Assessment of Algae as Mercury Bioindicators in Acid Mine Drainage waters and their potential for Phytoremediation

by

Rosamond Rosalie Marigold Setswa Tshumah-Mutingwende

A research report submitted to the Faculty of Science, University of the Witwatersrand in fulfilment of the requirements for the degree of Master of Science

(Msc. by Course work and Research report)

Johannesburg

2014

Dedication

To

My beloved mum; Bongie

Brothers; Rie, Rey and Ray

Sisters; Roe and Red

With all my love!!!

Declaration

I declare that this research report is my own unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other university.

Rosamond Tshumah-Mutingwende (Miss)

School of Chemistry, University of the Witwatersrand, Johannesburg, 2014

Abstract

The use of algae as heavy metal bioindicators in aquatic environments has received much attention. In this study, the performance of a common freshwater living green alga, *Cladophora* sp. as a mercury bioindicator and its potential for phytoremediation applications was assessed by various parameters which included the influence of contact time, pH, initial mercury concentration and the presence of competing metal cations. A rapid uptake of mercury by *Cladophora* sp. was displayed. More than 99% of mercury in solution was removed within the 5 min of contact and equilibrium was attained after 10 min. High adsorption capacities of 800 mg kg^{-1}, 530 mg kg^{-1} and 590 mg kg^{-1} at pH 3, 6.5 and 8.5 respectively were obtained at the optimum mercury concentration of 1.0 mg l^{-1}. Competitive adsorption studies showed that the selectivity of heavy metal cations by *Cladophora* sp. was in the following order: Hg^{2+} >Fe^{2+}>Cu^{2+}> Zn^{2+} > Co^{2+}. These results indicate that living *Cladophora* sp. algae are suitable for use as mercury bioindicators in AMD waters and are also suitable for the removal of mercury in AMD conditions.

Acknowledgements

With much appreciation, I would like to thank my supervisors Prof. Ewa Cukrowska and Dr. Julien Lusilao Makiese who tirelessly guided me through this project.

Many thanks to Prof. Luke Chimuka, Dr. Hlanganani Tutu, Dr. Elisee Nsimba and the Environmental and Analytical Chemistry team at large for their encouragements and constructive criticism throughout this work.

I am greatly indebted to my mother, siblings, nephews, nieces and friends for their unwavering support throughout this degree programme. Though I was away from home, it felt like I carried home with me due to your constant communication with great words of encouragement.

Many thanks to Anita Etale (School of Animal Plant and Environmental Sciences, University of the Witwatersrand) for all the provisions she made towards this research project.

Finally, my deepest thanks and appreciation goes to the Almighty God for being mindful of me and enabling me to reach this far, Ebenezer!

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AAS: Atomic Absorption Spectrometry

ABM: Acidic Bold-Basal Medium

AMD: Acid Mine Drainage

CCAP: Culture Collection of Algae and Protozoa

CCD: Coupled Charge Detection

CNS: Central Nervous System

CRM: Certified Reference Material

CVAAS: Cold Vapour Atomic Absorption Spectrometry

CVAFS: Cold Vapour Atomic Fluorescence Spectrometry

DRD: Durban Roodepoort Deep

DWA: Department of Water Affairs

ERPM: East Rand Proprietary Mines

FI: Flow Injection

FIA: Flow Injection Analysis

FIMS: Flow Injection Mercury System

FTIR: Fourier Transform Infra-red

GFAAS: Graphite Furnace Atomic Absorption Spectrometry

ICP-AES: Inductively Coupled Plasma Atomic Emission Spectrometry

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

ICP-OES: Inductively Coupled Plasma Optical Emission Spectroscopy

LOD: Limit of Detection

PTFE: Polytetrafluoroethylene (or Teflon ®)

RSD: Relative Standard Deviation

UNEP: United Nations Environment Programme

U.S EPA: United States Environmental Protection Agency

WHO: World Health Organization

Chapter One: Introduction

This is the introductory chapter and it gives a general overview of the scope of the project, an outline of the problem statement together with the motivation for the research. The general objective and the specific objectives which provide the roadmap for fulfilling the general objective together with the key questions are given.

1.1 General overview

Algae have been widely used in countries such as Malaysia, Poland, Spain, Turkey, United Kingdom, Bulgaria, Argentina and Canada as heavy metal biological indicators (bioindicators) in freshwater and marine environments due to their low cost, availability in every season, ability to uptake and accumulate metals to detectable levels, ease of identification, long life, abundance and presence at pollution sites (Omar, 2010; Al-Homaidan *et al.*, 2011). Heavy metal concentration is preferably measured in bioindicator organisms than direct measurement in water or sediment samples because bioindicators provide a time integrated measure (from periods of minutes to years) unlike direct analytical measurements which only provide information on the current condition of the sample (Cooper *et al.*, 2009). In addition, unlike analytical and electrochemical methods, bioindicators are a rapid and low cost method associated with minimum pre-treatment of samples and only the concentration of the bioavailable heavy metal is detected (Srivastava *et al.*, 2005).

Mercury (Hg), a naturally occurring element, is classified as a global pollutant because of its ability to travel extensive distances from the source. According to the World Health Organization (WHO), mercury is one of the top ten chemicals of major public health concern due to its ability to persist in the environment for longer periods (WHO, 2013). Mercury pollution has been of great environmental concern in South Africa as a result of the vast coal and gold reserves whose mining has generated voluminous quantities of Acid Mine Drainage (AMD) rich in mercury and other heavy metals such as zinc (Zn), lead (Pb), copper (Cu), cobalt (Co) and iron (Fe) amongst others. Minute quantities of mercury present in AMD volatilise into the atmosphere as the bulk of it is discharged into water bodies. Once present in water, mercury is methylated into its more toxic form, methyl mercury, which is a mutagenic, teratogenic and carcinogenic compound and has a great tendency to bioaccumulate and biomagnify at different trophic levels of the food chain thereby claiming the lives of aquatic organisms, birds, people and animals whose diet depend on the polluted water body (WHO, 2013; Durham *et.al.*, 1972). Current physical and chemical AMD remediation

technologies have proved to be ineffective due to the high investment and operational costs, limited capacity when compared to the volume of AMD generated and also the production of a secondary pollutant, that is, sludge rich in toxic and radioactive heavy metals thus making the sludge difficult to dispose as is the case of the radioactive waste sludge from the West Rand mines (Stuijt, 2010). These adverse global environmental and health impacts of mercury have fuelled the assessment of algae as mercury bioindicators in AMD waters and their potential use as an efficient, low cost but effective AMD remediation technology. Furthermore, the use of algae as mercury bioindicators in AMD will provide early warning signals of mercury pollution, thus solutions such as re-treatment of AMD are immediately considered before final disposal into water bodies, hence, protecting the dependants of that water body.

1.2 Problem statement and motivation

Despite the fact that South Africa is a water stressed country, AMD contributes about 88% of the total wastewater produced (Tandlich, 2012) and this accounts for up to 10% of the total potable water available in a metropolitan city (Tandlich, 2012). The ineffectiveness, that is, the high operation and maintenance costs, high energy demand, low capacity and the production of secondary pollutants (in the case of chemical neutralisation) of currently used physical and chemical AMD remediation technologies has led to the discharge of partially treated AMD into water bodies (Mulopo *et al.*, 2012), thus elevating the heavy metal concentrations in these water sources, thereby putting human, plant and animal life at a very high risk. The recommended total mercury concentration permitted in wastewaters before being discharged into a water body is 0.005 mg Hg l^{-1} (DWA, 2010) (Table 1.1).

Table 1.1: National Water Act waste discharge standards: DWA 2010 guidelines (Waste water limit values applicable to discharge of wastewaters into a water resource) (DWA, 2010)

Variables and substances	Existing general guidelines	Future all discharges
Chemical oxygen demand	75 mg l^{-1}	65 mg l^{-1}
Colour, odour or taste	No substance capable of producing the variables listed	No substance capable of producing the variables listed
Ionised and unionised ammonia (free and saline ammonia) (as N)	3.0 mg l^{-1}	1.0 mg l^{-1}
Nitrate (as N)	15 mg l^{-1}	15 mg l^{-1}
pH	Between 5.5 and 9.5	Between 5.5 and 7.5
Phenol index	0.1 mg l^{-1}	0.01 mg l^{-1}
Residual Chlorine (as Cl)	0.25 mg l^{-1}	0.014 mg l^{-1}
Suspended solids	25 mg l^{-1}	18 mg l^{-1}
Total aluminium (as Al)	-	0.03 mg l^{-1}
Total cyanide (as Cn)	0.02 mg l^{-1}	0.006 mg l^{-1}
Total arsenic (as As)	0.02 mg l^{-1}	0.01 mg l^{-1}
Total boron (as B)	1.0 mg l^{-1}	0.5 mg l^{-1}
Total cadmium (as Cd)	0.005 mg l^{-1}	0.001 mg l^{-1}
Total chromium III (as Cr III)	-	0.11 mg l^{-1}
Total chromium VI (as Cr VI)	0.05 mg l^{-1}	0.02 mg l^{-1}
Total copper (as Cu)	0.01 mg l^{-1}	0.002 mg l^{-1}
Total iron (as Fe)	0.3 mg l^{-1}	0.3 mg l^{-1}
Total lead (as Pb)	0.01 mg l^{-1}	0.009 mg l^{-1}
Total mercury (as Hg)	**0.005 mg l^{-1}**	**0.001 mg l^{-1}**
Total selenium (as Se)	0.02 mg l^{-1}	0.008 mg l^{-1}
Total Zinc (as Zn)	0.1 mg l^{-1}	0.05 mg l^{-1}
Faecal coliforms per 100 ml	1000 mg l^{-1}	1000 mg l^{-1}

Therefore, to ensure sustainability and also to protect human, plant and animal life the use of algae as biological indicators which will (1) provide early warnings on mercury pollution, (2) evaluate the effectiveness of remediation efforts in clean-up of contaminated AMD waters and (3) act as indicators of potential environmental and health hazards based on their response to environmental stressors (mercury pollution) (Belkin *et al.*, 2000) must be considered. In addition, the abundance, availability at low cost, ability to uptake and accumulate heavy metals to detectable levels makes algae a potential, low-cost but effective remediation technology which is most likely not to produce any secondary pollutants since there is no use of chemicals and the harvested algae biomass can be used to generate biodiesel or burned to produce heat and electricity after the recovery of the concentrated mercury (Oligae, 2009).

1.3 Objectives of the research

The general objective of this study was to assess the use of algae as mercury bioindicators in AMD waters and investigate their potential use as phytoremediation material for mercury in AMD waters. This general objective was fulfilled by satisfying the following specific objectives:

1. Optimization of a method for the extraction and measurement of mercury in algae
2. Investigation of factors that influence mercury uptake by algae

1.4 Research questions

This research project sought to answer the following key questions:

1. Are algae able to accumulate mercury present in AMD to quantifiable levels?
2. Can algae be used as mercury bioindicators?
3. What are the optimum conditions for the uptake of mercury by algae?

Chapter Two: Literature Review

This chapter briefly presents the sources of mercury and their environmental impacts. The generation and negative impacts of Acid Mine Drainage (AMD) in South Africa is looked at with a great emphasis on mercury in AMD. The convectional techniques used for AMD remediation are discussed together with their strengths and weaknesses. The use of algae for heavy metal remediation and their performance as heavy metal bioindicators are reviewed. Finally, optimization of the analytical method to be used for the determination of mercury in plants is also reviewed.

2.1. Mercury and its sources

Mercury occurs naturally as cinnabar, a mercury sulphide (HgS) ore, which is roasted to form elemental mercury (Hg^0). Hg^0 is a silver white, toxic heavy metal which is a liquid at room temperature and pressure (Gray, 2003). It is also found in certain ores containing compounds of zinc, tin and copper as well as rocks such as limestone, sandstone and basalt; and in coal and oil (The World Bank Group, 1998). Hg^0 is released into the atmosphere from natural and anthropogenic sources (Figure 2.1).

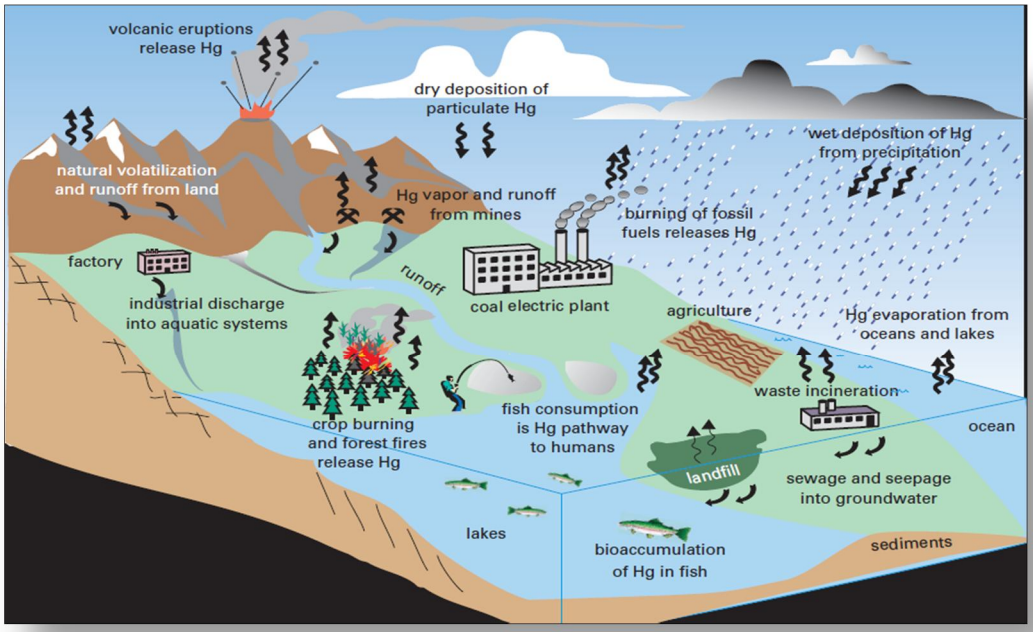

Figure 2.1: Schematic diagram of mercury cycle showing important contributions of mercury to the environment from land, water, air, and anthropogenic sources (Gray, 2003).

Natural sources account for 5207 Mg Hg yr^{-1} (Pirrone *et al.*, 2010) released globally and these include primary natural sources such as volcanic eruptions, springs and topsoil enriched with mercury as well as re-emissions from previously deposited mercury from vegetation, land or water surfaces. Anthropogenic point sources account for 2320 Mg Hg yr^{-1} (Pirrone *et al.*, 2010) and these are divided into four major groups which are: (1) combustion sources (e.g. emissions from

fossil fuel power plants such as coal, oil or petrol as well as crematories and municipal sludge incineration), (2) manufacturing sources (e.g. emissions from black carbon production, chlor-alkali plants, gold processing, cement manufacturing and pulp and paper manufacturing), (3) miscellaneous sources (e.g. emissions from explosives, geothermal power plants or pigments) and (4) area sources (e.g. emissions from electric lamp breakage, laboratory thermometers, paint or agricultural burning) (Keating *et al.*, 1997; The World Bank Group, 1998).

2.1.1 Properties and uses

Mercury and its compounds are highly volatile under ambient conditions and in the atmosphere, elemental mercury is more abundant in vapour form. It has a saturation vapour pressure of 14 mg m^{-3} which exceeds the average permissible concentrations of 0.05 mg m^{-3} for occupational or 0.015 mg m^{-3} for continuous environmental exposure (Rice *et al.*, 1997). Most mercury compounds are soluble in water and they exist in three oxidation states which are metallic (Hg^0), mercurous (Hg_2^{2+}) and mercuric (Hg^{2+}). The oxidation states of mercury compounds determine their properties, behaviour and toxicity. Mercury compounds including methyl mercury, an organic form of mercury, are quite stable so much that when they enter the environment they persist for long. Mercury species are easily convertible into one another by either biological or non-biological processes, thus, less toxic elemental mercury may be converted into highly poisonous methyl mercury (CH_3Hg^+) (Gray, 2003; Lodha, 1993).

Mercury finds application in different industries due to its unique physical and chemical properties. It responds rapidly to small changes in temperature and pressure thus it is used as a liquid in thermometers and pressure gauges. It readily forms alloys with most metals, for example, in gold processing, mercury alloys with gold to form a gold amalgam. It is highly mobile, a good conductor of electricity, and has a great tendency to be dispersed, hence, it is used in fluorescent lamps, wiring devices and the production of batteries (Keating, 1997).

2.1.2 Environmental impacts of mercury

Mercury is a non-biodegradable heavy metal, thus, it persists in the environment for a long. Mercury vapours in the air are deposited on land and water bodies in the form of rain drops, dust or by gravity and these settle in water bodies (streams, lakes, oceans, estuaries) thereby affecting water quality (U.S. EPA, 2008). Once in water bodies, mercury and its compounds tend to settle at the bottom of sediments where they are converted (methylated) by anaerobic bacteria into methyl mercury (CH_3Hg^+), a very toxic and soluble form of mercury, which is also easily taken up by plants and animals (Girard, 2010). Methyl mercury can also be discharged directly into water bodies from industrial wastewaters as was the case in the Minamata incident in Japan where the wastewater from an acetaldehyde production plant was discharged into a local bay (UNEP, 2002). Methylation and demethylation processes (Figure 2.2) are affected by factors such as temperature, pH, redox potential, and the presence of inorganic and organic complexing agents (Ullrich *et al.*, 2001).

Aquatic organisms such as algae and fish have the ability to take up mercurials from water to levels greater than the concentrations present in the water body. For example, mercury concentrations in algae are of the order 10^5-10^6 (Nordberg *et al.*, 2011) times higher than the concentrations in the surrounding water, thus the ability of mercury compounds especially methylmercury to biomagnify at different trophic levels of the food chain with the tertiary consumer being exposed to high levels of mercury. As a result, diet, especially fish and seafood consumption is the major pathway for human and animal exposure to mercury followed by consumption and use of contaminated water (Woodruff *et al.*, 2010).

Soil contamination by mercury is mainly due to anthropogenic activities. In soil, Hg^0 is more stable under reducing conditions and at increasing redox potential it precipitates as mercury sulphide. Hg^0 is also present as a result of the reduction of Hg^{2+}. The volatilization of mercury from the soil increases with increase in soil moisture content. Hg^{2+} cation is the most stable form of mercury found in the environment but it is rarely found in soil solution under natural conditions due to

its strong tendency to form complexes with anions such as Cl^-, OH^- and S^{2-} as well as humic matter (Selim, 2013).

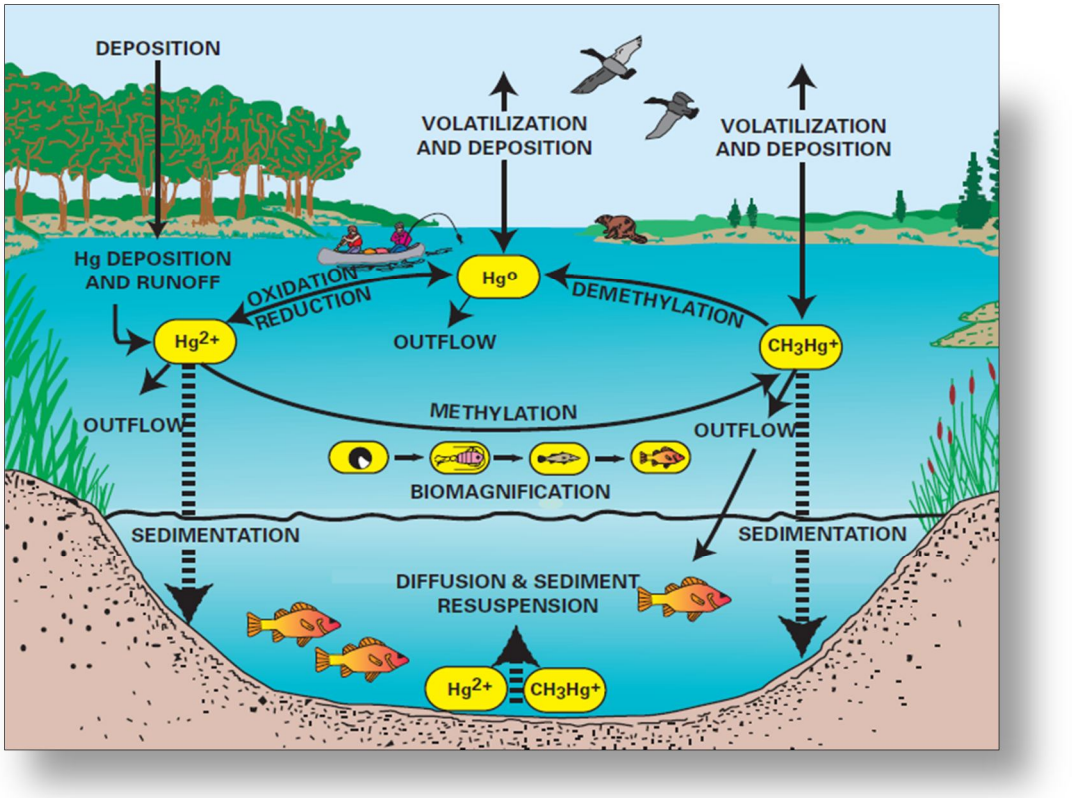

Figure 2.2: The aquatic mercury cycle showing important mercury species, mercury methylation and demethylation, and biomagnification of mercury in biota (Gray, 2003).

Inorganic mercury in the soil is methylated by abiotic and biotic processes in the soil system and the formed organic mercury is readily lost from the soil to the air. In surface soils, about 1–3% (Selim, 2013) of total mercury is in the methylated form with the rest predominantly as Hg^{2+} compounds. Plants take up mercury using their roots and this has a great potential of generating toxic vapours which are released during the evapotranspiration processes and some of the organic mercury present in soil can be deposited into water bodies as runoff water (Selim, 2013).

2.1.3 Health impacts of mercury

Inorganic mercury and its compounds are the least toxic compared to the organic forms of mercury. Acute inhalation of mercury vapour is characterised by severe chest pains, coughing and pneumonitis whereas chronic inhalation damages the Central Nervous System (CNS) over a longer period of time. Methylmercury poisoning is of great concern since 90-95% (The World Bank Group, 1998) of this compound is readily absorbed into the blood stream. Methylmercury poisoning readily affects the Central Nervous System (CNS) and the areas associated with the sensory, visual, auditory and coordinating functions and its effects are, in most cases irreversible because of the destruction of neuronal cells. Increasing doses result in paresthesia, ataxia, visual changes, dysarthria, hearing defects, loss of speech, coma and death. Prenatal life is more sensitive to methyl mercury exposure than adult life since elemental mercury and its organic compounds easily crosses the placental and blood-brain barriers. In addition, methyl mercury concentrations in umbilical cord blood tend to be higher than that in maternal blood (Woodruff *et al*., 2010).

2.1.4 Mercury in South-Africa

In the year 1886, gold was discovered in the Central Rand Goldfield of the Witwatersrand basin and it was mainly extracted by use of a mercury amalgam before the implementation of the McArthur-Forrest gold extraction process using cyanide (Mphephu, 2004; Naicker *et al*., 2003). Ore mined underground was brought to the surface, where it was milled to fine sand, during and after which it was exposed to a film of mercury spread on copper plates. These were periodically removed, and the mercury-gold amalgam scraped off and distilled to recover the gold. The tailings were then transported to tailings dumps near the extraction plant (Tutu, 2005).

Previously used gold extraction technologies had an average recovery rate of 8 g of gold per ton of ore (Tutu, 2005) and this led to minute quantities of gold,

typically in the range of 0.5 g ton^{-1} remaining in the tailings (Tutu, 2005). As a result, many of these dumps have been and are still being retreated to recover the remaining gold, and the tailings are pumped to disposal sites (Mpephu, 2004). It has been estimated that 240 tailings dams covering a surface area of 44 000 hectares are present in the Witwatersrand basin (Tutu, 2005). However, due to inadequate design, poor management and neglect, these tailings dams' original state has deteriorated due to water and wind erosion and this has resulted in water pollution as a result of AMD formation from the heavy metal and sulphides rich tailings dams. Airborne dust from untreated, partially treated and reprocessed tailings dams has resulted in significant amounts of air pollution (Mphephu, 2004).

Currently, mercury emissions from mining activities are still very high due to the uncontrolled artisanal and small scale gold mining activities which utilise mercury. Artisanal and small-scale gold miners highly depend on mercury for the extraction of gold due to its accessibility, ease of use and that it does not require any sophisticated equipment (Telmer *et al*., 2009). Most of the mercury released from artisanal and small scale gold mining activities is discharged into rivers, lakes, soils and tailings as some of it evaporates during the separation of gold from its alloy with mercury (gold amalgam). The uncontrolled mining activities by the artisanal and small scale gold miners disturbs mercury containing soils making them to erode more quickly thereby releasing more mercury into the environment than would otherwise have become available from controlled mining and natural erosion (UNEP, 2013). On the other hand, large scale mining and extraction of gold involves the use of mercury, cyanide or the two in combination. Cyanide has the ability to dissolve both mercury and gold forming cyano-mercury complexes which are soluble in and easily mobilized by rainwater into water bodies. From investigations carried out by the Global Mercury Project in Indonesia, Zimbabwe and Brazil; it is believed that cyano-mercury complexes are more bioavailable and easily biomethylated than elemental mercury, thus, highly threatening human and aquatic life together with organisms that depend on these water bodies (Telmer *et al*., 2009). When gold mining process water contaminated

with mercury and cyanide comes into contact with overburden rich in iron sulphide, AMD is formed resulting in more contamination as AMD has a great tendency of flowing into nearby rivers, lakes and dams.

In addition to the contribution of past and current mining activities to the mercury levels in South Africa, coal mining and combustion have also contributed significant quantities and mercury levels are estimated to be as high as 40 Mg yr^{-1} (Pirrone *et al.*, 2010). South Africa holds 96% (Scott, 2011) of Africa's coal reserves and it is ranked the world's sixth largest coal producer (Oosthuizen *et al.*, 2010). Coal is South Africa's main energy source, meeting about 90% (Scott, 2011) of the total primary energy needs and it is mainly combusted for the generation of electricity in the numerous power plants around the country. In addition to coal combustion, activities such as cement manufacturing, crude oil refining, gold and non-ferrous metal mining and processing contribute significant quantities to the total mercury emissions in South Africa (Leaner *et al.*, 2008, Pirrone *et al.*, 2010).

In an almost similar case to gold mining, effluent from a coal washing plant and coal mine tailings contain certain amounts of mercury. The effluent stream is usually recycled in the coal washer or stored in artificial dams where it is eventually discharged into nearby water bodies after almost all of the suspended solids have settled. Tailings which are also known to contain significant quantities of iron sulphide are usually stored in the tailings dump which in most cases is exposed to ambient environmental conditions thereby increasing the possibility of AMD generation. Coal combustion in the coal driers is another source of mercury within the coal mining and processing sector. Some of the mercury is released into the atmosphere as the rest remains trapped in the bottom and recoverable fly ash from the chimneys (UNEP, 2008). South Africa's coal is estimated to have a mercury content of 0.01-1.0 µg g^{-1} (Pirrone *et. al.*, 2010) and the fly ash has an estimated mercury content of 0.56-0.64 µg g^{-1}(Pirrone *et. al.*, 2010). The fly and bottom ash are commonly disposed of by mixing them with tailings before being discharged into the tailings dump so as to reduce the acidity of any generated

AMD since coal fly ash generates an alkaline leachate when contacted with water. However, this technique requires sophisticated engineering design for it to be a success (Shang *et al*., 2005). The formation of AMD, environmental concerns and possible remediation techniques are discussed in detail in the next section.

2.2. Acid Mine Drainage (AMD)

Acid mine drainage is acidic runoff mine water rich in sulphates, heavy metals and suspended solids formed when overburden rich in iron sulphide 'pyrite' (FeS_2) from mining processes such as coal and gold mining is oxidised into ferrous iron ($Fe^{2+}_{(aq)}$) and sulphuric acid as a result of uncontrolled exposure to atmospheric oxygen and rain water. The low pH of AMD favours the solubility of heavy metals such as zinc (Zn), cadmium (Cd), mercury (Hg), copper (Cu), lead (Pb) and even uranium (U) from their ores; thus, they are leached into solution from the soil and nearby rocks as AMD finds its course through the ground. According to the United States Environmental Protection Agency (US EPA) regulations, AMD is drainage water which before any treatment has a pH of less than 6 or a total iron concentration of 10 mg l^{-1} or greater (McElfish *et al*., 1990). The formation of AMD follows a sequence of four steps which are (McElfish *et al*., 1990):

1. The oxidation of pyrite into ferrous iron and sulphuric acid

$$2FeS_{2(s)} + 7O_{2(g)} + 2H_2O_{(l)} \rightarrow 2Fe^{2+}_{(aq)} + 4SO^{2-}_{4(aq)} + 4H^{+}_{(aq)} \quad (2.1)$$

2. Oxidation of ferrous iron into ferric iron.

$$4Fe^{2+}_{(aq)} + 4H^{+}_{(aq)} + O_{2(g)} \rightarrow 4Fe^{3+}_{(aq)} + 2H_2O_{(l)} \quad (2.2)$$

Equation 2.2 is catalysed by *Thiobacillus ferroxidans*, an iron oxidising bacterium which thrives at pH ranges below 3.5 and at higher pH ranges of 3.5-4.5, it is catalysed by a variety of bacteria which include *Metallogenium*, a filamentous iron oxidising bacterium.

3. Formation of ferric hydroxide

$$Fe^{3+}_{(aq)} + 3H_2O_{(l)} \rightarrow Fe(OH)_{3(s)} \qquad (2.3)$$

Ferric hydroxide $Fe(OH)_{3(s)}$ precipitates out of solution, thus, the red or yellowish colour of AMD.

4. At very low pH, ferric iron ion oxidises additional pyrite thereby greatly increasing the rate of acid generation.

$$FeS_{2(s)} + 14Fe^{3+}_{(aq)} + 8H_2O_{(l)} \rightarrow 15Fe^{2+}_{(aq)} + 2SO^{2-}_{4(aq)} + 16H^{+}_{(aq)} \qquad (2.4)$$

Once reactions (2.1-2.3) have been initiated, (2.4) becomes self-sustaining and does not require any constant supply of oxygen. Oxygen is only required by (2.2) to generate a constant supply of $Fe^{3+}_{(aq)}$ which oxidises $FeS_{2(s)}$ in (2.4).

In South-Africa, coal and gold mining activities are the major generators of AMD as a result of different mining activities such as land excavation, crushing, grinding and dumping of the pyrite rich rock or overburden on the tailings dump. Activities such as land excavation, crushing and grinding result in the formation of smaller particles with higher surface areas and as a result they are quickly oxidised. In coal mining, pyrite is present in both the overburden and coal whereas in gold mining, the conglomerate is the main bearer of pyrite and it can have concentrations as high as 3% (McCarthy, 2011). In both cases, voluminous

quantities of AMD are generated after mine closure when underground voids are filled with rainwater and seepages from groundwater (McCarthy, 2011).

AMD is believed to be the most neglected environmental disaster in South Africa and it has negatively influenced the livelihood of people living around the affected water bodies (Naidoo, 2009). The East (East Rand area, Boksburg, Brakpan, Springs and Nigel), Central (from Durban Roodepoort Deep (DRD)) in the west to the East Rand Proprietary Mines (ERPM) in the east) and the Western (Krugersdorp, Witpoortjie and Randfontein areas) basins of the Witwatersrand are the most polluted areas due to active and closed gold mining activities which generate voluminous quantities of AMD (Horak, 2011).

AMD is of serious environmental concern worldwide due to its low pH and high concentration of non-biodegradable heavy metals. The discharge of untreated AMD has negatively affected aquatic ecosystems/populations and other biological communities which depend on the polluted water body. The nature of AMD affects species diversity as it only favours the abundance of organisms that only thrive in low pH environments and those that are not sensitive to heavy metals. It also results in soil pollution and ground instability, with low pH being unfavourable to the growth of most plants (Fourie, 2009). In addition, AMD is a serious environmental threat as it does not only affect the area around the source but has the ability to travel extensive distances from the source and cause adverse environmental impacts especially when AMD is discharged into the main water stream. Also, the non-biodegradable heavy metals present in AMD have a great ability to accumulate in living organisms thus causing various diseases and disorders (Udayabhanu *et. al.*, 2010).

2.3 Prevention of AMD generation

It is inevitable; AMD will continuously be produced in mines even decades after their closure. However, several technologies which try to prevent its generation

have been developed and these are listed below (Johnson *et al.*, 2005; Strydom *et al.*, 2009):

- prevent contact of oxygenated water with pyrite by mechanism such as flooding or sealing of underground mines or diverting rainwater away from workings or dumps
- minimising the contact time of water and pyrite rock by removing water entering the pyritic mines as soon as possible

However, these technologies are only efficient if all AMD sources are known and there is no external seepage of oxygenated water (Johnson *et al.*, 2005). Therefore, in cases where AMD has already been generated, AMD remediation technologies must be sought and these are discussed in the following sections.

2.4 Remediation of AMD

Numerous physical and chemical AMD remediation technologies aiming at preventing, minimising the generation of AMD at the source or controlling the adverse environmental effects of AMD have been developed. These range from low cost technologies such as microbial remediation and phytoremediation to high cost technologies such as electrodialysis, reverse osmosis, ion exchange and chemical precipitation (Udayabhanu *et al.*, 2010). The conventional technologies mostly used in AMD are divided into two groups which are: (1) active and (2) passive treatment (Udayabhanu *et al.*, 2010; Gaikwad *et al.*, 2008).

2.4.1 Active treatment

Active treatment processes involve the use of chemicals for AMD neutralization and metal precipitation as well as the use of energy intensive processes such as reverse osmosis, electrodialysis and ion exchange. Though some active treatment technologies such as reverse osmosis are highly efficient with 95-99% heavy metal removal, they are labour intensive as they require constant maintenance for

them to be kept ‘active’, thus making their application expensive. To add, the use of chemicals for the precipitation of heavy metals results in the formation of a secondary pollutant, which is sludge rich in heavy metals some of which like uranium are radioactive thus causing devastating health and environmental impacts (Skousen *et al*., 1990; Udayabhanu *et. al*, 2010, Strydom *et al.*, 2009, Macingova *et al.*, 2012). Furthermore, active treatment techniques such as electrodialysis are not only energy intensive but result in the formation of metal hydroxides which clog the membrane thereby making the process inefficient (Ahalya *et al*., 2003).

2.4.2 Passive treatment

Passive treatment systems are natural chemical and biological reactions occurring in a controlled microbiological-chemical reactor without powered mechanical assistance (most of the time). Passive treatment technologies such as aerobic and anaerobic wetlands and anoxic limestone drains have lower operational and maintenance costs but require more land area. Passive treatment systems can be used on their own or in combination to treat different AMD effluents and the selection of a passive treatment system to be used is dependent on the AMD chemistry and the flow of discharge (Udayabhanu *et al.*, 2010; Ford, 2003). Common passive treatment systems are shown in Figure 2.3. The advantages and disadvantages of the passive treatment systems are listed below:

Advantages of passive treatment systems (Ford, 2003)

- do not require electrical power
- do not require any mechanical equipment, hazardous chemicals, or buildings
- do not require daily operation and maintenance
- are more natural and aesthetic in their appearance
- are less expensive

Disadvantages of passive treatment systems (Ford, 2003)

- may require complex discharge permits
- may not meet stringent water-quality-based effluent standards
- may fail because of poor design or severe winter conditions

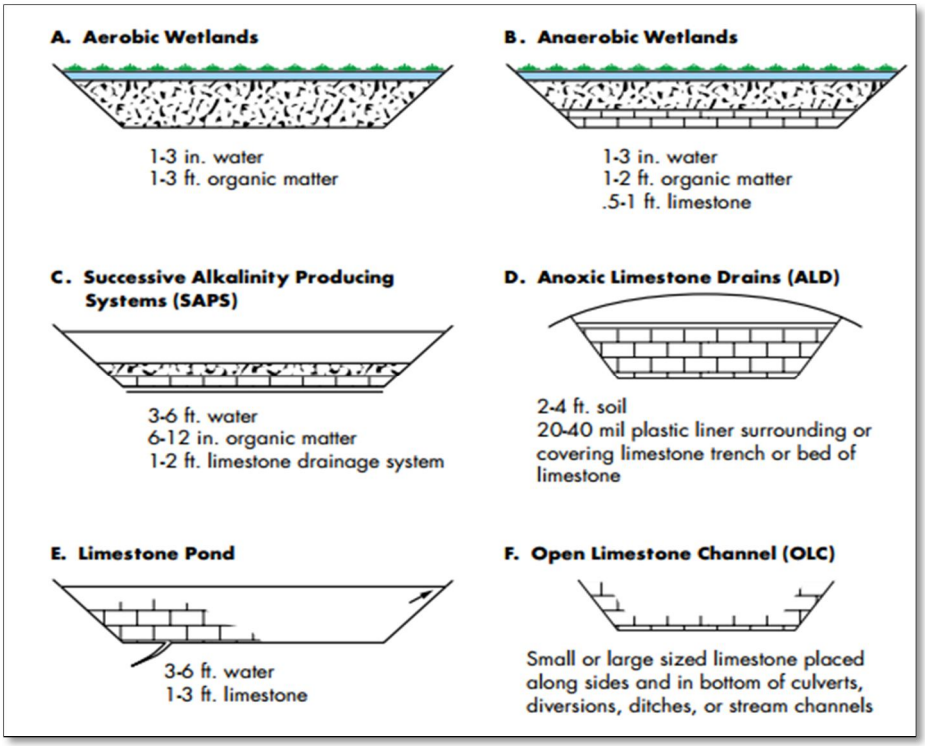

Figure 2.3: AMD passive treatment systems (Ford, 2003)

2.4.3 Phytoremediation

Phytoremediation is a process in which plants and microorganisms such as fungi, bacteria and algae are used to decontaminate soils and aquatic systems by the removal of heavy metals (Chekroun *et al.*, 2013). The removal of heavy metals from aquatic environments occurs by any of the three mechanisms: (1) phytoextraction which involves the use of plants or algae to remove contaminants such as heavy metals from soils, sediments or water into harvestable biomass, (2) phytostabilization which takes advantage of the ability of plants to decrease the amount of water percolating through the soil matrix, thus preventing soil erosion

and the transport of toxicants to the surrounding environments, and (3) rhizofiltration (or phytofiltration) which involves the use of aquatic and terrestrial plants for the remediation of extracted groundwater, surface water and wastewater with low contaminant concentrations (Nsimba, 2012, Henry, 2010; Sanghi *et al.*, 2012). The use of microorganisms such as algae, bacteria, fungi and yeasts in phytoremediation processes is also known as biosorption for both living and non-living biomass (Ahalya *et al.*, 2003; Nsimba, 2012; Davis *et al.*, 2003, Kumar *et al.*, 2012, Mehta *et al.*, 2005), phytosorption (Sekabira *et al.*, 2010) and also phycoremediation in the case of the removal of heavy metals from aquatic environments using algae only (Ambasht *et al.*, 2003). In some cases, the adsorption and absorption of heavy metals by organisms is simple referred to as heavy metal accumulation (or bioaccumulation) (Mehta *et al.*, 2005), therefore, unless stated otherwise, in the context of this research report, the mentioned terms will be used interchangeable referring to the use of living algae for the removal of heavy metals from aqueous environments. The disadvantages and advantages of phytoremediation are listed below:

Advantages of phytoremediation (Henry, 2000; Nsimba, 2012)

- It can be used for various organic and inorganic waste compounds
- In Situ/ Ex Situ application is possible
- It has low capital investment costs since expensive equipment or highly specialized personnel is not required
- It can be used to treat sites polluted with more than one type of pollutant

Disadvantages of phytoremediation (Henry, 2000; Nsimba, 2012)

- Possibility for environmental damage due to leaching of soluble contaminants
- Disposal of contaminated plant tissue is of environmental concern
- Dependent on climate conditions

- It is dependent on plant growth, when compared to other technologies, remediation time is long

The use of algae in phytoremediation processes is detailed in the following section.

2.5 Algae in phytoremediation

The term alga refers to a diverse group of eukaryotic photosynthetic organisms which contain chlorophyll *a* as their primary photosynthetic pigment. These organisms lack roots, stems and leaves and also their reproductive cells lack a sterile covering. The algal cell is surrounded by a thin and rigid cell wall. Some algae have an outer matrix lying outside the cell wall, similar to bacterial capsules. The nucleus has a typical nuclear envelope with pores; within the nucleus there are nucleolus, chromatin, and karyolymph. The chloroplasts have membrane-bound sacs called thylakoids that carry out the light reactions of photosynthesis. These organelles are embedded in the stroma where the dark reactions of carbon dioxide fixation take place. A dense proteinaceous area, the pyrenoid that is associated with synthesis and storage of starch may be present in the chloroplasts. Mitochondrial structure varies greatly in the algae. Some algae (euglenoids) have discoid cristae; some, lamellar cristae (green and red algae); and the remaining, (golden-brown and yellow–green, brown, and diatoms) have tubular cristae. The cell walls of the three most common types of algae; red, brown and green are different, that is; brown algae (*Phaeophyta*) generally contain three components: cellulose, the structural support; alginic acid, a polymer of mannuronic and guluronic acids (M and G) and the corresponding salts of sodium, potassium, magnesium and calcium; and sulphated polysaccharides (fucoidan matrix). Red algae (*Rhodophyta*) also contain cellulose, but their interest in connection with biosorption lies in the presence of sulphated polysaccharides made of galactanes (agar and carragenates). Green algae (*Chlorophyta*) are mainly cellulose, and a high percentage of the cell wall is proteins bonded to polysaccharides to form glycoproteins (Wang *et al*., 2009).

Algae sizes range from microscopic unicellular cells such as *Chlamydomonas* to huge tree like forms such as *Macrocystis pyrifera* which reaches a height of 60 m (Sambamurty, 2005). Algae fall under the plant kingdom but are distinguished from plants on the basis of their sexual reproduction. The differences between reproduction in the algae and that of plants is as follows: (1) in unicellular algae, the organisms themselves can function as gametes; (2) in certain multicellular algae, the gametes may be produced in special unicellular containers or gametangia; or (3) in others, the gametangia are multicellular, whereby every gametangial cell is fertile and produces a gamete (Nsimba, 2012).

Algae are usually classified according to their colour: *Cyanophyta*, blue-green algae; *Rhadophyta*, red algae; *Chrysophyceae*, golden algae; *Phaeophyceae*, brown algae; and *Chlorophyta*, green algae. In addition to colour, the nature of the chlorophyll(s), the cell wall chemistry, form in which food or assimilatory products of photosynthesis are stored, cell morphology, and reproductive structures are also used to classify algae (Wang *et al*., 2009).

The use of a common filamentous green algae *Cladophora* sp. was of great interest for the purpose of this research work. As a result, its properties and applicability for phytoremediation purposes is reviewed.

2.5.1 *Cladophora* sp.

Cladophora sp. is a green, filamentous (the cells are arranged end to end), multi-nuclei and hair-like alga that grows in fresh and salty water. *Cladophora sp*. falls under the Plantae kingdom. It is classified under the division *Chlorophyta* (green algae). Its class, *Chlorophyceae*, includes plants with cellulose or other polysaccharide cell walls having unicellular, colonial or filamentous cell arrangements, and containing a number of pigments, including chlorophylls *a* and b as well as α- and β- carotene. Its order, *Cladophorales*, specifies it is filamentous, with cells joined end to end in definite series, with or without branching of multinucleate cells. Each filament in the alga contains several cells.

Cytosis (cell division) occurs with parental division, while growth is both apical (from the growth tip) and intercalary (from expansion of the mid-section). *Cladophoraceae* is the only family in the order. Cell walls develop by the sequential layering of cellulosic chains. These cellulose chains combine to become crystalline microfibrils and have dimensions on the order of 10 nm (Johnson *et al.*, 1996). A high percentage of the cell wall is proteins bonded to polysaccharides to form glycoproteins. *Pyrenoids* or amylase-containing protein bodies occur in the chloroplasts of *Cladophorales*. *Cladophora* sp. reproduces in two ways: sexually by gametes and asexually by zoospores. All green algae have mitochondria with flat cristae. When present, flagella are typically anchored by a cross-shaped system of microtubules and fibrous strands, but these are absent among the higher plants and charophytes, which instead have a 'raft' of microtubules, the spline. Flagella are used to move the organism.

2.5.2 Classification of *Cladophora* sp.

The taxonomy of *Cladophora sp*. is summarised as follows (Kommineni, 2011):
Domain: *Eukaryota*
Kingdom: *Plantae*
Division: *Chlorophyta*
Phylum: *Chlorophyta*
Class: *Chlorophyceae*
Order: *Cladophorales*
Family: *Cladophoracae*

There are several advantages and disadvantages of using algae in phytoremediation and these are listed below.

Advantages of using algae in phytoremediation (Fu *et al.*, 2010; Omar, 2010)

- rapid reproduction rates, thus, wide availability
- low cost

- high metal sorption capacity
- reasonably regular quality
- potential to treat sites polluted with more than one type of pollutant

Disadvantages of using algae in phytoremediation (Nsimba, 2012)

- dependent on the growing conditions (climate, geology, altitude, temperature) required by the plant, hence success depends on tolerance of the plant to the pollutant
- possibility for environmental damage due to leaching of soluble contaminants

In the past decades, several researchers have reported on the ability of algae to absorb and adsorb nitrates, phosphorus and heavy metals from aqueous environments thereby improving the water quality (Ji *et al.*, 2011). However, less focus has been on the use of living algae for remediation purposes due to their potential to succumb to the toxic effects of heavy metals (unlike non-living algae) which at very high concentrations are most likely to result in (1) blockage of functional groups of biologically important molecules (e.g., enzymes and transport systems for essential nutrients and ions); (2) the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units; and (3) hinder other metabolic and photosynthetic activities of algae cells (Richmond *et al*., 2013). Various algae species have been discovered growing in aqueous environments polluted with heavy metals. For example, *Stigeoclonium* sp., a freshwater filamentous green alga, was discovered growing in mine water polluted with high concentrations of Zn^{2+} and it had relatively high sorption efficiency (Ji *et al.*, 2011). Also, another filamentous green algae *Mougeotia* sp. was discovered with a 'reddish rusty' colour growing in acidic mine voids of Collie, a coal mining town located 200 km southeast of Perth, the capital of Western Australia. This algae formed an extensive reddish mat, floating on some of the more shallow acidic mine voids and upon assessment it was discovered that up to 25% of the dry weight of this alga contained iron (Ambasht *et al.*, 2003).

Laboratory observations were also conducted in algae cultures and it was discovered that *Mougeotia* sp. thrived in the pH range of 3-6 and was capable of sequestrating iron and aluminium under these conditions (Ambasht *et al.*, 2003). This shows that there is a great potential by living filamentous algae to remove metal ions from aqueous solutions, yet, this remains largely unexplored. Following is a non-exhaustive discussion on the properties of algae which make them heavy metal adsorbents, mechanisms of heavy metal adsorption by algae and also the factors which influence the adsorption process.

2.6 Adsorption properties of algae

Adsorption of heavy metals in aqueous environments usually occurs on the cell surface (wall, membrane or external polysaccharides) and by binding to cytoplasmic ligands, phytochelatins and metallothioneins, and other intracellular molecules. The algal cell wall has many functional groups, such as, hydroxyl (-OH), phosphoryl ($-PO_3^{2-}$), amino ($-NH_2$), carboxyl (-COOH) and sulphydryl (-SH) which confer negative charge to the cell surface. Heavy metal ions such as Hg^{2+} and Pb^{2+} form strong bonds with CN−, R–S−, –SH−, NH_2 − and imidazole ($(CH)_2N(NH)C^-$) functional groups; all of which contain nitrogen and sulphur atoms (Wang *et al.*, 2009). Each functional group has a specific pKa (dissociation constant) and it dissociates into corresponding anion and proton at a specific pH. These functional groups are found associated with various cell wall components, e.g., peptidoglycan, teichouronic acid, teichoic acids, polysaccharides and proteins. The number and kinds of functional groups vary in different algal groups due to variation of distribution and abundance of cell wall components among the different algal groups. Among different cell wall constituents, polysaccharides and proteins have most of the metal binding sites. Cell wall of green algae contains hetero-polysaccharides, which offer carboxyl and sulphate groups for sequestration of heavy metal ions (Mehta *et al.*, 2005). In addition, several researchers have established that metals such as Ti, Pb, Mg, Zn, Cd, Sr, Co, Hg, Ni and Cu are sequestered in polyphosphate bodies in green algae. These bodies

perform two different functions in algae, that is; (1) provide a “storage pool” for metals and; (2) act as a “detoxification mechanism” (Dwivedi, 2012**)**.

2.7 Factors influencing the adsorption process

The adsorption of heavy metals by microorganisms is influenced by three main factors which are (Mamboya, 2007; Nsimba, 2012):

- characteristics of the metal ion (atomic weight, ionic ray, valence)
- environmental conditions (pH, temperature, ionic strength, contact time, biomass concentration)
- the nature of the biosorbent, which may determine differences in selectivity and affinity to metal ions

2.8 Mechanism of heavy metal uptake

The uptake of heavy metals by algae takes place through two processes which are: (1) passive uptake which is an initial rapid metabolism independent adsorption process which involves the adsorption of heavy metals on the cell surface and takes a relatively short time (from a few seconds or minutes) and (2) active uptake which is a slow metabolism dependant process which involves the transport of metal ions across the cell wall into the cytoplasm (Mehta *et al.*, 2005; Ahalya *et al.*, 2003). The biosorption mechanisms are further classified according to various criteria (Figure 2.4), although most of them are not yet fully understood (Nsimba, 2012).

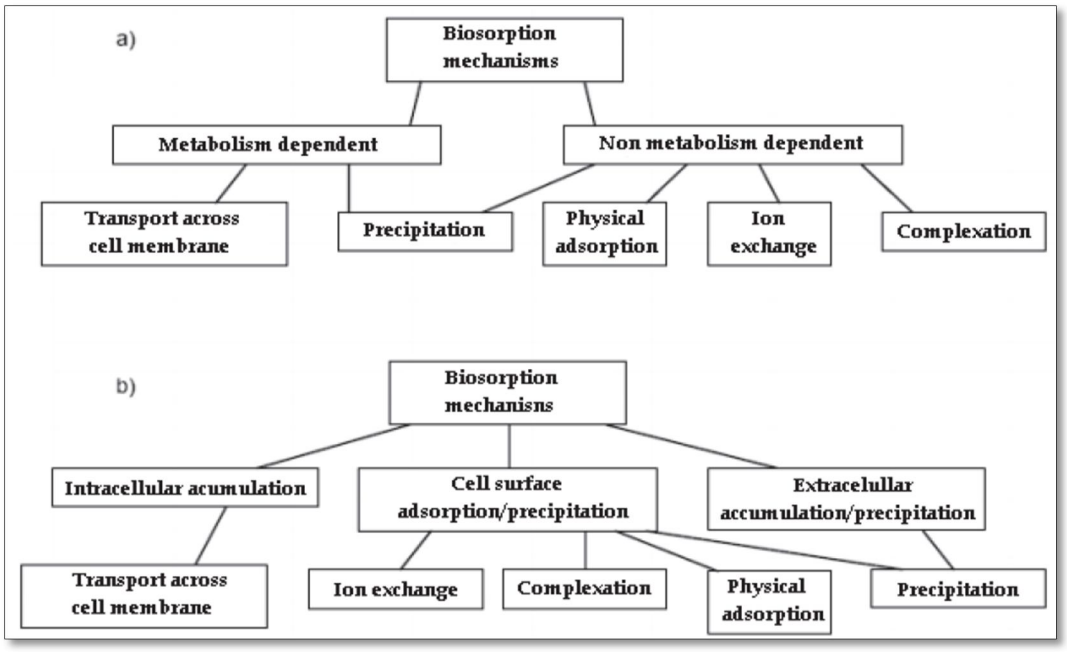

Figure 2.4: Mechanisms of biosorption, a) classification according to dependence on cell metabolism, b) classification according to the location within the cell and the metal removed (Nsimba, 2012)

2.8.1 Transport across cell membrane

Transport of metals across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism; as a result, only living cells are responsible for this kind of biosorption. It is often associated with an active defence system of the microorganism, which reacts in the presence of toxic metal. The transport of heavy metals across the cell membrane is by the same mechanism as the transport of essential ions such as potassium, sodium and magnesium. However, the metal transport systems may become confused by the presence of heavy metal ions of the same charge and ionic radius associated with essential ions. The two above mentioned mechanisms (active and passive uptake) are responsible for the uptake of metals by living algae (Ahalya *et al.*, 2003).

2.8.2 Physical adsorption

Physical adsorption takes place with the help of van der Waals' forces. The biosorption of heavy metals like uranium, cadmium, zinc, copper and cobalt by dead biomasses of algae, fungi and yeasts was assumed to take place through electrostatic interactions between the metal ions in solutions and cell walls of microbial cells. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chiarella vulgaris* for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger* (Ahalya *et al.*, 2003). Physical adsorption of heavy metals on the algal cell wall is a process which is greatly influenced by pH, that is, pH determines the availability and surface charge of the binding sites. At low pH, the availability of binding sites is decreased and as a result metal uptake is also decreased. At increased pH, an increased number of binding sites (amino and carboxyl groups of proteins and hydroxyl groups of polysaccharides) are replaced by negative charges, thereby increasing the attraction of metallic cations and adsorption on the cell surface (Nsimba, 2012; Dwivedi, 2012).

2.8.3 Ion exchange

Cell walls of algae contain polysaccharides and bivalent metal ions exchange with the counter ions of the polysaccharides. For example, the alginates of marine algae occur as salts of K^+, Na^+, Ca^{2+}, and Mg^{2+}. These ions can exchange with counter ions such as Co^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} resulting in the biosorptive uptake of heavy metals (Ahalya *et al.*, 2003).

2.8.4 Complexation

Metal removal from solution may also take place by complex formation on the cell surface after the interaction between the metal and the active groups. The biosorption of copper by C. *vulgaris* and Z. *ramigera* has been assumed to take place through both adsorption and formation of coordination bonds between

metals, amino and carboxyl groups of cell wall polysaccharides (Ahalya *et al.*, 2003). An extracellular complex is formed as a result of the electrostatic attraction between a metallic ion/ chelating agent and a polymer (excreted by a viable or non-viable micro-organism). These chelating agents maybe organic acids (e.g., citric, oxalic, gluonic, fumaric, lactic and malic acids) produced by microorganisms and these may chelate toxic metals resulting in the formation of metallo-organic molecules. These organic acids help in the solubilisation of metal compounds and their leaching from their surfaces. Metals may be biosorbed or complexed by carboxyl groups found in microbial polysaccharides and other polymers (Nsimba, 2012; Ahalya *et al.*, 2003).

2.8.5 Precipitation

Precipitation may be either dependent on the cellular metabolism or independent of it. In the former case, the metal removal from solution is often associated with active defence system of the microorganisms. They react in the presence of toxic metal producing compounds, which favour the precipitation process. In the case of precipitation not dependent on the cellular metabolism, it may be a consequence of the chemical interaction between the metal and the cell surface. The various biosorption mechanisms mentioned above can take place simultaneously (Ahalya *et al.*, 2003).

The ability of algae to adsorb and accumulate metals such as zinc, lead, cadmium and aluminium from mine waters makes them a useful tool for determining the concentrations of heavy metals available in those environments with time, thus their use as bioindicators. The use of algae as bioindicators is detailed in the following section.

2.9 Bioindicators

A bioindicator is an organism (or part of an organism or a community of organisms) that contains information on the quality of the environment (or a part

of the environment) and how it changes over time (Holt *et al.*, 2011, Gadzała-Kopciuch *et al.*, 2004). Bioindicators are useful in cases where; (1) the indicated environmental factor, such as climate change cannot be measured, (2) the indicated factor such as pesticides and their residues are difficult to measure and (3) where the environmental factor is easy to measure but difficult to interpret, for example, determining if the observed changes have ecological significance. Different types of bioindicators can be described from different perspectives (Figure 2.5). Bioindicators fall into three categories which are: (1) compliance, (2) diagnostic indicators and (3) early warning indicators based on their aims.

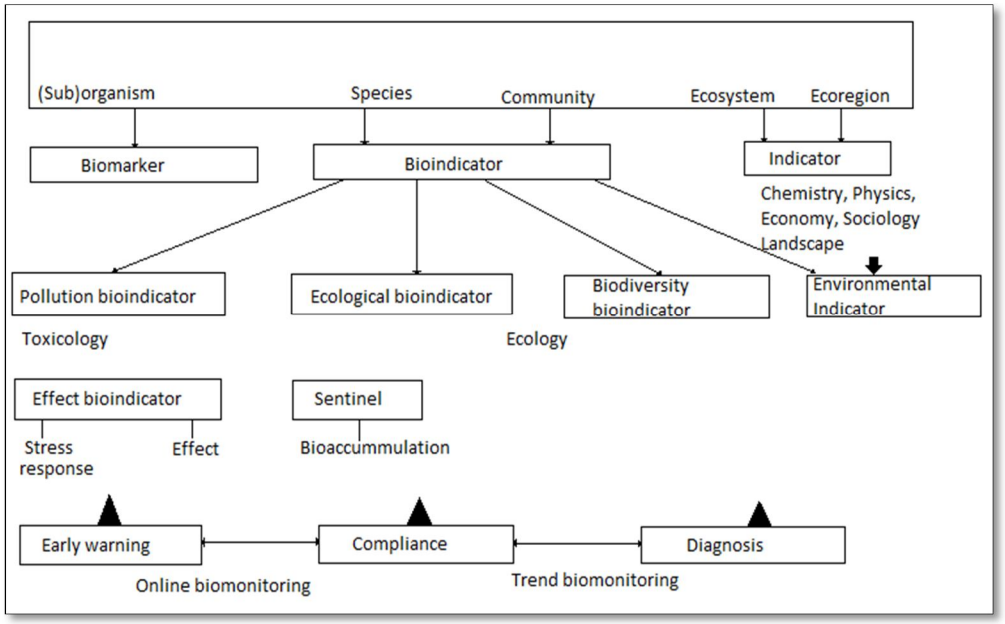

Figure 2.5 Types of bioindicators in the context of their use in biomonitoring (Modified after Gerhardt, 2002)

When using compliance indicators, organisms' attributes are measured at population, community or ecosystem level and are focused on issues such as the sustainability of the population or community as a whole. Diagnostic and early warning indicators are measured on the individual or suborganismal (biomarker) levels, with early warning indicators focusing on rapid and sensitive responses to environmental change (Gerhardt, 2002).

2.9.1 Algae as heavy metal bioindicators

Several organisms such as fish, lichens, bacteria, yeast and algae have been successfully used as heavy metal bioindicators in aquatic systems (Gadzała-Kopciuch *et al*., 2004, Al-Homaidan *et al.*, 2011). Diverse macroalgae species belonging to the divisions *Chlorophyta, Rhodophyta* and *Phaeophyta* have been selected as heavy metal bioindicators. For example, in the species of green algae (*Chlorophyceae*), the genera *Enteromorpha, Ulva, Cladophora, Codium, Caulerpa* and *Chaetomorpha*; in the species of red algae (*Rhodophyceae*) the genera *Gracilaria, Pterocladia, Gelidium, Gigartina* and *Porphyra;* and in the species of brown algae (*Phaeophyceae),* the genera *Dictyota, Scytosiphon, Colpomenia, Padina, Fucus, Ascophyllum* and *Macrocystis* (Conti, 2008; Al-Homaidan *et al.*, 2011).

2.9.2 Mode of operation of bioindicators

Bioindicators are biological indicators of environmental quality, characterizing environmental conditions. Their tolerance to stressors (e.g. heavy metals) is usually moderate; therefore, their presence or absence, and health state enable to determine some physical and chemical components of the environment without complicated measurements and laboratory analyses (Figure 2.6). In contrast, rare species (or species assemblages) which are known to have a narrow tolerance are often too sensitive to environmental change, or too infrequently encountered, to reflect the general biotic response. In a similar manner, ubiquitous species (or species assemblages) have very broad tolerances and are less sensitive to environmental changes which otherwise disturb the rest of the community (Gadzała-Kopciuch *et al*., 2004; Holt *et al*., 2011). Bioindicators may be divided into those responding to environmental changes in a visible way (morphological and physiological changes) and whose reactions are invisible, but which accumulate different substances (pollutants) whose concentrations may be determined (Gadzała-Kopciuch *et al.*, 2004). For example, two filamentous green algae *Enteromorpha intestinalis* (Linnaeus) Nees and *Cladophora glomerata*

(Linnaeus) Kutzing were used to determine the concentrations of manganese (Mn), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb) in Wadi Hanifah Stream, Riyadh, Saudi Arabia (Al-Homaidan *et al.*, 2011).

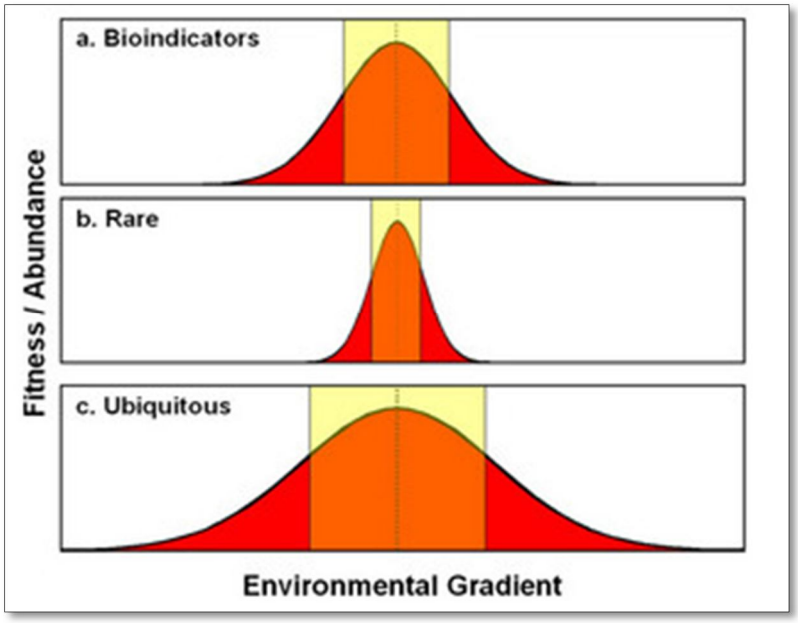

Figure 2.6 Comparison of environmental tolerances of (a) bioindicators, (b) rare species, and (c) ubiquitous species (Holt *et al.*, 2011)

In the above figure, the red areas represent portions of an environmental gradient (e.g. light availability and nitrogen levels) where an individual, species, or community, has fitness or abundance greater than zero. The dashed line represents the peak performance along this particular environmental gradient, while yellow boxes include the optimum range or tolerance. Bioindicators possess a moderate tolerance to environmental variability, compared to rare and ubiquitous species. This tolerance affords them sensitivity to indicate environmental change, yet endurance to withstand some variability and reflect the general biotic response (Holt *et al*., 2011).

2.9.3 Selection criterion of bioindicators

Bioindicators must be selected according to the following criterion (Gadzała-Kopciuch *et al*., 2004; Holt *et al*., 2011; Gerhardt, 2002)

- sedentary life
- abundance, wide distribution
- simple procedure of identification and sampling
- high tolerance for the pollutants analysed
- population stability
- high accumulating capacity
- species already being harvested for other purposes

There are numerous advantages and few disadvantages of using bioindicators for environmental monitoring when compared to the conventional physical and chemical methods of analysis and these are detailed below (Gadzała-Kopciuch *et al*., 2004; Holt *et al*., 2011, Omar, 2010, Nordberg *et al*., 2011).

Advantages of bioindicators

- bioindicators like algae accumulate pollutants to detectable levels even when their water concentrations are too low to be detected
- cheaper means of detecting pollutants in different environments compared to physical and chemical analysis which are expensive and require specialised laboratories
- less laborious process
- quicker means of detecting pollutants in the environment compared to traditional methods
- eco-friendly compared to chemical analysis which in the long term causes environmental damage
- the measured heavy metal concentration in the bioindicator organism reflects the bioavailable fraction of the polluting metal, that is, the fraction

available for uptake by organisms, thus providing a measure of the contaminant accumulated by the organism and this also helps in estimating the increase in the heavy metal concentration at different trophic levels of the food chain

Disadvantages of bioindicators

- the use of bioindicators in heterogeneous environments is limited due to the difficulty in discriminating natural variability from changes due to human impacts
- populations of indicator species may be influenced by factors such as disease, parasitism, competition or predation other than the disturbance or stress

2.10. Analytical procedures for mercury determination in plant and water samples

Proper sample collection and pre-treatment procedures as well as the selection of suitable preparation and measurement methodologies are some of the paramount factors which determine the accuracy of results obtained especially in trace metal analysis, therefore, these and other factors will be discussed in the following sections (Lusilao, 2012).

2.10.1 Sample collection, preservation and storage

Sampling, the first step in trace element analysis, must ensure the representativity of the sample in the context studied. Sampling is a common source of contamination if not done cautiously or with the use of recommended tools. In some cases, systematic and random errors of several orders of magnitude may occur. In addition, simple housekeeping procedures such as keeping the laboratory clean, providing appropriate ventilation; and rigorously washing all glassware,

tools and plastics can be adopted so as to minimise contamination especially when performing mercury analysis.

The best storage and preservation techniques for Hg^{2+} species must be adopted since these species are easily lost through vaporization. Hydrochloric acid has been so far preferable used as a preservative compared to HNO_3 since the chloride helps to complex the Hg^{2+}. However, regardless of acidification, mercury may still be lost through the walls; therefore, it is necessary to add an oxidizer to the original sample bottle prior to analysis for total mercury determination. In addition, proper instrument, glassware and plastic washing procedures must be developed and these must be used after each analysis. Usually, all materials are rinsed with deionized water (free of mercury) after acid washing. These materials are either dried, stored in double sealed plastic bags or as recommended by some authors stored in the nitric acid or hydrochloric acid bath until use.

For biological samples, knowledge of the trophic level of the organism to be analysed is important in developing a sampling strategy. In general, the samples are frozen immediately after the preliminary treatment. These samples are then analysed directly or after freeze-drying. The biotissues should be stored in the dark to avoid photodegradation.

On the other hand, water sampling strategy depends on the knowledge of the nature of the studied sites and the heterogeneity due to mixing of different water masses. The sampling and storage bottles have to be rinsed with the site water immediately before sampling. In the sampling of surface waters, pumps using polytetrafluoroethylene (PTFE or Teflon®) tubes can be used, however, in most cases, surface waters are collected “by hand” directly into the sampling bottle using long polyethylene gloves. The bottle has to be opened and closed under water to avoid mixing with the surface microlayer or oxidation of the sample.

2.10.2 Sample preparation for the determination of mercury in biological samples

The measurement of mercury in biological samples such as plants involves a pretreatment step, which is the conversion of the sample into solution before analysis. Technologies such as wet and dry ashing/ digestion and microwave digestion have been used for these purposes. The broad application of microwave digesters, shorter reaction times (usually minutes), reduced use of aggressive reagents, minimal contamination and lack of loss of volatile elements as well as the use of small amounts of reagents which reduces signals from the blank and increases accuracy of results makes them favourable for the digestion of biological samples containing mercury.

Microwave digestion is so far the most widely used technology for sample preparation. It involves the use of HNO_3 or its mixture with HCl or H_2SO_4 (with or without added H_2O_2) in the preparation of both organic and inorganic samples. The interaction of microwave radiation with samples and reagents results in fast heating of reaction mixtures and their efficient decomposition. Generally, a mixture of HNO_3 and H_2O_2 is used for most organic samples while a mixture of H_2SO_4 and H_2O_2 is mainly used for oily samples (Welna *et al*., 2011).

2.10.3 Analytical techniques

After the conversion of the biological sample into liquid form, technologies such as Cold Vapour Atomic Absorption Spectrometry (CVAAS), Graphite Furnace Atomic Absorption Spectrometry (GFAAS), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), and Cold Vapour Atomic Fluorescence Spectrometry (CVAFS) detection can be used to measure the total mercury concentration as well as other heavy metals present in the sample. However, samples handling and treatment for mercury determination have proved sometimes to be too cumbersome and complex with increasing risk of loss and/or contamination. This inconvenience was overcame by the introduction of the flow injection (FI) method

or flow injection analysis (FIA), which is computer compatible and allows automated handling of sample and reagent solutions with a strict control of reaction conditions. Generally, the FIA system (Figure 2.7) can be defined as the sequential insertion of discrete samples solution into an unsegmented continuously flowing stream with subsequent detection of the analyte.

Figure 2.7: Cold Vapour Flow Injection Atomic Absorption Spectrometry

The fast and intensive development of the FIA methodology was due to several factors essential for routine analytical determinations, such as very limited sample consumption, the short analysis time based on a transient signal measurement in a flow-through detector and an on-line carrying out difficult operations of separation, physicochemical conversion of analytes into detectable species (Parikh *et al.*, 2010). In this field, FIA offers unique features and advantages. Three outstanding attributes of this technology which ensured its rapid acceptance are: (1) the fundamental principles are easy to understand and implement, (2) instrumentation can readily be assembled from simple, inexpensive, off-the-shelf

components and (3) it provides a simple means of automating many manual wet chemical analytical procedures. The modern FIA system usually consists of: (1) injection valve, (2) high quality multi-channel peristaltic pump, (3) coiled reactor, (4) tubing manifold, (5) detector and (6) auto sampler. Additional components may include a flow through heater to increase the speed of chemical reactions, columns for sample reduction, de bubblers, and filters for particulate removal. FIA has been very successful in simplifying chemical assays. The main merits of FIA compared to conventional manual techniques are (Ruzicka, 2000):

- it is less labour intensive due to automation
- great precision due to mechanical performance of the assays
- high sampling rate
- smaller sample and reagent consumption and waste generation
- simplicity and low cost instrumentation
- availability of instrumentation in almost all laboratories
- reduced analyses cost when a lot of samples have to be analysed
- increased precision compared to batch methodologies
- automation in sample preparation and detection

FIA finds applications in a wide range of fields such as pharmaceuticals, environmental analysis, food analysis, biological material, mineral material, bioanalytical chemistry, on line monitoring in biotechnology and monitoring waste and its treatments. In a nutshell, FIA is a simple, rapid and versatile technique that is now firmly established. It is therefore a mature technique with well-defined and explored principles of operation (Ruzicka, 1998; Kuban *et al.*, 1998).

Chapter Three: Materials and Methods

This chapter addresses the research approach used, namely: the collection, identification, handling and storage of algae, metal analysis and the experimental procedure followed for the adsorption studies.

3.1 Reagents

Reagents used for the sorption studies were of analytical grade. Standards used for quantification were of high purity and were obtained from Sigma-Aldrich (Germany) and Merck (South Africa). As shall be discussed later in this chapter, super pure $SnCl_2$ and HCl were used as the carrier and reductant respectively for the Hydride generation Flow Injection Mercury System FIMS 400, (Perkin Elmer, USA) whose standards were prepared by serial dilution of 10 mg l^{-1} stock solution (De Bruyne, South Africa). For the ICP-OES, stock solutions were also supplied by De Bruyne (South Africa) at a concentration of 10 mg l^{-1}. All instrument standard solutions were prepared on the day of use.

3.2 Cleaning protocol

Contamination is a common source of error in all types of environmental analysis. It can be reduced by avoiding manual sample handling and by minimizing the number of discrete processing steps. However, the best way to assess and control the degree of contamination at any step of sample treatment is to use blank samples (Welna *et al.*, 2011). In addition, working under a clean environment is important, thus a rigorous cleaning procedure for all vessels used in sampling and sample preparation was adopted. The following cleaning procedure was used (Lusilao, 2012):

- running tap water was used to initially rinse all vessels (plastics and glassware) so as to remove all solid and liquid dirt
- all vessels were washed with soap after which they were rinsed thoroughly with running tap water and then de-ionized water with an electrical resistivity of 18.2 MΩ cm (Millipore, USA)
- all vessels were then soaked in a 10% (v/v) HNO_3 analytical grade (Merck) solution for 3 days after which they were thoroughly rinsed with deionized water

- all vessels were soaked in 10% (v/v) HCl analytical grade (Merck) solution for 3 days after which they were also thoroughly rinsed with deionized as described in the nitric acid step
- all vessels were dried in an oven after which they were stored in double sealed polyethylene bags until use

3.3 Algae collection and storage

The filamentous green algae strain used in this research was collected from Alexander dam, in Springs, on the East Rand of Gauteng Province, South Africa (Figure 3.1).

Figure 3.1: Site map of Alexander dam in Springs, Gauteng. (The red oval shape shows the area where the algae samples were randomly picked)

Algae samples were handpicked from the dam. To minimise the risk of contamination, nitrile gloves were used during sample collection (Figure 3.2) and in the removal of foreign material such as papers, grass and plant leaves which were attached to the algae. The dam water in which the algae were growing in was collected into polyethylene plastic containers in which the algae were placed. The

containers were tightly sealed and placed in refrigerated bags for transportation to the laboratory were some of the algae were treated by washing thoroughly with tap water and rinsed with de-ionized water until all the foreign material was removed. The rigorous washing of the algae also enabled the removal of metals attached to the algae surface. The rest of the algae which was not to be immediately used were stored in the refrigerator at 4°C until use.

Figure 3.2: Handpicking of algae from Alexander dam

3.4 Algae identification

Identification of the freshwater algae was carried out at the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand (Johannesburg, South Africa). The algae were also observed under a light microscope (Olympus Instruments, Melville, NY, USA) equipped with a DMX 1200 digital camera at 200 X magnification (Nikon, Japan). It was advantageous to use this light microscope with a digital camera attached to it than a simple microscope because it provided optical micrographs on a computer, thus, making the analysis of the algae easier.

3.5 Algae functional groups

The various functional groups present on the surface of *Cladophora* sp. before and after batch adsorption tests at pH 3, 6.5 and 8.5 were determined using Fourier Transform Infra-red (FTIR) analysis. FTIR spectra were measured directly from *Cladophora* sp. using a Tensor 27 (Bruker, Germany) device in the range of 4 000 to 400 cm^{-1}.

3.6 Algae culture

The algae samples were grown in an algae culture for 2-3 days and then acclimated in distilled water at room temperature and natural light for a day before use for experiments (Ji *et al*., 2012). The Acidic Bold-Basal Medium (ABM) algae culture was prepared as shown in Table 3.1 and 3.2 (CCAP, Accessed 20/06/13).

Table 3.1: Essential elements

Salt	Concentration (g l^{-1})
$NaNO_3$	25.0
$MgSO_4 \cdot 7H_2O$	7.5
NaCl	2.5
$K_2HPO_4 \cdot 3H_2O$	7.5
KH_2PO_4	17.5
$CaCl_2 \cdot 2H_2O$	2.5

To 800 ml of distilled water, 250 mg of $(NH_4)_2SO_4$ were added as well as 10 ml of each of the essential elements in Table 3.1.

Table 3.2: Trace elements

Salt	Quantity (mg)
$FeCl_3 \cdot 6H_2O$	97.0
$MnCl_2 \cdot 4H_2O$	41.0
$ZnCl_2 \cdot 6H_2O$	5.0
$CoCl_2 \cdot 6H_2O$	2.0
$Na_2MoO_4 \cdot 2H_2O$	4.0

To 1000 ml of deionised water, 0.75 g of Na_2-EDTA was added as well as the trace elements in the manner they appear in Table 3.2. To the 800 ml of deionized water in which 250 mg of $(NH_4)_2SO_4$ and 10 ml of each of the essential elements was added, 6.0 ml of each of the trace elements was also added. The resulting solution was made up to 1 l with deionised water and the pH was maintained at 6.5 since this was the pH of the dam water where the algae were collected. The solution was then autoclaved using a Precise Shaking Incubator WIS-30 (Daihan Scientific Co., Ltd, Korea) at 121^oC and 1000 Pa for 15 minutes so as to sterilise the culture media by inactivating microbial life such as bacteria (Barsanti *et al.*, 2006).

3.7 Batch adsorption tests

All batch experiments were conducted in triplicates and for each set a control which contained the metal contaminant but lacked algae was setup so as to determine if something besides algae caused the observed effects.

Stock solutions of 10 mg l^{-1} of Hg^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+} were prepared by weighing appropriate amounts of the following salts: $HgSO_4$, $Co(NO_3)_2 \cdot 6H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$ and $FeSO_4$. Appropriate aliquots were taken from these standards for subsequent dilution to the desired concentration level. The solutions were acidified using 1 mol l^{-1} HNO_3 to avoid the precipitation of the metals and stored in a refrigerator at 4^oC prior to the relevant experiments.

The adsorption of mercury on algae and the competitive adsorption of other heavy metals (Fe, Zn, Co and Cu) were done in batch mode. The following parameters were assessed: effect of pH, contact time and the presence of other heavy metal cations.

3.7.1 Effect of pH

The parameters used to assess the effect of pH on the adsorption of mercury by algae are shown in Table 3.3. The pH was adjusted by using a 0.1 mol l^{-1} solution of either NaOH or HNO_3. Batch experiment tests were performed in triplicates and 250 ml conical flasks were used. At different equilibrium times, 2 ml of the sample volume was withdrawn for analysis. This volume was such that the total volume drawn was less than 10% of the initial volume used so as to minimise the change in the ratio between the metal concentration and the sorbent mass (Nsimba, 2012).

Table 3.3: Parameters used to assess the effect of pH

pH	3.0, 6.5 and 8.5
Temperature (°C)	Room temperature (23.5 -25.5)
Residence time (min)	0-120
Metal ion concentration (mg Hg l^{-1})	0.5-1.0
Mass of algae (g)	2.0 ± 0.05
Volume (ml)	200
Agitation rate (rpm)	150

3.7.2 Effect of contact time (Kinetic studies)

As shall be explained in Chapter 4, the effect of contact time on the adsorption of mercury by algae was assessed at pH 3. Parameters used for this assessment are shown in Table 3.4 below. As explained in section 3.7.1, 2 ml of the sample volume was withdrawn for analysis.

Table 3.4: Parameters used to assess the effect of contact time

pH	3
Time intervals (min)	0, 5, 10, 20, 30, 60, 90, 120
Metal ion concentration (mg Hg l^{-1})	0.5-1.0
Mass of algae (g)	2.0 ± 0.05
Volume (ml)	200
Temperature (°C)	Room temperature (23.5 -25.5)
Agitation rate (rpm)	150

3.7.3 Effect of competing metal cations

Competitive adsorption tests were done using the same procedure as that for mercury adsorption on algae. As shall be explained in Chapter 4, the effect of competing heavy metal cations on the adsorption of mercury by algae was assessed at pH 3. Parameters used for this assessment are shown in Table 3.5 below.

Table 3.5: Parameters used to assess the effect of competing metal cations

pH	3.0
Temperature (°C)	Room temperature (23.5 - 25.5)
Residence time (min)	0-120
Metal ion concentration (mg l^{-1})	1.0
Metal cations used	Hg^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}
Mass of algae (g)	4.0 ± 0.05
Volume (ml)	500
Agitation rate (rpm)	150

3.8 Metal analysis

The analysis of metals in water samples involved a direct measurement on a Mercury analyser or ICP-OES and that on algae samples involved several treatment steps all of which shall be sequentially described.

3.8.1 Drying

After each experiment, the experimental solution containing algae was filtered through a 110 mm filter paper (ACE, South Africa). The treated algae samples were put into small polyethylene containers which were initially acid washed as described in section 3.1.2. The treated algae were either oven dried at 60°C (this temperature was selected so as to minimise the volatilization of Hg^{2+}) until constant mass or were freeze dried at -40°C for a period of 3 days using the Labconco freeze drier (Vacutec, South Africa). After drying, the dry mass and % moisture content of the treated algae was determined using the following equation:

$$\%\, Moisture\ content = \frac{w_2 - w_3}{w_2 - w_1} \times 100 \qquad (3.1)$$

$Where$;
$w_1 - weight\ of\ container$ (g)
$w_2 - initial\ weight\ of\ container\ with\ algae$ (g)
$w_3 - final\ weight\ of\ container\ with\ algae$ (g)

The treated algae samples were then crushed and homogenized using a mortar and pestle with the aid of liquid nitrogen.

3.8.2 Microwave digestion of algae

A mass of 0.25 ± 0.005 g of homogenized algae samples were weighed using an analytical balance (Precisa 180A, Switzerland) and placed into acid washed digestion tubes (PTFE-TFM liners) onto which 16 ml HNO_3 and 4 ml H_2O_2 were added before being placed into the Multiwave 3000 microwave digester (Anton Paar, Switzerland) shown in (Figure 3.3).

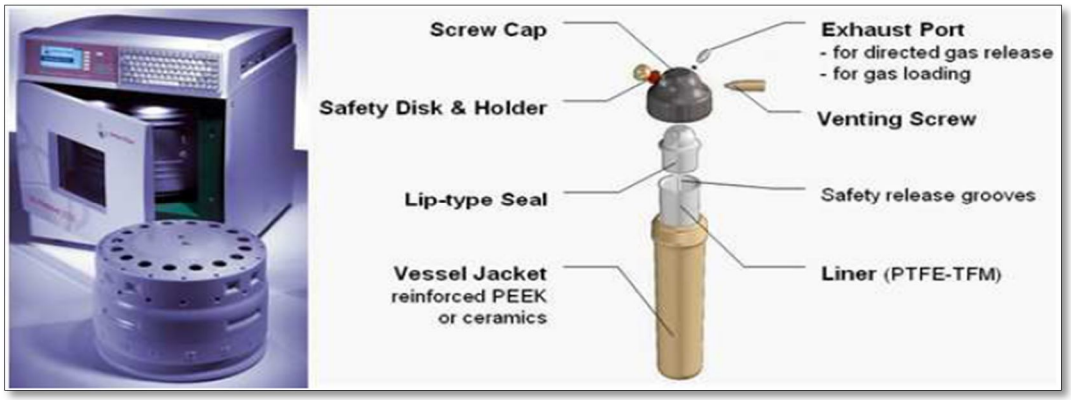

Figure 3.3: The Multiwave 3000 MAE system and the vessel design

Table 3.6 shows the microwave program for the sample digestion process. The digested samples were transferred into 50 ml acid washed volumetric flasks and the volumes were completed with distilled water after which they were kept at 4°C until further analysis.

Table 3.6: Microwave programme for sample extraction

Phase	Power (W)	Ramp (min)	Hold (min)	Fan
1	600	10:00	10:00	1
2	0	05:00	05:00	3

Sample weight: 0.25g; Reagent: HNO_3 (16 ml); H_2O_2 (4 ml)

3.8.3 Determination of total mercury concentration

The total mercury concentrations in the water and digested algae samples were determined using the Hydride generation Flow Injection Mercury System FIMS 400, (Perkin Elmer, USA) which uses a Cold Vapour Atomic Absorption Spectrometry (CVAAS) detection system (Figure 3.4). Quantification was with respect to reagent-matched mercury standard prepared by serial dilution of 10 mg l^{-1}. Of the 2 ml volume collected during the batch tests, 500 µl were used for analysis. This volume was placed in acid washed 15 ml centrifuge tubes and made up to 10 ml. The dilution factor was calculated and taken into account in determining the actual mercury concentration from the results obtained from the mercury analyser.

Tin chloride ($SnCl_2$) 1.1% (*wt.*) in 3% (v/v) hydrochloric acid (HCl) was used as a reductant and 3% (v/v) HCl as a carrier for the Hydride generation Flow Injection Mercury System. In this system, mercury is chemically reduced by the $SnCl_2$ to its free atomic state in a closed reaction system. The volatile free mercury is then driven from the reaction flask by bubbling argon through the solution. Mercury atoms are carried in the gas stream through tubing connected to an absorption cell, which is placed in the light path of the AA spectrometer. As the mercury atoms pass into the sampling cell, measured absorbance rises indicating the increasing concentration of mercury atoms in the light path. The highest absorbance observed during the measurement is taken as the analytical signal. The absorbance rises until an equilibrium concentration of mercury is attained in the system. The absorbance levels off, and the equilibrium absorbance is used for quantitation (Perkin Elmer, 2003).

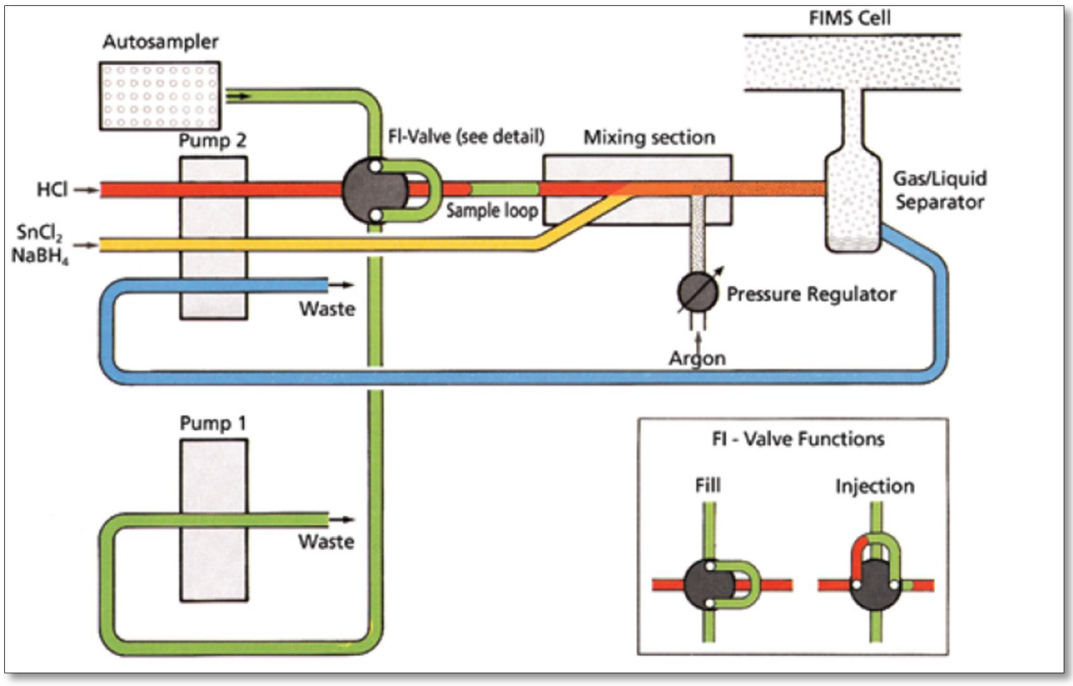

Figure 3.4: Hydride generation Flow Injection Mercury System (FIMS 400)

3.8.4 Determination of competing metal cations

Total metal concentrations of (Fe^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}) in water and digested algae samples were obtained by using an Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) instrument (Spectro, Kleve, Germany) (Figure 3.5) with coupled charge detection (CCD) system. The instrumental conditions (Table 3.7) were optimized to obtain sufficient sensitivity and precision and the concentration of each element was determined at various wavelengths. Wavelengths that represent a good compromise between maximum sensitivity and the ability to encompass a large range of concentrations are selected. The stock solutions supplied at a concentration of 10 mg l^{-1} were used to make working standards of 0.05 to 1 mg l^{-1}. Calibration curves were then constructed after the analysis of these standards and these are presented in Chapter 4.

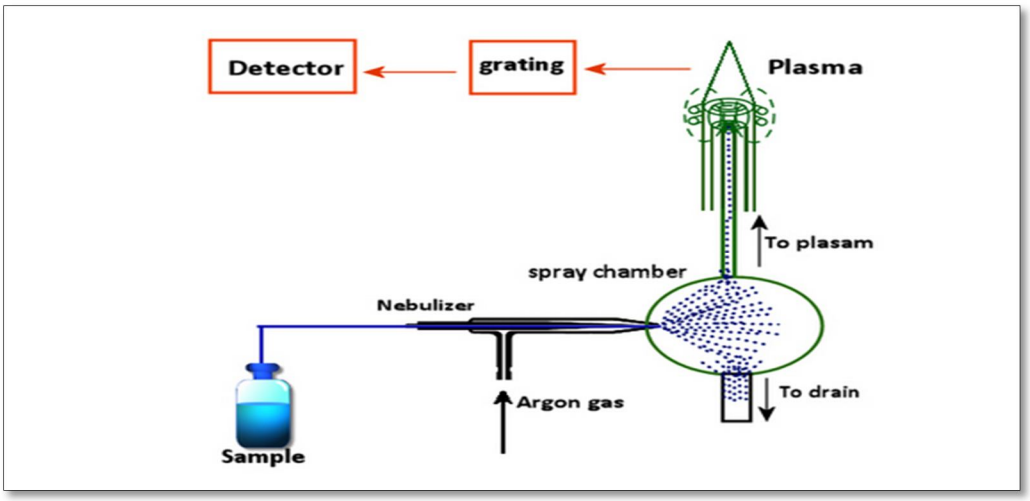

Figure 3.5: Process flow diagram of the ICP-OES

Table 3.7: Optimized parameters of the ICP-OES

Parameter	Value
Coolant flow	14 mL min^{-1}
Plasma power	1400 W
Auxiliary flow	1 mL min^{-1}
Nebulizer flow	1 mL min^{-1}
Type of nebulizer	Cross-flow
Injector tube diameter	0.889 mm

3.9 Parameters studied

Metal analysis results obtained from the batch adsorption tests on the effect of pH and contact time were used to calculate the extraction efficiency and adsorption capacity.

3.9.1 Extraction efficiency

The extraction efficiency (r) of Hg^{2+} by algae refers to the maximum amount of Hg^{2+} ions removed from solution compared to the initial concentration of Hg^{2+}

ions. The mass balance equation used to calculate the extraction efficiency is shown below (Ji *et al.*, 2012):

$$r = \frac{(C_0 - C_t)}{C_0} \times 100\% \qquad (3.2)$$

Where;

$r - extraction\ efficiency\ (\%)$

$C_0 - initial\ metal\ ion\ concentration\ (mg\ l^{-1})$

$C_t - equilibrium\ metal\ ion\ concentration\ (mg\ l^{-1})$

3.9.2 Adsorption capacity

The adsorption capacity (q_e) of an adsorbent material, in this case alga, refers to the maximum amount of metal ions that can be attracted to and held on the surface of a given amount of algae. This was calculated using the following equation (Ji *et al.*, 2008).

$$q_e(mg\ g^{-1}) = \frac{(C_0 - C_t)V}{M} \qquad (3.3)$$

Where;

$q_e - amount\ of\ metal\ adsorbed\ (mg\ g^{-1})$

$C_0 - initial\ metal\ ion\ concentration\ (mg\ l^{-1})$

$C_t - equilibrium\ metal\ ion\ concentration\ (mg\ l^{-1})$

$V - volume\ of\ media\ (l)$

$M - mass\ of\ biosorbent\ (algae)\ used\ (g)$

After determining the adsorption capacity, calculations were made to determine if the adsorption mechanism of mercury on algae was either a pseudo-first order or pseudo-second order reaction.

3.9.3 Lagergren's pseudo first order

The pseudo first-order rate equation is given by the following equation (Lagergren, 1898):

$$\frac{dq}{dt} = k_1(q_e - q_t) \qquad (3.4)$$

$Where;$

$q_t - amount\ of\ metal\ adsorbed\ at\ any\ time\ (mg\ g^{-1}), (mol\ g^{-1})$

$q_e - amount\ of\ metal\ adsorbed\ at\ equilibrium\ time\ (mg\ g^{-1}), (mol\ g^{-1})$

$k_1 - pseudo\ first\ order\ rate\ constant\ (min^{-1})$

Integrating and applying boundary conditions (t = 0 to t and q_t = 0 to q_e), (3.4) becomes:

$$log\ (q_e - q_t) = log(q_e) - \frac{k_1 t}{2.303} \qquad (3.5)$$

Simplifying (3.5) gives;

$$q_t = q_e(1 - e^{-k_1 t}) \qquad (3.6)$$

By plotting $log\ (q_e - q_t)$ against time t, q_e and k_1 can be determined from the intercept and slope of the plot respectively.

3.9.4 Ho pseudo second order

The pseudo second-order rate equation is given by the following equation (Ho *et al.*, 1999):

$$\frac{dq}{dt} = k_2(q_e - q_t)^2 \qquad (3.7)$$

Integrating and applying boundary conditions (t = 0 to t and q_t = 0 to q_e), (3.7) becomes:

$$\frac{t}{q_t} = \frac{1}{k_2 {q_e}^2} + \frac{1}{q_e} t \qquad (3.8)$$

$Where,$

$k_2 - rate\ constant\ for\ \ second - order\ model$

A plot of $\frac{t}{q_t}$ against t must give a straight line if the pseudo second-order kinetic model is applicable. q_e and k_2 are determined from the slope and intercept of the plot respectively.

3.9.5 Distribution coefficients

Metal analysis results for competitive adsorption obtained from the ICP-OES were used to calculate the distribution coefficients $K_d\ (l\ mol^{-1})$ of the different metal cations as shown in the following equation (US EPA, 1999):

$$K_d\ (l\ mol^{-1}) = \frac{(C_o - C_t)V}{C_t M} \qquad (3.9)$$

Where;

$C_0 - initial\ metal\ ion\ concentration\ (mg\ l^{-1})$

$C_t - equilibrium\ metal\ ion\ concentration\ (mg\ l^{-1})$

$V - volume\ of\ media\ (l)$

$M - mass\ of\ biosorbent\ (algae)\ used\ (g)$

3.10 Method validation

A Certified Reference Material (CRM) of lichens (BCR 482, Belgium) and digestion blanks were used for method validation and the results obtained are presented in Chapter 4.

3.11 Analytical figures of merit

The following analytical figures of merit were particularly assessed:

3.11.1 Method limit of detection

The Limit of detection (LOD) is the lowest analyte concentration which can be detected by the machine used (Armbruster *et al*., 2008). This was calculated using the following formula (Hutter, 2011):

$$LOD = 3 \times Standard\ deviation\ of\ a\ blank\ sample \quad (3.10)$$

3.11.2 Precision

Seven measurements were performed in each replicate sample so as to assess the repeatability and reproducibility of the analysis.

3.11.3 Accuracy

The accuracy of the analytical procedure used was evaluated by comparing results obtained from CRMs analyses to certified values.

Chapter Four: Results and Discussions

This chapter presents the results for the experimental work conducted. The results relate to: the optimization of analytical procedures, the adsorption of mercury by filamentous green algae, Cladophora sp.; competitive adsorption which involves the adsorption of mercury on Cladophora sp. in the presence of other heavy metal ions. Important kinetic models of the adsorption process are also included.

4.1 Method validation

An excellent linearity was obtained with the FIMS instrument for total mercury standards (Figure 4.1). Calibration was performed using standards in the range of 1-10 µg Hg l^{-1}. A method limit of detection of 0.009 µg Hg l^{-1} was obtained which makes the method suitable for ultratrace analysis of mercury.

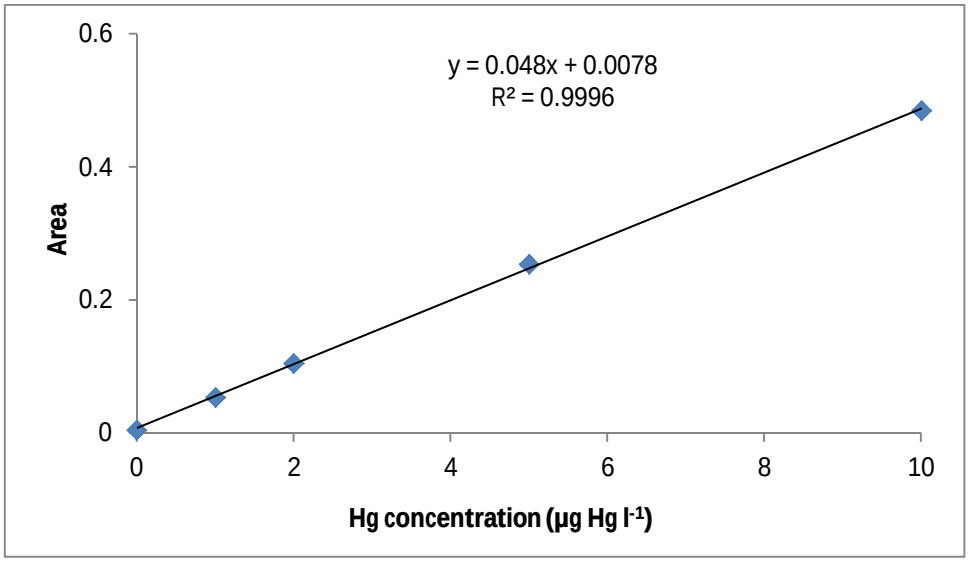

Figure 4.1: Calibration curve for the FIMS

The CRM BCR 482 of Lichens was analysed as described in Chapter 3 and the results shown in Tables 4.1 and 4.2 were obtained with the developed analytical methods. More than 99% of mercury recovery was obtained with the FIMS. This confirms that no considerable mercury loss or contamination occurred during samples preparation and subsequent analysis. The obtained results also demonstrated the excellent repeatability and reproducibility of the methodology with RSD always below 5%. Overall, the analysis of CRMs has shown that the optimised analytical method can be successfully used for the precise and accurate detection of mercury in plant materials.

Table 4.1: Flow Injection Mercury System (FIMS) CRM results

Sample ID	Hg ± SD ($\mu g\ kg^{-1}$)	Mean ± SD ($\mu g\ kg^{-1}$)	RSD (%)	Certified Value ($\mu g\ Hg\ kg^{-1}$)	% Recovery
CRM 1 CRM 2 CRM 3	456 ± 0.04 502 ± 0.67 471 ± 0.02	476 ± 23	4.8	480 ± 20	99

Table 4.2: ICP-OES CRM results

CRM BCR482	Al	Cd	Cu	Zn
Mean ($mg\ kg^{-1}$) (n = 3)	963 ± 20	0.66 ± 0.13	6.90 ± 0.31	91.5 ± 1.0
Certified Values ($mg\ kg^{-1}$)	1103 ± 24	0.56 ± 0.02	7.03 ± 0.19	100.6 ± 2.2
% Recovery	87	95	98	91

ICP-OES results of the same CRM generally exhibited the same trend since, with the exception of aluminium, all the analysed metals also showed a recovery beyond 90%.

4.2 Algae structure

Observations made on alga under a light microscope showed that the algae collected from Alexander dam was filamentous, with cells joined from end to end (Figure 4.1). Though this alga was not branched like the common *Cladophora* species (e.g. *Cladophora glomerata*), its hair-like nature and rough texture fitted well the description of *Cladophora* sp. In addition, the order of *Cladophora* sp., *Cladophorales*, stipulates that it is filamentous, with cells joined end to end in definite series, with or without branching of multinucleate cells (Johnson *et al.*, 1996).

Figure 4.2: Optical micrograph showing the filamentous nature of *Cladophora* sp. (wet filament)

4.3 *Cladophora* sp. functional groups

The FTIR spectrum of *Cladophora* sp. before the batch adsorption tests is shown in Figure 4.3 below. With reference to the obtained wavenumbers, the possible functional groups present on the surface of *Cladophora* sp. are given in Table 4.3 (Solomons Organic Drill Manual, 2007).

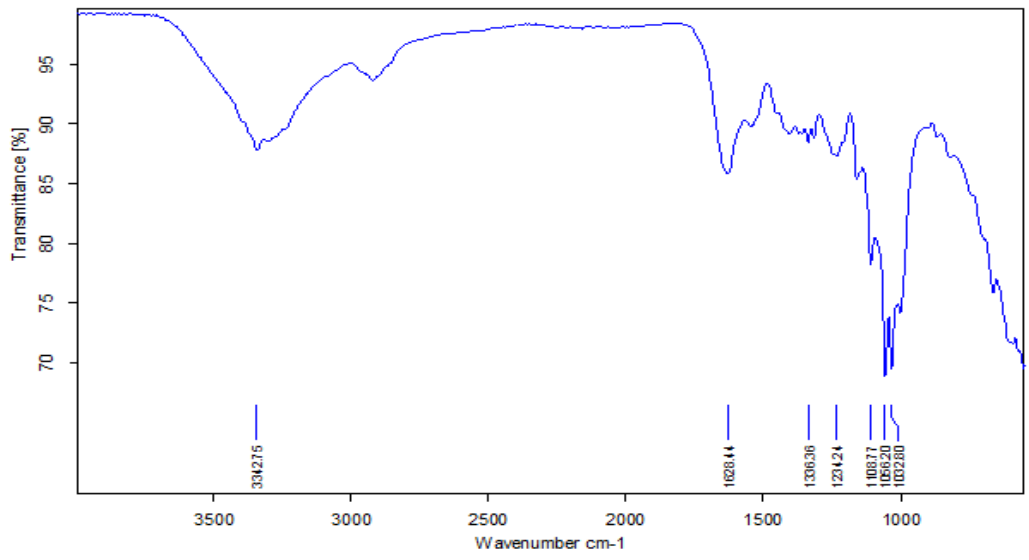

Figure 4.3: FTIR spectrum of *Cladophora* sp.

Table 4.3: FTIR vibrations of *Cladophora* sp.

Wavenumber cm^{-1}	Vibration type
3300-3600	O-H (alcohol)
3200-3500	N-H (amine)
2500-3000	O-H (carboxylic acids)
1700-1600	v(C=O)

It has been reported that the majority of the algae functional groups responsible for metal sorption such as the carboxyl are acidic and are available at low pH (Mehta et al., 2005). Also, at high pH values, the surface charge of algae is negative due to the presence of the hydroxyl (-OH) functional group thus facilitating metal adsorption due to attraction between the negative algae surface charge and the positively charged metal cation. Therefore, with reference to the FTIR results obtained, most of the metal adsorption is expected to occur at acidic and alkaline conditions.

4.4 Algae moisture content

The calculated average dry mass of algae used for the adsorption experiments in a single component and multi-component system was 0.31 g ± 0.06 g and 0.7 g ± 0.02 g respectively. The average moisture content was in the range of 82 – 84 %. The moisture content was high since the alga was filtered directly from the experimental solution. These results are shown in Table 4.4 below and Table 1 in Appendix A.

Table 4.4: Algae dry weight and moisture content results

Sample I.D	Average mass (g) ± SD	Average % moisture ± SD
pH 3: 0.5 mg Hg l^{-1}	0.35 ± 0.03	82.46 ± 1.63
pH 6.5: 0.5 mg Hg l^{-1}	0.33 ± 0.05	83.44 ± 2.73
pH 8.5: 0.5 mg Hg l^{-1}	0.35 ± 0.02	82.85 ± 1.02
pH 3: 1 mg Hg l^{-1}	0.36 ± 0.03	82.43 ± 1.27
pH 6.5: 1 mg Hg l^{-1}	0.34 ± 0.03	83.43 ± 1.36
pH 8.5: 1 mg Hg l^{-1}	0.32 ± 0.02	83.79 ± 1.16
pH 3.0: 1 mg l^{-1} (competitive adsorption tests)	0.69 ± 0.01	82.91 ± 0.17

4.5 Adsorption capacity, pH and isotherms

The effect of pH, contact time and the presence of other heavy metals on the adsorption of mercury by *Cladophora* sp. were assessed.

4.5.1 Effect of pH

The effect of pH on the adsorption of mercury by *Cladophora* sp. was studied at the conditions described in Table 3.3 (section 3.7.1). Higher adsorption capacities were obtained at a concentration of 1 mg Hg l^{-1} (Figure 4.4) compared to 0.5 mg l^{-1} (Appendix B). Mercury concentration of 1 mg Hg l^{-1} was therefore chosen as the optimum concentration for these studies. Adsorption capacities of 805 mg kg^{-1}, 538 mg kg^{-1} and 586 mg kg^{-1} were obtained for pH 3, 6.5 and 8.5 respectively.

Earlier studies have indicated that pH plays a major role on the adsorption of heavy metals by algae as it influences the solution chemistry of the metals, the activity of functional groups (carboxylate, phosphate, thiol and amino groups) on the cell wall as well as the competition of metallic ions for the binding sites (Rezaee et al., 2006, Pansamrit et al., 2012). In this study, the maximum adsorption was found at pH 3 (Figure 4.4), which is also the point of zero charge of algae (Yalçın et al., 2008). At this point, the surface charge of alga is neither positive nor negative, as a result, it has great tendency to either adsorb more or less of the mercury, but in this case, more of the mercury was adsorbed. This shows that under the given experimental conditions, the surface charge of alga at the point of zero charge developed as negative, thus more binding sites were available for mercury adsorption. Also, the majority of algae functional groups for metal sorption such as the carboxyl are acidic. Therefore, they are more available at low pH resulting in increased metal sorption though very low pH values have been reported to result in a decrease in metal sorption due to the increased concentration of the hydrogen ions (H^+) which are preferentially adsorbed onto the alga surface compared to metal ions (Mehta et al., 2005).

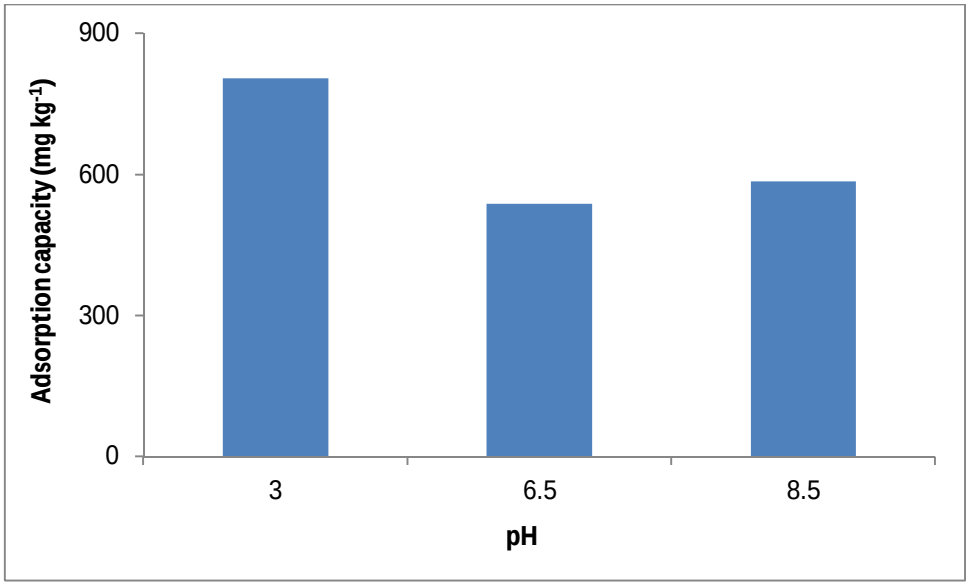

Figure 4.4: Effect of pH on the adsorption of mercury by *Cladophora* sp.

At pH 8.5, *Cladophora* sp. had a high adsorption capacity though lower than that observed at pH 3. This is mainly due to the decrease in the concentration of H^+ ions at high pH values; therefore there is less competition for binding sites between the metal cations and the H^+ ions. To add, at high pH values, the surface charge of alga is negative due to the presence of hydroxyl functional group, thus enhancing the adsorption process as a result of attraction between the positively charged metal cation and the negatively charged alga surface (Rezaee et al., 2006**)**. From these results, pH 3 was selected as the optimum pH for the uptake of mercury by alga and this suits well its intended purpose, that is, use in AMD which is characterised by low pH.

However, it should be noted that there could be a combination of precipitation and adsorption at pH 8.5. The solubility of many metals is amphoteric in the sense that the metals tend to dissolve and form cations at low pH and anions at high pH, with minimal solubility at intermediate pH, thus, low adsorption at pH 6.5 (Saad, 2011).

4.5.2 Effect of contact time

The effect of contact time on the adsorption of mercury by *Cladophora* sp. was studied at the conditions described in Table 3.4 (section 3.7.2). Higher adsorption capacities were obtained at an optimum concentration of 1 mg Hg l^{-1} and pH 3 (Figures 4.5 and 4.6) compared to 0.5 mg Hg l^{-1} (Figure 2, Appendix B). The effect of contact time on the adsorption of mercury by *Cladophora* sp. at 1 mg Hg l^{-1} and at different pH is presented in Figure 2, Appendix C.

The concentration of Hg^{2+} in solution rapidly decreased within the first 5 min, attaining greater than 99% Hg^{2+} removal. Here, a two steps adsorption mechanism can be proposed: the first step corresponding to the dissociation of the complexes formed between Hg^{2+} in solution and water hydronium ions followed by the interaction of the metal with algal functional groups. The rapid uptake of mercury by algae is mainly by physical adsorption, a metabolism-independent process.

This initial quick phase is believed to have been followed by slow attainment of equilibrium as a large number of vacant binding sites were initially available for sorption; but thereafter, the occupation of the remaining vacant sites were difficult to occupy due to the repulsive forces between the metal ions in the solid and bulk phases. The rapid uptake of mercury by algae is of great importance in environmental applications. This shows that algae can be efficiently used as mercury bioindicators as well as phytoremediation material at shorter retention times, thus resulting in huge operation cost savings. Equilibrium was attained within the first 10 min, therefore a contact time of 10 min was chosen as the optimum time.

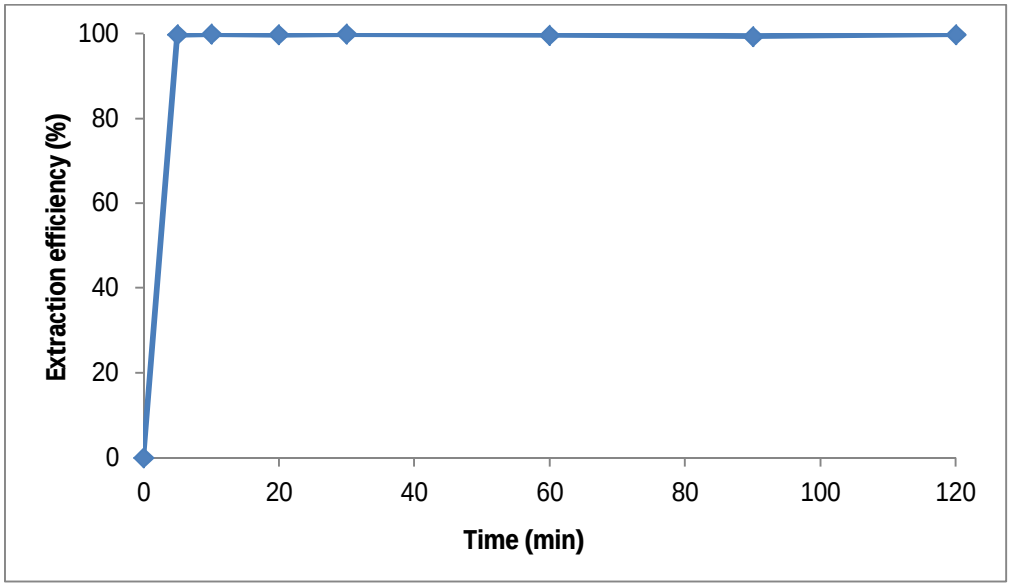

Figure 4.5: Effect of contact time on the extraction efficiency of *Cladophora* sp.

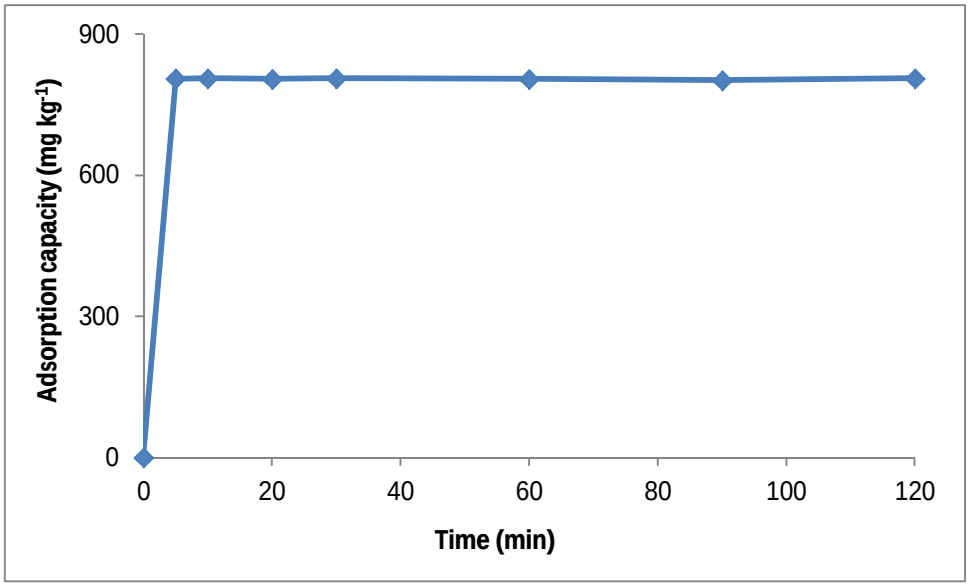

Figure 4.6: Effect of contact time on the adsorption capacity of *Cladophora* sp.

4.6 Kinetic modelling of the adsorption of mercury on *Cladophora* sp.

The pseudo first-order and second-order kinetic models were used to investigate the kinetics of adsorption of Hg^{2+} onto *Cladophora* sp. so as to gain insight into the mechanism and rate controlling steps affecting the kinetics of adsorption. The obtained experimental data were modelled onto the pseudo 1^{st} and 2^{nd} order equations (3.5 and 3.8). The working concentrations of 0.5 mg Hg l^{-1} (Figure 3 and 4, Appendix B) and 1.0 mg Hg l^{-1} (Figures 3 and 4, Appendix C) and different pH showed a better correlation with the pseudo 2^{nd} order model compared to the pseudo 1^{st} order model.

The pseudo 1^{st} and 2^{nd} order model results obtained at the chosen optimum conditions (pH=3 and mercury concentration = 1.0 mg Hg l^{-1}) are presented in Figures 4.7 and 4.8 respectively.

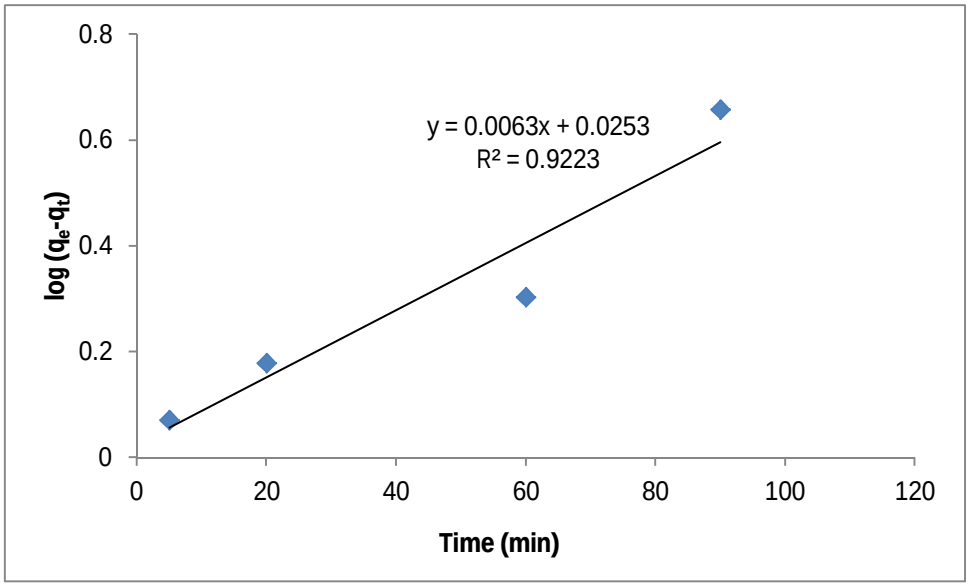

Figure 4.7: Pseudo first order model for the adsorption of mercury by *Cladophora* sp.

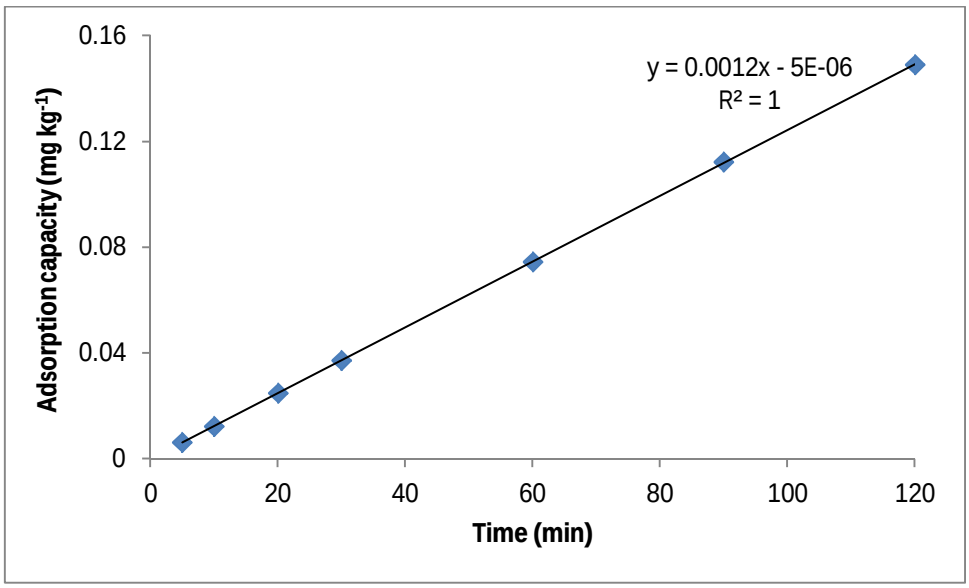

Figure 4.8: Pseudo second order model for the adsorption of mercury by *Cladophora* sp.

The pseudo 2nd order model showed a better correlation because of the high correlation coefficient, $R^2 = 1$ (Figure 4.8) compared to $R^2 = 0.9223$ in the pseudo 1st order model (Figure 4.7). In addition, the adsorption capacity (q_e) of 833 mg kg^{-1} calculated by the pseudo 2nd order model (Table 4.5) was close to the one obtained experimentally (805 mg kg^{-1}) as shown in Figure 4.4.

Table 4.5: Pseudo 1st and 2nd order model results

Parameter	Pseudo 1st order	Pseudo 2nd order
k_1 (min^{-1})	0.01	
k_2 (kg mg^{-1} min^{-1})		0.29
q_e (mg kg^{-1})	1.06	833

The obtained results indicate that the pseudo-second order mechanism was predominant and chemisorption might be the rate-limiting step that controlled the removal of mercury from solution by *Cladophora* sp. During the course of this process three possible mechanisms may take place: (1) an external surface mass transfer or film diffusion controlling the early stages of the biosorption process (2) the first stage is followed by a reaction or constant rate stage and (3) a diffusion stage in which the biosorption process slows down considerably (Satyanarayana et al., 2012).

4.7 Effect of other metal ions

In addition to Hg^{2+}, AMD waters contain other metal ions such as Fe^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+} whose presence is most likely to affect the adsorption of the target metal ion (Hg^{2+}) on *Cladophora* sp. The effect of these cations was investigated at the conditions mentioned in Table 3.5 and the obtained results are shown in Figure 4.9. It was observed that the removal efficiency of Hg^{2+} dropped from greater than 99% in a single component system to a maximum removal efficiency of 67% in a multi component system. However, this extraction efficiency is still high; therefore, algae can be still used as a biosorbent material in environmental applications.

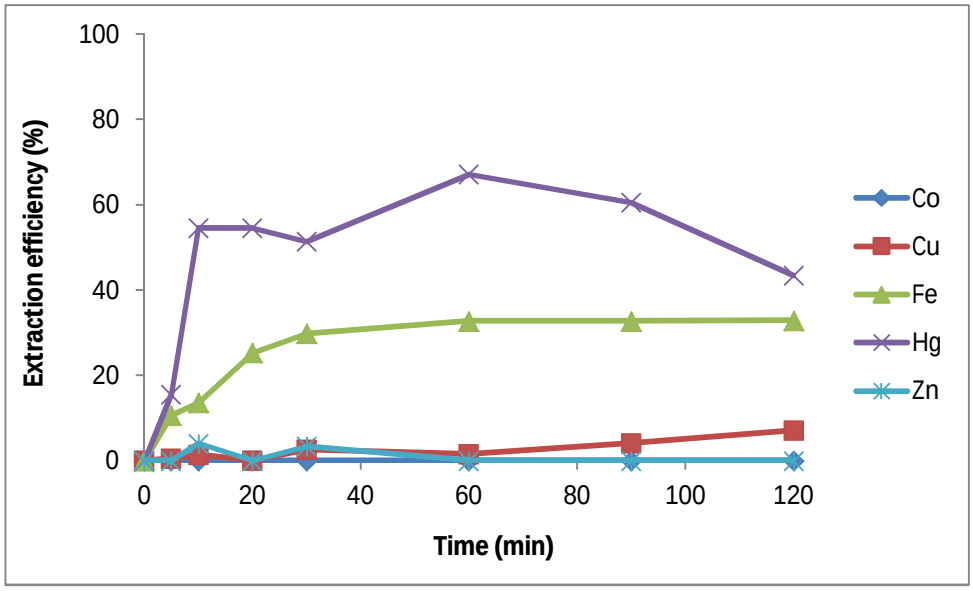

Figure 4.9: Effect of competing metal cations on the extraction efficiency of *Cladophora* sp.

The decrease in extraction efficiency in a multi component system can be attributed to the chemical interactions between the different metal cations present as well as with the algae thereby resulting in a competition on the active sites of the algae. In addition different metal cations compete for binding sites since most of the functional groups present on the algal cells wall are non-specific. It has also been reported that the preferential adsorption of metal cations in a multicomponent is also dependent on the ionic radii, that is, as the ionic radii increases, metal removal also increases. A larger ionic radius implies a larger spread of the electron configuration in space and a greater tendency for a metal to distort (polarize) the surface charge of the functional group in response to the electric field. This polarization is the necessary prerequisite for the distortion of the electron configuration leading to covalent bonding (Mohamed *et al*., 1998).

Thus for the competitive adsorption tests performed, this follows the order: Hg^{2+} >Fe^{2+}>Cu^{2+}> Zn^{2+} > Co^{2+}. To add, the difference in sorption affinities may also be attributed to differences in electronegativity of the atoms and this also follows the order: Hg^{2+} >Fe^{2+}>Cu^{2+}> Zn^{2+} > Co^{2+}. Therefore, the greater the electronegativity or ionic radii the higher the sorption affinity and this explains the high extraction efficiency of Hg^{2+} obtained in the presence of competing metal cations (Abu Al-

Rub *et al*., 2006, Seshadri *et al.*, 2002). The observed trend for Hg^{2+} adsorption (Figure 4.9) is most likely to be a result of adsorption and desorption occurring simultaneously. However, further work must be carried out inorder to validate this assumption.

The distribution coefficients for the Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} and Hg^{2+} cations used were calculated using equation (3.9). As shown in Table 4.6, the distribution coefficient was highest for Hg^{2+} (386.5 l mol^{-1}) followed by Fe^{2+} (241.5 l mol^{-1}) and Cu^{2+} (19.3 l mol^{-1}) and it remained at zero for both Co^{2+} and Zn^{2+}. These results show that *Cladophora* sp. has a higher sorption capacity for Hg^{2+} compared to Fe^{2+}, Cu^{2+}, Zn^{2+} and Co^{2+} under the same experimental conditions (Alloway, 1995).

Table 4.6: Distribution coefficient of competing metal cations

Distribution coefficient: K_d (l mol^{-1})				
Cu^{2+}	Co^{2+}	Zn^{2+}	Fe^{2+}	Hg^{2+}
19.3	0	0	241.5	386.5

4.8 Bioaccumulation and bioconcentration of mercury by *Cladophora* sp.

Inorder to assess the use of *Cladophora* sp. as a bioindicator of mercury in acid mine drainage waters, adsorption experiments were performed at longer residence times, from 1 hour to 1 week at different pH. Longer periods were chosen since the adsorption mechanism is believed to involve the transport of mercury across the cell wall into the cytoplasm, a slow metabolism dependent process known as chemisorption (Dwivedi, 2012; Ahalya et al., 2003**).** The concentrations of mercury accumulated by algae in the alga tissue as well as the water mercury concentration after each contact time were measured. After a contact time of 1 h, the measured mercury concentration was highest at pH 3 followed by pH 6.5 with the least adsorption at pH 8.5 (Figure 4.10). This measured tissue mercury

concentration was almost 30-40 times higher than the initial mercury concentration in solution.

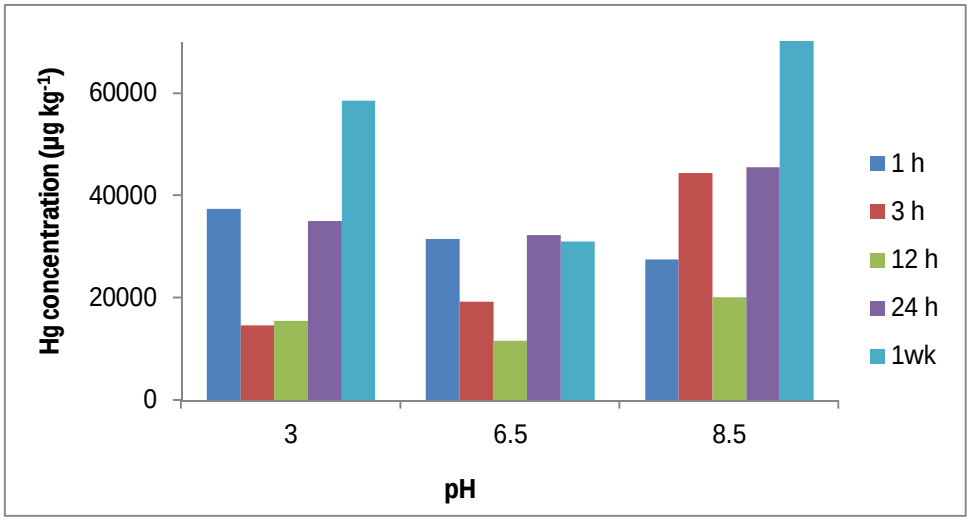

Figure 4.10: Effect of pH on the bioaccumulation of Hg by *Cladophora* sp.

This shows the ability of algae to bioaccumulate and bioconcentrate mercury into its tissues, thus, even at low water mercury concentrations, the mercury content in the algae tissue becomes measurable. Also, according to Nordberg et al., (2011), the concentration of mercury in phytoplankton organisms (e.g. algae) are typically 10^5-10^6 times higher than the concentration in the surrounding water, therefore, this result was expected. However, at 3 h and 12 h contact times the mercury concentration decreased at pH 3 and pH 8.5 with pH 6.5 maintaining an almost constant trend, that is, the concentration measured at the different contact times was always lower than that at pH 3 and pH 8.5 except after 3 h of contact where its concentration was higher than that for pH 3. This may be due to the increased desorption of mercury by *Cladophora* sp. at pH 3, since acidic conditions are known to be efficient desorbing agents for metal ions (Sanyahumbi, 1999). The mercury concentration at pH 8.5 was higher, as explained before, due to the negative charge on the algae surface thereby attracting more of the cations. As the contact time increased from 12 h to 1 week, the mercury concentration in the tissue of *Cladophora* sp. also increased. Also, as assumed from the FTIR results obtained (section 4.3), the trend observed, that is, the generally high adsorptions at pH 3 followed by pH 8.5 with the least adsorption at pH 6.5 may be due to the

abundance of carboxyl functional groups at pH 3 and the hydroxyl functional group at pH 8.5. Simultaneous adsorption and desorption at all pH values is assumed to be taking place as well. However, at all contact times, the measured mercury concentration in water was below the detection limit for the FIMS.

Chapter Five: Conclusions and Recommendations

This section gives the conclusions to the work presented in this report. The success and applicability of this research is highlighted. Recommendations for further studies are also given.

5.1 Conclusions

The aim of this project was to investigate the efficiency (performance) of green algae (*Cladophora sp.)* as Hg bioindicators and their potential use as phytoremediation material for Hg in acidic waters. In order to achieve this goal, a simple and reliable analytical procedure was developed and optimized for the determination of total Hg in plants using an automated FIMS. A CRM (BCR 482) of lichen was used for method validation and more than 99% Hg was recovered. Generally, recoveries of more than 90% were also observed for other metals with the ICP-OES technique. The FIMS has demonstrated a high sensitivity, with detection limits at picogram levels and also excellent repeatability and reproducibility with RSD values below 5%. The developed analytical procedure is, therefore, reliable for the accurate determination of mercury in plants and, hence, was used for the adsorption study carried out in this project.

On another hand, the different information obtained in this research indicated that filamentous, unbranched green alga; *Cladophora* sp. can be efficiently used as a bioindicator and low cost remediation material for mercury in AMD waters due to its abundance and easy sampling techniques. The ability of algae to bioaccumulate and bioconcentrate mercury into its tissue makes them good and efficient bioindicators. Mercury was accumulated to detectable levels into the algae tissue within the first 5 min of contact, hence, *Cladophora* sp. can be used to provide early warning signs by providing bioavailable concentrations of mercury in AMD waters. The measurement of the bioavailable mercury concentration will help in protecting organisms which could be affected by AMD waters.

More than 99% of mercury was removed by *Cladophora* sp. from mercury contaminated waters under acidic conditions (pH = 3) within the first 5 min whereas the equilibrium was attained within 10 min. This makes *Cladophora* sp. alga suitable for use in AMD waters due to its ability to sequestrate Hg^{2+} at low pH. The presence of metal ions such as Cu^{2+} and Fe^{2+} in a multi-component solution reduced the extraction efficiency of mercury by *Cladophora* sp. A decrease in the obtained maximum extraction efficiency (67%) was observed

when compared to the one obtained with the single component system (99%). This was mainly due to the competition of the limited binding sites between Hg^{2+}, Fe^{2+} and Cu^{2+}. The presence of Co^{2+} and Zn^{2+} did not have any significant effect on the adsorption of mercury by *Cladophora* sp.

5.2 Recommendations for future work

Pilot plant studies must be carried out so as to explore the economic feasibility of using *Cladophora* sp. as a bioindicator for mercury in AMD waters and also its ability to remediate mercury from polluted mine waters. Cost reductions for large scale use can be achieved by the use of *Cladophora* sp. for the removal of mercury together with precious metals such as gold and silver which are present in trace quantities and can be recovered from algae. This possibility must also be explored.

Full characterisation of this alga must also be done so as to understand the adsorption and desorption mechanisms at different pH. Bench scale experiments on the effect of different initial temperatures must also be carried out. In addition, speciation studies have to be conducted so as to observe the behaviour of *Cladophora* sp. in the presence of different mercury species such as Hg^{2+}, Hg_2^{2+} and CH_3Hg^{+}.

References

Abu Al-Rub F. A., El-Naas M. H, Ashour I., Al-Marzouqi M., (2006), Biosorption of copper on *Chlorella vulgaris* from single, binary and ternary metal aqueous solutions, **41**:2, 457-464

Ahalya N., Ramachandra T. V. and Kanamadi R. D., (2003), Biosorption of Heavy Metals, *Research Journal of Chemistry and Environment*, **7**:4

Al-Homaidan A. A., Al-Ghanayem A. A and Alkhalifa A. H., (2011), Green Algae as Bioindicators of Heavy Metal Pollution in Wadi Hanifah Stream, Riyadh, Saudi Arabia, *International Journal of Water Resources and Arid Environments*, **1**:1, 10

Alloway B. J., (1995), Heavy Metals in Soils, Blackie Academic and Professional, 2nd Edition, London, 23

Ambasht R. S. and Ambasht N. K., (2003), Modern trends in applied aquatic ecology, Kluwer Academic/ Plenum Publishers, New York, 135-137

Armbruster D. A. and Pry T., (2008), Limit of Blank, Limit of Detection and Limit of Quantitation, the Clinical Biochemist Reviews. Available online at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2556583/

Barsanti L. and Gualtieri P., (2006), Algae: Anatomy, Biochemistry, and Biotechnology, Taylor and Francis, United States of America, 235

Belkin S. and Gabbay S., (2000), Environmental challenges, Kluwer Academic Publishers, The Netherlands, 105

Boss B. C and Fredeen K. J., (1997), Concepts, Instrumentation, and Techniques in Inductively Coupled Plasma Optical Emission Spectrometry, the PerkinElmer Corporation, 2nd Edition

Chekroun K. B. and Baghour M., (2013), The role of algae in phytoremediation of heavy metals: A review, *Journal of Materials and Environmental Science*, **4**:6, 873-880

Conti M. E., (2008), Biological Monitoring: Theory and Applications, Bioindicators and Biomarkers for Environmental Quality and Human Exposure Assessment, WIT Press, Southampton, UK, 82-86

Cooper T. F., Gilmour J. P and Fabricius K. E., (2009), Bioindicators of changes in water quality on coral reefs: Review and recommendations for monitoring programmes, 1-2

Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban, Argyll, UK, Accessed 20/06/13. Available online at: www.ccap.ac.uk/media/recipes/ABM.htm

Dada A. O., Olalekan A. P., Olatunya A. M., Dada O., (2012), Langmuir, Freundlich, Temkin and Dubinin-Radushkevich Isotherm studies of equilibrium sorption of Zn^{2+} unto phosphoric acid modified rice husk, *IOSR Journal of Applied Chemistry*, **3**:1, 38-45

Davis T. A., Volesky B., Mucci A., (2003), A review of the biochemistry of heavy metal biosorption by brown algae, *Water Research Journal*, **37**, 4311–4330

de Guzman M. C., Cao P. E., (2010), Cadmium binding ability of the blue-green alga Hapalosiphon welwitschii Nägel under controlled conditions, Philippine Science Letters, **3**:1, 76-84

Durham W. F. and Williams C. H., (1972), Mutagenic, Teratogenic, and Carcinogenic Properties of Pesticides, Perrine Primate Laboratory, Pesticides Office, Environmental Protection Agency, Perrine, Florida, and Pesticides

Tolerances Division, Pesticides Office, Environmental Protection Agency, Washington, D.C, **17**, 123-128

Department of Water Affairs (DWA), (2010), National Water Act waste discharge standards: DWA 2010 guidelines; Available online at: http://www.wateronline.co.za/wastewater/downloads/dwa-general-standards-2010.pdf

Dwivedi S., (2012), Bioremediation of Heavy Metal by Algae: Current and Future Perspective, Journal of Advanced Laboratory Research in Biology, **3**:3, 195-199

Environment News South Africa, (2011), Tweelopiespruit Catchment Area – the evidence in pictures, Available online at: http://www.environment.co.za/acid-mine-drainage-amd/tweelopiespruit-catchment-area-the-evidence-in-pictures.html

Ford K. L., (2003), Passive Treatment Systems for Acid Mine Drainage, Technical Note 409, 3-8

Fourie M., (2009), Acid mine drainage for Witwatersrand Gold Mines: Environmental law remedies available to regulators and civil society, Wits School of Law Mandela Institute

Fu F. and Wang Q., (2010), Removal of heavy metal ions from wastewaters: A review, *Journal of Environmental Management*, **92**, 407-418

Gadzała-Kopciuch R., Berecka B., Bartoszewicz J. and Buszewski B., (2004), Some considerations about bioindicators in environmental monitoring, *Polish Journal of Environmental Studies*, **13**:5, 453-462

Gaikwad R. W. and Gupta D. V., (2008), Review on removal of heavy metals from acid mine drainage, Applied Ecology and Environmental Research, **6**:3, 81-87

Gerhardt A., (2002), Bioindicator species and their use in Biomonitoring, Environmental monitoring, **1**. Available online at: http://www.eolss.net/Sample-Chapters/C09/E6-38A-01-07.pdf

Girard J.E., (2010), Principles of Environmental Chemistry, 2nd ed., 384

Gray J. E., (2003), Geologic Studies of Mercury by the U.S. Geological Survey, U.S. Geological Survey, Circular 1248, Reston Virginia, 1-2, 6

Henry J. R., (2000), An Overview of the Phytoremediation of Lead and Mercury, 18-22

Ho Y. S, Wase D. A. J and Forester C. F., (1999) Kinetic studies of competitive heavy metal adsorption by sphagnum peat, Environ Technol. **17** 441–443.

Holt E. A. and Miller, S. W., (2011), Bioindicators: Using Organisms to Measure Environmental Impacts, Nature Education Knowledge, **3**(10):8

Horak S., (2011) Digby Wells Environmental, Background information document for the activities associated with the immediate and short term interventions regarding acid mine drainage in the western, central and eastern Witwatersrand basins, Available online at; http://www.amdshort.co.za/documents/BID%20Rev%2011%2023-02-2012%20for%20website.pdf

Hou X. and Jones B. T., (2000), Inductively Coupled Plasma/Optical Emission Spectrometry, John Wiley & Sons Ltd, Chichester

Hutter J. W., (2011), Performance characteristics of analytical tests, AL control laboratories, Intersol, 6-9

Ji L., Xie S., Feng J., Li Y. and Chen L., (2012), Heavy metal uptake capacities by the common freshwater green alga Cladophora fracta, *Journal of Applied Phycology*, **24**, 979-983

Johnson D. B. and Hallberg K. B., (2005), Acid mine drainage remediation options: A review, Science of the Total Environment, 338, 3-14

Johnson M., Shivkumara S. and Berlowitz-Tarrant L., (1996), Structure and properties of filamentous green algae, Materials Science and Engineering B38, 103-108

Keating M. H., (1997), Mercury Study Report to Congress Volume II: An Inventory of Anthropogenic Mercury Emissions in the United States, United States Environmental Protection Agency

Keating M. H., (1997), Mercury Study Report to Congress, Volume 1, United States Environmental Protection Agency

Kilborn INC., (1996), Mine Environment Neutral Drainage (MEND) Program, Review of Passive Systems for treatment of Acid Mine Drainage Phase II, 2-20

Kommineni V., (2011), Cladophora Algae, The Tracy Farmer, Institute for Sustainability and the Environment, College of Agriculture, Environmental and Natural Resource Initiative, University of Kentucky. Available online at: http://www.kentuckyawake.org/cladophora_algae

Kuban P. and Karlberg B., (1998), Interfacing of flow injection pre-treatment system with capillary electrophoresis: Trends in Analytical Chemistry, **17**:1, 34-41

Kumar J. I. N., Oommen C., (2012), Removal of heavy metals by biosorption using freshwater alga Spirogyra hyaline, *Journal of Environmental Biology*, **33**, 27-31

Lagergren S., (1898), Zur theorie der sogenannten adsorption gelöster stoffe. *Kungliga Svenska Vetenskapsakademiens. Handlingar*, Band **24:**4, 1–39

Leaner J., Dabrowski J., Mason R., Ashton P., Murray K., Resane T., Richardson M., Ginster M., Euripides R., Masekoameng E. and Gericke G., Mercury Emissions from Point Sources in South Africa, CSIR report, Available online at: http://www.htap.org/meetings/2008/2008_04/Presentations/07-04-08/5%20-%20J%20Leaner_UNEP%202008.pdf

Lodha R. M., (1993), Environmental Ruin: The Crisis for survival, Indus Publishing Co., New Delhi, India, 130-132

Lusilao J. G. M., (2012), Characterization and modelling of mercury speciation in industrially polluted areas due to energy production and mineral processing in South Africa, PhD Thesis, University of the Witwatersrand, Johannesburg

Macingova E. and Luptakova A., (2012), Recovery of Metals from Acid Mine Drainage, the Italian Association of Chemical Engineering, **28**, 109

Mamboya F. A., (2007), Heavy metal contamination and toxicity: Studies of Macroalgae from the Tanzanian Coast, Stockholm University, 11-13

McCarthy T. S., (2011), The impact of acid mine drainage in South Africa, *South African Journal of Science* **107** (5/6), 712, 2-3

McElfish J. M (Jr.) and Beier A. E., (1990), Environmental Regulation of Coal Mining: SMCRA's 2nd Decade, Environmental Law Institute, Washington, 132-133

Mehta S. K. and Gaur J. P., (2005), Use of Algae for Removing Heavy Metal Ions From Wastewater: Progress and Prospects. Available online at: http://www.redorbit.com/news/science/281074/use_of_algae_for_removing_heavy_metal_ions_from_wastewater/

Mohamed A. M. O. and Anita H. E., (1998), Geoenvironmental Engineering, Developments in Geotechnical Engineering, Elsevier Science B. V., The Netherlands, 90

Mulopo J., and Ikhu-Omoregbe D., (2012), Phosphogypsum Conversion to Calcium Carbonate and Utilization for Remediation of Acid Mine Drainage, **3**:129, 1-2

Mphephu N.F., (2004), Geotechnical environmental evaluation of mining impacts on the Central Rand, PhD. Thesis, University of the Witwatersrand, Johannesburg

Naicker K., Cukrowska E. and McCarthy T.S., (2003), Acid mine drainage arising from gold mining activity in Johannesburg, South Africa and environs, *Environmental Pollution Journal*, **22**, 29-40

Naidoo B., (2009), Acid Mine Drainage Single Most Significant Threat to South Africa's Environment, Mining Weekly

Nordberg G.F., Fowler B.A., Nordberg M., and Friberg L., (2011), Handbook on the toxicology of metals, 3rd ed., Academic Press, London, 262-272

Nsimba E. B., (2012), Development of a biophysical system based on bentonite, zeolite and micro-organisms for remediating gold mine wastewaters and tailings ponds, PhD Thesis, University of the Witwatersrand, Johannesburg

Oilgae Guide to Algae-based Wastewater Treatment: A Sample Report, (2009), 5-8. Available online at: http://www.fao.org/uploads/media/0911_Oilgae__Wastewater_Treatment_Using_Algae_Report_Preview.pdf

Omar W. M. W., (2010), Perspectives on the Use of Algae as Biological Indicators for Monitoring and Protecting Aquatic Environments, with Special Reference to Malaysian Freshwater Ecosystems, Tropical Life Sciences Research, **21**:2, 51–67

Oosthuizen A., John J. and Somerset V., (2010), Mercury exposure in a low-income community in South, **100**:6, 366

Pansamrit K., Ruangsomboon S., (2012), Effect of pH on biosorption of basic dye Malachite green, The 8th International Symposium on Biocontrol and Biotechnolgy, 220-222

Parikh A., Patel K., Patel C. and Patel B. N., (2010), Flow injection: A new approach in analysis, *Journal of Chemical and Pharmaceutical Research*, **2**:2, 118-125

PerkinElmer, (2003), Flow Injection Mercury System, Available online at: www.perkinelmer.com/CMSResources/Images/44-74440BRO_FIMS.pdf

Pirrone N., Cinnirella S., Feng X., Finkelman R.B., Friedli H. R., Leaner J., Mason R., Mukherjee A. B., Stracher G. B., Streets D. G., and Telmer K., (2010), Global mercury emissions to the atmosphere from anthropogenic and natural sources, *Atmospheric Chemistry and Physics Journal*, **10**, 5951-5952

Rezaee A., Ramavandi B., Ganati F., Ansari M. and Solimanian A., (2006), Biosorption of mercury by biomass of filamentous algae Spirogyra species, *Journal of Biological Sciences*, **6**:4, 695-700

Rice G.E. and Ambrose R.B., (1997), Mercury Study Report to Congress, Volume III: Fate and Transport of Mercury in the Environment United States Environmental Protection Agency

Richmond A. and Hu Q., (2013), Handbook of Microalgal Culture: Applied Phycology and Biotechnology, 2nd ed., Blackwell Publishing Ltd., 602-606.

Ruzicka J., Hansen E. H., (1998), Flow injection analysis – Where are we heading?, Trends in Analytical Chemistry, 17:2, 69-73

Ruzicka J., Hansen E. H., (2000), Flow Injection Analysis-From Beaker to Microfluids, Analytical Chemistry, 72:5, 212-217

Saad D.**M**., (2011), Development and application of polymeric materials for heavy metal ions recovery from industrial and mining wastewaters, Msc. Thesis, University of the Witwatersrand, Johannesburg

Saad D. M., Cukrowska E. M and Tutu H., (2013), Selective removal of mercury from aqueous solutions using thiolated cross-linked polyethylenimine, **3**, 527-529

Sambamurty A. V. S. S., (2005), A Textbook of Algae, I. K. International Pvt. Ltd., New Delhi, India, 23

Sanghi R. and Singh V., (2012), Green Chemistry for Environmental Remediation, Scrivener Publishing LLC, Salem, Massachusetts, Chapter 21

Sanyahumbi D., (1999), Removal of lead from solution by the non-viable biomass of the water fern Azolla Filiculoides, Msc. Thesis, Rhodes University, 35

Satyanarayana T., Johri B. N. and Prakash A., (2012), Microorganisms in Environmental Management, Springer Dordrecht Heidelberg London, 350

Scott G., (2011), United Nations Environment Programme, Reducing Mercury Emissions from Coal Combustion in the Energy Sector in South Africa, Final Project Report, Department of Environmental Affairs, Republic of South Africa, 3

Selim H. M., (2013), Competitive Sorption and Transport of Heavy Metals in Soils and Geological Media, Taylor and Francis Group, United States, 147-160

Seshadri R. and Basu R., (2002), Periodic Table of the Elements, Available online at: http://www.mrl.ucsb.edu/~seshadri/Periodic/

Skousen J., Hilton T. and Faulkner B., (1990), Overview of Acid Mine Drainage Treatment with Chemicals. Available online at: http://www.wvu.edu/~agexten/landrec/chemtrt.htm

Shang J. Q. and Wang H., (2005), Coal Fly Ash as Contaminant Barrier for Reactive Mine Tailings, World of Coal Ash, Lexington, Kentucky, United States of America

Solomons Organic Drill Manual, (2007), Simplified Table of Main IR Frequencies. Available online at: http://www.xula.edu/chemistry/crsorgleclab/Solomons_Organic_Drill_Manual_2007/Solomons_Organic_1_fifth_ed_2007_02nd_module_IR_table.pdf

Srivastava S., Singh P, Bhagat R and Tripathi V. N., (2005), Application of bacterial biomass as potential metal indicator, Research Communications, **89**:7, 1248-1250

Strydom H. A and King N. D., (2009), Environmental Management in South Africa, 2nd ed., 537-538

Stuijt A., (2010), Radioactive mine sludge poisons Crocodile/Limpopo river system, Available online at: http://censorbugbear-reports.blogspot.com/2010/06/radioactive-mine-sludge-poisons.html

Tandlich R., (2012), Bioremediation Challenges Originating from Mining and Related Activities in South Africa, *Journal of Bioremediation and Biodegradation*, **3**:3

Telmer K. H. and Veiga M. M., (2009), World Emissions from Artisanal and Small scale gold mining, 136-138

The World Bank Group in collaboration with the United Nations Environment Programme and the United Nations Industrial Development Organization, (1998), Pollution Prevention and Abatement Handbook: Toward Cleaner Production, The World Bank, The World Bank Group, Washington, D.C, 219-221

Tutu H., (2005), Determination and geochemical modelling of the dispersal of uranium in gold-mine polluted land on the Witwatersrand, PhD Thesis, University of the Witwatersrand, Johannesburg

Udayabhanu S. G. and Prasad B., (2010), Studies on Environmental Impact of Acid Mine Drainage Generation and its Treatment: An Appraisal, **30**:11, 953-967

Ullrich S.M., Tanton T.W., and Abdrashitova S.A., (2001), Mercury in the Aquatic Environment: A Review of Factors Affecting Methylation, Critical Reviews in Environmental Science and Technology, **31**:3, 241-293

United Nations Environment Programme (UNEP) Chemicals: Global Mercury Assessment, (2002), Geneva, Switzerland, 2

United Nations Environmental Program (UNEP), (2013), Global Mercury Assessment: Sources, Emissions, Releases and Environmental Transport, 7-12

United Nations Environmental Program: Mercury and Industry, (2008), Module 2, 4-7. Available online at:

http://www.unep.org/hazardoussubstances/Portals/9/Mercury/AwarenessPack/English/UNEP_Mod2_UK_Web.pdf

U.S. Environmental Protection Agency (U.S. EPA), (1999), Understanding Variation in Partition Coefficient, Kd, values, The Kd Model, Methods of Measurement, and Application of Chemical Reaction Codes, Volume 1, 3.1-3.10. Available online at: https://www.orau.org/PTP/PTP%20Library/library/Subject/Environmental/kdsa.pdf

U.S. Environmental Protection Agency (U.S. EPA), (2008), Risk-Based Prioritization Document: Initial Risk-Based Prioritization of Mercury in Certain Products, 1-7. Available online at: http://www.epa.gov/hpvis/rbp/Mercury_RBP_10.31.08_FINAL.pdf

Wang J. and Chen C., (2008), Biosorbents for heavy metals removal and their future, Biotechnology Advances, **27**, 195–226

Welna M., Szymczycha-Madeja A. and Pohl P., (2011), Quality of the Trace Element Analysis: Sample Preparation Steps, 54-56. Available online at http://cdn.intechopen.com/pdfs/23729/InTechQuality_of_the_trace_element_analysis_sample_preparation_steps.pdf.

World Health Organization (WHO), (2013), Mercury and Health, Available online at: http://www.who.int/mediacentre/factsheets/fs361/en/

Woodruff T. J., Janseen S., Guillette L.J. (Jr.) and Giudice L.C., (2010), Environmental impacts on reproductive health and fertility, Cambridge University Press, New York, 16-17

Yalcin E., Cavusosoglu K., Maras M and Biyikoglu M., (2008), Biosorption of Lead (II) and Cu (II) metal ions on Cladophora glomerata (L.) Kutz. (Chlorophyta) Algae: Effect of Algal surface modification, **55**, 228-232

Zaganiaris E. J., (2013), Ion Exchange Resins and Adsorbents in Chemical Processing, Books on Demand GmbH, Paris, France, 173

Ziemkiewicz P. F., Skousen J. G, Brant D. L., Sterner P. L., and Lovett R. J., (1997), Acid Mine Drainage Treatment with Open Limestone Channels, *Journal of Environmental Quality*, **26**:4, 1018

Appendices

Appendix A: Mass of *Cladophora* sp. used for adsorption tests

Table 1: Algae dry weight and moisture content results

Sample I.D	Wet mass (g)	Dry mass (g)	% moisture	Average mass (g)	Average % moisture
pH 3 (1): 0.5 mg Hg l^{-1}	2.01	0.39	80.77		
pH 3 (2): 0.5 mg Hg l^{-1}	2.00	0.32	84.02	0.35	82.46
pH 3 (3): 0.5 mg Hg l^{-1}	2.04	0.35	82.61		
pH 6.5 (1): 0.5 mg Hg l^{-1}	2.02	0.30	85.23		
pH 6.5 (2): 0.5 mg Hg l^{-1}	2.01	0.40	80.29	0.33	83.44
pH 6.5 (3): 0.5 mg Hg l^{-1}	2.03	0.31	84.79		
pH 8.5 (1): 0.5 mg Hg l^{-1}	2.04	0.36	82.51		
pH 8.5 (2): 0.5 mg Hg l^{-1}	2.04	0.37	82.06	0.35	82.85
pH 8.5 (3): 0.5 mg Hg l^{-1}	2.02	0.32	84.00		
pH 3 (1): 1 mg Hg l^{-1}	2.03	0.33	83.83		

pH 3 (2): 1 mg Hg l^{-1}	2.05	0.37	82.10	0.36	82.43
pH 3 (3): 1 mg Hg l^{-1}	2.05	0.38	81.36		
pH 6.5 (1): 1 mg Hg l^{-1}	2.05	0.34	83.33		
pH 6.5 (2): 1 mg Hg l^{-1}	2.04	0.31	84.84	0.34	83.43
pH 6.5 (3): 1 mg Hg l^{-1}	2.01	0.36	82.13		
pH 8.5 (1): 1 mg Hg l^{-1}	2.01	0.33	83.75		
pH 8.5 (2): 1 mg Hg l^{-1}	1.99	0.34	82.64	0.32	83.79
pH 8.5 (3): 1 mg Hg l^{-1}	2.02	0.30	84.97		
pH 3.0 (1): 1 mg l^{-1} (Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+})	4.01	0.68	83.04		
pH 3.0 (2): 1 mg l^{-1} (Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+})	4.05	0.70	82.72	0.69	82.91
pH 3.0 (3): 1 mg l^{-1} (Cu^{2+}, Hg^{2+}, Zn^{2+},	4.05	0.69	82.96		

Fe^{2+}, Co^{2+})

Appendix B: Batch adsorption test results at 0.5 mg Hg l^{-1}

a) Extraction efficiency

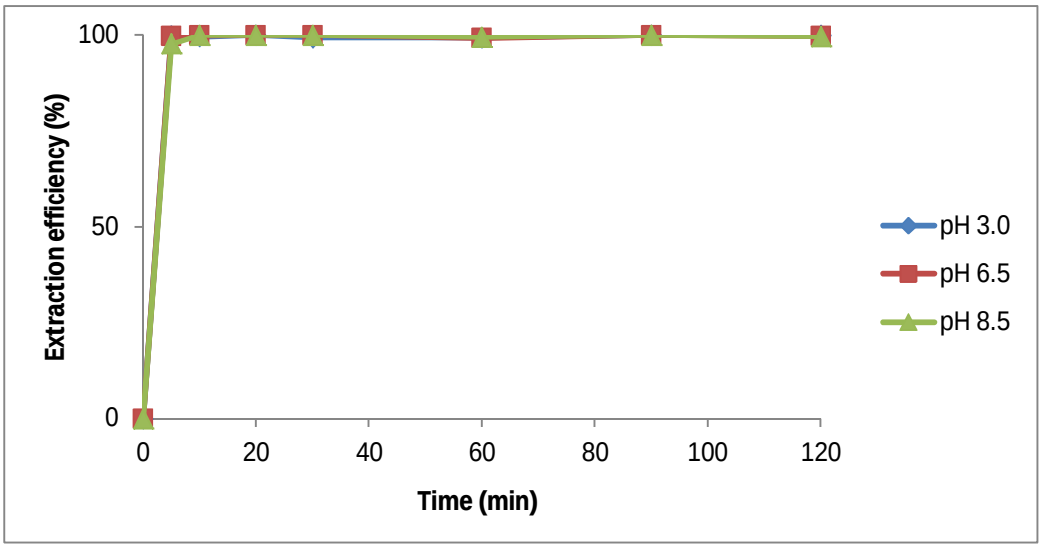

Figure 1: Effect of pH on the extraction efficiency

b) Adsorption capacity

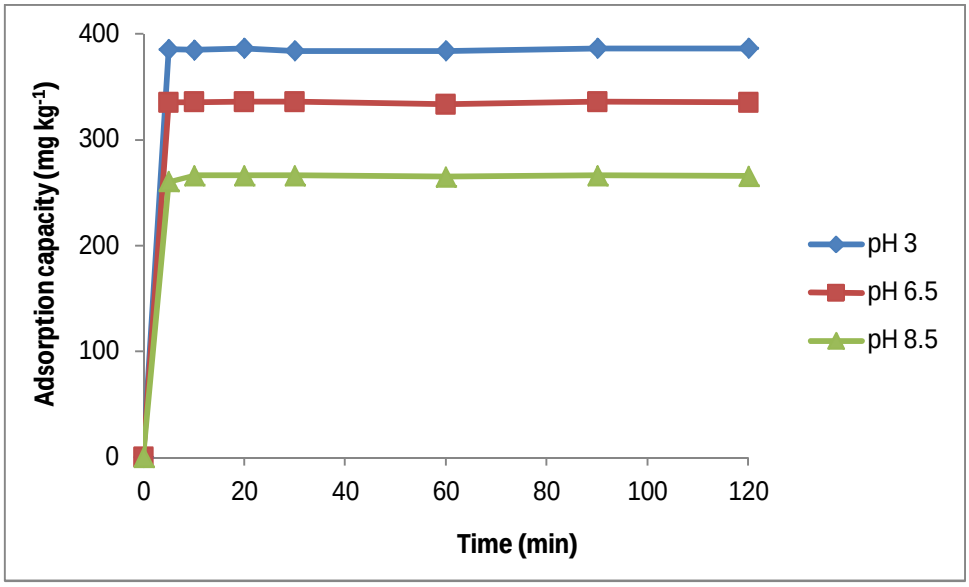

Figure 2: Effect of pH on the adsorption capacity

c) Pseudo first order kinetics

Data obtained in batch adsorption tests for pH 3 could not be modelled into pseudo first order reaction kinetics since the logarithm of the difference between the equilibrium and initial adsorption capacities could not be determined.

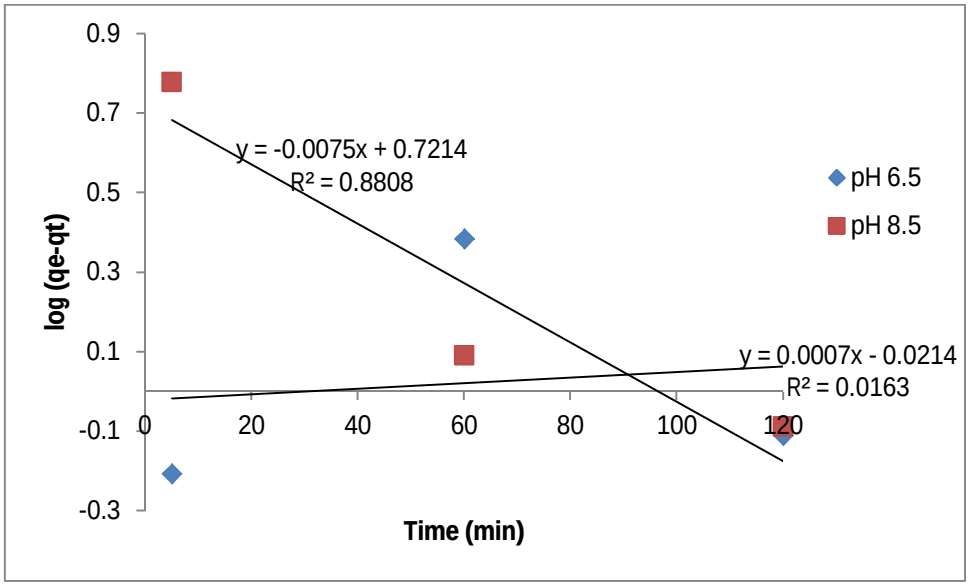

Figure 3: Pseudo first order model

Table 1: Pseudo 1st order kinetics

Parameter	pH 6.5	pH 8.5
k_1 (kg mg^{-1} min^{-1})	0.002	0.02
qe (mg kg^{-1})	0.95	5.27
R^2	0.02	0.88

d) Pseudo second order kinetics

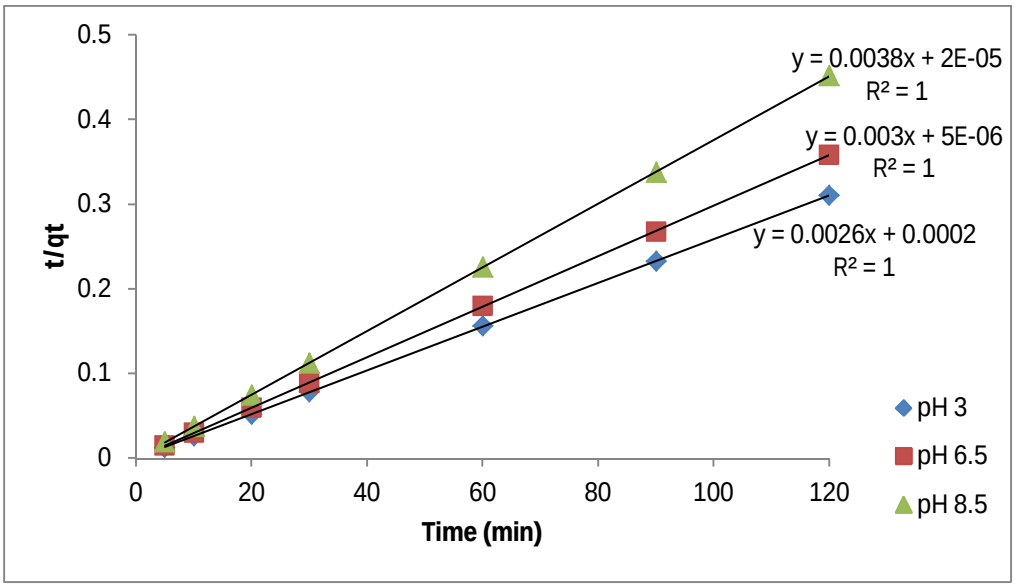

Figure 4: Pseudo second order model

Table 2: Pseudo 2nd order results

Parameter	pH 3.0	pH 6.5	pH 8.5
k_2 (kg mg min)	0.03	1.80	0.72
qe (mg kg^{-1})	384.62	333.33	263.16
R^2	1	1	1

Appendix C: Batch adsorption tests at 1 mg Hg l^{-1}

a) Extraction efficiency

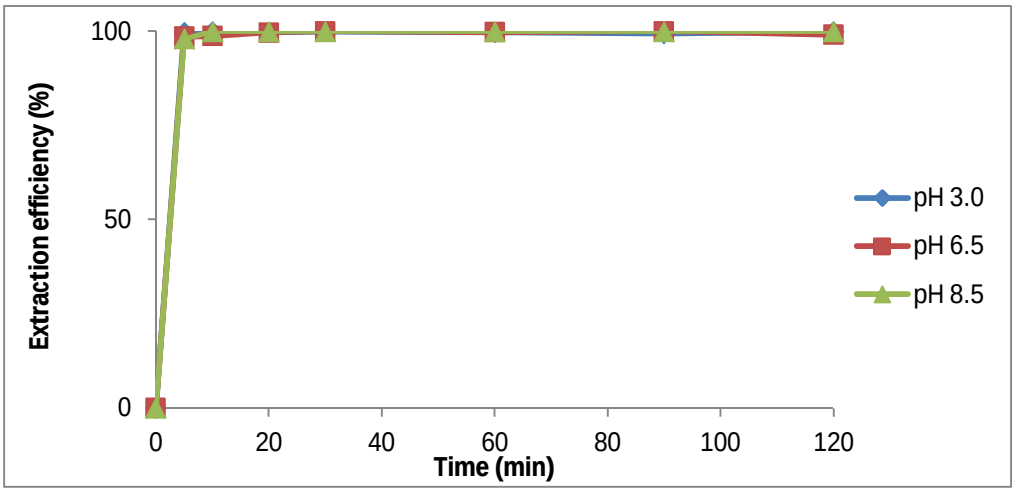

Figure 1: Effect of pH on the extraction efficiency of mercury on Cladophora sp.

b) Adsorption capacity

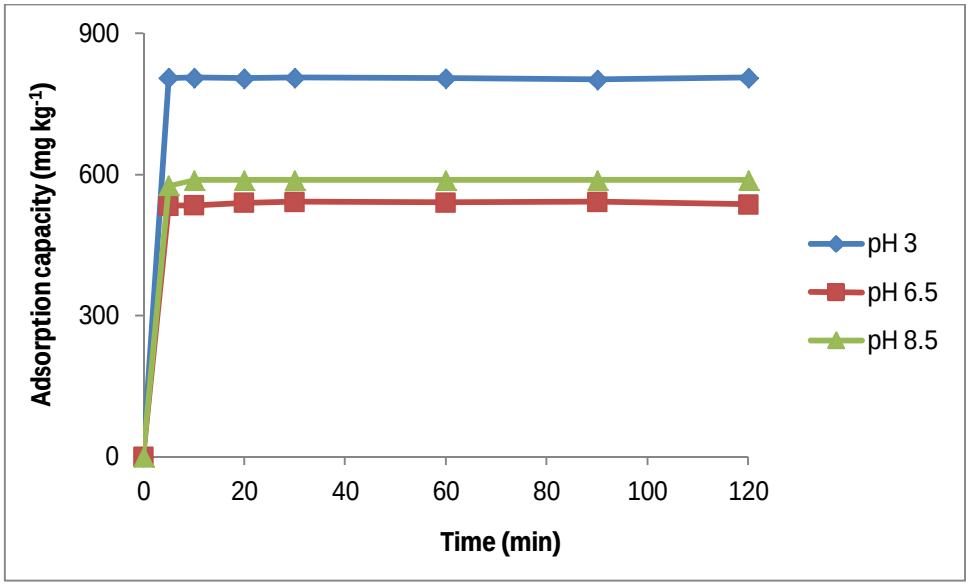

Figure 2: Effect of pH on the adsorption of mercury by *Cladophora* sp.

c) Pseudo first order kinetics

Data obtained in batch adsorption tests using pH 8.5 could not be modelled into pseudo first order reaction kinetics since the logarithm of the difference between the equilibrium and initial adsorption capacities could not be determined.

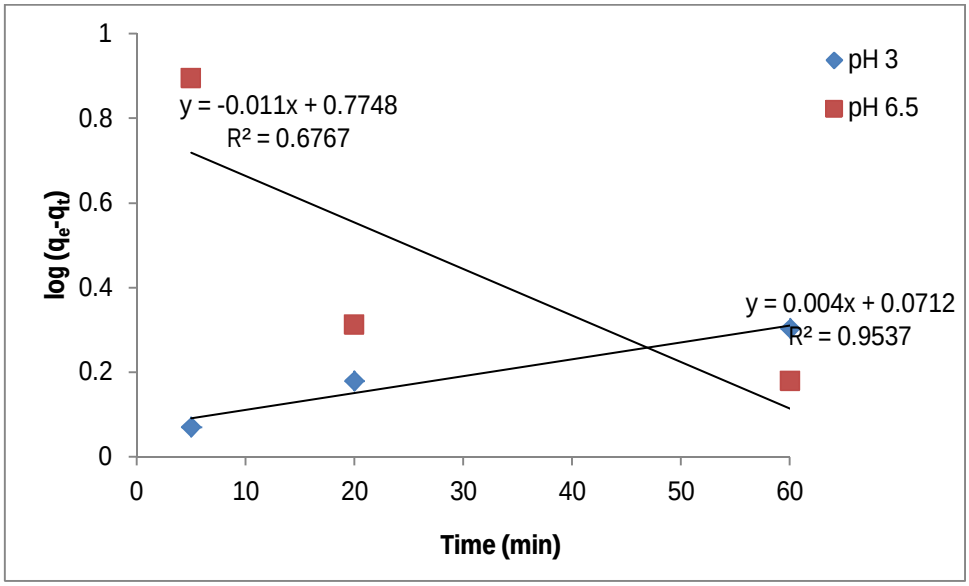

Figure 3: Pseudo first order model

Table 1: Results for pseudo 1st order kinetics

Parameter	pH 3.0	pH 6.5
k_1 (kg mg^{-1} min^{-1})	0.01	0.03
q_e (mg kg^{-1})	1.18	5.95
R^2	0.95	0.68

d) Pseudo second order kinetics

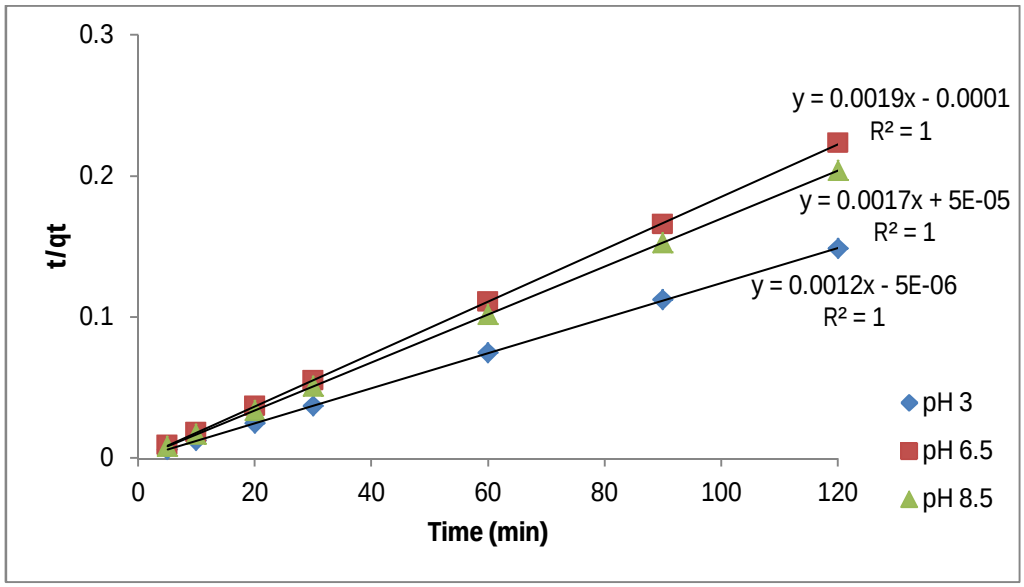

Figure 4: Pseudo second order model

Table 2: Results for pseudo 2nd order kinetics

Parameter	pH 3.0	pH 6.5	pH 8.5
k_2 (kg mg^{-1} min^{-1})	0.29	27.70	0.06
q_e (mg kg^{-1})	833.33	526.32	588.24
R^2	1	1	1