussed (Headley et al. 2013).

Fourier Transform Infra-Red (FT-IR) and Gas Chromatography Mass Spectrometry (CG-MS) Traditionally, FT-IR has been used for the rough estimation of TAO concentration in polluted water. It is still widely employed by industry because it is a simple method. The procedure involves the acidification (pH<2.5) of water samples followed by the extraction of TAO using dichloromethane (DCM) (Jiveraj et al. 1995).

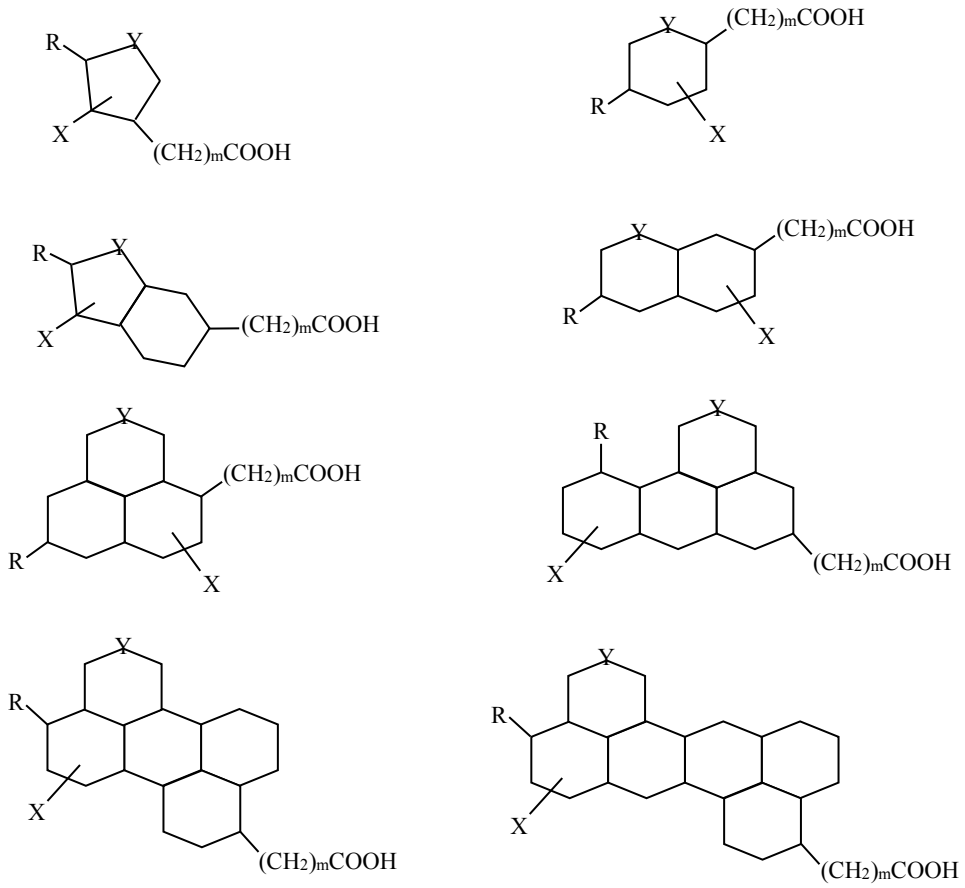


Figure 2-3 Representative molecular structures of NA fraction components (Headley et al. 2013)

Scott et al. (2008) suggested additional purification steps to separate organic acids from non-polar organic compounds. The TAOs are measured using the sum of absorbance intensities at 1743 and 1706 Cm^{-1}, representing the monomeric and dimeric forms of carboxylic groups (Jiveraj et al. 1995). Later Holowenko et al. (2002) developed an analytical method which comprised an additional derivatization procedure (using N-methyl-N-{tert-butyldimethylsilyl}-

trifluoroacetamide) followed by GC-MS analysis. This method has considerable advantages over the common FT-IR method. The m/z data provide information regarding the relative abundance of each compound with the known carbon number, hydrogen deficiency ($0<Z<-12$), and oxygen series. Presently, the GC-MS method is considered one of the easiest methods for the semi-quantification of the mixture of TAOs. Because GC-MS is affordable, and widely available in most laboratories, this instrument may be extensively employed instead of FT-IR to routinely measure TAO content. However, due to the low resolution of MS and the interference of components with similar molecular masses, the accurate quantification and characterization of organic acid mixtures is not feasible in this method. Martin et al. (2008) demonstrated the misclassifications and false positives in the results from the low-resolution MS analysis. The observation indicated that the response factor of TAOs (from oil sands PAW, analyzed using High-Pressure Liquid Chromatography/High-Resolution Mass Spectrometry [HPLC/HRMS]), was three fold lower than the response factor from Electrospray Ionization-low resolution MS (ESI-MS). This might be a result of the other organic compounds, including hydroxylated TAOs, interfering with the measurement. A similar misclassification in low-resolution GC-MS data was reported by (Bataineh et al. 2006). Despite misclassification and false positive results from low-resolution MS, the general trend in the distribution of compounds is similar to results from medium- and ultrahigh-MS (Headley et al. 2009).

GCxGC/MS This method is used for individual compound identification and confirmation by use of authentic standards. It is specifically suitable for quantifying and fingerprinting target compounds in transformation studies. However, MS employed using this technique has not yet had an ultra-high resolution and this may result in interference and misclassification of some components with a similar molecular mass. In 2011, the synthesis and identification of diamondoid compounds in oil sands PAW was reported using the GCxGC/MS method (Rowland et al. 2011; Rowland et al. 2011).

LCxLC/MS This method is suitable for separating isomeric molecules. Since the molecular structure plays a main role in the toxicity of organic compounds, this technique can be used for investigations related to the natural fate of recalcitrant organic acids in the environment (Headley et al. 2013).

Atmospheric Pressure Photoionization (APPI) and ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR MS) The high mass detection resolution is the main requirement for accurate characterization and fingerprinting of TAOs (Headley et al. 2009). Barrow et al. (2010) analyzed organic acids using FT-ICR MS in conjunction with ESI as well as APPI in both positive-ion and negative-ion modes. Results indicated that APPI in the positive-ion mode detected a greater number of compounds. In particular, naphthenoaromatic compounds in APPI appeared to be more intense than those in ESI. In addition, organic acids without heteroatoms or sulfur-containing species were less detectable in ESI.

Headley et al. (2011) employed high resolution ESI-FT-ICR MS to analyze the TAO content of samples from tailing ponds, interceptor wells, groundwater, rivers, and lake surface water. Principal Component Analysis (PCA) was used to identify the source of pollution according to the distribution of TAO species. It was reported that sulfur-containing species (O_nS n=1-10, and O_nS_2 n=1-8) may be used to distinguish the sources of PAW, while oxygen-containing species (O_n, n=1-16) and nitrogen-containing species (NO_n and N_2O_n, n=1-13) have the potential to be used to distinguish organic acids in PAW from organic acids in river and lake samples.

2.3.3. Toxicity of TAOs

TAOs and their salts have long been recognized as compounds in oil sands PAW that are toxic to many organisms, including fish, amphibians, zooplankton, phytoplankton, mammals, and bacteria (Corinne 2010).

Toxicity is a complex parameter. Toxicity observations may be influenced by many factors such as water chemistry, the type of organism used for the assay, and the molecular structure of the organic compound. TAOs in a non-ionized form (low pH) have shown more toxic potency to wetland plants than ionized form (high pH), which implies that water chemistry influences toxicity (Armstrong et al. 2009). Woodworth et al. (2012) also studied 21 indigenous phytoplankton candidates isolated from water containing TAOs. By adding the TAOs concentration (mg TAOs L^{-1}), growth inhibition was observed at 30 mg L^{-1} (for one stain), 100 mg L^{-1} (for one stain), 300 mg L^{-1} (for six stains) and 1000 mg L^{-1} (for six stains). Leung et al. (2003) reported that Chlorophyta tolerates a high TAO content (TAOs >20 mg L^{-1}) in PAW, whereas *Navicula cuspidata* (a diatom), *Gymnodinium* sp. (a dinoflagellate), and *Euglena acus* (a euglenoid) tolerate a high salinity of PAW.

Another factor influencing toxicity is the molecular structure of TAO compounds. Frank et al. (2009) employed proton nuclear magnetic resonance (1H NMR) to prove that TAOs with higher molecular mass possess a higher content of carboxylic acid. According to this article, the acute toxicity of surrogate NAs (measured using *D. magna* LC_{50}) increases with increasing molecular mass; however, compounds containing more of the carboxylic acid group showed lower toxicity. This result is verified by several publications reviewed by Corinne (2010). However, the observation by Frank et al. (2008) on molecular weight fractionation of tailing-associated TAOs was different. A toxicity assay using *Vibrio fischeri*, a bioluminescent bacterium that uses the Microtox® method, revealed that TAOs with lower molecular mass fractions possess higher toxic potency than TAOs with a higher molecular mass (Frank et al. 2008). This article did not explain why higher molecular mass TAOs, which are more hydrophobic, are less toxic. Holowenko et al. (2002) also reported a decrease in toxicity of PAW when the relative proportion of TAOs with large molecular weight (carbon number larger than 22) increases. Holowenko et al. (2002) suggested that toxicity mitigation might result from the biodegradation of low molecular weight TAOs and a reduction in the concentration of TAOs.

Toxicity is a molecular-structure dependent factor. Conducting experiments using a complex mixture of TAOs will not elucidate the influence of each individual compound on the toxicity potency of the TAO mixture. In addition, the presence of various compounds in the PAW matrix, such as polycyclic aromatic hydrocarbons and alkylphenols, make the toxicity even more complex. To address these challenges, Jones et al. (2011) synthesized and employed thirty-five pure individual organic acids and evaluated how structure of compounds affected toxicity. For Toxicity assay *Vibrio fischeri* (Microtox®) was used; and the target organic acid compounds involved following groups: n-acids, methyl branched n-acids, isoprenoid acids, monocyclic acids, branched monocyclic acids, bicyclic acids, triacyclic acids, and monoaromatic acids. In all groups, the toxicity (measured as EC_{50} mM) increased with an increase in the carbon number, and the toxicity mechanism was suggested to be non-specific narcosis.

Toxicity software Toxicity predictor software has been considered as a potential tool to predict the toxicity of TAOs to different organisms in the ecosystem. Scarlett et al. (2012) employed ADMET predictor™ software to model the toxicity of NAs using the effects of previously identified NA compounds (54 known compounds). It was found that polycyclic acids with an aromatic ring showed the most toxicity to fathead minnows. In addition, a list of compounds with predicted effects on human liver enzymes, the endocrine system and reproductive processes was reported. Frank et al. (2010) used an ECOSAR model from the U.S. Environmental Protection Agency (EPA) to predict the toxicity of TAOs. It was found that for the TAOs with the same molecular weight, the ECOSAR model estimated an increase in toxicity tendency for compounds containing fewer carbon rings. In addition, TAO molecules with a linear carbon ring structure showed higher toxicity than molecules with clustered rings.

2.3.4. Biodegradation and Mineralization of TAOs

Many factors contribute to the biodegradation of TAOs. These factors include nutrient availability, temperature, oxygen concentration, pH, salinity, redox potential, and sunlight (Corinne 2010). For instance, adding phosphate can increase the rate of removal of TAOs twofold; and reducing the temperature or the concentration of dissolved oxygen has been shown to decrease the TAOs' removal rate in oil sands TPW (Lai et al. 1996).

Misiti et al. (2013) investigated the biotransformation of a commercial naphthenic acid mixture (NA sodium salt; TCI chemicals) under aerobic conditions. Results indicated that 28.5% was mineralized to CO_2, 44% was utilized for bacterial biomass growth and 15 to 26% was persistent in all conditions. In addition, it was reported that in the presence of degradable synthetic wastewater, aerobic bacteria lost their ability to biotransform commercial NAs (Misiti et al. 2013). Using 16S rRNA gene clone library assay on aerobic microbial culture, it was identified that 80% of bacterial culture belong to *γ-Pro-teobacteria* class, which is closely related to known hydrocarbon degraders such as *Pseudomonas* and *Microbulbifer* (Misiti et al. 2013). A mixed culture of microorganisms is suggested to transform and biodegrade the recalcitrant TAOs (Johnson et al. 2011)

TAOs production by crude oil biodegradation Generally, organic acids in crude oil are the products of microbial activity (Watson et al. 2002; Meredith et al. 2000). Watson et al. (2002) studied the biodegradation of crude oil and reported the production of medium molecular weight carboxylic acids (C_{10} to C_{20}) during the biodegradation process. Medium molecular weight carboxylic acids were rapidly consumed by microorganisms; however, large, branched, and cyclic carboxylic acids (>C_{20}) were produced later. These are recalcitrant to biodegradation, and resulted from the extensive biodegradation of crude oil (Watson et al. 2002).

Influence of TAOs on biological activities TAOs may inhibit the microbial activity and biological processes. Misiti et al. (2013) reported the nitrification

inhibition at commercial NA concentrations of 80 and 400 mg L^{-1} for a mixed culture of aerobic heterotrophic/nitrifying as well as an enriched nitrifying culture, the latter of which contained a large population of nitrosofying and nitrifying bacteria (Misiti et al. 2013). For the denitrification process, no inhibitory effect was observed at concentrations lower than 400 mg L^{-1}. Commercial NAs also influenced anaerobic bacteria. Temporary inhibition took place for the methanogenesis at 80 mg L^{-1} and complete inhibition was observed at 200 mg L^{-1} and higher concentrations. However, CO_2 production was not affected during the anaerobic experiment (Misiti et al. 2013). This implies that fermentation and acidogenesis were not inhibited by commercial NAs.

Carbon number Molecular weight or the carbon number of organic acids is one of the most important influencing factors in the biodegradability of TOAs. Low and high carbon number TAOs are preferentially biodegraded by plants, with a minimum of around C_{14} and C_{15} (Headley et al. 2009).

Toor et al. (2013) used a microcosm, as an analogue of a proposed constructed wetland, and reached 64-74% TAOs removal for two PAW samples taken from Syncude Canada Ltd. and Suncor Energy Inc. However, the addition of nutrients (nitrate and phosphate) did not influence the removal rate of TAOs in a microcosm (Toor et al. 2013). In addition, TAOs with a lower carbon number (C# 11 to 16) or a small number of rings (Z# -2 to -4) biodegraded faster with half-lives between 19 and 28 weeks (measured by high-performance liquid chromatography quadrupole time of flight–mass spectrometry (HPLC-QTOF-MS)). The most recalcitrant TAOs possessed carbon numbers between 17 and 20 and Z series of -6 to -12 with half-lives as high as 37 and 52 weeks (Toor et al. 2013).

Holowenko et al. (2002) reported an increase in the relative proportion of TAOs with carbon numbers larger than 22 in aged PAW samples, implying the selective biodegradation of compounds with carbon numbers less than 21. According to this article, it was observed that there was an increase in the biodegradation rate and aging process of PAW in the wetland (Holowenko et al. 2002).

There is more evidence indicating that TAOs with low molecular weights are more biodegradable. Clemente et al. (2004) examined the aerobic biodegradation of commercial NAs (Kodak and Merichem) using bacterial cultures from the Mildred Lake Settling Basin (MLSB) at Syncrude Canada Ltd site. It was found that bacteria can biodegrade more than 90% of NAs (measured using GC-MS and HPLC). In addition, 60% of organic carbon associated with NAs molecules appeared in CO_2 which indicated the mineralization of NAs (Clemente et al. 2004). Results suggest that NAs with lower molecular weight (n=5-13) are more readily biodegradable.

Aromatic and aliphatic cyclic structure Holowenko et al. (2002) studied the aerobic biotransformation of alkyl-branched aromatic alkanoic NAs by Mycobacterium aurum. Their observations indicated a biotransformation through both β-oxidation and ω-oxidation mechanisms. The ω-hydroxylase activity of a cytochrome P450 enzyme (CYP124) played an important role in metabolizing methyl-branched lipids and oxidizing the chemically disfavoured ω-position. In this study, the aromatic ring was not catabolized. It was shown that the presence of polycyclic aromatic rings and alkyl branches in molecular structure increases the persistency of TAO compounds (Holowenko et al. 2002).

Han et al. (2009) observed lower concentration of TAOs in older PAW samples, implying the *in situ* biodegradation. However, there was no considerable difference among PAW samples in terms of TAO signatures and the cyclic fraction. The results suggested that the least cyclic fraction (Z series 0 to -2) of TAOs is rapidly biodegraded in the oil sands tailing ponds, and remaining steady-state factions are recalcitrant, with half-lives of 12.8 to 13.6 years (Han et al. 2009).

Branches in acyclic and cyclic structure For non-branched carboxylic acids, adding the methyl group will curb the β-oxidation mechanism. Moreover, *cis*-isomers are less biodegradable for bacteria than *trans*-isomers (Johnson et al. 2011). Herman et al. (1993) found that organic acid compounds with methyl substitutions on the cycloalkane rings appear resistant to biodegradation compared

to carboxylated cycloalkanes. Since the straight chain fatty acids are readily biodegradable, TAO compounds, with z series equal to zero, must possess a highly branched structure (Holowenko et al. 2002).

Oxygen series in TAOs. Ponds receiving fresh PAW contain TAOs with X=2 (X represents the number of oxygen atoms in molecular structure) as high as 19.5 to 35.6% (relative abundance), whereas ponds (Pond 9 and Demopond) under the reclamation process contain only 10.7 and 17.0%. This result implies that TAOs with X=2 (classical NAs) are more biodegradable that TAOs with X=3, 4, and 5 series (Grewer et al. 2010). The biological oxidation of TAOs will increase the oxygen number (X). Han et al. (2008) reported that aerobic bacteria biotransform classical NAs (X=2) to hydroxylated NAs (X=3), which results in an increase in the oxygen in the molecular structure of TAOs.

Biodegradation of TAOs by Algae. Algae, which are photosynthetic organisms, have been used for the biodegradation and biotransformation of NAs. *Dunaliella tertiolecta*, a marine alga, demonstrated tolerance to five model NAs (cyclohexanecarboxylic acid, cyclohexaneacetic acid, cyclohexanepropionic acid, cyclohexanebutyric acid and 1,2,3,4-tetrahydro-2-naphthoic acid) at 300 mg L^{-1} and metabolized four out of five model NAs. β-oxidation was recognized as the mechanism of biodegradation for cyclohexanebutyric acid and cyclohexanepropionic acid. In addition, *Dunaliella tertiolecta* demonstrated the capability of biodegradation of single ring tailings-associated TAOs in PAW. Headley et al. (2008) reported the complete phytodegradation of model NAs (4-methylcyclohexaneacetic acid) by the diatom algae *Naviculla sp*. However, no biodegradation was observed for tailing-associated TAOs. There is more evidence on the tolerance of various algae strains to TAOs in oil sands PAW (Leung et al. 2003; Quagraine et al. 2005; Goff et al. 2013).

Commercial NAs and tailings-associated TAOs. Although many articles have used commercial NAs as a surrogate model for the TAO mixture in PAW, an increasing number of publications are reporting huge differences between commercial NAs and tailing-associated TAOs in many aspects. For instance,

commercial NAs are more biodegradable than tailings-associated TAOs (Scott et al. 2005). In addition, West et al. (2011) identified toxic organic compounds in commercial NAs which are not carboxylic acids. Using two-dimensional comprehensive gas chromatography-mass spectrometry (GCxGC/MS), a range of C_{0-6} alkylphenols in a batch of commercial NAs was identified. This range possesses toxic potency without the carboxylic group (West et al. 2011).

CHAPTER 3: METAL REMOVAL FROM OIL SANDS TAILINGS POND WATER BY INDIGENOUS MICRO-ALGA*

*A version of this chapter has been published.
Mahdavi, H., Ulrich, A. C., and Liu, Y. (2012). Chemosphere, 89(3), 350-354.

3.1. Introduction

Considerable public and regulatory attention has focused on the unrecovered hydrocarbons fraction of TPWs as pollutants (Quagraine et al. 2005; Scott et al. 2005; Allen 2008; Allen 2008), but less attention has been paid to the metals in TPW that are of environmental concern. Allen (2008) reported the presence of trace metals, such as Cr, Cu, Pb, Ni, Zn, in concentrations ranging from ~0.001–3 mg L^{-1} in tailings ponds water, which exceeds the Canadian Council of Ministers of the Environment (CCME) and US Environmental Protection Agency (USEPA) surface water quality guidelines.

Algae have demonstrated metal removal by either passive sorption to dead biomass or removal by living cells. To date, most studies have focused on metal removal using dead biomass (Wang and Chen 2009), although viable algae have shown promise in metal removal from domestic wastewater (Ji et al. 2011) or synthetic media (Kaduková and Virčíková 2005; Terry and Stone 2002). Only two studies on algae and TPW have been reported to date, and these have mainly focused on removal of naphthenic acids, which compose the organic fraction responsible for most of the toxicity of TPW (Quesnel et al. 2011; Headley et al. 2008). To our knowledge, research has not been done on metal removal from TPW using algae. To address these knowledge gaps, this chapter sought axenic indigenous alga capable of survival in oil sands TPW, and assessed its capacity for metal removal. Removal of ^{53}Cr, Mn, Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, As, ^{88}Sr, ^{95}Mo and Ba from TPW was evaluated at two different nutrient levels (nitrate and phosphate). The objective of this research is to demonstrate metal removal under realistic conditions found in tailings ponds; and the results from this study are applicable to *in-situ* removal of trace metals from brackish water such as TPW or sewage using micro-algae.

3.2. Materials and Methods

3.2.1. Preparation of Materials

To ensure glassware was free of trace metals, it was washed with HNO_3 (30% v/v), and rinsed three times each with deionized and Milli-Q® water (Millipore; Billerica, MA) prior to use. All reagents were prepared with Milli-Q® water, and chemicals were either certified A.C.S or trace metal grade. Unless otherwise specified, the cultivations were performed in autoclaved glassware under continuous illumination at 4300 lux (cool light, color temperature of 3500K, 44W, Philips F48T8/HO/TL835 ALTO, Markham, ON) at 28°C with shaking (100 rpm). Algal cell counts were performed using a Sedgwick-Rafter (S-R) cell (Wildco, FL) according to the procedure outlined in the Standard Method 10200F (American Public Health Association. et al. 2005).

3.2.2. Strain Identification and Inoculum Preparation

Indigenous algae were discovered in samples of cyclone overflow (COF) water collected from bitumen extraction facilities in Syncrude Ltd Canada (Fort McMurray, AB). When green spots of algal growth were observed in a glass cylinder containing COF (see Appendix B), samples were transferred into fresh filtered COF water (0.45 µm; Stericup®, Millipore; Billerica, MA), and cultivated under the conditions described in section 2.1. Sub-cultures were stored at 15°C, with 12 hour night/day time intervals at 2000 lux.

To get an algal culture free of bacterial and fungal contamination, several modified protocols (Kim et al. 1999; Su et al. 2007) were performed. The algal culture was first treated with lysozyme (20 to1000 mg L^{-1}) at 37°C for 30, 60 and 90 minutes, followed by sub-culturing (section 2.1) in filter-sterilized COF containing the antibiotics ampicillin, streptomycin, norfloxacin, chloramphenicol and gramicidin (100 mg L^{-1} each), and the anti-fungal agent amphotericin B (2.5

mg L^{-1}). However, indigenous algal growth was only observed in cultures still containing very low numbers of bacteria.

Indigenous algal strains were identified by DNA sequencing. DNA was isolated from algal cells grown for seven days using the DNeasy Plant Mini Kit (Qiagen; Toronto, ON) according to the manufacturer's instructions. The algal universal primer pair p23SrV_f1 (5´ GGA CAG AAA GAC CCT ATG AA 3´) and p23SrV_r1 (5´ TCA GCC TGT TAT CCC TAG AG 3´) targeting the 23S plastid rRNA gene were used to generate a ~410 base pair amplicon using the method described by Sherwood and Presting (2007). The amplified 23S rRNA gene fragments were subjected to denaturing gradient gel electrophoresis (DGGE) using the DCode™ universal mutation detection system (Bio-Rad; Mississauga, ON). A gel containing 7.5% acrylamide and a 20 to 60 % gradient of formamide and urea was run for 2.5 h at 180 V (see Appendix C). Bands were cut from DGGE gel, and sent directly for DNA sequencing. Sequencing results were identified using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD). Twelve green algae homologues were retrieved from the database and compared with the strain in this study. A multiple sequence alignment based on the 23S rRNA genes and a neighbor-joining phylogenetic tree was calculated and constructed using MEGA5 (Tamura et al. 2011).

3.2.3. Confirmation of Metal Removal by Algae

To provide an inoculum that was not grown in the presence of the metals of interest, the identified alga was cultivated in simulated cyclone overflow water (0.153 mM KF, 0.03 mM LiCl, 0.181 mM KCl, 13.8 mM NaCl, 0.243 mM $Ca(NO_3)_2$, 0.170 mM $NaNO_3$, 0.323 mM $MgSO_4$, 3.34 mM Na_2SO_4, 14.4 mM $NaHCO_3$) for seven days prior to experiments. For the metal uptake experiment, two TPW samples one from Syncrude (West In-pit pond) and another from Albian (the External Tailings Facility pond) were used (5 meter depth). Prior to

use, TPW was filter-sterilized (0.22 µm Stericup®; *Millipore, Billerica, MD*) and enriched with nitrogen and phosphorus to determine the effect of nutrient addition. Previous experiments demonstrated that nutrient supplementation is required to achieve a suitable algal growth (>10^6 cells) in oil sands TPW (Appendix F); thus two nutrient levels were chosen. The nutrient concentrations were either "low" (0.24 mM NO_3^- and 0.016 mM PO_4^{3-}) as suggested by Levy et al. (2009) or "high" (1.98 mM NO_3^- and 0.201 mM PO_4^{3-}) as found in general purpose medium (GPM) (Andersen 2005). For each TPW sample and nutrient level treatment, three 1000-mL Erlenmeyer flasks containing 200 mL TPW were inoculated with algae to a final concentration of 10^5 cells mL^{-1} (22.4 mg L^{-1} dry biomass). Duplicate uninoculated controls were included for each treatment. Samples were taken for metal analysis, cell counts following four hours, and after one, four, eight and 14 days of incubation.

3.2.4. Water Chemistry Analysis

To determine the amount of metal removed, TPW samples were centrifuged (3700 × *g*, 20 min), and the supernatant was employed for metal analysis. The removal percentage was calculated using the following equation:

Removal (%)= (A-B)/A × 100

A: metal concentration in uninoculated control, B: metal concentration in inoculated TPW

Metal analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) (ELAN 9000; PerkinElmer/SCIEX, Waltham, MA). The TPW samples were filtered (0.45 µm PTFE with luer-lok, Fisher chemical, Canada) and five times diluted by 1% nitric acid solution (trace metal grade, Fisher chemical, Canada). Calibration was performed using standards containing multi-elements (Pure Plu, PerkinElmer; Waltham, MA) and Mo (SPEX certiPrep; Metuchen, NJ). An internal standard containing ^{45}Sc, ^{89}Y, and ^{159}Tb (100 µg L^{-1}; VHG Labs;

Manchester, NH) was added to all samples. A multi-element standard solution (20 µg L^{-1}), demineralised water rinse and a 20 µg L^{-1} multi-element spiked TPW sample were included to ensure quality control. The ICP-MS instrument was operated in the following conditions: vacuum (9.8x10-6 torr), 0.96 L min^{-1} nebulizer gas flow, 1200 W ICP RF power, 6V lens voltage, -1850V analog stage voltage and 1400V pulse stage voltage.

Cations and anions in TPW samples were measured using ion chromatography (IC). A Dionex ICS-2000 (Dionex Corporation, CA) equipped with an IonPac® CS12A (4×250mm) column (Dionex Corporation, CA) was used for measuring cations with an eluent of 8 mM sodium carbonate and 1 mM sodium bicarbonate. A Dionex 2500 (Dionex Corporation, CA) equipped with an IonPac® AS14A (4×250mm) (Dionex Corporation, CA) column was used for measuring anions with an eluent of methanesulfonic acid (>99% purity). An eluent flow rate of 1 mL min^{-1} was used for both eluents.

The pH and conductivity measurements were conducted using Accumet® Research, AR50, Fisher Scientific and ThermoOrion Cond (model 130A, Germany).

3.2.5. Statistical Analysis

To determine the significance of the two factors tested (TPW source and nutrient level) on metal removal, analysis of variance (ANOVA) was performed using two-factors with replication (Excel; Microsoft, Redmond, WA). T-tests were performed to determine the significance of nutrient concentrations ($p < 0.05$). Error of the mean is indicated using plus and minus one standard deviation in graphs and text, respectively.

3.3. Results and Discussion

3.3.1. Identification of Algae

Based on DNA sequencing results for amplified 23S rRNA gene fragments, the unknown strain was identified as *Parachlorella kessleri* with a maximum similarity of 99 %. *P. kessleri,* or *Chlorella kessleri* (Graham et al. c2009), are unicellular freshwater green micro-algae classified as Phylum *Chlorophyta*, Class *Trebouxiophyceae*, and Order *Chlorellales* (Graham et al. c2009).

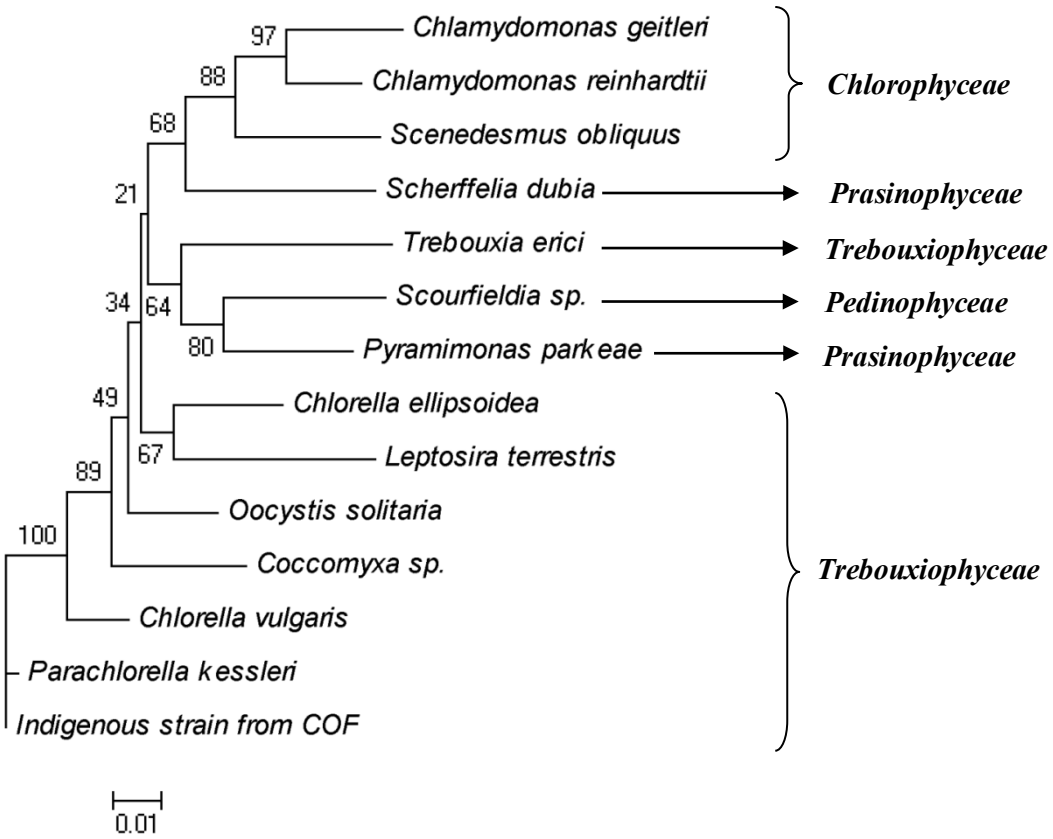


Figure 3-1 Phylogenetic tree relating *Parachlorella kessleri*, isolated from oil sands process-affected water, to blue-green algae known for metal removal or tolerance to TPW. The evolutionary history was constructed using the Neighbor-Joining method (Saitou and Nei 1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method, both conducted in MEGA5 (Tamura et al. 2011). Bootstrap of 2000 replicates is shown next to the branches.

The ability of *Chlorella* to remove trace metals by biosorption or bioaccumulation has previously been identified (Huang et al. 2009; Hassler et al. 2005; Kaduková and Virčíková 2005; Ngo et al. 2009; Wang and Chen 2009).

Figure 3-1 shows the phylogenetic relationship between *P. kessleri* and selected green algae that have either been investigated for metal removal from aquatic media (Backor and Váczi 2002; Bajguz 2011; Bonnineau et al. 2011; Levy et al. 2007; Radu et al. 2010; Sabatini et al. 2011) or are tolerant to oil sands TPW such as *Scourfieldia* spp. and *Oocystis* spp. *(Leung et al. 2003)*. As seen from the phylogenetic tree, *P. kessleri* is closely related to both *Chlorella vulgaris*, which has been widely studied for metal removal (Wang and Chen 2009; Muñoz and Guieysse 2006), and *Oocystis* spp., which has shown tolerance to TPW. *Oocystis* spp. can tolerate high naphthenate concentrations (>20 mg L^{-1}) along with high major ion concentrations (>1000 ΦS cm^{-1} conductivity) *(Leung et al. 2003)*.

3.3.2. Growth of *P. kessleri* in TPW with Nutrient Amendment

To determine if nutrient amendment affected growth of *P. kessleri* in TPW, samples of TPW originating from Syncrude or Albian were inoculated with *P. kessleri*, and enumeration was performed at selected time points. A maximum of 10^7 cells mL^{-1} was reached by day 8 and day 12 in TPW from Albian and Syncrude sites, respectively (Figure 3-2). In general, no significant differences in counts of *P. kessleri* were observed between any treatments at any time point (Figure 3-2), which indicates that the two sources of TPW supported similar levels of *P. kessleri*, and that the addition of higher levels of nitrogen and phosphorus does not significantly enhance growth in comparison with the lower concentration. The initial pH of both Syncrude and Albian TPWs was 8.3 and their initial electron conductivities were 3.940 and 1.517 ms cm^{-1} respectively. The final pH after 14 day algae cultivation reached 9.16±0.10 and 9.06±0.02 in Syncrude and Albian TPWs. In the present study, the photosynthesis rate was not monitored; however the observed pH increase might be as a result of

photosynthesis process, since the algal photosynthesis process consumes soluble carbon dioxide in equilibrium with bicarbonate species (Mara and Horan 2003).

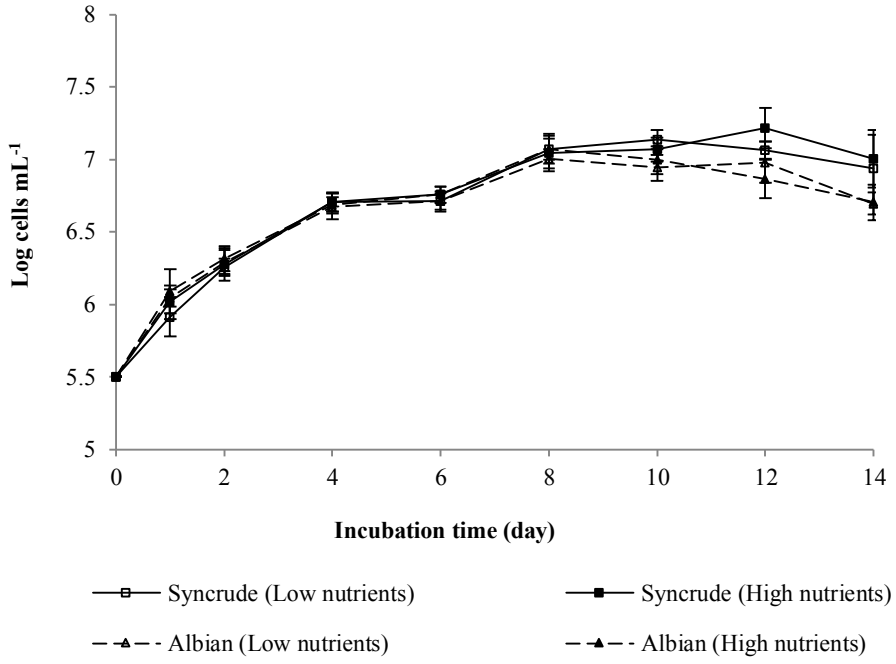


Figure 3-2 Growth of *Parachlorella kessleri* in tailings pond water from different sources with supplementation with low or high levels of nitrogen or phosphorus. Data points indicate mean of n = >15, and error bars represent ± standard deviation.

3.3.3. Removal of Metals from TPW Using *P. kessleri*

To determine the efficiency of *P. kessleri* in removing metals, target metals in two TPW sources containing low and high levels of nutrient augmentation were quantified pre- and post-treatment with the algae. After 14 days of incubation with *P. kessleri*, metal concentrations decreased in both Syncrude and Albian (Table 3-1a) TPWs at both nutrient concentrations. In the Syncrude TPW, the maximum removal efficiencies were observed for ^{66}Zn and Mn, with levels near 100 %.

Table 3-1 (a) Initial metal concentration of tailings pond water samples collected from Syncrude and Albian and removal percentage at 14 d of target metals from tailings pond water originating from a) Syncrude or b) Albian, and augmented with different levels of nitrogen and phosphorus. Data represent the average of replicates ± standard deviation (n = 3 for removal % and n=4 for initial metal concentration), and **(b)** major anions and cations.

(a)

Initial trace metal concentration	Syncrude			Albian		
	Initial concentration (µg L^{-1})	Low nutrient (removal %)	High nutrient (removal %)	Initial concentration (µg L^{-1})	Low nutrient (removal %)	High nutrient (removal %)
^{53}Cr	6.7±1.0	25.15±12.88	27.37±5.90	3.8±0.3	13.41±5.34	0.00±0.00
Mn	44.01±3.66	99.91±0.16	97.99±1.54	7.20±0.65	99.93±0.12	98.94±1.14
Co	2.13±0.20	18.61±13.40	30.47±4.19	1.43±0.14	42.15±4.66	13.16±11.41
^{60}Ni	9.90±0.89	34.05±2.38	45.88±5.14	10.33±0.93	49.97±2.22	34.97±3.58
^{65}Cu	15.68±2.93	47.09±2.77	58.50±2.37	20.50±6.04	66.41±1.19	76.19±30.91
^{66}Zn	24.35±9.73	100.00±0.00	100.00±0.00	20.14±2.06	92.31±13.32	100.00±0.00
As	5.11±0.22	21.56±5.96	46.24±1.51	3.57±0.10	49.79±1.25	33.94±7.48
^{88}Sr	698.53±9.60	32.19±4.78	53.35±0.08	568.44±5.46	51.50±1.44	35.68±10.84
^{95}Mo	122.94±4.50	0.12±0.20	21.08±1.57	53.05±1.09	27.23±2.13	2.05±3.10
Ba	135±6.5	45.85±4.73	66.86±0.79	240±3.4	69.15±1.57	55.53±10.43

* Removal percentage for Zn in high nutrient concentration was calculated from data from d 8.

(b)

Sample	Cations (mg L^{-1})						Anions (mg L^{-1})					
	Li^+	Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	NO_2^-	Br^-	NO_3^-	SO_4^{2-}
Syncrude	0.21	842	30.2	17.4	10.6	18.7	2	635	5	< 1	17	565
Albian	0.14	299	1.2	16.7	11.3	22.1	2	171	< 1	< 1	< 1	159

Addition of high concentrations of nitrogen and phosphorus significantly ($p < 0.05$) improved removal of ^{60}Ni, ^{65}Cu, As, ^{88}Sr, ^{95}Mo, and Ba in Syncrude TPW; however, for the other metals, no significant removal was observed ($p > 0.05$) with high nutrient levels (Table 3-1a). In contrast, in Albian TPWs inoculated with *P. kessleri*, high nutrient concentrations adversely affected metal removal (Table 3-1a). This negative effect was significant for the removal of Co, ^{60}Ni, As, ^{88}Sr and ^{95}Mo ($p < 0.05$). The reason for these conflicting results is not known;

thus, future work should explore differences between the two TPW sources that may account for these results.

The absorption of metal ions by *C. kessleri* in diluted Gambrog's B5 medium was studied by Fujiwara et al. (2008). The highest removal efficiency was reported for Cr^{3+} was 31±1.2 %, followed by Mn^{+2} (9.89±5.4%), Co^{2+} (12.8±2.9%), and Mo^{6+} (13.0±5.0%). The results of the current study surpassed these removal percentages for Mn and Co, demonstrated similar removal of Mo, and showed a lower removal for Cr; however, the initial concentrations of the metals in the current study were considerably lower (see Table 3-1(a)). Ji et al. (2011) studied Cu^{2+} and Zn^{2+} removal using freshwater green alga *Cladophora fracta* at initial concentrations in the range from 1 to 10 mg L^{-1}, which are considerably higher than both TPWs. The maximum removal percentages reached were 99% and 85% for Cu^{2+} and Zn^{2+} respectively, when algal wet biomass weight was 8 g L^{-1}. In this study, the algal wet biomass was lower, reaching 1.18±0.44 and 1.50±0.33 g L^{-1} in Syncrude and Albian samples, respectively, after 14 day cultivation. While a lower concentration of algae may be one factor in the lower removal efficiency observed in this study, another factor might be the high cation content in TPW. TPW is brackish water containing high concentrations of sodium, calcium and magnesium (Table 3-1(b)). These cations can compete with metals for binding with negatively charged functional groups on the algal cell surface, thereby reducing the metal uptake (Andrade et al. 2005).

In Table 3-1a, a lower As removal for Albian TPW samples was observed at high nutrient concentration, which may be explained by the competition between phosphate ions and As for extracellular bioaccumulation (absorption on the cell surface). It has been reported that the high phosphate ion concentration can reduce As uptake by algae cells by competing with As for extracellular bioaccumulation (Reuther 1992).

3.3.4. ANOVA Two-Factor Analysis

The effects of nutrient concentration and TPW type on metal removal by *P. kessleri* were analysed with an ANOVA two-factor test (Table 3-2). Based on this analysis, removal of Co, ^{60}Ni, As, ^{88}Sr, and Ba was significantly influenced by the interaction of the nutrient concentration and the TPW type. For these metals, a high nutrient concentration in Syncrude TPW enhances metal removal; whereas in Albian TPW it has an adverse effect on metal removal. Therefore, the interaction between nutrient concentration and TPW type is considered a significant factor when determining the appropriate nutrient concentration to achieve the maximum metal removal in TPW (see Table 3-2). Nutrient concentration was found to be the most significant parameter for Mn removal, as maximum Mn removal occurred in the samples with low nutrient concentrations in both Syncrude and Albian TPWs. Removal of ^{65}Cu from Syncrude and Albian TPW samples showed no significant interfering parameter ($p > 0.05$), indicating that ^{65}Cu removal was not influenced by TPW type or by nutrient concentration.

Table 3-2 Anova analysis of metal removal from TPW to determine interaction effects of nutrient concentration and TPW. Significant factors ($p < 0.05$) are underlined.

Factors	P-values for metals of environmental concern							
	Mn	**Co**	**^{60}Ni**	**^{65}Cu**	**As**	**^{88}Sr**	**^{95}Mo**	**Ba**
Nutrient concentration	**0.031**	0.070	0.457	0.273	0.156	0.460	0.96	0.302
TPW type	0.407	0.363	0.254	0.074	**0.022**	0.818	0.82	0.112
Interaction	0.430	**0.035**	**0.0002**	0.930	**0.0001**	**0.001**	N/A[a]	**0.001**

[a] Not Applicable

3.4. Conclusions

The indigenous micro-alga *P. kessleri* was isolated from cyclone overflow water (Syncrude) and its ability to grow in and remove metals from oil sands TPW was evaluated. The maximum algal cell concentration of $10^{7.1}$ cells mL^{-1} was achieved in the TPW sample from Syncrude after 12 days incubation period. The removal

of ^{60}Ni, ^{65}Cu, As, ^{88}Sr, ^{95}Mo, and Ba from TPW sample from Syncrude was significantly higher at high nutrient concentrations compared to low nutrient concentrations, whereas in samples of tailings pond water from Albian, high nutrient concentrations adversely affected the removal rate of Co, ^{60}Ni, As, ^{88}Sr, and Mo. Low removal efficiency in this study is believed to be a result of insufficient algal biomass, a high concentration of cations in TPWs, or both. Based on ANOVA two-factor test analysis, a high nutrient concentration does not always result in higher metal removal. To achieve maximum metal removal, the TPW source, along with nutrient concentration, must be taken into account.

CHAPTER 4: PARTITIONING AND BIOACCUMULATION OF METALS FROM OIL SANDS PROCESS AFFECTED WATER IN INDIGENOUS *PARACHLORELLA KESSLERI**

*A version of this chapter has been published.
Mahdavi, H., Liu, Y., and Ulrich, A. C. (2013). Chemosphere, 90(6), 1893-1899.

4.1. Introduction

Metals can be a threat to human health, aquatic life and the environment (Febrianto et al. 2009), yet there has been little research to find efficient methods for removing metals from TPW. According to chapter 3, The concentration of dissolved metals As, Cr, Mo and Cu in Syncrude's TPW and Cr and Cu in Albian's TPW exceeded Canadian Water Quality Guidelines for the protection of aquatic life (http://ceqg-rcqe.ccme.ca). Biological methods are believed to be more economical for metal removal from TPW, because there is no cost for operation and maintenance or addition of chemicals and equipment use. In addition, microorganisms (biomass) can be regenerated through cell division and reproduction.

Metal bioaccumulation process in algal cells occur through two mechanisms; extracellular where metals bind to functional groups on the cell wall, and intracellular where metals are taken inside the cell body. Ionic strength, pH, temperature, organic matter, multi-metal interaction and algal biomass weight impact the efficiency of metal removal and consequently the total bioaccumulation capacity (Hassler et al. 2005; Worms et al. 2010; Chojnacka 2007; Peña-Castro et al. 2004; Terry and Stone 2002; Andrade et al. 2005).

In chapter 3, *Parachlorella kessleri*, an indigenous alga isolated from cyclone over flow, was used for the removal of ten target metals of environmental concern (^{53}Cr, Mn, Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, As, ^{88}Sr, ^{95}Mo and Ba) from two oil sands TPWs (taken from Syncrude's and Albian's ponds). However, the fate of these removed metals, partitioning among algal cells and mechanism of removal, are still unknown. To address these knowledge gaps, chapter 4 considers the functional groups associated with metal removal on *P. kessleri's* cell wall, and then investigates the intracellular and extracellular bioaccumulation of target metals and corresponding bioaccumulation parameters. The possible role of organic and inorganic fractions of TPW and their interference on the bioaccumulation and partitioning of target metals will be discussed. The results of this study elucidate

many unknown aspects of metal uptake in algae and can be applied for further algae related investigations on metal removal.

4.2. Materials and Methods

The preparation of material and facilities, *P. kessleri* identification and inoculum preparation, experimental condition, and the methodology of target metal removal confirmation have been described previously in chapter 3. Water chemistry analysis is shown in Table 4-1.

Table 4-1 Initial chemical composition of tailings pond water samples collected from Syncrude and Albian. (a) metals (data represent the average of four replicates ± standard deviation), and (b) major anions and cations.

(a)

Sample	Initial trace metal concentrations (µg L^{-1})									
	^{53}Cr	Mn	Co	^{60}Ni	^{65}Cu	^{66}Zn	As	^{88}Sr	^{95}Mo	Ba
Syncrude	6.7 ±1.0	44.0 ±3.66	2.13 ±0.20	9.90 ±0.89	15.7 ±2.9	24.3 ±9.73	5.11 ±0.22	698 ±9.60	123 ±4.50	135 ±6.5
Albian	3.8 ±0.3	7.20 ±0.65	1.43 ±0.14	10.3 ±0.93	20.5 ±6.0	20.1 ±2.06	3.57 ±0.10	568 ±5.46	53.1 ±1.09	240 ±3.4

(b)

Sample	Cations (mg L^{-1})						Anions (mg L^{-1})					
	Li^+	Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	NO_2^-	Br^-	NO_3^-	SO_4^{2-}
Syncrude	0.21	842	30.2	17.4	10.6	18.7	2	635	5	< 1	17	565
Albian	0.14	299	1.2	16.7	11.3	22.1	2	171	< 1	< 1	< 1	159

A short explanation of the experimental set-up follows: Two oil sands TPWs taken from Syncrude and Albian ponds were filter sterilized (0.22µm, Stericup® *Millipore*) and each TPW was enriched with two concentration levels of nitrate and phosphate (as low as 0.24 mM NO_3^- and 0.016 mM PO_4^{3-} or as high as 1.98 mM NO_3^- and 0.201 mM PO_4^{3-}). Three of these enriched TPW samples were

inoculated with *P. kessleri* cells to a final concentration of 10^5 cells mL^{-1} (22.4 mg L^{-1} dry biomass), and two were left as sterilized blanks. The cultivations were performed in autoclaved 1000 mL flasks under continuous illumination at 4300 lux (cool light, 44W, Philips, Markham, ON) at 28°C with shaking (100 rpm). Over the 14 day cultivation many parameters including alga cell count, dry biomass, pH, and metal content in TPW and alga cells were monitored. Algal dry biomass was established by filtering (1µm pore size, Whatman GF/B) at least 3 mL TPW containing *P. kessleri* cells, followed by drying for 24 hr at 55 °C. Dried cells were weighed using a Mettler Toledo, AB204-S/Fact scale. The pH of aliquots from the experimental flasks was measured with a probe from Accumet® Research, AR50, Fisher Scientific. Since the results showed a pH increase in blanks, the actual pH increase as a result of algae growth was determine from the following calculations:

$$A = B - C,$$

Where A is pH increase due to abiotic characteristics of TPW, B is pH of TPW blank sample and C is initial pH of the TPW blank sample at the beginning of experiment. The actual pH increase due to algae growth was then calculated by subtracting pH increase due to abiotic characteristics of TPW (A) from pH observed in algae containing sample (F):

$$E = F - A,$$

Where E is the actual pH variation due to algae growth (without abiotic interference). Figure 4-2 shows the E values (the actual pH increase) in both TPWs.

4.2.1. Metal Partitioning Analysis

Metal analysis was performed with an inductively coupled plasma-mass spectrometry (ICP-MS) (PerkinElmer SCIEX, ELAN 9000). The technical details of the ICP-MS method have been described in Chapter 3. In this research for the

target metals which possess more than one isotope, the number of nucleons was reported at the upper left of the chemical symbol (e.g. ^{53}Cr). To measure metal removal percentage, TPW containing algae was centrifuged (3700×g for 20 min) and the supernatant was utilized for metal removal confirmation (see chapter 3). The remaining algal pellet was resuspended in 0.2 M phosphate buffer (pH 7.5), which dissolves the precipitated metals (Matsunaga et al. 1999). This mixture was recentrifuged (3700×g for 20 min) and the supernatant was analysed by ICP-MS. The mass of the precipitated metals was determined by measuring metal concentrations in the phosphate buffer before and after contact with the algal pellet (Matsunaga et al. 1999). Afterward, the remaining algal pellet was resuspended in 5 mM EDTA/HEPES buffer, which stripped the metals attached to the extracellular functional groups (Hassler et al. 2005). ICP-MS analysis was completed on the supernatant to determine the extracellular bioaccumulated metals.

To determine intracellular bioaccumulated metal concentrations, the algal pellet was dissolved and digested in 30% HNO_3, left for 24 hrs at 40°C and then analyzed for metal content. To validate the results of the above employed digestion method, a second microwave-assisted digestion method was used: 4 mL concentrated nitric acid (70%, trace-metal grade Fisher Scientific), 2 mL H_2O_2 (30%, Fisher Scientific) and 4 mL DI water (Moreda-Piñeiro et al. 2007) was added to the algal pellet, biological Certified Reference Material (supplied from National Institute of Standards and Technology, NIST 1547, peach leaves) and blanks (see Appendix H). Microwave-assisted digestion was performed at 1000W (Milestone Ethos Touch Control) according to the following temperature gradient: 10 min ramp from room temperature ~20°C to 90°C, held at 90°C for 5 min, 10 min ramp from 90°C to 120°C, 10 min ramp from 120°C to 190°C and held at 190°C for 20 min (Llorente-Mirandes et al. 2010). No significant difference for target metal content was observed between the microwave digested method and the employed method ($P>0.05$), except for As, which was corrected with a correction factor of 1.23 (derived from the method comparison test).

4.2.2. Fourier Transform Infrared Spectroscopy

The main functional groups present on the surface of the algal cells were determined by solid phase FT-IR analysis. Algal cells were washed with 5 mM EDTA/HEPES buffer three times, rinsed with Milli-Q water three times and the centrifuged biomass (3700×g for 5 min) was freeze-dried at -20°C for two days. Infrared spectra were obtained with BIO-RAD, FTS 6000 and Varian Resolutions Pro software with a N_2 purging system equipped with a Mercuric Cadmium Telluride (MCT) detector. The dried biomass and KBr powder were mixed, keeping a weight ratio of 2:100 and then scanned under the following conditions: 20 kHz speed, 5 filter, 4 cm^{-1} resolution, 8 sensitivity and 128 scans. The background was obtained with the scan of pure KBr powder and was automatically subtracted from the sample spectra. The Kubelka-Munk formula (molar absorption coefficient over the scattering coefficient) was used to determine the Kubelka-Munk absorption, which correlates to the abundance of the measured chemical bond.

4.2.3. Total Concentration of Ionisable Groups And Functional Group pKa

An acid-base titration experiment was conducted to characterise functional group pKa values and quantify ionizable sites on the surface of *P. kessleri*. Algal cells were freeze-dried for 5 days, weighed and then suspended in 10 mL of $NaNO_3$ 0.1 mol L^{-1} (pH=3). Titration was performed using NaOH 0.1 N (standardized by HCl 0.1 N) as titrant in the presence of N_2 (ultra-pure grade) up to pH 11 (Andrade et al. 2005). Titrant was added with a micro pipet and pH was monitored using a probe from Accumet® Research, AR50, Fisher Scientific. To validate equilibrium condition at each point, titrant was added when a steady pH was obtained (time intervals ranged from 3 to 20 min). A titration under the same conditions was performed for 10 mL of $NaNO_3$ 0.1 mol L^{-1} (pH=3) without algae as a blank. The achieved titration data were analyzed using the Gran's plot and the non-linear regression method (Hofstee 1960).

4.2.4. Data Analysis and Statistics

In order to establish accuracy, reliability, and reproducibility of the collected data, all samples were studied in triplicates (except blanks that were in duplicates). Also the one-simple *t-Test* (using the data analysis function of *Excel MS Software*) was selected to evaluate the significance of factors. The threshold chosen for the statistical significance was 5 percent or $P<0.05$. All error bars and error values were plus and minus one standard deviation in graphs and text, respectively.

4.3. Results and Discussion

4.3.1. Functional Groups on *P. kessleri* Cell Walls

FT-IR spectra have been widely used for detecting vibrational frequency changes that indicate metal adsorption capacity on algal cell surfaces. In green algae, the cell wall matrix comprises complex and branched heteropolysaccharides (a galacto-arabinorhamnside core with peripheral xylose and galactose), offering carboxyl and sulphate groups and protein (10-70% of cell wall) (Schiewer and Wong 2000; Siegel and Siegel 1973). The algal cell surface, which can be considered a poly-functional macromolecule, behaves like a heterogeneous ligand and contributes to extracellular bioaccumulation. In fact, the deprotonated functional groups possess negative charges which attract positive ions, such as metal ions, and create electrostatic binding with target metals (Andrade et al. 2005). Therefore quality and quantity of functional groups on the cell wall directly contribute to metal uptake, especially extracellular bioaccumulation.

The FT-IR spectra shown in Figure 4-1 was performed to evaluate the potential metal binding capacity of indigenous *P. kessleri* through the quality of its functional groups. The FT-IR assignments (boxed in the figure) indicate the wave number for detected functional groups in *P. kessleri's* cell wall. The assigned FT-IR bands and range of the corresponding wavenumber were chosen according to a

review by Murphy et al. (2007). Twelve functional groups in the FT-IR spectra of indigenous *P. kessleri's* cell surface were detected (Fig 4-1), but only those important in metal sorption are further discussed. The Kubelka-Munk absorption peaks at 1542 and 1655 cm^{-1} correspond to the amide II and I bands, respectively, as reported by Han et al. (2007) for *Chlorella miniata*. A stretching band of the free carbonyl double bond from the carboxyl functional group appeared at 1741 cm^{-1} (Fourest and Volesky 1996). In our study, we observed a relatively small and unclear peak at 1741 cm^{-1}, indicating the low density of the carboxyl functional group on the cell surfaces. The carboxylic groups (contributed by uronic acids) and their homogenous distribution in the polysaccharides, attached to the *Chlorella* species cell walls, were reported as an important factor in metal-complexing (Kaplan et al. 1987). The region between 3200 and 3500 cm^{-1} results from the overlapping peaks of O–H and N–H stretching frequencies. These bands have been detected in various algae types (Murphy et al. 2007), and the latter group are involved in metal binding (Sheng et al. 2004). As a result of the cell surface analysis by FT-IR, it is concluded that indigenous *P. kessleri* possesses the necessary functional groups (amide I and amide II bands as well as the N–H stretch) for metal binding.

An acid-base titration was used to determine the actual proton binding capability of functional groups. Total concentration of ionisable sites in *P. kessleri* was 0.026 mmol H^+ g^{-1} (biomass) which is considerably smaller in comparison with *Chaetophora elegans* of 1.64 mmol H^+ g^{-1} (biomass) reported by Andrade et al. (2005). Moreover, only one dissociation constant (pKa=6.6 with ionisable group concentration of C[AH]=0.016 mmol H^+ g^{-1} (biomass)) was detected which can be attributed to carboxylic groups.

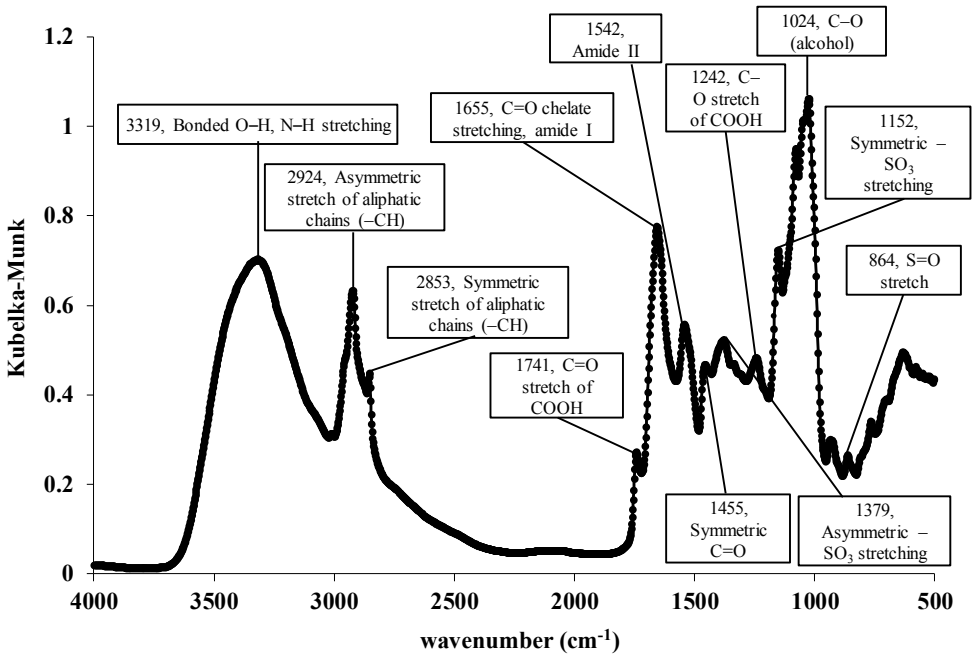


Figure 4-1 FT-IR spectra of freeze-dried *P. kessleri* (4 cm^{-1} resolution, 8 sensitivity and 128 scans).

4.3.2. pH Change In TPW Samples Containing Algae

The pH was monitored in filter-sterilized TPWs (blanks) as well as TPW samples containing algae. The pH is an important factor in metal removal, since at high pH metals precipitate and therefore higher total metal removal can be achieved (Perales-Vela et al. 2006). An increase in pH was observed in blanks, which may be due to the abiotic characteristics of TPW (complex mixture of metals, organics and salts). Figure 4-2 illustrates the actual pH variation resulting from algal activities (calculation explained in material and methods).

After introducing *P. kessleri* cells, pH increased in all water samples and reached the highest pH (~9) on day 4, followed by a decrease to 8.3 (close to initial pH) on day 14. In the present study, the algal photosynthesis rate was not monitored; however the observed increase in pH may imply an increase in photosynthesis rate since the algal photosynthesis process consumes soluble carbon dioxide in

equilibrium with bicarbonate species (Mara and Horan 2003). A similar trend was observed in alga cell counting results (see Figure 3-2), where cells number increased by more than one log unit in the fourth day of the experiment.

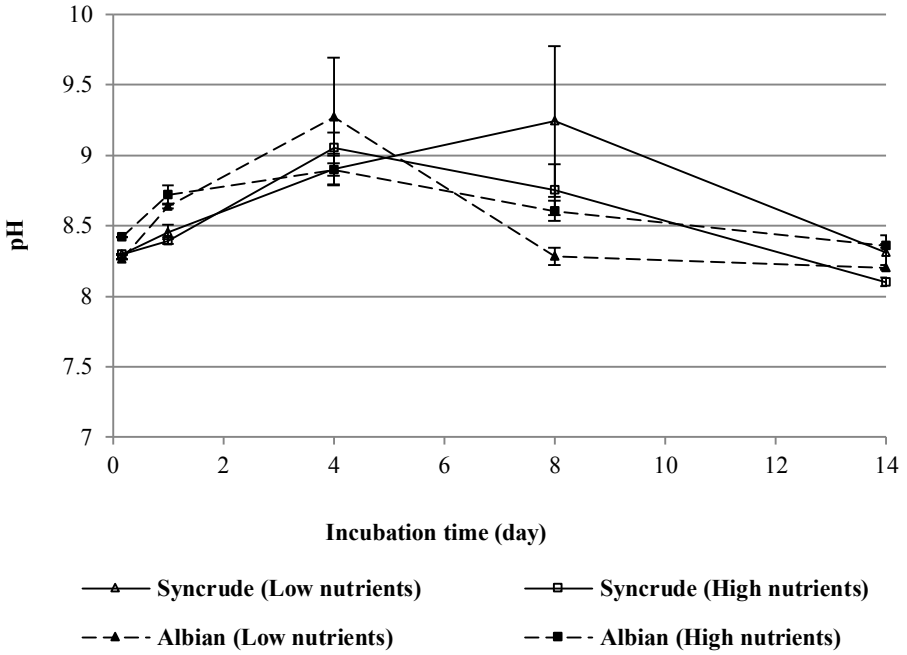


Figure 4-2 pH changes due to *P. kessleri* during fourteen day cultivation period for low (0.24 mM of NO_3^- and 0.016 mM of PO_4^{3-}) and high (1.98 mM of NO_3^- and 0.201 mM of PO_4^{3-}) nutrient concentrations in Syncrude's and Albian's TPWs.

4.3.3. Partitioning of Target Metals

The role *P. kessleri* played in removing metals from the TPWs was determined by analyzing partitioning of the metals. The partitioning of metals in this study involves the fractions of metals that are precipitated or bioaccumulated inside and on the surface of *P. kessleri* cells (intracellular and extracellular bioaccumulation). In Figure 4-3, the relative percentage of precipitated target metals as well as extracellular and intracellular bioaccumulation after 14 days are illustrated. Based on Fig 4-3, intracellular bioaccumulation plays the main role in metal removal,

whereas extracellular bioaccumulation occurred to some extent for Mn, ^{88}Sr, ^{95}Mo and Ba for Syncrude TPWs and Mn, Co, Ni, ^{65}Cu, ^{88}Sr, ^{95}Mo and Ba for Albian TPWs. Except for Mn, the partitioning of target metals was generally independent of the nutrient concentration for both TPWs. Intracellular bioaccumulation has been reported as the main mechanism of metal removal in other studies. Matsunaga et al. (1999) found Cd partitioning of 67% and 25% (intracellular and extracellular bioaccumulation respectively) in *Chlorella kessleri* after 12 days of cultivation, demonstrating the importance of intracellular bioaccumulation in metal removal.

Low extracellular bioaccumulation in this study results from the poor proton binding capability of *P. kessleri's* surface. As discussed in section 4.3.1, only one functional group on the cell surface was detected (carboxylic group) and the total concentration of ionisable sites in *P. kessleri* was small, which resulted in lower metal binding capacity. However, other interfering factors may reduce extracellular bioaccumulation of *P. kessleri* in TPWs.

Low extracellular bioaccumulation might result from the high sodium content in TPWs. According to previous studies, high sodium concentration and high ionic strength reduce the metal binding efficiency of functional groups on the algal cell walls (Chojnacka 2007; Andrade et al. 2005). High concentration of sodium (842 mg L^{-1} and 299 mg L^{-1} in Syncrude and Albian TPW samples, respectively, Table 4-1) and high ionic strength in TPWs adversely affect extracellular bioaccumulation through sodium competition with positively charged metals for electrostatic binding. Deprotonated functional groups on the cell surface possess a negative charge that attracts cations (such as metals and sodium) in the solution. A high concentration of sodium leads to a narrower electrical double layer which decreases the attraction of metals ions and consequently the metal binding capacity of cell wall reduces (Andrade et al. 2005).

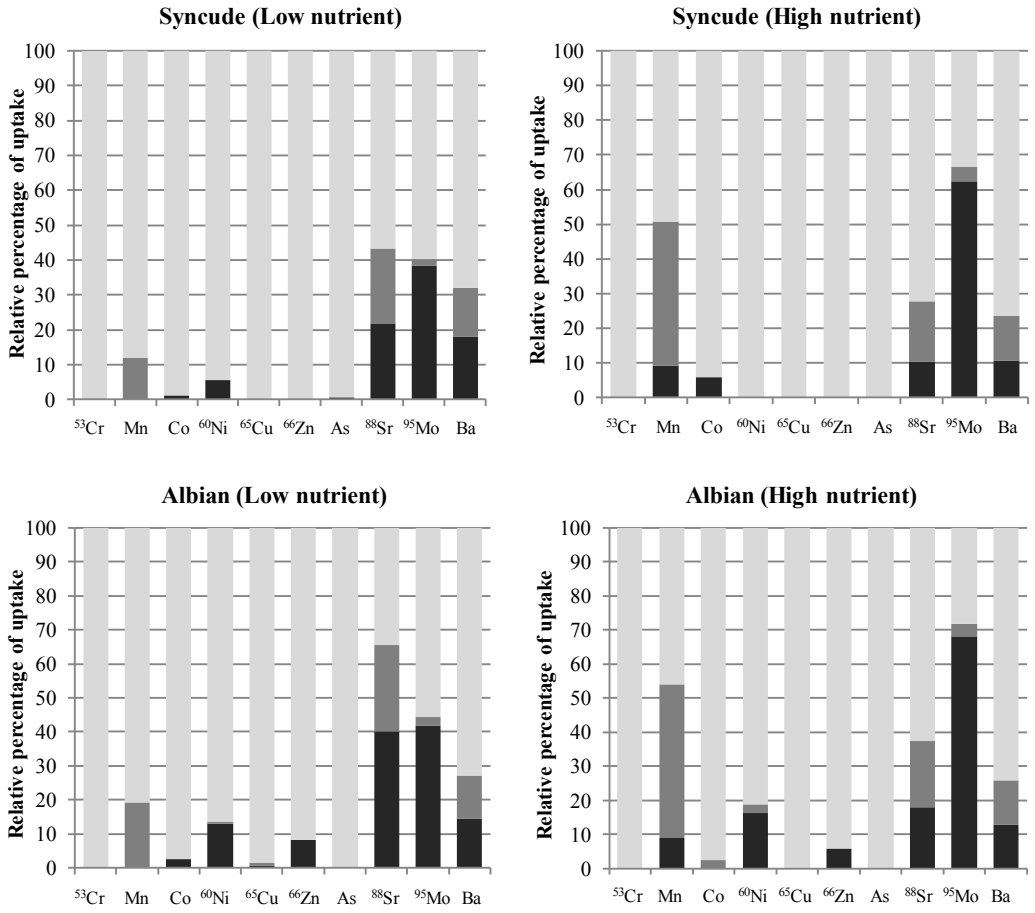


Figure 4-3 Percentages of precipitated as well as intracellular and extracellular bioaccumulated metals for Syncrude's and Albian's TPWs in low (0.24 mM of NO_3^- and 0.016 mM of PO_4^{3-}) and high (1.98 mM of NO_3^- and 0.201 mM of PO_4^{3-}) nutrient concentrations. (■ precipitated metals, ■ extracellular bioaccumulation, □ intracellular bioaccumulation)

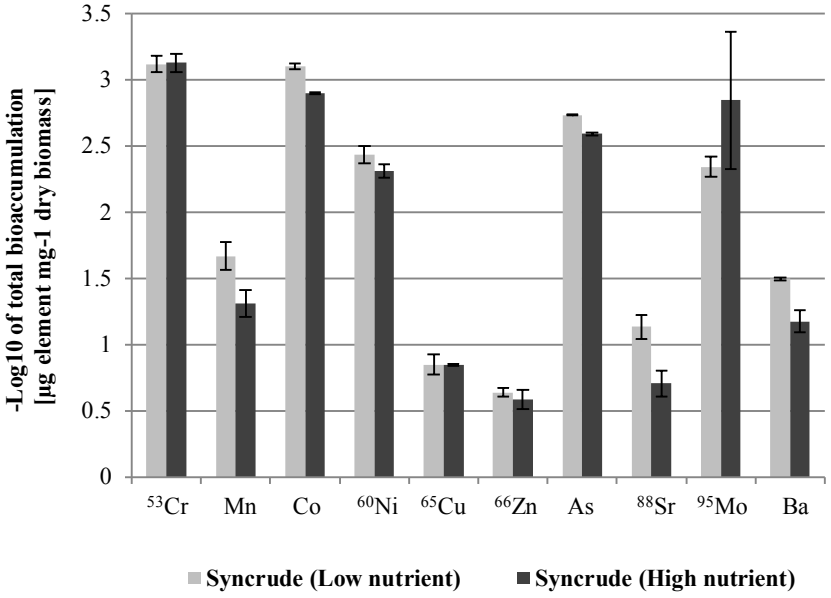
Dissolved organic ligands in TPW can also lower extracellular bioaccumulation as they compete with microorganisms for metal uptake (Di Toro et al. 2001; Gorski et al. 2008; Worms et al. 2010). Syncrude and Albian TPWs contain unrecovered hydrocarbons and dissolved naphthenic acids (~50-70 mg L^{-1}) (Allen 2008) that act as chelating agents and reduce bioavailability of metals to be absorbed by transport sites on the algal cell wall.

Intracellular bioaccumulation of metals, which played the main role in metal removal in this study, takes place via mechanisms which are employed to maintain appropriate metals concentration inside cells. For example, microalgae synthesize peptides capable of binding with heavy metals to form organometallic complexes. These organometallic complexes are accumulated inside vacuoles to control the cytoplasmic metal concentration and prevent or neutralize the potential toxic effects of the metals. Examples of metal binding peptides and proteins that have been identified in algae and higher plants are: phytochelatins (class III metallothioneins, MtIII), which are short-chain polypeptides that are enzymatically synthesized, and the gene-encoded class II metallothionein proteins (Perales-Vela et al. 2006). Production of MtIII in an algal cell depends on the presence of the heavier metals (such as Cd^{2+},As^{3+}, Ag^{+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Au^{2+}, Cu^{2+}, Hg^{2+}), glutathione, cysteine, and sulfide ions (S^{2-}). Sulfide ions contribute to stabilization and nanocrystallization of metal-MtIII complexes inside the cytoplasmic vacuole where all metal-MtIII complexes finally end up (Perales-Vela et al. 2006).

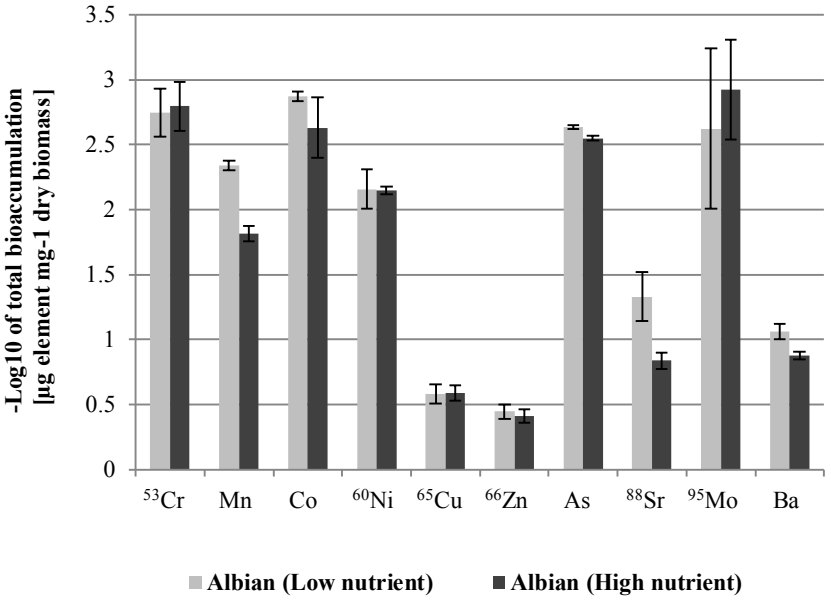
Based on Eh-pH diagrams for target metals (Takeno 2005), the precipitation of Cr_2O_3(s), $HCrO_2$ (s), $Co(OH)_2$(s), CoO(s), Cu_2O(s) and $Cu(OH)_2$(s) might occur for a simple Metal-H-O system. But in the case of a complex mixture such as TPW, many interfering inorganic and organic compounds may affect solubility or precipitation of target metals.

4.3.4. Total Bioaccumulation

Figure 4-4 shows total bioaccumulation (units of –log), comprising intercellular and extracellular metal uptake per dry biomass weight, after 14 days of cultivation for both TPW samples in low and high nutrient concentrations.



(a)



(b)

Figure 4-4 Bioaccumulation comprising intracellular and extracellular uptake per dry biomass (bioaccumulation capacity) on day 14 for Syncrude's (top) and Albian's (bottom) TPWs in low (0.24 mM of NO_3^- and 0.016 mM of PO_4^{3-}) and high (1.98 mM of NO_3^- and 0.201 mM of PO_4^{3-}) nutrient concentrations. Error bars indicate ± one standard deviation (n = 3).

Dry biomass, which indicates the weight of algal cells in TPWs, contributed to algal total bioaccumulation along with intra- or extracellular metal uptake. The total bioaccumulation average for Mn in Syncrude TPW was significantly more than Albian TPW ($P=0.02$); whereas for other target metals no significant difference was observed ($P>0.05$).

Based on *t-Test* analysis, the low nutrient concentration in Syncude TPW resulted in higher total bioaccumulation for Mn, Co, ^{60}Ni, As, ^{88}Sr and Ba ($P<0.05$, Figure 4-4 (a)). For the remaining metals, the nutrient concentration did not affect the total bioaccumulation. In a similar manner, a low nutrient concentration in Albian TPW resulted in a significantly higher total bioaccumulation for Mn, As, ^{88}Sr and Ba ($P<0.05$, Figure 4-4 (b)). It is hypothesised that the lower nutrient concentration causes higher transport sites on the cell surface for nitrate and phosphate uptake (Hassler et al. 2005). These transport sites might contribute to metal uptake as well and consequently increase the bioaccumulation of some metals at lower nutrient concentration. It was previously reported that As and phosphate share similar transportation sites and compete with each other for adsorption on extracellular biotic surfaces (Reuther 1992).

4.3.5. Time-Dependence of Total Bioaccumulation

For all the target metals, total bioaccumulation varied with time during the 14 day cultivation. Except for As and Ni, all target metals peaked in total bioaccumulation between the first and eighth day of the incubation (see Appendix G). This time period corresponds to the exponential growth phase of *P. kessleri* over a 14 day cultivation in this experiment. Fujiwara et al. (2008) reported 3- to 7-fold greater absorption of positively charged metals in the exponential phase in comparison with the stationary phase. This might be related to metal transporters or surface charge of cell walls in the exponential and stationary phases (Fujiwara et al. 2008). The accumulation of each metal is regulated by the activity of their respective metal transporter. The number of metal transporters on the cell surface

changes in different growth regimes and can then affect total bioaccumulation (Fujiwara et al. 2008). Therefore, total bioaccumulation can be considered an active and dynamic factor that changes over a cultivation period, as the results of this study indicate and other reports have verified (Yan and Pan 2002; Hassler et al. 2005; Fujiwara et al. 2008).

4.3.6. Short-Time Exposure Experiment

A short time exposure experiment showed that within the first four hours of introducing *P. kessleri* cells into TPWs, the highest total bioaccumulation capacities were achieved for ^{53}Cr, Co, ^{65}Cu and ^{66}Zn as follows 0.018±0.006, 0.016±0.010, 1.64±0.16 and 4.03±0.52 μg mg^{-1} dry biomass, respectively (see Appendix G). Hassler et al. (2005) found that *C. Kessleri,* grown under limiting Zn^{2+} conditions (i.e. 16pM), was able to accumulate Zn^{2+} in a short-time exposure (≤30 min) 50 times more than when *C. kessleri* were grown in non-limiting Zn^{2+} conditions (i.e. 1.6 μM). This observation might be attributed to the acclimation mechanism under limiting conditions that leads to the greater metal transport sites. In the present study, the inoculums had been cultivated in an artificial cyclone overflow without trace metals (see chapter 3, section 3.2.3), therefore, it is possible that an increase in transport sites on the cell surface (rapid acclimation) occurred in response to metal deficiency. More transport sites on the cell wall lead to higher metal uptake; consequently in the first few hours of exposure the bioaccumulation rate dramatically increases.

Short-time accumulation did not take place for Mn, ^{60}Ni, As and ^{88}Sr in this study. These metals exhibit an increasing trend of total bioaccumulation over the course of the 14 day cultivation period. A similar result has been reported by Fujiwara et al. (2008) for In (Indium) bioaccumulation in *Chlorella kessleri* over the 48 hrs cultivation. Although the number of transport sites was not monitored in this research, it might be assumed that higher transport sites (due to rapid acclimation to metal deficiency) did not increase short-time accumulation for these metals.

4.3.7. Bioconcentration Factor [BCF]

Table 4-2 presents the bioconcentration factor (BCF) for target metals in Syncrude and Albian TPW samples containing low and high concentrations of nutrients. The BCF is equal to the metal concentration in algal biomass [µg {g dry biomass}$^{-1}$] per metal concentration in aqueous medium [µg L^{-1}]) and it is used to compare bioaccumulation results in various organisms. Because of the lower initial concentrations of target metals in Albian TPW as compared to Syncrude TPW (see chapter 3), the average BCFs are higher for the Albian TPW samples. The high nutrient concentration condition provided the maximum BCF in Albian TPW for all metals except ^{53}Cr and ^{95}Mo, whereas no apparent trend was observed in Syncrude TPW.

Table 4-2 Bioconcentration factor (BCF) for low (0.24 mM of NO_3^- and 0.016 mM of PO_4^{3-}) and high (1.98 mM of NO_3^- and 0.201 mM of PO_4^{3-}) nutrient concentrations in Syncrude's and Albian's tailings pond water (TPWs). Data represent the average of three replicated experiments ± one standard deviation.

Bioconcentration Factor [BCF]	^{53}Cr	Mn	Co	^{60}Ni	^{65}Cu	^{66}Zn	As	^{88}Sr	^{95}Mo	Ba
Syncrude										
Low nutrient	0.12 ±0.02	0.51 ±0.12	0.38 ±0.02	0.38 ±0.06	10.11 ±1.77	11.62 ±0.85	0.35 ±0.00	0.11 ±0.02	0.04 ±0.01	0.23 ±0.01
High nutrient	0.10 ±0.02	1.11 ±0.26	0.58 ±0.01	0.49 ±0.06	8.37 ±0.17	9.12 ±1.46	0.52 ±0.01	0.29 ±0.07	0.02 ±0.02	0.52 ±0.10
Albian										
Low nutrient	0.49 ±0.22	0.61 ±0.05	0.90 ±0.08	0.67 ±0.25	10.50 ±1.71	17.87 ±2.18	0.63 ±0.02	0.09 ±0.03	0.09 ±0.11	0.37 ±0.05
High nutrient	0.47 ±0.19	2.23 ±0.32	1.91 ±1.11	0.73 ±0.05	16.45 ±2.21	19.23 ±2.13	0.81 ±0.03	0.26 ±0.03	0.03 ±0.02	0.55 ±0.04

Bioconcentration factor [BCF] = metal concentration in algal biomass [µg (g dry biomass)$^{-1}$] / metal concentration in aqueous medium [µg L^{-1}].

BCFs for *Chlorella* sp. have been reported in other studies. Becker (1983) studied the removal of metals from municipal wastewater using algae (*Chlorella Scenedesmus* and *Chlorella* sp.*)* and reported maximum BCFs of 0.86 Lg^{-1} (Zn) and 0.32 Lg^{-1} (Cr) for *C. Scenedesmus* and 1 Lg^{-1} (Zn) and 0.62 Lg^{-1} (Cr) for *Chlorella* sp. In addition, Maeda et al. (1990) reported a BCF of 3.5 Lg^{-1} (Zn) for *Chlorella vulgaris*. Yan and Pan (2002) found the BCF to be a dynamic parameter over a six day cultivation of *Chlorella pyrenoidosa*, reaching 10.1 Lg^{-1} (Cu). Except for Zn, BCF values for all other metals are in the similar range as those reported in the literature, while Zn achieved a BCF that is higher than the reported value in the literature.

4.4. Conclusion

The indigenous micro-alga *P. kessleri*, was enriched in oil sands TPWs (taken from Syncrude's and Albian's tailing ponds) to study target metal partitioning over a 14 day cultivation period. The intracellular (internal uptake), extracellular (on the cell surface adsorption) bioaccumulation and precipitation of target metals comprising ^{53}Cr, Mn, Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, As, ^{88}Sr, ^{95}Mo and Ba was investigated. FT-IR analysis showed that the cell wall of indigenous *P. kessleri* possessed functional groups comprising amide I and amide II bands and carboxylic groups known to act in metal adsorption. The titration of freeze-dried *P. kessleri* cells revealed that only one of the functional groups (carboxylic group) is involved in proton binding and that the total concentration of ionisable sites on the surface of *P. kessleri* is smaller than those reported by Andrade et al. (2005).

Intracellular bioaccumulation played the main role in metal removal, while extracellular bioaccumulation was observed to some extent for Mn, ^{88}Sr, Ba and ^{95}Mo in Syncrude TPWs and Mn, Co, Ni, ^{65}Cu, ^{88}Sr, Ba and ^{95}Mo in Albian TPWs. Based on the FT-IR scan and functional group titration results, low extracellular bioaccumulation is likely due to the low total concentration of ionisable sites on *P. kessleri*. However, other interfering factors in TPW such as

high cation concentration, unrecovered hydrocarbons and naphthenic acids might reduce extracellular bioaccumulation. High cation concentration in TPW decreases the metal binding on the cell wall, while unrecovered hydrocarbons and naphthenic acids can act as chelating agents and compete for metal uptake with the cell surface, therefore, lowering extracellular bioaccumulation. The total bioaccumulation was more efficient at low nutrient concentrations. The maximum bioaccumulation of ^{53}Cr, Co, ^{65}Cu and ^{66}Zn took place in the first 4 hours of exposure, which implied rapid acclimation occurring in response to metal deficiency during cultivation of inoculums. Total bioaccumulation and bioconcentration factor (BCF) were found to be dynamic parameters which varied over the cultivation period under different growth regimes.

CHAPTER 5: *IN SITU* NAPHTHENIC ACID REMOVAL FROM OIL SANDS TAILINGS POND WATER USING INDIGENOUS ALGAE-BACTERIA CONSORTIUM

5.1. Introduction

Process-affected water contains sand and clay, unrecovered hydrocarbons, dissolved metals, and a complex mixture of organic acids which are historically called naphthenic acids (NAs) (Grewer et al. 2010). In chapter 3 and 4, the removal of metals from oil sand tailings pond water (TPW) and the mechanism of metal bioaccumulation in *Parachlorella kessleri*, an indigenous alga, were studied. However, metals are not the only source of toxicity in oil sands TPW and unrecovered hydrocarbons, especially naphthenic acids (NAs), contribute considerably to the acute toxicity. Based on the classical definition of NAs, they are "alkyl-substituted acyclic and cycloaliphatic carboxylic acids" which are characterized by the general formula of $C_nH_{2n+Z}O_2$, where n indicates the number of carbon atoms and Z is a negative integer that refers to hydrogen deficiency resulting from the cyclic structure (Clemente and Fedorak 2005). Recent publications have reported tailings-associated NAs with a molecular structure that is different from the general formula; they suggest the use of the term total acid-extractable organics (TAOs) instead of NAs (Grewer et al. 2010).

Previous studies have demonstrated that an algae-bacteria consortium can be utilized for the treatment of aromatic pollutants (Borde et al. 2003), piggery wastewater (Gonzalez et al. 2008), pulp and paper industry wastewater (Tarlan et al. 2002), and generally hazardous contaminants (Muñoz and Guieysse 2006; Bahr et al. 2011). In the synergistic relationship between algae and bacteria, algae are responsible for providing oxygen (through photosynthesis), which is required by aerobic bacteria in the biodegradation of toxic organic pollutants. In addition, some studies have indicated that certain algal strains contribute directly to the biotransformation or mineralization of recalcitrant organic compounds (Warshawsky et al. 2007; Semple 1997). For instance, some stains of algae and diatoms demonstrate a partial biodegradation of NAs (Quesnel et al. 2011; Headley et al. 2008). It was reported that *Navicula* sp (a diatom) phytodegraded and consumed a model NA (4-methylcyclohexaneacetic acid) completely (Headley et al. 2008); whereas it is known that the commercial and model NAs are more biodegradable than tailings-associated NAs in TPW (Corinne 2010).

Aerobic bacteria have proven to be capable of oxidizing recalcitrant NAs (Hwang et al. 2013). For example, NAs branched with methyl groups show limited biodegradability; however, a mixed bacterial consortium has been reported to degrade NAs that are

recalcitrant due to methyl substitution on their cycloalkane rings (Headley et al. 2002; Smith et al. 2008).

In this research, an indigenous microbial culture containing bacteria and algae, taken from the surface of tailings ponds, was exploited to remove tailings-associated NAs in the toxic environment of TPW (thus emulating the most realistic scenario). In addition, the contribution of several factors to the biodegradation of NAs, including light, the presence of *Navicula pelliculosa* (a diatom), and the absence of oxygen, was assessed. The results from this chapter elucidated the rate of natural biodegradation of NAs due to indigenous microbial activities on the surface of tailings ponds and clarified the feasibility of *in situ* NA removal using an algae-bacteria consortium.

5.2. Material and Methods

5.2.1. Preparation of materials

All chemicals were certified A.C.S., reagents were prepared with Milli-Q® water, and glass-made containers were used. *Navicula pelliculosa* (strain 552) was purchased from the Canadian Phycological Culture Centre (CPCC). A fresh TPW sample was taken from the surface of an oil sands tailings pond in northern Alberta and stored in 4°C in a glass carboy for two months. 18 clean and autoclaved clear-glass bottles (3.78 L each) were filled with 1.5 L TPW to create 6 different experimental conditions, as follows: 3 were autoclaved in 3 consecutive days as blanks (*Blank*); 3 bottles were left as original samples for algae and bacteria growth (*A-B* samples); 3 bottles were wrapped with aluminum foil preventing exposure to light, and thus creating conditions suitable only for bacteria growth (*B* samples); 3 bottles were inoculated with *N. Pelliculosa* stain for algae and bacteria and *N. Pelliculosa* growth (*A-B-N* samples); 3 bottles were inoculated with *N. Pelliculosa* stain and flushed with N_2 gas (*A-B-N (purged with N_2 samples)*); and finally 3 were autoclaved over 3 consecutive days and inoculated with *N. Pelliculosa* strain after adjusting the pH to 8.3 (*N* samples). TPW samples containing *N. Pelliculosa* were enriched with 0.521 mM NO_3^- and 0.0880 mM PO_4^{3-}, and all remaining samples were enriched with 0.521 mM NO_3^- and 0.0212 mM PO_4^{3-}. Bottles were tightened with caps attached to a Swagelok® valve connected to a Puresep-T® Septum (9.5 mm) for sampling (see Appendix P). All bottles were incubated for 8 hrs and shaken at a rate of 90rpm

under light (4300 lux, Philips F48T8/HO/TL835 ALTO, Markham, ON), followed by 16 hrs of darkness in a still condition.

60 mL aliquot of TPW was sampled from each bottle using a 60 mL luer-lok sterile BD syringe and an autoclaved deflected non-coring septum penetration needle. 5 mL was utilized for the genomic isolation of all microorganisms, and for the measurement of total Chemical Oxygen Demand (total COD). The remaining sample was filtered for further water chemistry analysis.

5.2.2. Water chemistry analysis

The pH and DO measurements were conducted immediately after sampling using Accumet® Research, AR50, Fisher Scientific and YSI-52 Dissolved Oxygen Meter, respectively. Total COD and Dissolved COD were measured based on the closed reflux colorimetric method (American Public Health Association. et al. 2005).

Dissolved Organic Carbon (DOC) and Dissolved Inorganic Carbon (DIC) measurements were performed using a TOC Analyzer (Shimadzu TOC-L CPH, Japan). For DOC analysis, the inorganic fraction was sparged by adding 2.3% (v/v) 2N HCl reagent and air flow rate of 50 mL min^{-1} for 3 minutes and organic carbon faction was decomposed at 680^{o}C. Standards, samples and check controls were analysed through triplicate injections with a standard deviation of less than 5%.

Total acid-extractable organics (TAO) content was measured using Fourier transform infrared (FT-IR) spectroscopy (Jiveraj et al. 1995). The method was modified as follows: 4.5 g NaCl was dissolved in 30 mL of filtered TPW sample (0.45 µm nylon membrane, Millipore, Ireland), followed by adding concentrated HCl (pH<2). TAOs was extracted using three extractions of 10 mL dichloromethane each, and then dried overnight. Standard solutions were prepared by making known concentrations of Merichem naphthenic acids in dichloromethane. The FT-IR scan was performed using a PerkinElmer® Spectrum 100 instrument and PerkinElmer® Spectrum 10 software. For each sample, 32 scans with a spectral resolution of 4 cm^{-1} were obtained, and absorbance at 1743 and 1706 cm^{-1} were summed for calculating the total NA concentration.

Concentrations of cations and anions were determined using Inductively Coupled Plasma/Mass Spectrometry and Ion Chromatography instruments respectively. The detailed procedure for the analysis is provided in the supporting information.

To monitor toxicity, a Microtox^{TM} assay was conducted using a Model 500 Analyzer (AZUR Environmental Corp.), and a bacterial reagent (*Vibrio fischeri*) was used up to 3 h after hydration. The 81.9% basic toxicity method was employed for the calculation of IC20 using Microtox^{TM} Onmi software (AZUR Environmental Corp.). pH in all filtered TPW samples was adjusted around 7.5 and the assay was performed according to the Microtox^{TM} protocol. For quality assurance, a phenol standard solution as a positive control, was run for each experimental set with EC50 expected to fall 13 and 26 mg L^{-1}.

The methods used for NH_4^+, NO_3^-, and PO_4^{3-} measurement involved the 4500-NH_3 F method, the 4500-NO_3 I method, and the 4500-P F method, according to (American Public Health Association. et al. 2005). Anions including (Cl^-, SO_4^{2-}, F^-, NO_2^-, and Br^-) were determined using a Dionex DX600 Ion Chromatography instrument (Dionex, Sunnyvale, CA, USA) equipped with a Dionex IonPac AG9-HC guard column (4 x 50 mm) and an IonPac AS9-HC analytical column (4 x 250 mm), according to EPA method 300.1. For cation measurement (including metals), an Inductively Coupled Plasma/Mass Spectrometry (Elan 9000, PerkinElmer) instrument was used and 25µg L^{-1} of ^{45}Sc, ^{89}Y, ^{103}Rh, ^{115}In and ^{159}Tb were added to samples as internal standards. The operating conditions was as follows: vacuum 5.7E-6, 0.96L min^{-1} nebulizer gas flow, 1200 W RF power, 9.00V lens voltage, -1700V analog stage voltage, 1050V pulse stage voltage.

5.2.3. Microbial quantification using molecular biology techniques

DNA isolation. Total microorganisms' genomic DNA was isolated using the Dynabeads DNA Direct Universal Kit (DynalBiotech, Norway). 250 µL of each TPW sample was centrifuged at 14000*g for 10 min; pellet was re-suspended in a 10 µL lysis buffer, following a procedure included in the DNA isolation protocol suggested by the manufacturer.

Quantitative PCR (qPCR). To quantify the total algae and bacteria population, the qPCR technique was performed using a C1000 Thermal Cycler with a CFX96 *in situ* detection system (Bio-Rad, Singapore). All samples and standards were in triplicate, and the

amplification data were analyzed using Bio-Rad CFX Manager™ 3.0 software. To enumerate the total bacteria count without the interference of algal genomic DNA, a primer set targeting rpoB gene was employed (Takahashi et al. 2006). The qPCR amplifications were carried out in 20 µL of total reaction volume containing 1 µL genomic DNA, 200nM of each primer and 1× of SsoFast™ EvaGreen® Supermix (Takahashi et al. 2006). Algal population was elevated using a primer pair targeting the 23S rDNA plastid gene (Sherwood and Presting 2007). The reaction was performed in 1000nM of each primer, and other conditions were the same as the conditions used in calculating qPCR for bacteria. The thermal cycling involved 40 cycles of the PCR conditions (Sherwood and Presting 2007).

Algae identification. The biological techniques described in chapter 3 were used, including Polymerase Chain Reaction (PCR), followed by Denaturing Gradient Gel Electrophoresis (DGGE) and Sanger DNA sequencing.

5.2.4. Data analysis

In order to evaluate the homogeneity of data, the Levene test was used. TAO results from day 120 were analysed using a one-way analysis of variance (ANOVA) to confirm whether there was a significant difference among treatments ($P<0.05$). To determine the specific differences between each treatment means, a Tukey's test (post hoc analysis) was conducted using SPSS® (Ver 21;SPSS) software.

Principal component analysis (PCA) was used to classify treatments according to FT-IR spectra using MATLAB (R10) software (Abdi and Williams 2010). A singular value decomposition was employed to obtain the principal components and the scores and loadings of the data matrix. The scores were used to evaluate the similarity between the samples (treatments), and the loadings indicate the factors (wavenumbers) that have dominant contributions to the variation between the samples. In addition, hierarchical clustering was used to classify wavenumbers and identify similarity between dominant factors (Kaufman and Rousseeuw 2005).

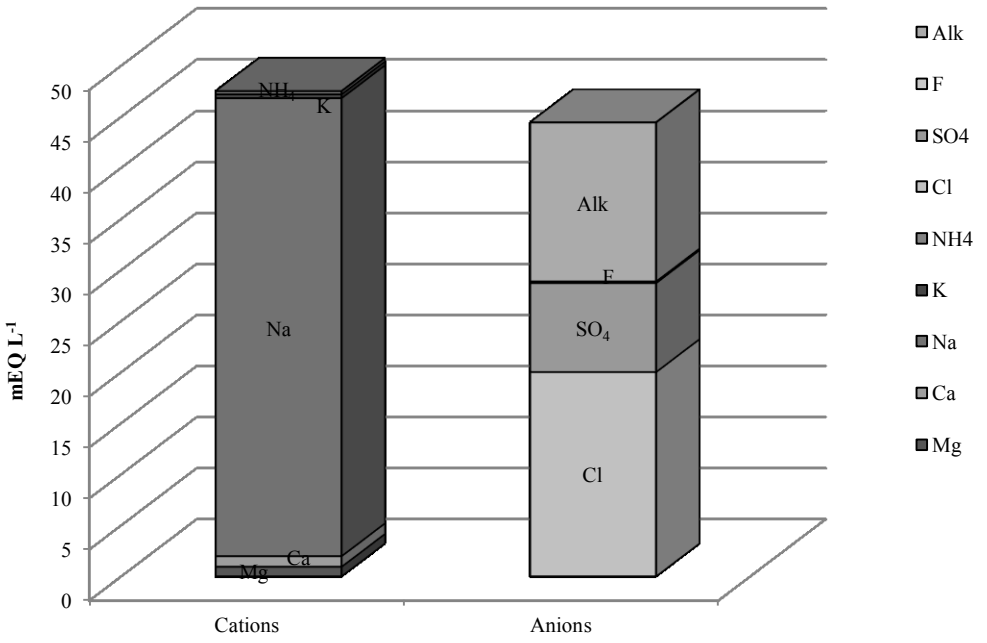
Biodegradation kinetics of TAOs were fit to the first-order kinetic model ($Ln(C/C_0) = kt$) where C_0 is the initial concentration of TAOs (mg/L), C is the concentration at time (t), k

is the degradation rate coefficient, and *t* is time (days). Half-live ($t^{1/2}$) was calculated as follows: $t^{1/2} = 0.693/k$.

5.3. Results and discussion

5.3.1. Water chemistry

The electroneutrality balance of major anions and cations, for the raw TPW sample used in this experiment, indicated that sodium was the dominant cation; whereas the major anions comprised chloride, sulfate and bicarbonate species (See Figure 5-1). The total electroneutrality of anions was slightly lower than that of cations; this might be due to the presence of dissolved organic acids such as NAs in the TPW. Concentrations of NO_2^-, NO_3^- and PO_4^{4-} were lower than the detection limit.



Metals	Cr	Mn	Co	Ni	Cu	Zn	As	Se	Cd
µg L^{-1}	6.36	40.70	1.33	9.47	5.37	7.07	13.24	5.28	0.11

Metals	Sb	Ba	Si	Mo	Ti	Al	B	Pb
µg L^{-1}	1.46	356.3	3	118	8.90	4.36	2.89	0.05

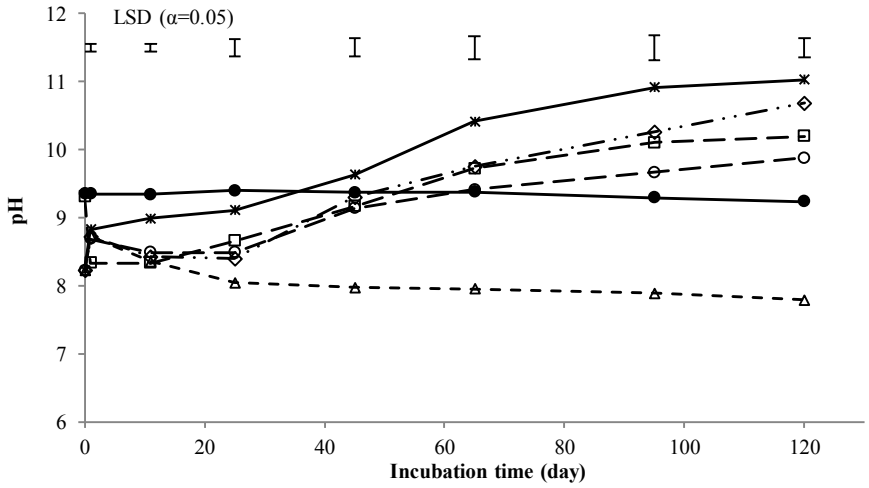
Figure 5-1 Electroneutrality balance of major anions and cations in the TPW sample taken from the surface of the West In-Pit pond (Syncrude Canada Ltd.).

Figure 5-2(a) illustrates the pH change over the 120-day incubation period. Regarding the dissociation constant of NAs (pKa range between 5 and 6), it can be concluded that NA molecules were predominantly ionized in this experiment.

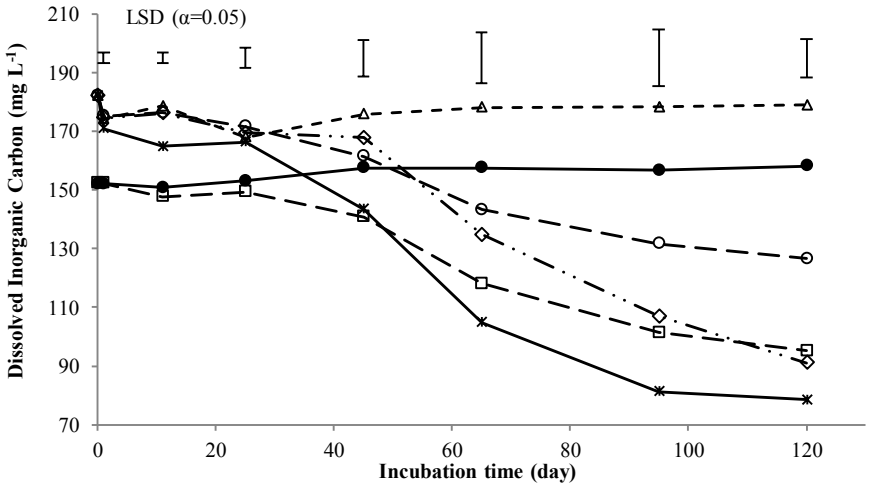
In the samples with bacteria growth only (*B* sample), a slight reduction in pH was observed. It is hypothesized that the slight pH reduction was due to CO_2 emission from bacteria respiration which was dissolved and trapped in water (pH>8). As a result, the dissolved inorganic carbon (DIC) content in *B* sample slightly increased, from 174.16 mg L^{-1} to 179.0 mg L^{-1} (see Figure 5-2(b)). Conversely, all algae containing samples experienced a statistically significant pH increase (p<0.05) which may be attributed to algal photosynthesis. Our results showed that algae and bacteria can impact solution pH levels differently; algae utilize the dissolved carbonate species in water as a source of inorganic carbon for photosynthesis reaction and raise the pH level, whereas bacteria produce carbon dioxide through respiration and reduce the level of pH.

The highest pH increment was observed in *A-B-N (purged with Nitrogen)*. Although respiration rate was not measured in this study, the lack of oxygen in the first 45 days might have reduced the respiration rate of bacteria or increased the rate of photosynthesis. The *A-B-N (purged with Nitrogen)* sample experienced the greatest reduction in DIC, which confirms that the highest photosynthesis rate occurred in this sample (see Figure 5-2(b)). Dissolved oxygen (DO) concentration confirmed that photosynthesis took place in algae-containing samples and also there was enough oxygen for aerobic bacteria in *B* sample over the course of the experiment (see Figure 5-2(c)).

Although the pH level remained constant over 120 days in the *Blank* sample, a considerable increase in pH (8.3 to 9.3) was observed after autoclaving three times. The low DIC in the *Blank* sample (autoclaved) suggests that a pH increase may have resulted from losing inorganic carbon owing to high pressure and high temperature in the autoclave.



(a)



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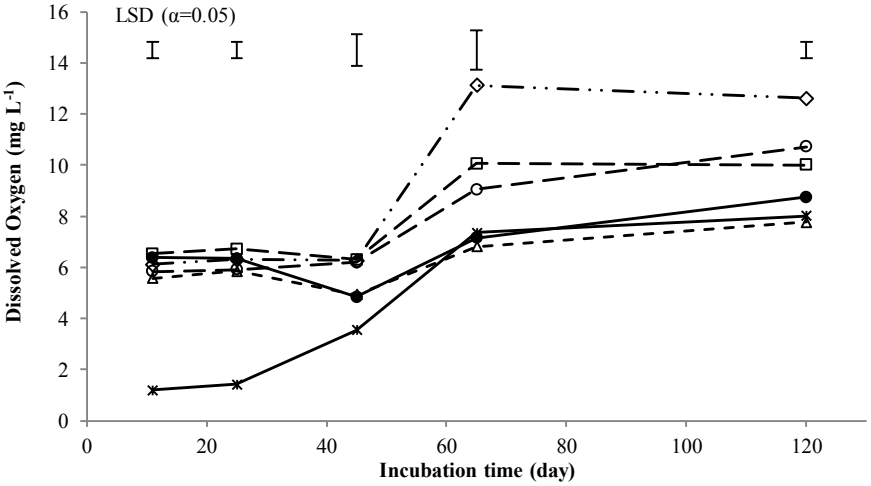


(c)

{—●— Blank, - -△- - Bacteria (B), —○- - Algae-Bacteria (A-B), —◇— Algae-Bacteria-N. Pelliculosa (A-B-N), —✳— A-B-N (purged with Nitrogen), —▫- - N. Pelliculosa (N)}

Figure 5-2 (a) pH variations, **(b)** dissolved inorganic carbon (DIC), and **(c)** dissolved oxygen (DO) over the 120-day incubation period. Error bars indicate the least significant difference (LSD) for each sampling day (α=0.05)

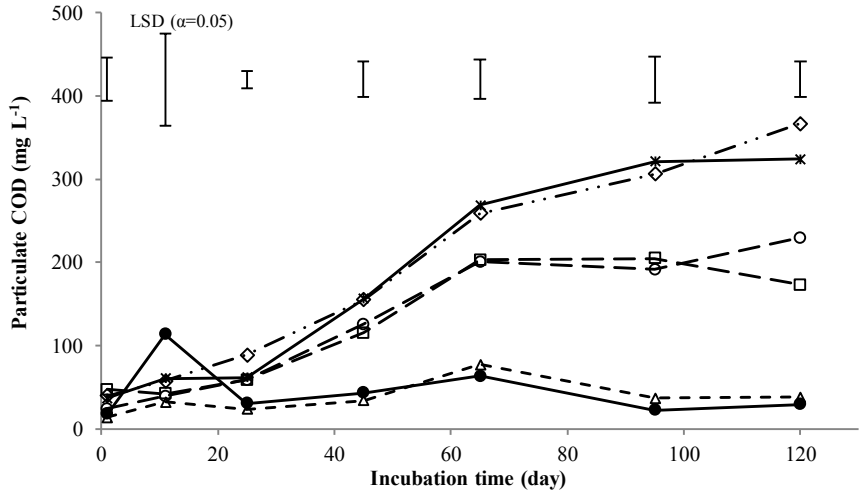
5.3.2. Population of microorganisms

The overall growth in the population of microorganisms may be explained in light of the particulate Chemical Oxygen Demand (particulate COD), which is the COD of biomass.

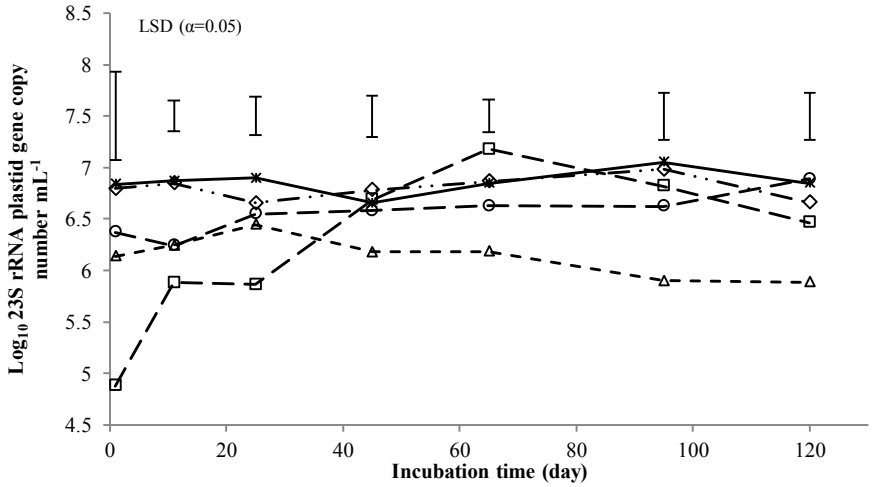
Although particulate COD does not depict the quality and quantity of microorganism populations, it still provides an estimation of biomass growth and is used extensively in environmental engineering publications (Tchobanoglous 2003). Figure 5-3(a) shows particulate COD in samples over a 120-day incubation period. Figure 5-3(b) and Figure 5-3(c) illustrate the $\log_{10}$ copies number of the 23S rRNA plastid gene (in algae) and the rpoB gene (in bacteria) in TPW respectively.

Regarding Figure 5-3(a), the highest particulate COD, inducing algae and bacteria biomass, was observed in *A-B-N* and *A-B-N (purged with Nitrogen)*, followed by *A-B*. Particulate COD in the *B* sample is sharing similar trend with that in the *Blank* sample; this implies a small amount of bacterial biomass growth in these samples.

As shown in Figure 5-3(b), the algae population in all light-receiving samples showed an increase in the number of copies of 23S rRNA plastid gene, as well as slight decrease in the *B* sample (no light). The largest fluctuation in 23S rRNA plastid gene copy number was observed in the *N* sample, which showed a clear exponential growth phase and death phase. In the other samples, which contained a diverse population of indigenous algae and bacteria, the growth and death phases were not as clear and sharp.



(a)



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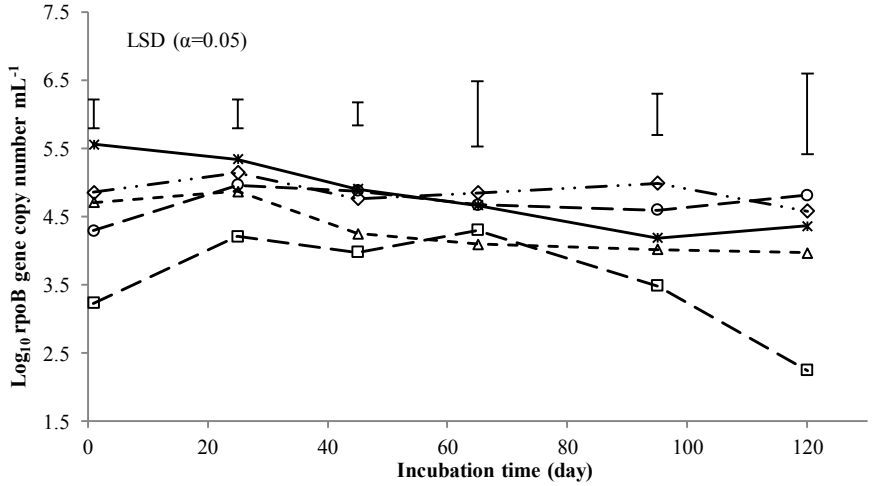


(c)

{Blank, Bacteria (B), Algae-Bacteria (A-B), Algae-Bacteria-N. Pelliculosa (A-B-N), A-B-N (purged with Nitrogen), N. Pelliculosa (N)}

Figure 5-3 The growth of microorganisms over the 120-day incubation period. **(a)** Particulate COD (=Total COD-Filtered COD), which indicates biomass growth, **(b)** Log_{10} 23S rRNA plastid gene copy number mL^{-1} (total algae), and **(c)** Log_{10} rpoB gene copy number mL^{-1} (total bacteria). Error bars indicate the least significant difference (LSD) for each sampling day (α=0.05).

This moderate and stable algal population growth over the course of the experiment might have resulted from the diversity of microorganisms and their synergistic relationship. Sanger DNA sequencing of the amplified 23S rRNA gene identified the *Parachlorella kessleri* as the dominant indigenous alga on the surface of TPW. *P. kessleri* is a unicellular freshwater green alga, which can tolerate harsh conditions. It was identified in cyclone overflow (COF) water from the extraction facilities (see chapter 3). Although *P kessleri* has demonstrated the ability to remove toxic metal (Mahdavi et al. 2012; Hassler et al. 2005; Fujiwara et al. 2008), there is no evidence for the biodegradation of NAs by *P kessleri*.

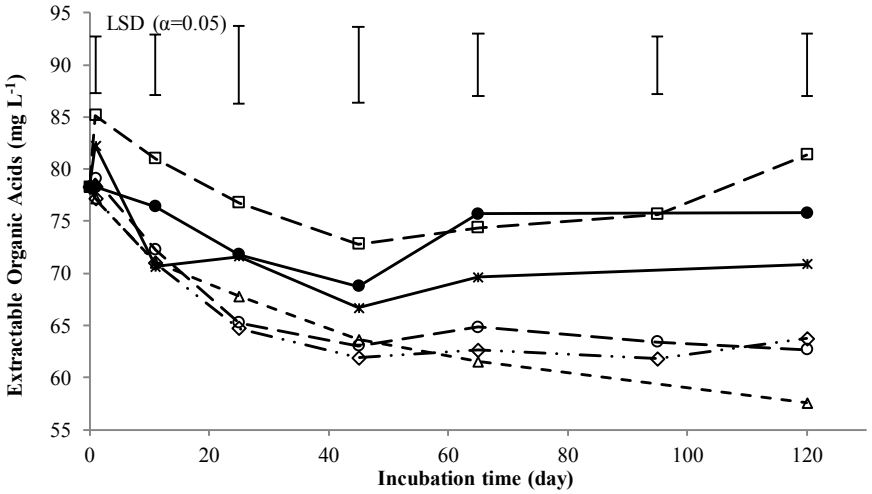
As shown in Figure 5-3(c), only very small bacterial population growth can be observed in *B* samples, which corresponds to limited changes in particulate COD concentrations in these samples. At day 120, the largest copies number of rpoB gene was observed in the *A-B* and *A-B-N,* followed by *A-B-N (purged with Nitrogen)*. Although all of the samples, except *B,* experienced high levels of pH (as high as 9.88 to 11.2; see Figure 5-2(a)), copies number of rpoB gene reveals active bacterial growth, which indicates that indigenous bacteria were highly adapted to high pH. Aerobic bacteria comprising *Pseudomonas, Alcaligens, Acinetobacter, Nocardia, Kurthia,* and *Xanthomonas* species have been identified in enrichment cultures derived from tailings ponds (Quagraine et al. 2005). Depending on depth in oil sands tailings ponds, the aerobic bacterial community was dominated by *Alcaligens* sp. or *Acinetobacter* sp. (Quagraine et al. 2005); it has been reported that the latter strain is capable of degrading recalcitrant cyclopentyl and cyclohexyl carboxylic acids (Herman et al. 1993).

5.3.3. Total Acid-extractable Organics (TAOs)

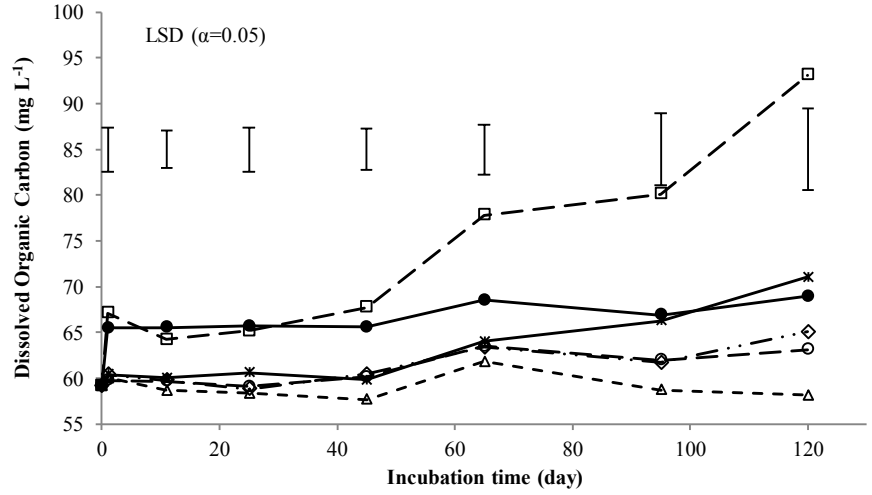
Due to the possible interference of organic acids with naphthenic acids (NAs) measured by FT-IR, in this study the term "total acid-extractable organics (TAOs)" was used instead of "NAs" (Grewer et al. 2010; Scott et al. 2008). Figure 5-4(a) depicts the concentration of TAOs over a 120-day incubation period. In all samples except *Blank*, *N* and *A-B-N (purged with Nitrogen)*, a sudden decreasing trend in the concentration of TAOs was observed until day 40, followed by a slight decrease. Although the *B* sample showed the lowest respiration activity and biomass growth (See Figure 5-2(b) and Figure 5-3(a)), it possessed the highest level of TAO removal (24.06%) among the samples (see Table 5-1).

The *A-B* and *A-B-N* samples demonstrated the next highest rates of TAO removal (17.31 and 15.88 % respectively). It was reported that aerobic bacteria lost their ability to biodegrade commercial NAs in presence of additional readily biodegradable compounds (Misiti et al. 2013). According to algae-bacteria (heterotrophic) biofilm study (Headley et al. 2010), biodegradation rate of TAOs deceases in the presence of algal materials, which are preferable organic carbon for bacteria. In this study, lack of light exposure in *B* sample curbed the algal growth and accelerated TAO biodegradation. Based on biodegradation kinetics modeling, *B* sample possesses the highest removal rate coefficient (k) of 0.0034 day^{-1} and lowest half-life ($t^{1/2}$) of 203 day among the samples (see Table 5-1). According to Table 5-1, presence of *N. Pelliculosa* did not improve the removal rate of TAOs from tailings pond water.

All samples containing indigenous bacteria showed the removal of TAOs, which implies that indigenous bacteria (likely aerobic species) played the main role in the removal of extractable acids. Small TAO removal in the *A-B-N (purged with Nitrogen)* sample may be resulted from the absence of oxygen in the first 45 days and its consequent adverse effects on the aerobic species. Tukey HSD analysis data in Table 5-1 implies that difference in TAO removal between *B* and *A-B* samples is not significant (P=0.454); in other words, aerobic bacteria performed the major contribution to TAO removal.



(a)



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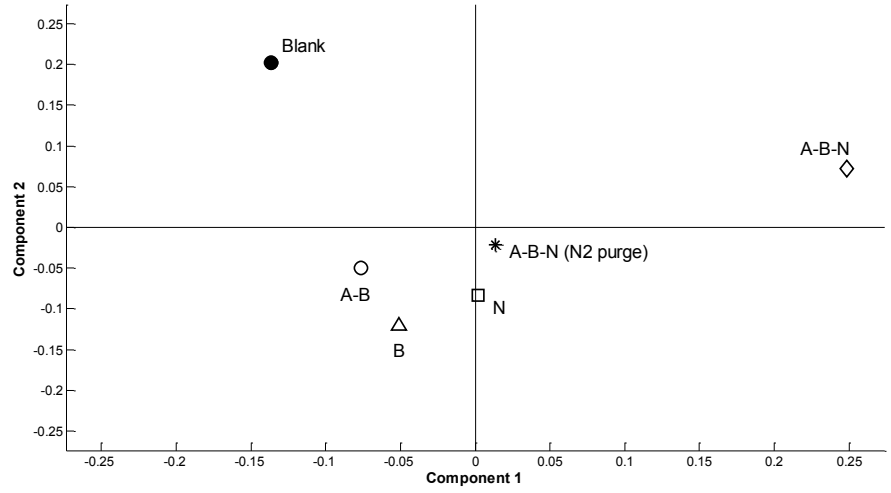


(c)

{Blank, Bacteria (B), Algae-Bacteria (A-B), Algae-Bacteria-N. Pelliculosa (A-B-N), A-B-N (purged with Nitrogen), N. Pelliculosa (N)}

Figure 5-4 (a) Extractable acids measured over the 120-day incubation period using Fourier Transform Infrared Spectroscopy (FT-IR), **(b)** Dissolved organic carbon over the 120-day incubation period. Error bars indicate the least significant difference (LSD) for each sampling day (α=0.05), and **(c)** The score plot of principal component analysis (PCA) for the first two principal components for the data set of all treatments at the end of the 120-day incubation period.

The increase in the concentration of TAOs in the *N* sample may be resulted from the excretion of organic acids from *N. Pelliculosa* cells. A similar increasing trend in dissolved organic carbon (DOC) was observed in the *N* sample over a 120-day cultivation period (see Figure 5-4(b)). *Navicula sp* has been reported to phytodegrade both the *cis*- and *trans*-isomers of a model NAs (4-methylcyclohexaneacetic acid) at ~5.5 mg/L over the course of 14 days (Headley et al. 2008). However, our observations indicate that *N. Pelliculosa* does not contribute to the removal of tailings-associated TAOs.

Table 5-1 P-values (Post Hoc Tests, Tukey HSD), percentages of extractable acid removal for each sample (n = 3), and TAO biodegradation first order kinetic model (removal rate coefficient (k), Half-life ($t^{1/2}$), and the coefficient of determination (r^2))

	Sample Compared	P-value	Removal (%)	k (day^{-1})	$t^{1/2}$ (day)	r^2
Blank	Bacteria (B)	0.000	24.06	0.0034	203	0.93
	Algae-Bacteria (A-B)	0.004	17.31	0.0030	231	0.69
	Algae-Bacteria- Navicula (A-B-N)	0.008	15.88	0.0032	216	0.78
	A-B-N (purged with Nitrogen)	0.495	6.48	0.0022	315	0.50
	Navicula (N)	0.364	-7.39			
Bacteria	Algae-Bacteria (A-B)	0.454				

Measuring the concentration of naphthenic acids using Fourier transform infrared (FT-IR) spectroscopy involves summing the absorbance at 1743 and 1706 cm^{-1} which represent the monomers and hydrogen-bonded dimers of the carboxylic group, respectively (Jiveraj et al. 1995). Although this methodology has been used extensively in industry, it involves certain inherent issues in terms of overestimating the concentration of naphthenic acids

(Scott et al. 2008). In addition, FT-IR spectroscopy does not specify the molecular structures of NAs in terms of carbon number, Z families or oxygen content. Due to the gross quantification of NAs by FT-IR spectroscopy, excreted organic acids from microorganisms can easily interfere with the measurement of NAs. Using ultrahigh-resolution instruments, such as Ultrahigh Resolution Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR MS), for characterization of TAOs, it is possible to verify the removal or production of organic acid compounds in terms of carbon number and hydrogen deficiency. It is assumed that microorganisms preferentially biodegrade or mineralize NAs with lower molecular weight, and the addition of methyl branches or rings (recalcitrant NAs) curbs the biodegradation process (Corinne 2010). For example, *Dunaliella tertiolecta* (a unicellular alga) was reported that is capable of removing most of the tailings-associated NAs with carbon numbers between 11 to 17 and a Z number (hydrogen deficiency) of -2, which indicates the presence of one ring in the NAs' molecular structure (Quesnel et al. 2011). In that study, the mixture of NA compounds with up to 6 rings (Z=-12) was detected and monitored over 6 weeks of incubation in 60% TPW and 40% f/2 medium.

5.3.4. Principal Component Analysis (PCA) and biotransformed bonds

PCA as a statistical technique is well-suited to identify patterns and similarities in high dimensional data such as FT-IR spectra. PCA was performed on the data set including the FT-IR spectra at the end of 120 days for all treatments. The first two principal components were found to explain 82% of the variance in the data set. Figure 5-4(c) illustrates the score plot of PCA for the first two principal components for vibrational spectra between 400 cm^{-1} and 4000 cm^{-1}. This figure shows that the *Blank* and *A-B-N* samples differ from other samples in terms of functional groups and bonds. In addition, the *B* and *A-B* samples are close on the score plot, indicating their similarity, which is consistent with the analysis of the Tukey HSD analysis based on the calculation of concentrations using the method described in section 2.2.

In the PCA shown in Figure 5-4(c), there are factors (wavenumbers) that have a significant loading (absolute value greater than 0.05). To see the loading plots for each principal component refer to Appendix Q.The functional groups and bonds corresponding

to those significant wavenumbers may imply molecular transformation of TAO during the biodegradation process. Hierarchical clustering analysis was used to group wavenumbers that correlate to each other, and it was consistent with the significant loadings in the PCA. The functional groups and bonds corresponding to these significant wavenumbers include (Crews et al. 2010): (1) O-H stretch involving H-bonded (broad 3500 cm^{-1}) and free hydroxyl groups (sharp at 3600 and 3685 cm^{-1}) belonging to alcohols and phenols; (2) C-H stretch belonging to alkanes (2970 to 3000 cm^{-1}), aryl-H stretch (3000 to 3100 cm^{-1}) or COOH functional group (2950 to 3100 cm^{-1}); (3) 1400 to 1455 cm^{-1} that might be free amino acids or amid bends C(=O)N-H. The *B* sample displayed the highest intensity in this region; (4) 1245 to 1260 cm^{-1} that might be aryl-OH bends and O-H in-plane bends belonging to alcohols and phenols; and (5) 640 to 812 cm^{-1} that might be N-H and aryl-OH bends.

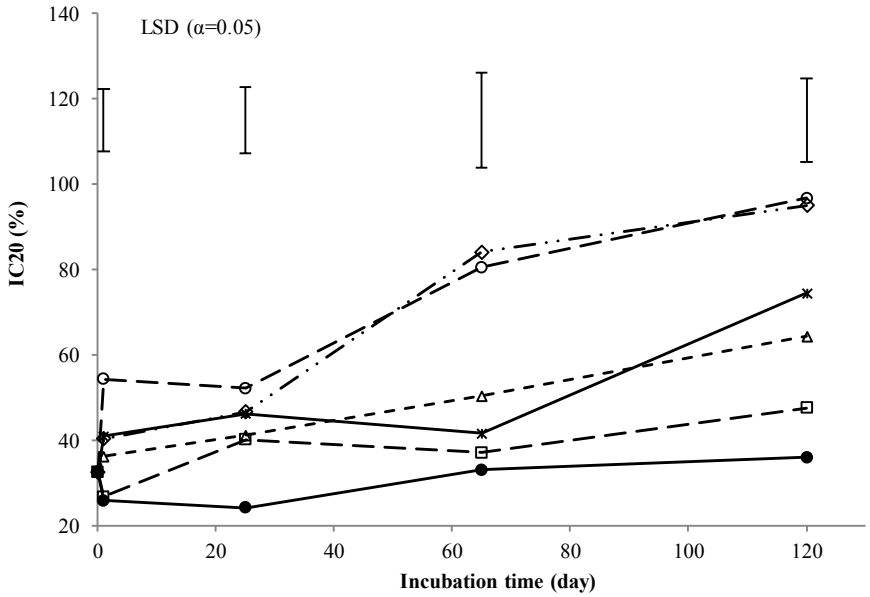
These wavenumbers and corresponding functional groups might be considered as potential markers for biotransformation process. Due to the complex nature of the TAO mixture, vibrational spectra from various bonds may overlap and make it difficult to interpret bonds accurately.

5.3.5. Toxicity

NAs have been shown to be both acutely and chronically toxic to many organisms, including plants, fish, amphibians, zooplankton, phytoplankton, mammals and bacteria (Whitby 2010). Figure 5-5 illustrates toxicity (IC20) over a 120-day incubation time. Because IC50 values in some samples reached more than 100% during the experiment, the IC20 value was selected for the toxicity test. Reduction in toxicity (increase in IC20 value) was observed in all treatments; however, *A-B* and *A-B-N* samples experienced the highest levels of detoxification, followed by *A-B-N (purged with Nitrogen)* and *B* samples. It is hypothesized that toxicity reduction may correlate with an increase in microorganism diversity and population. Because of the presence of oxygen and light, it is expected that *A-B* and *A-B-N* samples possess the greatest diversity of microorganisms among the samples, whereas the absence of oxygen in the first 45 days for the *A-B-N (purged with Nitrogen)* sample, and the lack of light for the *B* sample, might limit aerobic bacterial activity and the numbers of photosynthetic microorganisms respectively. This

result suggests the importance of synergistic relationship between algae-bacteria in the detoxification of TPW.

The order of samples ranked in terms of TAO removal, as shown in Figure 5-4(a), differs from the order of samples ranked in terms of detoxification (Figure 5-5). This difference suggests that some factors in addition to the TAOs measured by FT-IR might influence toxicity. For instance, the *B* sample experienced the highest level of TAO removal, even though it was not the least toxic sample. In fact, toxicity is highly dependent on the molecular structure of organic compounds (Rowland et al. 2011); and regards the diversity of compounds, the measurement of mixture of TAOs by FT-IR will not clarify the contribution of abundance of compounds to the toxicity. Further fingerprinting techniques is required to elucidate the rate of removal for each group of compounds (in terms of carbon number and hydrogen deficiency) and correlate toxicity to TAO composition.



{—●— Blank, - -△- - Bacteria (B), —○— Algae-Bacteria (A-B), —◇— Algae-Bacteria-N. Pelliculosa (A-B-N), —✳— A-B-N (purged with Nitrogen), —▫— N. Pelliculosa (N)}

Figure 5-5 Toxicity (IC20, measured using Microtox) over the 120-day incubation period.

5.4. Conclusions

An indigenous algae-bacteria consortium was used as a biological method to remove recalcitrant tailings-associated NAs. A TPW sample taken from the surface of oil sands tailings pond was incubated for 120 days and the influence of light, oxygen and *Navicula Pelliculosa* on the removal of TAOs was investigated. pH and DIC results indicated that the algal photosynthesis process had occurred in the light-receiving samples, while a small change in DIC and copy number of the rpoB gene in *B* sample (a sample without light suitable for indigenous bacteria growth) was observed, implying a low respiration rate and low bacterial growth. Low particulate COD confirmed a negligible biomass growth in the *B* sample, even as it experienced the highest rate of TAO removal (24.06%), followed by the sample containing Algae-Bacteria, *A-B* (17.31%) and the sample containing Algae-Bacteria-*N. Pelliculosa*, *A-B-N* (15.88%). Indigenous bacteria (aerobic strains) demonstrated most significant contribution to TAO removal, whereas the presence of *N. Pelliculosa* did not enhance the removal rate. The highest TAOs removal rate was observed in *B* sample with a half-life of 203 days, followed by samples with exposure to light. By conducting principal component analysis on the FT-IR results, the significant loading wavenumbers, presenting modified functional groups and bonds among the samples, were identified. These functional groups and bonds indicate the biotransformation of TAOs and involve hydroxyl, carbocyclic and amid groups along with C-H, aryl-H, aryl-OH and N-H bonds.

The indigenous algae-bacteria consortium reduced the toxicity of TPW significantly; however, our results suggest that TAO removal rate does not necessarily correlate to detoxification, suggesting that other factors may contribute to toxicity. In addition, concentration of TAOs measured using FT-IR is not sufficient in itself to study toxicity resulted from NAs. The structure of individual molecules in the mixture of NAs influences the toxicity action; and by using fingerprinting techniques, it is possible to correlate the abundance of NA groups (in terms of carbon number and hydrogen deficiency) to the toxicity level.

CHAPTER 6: CONCLUSIONS, ENGINEERING SIGNIFICANCE AND RECOMMENDATIONS FOR FUTURE WORK

6.1. Introduction

In this chapter, summaries of theory, knowledge gap and objectives for each experiment, including the removal of metals and biodegradation of total acid-extractable organics (TAOs), will be described. In addition, the engineering significance and application along with recommendations for future experiments will be discussed for each test.

6.2. Removal of Metals from Oil Sands Tailings Pond Water Using Algae

6.2.1. Summary

The oil sands industry is currently experiencing rapid development in Alberta. Oil production is expected to increase from 1.9 million barrels per day in 2012 to 3.8 million in 2022, which indicates considerable growth in the near future (www.energy.alberta.ca). Both bitumen recovery methods, namely surface mining and steam-assisted gravity drainage (SAGD), utilize water for the extraction process. Consequently, process-affected water (PAW) must be safely collected, reused and reclaimed. The large volume of water collected in the tailings ponds covers an area of 176 km^2 (67 mi^2). Among the pollutants in tailings pond water (TPW), metals are important inorganic contaminants. Metals threaten the ecosystem and human health at a certain concentration level. In this research, the potential of indigenous alga, *Parachlorella kessleri*, for use in the *in situ* removal of metals including ^{53}Cr, Mn, Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, As, ^{88}Sr, ^{95}Mo and Ba has been investigated. In this research, the feasibility and efficiency were evaluated for further engineering applications. The design parameters obtained in this research, such as bioconcentration factor (BCF), total bioaccumulation, and removal percentage, can be employed in real engineering practice. In addition, the mechanism of metal uptake, in terms of intracellular and extracellular bioaccumulation, was determined, and the functional groups, which contribute to metal uptake were identified. These observations help elucidate the mechanism and theory behind the algal metal removal process from TPW.

6.2.2. Conclusions and Engineering Significance

Indigenous alga from cyclone overflow water (in a bitumen recovery facility at Syncrude Canada Ltd.) was isolated and used for the experiments. For strain identification, molecular biology techniques were used, including amplification of 23S rRNA gene fragments using a polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE). Using the Sanger Sequencing method, the unknown strain was identified as *Parachlorella kessleri*. To the best of our knowledge, this is the first investigation of the removal of metals from TPW. Two TPW samples were taken from Syncrude (West In-pit pond) and Albian (External Tailings Facility pond), and each was enriched with a "low level" (0.24 mM NO_3^- and 0.016 mM PO_4^{3-}) as well as a "high level" (1.98 mM NO_3^- and 0.201 mM PO_4^{3-}) of nutrient supplements. The algal cell count observations indicated that a high level of nutrients did not improve the algal growth rate in either TPW sample.

The removal of ^{60}Ni, ^{65}Cu, As, ^{88}Sr, ^{95}Mo, and Ba was significantly higher ($p < 0.05$) in the Syncrude TPW sample when the level of nutrients was high, while in the Albian TPW sample, the highest removal of Co, ^{60}Ni, As, ^{88}Sr and ^{95}Mo ($p < 0.05$) was achieved when the level of nutrients was low. This observation implies that for an engineering application, the nutrient level must be selected based on the TPW source and water chemistry; moreover higher nutrient concentration does not necessarily increase metal removal.

The algal wet biomass concentrations were 1.18±0.44 and 1.50±0.33 g L^{-1} in the Syncrude and Albian samples, respectively. The algal wet biomass concentration and metal removal in this study are lower than those reported in other publications. This suggests that operating at higher algal biomass weight increases the efficiency of the metal removal process. In addition, it has been shown that the high concentration of sodium in TPW adversely affects the process of extracellular bioaccumulation, and consequently that reduces total bioaccumulation (Chojnacka 2007; Andrade et al. 2005).

FT-IR spectra identified some functional groups on the surface of the cell wall in *P. kessleri,* namely amide I, amide II bands, and carboxylic groups. These functional groups act as metal chelating sites and contribute to extracellular metal bioaccumulation. However, an acid-base titration of freeze-dried cells demonstrated that carboxylic groups (pKa=6.6 with an ionisable group concentration of C[AH]=0.016 mmol H^+ g^{-1} (biomass)) are the only active proton binding site on the surface of *P. kessleri* which may possibly contribute to metal binding.

Intracellular bioaccumulation was the main metal removal mechanism. Unlike extracellular bioaccumulation, intracellular bioaccumulated metals are stable. Because metals will not be desorbed in the solution by altering pH or other water chemistry parameters, and this elevates the reliability of this metal removal by algae. The total bioaccumulation as well as the bioconcentration factor (BCF) varied over the course of incubation period in different growth regimes, indicating that these parameters are dynamic factors.

6.2.3. Future Research and Recommendations

1. Metals can also be adsorbed on the surface of suspended solids and clays in TPW. In this experiment, all TPW samples were filtered (0.45 µm), and therefore suspended solids could not interfere with the metal removal process during the incubation period. Future research may determine the capacity and the mechanism of metal adsorption by the suspended solids in TPW, and its interaction with metal removal by algae. This can be done by running an experiment on two samples of TPW, one sample containing algae and suspended solids and the other containing only algae. The observations will explain the interference of solids during metal bioaccumulation by algae. In addition, the absorption rate of metal on the surface of suspended solids can be determined by conducting an experiment at different metal concentrations. The adsorption equilibrium

constant for each metal can be calculated using common models such as the Langmuir adsorption model.

2. In this research, extracellular bioaccumulation was measured by washing the algal cells using a 5 mM EDTA/HEPES buffer. The concentration of metals in the EDTA/HEPES buffer indicated the extracellular bioaccumulation. Intracellular bioaccumulation was measured by acid digestion of algal cells, which were washed with EDTA/HEPES buffer. However, the difference in the metal content of digested algal cells after and before washing with EDTA/HEPES buffer will give the extracellular bioaccumulation. In this new approach for extracellular bioaccumulation measurement, the EDTA and HEPES content of the buffer will not interfere with the metals measurement in the ICP-MS instrument, and accuracy of the extracellular bioaccumulation measured will increase. In addition, the extracellular and intracellular bioaccumulation data will be more comparable, because both are calculated by the measurement of metals in digested algal cells.
3. An increase in algal growth rate (total biomass) may enhance metal removal efficiency. Several factors contributing to algal growth, such as light, temperature, and various nutrient supplements, can be examined and optimized. In addition, the metal adsorption rate at each growth stage, including the lag, log, stationary, and dead phases, can be determined. In engineering applications, the wastewater treatment plants for metal removal must operate in the algal growth stage, which results in the highest metal removal.
4. In this research, it was observed that the level of nutrient concentration affects the metal removal rate differently in samples from Syncrude and Albian. It is hypothesized that water chemistry contributes to the complex mechanism of metal removal and alters the results. More investigations are required to elucidate the unknown aspects of metal removal from TPW.
5. It was reported that a high concentration of sodium in TPW reduces extracellular bioaccumulation through sodium competition with positively

charged metals for functional groups on the algal cell walls (Chojnacka 2007; Andrade et al. 2005). To study the influence of sodium ions on extracellular bioaccumulation, it is suggested that several batch tests are conducted on a series of TPW samples with various sodium concentration. Extracellular bioaccumulation is expected to reduce if the theory of sodium competition with metals is dominant.

6. Phosphate is considered a nutrient in this experiment; however, it was reported that phosphate ions share similar transportation sites with some metals, such as arsenic, for bioaccumulation. Consequently, phosphate ions may compete for adsorption on extracellular biotic surfaces and may reduce metal removal efficiency (Reuther 1992). The influence of phosphate on metal removal in algae can be studied in the future.

6.3. Removal of Total Acid-Extractable Organics (TAOs) from Oil Sands Tailings Pond Water Using Algae-Bacteria Consortium

6.3.1. Summary

Total acid-extractable organics (TAOs), commonly called naphthenic acids (NAs), are an important group of organic pollutants that dissolve in the PAW during the recovery of bitumen from oil sands ore. It is believed that TAOs are the main source of acute and chronic toxicity in oil sands TPW, and that they adversely affect a variety of organisms including plants, fish, amphibians, phytoplankton, mammals, and bacteria. Regarding the zero liquid discharge (ZLD) policy, it is essential to treat and reclaim the TPW by using environmental friendly and economically feasible treatment methods. In this research, the *in situ* biodegradation of TAOs from oil sands TPW was investigated using an algae-bacteria consortium. The objectives of this research involve the removal of recalcitrant TAOs and evaluation of the biodegradation mechanism using indigenous bacteria, indigenous algae, and *Navicula pelliculosa* (a diatom). In addition, the influence of light and the presence of oxygen on the biodegradation

rate were studied. The results from this research elucidate the mechanism and efficiency of *in situ* TAO biodegradation for future engineering applications.

6.3.2. Conclusions and Engineering Significance

A water sample, containing indigenous microorganisms (bacteria and algae), was taken from the surface of an oil sands tailings pond and used for the experiment. Using genetic molecular techniques, the dominant alga strain was determined to be *Parachlorella kessleri*. Six TPW samples were prepared, namely: (1) Bacteria (sample *B*, without light to prevent algae growth), (2) Algae-Bacteria (sample *A-B*, with light), (3) Algae-Bacteria-Nacivula (sample *A-B-N*, TPW inoculated with *Navicula Pelliculosa* strain), (4) *A-B-N* sample purged with N_2, (5) autoclaved TPW incubated with *Navicula Pelliculosa* strain (sample *N*), and (6) autoclaved TPW (sample Blank).

According to observations, the dissolved inorganic carbon (DIC) content of TPW was used as a carbon source in the photosynthesis process by algae, and consequently, pH increased. However, the respiration rate (carbon dioxide production) of bacteria in sample *B* did not considerably reduce pH or increased DIC. A similar pH increase is expected to occur in a real engineering application in the case of using algae for treatment; therefore, pH adjustment might be required to maintain pH in the appropriate range to protect microorganisms, especially bacteria.

The copy number of the rpoB gene, which indicates the bacteria population, and the particulate COD concentration, implied negligible bacterial growth as compared to algal growth; however, bacteria played the main role in the removal of TAOs. The highest TAO removal rate was observed in the *B* sample (24.06%), followed by the *A-B* (17.31%) and *A-B-N* (15.88%) samples. Based on first-order kinetics, the half-life removal rate in the *B* sample was 203 days. The half-life removal rate can be employed as a key parameter in the design of bioreactors,

such as wetlands or lagoons, for the treatment of TPW. According to our observations, *N. Pelliculosa* did not contribute to TAO removal.

Principal component analysis (PCA) on the FT-IR spectra between 400-4000 cm^{-1} was used to identify the wavenumbers with significant loading. These wavenumbers indicated the modified functional groups and bonds among the samples. The biotransformed functional groups and bonds were:

(1) O-H stretch involving H-bonded (broad 3500 cm^{-1}) and free hydroxyl groups (sharp at 3600 and 3685 cm^{-1}) belonging to alcohols and phenols;

(2) C-H stretch belonging to alkanes (2970 to 3000 cm^{-1}), aryl-H stretch (3000 to 3100 cm^{-1}) or COOH functional group (2950 to 3100 cm^{-1});

(3)1400 to 1455 cm^{-1} that might be free amino acids or amid bends C(=O)N-H. The *B* sample displayed the highest intensity in this region;

(4) 1245 to 1260 cm^{-1} that might be aryl-OH bends or O-H in-plane bends belonging to alcohols and phenols; and

(5) 640 to 812 cm^{-1} that might be N-H or aryl-OH bends.

In terms of detoxification, the most efficient treatment was observed in samples containing the indigenous algae-bacteria consortium. However, toxicity reduction did not correlate with the TAO removal rate, implying that some other factors may contribute to toxicity. Therefore, it is strongly suggested that the industry directly measures and monitors the toxicity in environmental samples, because the TAO measured by FT-IR (which is a common method used in the oil sands industry to monitor pollution in water) does not represent the toxicity level.

6.3.3. Future Research and Recommendations

1. In this research, an FT-IR instrument was used for the quantification of TAO. This method provides a rough estimation of TAO content, which

involves calculating the sum of intensities in two wavenumbers of 706 and 743 cm^{-1}. However, using ultrahigh resolution characterization and fingerprinting methods, such as Ultrahigh Resolution Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR MS), it is possible to categorize TAO compounds based on their carbon number and hydrogen deficiency. Therefore, the dynamic change in TAO composition in each treatment is observed and the preferable compounds for biodegradation are identified.

2. Although the bacterial population was measured using qPCR (the copies number of rpoB gene), the diversity of bacterial species and the types of strains remained unknown. For future research, pyrosequencing techniques can be used to sequence the bacterial genomic DNA and eventually identify the microbial diversity and dominant species. The results from pyrosequencing will reveal the tolerant bacteria to toxicity from naphthenic acids, and dominant bacteria in the algae-bacteria consortium. Conducting an experiment using axenic culture of each identified bacterium may elucidate the contribution of the target individual bacterium to biodegradation of naphthenic acids.
3. Fixed growth bioreactors have demonstrated higher efficiency in the treatment of wastewater. These bioreactors can tolerate hydraulic and toxic contamination shocks and provide a stable removal rate. It is suggested to use immobilization techniques (de-Bashan and Bashan 2010) to form biofilms of the algae-bacteria consortium for treatment of contaminants from tailing pond water. Combining such an algae-bacteria fixed growth bioreactor with a pre-ozonation unit (to decompose the recalcitrant naphthenic acids) is suggested for future engineering applications. It is proven that ozonation improves the biodegradation of naphthenic acids with large molecules, by decomposing them into small and biodegradable molecules. However, it has been also proven that indigenous bacteria population in tailings pond water will survive after ozonation (Brown et al. 2013).

4. Carbon fixation by algae may reduce the carbon dioxide emission from the oil sands industry. In addition, biodiesel production from algal cells can be considered as a future engineering application for algae in the oil sands industry.

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APPENDICES

APPENDIX A

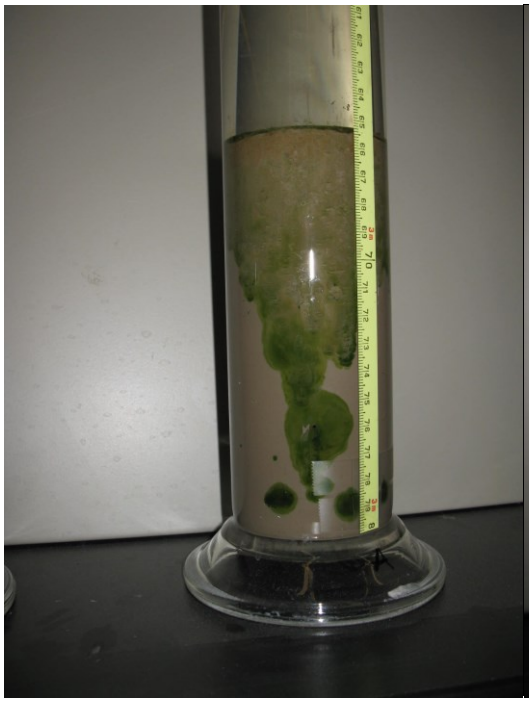
Algae contain lakes with high total dissolved solids (TDS), suitable for algae isolation

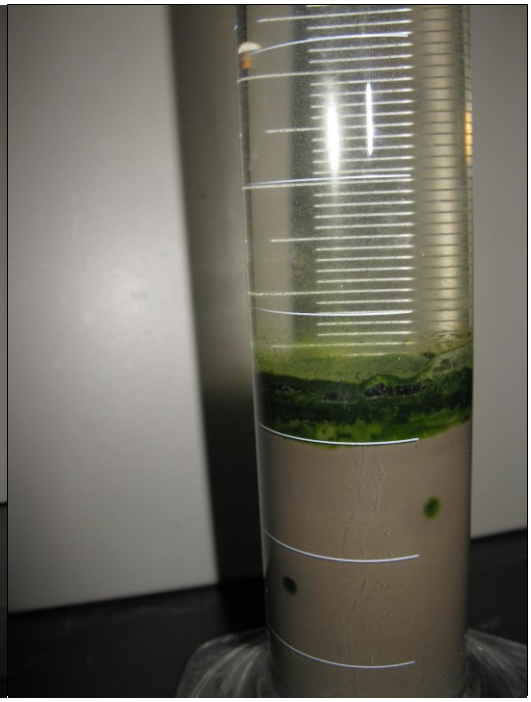
This table shows the algae species in some lakes, located in Alberta region. Indigenous algae from each lake are adapted to the corresponding total dissolved solids (TDS). These algae can be isolated and used for future experiments for the treatment of wastewater with similar TDS.

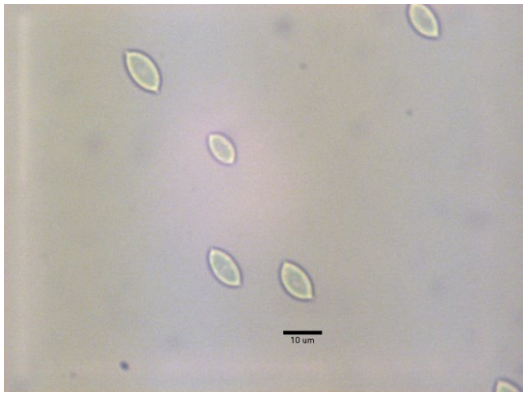
Lake	TDS (mg/L)	Algae Species in Lakes	Best time for sampling
Buffalo Lake	1595	*Anabaena flos-aquae*	-
		Microspora tumidula	-
		Synechocystis sp	-
		Gomphosphaeria aponina	-
		Gomphosphaeria lacustris	-
		Chaetoceros elmorei	-
Eagle Lake	1231	*Aphanizomenon sp*	-
Miquelon Lake	5402	*Gomphosphaeria sp.*	May
		chlorophyta, Eudorina	June-Aug
		chlorophyta, Trachelomonas	June-Aug
		chlorophyta, Monoraphidium	June-Aug
		chlorophyta, Chlamydomonas	June-Aug
Olivia Lake	12364	*cryptomonad (Cryptomonas sp.)*	Aug
		Fragilaria spp.	May-July
		Navicula spp	May-July
		Aphanizomenon flos-aquae	Aug
		Aphanothece clathrata	Aug
Peninsula Lake	9600	*Microcystis aeruginosa*	-
		Lyngbya Birgei	-
		Rhizoclonium hieroglyphicum	-
		Rhodomonas minuta	May
		Monoraphidium contortum	May
		Chrysomonad sp	July
		Oscillatoria angustissima	Aug
		Chroococcus dispersus	Aug

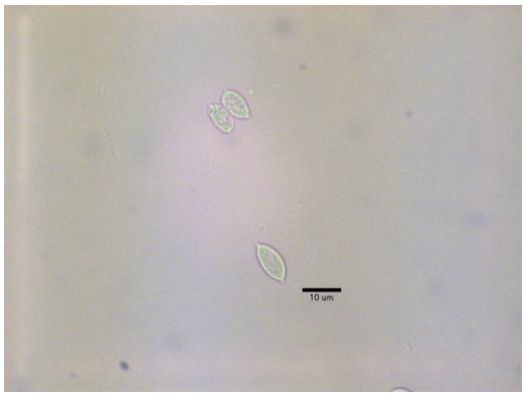
APPENDIX B

The indigenous alga, *Parachlorella kessleri,* found in a cylinder, containing cyclone over flow and cellular image





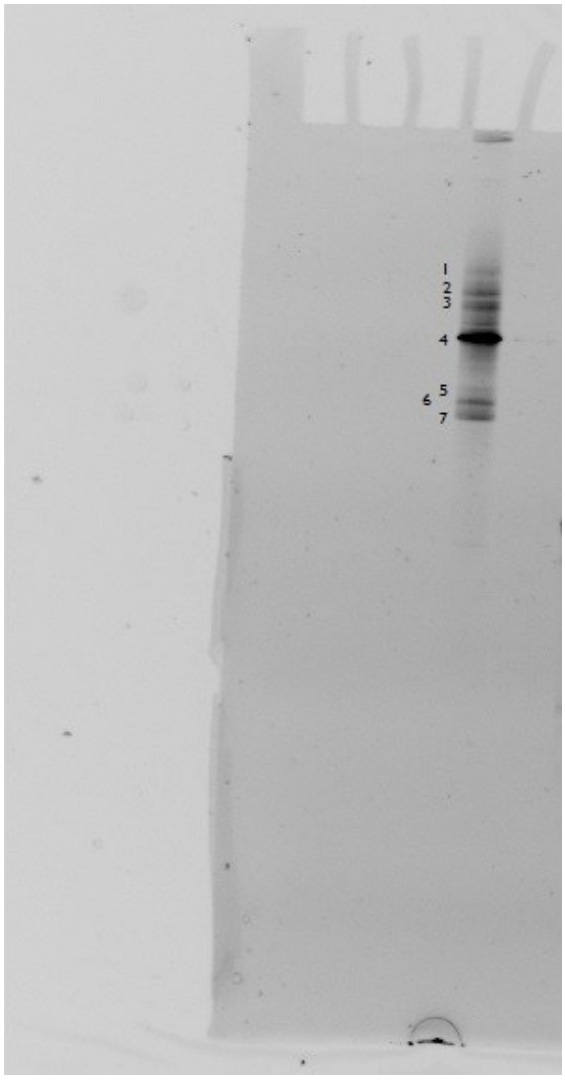




APPENDIX C

Denaturing Gradient Gel Electrophoresis (DGGE) for Identification of Unknown alga

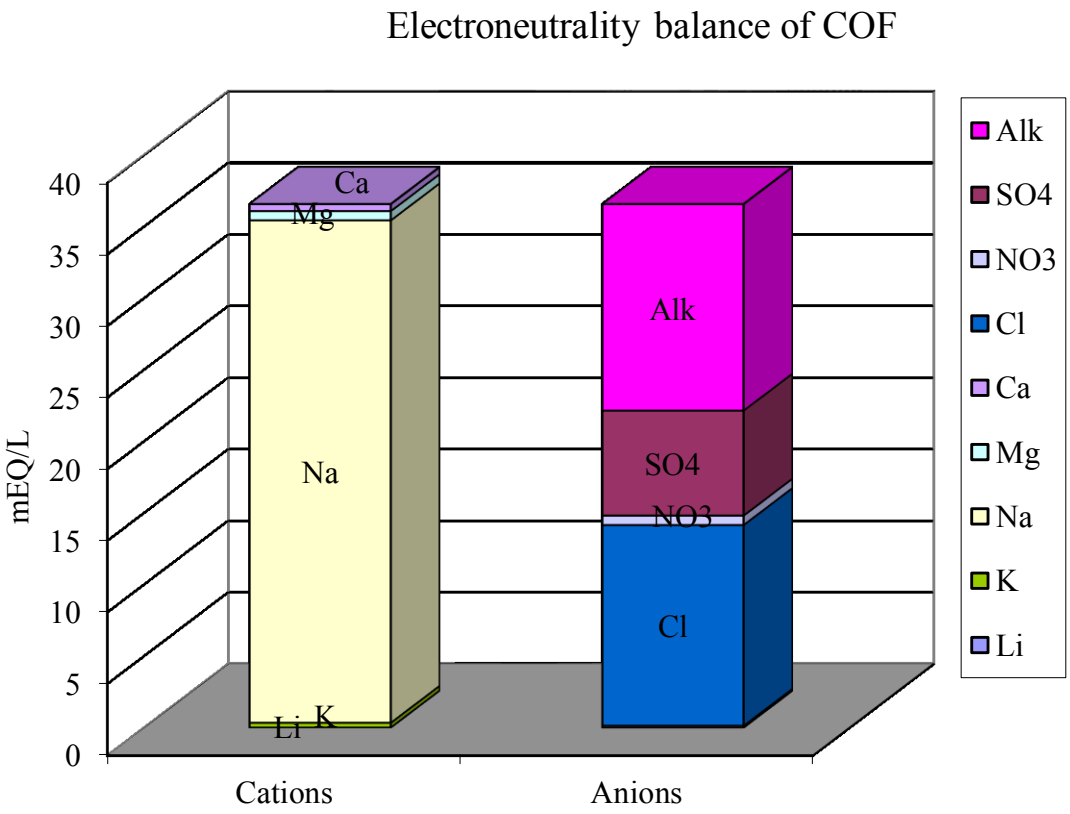
By amplification of 23S rRNA gene fragments using a polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE), the unknown indigenous alga was identified as *Parachlorella kessleri* and was used for further experiments including the removal of metals and biodegradation of total acid-extractable organics (TAOs). In the DGGE gel, each single band starting from 1 to 7 (see Figure below), was cut and sent for DNA sanger sequencing. Sanger sequencing results for all bands (fragments) resulted only one species (*Parachlorella kessleri*).



APPENDIX D

Electroneutrality balance for cyclone over flow (COF).

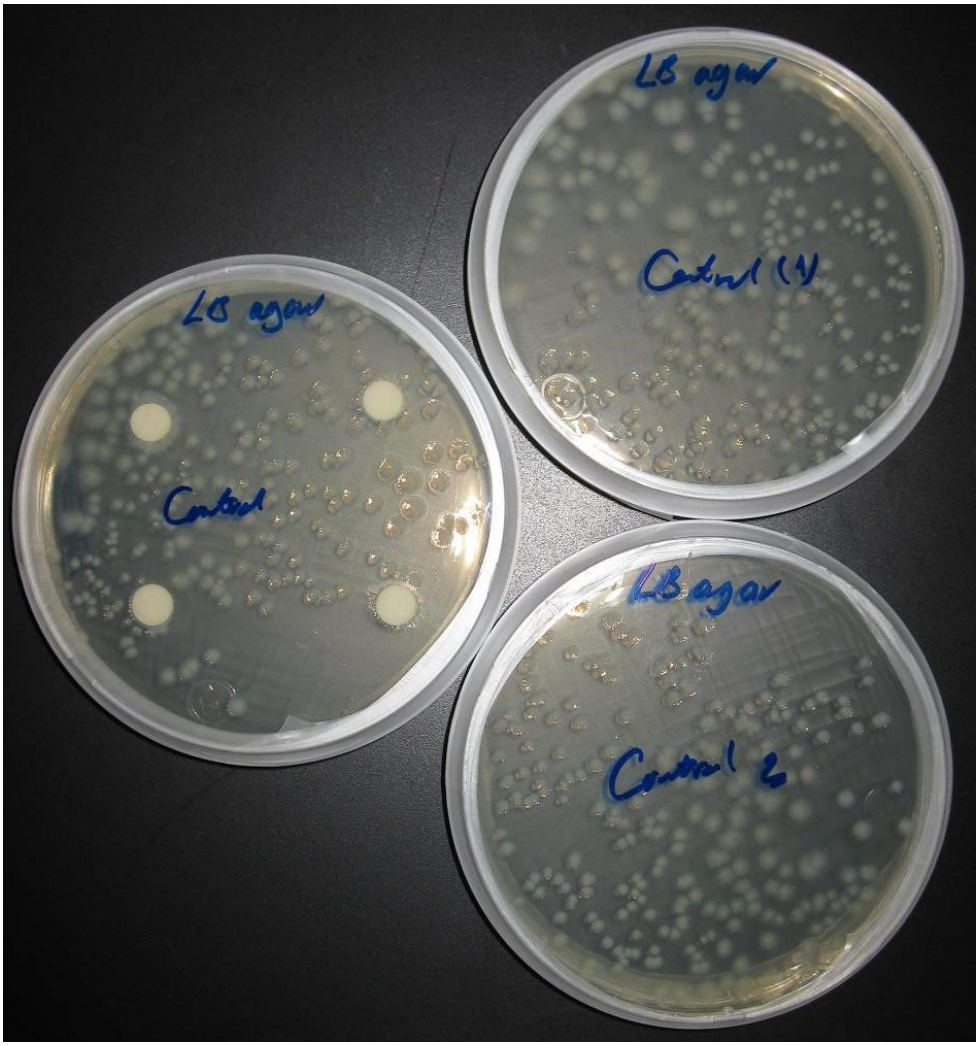
For the method of measurement of cations and anions, see chapter 5.



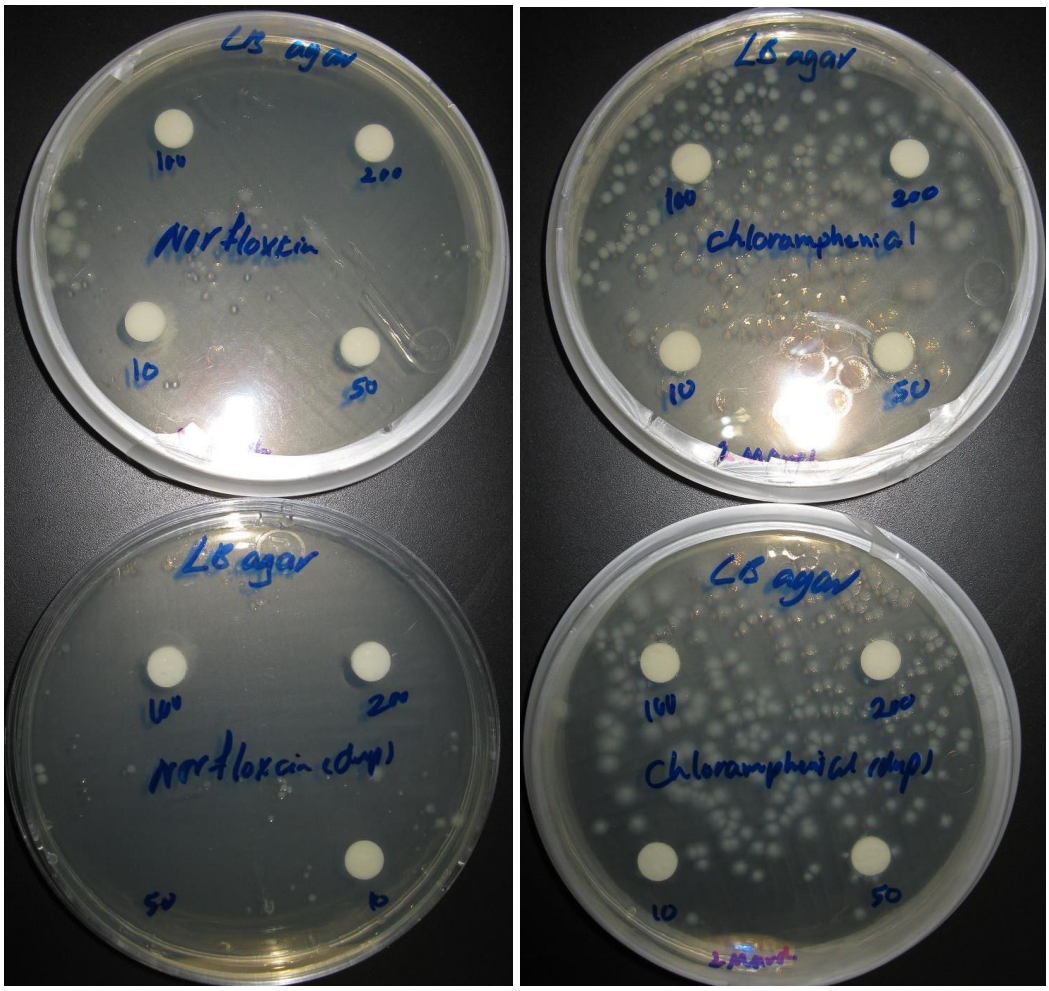
APPENDIX E

Antibiotic experiment for making an axenic culture of *Parachlorella kessleri*

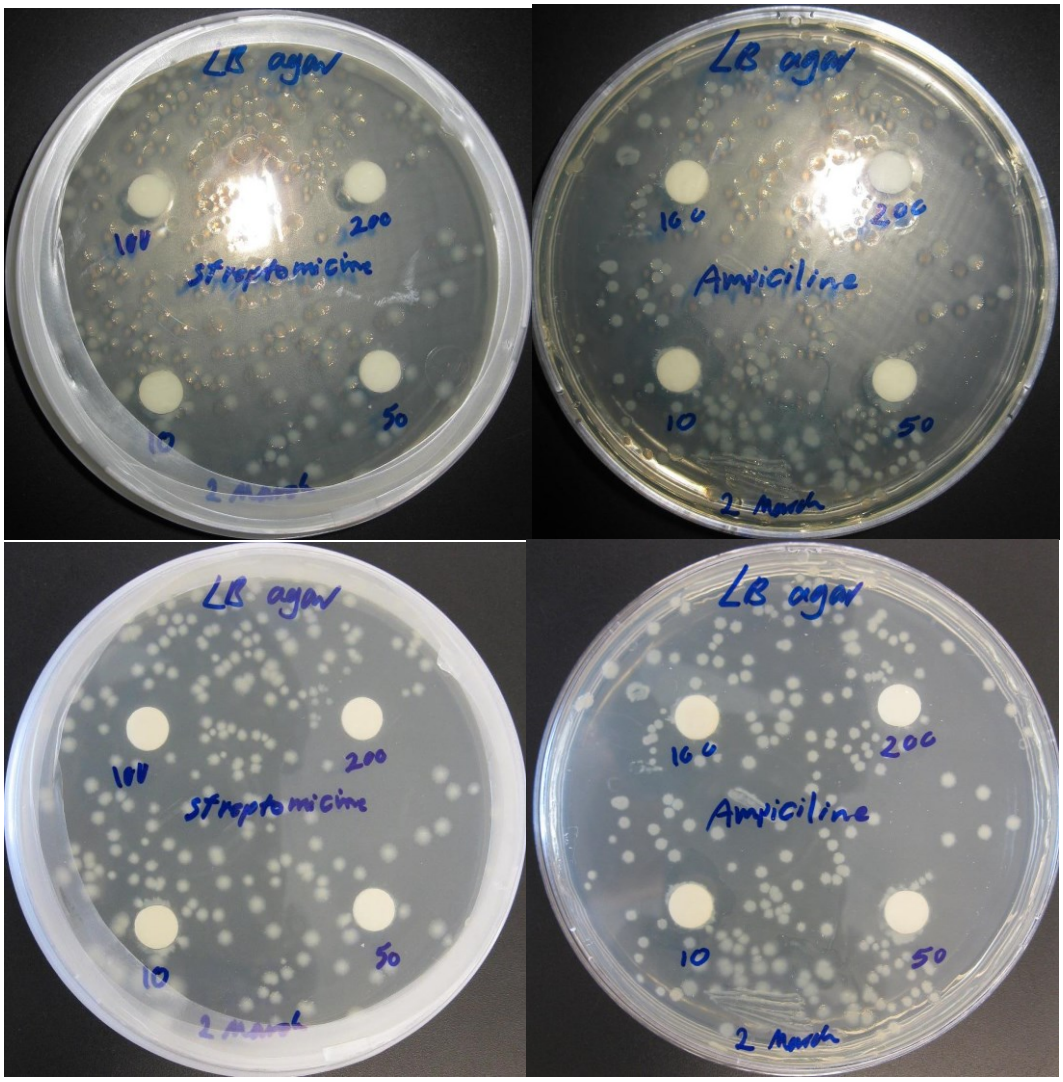
For making an axenic culture of *Parachlorella kessleri*, an antibiotic must be used along with lysozyme and sodium dodecyl sulfate (SDS) solution. Despite *P. kessleri* could not survive in absence of indigenous bacteria and production of its axenic culture did not accomplished, the series of antibiotic tests proved that Norfloxacin had the most influence on the indigenous bacteria in TPW. However, after two weeks, slow growing bacteria appeared on the agar which could resist Norfloxacin. Indigenous bacteria showed a strong resistance to Chloramphenicol, Streptomycin, and Ampicillin



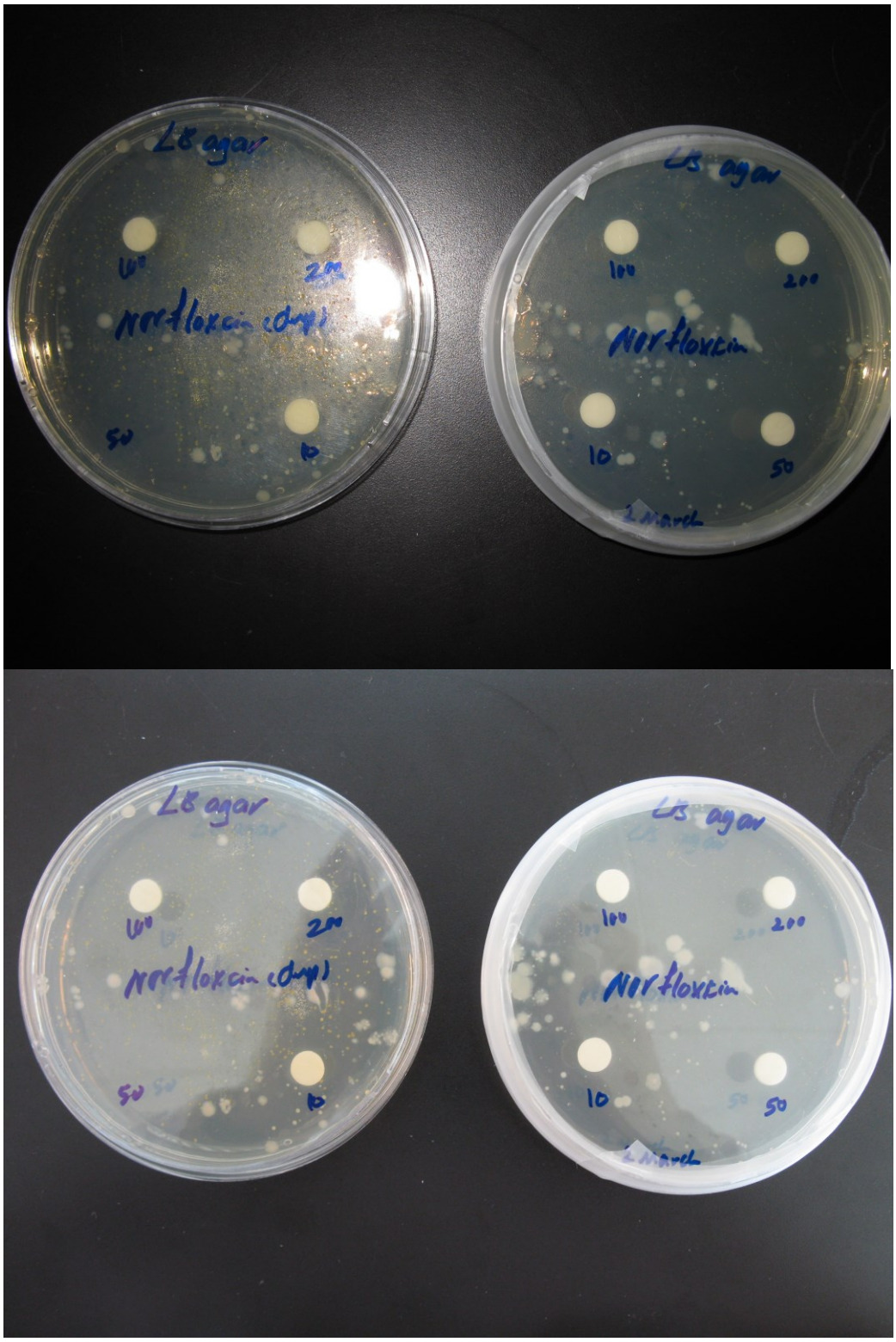
Negative control (Blank) sample



Norfloxacin and Chloramphenicol after one week incubation. Various volumes of antibiotic solutions (10, 50, 100, and 200 µL) were added to the discs on the agar to see the influence of antibiotics on the bacteria.



Indigenous bacteria fromTPW were resistant to Chloramphenicol, Streptomycin, and Ampicillin.

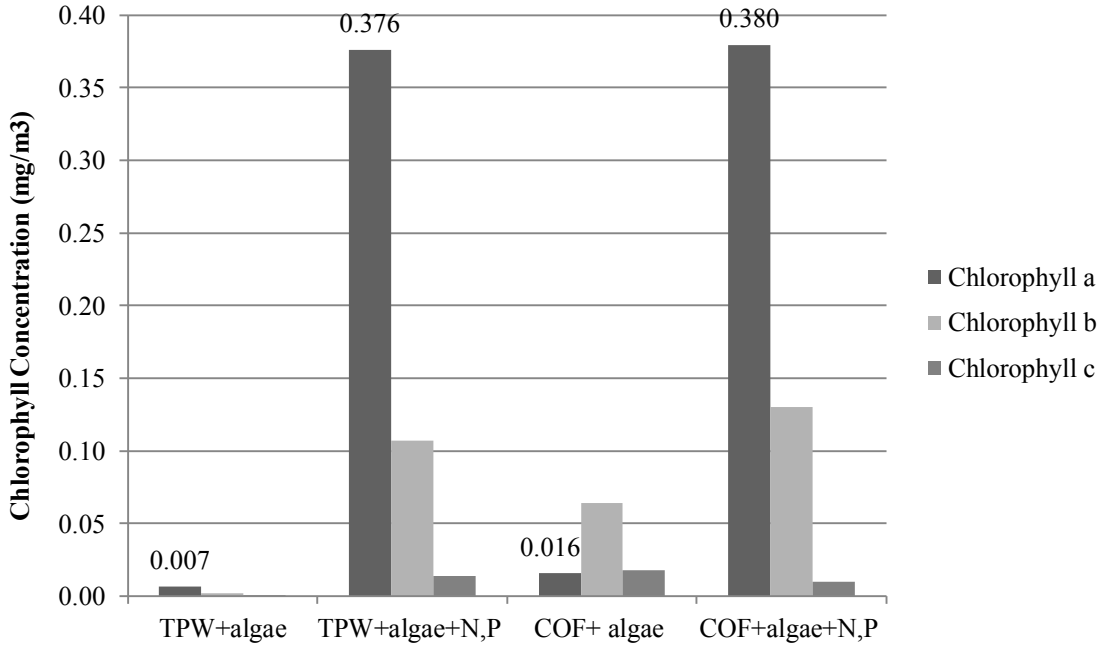


In the second week, slow growing bacterial colonies appeared on the agar, which could tolerate Norfloxacin.

APPENDIX F

Influence of nutrient supplements of nitrate and phosphate (N,P) on the growth rate of *Parachlorella kessleri*

This quick experiment was conducted to verify if addition of nutrient supplements of nitrate and phosphate (N,P) is required to accelerate growth rate and activities of *Parachlorella kessleri* in TPW. Chlorophyll concentration was measured according to Standard methods for the examination of water & wastewater (2005); and was used as an indicator of algal activities. Two samples of tailing pond water (TPW) and cyclone over flow (COF) from Syncrude were used for this experiment and some samples were enriched with 0.24 mM NO_3^- and 0.016 mM PO_4^{3-}.



Conclusion: Results indicated that addition of nutrient supplements of nitrate and phosphate (N,P) significantly increases chlorophyll concentration and algal activities.

Protocol used for Chlorophyll measurement:

Reagents

1. 0.1N HCl
2. Saturated magnesium carbonate solution: Add 0.25 g finely powdered MgCO3 to 25 mL distilled water. Mix the solution vigorously. Let the solids be settled (for 10 min) and then filter supernatant using syringe and 0.22 µm filter.
3. Aqueous acetone solution: Mix 90 mL acetone (reagent-grade BP 56°C) with 10 mL saturated magnesium carbonate solution.

Extraction:

1. All facilities must be acid free.
2. Take a 0.45 nylon filter and prepare a filtration installation.
3. Filter a certain amount of sample, and record the volume. The algae cells should not attach to the around filter holder's wall. If it attached, wash the wall by DI water.
4. Remove the filter on filter holder and push into the glass-glass cell grinder. Add 3 mL of aqueous acetone solution and macerate and grind for a least 10 min.
5. Transfer sample to 15 mL screw cap centrifuge tube and adjust total volume to 10 mL, with aqueous acetone solution. Close the cap and steep sample at 4 oC for 2 hr.
6. Centrifuge the sample for 20 min at 500.*g. Decant the clarified extracted into a clean 10 mL cylinder and measure volume.

Measurement of chlorophyll a

1. Transfer 3 mL of clarified extrac to a 1-cm cuvette and read optical density (OD) at 750 and 664 nm.
2. Add 0.1 mL of 0.N HCl and gently agitate and read the OD at 750 and 665 nm exactly after 90 s.
3. Volume of extract, acide and the time after acidification are critically important.
4. The value of OD 664 before acidification must be between 0.1 and 1.
5. For low concentration of chlorophyll, use 4 and 10 cm cuvettes.
6. For 4 and 10 cm cuvettes add a proportionally larger volume.
7. subtract the 750-nm OD value from the readings before (OD 664 nm) and after acidification (OD 665 nm)

$$Chlorophyll\ a\ , \frac{mg}{m^3} = \frac{26.7 * (664_b - 665_a) * V_1}{V_2 * L}$$

$$Pheophytin\ a\ , \frac{mg}{m^3} = \frac{26.7 * [1.7 * (665_a) - (664_b)] * V_1}{V_2 * L}$$

where:

$V1$ = volume of extract, L,

$V2$ = volume of sample, m3,

L = light path length or width of cuvette, cm, and

664b, 665a = optical densities of 90% acetone extract before and after acidification, respectively.

Chlorophyll a, b and c measurement

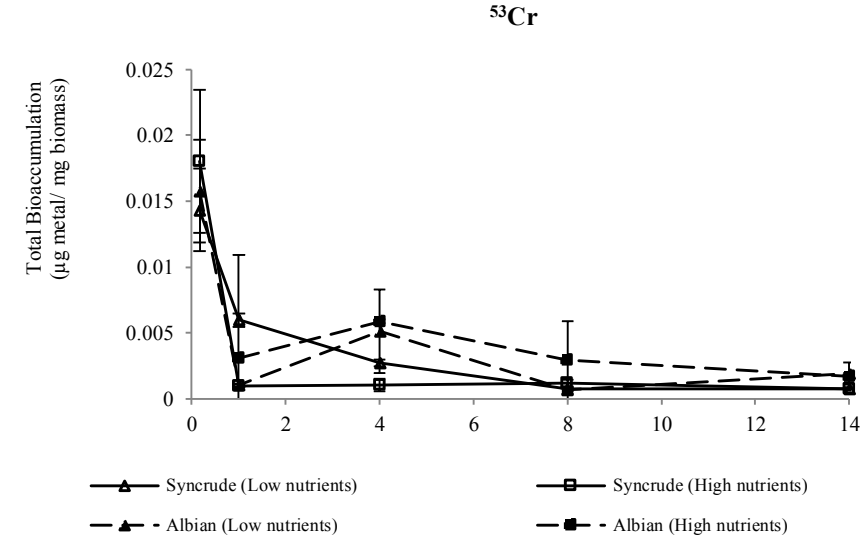
1. Transfer 3 mL of clarified ectract to a 1-cm cuvette and read optical density (OD) at 750 and 664, 647 and 630 nm.
2. Substract the 750-nm OD value from the readings.
   a) Ca = 11.85(OD664) − 1.54(OD647) − 0.08(OD630)
   b) Cb = 21.03(OD647) − 5.43(OD664) − 2.66(OD630)
   c) Cc = 24.52(OD630) − 7.60(OD647) − 1.67(OD664)
3. After determining the concentration of pigment in the extract, calculation the amount of pigment per unit volume as follows:

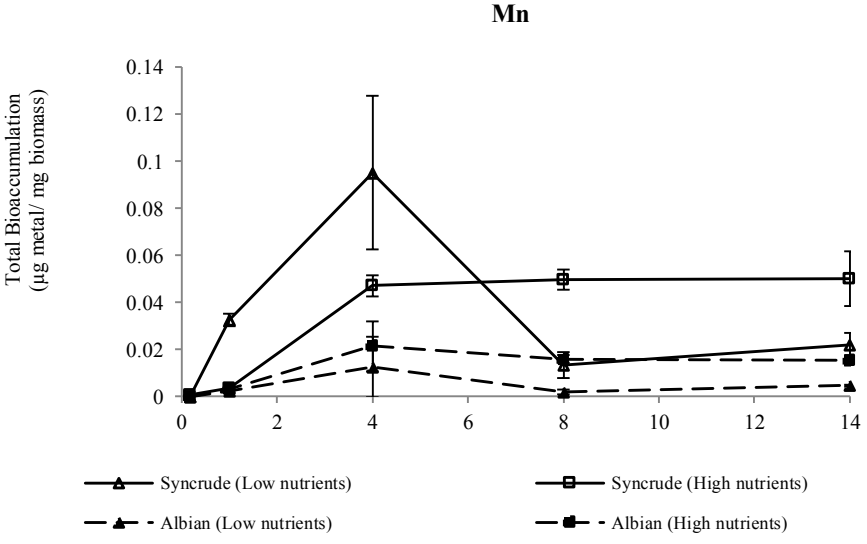
$$Chlorophyll\ a\ , \frac{mg}{m^3} = \frac{C_a * extract\ Volume, L}{volume\ of\ sample, m^3}$$

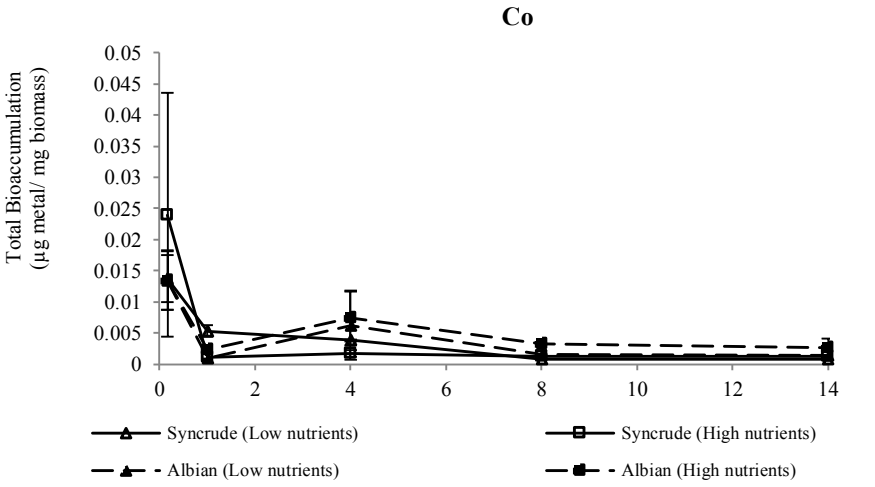
APPENDIX G

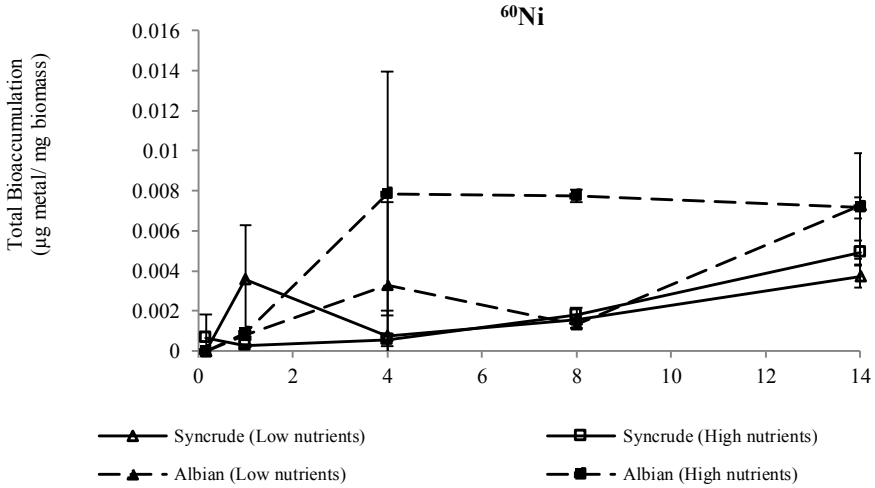
Total Bioaccumulation during 14 days cultivation

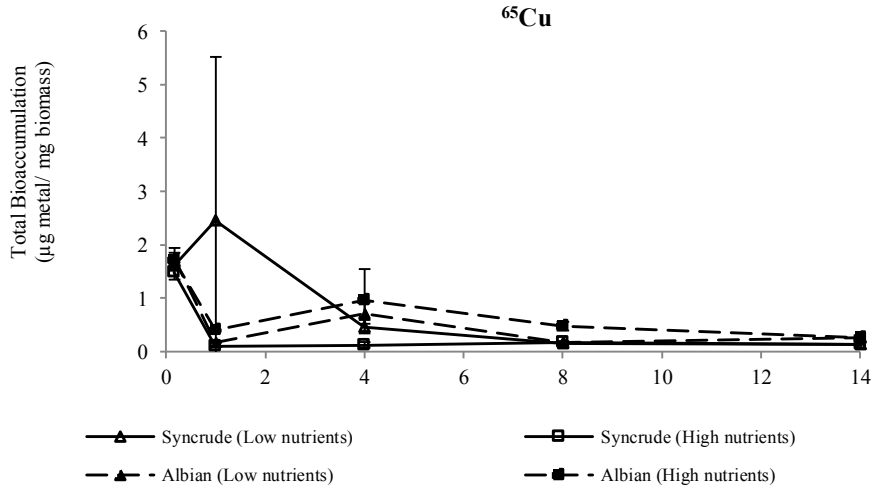
Total bioaccumulation (includes intracellular and extracellular bioaccumulation) during 14 days cultivation in Syncrude's and Albian's TPWs and in low (0.24 mM of NO_3^- and 0.016 mM of PO_4^{3-}) and high (1.98 mM of NO_3^- and 0.201 mM of PO_4^{3-}) nutrient concentrations. Error bars indicate ± one standard deviation (n = 3)

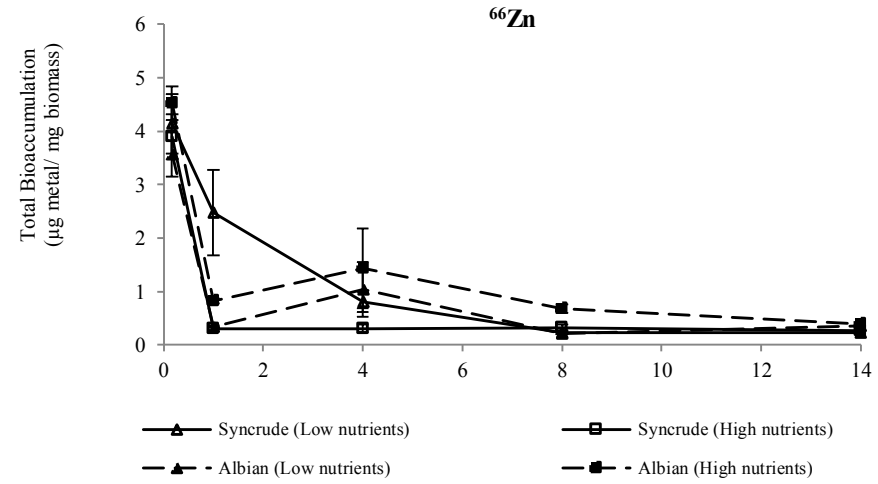


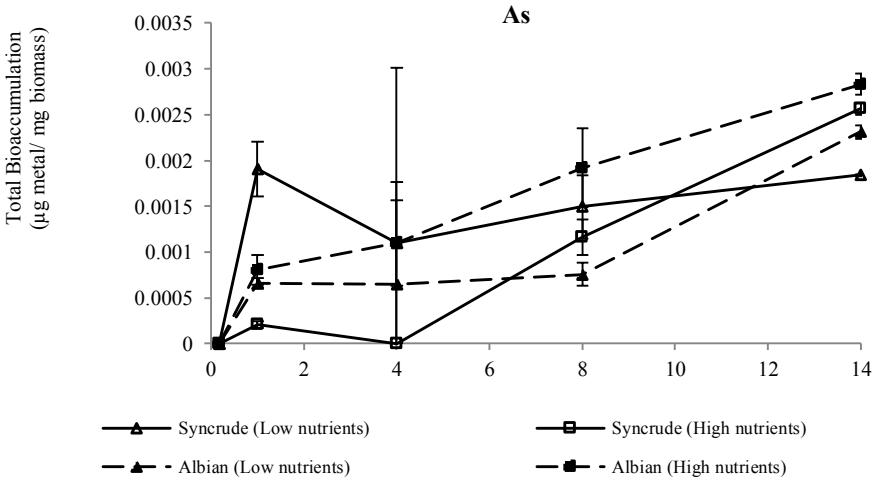


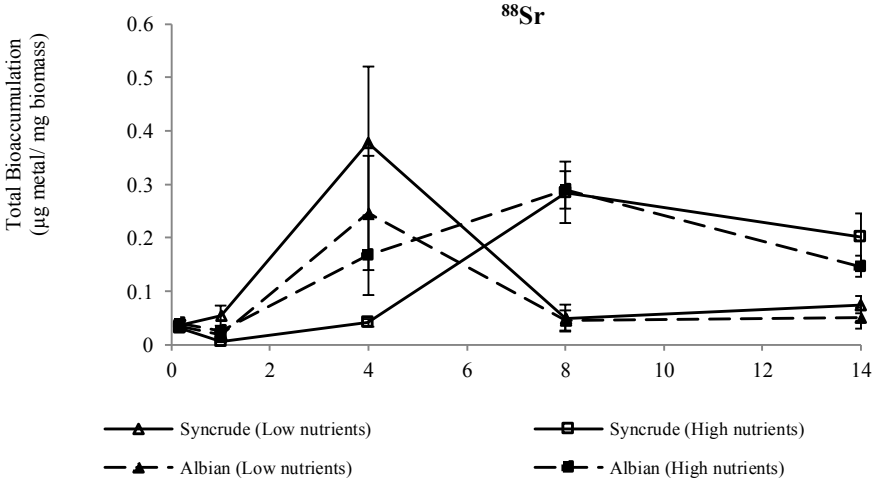

Co
Total Bioaccumulation (µg metal/ mg biomass)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)

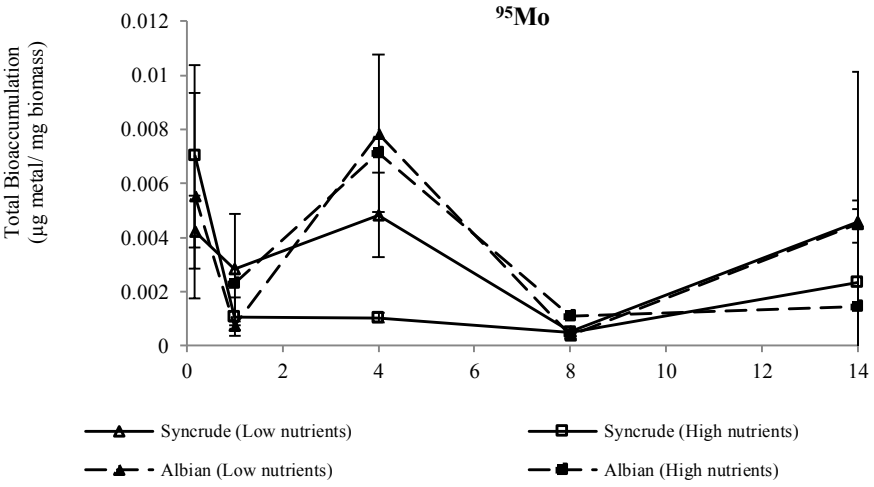

60Ni
Total Bioaccumulation (µg metal/ mg biomass)
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)

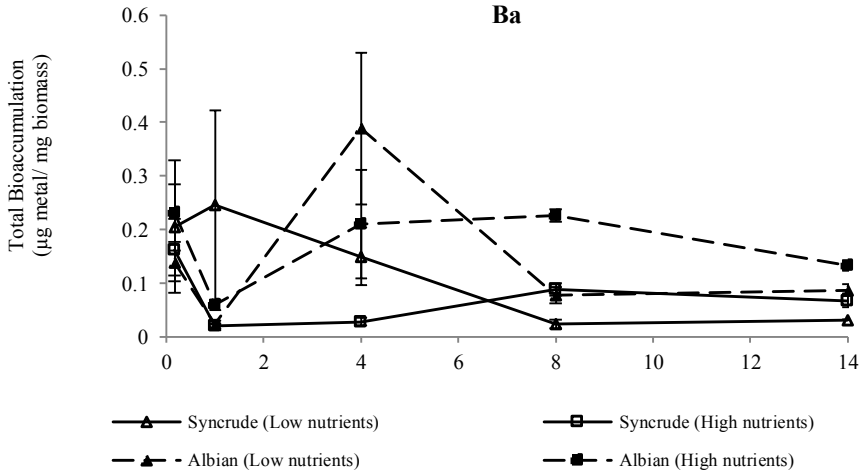

65Cu
Total Bioaccumulation (µg metal/ mg biomass)
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)


66Zn
Total Bioaccumulation (µg metal/ mg biomass)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)


As
Total Bioaccumulation (µg metal/ mg biomass)
0
0.0005
0.001
0.0015
0.002
0.0025
0.003
0.0035
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)


88Sr
Total Bioaccumulation (µg metal/ mg biomass)
0
0.1
0.2
0.3
0.4
0.5
0.6
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)


95Mo
Total Bioaccumulation (µg metal/ mg biomass)
0.012
0.01
0.008
0.006
0.004
0.002
0
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)


Ba
Total Bioaccumulation (µg metal/ mg biomass)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
Syncrude (Low nutrients)
Syncrude (High nutrients)
Albian (Low nutrients)
Albian (High nutrients)

APPENDIX H

NIST certified reference material (CRM) for validation of digestion method

Observed concentrations of metals from the NIST certified reference material (CRM) and expected concentrations, along with deviation percentage from expected concentration

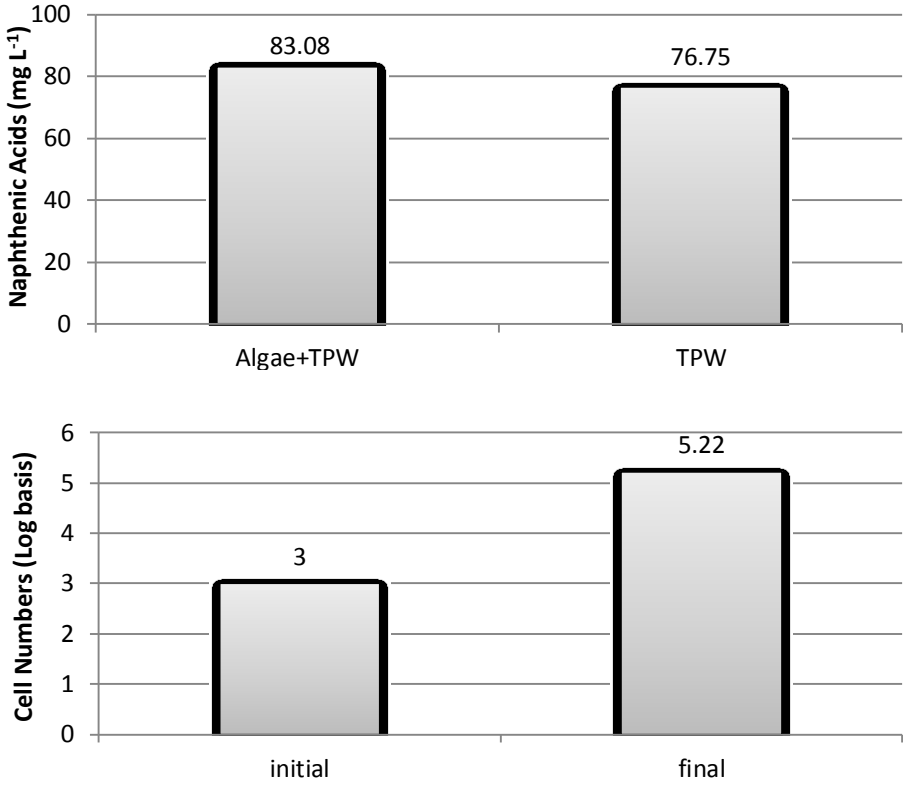
		^{53}Cr	Mn	Co	^{60}Ni	^{65}Cu	^{66}Zn	As	^{88}Sr	^{95}Mo	Ba
Expected Concentration (µg/g)	Mean	1	98	0.07	0.69	3.7	17.9	0.06	53	0.06	124
	STD		3		0.09	0.4	0.4	0.018	4	0.008	4
Measured metals' concentration in NIST certified reference material (µg/g)	CRM 1	1.29	103.12	0.08	0.89	3.60	18.09	0.06	49.45	0.045	110.76
	CRM 2	1.41	101.64	0.07	0.82	4.21	17.57	0.08	51.30	0.04	115.51
	CRM (Mean)	1.35	102.38	0.08	0.85	3.91	17.83	0.07	50.38	0.043	113.14
Deviation Percentage from expected concentration (%)		35.12	4.47	11.71	23.91	5.64	-0.37	14.67	-4.96	-28.67	-8.76

$$\text{Deviation Percentage from expected concentraiton (\%)} = \frac{\text{measured concentraiton - expected concentraiton}}{\text{expected concentraiton}} * 100$$

APPENDIX I

Quick experiment on the Naphthenic acids (NAs) removal using *Parachlorella kessleri*

This quick experiment was conducted to evaluate the ability of *Parachlorella kessleri* for the removal of Naphthenic acids (NAs). Two 300 mL samples of tailings pond water (TPW) from Syncrude were filtered (0.45 µm, Nylon membrane) and used for this experiment. One sample was used as a blank (negative control) and second one was inoculated with 10^3 *P. kessleri* cells. The total algae cell number and NA concentration were measured after 14 days incubation period (30 ^oC at 110 rmp shaking rate and 4300 Lux cool fluorescent light). NA concentration was measured using FT-IR instrument (for the measurement protocol see chapter 5).



Conclusion: *Parachlorella kessleri* did not biodegrade NAs in the absence of bacteria. A slight increase in NA concentration in algae containing sample was observed which might be resulted from the release of extracellular organic compounds.

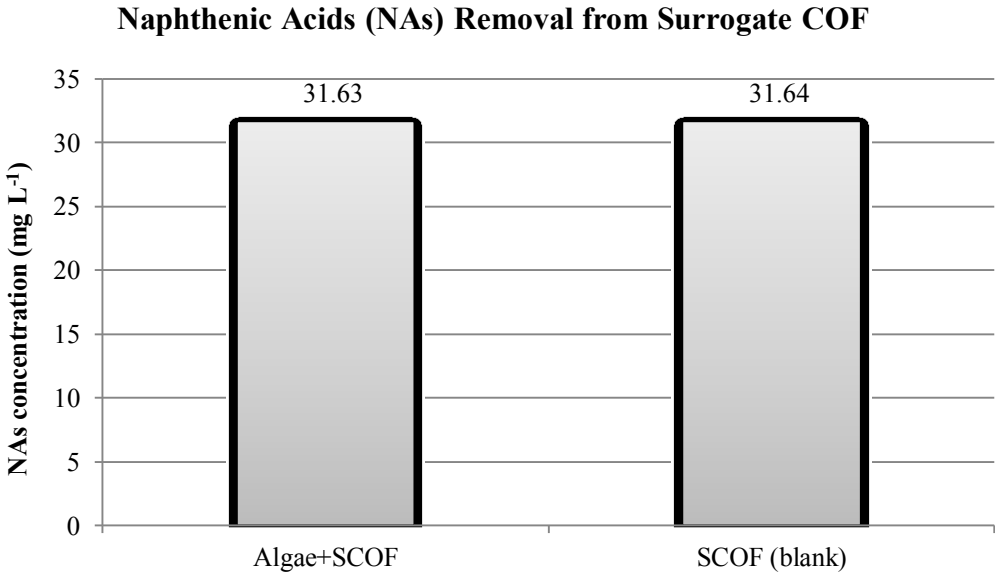
APPENDIX J

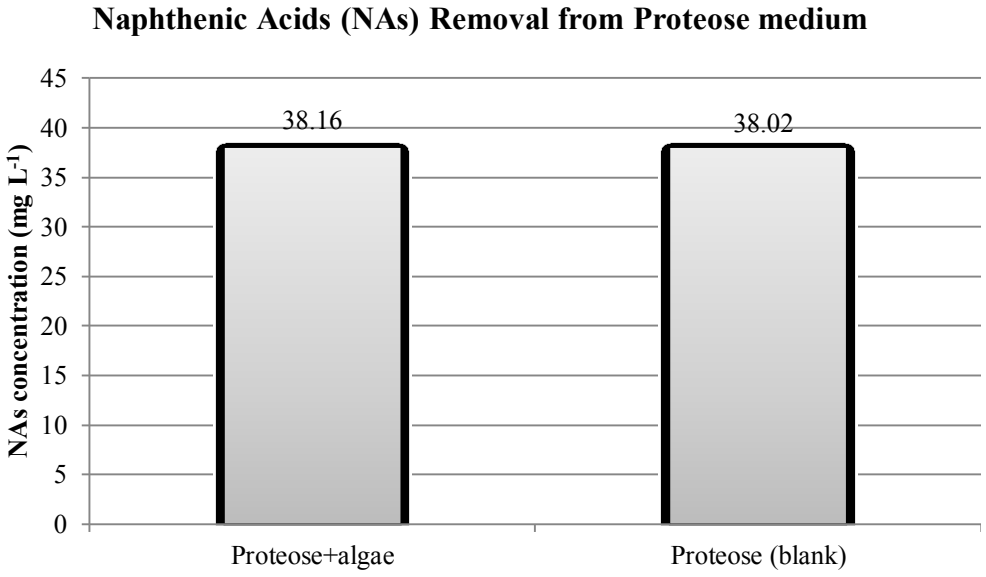
Removal of naphthenic acids (NAs) as a sole organic carbon source in media

This quick experiment was conducted to verify if *Parachlorella kessleri* can remove naphthenic acids (NAs) from media without any organic source (except NAs). In this experiment, it is hypothesized that *P kessleri* will consume NAs as a sole organic carbon from the medium. Two media were used in this experiment:

(1) Surrogate cyclone over flow (surrogate COF), this medium was made to mimic inorganic composition of COF. A certain concentration of NAs was added to solution as a sole source of organic carbon.
(2) Proteose medium (all minerals except Proteose): According to a recipe, all the inorganic fractions were used to prepare the medium; however instead of Proteose, NAs was added to solution.

Both media were enriched with nutrient supplements of nitrate and phosphate (0.24 mM NO_3^- and 0.016 mM PO_4^{3-}). For each medium, 250 mL of solution in 1 L flask was used for the experiment. All the samples were duplicated.



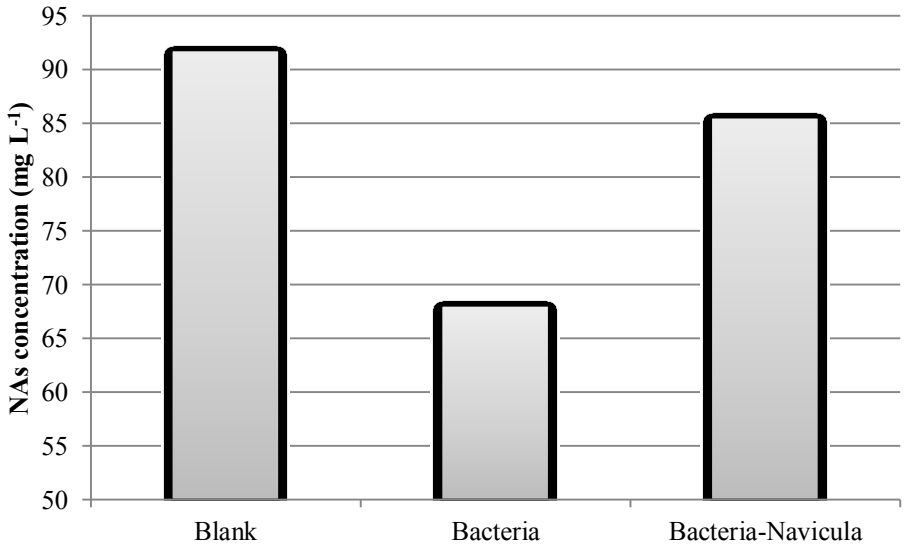


Conclusion: Results indicated that *P. kessleri* does not biodegrade NAs, even in a medium containing NAs as a sole organic carbon.

APPENDIX K

Quick experiment on the Naphthenic Acids (NAs) removal using indigenous algae-bacteria consortium

An aged sample of TPW from Syncrude was enriched with 1.44 mM nitrate (0.44 mM of $Ca(NO_3)_2$ and 1 mM of $Mg(NO_3)_2$) and 0.32 mM phosphate (0.17 mM of KH_2PO_4 and 0.15 mM of Na_2HPO_4). 50 mg L^{-1} of Merichem naphthenic acids was added to the TPW sample, followed by autoclaving. Two bottles containing autoclaved TPW were used as blank (negative control). Slurry of sediments from tailings pond (indigenous bacterial inoculum) was added into four autoclaved TPW bottles; and two out of four bottles were also inoculated with *Navicula pelliculosa* (a diatom) and purged with Argon. The cap for all six bottles, including two blank samples, two bacteria samples, and two bacteria- *N. pelliculosa* samples, were tightened.

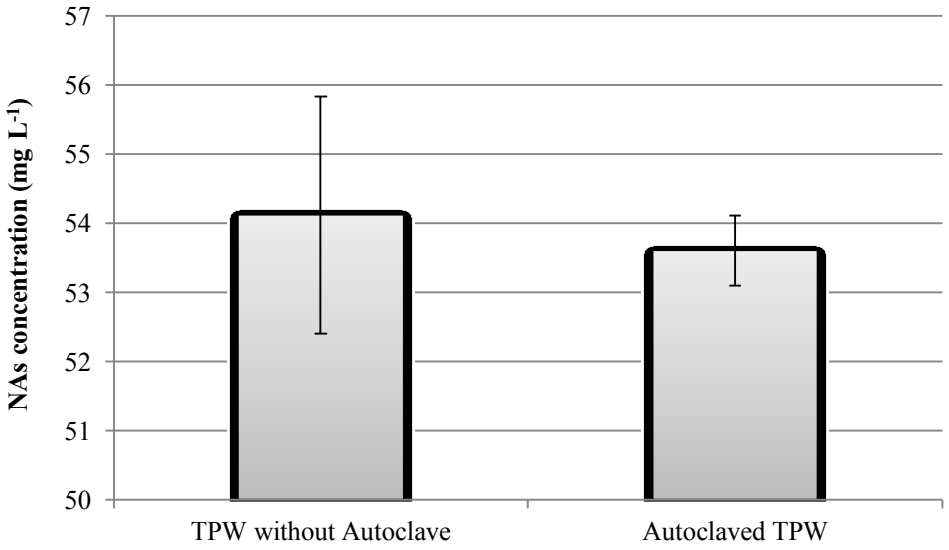


Conclusion: This quick test proved that bacteria can remove NAs significantly; however, the lack of oxygen in the sample containing *Navicula* might influence the biodegradation rate in the *Bacteria-Navicula* sample.

APPENDIX L

A quick test to validate the effect of autoclave on Naphthenic Acids concentration (NAs, measurements by FT-IR)

In this experiment, three out of six bottles, containing 100 mL of fresh TPW sample from Syncrude, were autoclaved. NA concentration in three autoclaved TPW samples and intact samples were measured using FT-IR.

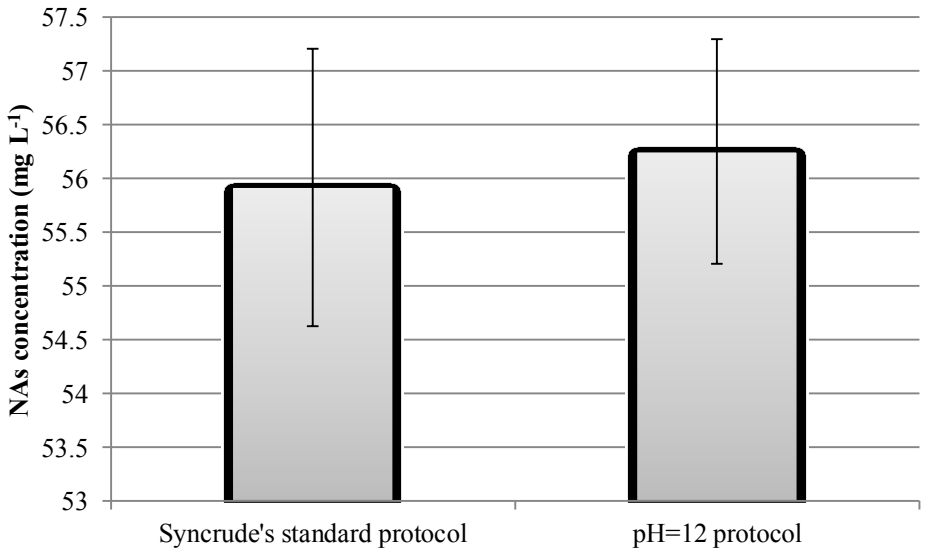


Conclusion: A simple t-Test revealed that there is no significant difference between TPW sample without autoclave and autoclaved TPW (p-value= 0.64).

APPENDIX M

A quick test to validate the attachment of Naphthenic Acids (NAs, measurements by FT-IR) on the suspended solids in TPW

In this experiment, six bottles, which contained 250 mL of fresh TPW sample from Syncrude, were used. NAs from three TPW samples were extracted using Syncrude's standard protocol, which involves: filtration (0.45 µm Nylon membrane) and pH reduction (<2) by adding concentrated HCl, followed by three times extraction of NAs by dichloromethane (5 mL each). For three remaining samples, at first pH increased to 12. This allows that adsorbed NAs on the suspended solids completely dissolved in water. After separation of suspended solids (filtration, 0.45 µm), pH was reduced to 2 by adding concentrated HCl followed by three times extraction of NAs by dichloromethane (5 mL each).



Conclusion: A simple t-Test revealed that there is no significant amount of NAs attached on the surface on suspended solids in TPW (p-value= 0.74).

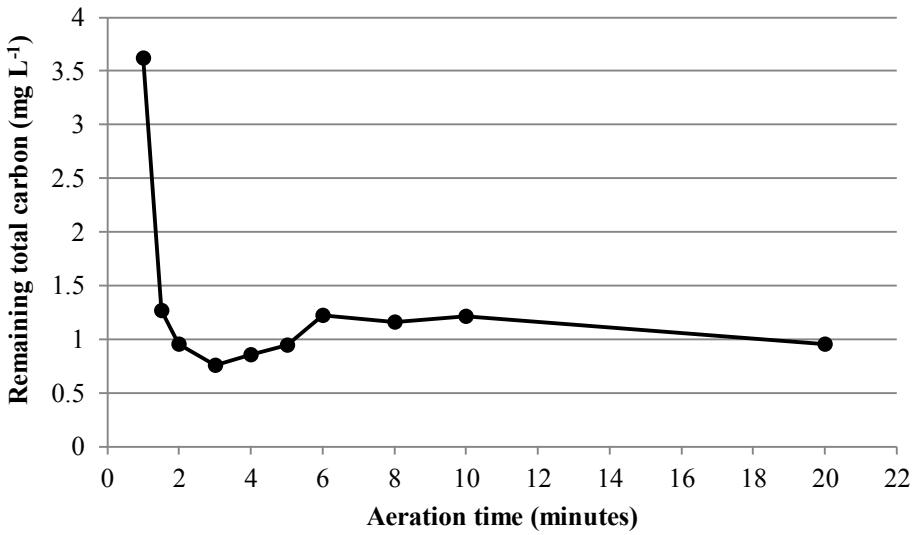
APPENDIX N

A quick test to determine the minimum aeration time to remove inorganic carbon after acidification in TOC-L instrument

For measurement of organic carbon fraction, the sample must be acidified to pH less than 3 followed by purging to remove all inorganic carbons. During the purging period, the volatile organic compounds lost as well as foaming due to surfactant properties of NAs may take place, that result in a reduction in the organic carbon measured. The objective of this quick test is to determine the optimized purging period to remove the inorganic carbon, with least volatile organic carbon lost.

Analysis was conducted using a Shimadzu TOC-L (CPH, Japan). The instrument uses 2N HCl reagent for acidification with an adjustable purging flow rate (ranged 50 to 200 mL min^{-1}) and time, and furnace temperature of 680 ^{o}C. Standards were prepared according to American Public Health Association (2005). Standards, samples and check controls were analyzed through triplicate injections with a standard deviation of less than 5%.

To determine the optimum time for purging inorganic carbon, a sample containing 0 mg organic-C/L and 600 mg inorganic-C/L was prepared by adding a certain amount of sodium bicarbonate and sodium carbonate in high pure water according to the standard preparation protocol in American Public Health Association (2005). The amount of required 2N HCl acid reagent to reduce pH to < 2 was determined by sample titration (7% v/v of acid solution was required). Results indicated that after 1, 1:30, 2 and 3 minutes the remaining inorganic carbon in solution will drop to 3.62, 1.27, 0.96 and 0.76 mg inorganic-C/L, respectively. For purging times more than 3 min the remaining inorganic carbon content slightly increased. In this experiment, the minimum possible air flow rate (50 mL min^{-1}) was used.



Conclusion: Results suggest that the rate of inorganic carbon reduction is considerable; and even 1 min purging period is enough to reduce the inorganic carbon content from 600 to 3.62 mg inorganic-C/L. It should be considered the rate of inorganic carbon removal may decrease in the presence of dissolved solids content and organic compounds in tailing ponds. It is suggested that the sample should be purged for 3 min with the minimum air flow rate (50 mL min^{-1}) to ensure the efficient removal of carbonate species, while minimizing the rate of volatile organic compounds lost as well as reducing the risk of foaming during the purging period.

APPENDIX O

A quick test to determine the volatility of commercial (Merichem) and tailings-associated Naphthenic Acids (NAs)

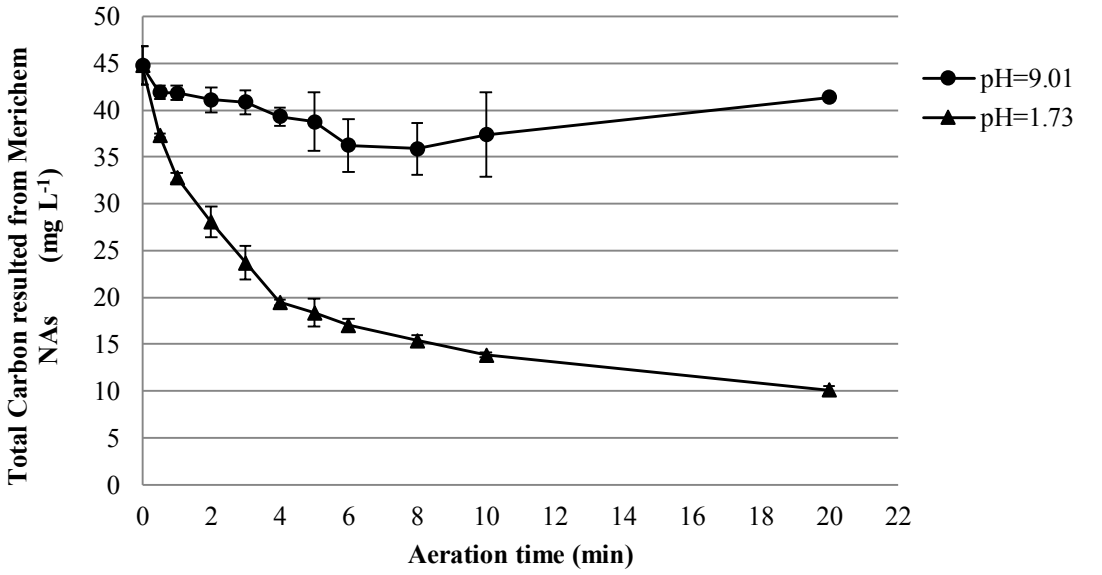
For measurement of organic carbon fraction, the sample must be acidified to pH less than 3 followed by purging to remove all inorganic carbons. During the purging period, the volatile organic compounds lost as well as foaming due to surfactant properties of NAs may take place, that result in a reduction in the organic carbon measured. The objective of this quick test is to determine the rate of NA lost (from Merichem (commercial) and tailings-associated) during the purge period.

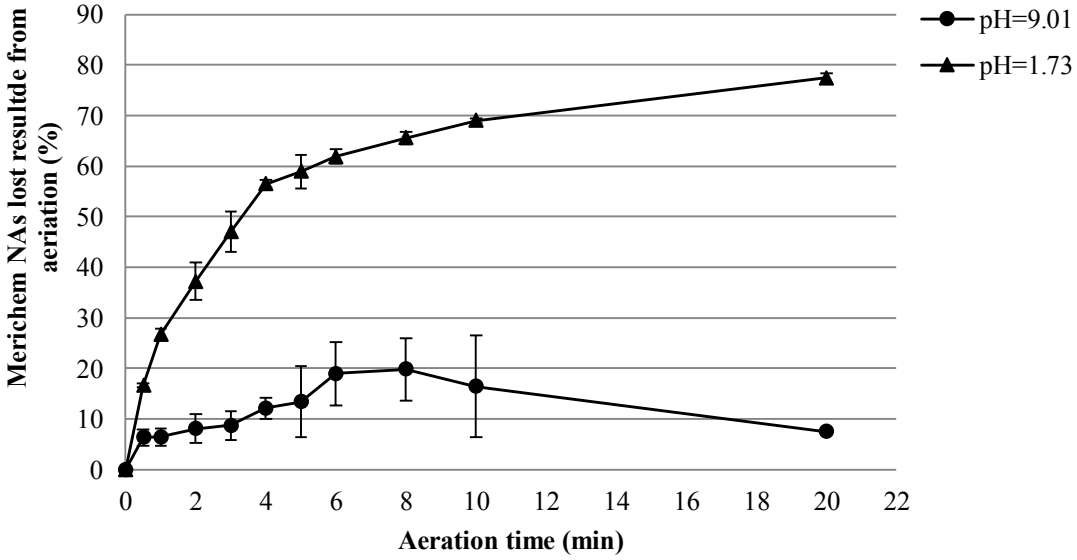
A: Merichem NA Lost:

Merichem NAs was dissolved in high pure water by adding NaOH pellets and final pH was adjusted at 9.01 using 1N HCl. This solution was analyzed by TOC-L instrument using two different methods. In the first method, the solution at pH 9.01 was directly injected to furnace (680 ^{o}C) to measure the total carbon concentration in the sample after various aeration time, ranged between 0 to 20 minutes.

In the second method, 2N HCl was added to sample to reduce the pH to 1.73, and the sample was injected to furnace to measure the total carbon content after various aeration time, ranged between 0 to 20 minutes.

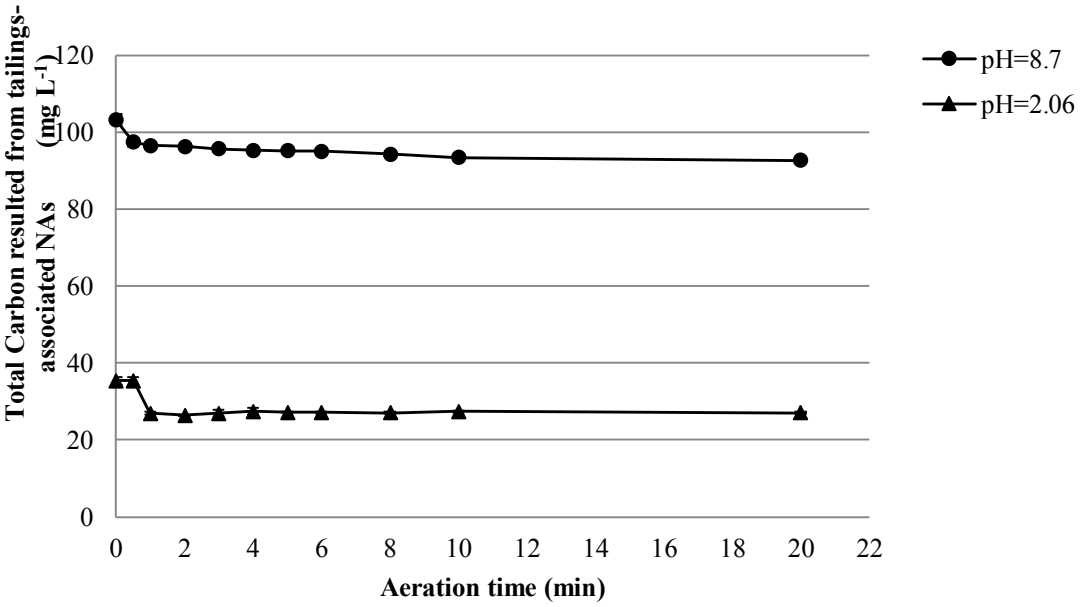
In both methods aeriation flow rate was 50 mL min^{-1}.

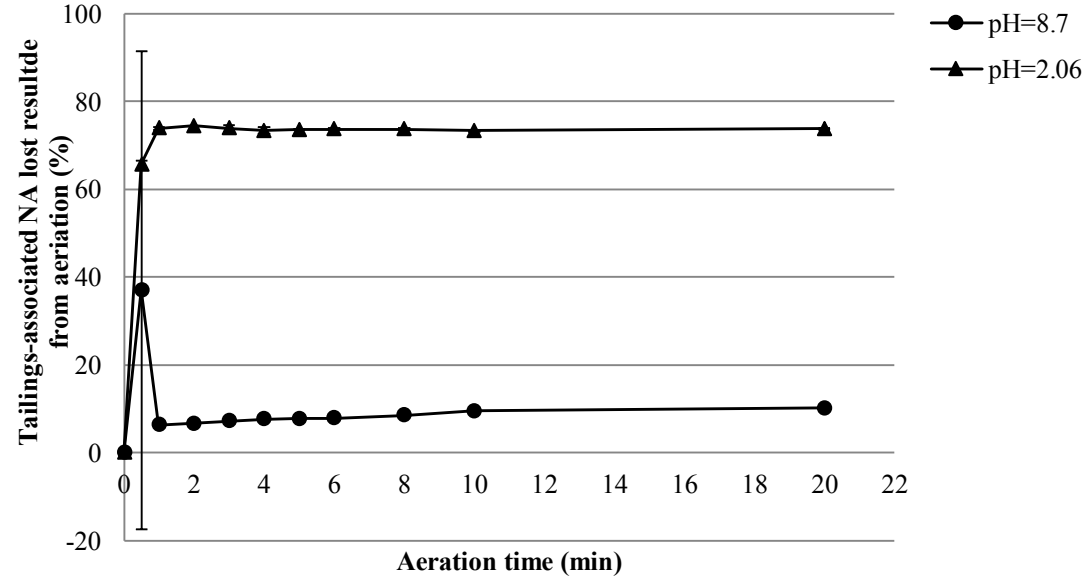




B: Tailings-associated NA lost:

Similar to the stage A, two mentioned methods were used to analyzed the fresh TPW from Syncrude. However, TPW contained inorganic carbons in the form of carbonates and bicarbonates, which was measured in the method one (without acidification)





Conclusion: The results indicated that Merichem NAs is volatile at low pH and will be lost by 80% during aeration period. However, at high pH, the molecules of Merichem NAs dissociate in water, and this reduces the NA lost through aeration. Unlike Merichem NAs, tailings-associated NAs showed low volatility, and after 3 and 20 min aeration, 7.25% and 10% of tailings-associated NAs was lost.

APPENDIX P

Bottles used in the experiment of TAO biodegradation

Bottles tightened with caps attached to a Swagelok® valve connected to a Puresep-T® Septum (9.5 mm) for sampling.

APPENDIX Q

Loading plot for each principal component for the analysis of FT-IR spectra using principal component analysis (PCA).